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Full chapters of the following presentations will be published in a Symposium Book - *Microbial Subversion of Host Cells* - published for the Society for General Microbiology by Cambridge University Press.

Monday 7 April 2003

0900 Current perspectives of the integrin field and their relationship to cellular microbiology

M.J. HUMPHRIES
University of Manchester
Abstract not received

0945 The structure and function of the enteropathogenic *Escherichia coli* type III secretion system

GADI FRANKEL
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Type III secretion systems (TTSS) are utilised by many Gram-negative pathogens to deliver virulence proteins directly into host cells. Although there is little homology between the virulence proteins secreted by these bacteria, there is considerable homology between components of the secretion apparatus. We study the TTSS of enteropathogenic *Escherichia coli*, a major cause of infantile diarrhoea and mortality in developing countries, which is shared with enterohaemorrhagic *E. coli* (EHEC), *Citrobacter rodentium*, and *E. coli* strains pathogenic for animals. The EPEC secretion apparatus includes the conserved components found in all systems but is unique in that it possesses a long filamentous structure attached to the distal end of the secretion apparatus, which links the bacterium to the host cell. Although the components of most TTSSs are well known, the exact structure of the secretion apparatus remains elusive; here we describe recent work undertaken in our laboratory aimed at addressing the role, structure and location of the individual components of the EPEC TTSS. In some cases these are proteins unique to the EPEC system that highlight differences between systems, in others they are conserved proteins that reveal details of TTSSs in general.

1100 Setting up a nest and maintaining it: intracellular replication of legionella pneumophila

RALPH R. ISBERG
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Shortly after ingestion of enteropathogenic *Yersinia* the bacteria are internalized by M cells, specialized intestinal cells overlying the Peyer's patches. Once localized within these lymph nodes, the microorganism maintains an extracellular lifestyle. Entry into host cells is primarily promoted by the bacterial invasin protein, which recognizes multiple members of the integrin family of mammalian cell adhesion molecules. The site on the integrin receptor recognized by invasin is apparently identical to the site recognized by natural host substrates of the receptor, and the region of invasin that binds the integrin receptor has many features in common with natural substrates. Invasin promotes uptake by engaging the integrin receptors with a high affinity and promoting clustering of the receptors. After clustering, signals are sent from the cell surface to host cell cytoskeletal components that direct the uptake process. Proteins involved in this cascade include tyrosine phosphorylated proteins and proteins that are activated by the small GTPase Rac1, which is involved in directing organization of the host cell cytoskeleton.

1145 The entry of *Listeria monocytogenes* into mammalian cells, a tightly controlled process

PASCALE COSSART
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Listeria monocytogenes is a facultative intracellular pathogen which uses at least two proteins to invade mammalian cells. Internalin interacts with the transmembrane protein E-cadherin. InlB interacts with the transmembrane tyrosine kinase C-Met, the hepatocyte growth factor, gC1qR, an ubiquitously expressed surface molecule and proteoglycans. We are dissecting these two pathways independently using latex beads coated with internalin or InlB. Our recent results indicate that for the internalin pathway, in addition to α - and β -catenins, a critical factor is the unconventional myosin, myosin VIIa and the transmembrane protein vezatin. For the InlB pathway, we have highlighted that in addition to PI3 kinase, key components include rac, Arp2/3, with cofilin and VASP playing key regulatory roles in the control of actin rearrangements. Whether the two entry pathways are interconnected is under current investigation.

1330 Vaccinia virus movement in cells

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Vaccinia virus is a large DNA virus that replicates in the cytoplasm of infected cells and exploits components of the cytoskeleton for its replication and dissemination. Each infected cell produces morphologically and functionally distinct virions called intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV). IMV and EEV have different functions during the virus life cycle, bind to different cell surface receptors and enter cells in different ways. During virion egress, IMV are transported on microtubules from virus factories to sites near the microtubule organising centre (MTOC) where they become wrapped by intracellular membranes forming IEV. IEV are transported by microtubules from near the MTOC to the cell surface where the outer membrane fuses with the plasma membrane to form CEV. CEV induce the polymerisation of actin from beneath the plasma membrane and these growing actin tails drive the virions away from the cell, aiding cell-to-cell transmission of virus. EEV are formed by release of CEV from the cell surface and mediate longer-range spread of virus in cell culture and *in vivo*.

1415 Induction of pro-inflammatory signals by *Salmonella*-epithelial cell interactions

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Salmonella infection of both humans and food-producing animals causes major public health, economic and welfare problems throughout the world. Understanding the pathogenesis of *Salmonella* infections is of great importance in helping alleviate these problems.

Recent studies on the molecular mechanisms of *Salmonella* virulence using relevant model systems have improved our understanding of how *Salmonella* causes inflammatory gastrointestinal disease. The first set of host cells that *Salmonella* interacts with in the intestinal tract are epithelial cells. Signals elicited by this bacteria-host cell interaction result in invasion of the cells by the *Salmonella*, disturbances in electrolyte balance and accompanying fluid secretion, and a massive pro-inflammatory response involving secretion of chemoattractant

molecules including cytokines and an influx of polymorphonuclear leukocytes into the infected area. Type III secretion systems (TTSS) play an important role in the induction of these pro-inflammatory responses by *Salmonella*. TTSS are used by bacteria to secrete and translocate a range of proteins, known as effectors, directly into the host cell cytosol where they elicit many wide-ranging effects.

The interaction of *Salmonella* with epithelial cells, and the signals elicited by this interaction that ultimately lead to the onset of inflammatory gastrointestinal disease will be discussed in this presentation.

1530 A52R and A46R from vaccinia virus antagonise toll-like receptor signal transduction

ANDREW BOWIE

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Toll like receptors (TLRs), part of the interleukin-1 receptor (IL-1R)/TLR superfamily of proteins, participate in the first step of host defence by recognising pathogens and activating innate immunity. We previously identified A46R and A52R as two Vaccinia Virus (VV) proteins that could inhibit IL-1/TLR-induced activation of the transcription factor NFκB. Antagonism of TLRs by VV proteins was predictive of a role for TLRs in anti-viral innate immunity, and indeed TLR3 has subsequently been proposed as a receptor for viral dsRNA. A46R has significant sequence similarity to the Toll/Interleukin-1 receptor homology (TIR) domain, the cytosolic signalling domain of the IL-1R and TLRs. A46R was shown to target MyD88, a TIR-containing adaptor molecule important in many IL-1R/TLR downstream signals. Consistent with this, A46R was capable of inhibiting multiple signals emanating from the IL-1R and TLR4. Further, poly(I:C) induced NFκB activation via TLR3, which is independent of MyD88, was not blocked by A46R. In contrast, A52R targeted two signalling molecules downstream of TIR receptor complexes, TNF receptor associated-factor 6 (TRAF6) and IL-1R-associated kinase 2 (IRAK2), and was capable of inhibiting NFκB activation by multiple TLRs, including TLR3. Thus targeting of IRAK-2 and/or TRAF6 by A52R may prevent TLR3 escaping from VV inhibition *in vivo*. Further analysis of A46R and A52R will shed light on the interaction between viruses and the TLR host defence system.

1615 Co-ordinate subversion of host cellular processes by enteropathogenic *E. coli* (EPEC)

BRENDAN KENNY

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The virulence of enteropathogenic *E. coli* is dependent on its ability to transfer effector molecules into host cells to subvert cellular processes. EPEC, like many gram-negative pathogens, encode a specialised organelle to translocate such molecules directly into the host cell. EPEC injects at least five proteins into host cells of which one, Tir (Translocated intimin receptor), has been shown to be essential for virulence. Tir is inserted into the host plasma membrane where its ability to trigger actin reorganisation – a process correlated with disease – is dependent on its modification by host kinases and interaction with the bacterial outer membrane protein, intimin. A second protein, Map (Mitochondria associated protein) is targeted to host mitochondria and induces dysfunction. Recently, we reported that both Tir and Map have additional functions and act antagonistically to modulate the activity of a host ‘molecular switch’. The identification of a role for intimin in this process suggests that EPEC can control effector function from an extracellular location to direct an outcome that favours survival, replication and subsequent transmission.

Tuesday 8 April 2003

0900 Regulation of viral glycoprotein traffic and virus-induced membrane fusion

R.W. COMPANS, MIN LI, SHAGUNA SETH, ANDREI VZOROV, SUXIANG TONG, ARMIN WEIDMANN & CHINGLAI YANG

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Many viral glycoproteins are preferentially associated with cell membrane microdomains that are enriched in cholesterol and sphingolipids, and evidence has been obtained that this association may play an important role in viral assembly. Such association could play a role in the selective incorporation of viral envelope glycoproteins, and the exclusion of most cellular membrane glycoproteins, from the envelopes of lipid-containing viruses. Further, such lipid raft association could provide a basis for incorporation of heterologous viral envelope proteins into phenotypically mixed virions. We found that the envelope protein of murine leukemia virus (MuLV) requires palmitoylation for its lipid raft association. In contrast, the association of the SIV Env protein with lipid rafts does not require such modification, but appears to be a function of its long cytoplasmic domain. Association with lipid rafts is not required for membrane fusion activity of the MuLV or SIV Env proteins, although the clustering of viral fusion proteins or their receptors in specific microdomains could potentially play a role in facilitating the fusion process. We also found that fusion activity of retroviral Env proteins as well as a paramyxovirus F protein can be suppressed by the presence of an elongated cytoplasmic domain. This fusion-suppressive effect is sequence dependent, and results in stabilization of the metastable pre-fusion conformation of the protein. These results show an unexpected role of the cytoplasmic domain in regulation of viral glycoprotein-mediated membrane fusion.

0945 Subversion of the early secretory pathway by *Legionella pneumophila*

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Legionella pneumophila is a gram-negative bacterial pathogen that replicates inside of host vacuoles derived from the endoplasmic reticulum. Transport of phagosomes containing *L. pneumophila* is controlled by a bacterial type IV secretion system encoded by the *dot/icm* genes. This type IV secretion system injects a protein into host cells called RalF. The bacterial RalF protein functions as an exchange factor that activates eukaryotic GTPases belonging to the ADP ribosylation factor (ARF) family. ARF activity is required for the transport of endoplasmic reticulum-derived vesicles to phagosomes containing *L. pneumophila*. Interfering with ARF activity during the early stages of infection prevents the conversion of phagosomes containing *L. pneumophila* into endoplasmic reticulum-like organelles and leads to delayed fusion of these phagosomes with lysosomes. Thus, ARF function is important for cellular processes that promote the establishment of a vacuole that allows intracellular replication of *L. pneumophila* and prevents these phagosomes from being delivered to lysosomes.

1100 Molecular mechanisms involved in phagocytosis: lessons from the intracellular pathogen *Mycobacterium* spp

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Macrophages are professional phagocytes whose function is to clear the circulation from microbial organisms as well as debris resulting from apoptotic events. Following phagocytosis, such material is transferred to the endocytic pathway for degradation and generation of immune responses.

Pathogenic mycobacteria, including *M. tuberculosis*, are phagocytosed by macrophages but have gained the capacity to circumvent destruction within lysosomes through inhibition of lysosomal delivery. This resistance to lysosomal delivery occurs for living, but not killed mycobacteria and represents a key mechanism in mycobacterial survival and significantly contributes to the pathology of diseases such as tuberculosis.

We are analyzing the host factors involved in mycobacterial survival within macrophages. Our results show that both at the point of entry as well as in intracellular phagosomes a specific subset of molecules are recruited by mycobacteria that allow these pathogens to circumvent destruction in lysosomes.

Survival of mycobacteria within phagosomes does not only allow them to escape destruction in lysosomal organelles, but also results in these pathogens to become sequestered from immune recognition, due to the inefficient degradation of mycobacteria in lysosomes. Nonetheless, MHC restricted responses do occur during mycobacterial infection. We are currently investigating the mechanisms leading to mycobacterial degradation allowing epitopes to be presented in the context of MHC molecules.

1330 Cellular and inflammatory activities of virulence factors of *Helicobacter pylori*

CESARE MONTECUCCO

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Helicobacter pylori chronically infects the stomach of the majority of the human population and it is associated with severe gastroduodenal diseases. It resides within and below the mucus layer which protects the stomach epithelial lining. This ecological niche is characterized by a paucity of nutrients. Here, I will discuss the mechanism of action of two main virulence factors of *H. pylori* which alter the stomach mucosa to induce release of nutrients necessary to bacterial growth.

The vacuolating cytotoxin VacA induces vacuolization of late endosomal compartments with mis-targeting of acid hydrolases and degradation of the mucus layer, which becomes more permeable to nutrients from the stomach lumen. VacA makes transmembrane anion selective channels, which increase the luminal osmotic pressure thereby causing swelling of late endosomes. In addition, VacA causes a selective increase in the paracellular route of permeability of polarized epithelial monolayers to small molecules, permitting the passage of nutrients from the basolateral side to the apically located bacteria thus supporting their growth.

The neutrophil activating protein HP-NAP was found to activate mast-cells, neutrophils and monocytes via a pertussis toxin sensitive G protein-coupled receptor. A limited tissue inflammation makes additional nutrients available to the bacterium. At the same time, oxygen radicals can generate mutagenic substances which could contribute to the increased risk of developing cancer.

1415 Invasion and inflammatory destruction of the intestinal epithelium by *Shigella*

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The pathogenesis of bacillary dysentery can be studied at different levels of integration of the cellular components that constitute the colonic mucosal barrier. Interaction of *Shigella flexneri* with individual epithelial cells shows a series of events in which the bacterium, upon contact with the rafts of cell surface that allows engagement of the eukaryote receptor CD44, releases Ipa proteins (i.e. invasins) through a specialized, activable, type III secretory apparatus (i.e. Mxi/Spa). Via a complex signaling process that involves both the cascade of signals elicited by the three GTPases of the Rho family (Cdc42, Rac and Rho) and pp60 c-src, these invasins cause major rearrangement of the subcortical

cytoskeletal network, thereby allowing bacterial entry by a macropinocytic event. Then the bacterium lyses its phagocytic vacuole and initiates intracytoplasmic movement, due to polar nucleation and assembly of actin filaments caused by a bacterial surface protein, IcsA. The cytoskeletal-associated protein N-WASP and Arp2/3 play an essential role in initiation of actin polymerization. This allows very efficient colonization of the host cell cytoplasm and passage to adjacent cells via protrusions which are engulfed by a cadherin-dependent process. Recent evidence indicates that expression of connexins that form both gap junctions and hemiconnexons in polarized epithelial cells increase permissivity of the cells adjacent to the infected one to endocytose the protrusion and to undergo invasion by extracellular microorganisms. Connexin-mediated paracrine transfer of ATP appears essential to mediate increased permissivity of epithelial cells to invasion. ATP-mediated signalling also causes recurrent Ca⁺⁺ fluxes whose function in the invasive and inflammatory process remain to be analysed. However, when invasive *Shigella* are deposited on the apical side of polarized monolayers of human colonic cells, they are unable to invade, indicating that bacteria need to reach the subepithelial area to invade the epithelium.

Initial bacterial crossing of the epithelium occurs essentially via the M cells present in the follicle associated epithelium (FAE). Entering bacteria are then taken up by resident macrophages of the dome area located atop the solitary lymph nodes in the colonic mucosa (i.e. the inductive sites of the mucosal immune response). In order to survive the phagocytic event, *Shigella*, through its secreted invasin IpaB, causes apoptotic death of the macrophages. This killing event which is essential for the bacteria to escape and invade the epithelial lining baso-laterally is caused by activation of the cysteine protease caspase-1, a pro-apoptotic caspase that also achieves hydrolysis of pro-interleukin-1 and pro-interleukin-18, two strong pro-inflammatory cytokines. Current evidences indicate that *Shigella*-induced apoptosis of mucosal macrophages is therefore both a survival mechanism of the invading microorganism and an early pro-inflammatory event in the course of the disease. This early event, particularly via IL-1 β release, triggers an inflammatory response that destabilizes the epithelial barrier and facilitates further bacterial invasion at distance of the FAE. This signaling loop that appears to establish initial conditions for proper *Shigella* invasion may also play a role as triggering events in IBD's.

Recent evidence also indicates that invaded epithelial cells are able to sense intracellular presence of bacterial peptidoglycan through proteins of the NOD/CARD family that are able to activate the NF- κ B pathways in these invaded epithelial cells, thus reprogramming them to produce pro-inflammatory chemokines and cytokines such as IL-8. As an increasing number of epithelial cells are invaded by *Shigella* (i.e. direct invasion considerably amplified by cell to cell spread), the colonic epithelium becomes a major provider of IL-8, thereby inducing massive recruitment of polymorphonuclear leukocyte that account for the destructive inflammatory process that is so characteristic of shigellosis.

Recent demonstration that mutations leading to functional alterations of NOD2/CARD15 are found in familial cases of Crohn's disease, appears to validate the existence of a parallel between shigellosis and IBD's through alterations of similar signalling pathways.

We hope that further understanding of *Shigella* pathogenesis will help deciphering the cross talks between intestinal microbes and the mucosal innate immune response that lead to intestinal inflammation.

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activation by invasive *Shigella flexneri*. EMBO Reports 2:736-742 / PHILPOTT, D.J., GIRARDIN S.E., SANSONETTI, P.J., 2001 Innate immune responses of epithelial cells following infection with bacterial pathogens. Curr Opin Immunol, 2001 13:410-416 / d'HAUTEVILLE, H., KHAN, S., MASKELL, D., KUSSAK, A., WEINTRAUB, A., MATHISON, J., ULEVITCH, R., WUSCHER, N., PARSOT, C., SANSONETTI, P.J., 2002 Two *msbB* genes encoding maximal acylation of lipid A are required for invasive *Shigella flexneri* to mediate inflammatory rupture and destruction of the intestinal epithelium. J. Immunol., 168: 5240-5251 / GIRARDIN, S., GOMPERTS-BONECA, I., VIALA, J., LABIGNE, A., PHILPOTT, D.J., SANSONETTI, P.J., 2002. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. J. Biol. Chem., in press.

1530 Cell death on demand: Herpes simplex viruses and apoptosis

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A common feature of viral gene expression is the perturbation of cellular metabolism resulting in activation of programmed cell death. While some viruses benefit from the pro-apoptotic responses elicited by their gene products, others have evolved elaborate strategies to preclude programmed cell death in order to maximize viral replication. Herpes simplex viruses have evolved several multifunctional proteins designed to delay activation of programmed cell death induced by both exogenous agents and perturbations of cellular metabolism caused by viral proteins. The anti-apoptotic activity begins virtually from the first contact of the virus with the infected cell and continues through the release of progeny virus. Among the viral proteins with anti-apoptotic activity are two glycoproteins and a protein kinase. These proteins target different cellular pro-apoptotic pathways with remarkable efficiency.

1615 Toll-like receptor-toxin interactions in *Shigella*

A. ZYCHLINSKY

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Toll-like receptors (TLR) are involved in the recognition and initiation of host defenses against pathogens. TLR signal in response to conserved and essential regions of bacterial components which are present in many, if not all, bacterial species. Upon activation by bacterial ligands, TLR signal for inflammation through cytokine production as well as activating antibacterial defenses such as the production of reactive oxygen species.

We have studied the pro-inflammatory signaling pathways of TLRs in response to diverse stimuli. We discovered that TLR can signal for apoptosis, and identified the apoptotic signaling pathway downstream of TLR. Furthermore, we believe that this pathway is critical for the release of the potent pro-inflammatory cytokines IL-1 β and IL-18. In addition, we are currently testing the role of TLR in *Salmonella* infections.

POSTERS

MS 01 *Campylobacter* induced proinflammatory signals from human monocytes

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Campylobacter is known to cause inflammatory enteritis, during the course of disease it can penetrate the epithelial barrier and may interact with leucocytes. We studied the interaction of *Campylobacter* with the human monocytic cell line, THP-1. We show that *Campylobacter* can stimulate a range of inflammatory

cytokines and that this stimulation is not dependent on the cytolethal-distending toxin. *Campylobacter* infection of THP-1 cells can stimulate the degradation of I- β Ba and trigger translocation of functional NF- κ B. Additionally we have shown differential IL-1 induction which would indicate that an NF- κ B-independent stimulation may also be occurring. The extent of pro-inflammatory cytokine stimulation suggests that monocytes could significantly contribute to inflammation and disease pathology.

MS 02 Probing the regulation and function of the *E. coli* *psp* operon*

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The phage shock operon (*psp* operon) in *Escherichia coli* is a stress response operon conserved amongst Gram negative bacteria which can be activated by a range of membrane-damaging cues such as secretin insertion, heat shock and treatment with ethanol. The *psp* operon is therefore thought to act as an adaptation to virulence factor export as this process is dependent on the functioning of secretins. Phage shock genes are organised into an operon comprising 5 genes (*pspABCDE*) divergently transcribed from the transcriptional bacterial enhancer binding protein PspF, which together with the σ^{54} holoenzyme mediates transcription of *psp* genes. The precise inducing stimulus and the basis for the adaptive value of the *psp* operon is unknown. Here we explore the role and functioning of the *E. coli* *psp* operon using RNA and metabolic profiling methods and GFP fusions to key components of the *psp* transcription control proteins. RNA profiling shows there to be global changes in gene expression on induction of the *psp* operon and these changes can be linked to phenotypes seen in the metabolic profiling. Stable GFP fusions to *pspA* and *pspF* have been constructed which can be used to visualise these key proteins in the cell under inducing and non-inducing conditions. *This work was funded by the Wellcome Trust.

MS 03 The characterisation of an avirulence gene, *avrPpiG1*, from the pea pathogen, *Pseudomonas syringae* pv. *pisi*

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Avirulence gene, *avrPpiG1*, was first isolated by PCR amplification from a race 4A strain of *Pseudomonas syringae* pv. *pisi*. The cloned gene was used as a probe to isolate cosmid clones carrying *avrPpiG* homologues from gene libraries of two further races, 2 and 3A. When these cosmids and the original cloned gene were conjugated into a suitable recipient strain of *P. syringae* pv. *phaseolicola* (pathogenic on all bean cultivars) and tested in bean, a differential reaction was observed between cultivars. The cloned gene produced a hypersensitive response (HR) in all cultivars tested, while the cosmids produced a HR in cv. Red Mexican, but a disease in cvs. A43, Canadian Wonder and Tendergreen. Disruption of the *avrPpiG* homologues in the cosmids produced a HR in all bean cultivars. These results suggest that *avrPpiG* may interact with other, as yet undefined, effector genes in the *Pph*-bean interaction.

MS 04 Adhesion and invasion by *Escherichia coli* K1 R. FAGAN & S.G.J. SMITH

Dept of Microbiology, Trinity College Dublin, Dublin 2, Ireland

Escherichia coli is a major cause of septicaemia and meningitis in neonates. Sepsis occurs in approximately one in every thousand live births, with 10% progressing to develop meningitis. A disproportionate number of these infections are caused by strains expressing the K1 capsule; up to 40% of septicaemia isolates and 80% of meningitis isolates. To date, most studies

have focussed on immune evasion and bacterial penetration of the blood-brain barrier and more than 15 *E. coli* proteins have been shown to contribute to these events, including: OmpA, YijP, IbeA and IbeB. However it is still unclear how the bacteria first colonise and then invade the epithelial surfaces prior to septicaemia.

We have cloned a gene from *E. coli* K1 strain RS218 that contributes to adhesion. The protein encoded by this gene is 90% identical to Hra1 and is also highly homologous (63% identical) to Tia, another adhesin/invasin from ETEC. This protein, designated Hek (Hra1 from E. coli K1), is a 26kDa outer-membrane protein and is predicted to form a β -barrel in the outer membrane consisting of 8 anti-parallel, amphipathic β -sheets with four short periplasmic turns and four longer external loops. Expression of multicopy Hek in an *E. coli* K12 strain confers a heat-resistant haemagglutination phenotype, similar to that observed with Hra1. Furthermore, bacteria expressing Hek display an auto-aggregation phenotype similar to that reported for the Ag43, FimH and Cah proteins. Auto-aggregation is important in bacterial biofilm formation and may have a role in intestinal colonisation. With its high level of homology to well characterised virulence factors such as Tia and Hra1 and its ability to promote attachment both to mammalian cells and to other bacteria, Hek is an interesting candidate protein which may be responsible for adhesion to, and perhaps invasion of, the intestinal epithelium. Here we present data supporting this hypothesis.

MS 05 Assessment of the intracellular activities of the secreted effector protein SopA from *Salmonella*

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Salmonella secretes and translocates a range of proteins known as effectors into host cells, through the use of two virulence-associated type III secretion systems. These effector proteins elicit a variety of responses within the host cell including rearrangement of the actin cytoskeleton and induction of pro-inflammatory responses. As part of our on-going effort to elucidate the functions of some of these effector proteins and to understand how they contribute to *Salmonella* pathogenesis, we created HEK-293 cells stably transfected with plasmids containing individual *Salmonella* outer proteins (Sops), under the control of a tetracycline-regulated promoter. The cells were used in a variety of assays to study the effector proteins and their cellular responses.

Flow cytometric analysis of surface markers on the transfected cells revealed that ICAM-1, a cell adhesion molecule, was up-regulated on cells expressing the effector protein SopA. In addition, monocytes isolated from human blood demonstrated increased adherence to SopA-transfected cells when compared with non-transfected cells. Thus it appears that the effector protein SopA acts to increase expression of ICAM-1, and possibly other receptors, on host cells which allows stronger binding of monocytes during the inflammatory response. Work is continuing to confirm this data in the *in vivo* situation using mutant *Salmonella* strains and to ascertain the relevance of this finding during infections.

MS 06 Immunopathology of *Chlamydomphila abortus* infection in a pregnant mouse model

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Chlamydomphila abortus is an obligate intracellular bacterium that targets the placenta, causing tissue damage, inflammation and abortion. Ruminants are the principal hosts, although humans are susceptible to infection. A mouse model has been developed to study the immunopathogenesis of chlamydial abortion. Infection

was monitored by recovery of the organism and by immunohistochemical labelling for chlamydial antigen and immune cells in tissue sections. Pregnant mice were infected at mid-gestation (day 11) with *C. abortus* (strain S26/3) to determine the kinetics of infection. Infected cells were found at the maternal-foetal interface, specifically maternal giant cells and the trophoblast cells within the foetal labyrinth, on days 3 and 5 post-infection (p.i.). Chlamydial inclusions were scattered throughout the labyrinth between days 3 and 7 p.i., which immediately preceded abortion. Infection was accompanied by a maternal mononuclear inflammatory cellular infiltrate. The role of this inflammatory response in disease pathogenesis and the characterisation of cellular phenotypes and cytokine expression *in situ* are under investigation.

MS 07 Mitochondrial associated protein (Map): an EPEC effector protein

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Enteropathogenic *Escherichia coli* (EPEC) is a pathogenic bacterium that causes diarrhea in humans. After binding to gut epithelium, EPEC injects virulence factors into the host cell using a Type III secretion apparatus. One such injected effector protein that has been recently identified is Map (mitochondrial associated protein) which has been found to accumulate in punctate structures within the host cell that have been identified as mitochondria. Data to date suggest that Map carries a mitochondrial targeting sequence that directs this protein to these organelles where it appears to undergo N-terminal cleavage, a process indicative of mitochondrial import. Map interaction with mitochondria in host cells also appears to result in mitochondrial swelling and disruption of mitochondrial membrane potential. Given that mitochondria play essential roles in many aspects of cellular physiology, including the production of ATP and the control of programmed cell death (apoptosis), the manipulation of these processes by EPEC may give this pathogen increased control over the host cell. Map appears to be a multifunctional protein as it has also been shown to regulate Cdc42 activity, independent of mitochondrial association. The present work however, focuses on recent findings on the interaction of purified Map with isolated mitochondria.

MS 08 Stimulation of TLR4 by lipopolysaccharide during cellular invasion by live *Salmonella typhimurium* is a critical but not exclusive event leading to macrophage responses

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Macrophage activation by salmonellae is likely to involve cellular recognition of a number of pathogen associated molecular patterns (PAMP).

Lipopolysaccharide (LPS) is a key PAMP, but its precise role in macrophage activation, especially when presented as a component of live infecting bacteria, is unclear. Using the lipid A antagonist E5531, we investigated the role of LPS during *Salmonella* infection in the macrophage-like cell line RAW 264.7 and primary macrophage cultures from C3H/HeJ and Toll-like receptor 4 (TLR4) $-/-$ mice.

We show that LPS presented on live salmonellae activates TLR4 receptors on macrophages to produce nitric oxide (NO) and tumour necrosis factor (TNF)- α . Both purified LPS and bacterial invasion cause translocation of NF- κ B, but whereas E5531 inhibited the effects of purified LPS, signalling activated by bacterial invasion was only inhibited at later time points. In conclusion, NO and TNF- α production in response to bacterial infection with salmonellae is critically dependent on LPS activation of TLR4, but activation of intra-cellular signalling pathways in macrophages may differ between purified LPS and live salmonellae.

MS 09 Proteomic analysis of osmotic stress responses in *Salmonella*

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Foodborne pathogens such as *Salmonella* face a range of environments during their life cycles and gain selective advantages through their adaptive responses. Changes in osmolarity play a particularly important role as they impose a significant stress and many genes necessary for virulence are under osmotic control. In this study, proteomics was used to study the effect of hyperosmotic conditions on protein expression in *S. typhimurium*.

Both wild-type and an isogenic *ompR* null mutant were used to define targets controlled by the EnvZ/OmpR two-component system and those that were osmotically regulated by other mechanisms. The results can be summarised as follows: (i) 11 proteins changed in both the wild-type and *ompR*-*Salmonella* strains. Generally, their levels in the latter strain were higher than in the wild-type; (ii) A further 9 proteins changed expression in the wild-type strain but not in the *ompR*- mutant on hyperosmotic stress.

These are therefore regulated by the EnvZ/OmpR system; (iii) 6 proteins only changed expression in the *ompR* mutant. Thus, other systems compensate for or are affected by the loss of OmpR; (iv) The most highly induced protein was YgaU (STM2795), a hitherto hypothetical protein of unknown function; (v) Other induced genes included asparaginase, DnaK and MreB. The last is of particular interest as it forms actin-like filaments and determines shape. We propose that MreB directs a shape change under osmotic stress conditions, e.g., by acting as a structural brace.

MS 10 Phenotypic characterisation of *relA* and *hpt* mutants of the intracellular pathogen *Listeria monocytogenes*

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It has been previously shown that *relA* and *hpt* Tn917-LTV3 insertion mutants of *Listeria monocytogenes* that are unable to accumulate (p)ppGpp in response to amino acid starvation are avirulent in a mouse infection model. To further investigate the role of the stringent response in the virulence of *L. monocytogenes* the ability of these mutants to infect and replicate within epithelial (Caco-2), endothelial (HBMEC) and macrophage (J774.A1) cell lines was investigated. Both mutants were able to infect and replicate within all three cell lines in a manner indistinguishable to that of the wild type. Dramatic cytopathic effects were observed during the course of infection with the progenitor wild type strain C52. As a consequence the nucleotide sequence of the *prfA* master virulence regulator gene was determined. This established that strain C52 carries the *prfA** mutation resulting in constitutive expression of PrfA. This indicates that the avirulence observed in the murine model of infection is not a consequence of ppGpp dependent activation of *prfA* gene expression.

MS 11 Curing native plasmids in *Pseudomonas syringae* pathovar *pisi* to investigate pathogenicity determinants

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Pseudomonas syringae pv. *pisi* (*Ppi*) is a seedborne pathogen which causes bacterial blight of pea (*Pisum sativum*). Seven races, comprising two distinct genomic groups (GG), have been

identified and these interact with a series of eight differential pea cultivars (cv.). We have cloned plasmid replication genes from strains of *Ppi* and used them to cure *Ppi* strains of native plasmids by incompatibility. Race 3A (strain 870A, GG II) was successively cured of the three native plasmids it carries and the resulting cured strains were tested in pea. Pathogenicity to pea appears to be determined primarily by a large plasmid, pAV232 (106 kb) and the avirulence gene, *avrPpiB1.R3*, was confirmed to be located on the smallest plasmid, pAV231 (42 kb). Race 1 (strain 299A, GG I) was successively cured of its two native plasmids. Loss of pAV212 (60 kb) resulted in compatibility to pea cvs. carrying *R3*, due to the loss of *avrPpiB2*. Loss of both pAV213 (55 kb) and pAV212 resulted in a novel pathogen phenotype, avirulence on cv. Belinda, that was not due to *avrPpiB*. Pathogenicity factors involved in these phenotypic changes are being investigated.

MS 12 Capsular polysaccharide export in *Escherichia coli*: a role for KpsD, an outer membrane protein

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Capsular polysaccharide expression is an important virulence factor for many invasive bacterial pathogens of man. The capsule confers resistance to non-specific host defences. *Escherichia coli* offers a model system to study the mechanisms by which capsular polysaccharides are synthesised and exported out of the cell. The export pathway for group 2 capsular polysaccharides is conserved in *E. coli*, where *E. coli* are capable of exporting chemically distinct polysaccharides irrespective of the polysaccharide repeat structure. The KpsD protein plays a role in the final steps of the export pathway across the outer membrane, such that mutations in *kpsD* result in periplasmic polysaccharide. Initially KpsD was thought to be a periplasmic protein but recent evidence from our laboratory would suggest that this is not the case. Firstly, KpsD is detectable in outer membrane fractions of *E. coli*. Secondly, through FACS analysis KpsD was shown to be exposed on the cell surface. Thirdly, KpsD can form pores in lipid bilayers suggesting it may play a role in pore formation in the outer membrane. Therefore our current model is that KpsD participates in the export pathway of the K5 polysaccharide by forming pores in the outer membrane, through which the capsular polysaccharide is extruded.

MS 13 Investigation of protein interactions in the type III secretion system of Enteropathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC) is an enteric human pathogen that utilises a type III secretion system (TTSS) for colonisation and pathogenesis. TTSSs are found in many gram-negative pathogens and serve to transport bacterial effector proteins directly into the cytoplasm of host cells. Although there is little conservation of effector proteins, the delivery machinery is highly conserved. This investigation utilised the yeast two-hybrid system to identify novel protein interactions between components of the EPEC TTSS to enhance the understanding of the structure and function of this system. Twenty novel interactions were identified including interactions between structural components, between chaperones and effectors, between the translocon protein EspD and the type III machinery and between proteins of unknown function. Some interactions involving chaperones or putative chaperones and secreted proteins have been studied in more detail using standard biochemical and molecular biology techniques. Because of the high degree of homology, the results obtained in this

investigation reveal information relevant not only to the EPEC TTSS but also to TTSSs in general.

MS 14 Epithelial cell responses to verotoxin and VTEC

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Verotoxin (VT) is the crucial pathogenicity determinant in human infection caused by verotoxigenic *E. coli* (VTEC). Although potentially cytotoxic, VT also acts as an immunomodulator. VT induces and augments the transcription and release of multiple chemokines from human intestinal epithelial cells thereby mediating pro-inflammatory responses. Cattle, the major reservoir host for VTEC (particularly serotype O157), are colonised asymptotically, however the basis to avirulence in this host remains unclear. Our findings indicate that Gb3 is expressed by a sub-population of epithelial cells in the bovine intestinal tract. Despite receptor expression, VT exhibited no cytotoxic activity against bovine epithelial cells. Sub-cellular localisation of VT indicated that this toxin was localised to lysosomes, corresponding with abrogation of cytotoxicity. Although not susceptible to VT toxicity, we showed that VT1 suppresses the stereotypical pro-inflammatory responses of epithelial cells. These findings are consistent with an immunomodulatory role for VT in cattle, suggesting that VT may aid colonisation of VTEC in the reservoir host.

MS 15 Up-regulation of human beta defensins by bacteria colonising the colonic epithelium and their bactericidal activities on other mucosal species

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Human beta defensins (HBD) 1 and 2 are secreted by the colonic epithelium. HBD1 is constitutively expressed, whereas HBD2 is only expressed by the inflamed colon. These molecules constitute the first line of immune defence against many pathogenic Gram negative bacteria in the large bowel, but how they affect species belonging to the normal colonic microbiota is unknown. Moreover, it is not clear whether the commensal microbiota can induce or suppress production of these bactericidal molecules. This study has investigated the abilities of HBD1 and HBD2 to kill bacteria isolated from the rectal mucosa. Results show marked variations in susceptibilities of different members of the commensal microflora to these peptides. For example, bacteroides are highly susceptible to killing by defensins, whereas bifidobacteria are largely refractile to these substances. It has further been demonstrated that *Bacteroides fragilis* can up-regulate mRNA for these molecules (>100-fold), in HT29 colonic epithelial cells. Interactions between commensal bacteria in the large intestine and the host epithelium, to direct bacterial colonisation of the mucosa by defensins, may play an important role in maintaining a functioning mucosal barrier.

MS 16 Identification of 3'cis-acting elements essential for the temperature regulation of transcription of group 2 capsule gene clusters in pathogenic *Escherichia coli*

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Group 2 capsular polysaccharides of extra-intestinal *Escherichia coli* have a conserved genetic organisation, composed of 3 functional regions. Transcription occurs from 2 convergent promoters, P1 and P3, which are temperature regulated. Maximal transcription occurs at 37°C, with repression at 20°C. Studies of the *E. coli* K5 gene cluster have revealed that temperature regulation is mediated via H-NS and BipA.

To study regulation of the P3 promoter, fragments were amplified by PCR and cloned into the *lacZ* promoter probe vector pRS415, generating *lacZ* transcriptional fusions. Activity of the promoter was measured under a variety of conditions by

measuring β -galactosidase activity. Previous work has located the P3 promoter 741bp's 5' the ATG of the first region 3 gene; *kpsM*. Initial β -galactosidase data suggests that the large untranslated region 3' to the promoter contains *cis*-acting elements required for H-NS mediated temperature regulation. On the basis of this data studies are to be carried out in the future to further dissect P3 promoter.

MS 17 Characterisation of the lipopolysaccharide biosynthesis *rfaJ* genes of *Helicobacter pylori*

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Helicobacter pylori is the aetiological agent of a range of gastric diseases, including gastric ulcer and adenocarcinoma. A number of virulence factors are associated with pathogenesis, including LPS. Several putative LPS biosynthesis genes have been identified on the sequenced genome including three *rfaJs* with identity to the α 1,2-glucosyltransferase-encoding *rfaJ* of *E. coli*. One of them (*rfaJ1*) has a repeating GA tract in the 5' region suggesting that it is potentially phase variable. To elucidate the role of the *H. pylori rfaJ* genes insertional mutants have been engineered and characterised. In addition a constitutively phase 'ON' *rfaJ1* mutant has been generated and the *H. pylori* genes have been expressed in *S. typhimurium*. Results suggest that two of the *rfaJs* (including *rfaJ1*) are involved in synthesis of core LPS and that the third may be required for O antigen elongation. None of the *H. pylori rfaJs* complement a *S. typhimurium rfaJ* mutant. In conclusion, the *H. pylori rfaJ* genes encode LPS biosynthesis enzymes with differing functions and distinct specificities to *S. typhimurium* RfaJ.

MS 18 A luminescent *E. coli* assay used to study predator-prey interactions in *Bdellovibrio bacteriovorus*

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Bdellovibrio bacteriovorus is a Gram-negative motile bacterium that preys upon other Gram-negative bacteria, entering the periplasm of the host cell, where it grows, replicates and then lyses the host cell to release the progeny. Active motility seems important in locating prey but motile behaviour & taxis have been little studied in *Bdellovibrio*. Degradative enzymes are also secreted by *Bdellovibrio*, and may have a several roles; entry into the periplasm, killing the host, modifying the bdelloplast, digesting host cell contents for use as nutrients and lysing the host. The aim of this work is to identify *Bdellovibrio*- encoded motility & taxis proteins and proteases and to elucidate roles for these in the predatory life cycle.

Using a modification of a suicide plasmid conjugation system, one serine protease (*spb*) gene and one methyl-accepting chemotaxis protein (MCP) gene were sequenced and disrupted in *Bdellovibrio*. A novel assay was used in which light emission was measured as luminescent planktonic *E. coli* were attacked and killed by wild-type, MCP⁻ and protease⁻ mutants of *Bdellovibrio*. It was shown that both wild-type and *spb*⁻ strains were equally efficient at killing *E. coli* in planktonic conditions but that an MCP⁻ mutant was less efficient.

MS 19 Interactions of streptococcal antigen I/II proteins with salivary agglutinin (gp-340)

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Salivary agglutinin glycoprotein, which binds streptococci in the oral cavity, has recently been identified as gp-340, a member of the scavenger receptor cysteine-rich (SRCR) superfamily. SRCR

glycoproteins are involved in a range of interactions with bacteria and host cells that contribute to host innate and adaptive immune responses. The receptors for gp-340 in oral streptococci are antigen I/II polypeptides. We hypothesized that different affinities of antigen I/II proteins for gp-340 might influence the distribution of different streptococcal species in the oral cavity. Genes encoding antigen I/II molecules from *S. gordonii* DL1 (SspA and SspB), *S. mutans* NG8 (Pac) Guy's c (SpaP_G) and Ingbritt (SpaP_I), and *S. intermedius* ATCC#27335 (Pas) were independently cloned in pUB1000, a novel vector for expression of proteins on the cell surface of Gram-positive cocci. Recombinant plasmids were transferred to *S. gordonii* UB1360, an antigen I/II deletion mutant, and subsequently to *Lactococcus lactis* MG1363. Expression of antigen I/II was constitutive in *S. gordonii*, but was regulated in *L. lactis* during batch growth, reaching a maximum in late exponential phase. With the exception of SpaP_I, all antigen I/II proteins bound to purified gp-340, although SspA was three-fold more effective than other polypeptides. The heterologous expression systems will be exploited in future to analyse in greater detail the host cell and tissue binding functions of antigen I/II polypeptides. *Supported by the Wellcome Trust.*

MS 20 Role of Efa1 homologues in the colonisation of calves by *Escherichia coli* O157:H7

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Shiga toxin-producing *Escherichia coli* (STEC) comprise a broad group of bacteria some of which cause attaching and effacing (AE) lesions and enteritis in animals and humans. Cattle are a major reservoir of STEC. Non-O157 STEC contain a gene (*efal*) that influences adherence to cultured cells. An almost identical gene in enteropathogenic *E. coli* (*lifA*) mediates inhibition of lymphocyte proliferation and proinflammatory cytokine synthesis. We have reported that STEC O5 *efal* and O111 *efal* mutants are shed in significantly lower numbers than the parent strains following inoculation of calves. *E. coli* O157:H7 strains lack *efal* but contain a truncated version of the gene (*efal'*) and an *efal* homologue on pO157 (*toxB*). Here we report that *E. coli* O157:H7 *toxB* and *efal'* mutants exhibit reduced adherence to cultured cells and express and secrete lower levels of locus for enterocyte effacement (LEE)-encoded proteins required for AE lesion formation. The activity of LEE promoters was not altered in the *toxB* or *efal'* mutants, indicating that expression of LEE-encoded proteins may be affected at a post-transcriptional level. Despite this, mutation of *toxB* and *efal'* did not significantly affect the course of faecal excretion of *E. coli* O157:H7 following inoculation of 10-14 day old conventional calves.

MS 21 Proteomic identification of proteins of *Mycobacterium tuberculosis* that are differentially expressed in the host

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Following infection by *Mycobacterium tuberculosis* (*Mtb*), most people mount an immune response that blocks direct progression to active tuberculosis but leaves them susceptible to disease in later life. During active disease, the immune response tends to damage tissues, thereby aiding the subsequent spread of the infection. A better understanding of the survival mechanisms of *Mtb* within the host would significantly facilitate more rational intervention strategies for its elimination. However, little is

known about the proteins that are switched on or off in the host environment. To address this question, efficient procedures for recovering *Mtb* from infected tissue have been developed, thereby allowing the comparative proteomics of *Mtb* grown in vivo and in vitro. Analysis of *Mtb* taken at different stages of growth in vitro identified several growth phase-regulated proteins. *Mtb* from an acute infection model resembled in vitro-grown cells at Day 7, even though they had been in the animal for 14 days. However, their protein expression profile was not identical and a range of proteins that are up-regulated in vivo were identified. *Mtb* cells from a chronic model of infection were also recovered. While the number of cells available precluded comprehensive analyses, proteins with reproducibly different levels of abundance in acute and chronic infections were identified. These results highlight the potential of proteomics to provide insights into the host cell subversion strategies of a key pathogen.

Thursday 10 April 2003

0900 An introduction to protein secretion systems, and current status of Type IV systems

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Evolution has invented a limited repertoire of protein secretion mechanisms in Gram-negative bacteria. However, these protein targeting systems are widespread in pathogens of man, animals and plants - but also in saprophytes. Although other secretion systems have been identified, there are currently five major, widely-distributed systems: Types I, II, III, IV and V. The various secretion systems show some similarities and important differences regarding their Sec, Tat or Dsb-dependence; the nature and function of the outer membrane components (e.g. "secretins"); the presence of ABC proteins; the role of molecular chaperones and the nature of the secreted proteins involved. In recent years there have been important advances in understanding of the structures of some individual components and macromolecular complexes involved in some secretory systems. The "original" Type IV system discovered was the Ti targeting machinery of the plant pathogen, *Agrobacterium*. But the discovery of a homologous system in *Bordetella* suggested it was more widespread. As expected, the genomics revolution has provided evidence for the existence of Type IV systems in various pathogens.

0940 Biochemical analyses of the type IV secretion systems from *Agrobacterium tumefaciens* and *Brucella suis*

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The VirB/D4 T4SS from *Agrobacterium tumefaciens* translocates virulence factors (VirE2, VirF and the VirD2-ssDNA complex) to plant cells. The membrane-bound translocation machinery consists of 12 proteins (VirB1 to VirB11 and VirD4), which are required for substrate translocation. Protein-protein interactions in the membranes were analyzed after extraction with the mild detergent dodecyl maltoside, followed by separation under native conditions. Separation of solubilized proteins by blue native electrophoresis and gel filtration chromatography separated pilus-associated proteins (VirB2, VirB5 and VirB7), which may constitute a pilus pre-assembly complex, and T4SS core components. Based on previously described VirB protein interactions and the discovery of different subassemblies in the membranes, a model of T-pilus assembly in *A. tumefaciens* is suggested. The T4SS of *Brucella suis* is required for intracellular survival of the bacteria in mammalian cells, but large-scale growth for biochemical experiments is difficult to achieve. To approach this question, the *virB* operon from *B. suis* was expressed in *A. tumefaciens*, and analyses with specific antisera indicated VirB protein interactions in the heterologous host. The *B. suis* T4SS stimulated *Agrobacterium*'s ability to serve as a recipient for IncQ plasmids, demonstrating that it is at least partly functional. The results suggest that *in vivo* studies in a heterologous host are suited to analyze the function(s) of the *B. suis* T4SS.

1020 Recruitment of plasmid conjugal transfer systems for bacterial pathogenesis

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The genus *Bartonella* comprises human-specific and zoonotic pathogens responsible for a wide range of clinical manifestations. The hallmark of infection in the human or animal reservoir host is the intracellular colonisation of erythrocytes, while vascular endothelial cells are major target cells in incidentally infected humans. Here we report on the identification and characterisation of two distinct type IV secretion systems (T4SS), Trw and VirB, in *Bartonella*, which display an unprecedented level of sequence similarity with the conjugation machineries Trw of the *Escherichia coli* antibiotic resistance plasmid R388 and Avh of the *Agrobacterium tumefaciens* plasmid pATC58, respectively. For the homologous Trw systems of *Bartonella* and *E. coli* we could moreover demonstrate the interchangeability of individual structural and regulatory components, demonstrating that conservation is not only structural but also functional. Strikingly, mutants in either VirB or Trw are unable to establish erythrocyte infection in an animal model, demonstrating that both T4SS are *bona fide* pathogenicity factors in *Bartonella*. These examples of adaptive evolution reveal the capacity of T4SS to assume dedicated functions in either plasmid transfer or pathogenesis over rather short evolutionary distance and imply a novel role for the conjugation systems of widespread broad-host-range plasmids in the evolution of bacterial pathogens.

1330 VirB like type IV secretion and virulence in the facultative intracellular pathogen *Brucella*

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Brucella is a member of the α -proteobacteria which is highly pathogenic for animals and man. This bacterium is a facultative intracellular pathogen which is able to survive and multiply within phagocytic cells such as macrophages, the very cell type which normally plays a role in defending the host from bacterial infections. Within macrophages, virulent *Brucella* perturb the endocytic pathway and create a novel intracellular compartment in which they multiply.

A type IV secretion system is an essential virulence factor for *Brucella*, since mutants are unable to multiply in eucaryotic cells or to cause disease in animals (mice and goats). The *virB* operon of *Brucella* is specifically induced rapidly after being phagocytosed by macrophages and acidification of the phagosome is the major signal. We believe that the VirB system in *Brucella* secretes factors into the phagosome which block endosomal trafficking and allow *Brucella* to create a novel intracellular niche in which it replicates. The nature of protein secreted by *Brucella* and their effects in the cell are still unknown. We have developed an original system that permits the identification of putative secreted proteins. One candidate protein identified was shown to be essential for *Brucella* virulence.

1410 Pertussis toxin: biogenesis and secretion from *Bordetella pertussis*

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Pertussis toxin (PT), a critical virulence factor of *Bordetella pertussis*, is secreted from the bacteria by a type IV secretion system known as the Ptl system. PT has an A-B structure that is typical of many bacterial protein toxins. It is composed of an

enzymatically active S1 subunit and a binding component, known as the B oligomer, that is made up of one copy each of subunits S2, S3, and S5 as well as two copies of subunit S4. We have examined the capability of partial assemblies of toxin subunits to form *in vivo* in order to better understand the assembly of the toxin and its interaction with the Ptl proteins. We expressed *ptxS1*, *ptxS2*, and *ptxS4*, either individually or in combination, in strains of *B. pertussis* that did not produce PT and which either did or did not produce the Ptl proteins. We then examined the stability of the PT subunits and their localization with the bacterial cell. Using this approach, we were able to detect stable subassemblies of the toxin and found evidence for the interaction of certain toxin subunits with the Ptl transport apparatus. This information will shed light on the assembly of PT within *B. pertussis* and its secretion from the bacterium.

1450 Translocation of effectors by the Icm/Dot Type IV secretion system of *L. pneumophila*

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Legionella pneumophila is a gram-negative bacterium that survives and replicates within protozoan and mammalian phagocytic cells by establishing an unusual vacuole (*Legionella* specific vacuole, LSV) that serves as a site for growth. In people the organism causes a variety of pneumonia-like acute diseases, the most severe being legionnaires' disease. Proliferation of *L. pneumophila* within protozoan hosts is thought to provide large numbers of *L. pneumophila* during outbreaks. The Icm/Dot translocator is a type IV secretion system that is essential for the ability of *L. pneumophila* to form the LSV. We have identified two effectors that are translocated to host cells from *L. pneumophila* by the Icm/Dot type IV secretion system. Mutants that lack these two effectors, LepA and LepB (*Legionella* effector protein), multiply intracellularly and kill host cells, but exhibit unusual properties. The primary defect in these mutants is a decreased ability to exit protozoan host cells following intracellular growth. However other Icm/Dot dependent functions such as contact dependent cytotoxicity and enhanced phagocytosis are increased in the mutant strains. These results suggest that other effectors may be translocated more efficiently by the Icm/Dot translocator in the absence of the Lep proteins.

1600 "Bacterial" oncogenes?

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Type I strains of *Helicobacter pylori* possess the *cag* pathogenicity island to deliver virulence factors. *cag* is a specialized Type IV secretion machinery that is activated during infection of an appropriate animal model or by co-cultivation with different cell lines, including red blood cells or *Drosophila* tumor cell lines. The *cag* locus comprises 31 genes originated from a distant event of horizontal transfer involved in cellular responses like reshaping, secretion of pro-inflammatory chemokines and phosphorylation of proteic targets. It has previously been reported that co-cultivation of epithelial cells with *Helicobacter pylori* triggers signal transduction and tyrosine-phosphorylation of a 145 kDa putative host cell protein. This protein is not derived from the host but rather is the bacterial immunodominant antigen CagA, a virulence factor commonly expressed in peptic ulcer disease and thought to be orphan of a specific biological function. Following transfer CagA is phosphorylated on tyrosine residues restricted to a previously identified repeated sequence called D1. This sequence is located in the C-terminal half of the protein and contains the five amino acid motif EPIYA, which is amplified by duplications in a large fraction of clinical isolates. Tyrosine-phosphorylation of CagA is essential for the activation process that leads to dramatic changes in the morphology of cells growing in culture. In addition, we observed that two members of the src kinases family, c-Src and Lyn, account for most of the CagA-specific kinase activity in AGS cell lysates. Translocated CagA interacts with the ZO-1 protein causing disruption of the apical junctional complex

promoting a growth factor-like type response with intense cytoskeletal rearrangements, cell elongation effects and increased cellular motility.

1640 Environmental distribution of type IV secretion systems

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Sequence analysis of a plasmid, pQBR103 (*tra*⁺, *Hg*^r, 330kbp) indigenous to the plant associated pseudomonad community at an Oxford, UK agricultural site, reveals only limited homology to existing bacterial genome data bases. Although interesting alignments have been observed, functional analysis of pQBR103 by promoter probe methods reveal a number of genes expressed only by the plasmid when host bacteria colonise the plant surface. To date a high proportion of the orfs identified are novel, and a putative region associated with conjugative transfer has been identified, though homologies to other well described *Tra* genes have not been recorded. The objective of the presentation will be to review the available information on the distribution of known Type IV systems in an functional and evolutionary context and relate these data to the study of the ecology of other well studied bacterial systems. By the molecular and ecological analysis of plasmids, mobile genetic elements and "islands" associated with horizontal gene transfer we can characterise those encoded functions that enhance host fitness, and identify the environmental signals that promote cell-cell interactions and transfer. Studies of the genetics of natural populations of bacteria reveal the relevance of the horizontal gene pool in the generation, replication and dissemination of the genes associated with host fitness. In plant and soil habitats plasmid transfer is an important mechanism of gene mobilisation that is directly linked to the spread of adaptive and symbiotic traits important in host survival. Though plasmid transfer is rare in bulk soil, microcosm and field studies have shown that conditions on roots and leaves promote conjugation. Though few data are available that quantify these events. To address these issues we have combined the analysis of the population genetics of natural populations of bacteria and their plasmids with the functional genetic analysis of key plasmid isolates. The aims of the study have been to establish the genetic diversity of plasmids and their hosts, the ecological role of adaptive traits carried by these mobile genetic elements and determine the role of the horizontal gene pool in bacterial population biology.

CELLS & CELL SURFACES GROUP - POSTERS

CCS 01 Internalization of *Staphylococcus aureus* by keratinocytes

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In healing wounds, keratinocytes migrate on a fibronectin (FN)-rich granulation tissue matrix and may phagocytose FN-coated debris. *S. aureus* is a common pathogen in wound infection, and is often isolated from oral lesions. It can be internalized by many non-phagocytic host cells, a process thought to involve bacterial FN-binding proteins (FnBPs), FN and host cell $\alpha 5\beta 1$ integrin. The aim of this study was to investigate factors affecting *S. aureus* internalization by keratinocytes. Examination of isogenic mutants of *S. aureus* lacking FnBPs revealed a significant reduction in the adhesion (50%-70%) and internalization (80%-94%) of these strains by an immortalized keratinocyte cell line (UP), but not by primary normal oral keratinocytes (NHK). A recombinant protein encompassing the D1-D4 repeat region of FnBPB inhibited internalization of *S. aureus* by UP cells, but not by NHK. Blocking antibodies to $\alpha 5$ (P1D6) and $\beta 1$ (P4C10) integrin subunits had no effect on the uptake of *S. aureus* by

either UP or NHK. These results indicate that the FnBPs are essential for the uptake of *S. aureus* by immortalized keratinocytes, although, host factors other than FN-binding integrins may be involved in the internalization process. In primary keratinocytes, *S. aureus* internalization occurs independently of FnBPs.

CCS 02 Oligosaccharide inhibition of bacterial attachment to respiratory epithelial cell lines

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Many bacteria attach by receptor-ligand interactions to host cell surfaces prior to producing disease. The ligands involved are often oligosaccharide structures located on the cell surface. Providing mimics of these ligands in solution could inhibit bacterial attachment and subsequently prevent infection. Several pathogens that can cause pulmonary disease (*Legionella pneumophila*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, and *Yersinia pestis*) were examined for oligosaccharide inhibition of attachment to the A549 type II pneumocyte cell line. The inhibition profiles of *L. pneumophila*, *Ps. aeruginosa* and *Burk. cepacia* differed slightly with respect to the degree of inhibition by the various oligosaccharides, however generally GalNAc/Gal β 1-4 containing saccharides were more inhibitory than other types. In contrast, *Y. pestis* was only inhibited to a great degree by *p*-nitrophenol (75%), GalNAc β 1-4Gal (54%) or GalNAc β 1-3Gal (55%). Therefore, disaccharides (GalNAc β 1-4Gal, GalNAc β 1-3Gal) or *p*-nitrophenol can interfere with the attachment of some respiratory pathogens. Additionally, pre-treatment of the cell line was examined for effects on *Legionella pneumophila* attachment. Carbohydrate moieties are important in the attachment of *L. pneumophila* as evidenced by a 74% decrease in adherent bacteria when the cell lines were treated with tunicamycin. Treatment of the cell line with β 1-4-galactosidase, but not β 1-3/6-galactosidase resulted in decreased bacterial adhesion indicating the importance of the Gal β 1-4 linkage in attachment.

CCS 03 Regulation of *LsaA*, a major virulence factor of *Lawsonia intracellularis*

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Lawsonia intracellularis is an obligate intracellular Gram-negative bacterium which is the etiological agent of proliferative enteropathies. A *Lawsonia* surface antigen involved in cell attachment and invasion, *LsaA*, is expressed during infection *in vitro* and *in vivo* suggesting it may be activated by contact with eukaryotic cells. Analysis of upstream regions did not identify canonical promoter sequences found in other bacteria. *LsaA* and 16S rDNA promoters were assessed by 5' RACE amplification. To investigate promoter activity of this gene, *LsaA-lacZ* fusions were constructed using reporter plasmids. β -galactosidase activity was assayed under a range of pH, temperature, and iron restriction conditions: acidic pH, low temperatures and iron depletion all impaired expression. To analyse expression in *L. intracellularis* several plasmids were constructed containing promoterless dual reporter cassettes *gfp-cat* (pMPAV3, chloramphenicolR) or *gfp-gen* (pMPAV4, gentamicinR). *LsaA* promoters were ligated into pMPAV3 and 4. *Lawsonia intracellularis* was transformed by electroporation and *gfp* and/or *gen/cat* expression was monitored. This study has identified functional promoters located upstream from the translation initiation site of both *LsaA* and the housekeeping gene 16S rDNA of *Lawsonia intracellularis* that do not appear to follow the consensus sequence for the -10 and -35 promoter elements observed in other bacteria.

CCS 04 Effects of *E. coli* O157 and verotoxins on expression of intestinal epithelial antimicrobial peptides and their anti-bacterial action against EHEC

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Antimicrobial peptides form an important component of epithelial defences both in terms of their direct anti-bacterial action and by alerting and activating innate and adaptive host defences. Their role during infection caused by enterohaemorrhagic *E. coli* (EHEC) such as *E. coli* O157, has not been established to date. Expression of members of the β -defensin family by epithelial cells of both human and bovine origins in response to EHEC and purified verotoxins have been examined. In addition to established cell lines, responses of bovine primary intestinal epithelial cell cultures have been assessed. Differential expression of EBD, a bovine β -defensin, was observed between primary, sub-confluent (immature) rectal epithelial cells and established (mature), monolayer-forming cells. Up-regulation of epithelial expression of β -defensins resulted from stimulation by both bacteria and purified verotoxin, indicating that this innate response may be important in interaction of *E. coli* O157 with the host. Since this bacterium elicits β -defensin expression *in situ*, the antibacterial activities of synthetic or recombinant human and bovine are examined to establish sensitivity of *E. coli* O157 to these antimicrobial peptides.

CCS 05 *VacA* b-barrel mediates secretion of EspC **LOUISE ARNOLD, THOMAS BALDWIN & KIM R. HARDIE** Institute of Infections and Immunity, Queen's Medical Centre, Nottingham NG7 2UH

Characterization of the IgA protease secreted from *Neisseria meningitidis* heralded the discovery of a family of autotransporter proteins which include virulence factors of (i) pathogenic *E. coli* such as EspC (secreted by some strains of Enteropathogenic *E. coli*, EPEC) and PET (secreted by Enterotoxigenic *E. coli*, EAggEC), and (ii) the vacuolating toxin (*VacA*) secreted by the gastric pathogen, *H. pylori*.

Autotransporters are translated as large precursor polypeptides in the cytoplasm. An N-terminal targeting signal directs the translocation across the cytoplasmic membrane and is cleaved in the process. This is proposed to occur with the aid of the Sec machinery, and subsequently the C-terminal domain of the polypeptide inserts into the outer membrane forming a β -barrel through which the central passenger domain is transported. Once at the cell surface, the mature passenger domain is released following cleavage which is achieved either by cell associated proteases, or via the autoproteolytic activity of certain autotransporters e.g. IgA protease.

This study has demonstrated that the β -barrel domains of EspC, PET and *VacA* are functionally interchangeable. Moreover, EspC, PET and hybrid autotransporters could successfully translocate across the two membranes of laboratory adapted *E. coli* strain MG1655, indicating that no accessory factors specific to the original pathogenic host are required for translocation.

CCS 06 *Salmonella enterica* serovar Typhimurium produces a type IV pilus that influences intestinal colonisation

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Type IV pili are filamentous appendages produced by several gram-negative pathogenic bacteria. Recently a type IV pilus was shown to be encoded by *Salmonella* Pathogenicity Island-7 of serovar Typhi and influences invasion of human epithelial cells. The sequence of serovar Typhimurium LT2 suggests that this serovar does not carry SPI-7. During a screen of signature-tagged mutants of serovar Typhimurium 4/74 to identify genes influencing colonisation of bovine intestinal mucosa, we

identified a mutant with a Tn5 insertion in a gene almost identical to the *pilO* gene of serovar Typhimurium plasmid R64. Analysis showed that a *pil* operon is carried on an R64-related plasmid in serovar Typhimurium 4/74. The *pil* operon of R64 encodes a type IV pilus which was previously thought to be required only for conjugation. The *pilO* mutant was attenuated following oral infection of *ity*^S mice, but virulent following intraperitoneal infection of *ity*^R mice. A calf ileal loop model, however, showed that the *pilO* mutant elicited an intestinal secretory and inflammatory response identical to that of the wild-type strain. These results suggest that the type IV pilus of serovar Typhimurium is required during colonisation of the gastrointestinal tract but does not contribute to *Salmonella*-induced enteropathogenesis or systemic pathogenesis.

CCS 07 Whole genome expression profiling of biofilm forming *Salmonella enterica* serovar Typhimurium

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The success of *Salmonella enterica* serovar Typhimurium to survive and overcome environmental stress is reflected by the numerous sources they are isolated from. The ability of the bacterium to colonise and persist on surfaces is of particular importance to the medical and food industries. During colonisation of both biotic and abiotic surfaces the bacteria form an aggregate within a hydrated polymeric matrix termed a biofilm. It is considered that this sessile mode of growth provides an environment that serves as a protective barrier from antibiotics and host defences making eradication of this pathogen difficult. Currently, it is not clear which genes are specific to biofilm formation, maturation and persistence, and whether they are differentially expressed in comparison to free living (planktonic) cells. To identify genes that are involved in the *Salmonella* biofilm we have determined whole genome expression profiles using microarrays. Analysis of the array data revealed that many genes involved in chemotaxis and motility were affected. Our data confirmed the upregulation of genes known to encode proteins involved in biofilm formation, such as fimbriae. In addition, many unknown and uncharacterised genes appeared to be affected. Biofilm formation assays, western blot analysis, multiplex primer extension analysis (MPEA), and mutants were used to confirm the array results.

CCS 08 Differential protein production by planktonic and biofilms of *Salmonella enterica* serovar typhimurium

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This study explored the reproducibility of different methods to study biofilms. Normally the media feeding the flow cell is inoculated with the organism to be studied, in this case *Salmonella enterica* serovar Typhimurium. This study looked at the difference in biofilm formation when the media is kept sterile and the flow cell is 'seeded' with *S. enterica* for one hour before pumping through the sterile media, a method first devised by Sauer. This method is more indicative of a real environment, where any fluid flow past the biofilm is not likely to be saturated with stationary phase or dead cells. The biofilm that forms in this flow cell is different from the standard method. The most striking difference was the filamentation of the bacteria after approximately 20h. On the bottom surface a grid-like pattern was formed with cells flowing in channels around the biofilm, while on the top surface clusters of filamenting cells developed. Percentage surface coverage reached 20% after 24h, 15% more than in the standard method. The study also investigated the differential protein expression between the planktonic and the

biofilm populations obtained from the Sauer method flow cell in an effort to determine which proteins are produced preferentially when the cells are in the biofilm phase.

CCS 09 Mutagenesis reveals assembly pathway and structure of *Y. pestis* polymeric capsule

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F1 antigen forms an amorphous capsule on the surface of *Yersinia pestis*. It is antiphagocytic, highly immunogenic and a key component of anti-plague vaccines. The major capsular subunit, Caf1, a 15.6 kDa polypeptide, is encoded within the *caf* operon. Although no defined structure has been resolved by EM within the capsule, the two accessory genes of the *caf* operon belong to the chaperone/usher family of pili assembly genes. Using a combination of mutagenesis and biochemical assays we have demonstrated that polymer assembly proceeds via a series of donor strand complementation interactions that stabilise the incomplete immunoglobulin fold of the subunit. Mutagenesis also permitted isolation and crystallisation of chaperone:subunit and chaperone:subunit:subunit complexes, providing for the first time high resolution X-ray structures and snapshots of the assembly process. Comparison of the chaperone-bound subunit with the subunit in the growing fibre indicates that chaperone traps a high energy folding intermediate. Release of the subunit allows completion of folding to form a more closely packed hydrophobic core which drives subunit polymerisation. This provides an explanation of how chaperone:usher systems assemble surface adhesins in the absence of cellular energy. The same principles are likely to be common to all non-pili adhesins assembled by this pathway.

CCS 10 Identification of a Type III system in the fish pathogen *Yersinia ruckeri*

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By injecting toxic proteins directly into the host cytosol, the type III secretion system (TTSS) enables bacteria to interfere with host signalling pathways. It has been identified as a key virulence determinant in many pathogens of mammals as well as a number of plant pathogens. For most bacterial fish pathogens understanding of the factors involved in pathogenesis is very limited. Therefore in this study the prevalence of TTSS in bacterial pathogens of fish was investigated.

Degenerate oligonucleotides were designed against two suitable regions within the gene for one of the most highly conserved TTSS proteins, type III ATPase. Following touchdown PCR, target fragments were cloned and sequenced. All positive controls confirmed amplification of the type III ATPase rather than the closely associated flagella ATPase genes. The amplified product from *Yersinia ruckeri* NCIMB 1315 showed close homology to the newly identified *ysaN* ATPase gene of the chromosomally encoded Ysa system in *Yersinia enterocolitica*. *Y. ruckeri* is the causative agent of enteric redmouth disease a serious disease endemic in salmonid fish. There was no evidence of a plasmid encoded Ysc system in *Y. ruckeri*, making this an ideal pathogen to investigate the function and significance of this newly identified class of Yersinia TTSS.

CCS 11 *Salmonella* Typhi pathogenicity Island-7 encoding the ViaB capsule and type IVB pili, is a putative conjugative transposon with synteny to the DNA transfer regions of soil and plant bacteria 'gene islands'

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Salmonella enterica serovar Typhi has recently been sequenced. This enabled detailed analysis of SPI-7, one of the large pathogenicity islands of this serovar. Few *S. enterica* species contain this island. Those that do, including *S. Paratyphi C* and some *S. Dublin* isolates, can be characterised by the expression of the ViaB exopolysaccharide on the bacterial surface. Along with the presence of the type IVB pili, these gene clusters constitute the major identifiable regions of SPI-7. The island in these serovars were compared to *S. Typhi* by DNA sequencing. A number of genes found in SPI-7 also have known roles in plasmid conjugation. These include a single-stranded DNA binding protein and a putative Anti-restriction protein C. Further analysis of the large number of hypothetical genes within SPI-7, revealed extensive synteny to potential DNA transfer regions of a variety of plant and soil bacteria 'gene-islands'. These bacteria included the Plant pathogen *Xanthomonas axonopodis* pv. citri, the soil bacterium *Burkholderia fungorum*, and the ubiquitous bacterium, *Pseudomonas aeruginosa*. In the latter case, the 'gene-island' was localised to a known 110kbp horizontally transferred region within duplicated tRNA_{glyU} sites. No mating-pair formation structures (mpf) could be discerned in this region to facilitate mobility. A possible mechanism is postulated.

CCS 12 Systematic characterization of type IV secretion system proteins of human pathogenic *Bartonella*

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The type IV secretion system is ancestrally related to the core components of conjugation machine. The type IV secretion system is able to transport diverse types of macromolecules including DNA and proteins. Historically, the *Agrobacterium tumefaciens*, a phytopathogen, has been the reference point for the type IV systems. This system is assembled from products of the *virB* operon, responsible for the delivery of the tumour-inducing DNA (T-DNA) to plant cells. Recently, a putative type IV secretion system of unknown function, a *virB* locus, has been described for *Bartonella henselae*, the agent of Cat Scratch Disease. The *virB* operon of *B. henselae* encodes 10 genes, of which 8 have significant homology to the components of the type IV secretion system of *A. tumefaciens*. So far, the knowledge about different components of the *B. henselae* vir-encoded type IV secretion has been limited. Here, we have applied a yeast two-hybrid system to identify pair protein interactions between components of this system. We have been able to establish several interactions between proteins associated with the core secretion apparatus (VirB8, VirB9, VirB10 and VirB11). Also, the data illustrated a possible network of interactions between the core complex and the putative pilus components (VirB2, VirB5, VirB7 and VirB9). Moreover, the detected VirB3-VirB5 protein interactions might be involved in stabilising the pilus component. Identifying these protein-protein interactions could be decisive in deciphering the structure of the type IV secretion system in *Bartonella*.

Monday 7 April 2003

0910 Changing aetiology of septicaemia

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Septicaemia, increasingly referred to as bloodstream infection, remains an important life-threatening condition. The aetiology is determined by the source of infection and the clinical category of the patient. Septicaemia is now classified as community-acquired, healthcare-associated and hospital-acquired.

Whilst *Escherichia coli* (20%) and *Staphylococcus aureus* (18%) remain the most common causes, the proportion of cases due to coagulase-negative staphylococci (CNS) is increasing. Emerging pathogens causing septicaemia include *Acinetobacter* species (3%), enterococci (4%) including glycopeptide-resistant enterococci, and *Candida* species (3%). Their importance reflects the increasing proportion of immunocompromised patients and the widespread emergence of antibiotic resistance, especially amongst Gram positive bacteria.

Risk factors for septicaemia include chronic underlying medical conditions such as renal failure, the presence of an invasive device and underlying immunosuppression such as neutropenia. The mortality will depend upon the source of septicaemia, the underlying health of the infected patient, the speed of diagnosis and the appropriateness of initial treatment. However, approximately 15-20% of patients die from septicaemia. The future is likely to see developments in earlier diagnosis, to facilitate the commencement of more appropriate antibiotics earlier, and prevention strategies, such as the use of antimicrobial impregnated medical devices.

0950 The development of automated blood cultures: from plates to computers

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Blood cultures are one of the more useful diagnostic tools for a microbiology laboratory, giving direct evidence of bacteraemia and allowing further investigation into appropriate therapy. Traditional culture methods were slow and time consuming; mortality was high. Through the 1970s and 80s experimentation with metabolic bi-products of microbial respiration, notably CO₂, as a marker of bacterial growth gave rise to a number of semi-automated techniques, including atmospheric pressure, radio-labelling and infrared detection systems. With the challenge for increasing the quality of results for less (time, labour, finance), the current generation of continuous monitoring automated culture systems employing colourimetric and fluorimetric sensors was born. The compromise associated with the standardisation of cultures; broth types, temperatures, blood volumes and transportation will be discussed.

1020 Streptococcal toxic shock syndrome: causation and management update

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Streptococcal toxic shock syndrome (STSS) with/without necrotizing fasciitis is caused primarily by superantigen producing group A streptococci, but groups C and G strains also cause the illness. Children are less likely than adults to develop necrotizing fasciitis in association with STSS, possibly because they are more susceptible to streptococci. Our epidemiology studies continue to show that the SAg streptococcal pyrogenic exotoxins A and C (SPEs A and C) are primarily associated with STSS, but new SAg continue to be identified that are likely to

contribute. Rabbit model studies suggest that necrotizing fasciitis depends on SAg and peptidoglycan but not production of hemolysins and SPE B, though these proteins may contribute. SAg production by STSS strains is inhibited by sub-bacterial inhibitory concentrations of antibiotics that inhibit protein synthesis, suggesting their use in management of the illness. Several studies have shown that intravenous immunoglobulins (IVIG) have high levels of antibodies against group A streptococcal SAg which neutralize the toxins. The use of IVIG significantly reduces the case:fatality rate of STSS. Recent studies have shown that certain HLA make-ups allow prediction of whether patients will develop more or less serious streptococcal disease.

1130 Gram-negative endotoxic shock

T. EVANS

Imperial College, London

Abstract not received

1210 Characterisation of blood culture isolates of *Staphylococcus aureus* in Scotland

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Introduction: Antimicrobial resistance is an emerging problem in *S.aureus*, however, comprehensive data is not available. The Scottish MRSA Reference Laboratory (SMRSARL) requested Scottish laboratories to refer *S.aureus* from blood cultures and we present the preliminary findings.

Methods: 192 *S.aureus* isolates from 11 laboratories were characterised by phenotypic and molecular methods.

Results: 34% of blood culture *S. aureus* were MRSA. All MRSA isolates except one (Scottish MRSA type 122) were EMRSA15 (61%) or EMRSA16 (38%). PFGE identified 10 and seven clonal variants of EMRSA15 and EMRSA16, respectively. In contrast, EMRSA16 isolated from "all sites" accounted for only 20% of MRSA. 16% of MSSA from blood were methicillin sensitive (MS) variants of the E16 clone (MS-E16) and 5% were MS-E15. Fifteen other clones were identified by PFGE, three of which (SMRSA105, SMRSA111 and SMRSA127) each accounted for 8% or more of the total.

Conclusion: The higher proportion of EMRSA16 from blood compared to "all sites" suggest that this clone may be more virulent than EMRSA15. The finding that methicillin sensitive variants of the E16 clone are the most prevalent MSSA isolated from blood supports the greater pathogenic potential of this clone.

1225 Rapid extraction and characterisation of *Staphylococcus* species from BACTEC blood culture bottles by the Polymerase Chain Reaction

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common causes of nosocomial infections and bacteraemia. Standard bacterial identification and sensitivity testing frequently require as long as 72 h to report results. This study aimed to reduce the turn-around time for results to facilitate rapid diagnosis and initiation of appropriate antibiotic therapy. A multiplex PCR was developed to detect the *Staphylococcus* 16S rRNA gene sequence, the *mecA* gene and the *coag* gene for coagulase. Detection of these respectively confirmed the presence of staphylococci, ascertained resistance to methicillin and differentiated between *S.aureus* and coagulase-negative staphylococci. The method was validated against 40 clinical

isolates, including methicillin-sensitive *S.aureus*, MRSA, methicillin resistant coagulase-negative staphylococci and methicillin-sensitive coagulase-negative staphylococci, and showed complete agreement with standard laboratory methods. Different methods of DNA extraction from positive blood culture bottles were compared and a modified version of the Wizard Genomic DNA Purification Kit (Promega) performed most satisfactorily.

Clinical evaluation determined that this procedure is accurate, rapid and fitted well into laboratory workflow. Rapid detection and differentiation of staphylococci from positive blood culture makes a satisfactory and valuable test for use in the routine laboratory. Turnaround time was reduced to as little as 5 hours from a blood culture signalling positive.

1400 Diagnosis of invasive *Streptococcus pneumoniae* infection by serotype-specific ELISA

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Pneumococcal infection is currently under-diagnosed in the laboratory; suitable samples are often not submitted and when they are, prior antibiotic treatment can compromise viability. With the recent development of conjugate polysaccharide vaccines (which promise better immunogenicity but cover fewer serotypes than conventional vaccines), it is important to improve the diagnostic repertoire available to monitor the likely changes in epidemiology of pneumococcal infection.

We have developed a series of solid-phase indirect sandwich ELISAs for detection of 13 of the commonest pneumococcal capsular antigens. These assays were evaluated by testing 208 urine samples collected from patients with blood culture proven invasive pneumococcal disease (n=134) and patients with non-pneumococcal bacteraemia (n=74). All assays were done blind to the expected result.

The 13 serotypes in the assay panel covered 81% of patient isolates. For urine from these patients, the ELISA tests had a sensitivity (correct serotype detected) of 83%. Specificity was 98.6%.

Urine is a simple specimen to obtain and we conclude that this test has the potential to improve rates of laboratory diagnosis and to contribute to enhanced surveillance of serotype distribution in pneumococcal disease as new vaccines are introduced.

1415 Prevalence and identification of oral bacteria associated with endocarditis that are resistant to the recommended prophylaxis for dental procedures

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The British National Formulary recommends the use of antibiotic prophylaxis (amoxicillin) before dental procedures for the prevention of endocarditis in children with specific congenital heart diseases. However, other workers have stated that antibiotic prophylaxis during dental procedures has no clinical benefit. The aim of this study was to determine the prevalence and proportion of bacteria present in the oral cavity that are associated with endocarditis (species of *Haemophilus*, *Neisseria*, *Prevotella*, *Staphylococcus*, *Streptococcus*, *Stomatococcus* and *Veillonella*) and are resistant to the recommended prophylactic antibiotic amoxicillin. Thirty-five healthy children were screened, 97% harboured amoxicillin-resistant bacteria. The median percentage of the oral microflora resistant to amoxicillin was 2% (range 0-34%). A total of 122 amoxicillin-resistant bacteria were isolated and consisted of 17 different species. Amoxicillin MICs ranged from 8 µg/ml to 128 µg/ml and a multi-resistant phenotype was common with 79% of the isolates also being penicillin-resistant and 49% of them erythromycin-resistant.

This study has demonstrated that bacteria associated with endocarditis and resistant to amoxicillin can be readily isolated from the oral cavity of healthy children.

1430 Toll-like receptors and the innate recognition of bacteria

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The immune response to microbial pathogens relies on both innate and adaptive components. Adaptive immunity is mediated by clonally distributed T and B lymphocytes that provide immunological specificity and memory. In contrast, the innate immune response traditionally has been considered non-specific. In recent years, it has become clear that the innate immune system can specifically recognize groups of microorganisms, and that it not only provides a first line of antimicrobial host defense, but also has a profound impact on the establishment of adaptive immune responses.

The innate immune system discriminates potential pathogens from self through a series of receptors that recognize conserved motifs on pathogens that are not found in higher eukaryotes. These motifs have been termed "pathogen-associated molecular patterns" or PAMPs, whereas their cognate binding partners on host cells involved in the innate immune response have been named "pattern-recognition receptors" or PRRs. Examples of PAMPs include lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, peptidoglycan (present in most bacteria), lipoteichoic acid (in many Gram-positive bacteria) and mannans in the yeast cell wall.

The Toll family of receptors, which is conserved throughout evolution from flies to humans, has been implicated to play a central role as PRRs in the initiation of cellular innate immune responses. The Toll receptor family most likely represents the connection between the extracellular compartment, where contact with and recognition of pathogens occurs, and the intracellular compartment, where signaling cascades leading to cellular immune responses are triggered. First discovered in the fruit fly, at present 10 human homologs of *Drosophila* Toll have been identified. This human receptor family has been designated Toll-like receptors or TLRs. TLRs are distinguished from other PRRs by their ability to recognize, and more significantly, discriminate between different classes of pathogens. Ligands for at least 8 mammalian TLRs have been described, among which TLR4 is the signaling receptor for LPS.

The LPS receptor complex provides an example of how PAMPs are recognized by the host in general and by TLRs in particular. LPS is bound in the extracellular environment by an acute phase protein called LPS binding protein (LBP), which delivers LPS to the surface receptor CD14. CD14 then physically associates with a complex that includes TLR4 and an extracellular protein, MD-2. Each component of this LPS receptor complex is required for efficient LPS signaling. Recent evidence suggests that MD-2 may also play a role in efficient TLR2 activation by Gram-positive bacteria. In addition, CD14 is known to bind a variety of bacterial components besides LPS, including lipoteichoic acid and peptidoglycan. Hence, CD14 and MD-2 can be considered as components of the TLR signaling pathway, although they likely are involved in signaling by a limited number of TLRs. TLR1 recognizes bacterial lipopeptides and mycobacterial 19-kD lipoprotein, TLR3 recognizes poly(I:C) (double-stranded viral RNA), TLR5 is responsible for cellular activation by flagellin and flagellated bacteria, and TLR9 for responses induced by CpG bacterial DNA. TLR2 has a broad specificity for the recognition of PAMPs present in (Gram-positive) bacteria, yeasts, mycobacteria and parasites.

This talk will focus on in vivo models of bacterial infection, and the role of the LPS receptor complex in host defense against pneumonia and peritonitis, the two most common causes of sepsis.

1540 Managing the septic patient

DILIP NATHWANI

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In recent years the recognition and management of sepsis has been confounded by poor definitions and recognition, a blunted response to infection in a variety of populations and a lack of appreciation of the principles of antimicrobial and supportive managements.

This presentation is confined to the management of the immunocompetent adult patient with sepsis and aims to:

1. Highlight the modern definitions of sepsis and their limitation
2. Discuss the evolving role of evidence based clinical decision making in infection
3. Provide the scientific basis of empiric antimicrobial management
4. Introduce the important role of antibiotic prescribing guidelines in acute infection management & the role of continuing education in sustaining a change in behaviour
5. Briefly review the potential role of supportive and adjunct therapy

Key words: sepsis, adult immunocompetent, guidelines & education, adjunct therapy

1610 Differential human gene expression and immune activation mediated by *Neisseria meningitidis*

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During invasive meningococcal disease, high concentrations of inflammatory factors can be detected in serum and CSF. Using expression arrays, we sought to determine the effect of viable meningococci and their secreted proteins (SP) upon human gene expression in meningeal-derived epithelial cells.

Viable meningococci or SP (LOS-depleted) were added to test monolayers of meningotheial cells, whilst medium alone was added to control cells. After incubation, RNA was extracted, radio-labelled probes were synthesized, and used to probe human cDNA expression arrays. RT-PCR was used to confirm array data. Expression of human cytokines and other gene products were quantified by ELISA or immunocytochemistry.

In response to either meningococci or SP, up-regulated expression of pro-inflammatory factors including TNF α , IL-6, and IL-8, and adhesion molecules ICAM-1 and VCAM-1 was detected. Changes in apoptosis-related gene expression were also observed. Up-regulated expression of anti-apoptotic genes, including FLIP and IAP-1, and pro-apoptotic genes including BAD and DAXX, was detected.

These data indicate that cells of the meninges take measures to resist the damaging effects of meningococci. Leukocytes would be recruited, via chemokines and adhesion molecules, to the area of infection, promoting the removal of invading pathogens. Such responses may ultimately have a role in maintaining the integrity of the blood-CSF barrier during meningitis.

Tuesday 8 April 2003

0900 The role of innate immunity to endotoxin in outcome following major surgery

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According to one theory of organ failure, during critical illness and surgery underperfused gut mucosa becomes permeable allowing bacteria or endotoxin contained within the lumen to leak out into the circulation with consequent effects. Being a 'foreign' substance, endotoxin induces natural antibodies to the O-Polysaccharide chain, Core or Lipid-A portions. All humans have endogenous serum antibodies directed against the endotoxin

core (EndoCAb) which varies between people by over three hundred-fold. Several studies have noted that those patients with higher natural levels of antibodies to endotoxin 'core' have a better outcome. This finding, in cardiac, general surgery, sepsis and pancreatitis has led to a renewed interest in anti-endotoxin therapies. For planned events such as elective surgery, we are able in theory, to give anti-endotoxin therapies before endotoxin exposure. There are two ways to increase antibody levels: passively (giving antibodies) or actively. Vaccination requires the patient to be able to mount an antibody response, but if successful, levels are sustained for a greater time than that acquired by passive immunity.

References: Relationship of preoperative antiendotoxin core antibodies and adverse outcomes following cardiac surgery following cardiac surgery. JAMA 1997; 277(8): 646-50 / Preparation and preclinical evaluation of a novel liposomal complete-core lipopolysaccharide vaccine. Infect Immun. 2000;68:6202-8.

0945 Toll-like receptor 4 mutations in sepsis

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Two common co-segregating missense mutations in the Toll-like receptor 4 gene, Asp299Gly and Thr399Ile, occur in the Caucasian population at a frequency of around 10%. It has been previously reported that individuals heterozygous for these mutations show blunted airway responses to LPS. We studied the immunological responses towards LPS of 40 patients admitted to the Edinburgh Royal Infirmary emergency surgical unit with symptoms of abdominal inflammation presumed to have an infective origin, and 40 age and sex matched healthy volunteers. 4 individuals were found to be heterozygous for both mutations in each group. No significant differences could be observed in terms of patient outcome, T-cell or antibody responses to LPS between carriers of wild type and mutant alleles. We therefore compared the capacity of wild type and heterozygous monocytes to respond to LPS. No difference in LPS signalling was observed, suggesting that these mutations do not appreciably affect responses to Gram-negative LPS.

1000 The group A streptococcus: new virulence attributes of a flesh eating pathogen

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Streptococcus pyogenes has re-emerged as a major human pathogen, causing diseases as diverse as necrotising fasciitis, toxic shock, and rheumatic fever. Molecular genetics and genomics have revealed an array of novel pathogenetic mechanisms which enable this bacterium to survive in the host and cause disease. Entry into and penetration of epithelial cell barriers may be key determinants of initial infection, exploiting host cell surface molecules such as integrins and CD44. However, *S. pyogenes* has developed an additional array of broad spectrum countermeasures which target the host immune response. These countermeasures may well have co-evolved with humans, and include complement binding proteins, novel proteases which degrade human immunoglobulins and cytokines and, intriguingly, novel superantigens with unprecedented potency.

Above presentation replaced: Recent insights into Streptococcus pyogenes pathogenesis - M. LLEWELYN (Imperial College, London)

1015 Staphylococcal sepsis – microbial factors important in systemic disease

MATHIAS HERRMANN

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The impact of staphylococci as major pathogens causing bacteremia and sepsis is largely unabated. Particularly in the

hospital setting, these infections have to be viewed as a consequence of the use of vascular access devices, invasive procedures, and/or impairment of host defence mechanisms; they are associated with considerable morbidity and, particularly for *Staphylococcus aureus*, high mortality. Among various reasons for the unique role of these organisms, their adaptivity as a colonizer of human skin and mucous membranes combined with an armamentarium of pathogenicity and virulence factors causing invasive disease may be of foremost importance. *S. aureus* provides of various factors allowing for attachment both to host factor-adsorbed foreign material as well as to biologic substrates including the MSCRAMM ('microbial surface components recognizing adhesive matrix molecules') family of cell wall adhesins and various secreted molecules equally contributing to bacterial adhesion and cell invasion. While the coagulase-negative species lack many of the *S. aureus* virulence factors, they contain a complex machinery for biofilm formation particularly on foreign material such as catheters. A global regulator network including the Sar regulatory protein superfamily, the *agr* autoinducing two-component regulator, and various other elements including the stress-dependent alternate sigma factor B concert the expression of adhesins and toxins and confer the versatility of the microorganisms also involved in major phenotypic alterations such as the 'small colony variant'. For the topic of this contribution, recent findings of our group will be presented and discussed in the context of evidence from other researchers.

CLINICAL MICROBIOLOGY GROUP – POSTERS

CM 01 Heteroresistance in mycobacteria

D. O'SULLIVAN, T.D. McHUGH, M.M. MacKINNON, A. ROSSER & S.H. GILLESPIE

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Some authors have suggested that sub-populations of antibiotic resistant organisms naturally occur in the absence of drug exposure (heteroresistance). PCR based studies of clinical specimens from patients with pulmonary tuberculosis have found evidence of more than one sequence in amplification product (mixed populations demonstrated in 2/11 patients). To investigate this further we studied the distribution of minimal inhibitory concentrations using *M. fortuitum* as a model system.

M. fortuitum NCTC10394 and 2 clinical isolates were grown in broth and plated for single colonies. A total of 40 of the colonies were tested for MIC to 3 drugs: amikacin, minocycline and moxifloxacin. If heteroresistance was present then some of the colonies would be found to have an MIC more than twice the mean value. For all three drugs up to 10% of colonies had a MIC more than x2 MIC. This number was too high to be explained by the intrinsic mutation rate (usually 10^{-9} per cell division) or variation in the experimental method. When individual highly resistant colonies were subjected to the same procedure reversion towards the mean MIC was seen for the colonies with a high tetracycline MIC but neither moxifloxacin or amikacin. This procedure was performed on 2 separate occasions.

To investigate the mechanism of heteroresistance the genes associated with resistance and members of the *mufT* family of mutator genes were sequenced.

CM 02 Serotyping of *Streptococcus pneumoniae* using long-range PCR and RFLP

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Streptococcus pneumoniae claims the lives of some three to four million children in developing countries each year, and the disease burden is being increased by the spread of antibiotic resistant organisms. Following the release of the 7- and 9-valent conjugate vaccines as a means of protecting against carriage as well as disease in children, serotyping is as important as ever.

However, small epidemiological projects in developing countries designed to assess potential vaccine coverage may not have resources to cover the high cost of pneumococcal antisera.

Using Accutaq Red (Sigma) the whole capsulation locus can be amplified targeting primers to the homologous regions of *dexB* and *aliA* genes (which flank the capsulation locus). The PCR has high reproducibility with every serotype tested so far. The PCR amplimers can be digested with *HinfI* and all serotypes produce a unique pattern. Distinguishing patterns have been generated for serotypes/groups 1, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 18, 19, 20, 23 and 38 and a database created using Bionumerics (version 2.0). Sub-types e.g. 6A and 6B can be differentiated. The database has been used to identify the serotypes of new strains, and is in agreement with types identified using the pneumococcal antisera. Updating the database with more serotypes is the focus of ongoing work.

CM 03 Serotypes and transmission of *Streptococcus pneumoniae* nasopharyngeal isolates in village children in Northern Tanzania over a six month period

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Serotype specific immunity is essential for recovery from pneumococcal disease and it is now possible to protect children using the Wyeth / Lederle 7-valent and 9-valent conjugate vaccine. The serotypes included in current conjugate vaccines are based largely on epidemiological data from Europe and America, and there is little data about prevalent serotypes in Africa. Epidemiology of the pneumococcus varies with geography and countries may require individual vaccine formulations.

We have conducted a longitudinal study of carriage of *Streptococcus pneumoniae* in a northern Tanzanian village. 3724 throat swabs were taken at 3 collection periods, month 0, month 2 and month 6, from which 382 strains were isolated and serotyped. Overall the most frequently isolated serotypes were 19, 6, 23, 9, 3 and 18.

There were 465 children sampled three times. Of these, 11 individuals were colonised on more than one occasion, all but one by different serotypes. Congruent carriage was common and accounted for 24% of strains. There were 9 cases of multiple colonization, four of which included a serotype 3 strain suggesting that serotype 3 may promote carriage by another serotype. The pattern of most frequently isolated serotypes differs from those in the UK, most significantly with serotype 14 only being isolated on two occasions. The Wyeth / Lederle vaccine would cover only 47% (7-valent) and 48% (9-valent) of isolates in this study, demonstrating the need for epidemiological data from Africa to be included in potential new vaccine formulations.

CM 04 Allelic variation in the secretory-component binding domain of pneumococcal CbpA: evolutionary significance and functional implications

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University College London, Royal Free Campus

The 75kDa 663 amino acid pneumococcal surface protein choline-binding protein A (CbpA) has recently been shown to play a major role in the pathogenesis of invasive infection by binding secretory component (SC). This allows pneumococci to co-opt the polymeric IgA receptor (pIgR) transcytosis pathway in order to cross epithelial barriers. Our studies have demonstrated allelic variation in the 123 amino acid SC-binding domains of CbpA in isolates of *Streptococcus pneumoniae* belonging to a range of serogroups isolated from blood cultures of patients with pneumococcal bacteraemia. All but one of the ten isolates examined had alleles not previously described. When compared with strain R6x, these isolates showed variation in the predicted peptide sequence of between 4 and 14%, and variation in the

charged amino acids of up to 3.3%. Our modelling of secondary structures of SC-binding proteins by Discrimination of protein Secondary structure Class and Simpa96 predicts a modular organisation, suggesting that the molecular evolution of these genes has largely entailed substitutive recombination. The insertion/deletions seen in these genes support this. However, the random patterns of base pair substitutions seen between isolates suggest additional ongoing allelic variation being produced by point mutation. This random variation in a domain involved in critical interactions with the pneumococcal hosts may be highly advantageous, allowing rapid response, by natural selection, to unpredictable changes in the environment, whilst also ensuring the conservation of essential function. Real-time binding kinetic analysis using a resonant mirror biosensor has provided evidence of variation in binding kinetics between isolates with different *cbpA* alleles and secretory IgA.

CM 05 Evaluation of different media types for the cultivation of A39, a novel mycoplasma

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A39 is a novel *Mycoplasma* species which was first isolated from a sputum sample of a primary antibody deficient (PAD) patient. Subsequently A39-like organisms have been isolated from seven more PAD patients attending the RFH and has been implicated in respiratory disease. All of these isolations were made using a commercially available medium. A39 colonies do not have the typical 'fried egg' appearance typical of other *Mycoplasma* species, but are small and featureless. It is possible that A39 was previously unrecognised due to its inability to grow on other media types or due to it being overlooked because of its atypical colony appearance. We have evaluated different media quantitatively for their ability to support the growth of A39. So far we have found that A39 growth is not supported by Oxoid mycoplasma media, but is supported by SP4, a medium made in-house and used for fastidious mycoplasma. Therefore, A39 may not have been isolated previously due to its atypical colony appearance on solid media.

CM 06 Effect of sub-MIC concentrations of antibiotics on *Clostridium difficile* growth and toxin production

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Some antibiotics are more likely to precipitate *Clostridium difficile*-associated disease (CDAD) than others, presumably by disruption of the normal bowel flora. Antibiotics are also used for treatment of CDAD. The two toxins (A and B) are the established virulence factors of *C. difficile*. Their production has been shown previously to be influenced by environmental stimuli including antibiotics.

This study investigates the effect of antibiotics on growth of *C. difficile* and their production of toxin. Three strains were grown in the presence of sub-lethal levels of i) the agents used to treat CDAD; vancomycin and metronidazole and ii) antibiotics commonly precipitating the disease; amoxicillin, clindamycin, cefoxitin and ceftriaxone. Growth (OD 600nm) and toxin production (ToxA ELISA) were measured over 104 hours. Most antibiotics tended to induce a longer lag phase, with the exception of a clindamycin-resistant strain in the presence of clindamycin. Toxin production was also affected in that it tended to be produced earlier in the growth curve in comparison to the control. Metronidazole, amoxicillin and clindamycin were noticeably associated with this effect. Strain differences were also apparent with effects on toxin and growth differing for the same antibiotics.

This study demonstrates that antibiotics appear to have a direct effect on the growth and toxin production of *C. difficile*, as well as the recognised effect on other gut bacteria.

CM 07 The role of Group III *Clostridium botulinum* in animal disease

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Group III *C. botulinum* consists of the organisms capable of producing the botulinum neurotoxins type C and D and the non-neurotoxic variant, *C. novyi* type A. These bacteria are phenotypically similar and can only be distinguished by the toxin they produce.

Clostridium botulinum type C/D has never been implicated in human disease. However, the bacterium causes outbreaks of botulism in several species worldwide, including cattle, mink, birds and horses. Circumstantial evidence suggests that it is also involved in dysautonomias of horses and cats.

Recently we have investigated cases of equine and feline dysautonomia both microbiologically and serologically. Botulinum neurotoxin type C has been detected in ileal and faecal samples of equine dysautonomia cases. Toxin was also detected in 87.5% of faecal samples from dysautonomic cats after enrichment.

Specific IgA to both toxin and a type C toxoid complex has been found to be much higher in equine and feline dysautonomia cases than in healthy controls. Conversely, specific IgG levels in healthy controls of both species are found to be higher than in affected animals, indicating that some of the equine and feline populations are susceptible. We suggest that animals that are susceptible to type C/D botulism are naturally protected by humoral immunity, and only get disease when this immunity is deficient or overwhelmed.

CM 08 Effects of symbiotic feeding on faecal bifidobacteria and lactobacilli in healthy elderly volunteers

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The colonic microbiota plays an important role in host health. However, detailed knowledge of compositional changes in the microflora and its metabolism during ageing is limited, though a few studies have suggested that degenerative changes occur in the microbiota in elderly people that may have health implications. Human feeding trials were done to determine how beneficial bacterial species (lactobacilli, bifidobacteria) in the microbiota in the elderly could be promoted by synbiotic feeding. An eight week double-blinded feeding study using freeze dried *B. bifidum* and *B. lactis*, in combination with fructooligosaccharides was carried out with 18 volunteers, aged 65 and over. Lactobacillus and bifidobacterial populations were enumerated and identified to species level on the basis of their cellular fatty acid profiles. *Bifidobacterium bifidum* was isolated from faeces more frequently, and in higher numbers in the group receiving the synbiotic compared to the placebo group, during and after feeding, resulting in an increase in total bifidobacteria. No changes in colonisation resistance towards *Salmonella typhimurium* in faecal cultures resulted from synbiotic feeding. Faecal SCFA, as well as harmful metabolic products such as ammonia, indoles and phenols were also measured, but no significant changes were found due to the synbiotic.

CM 09 Analysis of clinical pneumococcal isolates using an automated MLST protocol

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Multi-locus sequence typing (MLST) is a sequence based typing method, which uses data from multiple housekeeping genes. It provides molecular typing data that is highly discriminatory and electronically portable between laboratories. MLST is therefore a suitable tool to use to investigate the relatedness of large populations of microorganisms and has been validated using some important bacterial pathogens including *Neisseria meningitidis* and *Streptococcus pneumoniae*.

The Scottish Meningococcus and Pneumococcus Reference Laboratory (SMPRL) provides a national service for the confirmation of pneumococcal, meningococcal and *Haemophilus influenzae* disease in Scotland. The development of an automated MLST method for characterisation of *N. meningitidis* has been reported previously. Here we outline a method for the analysis of large numbers of *S. pneumoniae* isolates using an improved automated MLST protocol and high throughput capillary sequencing unit. Using this protocol we present MLST data from a set of 120 pneumococcal isolates chosen from the SMPRL database to maximise diversity with respect to serotype and geographical location within Scotland. We also show how analysis of this data using BURST can lead to the identification of clonal complexes, important in epidemiology and public health studies.

CM 10 Detection of *Vibrio parahaemolyticus* in seafood using PCR and phage antibody display

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Vibrio parahaemolyticus has been implicated in outbreaks of gastroenteritis linked to the consumption of seafood in several countries. Most commonly implicated seafoods are undercooked or raw oysters, crabs, shellfish and lobsters. In recent years there has been a growing interest in developing a rapid detection methods for this organism. Conventional methods are laborious and time-consuming. PCR is normally the technique of choice as a rapid assay method. In our laboratory, we have used PCR, and a novel bacteriophage antibody display approach to detect this organism in seafood. Bacteriophage antibody display is a powerful tool that can be used to differentiate between pathogenic and non-pathogenic strains of bacteria. This technique can be modified to give a rapid and sensitive assay for detection of pathogenic bacteria in food samples. The main advantage of this technique is that antibodies can be generated without immunization into animals.

In our study we used both PCR and phage display to differentiate between pathogenic and non-pathogenic strains of *V. parahaemolyticus*. The sensitivity and specificity of both the methods were compared. Seafood samples were then seeded with the organism, and their recovery was estimated using both techniques. Even though PCR is a rapid technique, it was found that with suitable modifications, the phage display method was also highly sensitive and could adopted for routine use in the laboratory.

CM 11 The relationship between alcohol consumption, hepatocellular carcinoma and HCV infection

MASOUD SABOURIGHANNAD

Viral hepatitis and alcohol drinking are recognised as important reasons for liver disease in the world. Chronic alcoholism in patients with chronic hepatitis C appears to cause more severe and rapidly progressive liver disease leading more frequently to cirrhosis of the liver and hepatocellular carcinoma.

Excess alcohol consumption among patients with chronic hepatitis C is likely to result in more severe hepatic injury, promoting pathologic progression to cirrhosis and increasing the risk for development of HCC. Although the exact mechanisms involved in progression of chronic hepatitis C in alcoholic patients have not been definitely established, possible alcohol-induced enhancement of viral replication, iron overload, immunologic suppression, the role of NFκB and the signalling pathways involving in its activation have been suggested. Significant correlation between HCV RNA levels and amount of alcohol consumed has been reported.

Interferon therapy is less effective among alcoholic than non-alcoholic patients, even after a period of abstinence. The obtained data suggest that HCV infection is an important cofactor in the pathogenesis of liver disease among alcoholic patients.

In light of a possible synergistic effect between alcohol and HCV replication, total abstinence ought to be recommended. Because alcohol inhibits the effect of interferon therapy, alcoholics should not be treated until they stop drinking. Moreover a 6-month period of avoidance may not be sufficient to resolve this negative effect on treatment outcome.

CM 12 The variable region of *Campylobacter fetus* surface layer proteins is immunodominant over the conserved regions

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The surface layer of *C. fetus* is comprised of multiple copies of a single protein: the surface layer protein (SLP). There are up to nine different gene homologues encoding SLPs in any given strain. Only one of these is expressed at any time but switching between homologues is continuous throughout infection. This is hypothesised to be an antigenic variation mechanism. An immunogenic region that is completely conserved is present at the amino-terminal region of all of the SLPs, whereas the region beyond this conserved region is highly variable between homologues. This conserved region is inconsistent with the antigenic variation hypothesis. What is the reason for switching an antigen if some epitopes remain constant? A hypothesis to explain this phenomenon is that the variable regions are immunodominant over the conserved region. This was tested using a series of recombinant ELISAs to measure levels of antibodies produced against the conserved and the variable regions of the SLPs. Antibody levels were significantly higher against the variable regions of SLPs.

CM 13 Bactericidal activity of honey on MRSA

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The increasing prevalence of MRSA in hospital environments is a major cause for concern. Unsuccessful attempts to eliminate strains increase the risk factors for cross-infection and nosocomial infections. The continued emergence of new strains with patterns of multiple resistance to systemic and topical antibiotics, or even disinfectants and antiseptics is a cause for concern. In response to this, new solutions for eradication are being sought and honey may prove to be an option, as it has broad-spectrum antimicrobial activity, although the mechanism has not yet been fully elucidated. Recent studies have demonstrated that selected honeys prevent the growth of staphylococci when diluted by a factor of at least 30 in *in vitro* tests. This study aims to investigate the inhibition of a selected New Zealand (manuka) honey on MRSA strains. Time to kill studies with 5 clinical isolates of MRSA recovered from wounds, MRSA NCTC 10442 and *Staph. aureus* NCTC 6571 were performed using 10% (w/v) manuka honey in nutrient broth. All cultures showed at least 10⁵ decimal reductions within 24 hours, and so bactericidal activity of honey was indicated. These results support the clinical evaluation of honey to eradicate MRSA in colonised wounds.

CM 14 Cell wall deficient bacteria mediate stable changes in antibiotic susceptibility

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Cell wall defective bacteria have been associated with a number of disease processes from carcinoma to chronic infection. This study investigates the rate at which cell wall deficient variants

(CWDV) of *Staphylococcus aureus* arise, and the changes to antibiotic susceptibility profiles that are mediated by exposure of CWDV to sub-lethal doses of penicillin. The loss of cell wall was confirmed by colonial morphology, loss of Gram stain reaction and electron microscopy; sensitivity testing was carried out following BSAC guidelines.

On a supplemented medium, with increased osmolarity, CWD bacteria arose with a high frequency. These cells had an indistinct margin, when visualised by transmission electron microscopy, stained as Gram negative and lost the typical staphylococcal arrangement. Passage of the cells on increasing concentrations of penicillin led to an altered antibiotic sensitivity profile for a number of cell wall and non-cell wall active antibiotics. Cells that were allowed to recover their cell wall, by passage in the absence of penicillin, stably maintained the altered antibiotic sensitivity phenotype.

In the presence of sub-lethal doses of penicillin, *S. aureus* easily becomes cell wall defective, which confers a stable penicillin resistance even after the cell wall has been regained. This could have profound consequences for future antibiotic regimes and patient therapy.

CM 15 Optimised random amplification of polymorphic DNA (RAPD) typing for genotyping clinical strains of *Propionibacterium acnes*

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Traditionally considered as non-pathogenic, *Propionibacterium acnes* is increasingly being associated with conditions such as acne vulgaris, endophthalmitis, endocarditis, prosthetic joint infections and most recently sciatica. Typing schemes for isolates of *P. acnes* are limited. We investigated the potential of random amplification of polymorphic DNA (RAPD) to genotype strains of *P. acnes* isolated from clinical samples. Thirty-eight strains recovered from microdissection samples from patients with sciatica, 12 from prosthetic hip infections, 16 from blood cultures and 12 strains isolated from the skin were subjected to genotyping by optimised RAPD. UPGMA dendrogram analysis revealed 4 major RAPD profile clusters of *P. acnes*, two clusters of which consisted of *P. acnes* isolated from patients with sciatica. A further 2 distinct clusters contained strains recovered from either blood cultures or prosthetic hip infections. Skin commensal strains of *P. acnes* did not cluster and occurred sporadically throughout the dendrogram. Typing of *P. acnes* by optimised RAPD revealed that certain genotypes of *P. acnes* may be correlated with specific types of infection.

CM 16 Determination of the synergistic effects of antibiotic combinations used in the treatment of cystic fibrosis lung infection

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Treatment of respiratory infection caused by either *Pseudomonas aeruginosa* or *Burkholderia cepacia* in patients with cystic fibrosis (CF) usually requires the use of a combination of two or more empirically selected antibiotics. The aim of the present study, was to determine whether the empiric antibiotic combinations used to treat lung infection in individual patients demonstrated synergy *in vitro*. The susceptibility of 6 *P. aeruginosa* and 3 *B. cepacia* strains to a number of antibiotics were determined and the effect of antibiotic combinations was interpreted as synergistic, additive, indifferent or antagonistic. Lung infection in 8 of the 9 patients was treated with a combination of tobramycin and ceftazidime and synergy testing of this combination against the bacteria isolated from these patients revealed an indifferent effect for 2 strains, an additive effect for 2 strains and an antagonistic effect for 4 strains. Infection with *B. cepacia* in the other patient was treated with a

combination of tobramycin, meropenem and chloramphenicol and synergy testing revealed that this combination had an indifferent effect for this strain. The results highlight the importance of using synergy testing to determine which antibiotic combinations are synergistic prior to commencing antibiotic therapy for the treatment of CF lung infection.

CM 17 Serotype distribution in *Streptococcus Pneumoniae* strains, gained at the Far East of Russia

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Background: *Streptococcus pneumoniae* continues to be a major cause of morbidity through all the world. The emerging resistance to some common antibiotics compounds this problem. There arises a need to monitor the resistance pattern and map serotype distribution in different geographic locations. The present study was undertaken to determine the serotype prevalence and antibiotic susceptibility of clinically significant *S. pneumoniae* isolated from the patients of young age (18-20 years) with pneumonia in the Far East of Russia.

Methods: A total of 120 clinical isolates from clinically significant pneumococcal infections were serotyped and screened for susceptibility to commonly used antibiotics by the NCCLS standards.

Results: Majority (59.3%) of the isolates belonged to one or other of the serotypes 1, 6, 19, 5, 23 and 7. Serotype 1 was the commonest isolate from patients of empyema followed by pneumonia. Eleven (7.3%) isolates were relatively resistant to penicillin (MIC was 0.1 - 1 mg/ml) and 16.4% were resistant to one or more antibiotics. Resistance was distributed equally among the predominant serotypes.

Conclusion: The common serotypes responsible for significant infections were similar to those reported in some other studies from Russia, with minor variations. Furthermore, it calls for monitoring of resistance and serotype distribution.

CM 18 Ecoimmune correction of the intralumen homeostasis of large intestine in acute abdominal pathology

KYRYK TARAS

Ecoimmune correction of microflora composition of large intestine is a significant part of treatment for peritonitis and pancreatitis. Imbalance of microflora composition in the lumen of large intestine is a persisting symptom of these pathologies. Thus, ecoimmune correction of the endogenous microflora should be focused on as a perspective component of the surgical and therapeutic management of peritonitis and pancreatitis. Ecoimmune correction is conducted with the use of various medical preparations which contain different probiotic bacteria as lactobacilli, bifidobacilli and other and colonic food that is dietary fibers fermented by probiotic bacteria in the lower section of the digestive tube with production of synbiotics.

Thursday 10 April 2003

1400 Microbiology overview

J.V. LEE (CPHL London)

Central Public Health Laboratory, London

Abstract not received

1430 Viruses in water - hazards and risks

JANE SELLWOOD

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Many virus types are capable of multiplication in the human gastro-intestinal tract. Any of the enteric viruses may be present in water contaminated with sewage; the greater the concentration of sewage the larger the potential number of viruses. Sewage that has undergone primary and secondary treatment will contain significantly fewer viruses than untreated sewage. However viruses will be present and will reflect the range of organisms in circulation within the community at any one time. Recreational water, fresh or marine may contain sewage and hence some viruses. Drinking water after chlorination is unlikely to contain any infectious virus particles.

Enteric viruses fall into three main categories: those that cause sufficient damage to the gastro-intestinal tract to produce local symptomatic disease of gastro-enteritis, those that replicate in the gut causing no discernable local disease and those viruses that infect the liver, Hepatitis A and E virus. Investigation of the distribution of enteric viruses in recreational water demonstrates that these hazards are present but because of the epidemiology of the different virus groups only a few present a risk to human health when found in water.

1500 Virological quality of the drinking water supply

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The occurrence and health risk from human enteric viruses is increasingly becoming a concern for drinking water utilities, particularly because the availability of molecular techniques makes it possible to monitor for these organisms.

Viruses are a concern in groundwater because of their small size and mobility in porous media. A national study was conducted to monitor the occurrence of enteric viruses in the continental United States. Groundwater samples from 448 sites, from 35 states were assayed for enteric viruses by cell culture and RT-PCR. Twenty-one sites (4.8 %) were positive for virus by cell culture and 141 sites (31.5%) were positive for viruses by RT-PCR. Routine monitoring of groundwater for fecal contamination may soon be a requirement for water utilities under the proposed Ground Water Rule by USEPA.

Choosing the appropriate indicator system to determine the vulnerability of groundwater supplies to viral contamination is important to the successful implementation of the Ground Water Rule. Twenty groundwater sites from the national study were monitored monthly, for 1 year to develop a useful microbial indicator for assessing the vulnerability of groundwater at risk of fecal contamination. Human enteric viruses were detected either by cell culture or RT-PCR on at least one occasion in 17 of the 20 wells. The results suggest that both bacterial indicators and coliphage would be useful indicators for monitoring the vulnerability of groundwater sources. The detection of microbial indicators on at least one occasion in all of the wells tested supports the need for disinfection of all groundwater supplies.

Within distribution systems, viruses have the opportunity to contaminate potable water supplies through transient negative pressure events. Monitoring of 66 soil and water samples immediately exterior to drinking water pipelines from 8 utilities in 6 states showed that human enteric viruses were detected in

56% of the samples. It is likely that the pathogens originated from nearby leaking sewer lines. The result suggests that opportunities exist for pathogens intrusion into distribution systems, and supports the need for maintenance of a disinfectant residual in piped systems.

Noroviruses are candidates for future regulatory consideration for drinking water. An international workshop held in 2001 outlined research needs and methods development for addressing these important pathogens. Drinking water professionals need to be familiar with viral risks to potable water supplies and the availability of molecular tools makes it increasingly affordable for environmental monitoring for waterborne viruses.

1600 Risk of gastroenteritis in EU-controlled German fresh water bathing sites: results of a randomised epidemiological prospective study

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K. BOTZENHART

Eberhard-Karls-Universität Tuebingen, Germany

During the bathing seasons 2000 and 2001 epidemiological studies were performed at five different German fresh water bathing sites. All sites had been complying for years with the EU bathing water directive of 1976. The studies were performed according to a "randomised controlled trial" design which had previously been adopted by similar studies at British sea water bathing sites from 1989-1992. Dose-response relationships between indicator organism concentrations and the incidence of gastroenteritis could be established for intestinal enterococci (IE) and *Escherichia coli* (EC) using a simple step-model. The model is described by only three parameters: the gastroenteritis incidence of non-bathers, the maximum gastroenteritis incidence of bathers and a threshold concentration. This threshold concentration is chosen such that a hypothesis test about the difference of the two incidences yields a minimum p-value. Depending on the definition of gastroenteritis (UK, UK modified, and Netherlands), thresholds for IE were 23, 21 and 23 IE/100ml and thresholds for EC were 180, 78 and 167 EC/100ml, respectively. Based on these results a new way for classifying beaches is proposed which is more risk-related than the current one using percentages and percentiles.

1630 Microbial hazards of sea-water: just when you thought it was safe to go back in the water

DAVID KAY

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WHO have developed microbiological guidelines for recreational water environments and a new management approach designed to safeguard the health of bathers. The scientific basis of the WHO Guidelines and the rationale for 'beach management' as proposed by WHO is explained. The WHO Guidelines are compared with the European Commission proposal for an amended Bathing Water Directive which was presented in October 2002.

Significant differences are identified and the implications of this dichotomy of approach between the WHO and EU is outlined in terms of UK bathing water compliance.

UK case studies are presented to outline the potential for full implementation of the WHO Guidelines. These combine health based numerical microbiological standards with 'real time' prediction of water quality to facilitate public health protection for users of bathing waters which exhibit significant but predictable variability in natural background faecal indicator concentrations. Finally, a series of research needs are identified for the complete implementation of the WHO approach in a range of recreational environments.

Wednesday 9 April 2003

0915 The laboratory management of outbreaks

WILLIAM F. CARMAN

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There are a number of aims and issues concerning examining samples from outbreaks of viral infections. First, an aetiological diagnosis has to be made. Second, if there is a possibility of a human point source, it may be useful to identify that person. Third, it may be important to identify an environmental source. For both types of sources, sufficient samples need to be tested from cases to prove there has been a common contact. Fourth, protocols need to be established for database management and communicating with hospital outbreak committees. Finally, on the technical side, one needs SOPs, algorithms for defining relatedness of viruses, a reliable sequencer and easy to use sequence manipulation software. Having good, well-trained staff helps, too.

0955 The management of high hazard viral outbreaks

STUART T. NICHOL

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Over the past decade, the Special Pathogens Branch at CDC, has been involved in the management of numerous high profile infectious disease outbreaks involving viruses such as Ebola and Marburg hemorrhagic fever viruses, Rift Valley fever virus, HPS-associated hantaviruses and Nipah virus. The high case-fatality associated with these infections, together with the ability to generate infectious aerosols has led to their notoriety and inclusion on various lists of potential bioterrorism threat agents. The general control measures utilized during such outbreaks and their specific application in a variety of disease settings will be discussed.

1105 Bioinformatics in outbreak reporting and investigation

J. GREEN

Central Public Health Laboratory, London

Developments in the last ten years in technologies of molecular microbiology have provided us with tools that offer forensic levels of accuracy and discrimination in the analysis of microbial pathogens. In employing the exciting technological developments of microbial genomics (high-throughput DNA sequencing, DNA arrays) and proteomics (MALDI-TOF, SELDI-TOF) increasingly large and complex types of biological data are generated, the scale and breadth of which represents a challenge in terms of data handling and analysis. Hence the rise in Bioinformatics, the development and application of computational tools to acquire, store, organize, archive, analyse and visualise biological data.

In the public health arena, there is a need to extend bioinformatics further using approaches that not only integrate complex biological and/or experimental data from microorganisms but also the clinical and epidemiological data. It is through the appropriate integration of these data sets that true understanding of microbial pathogenesis can be understood. Exploiting the developments in bioinformatics, in combination with the parallel developments in IT and the WWW, offers huge potential for 'early warning' systems for outbreak identification, new understanding of infectious disease processes, identification of new disease associations and consequently a direct contribution to the prevention and control of infectious diseases. The presentation will review bioinformatics developments in the context of public health microbiology and outbreak investigation.

1145 Confidentiality, ethics and legal implications of outbreak investigation

SHEILA M. BIRD

MRC Biostatistics Unit Cambridge & Visiting Professor at University of Strathclyde's Dept of Statistics and Modelling Science

Outbreak investigations should take place, most often by informed consent, to guide action. When public health solutions are obvious, investigation should not displace action.

For epidemiological purposes, there may be reason to afford 'demonstrable anonymity' to elicit high volunteer rate in provision of behavioural, biological or incriminating information by those who may have been at risk, not all of whom will consent to investigation if results are attributable to individual participants - because of fear of professional or legal recrimination.

Participants in outbreak investigations should be informed about how their data will be labelled in laboratories and by statistical centres. The legal case of *R. v. Kelly* raises the question of quality assurance in HIV counselling for the dual protection of counsellors and counselled.

Examples of design considerations that can assist or hinder investigation of outbreaks of blood-borne viruses are given, illustrated by: Hepatitis B in Edinburgh's healthcare workers in the early 1970s, HIV and hepatitis B transmissions at Glenochil prison in 1993, injection-related hepatitis B outbreak in Grampian, HIV infection in healthcare workers from high HIV prevalence countries, and the role of partner notification to curtail the transmission of drug-resistant HIV or to interrupt HIV transmission networks around recent seroconverters. Other outbreak investigations, such as of BSE in cattle born after the real feed ban of 1 August 1996, are briefly considered.

1345 Measles outbreaks following the fall in MMR vaccine uptake rates

ELIZABETH MILLER

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Following the introduction in the UK of the combined measles, mumps and rubella (MMR) vaccine in 1988, and the measles/rubella (MR) booster campaign in 1994, measles has been virtually eliminated from the community, with most cases following imported infection and small outbreaks occurring only in groups of unvaccinated individuals. National surveillance since 1994 by oral fluid (saliva) antibody testing, genomic detection and sequence analysis, together with epidemiological information, has enabled the incidence of measles importations and their probable origins to be monitored.

A decline in MMR vaccine coverage in the UK following recent adverse publicity contributed to the occurrence of outbreaks in young children during the winter of 2001-2002, many in inner London where coverage rates are lowest, and involving children in primary school. (<http://www.phls.org.uk/publications/cdr/archive02/News/news1302.html#measles>). Other cases which occurred were primarily in young unvaccinated adults. About 10% of notified measles cases submitted for oral fluid testing during this period have been confirmed by the detection of measles-specific IgM, and over 50% of confirmed cases have yielded genomic sequence data. Genotypic analysis has revealed the emergence of measles genotype D5 as the predominant measles strain currently circulating in the UK, a strain distributed in Japan, Namibia and Thailand and rarely recorded in the UK until recently.

1425 Protection from VZV: the role of live attenuated varicella vaccine

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Varicella vaccine is not a new vaccine, although it may be thought to be new because it is still in the developmental stages with regard to its use. Although the vaccine was developed in the early 1970s, it has been in common use in only a few countries, and only since the mid 1990s. This might seem surprising, but is probably related to the misconception that both varicella and zoster are relatively mild diseases for which a vaccine is unnecessary.

The most extensive use and study of varicella vaccine comes from the United States, where it was licensed for routine immunization of healthy susceptible children and adults, in 1995. Today over 70% of children between the ages of 1 and age 3 years are routinely given varicella vaccine in the United States. The vaccine, distributed by Merck in America, has been given to an estimated 25 million individuals. This presentation will review the experience to date with this vaccine in the United States.

The varicella vaccine is extremely safe, as shown in both pre- and post-licensure studies. The "major" reported adverse reactions are brief tenderness at the injection site in about 20% and a transient very mild rash in about 5% of vaccinees. Spread of the vaccine virus to contacts is exceedingly rare and has only been reported in only 3 recipients of the Merck vaccine. Temporally related adverse events involving the nervous system (ataxia, encephalitis) have been observed rarely, but no case has been shown to be caused by the vaccine virus.

About 90% of vaccinated individuals are completely protected against varicella, and the remainder develops mild breakthrough infections, for the most part. Occasionally there appears to be a "no take" from the vaccine. For adolescents and adults, this is less likely to occur because they are given 2 doses of vaccine, 4 to 8 weeks apart. Currently, there is interest in determining whether 2 doses given routinely to children will provide increased protection.

It appears that zoster is less likely to occur after vaccination than after natural infection. This has been most clearly shown in vaccinated immunocompromised children, but the rarity of zoster in healthy vaccinees up to 7 years after immunization suggests this will also be true for patients who are not immunocompromised. It is still unclear whether this might be due to less latency, less potential for the vaccine virus to reactivate, or both. There has been, however, increasing concern that by decreasing the opportunity for boosting of immunity due to exposure to the natural virus, that the incidence of varicella and zoster may increase in the next 50 years or so. It remains unclear whether this will be a significant problem or not, and it is being intensively studied. Small studies have indicated that zoster is more common when there is no exposure to the natural virus, but how many actual cases of zoster and their potential severity remains unknown. This subject continues to be a subject of intense investigation. In the meantime, a randomized clinical trial of immunizing older individuals with latent infection due to the wild type virus has enrolled over 30,000 subjects and is entering the phase of data analysis and should indicate whether it is feasible to prevent zoster in older individuals by boosting their immunity to the virus.

1535 Human caliciviruses on the run: the molecular tracing of a happy replicator

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Caliciviruses are among the most common infections in humans, and are a major cause of illness in the community, and of outbreaks of gastro-enteritis in institutions such as nursing homes and hospitals. While the course of illness is generally mild, the

sheer numbers of infections, and the high attack rate result in a substantial burden of illness. In recent years, molecular detection techniques have been developed and used initially to study the incidence of caliciviruses. With increasing refinement, strain typing is now being used to further our understanding of the epidemiology of calicivirus infections. By the powerful combination of epidemiological investigations and molecular strain typing, novel information has emerged which adds to our knowledge of the modes of transmission of these viruses, and their evolutionary mechanisms. This presentation will provide a global overview of the state-of-the-art of molecular epidemiology of caliciviruses and will include unpublished data from our European research and surveillance network.

1615 Hepatitis outbreaks in the community and in hospital

C.G. TEO
Central Public Health Laboratory, London
Abstract not received

CLINICAL VIROLOGY GROUP – POSTERS

CV 01 Molecular epidemiology of Rift Valley fever virus isolates from Zimbabwe

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Rift Valley fever virus, a phlebovirus belonging to *Bunyaviridae* family, is a mosquito-borne virus, which emerges periodically, usually in Africa to cause epidemics in animals and humans.

Aim: To determine the heterogeneity among 27 rift valley fever virus isolates from Zimbabwe by nucleic acid sequencing of regions of L, M and S segments in order to evaluate the virus strains from Zimbabwe.

Material and methods: Viral RNA of 27 stored Zimbabwean rift valley fever virus isolates from various sources, were amplified by RT-PCR and sequenced in ABI 377 across all three L, M and S RNA segment regions. Sequences were aligned using a Sequencher V, phylogenetic analysis and networking was performed.

Results: DNA maximum likelihood (DNAML) tree of sequences showed the existence of two major groups A and B, with group B further divisible into subgroups B1, B2, B3 and B4. Nearly half (13/27, 48%) of all isolates studied were in subgroup B1 across all three-segment regions, of which the majority, 10/13 (76.9%) were isolated from 1978-79 Zimbabwean outbreak. Isolates from subgroups B2, B3 and B4 were also seen in the 1978-79 epidemic. Group A isolates do not feature in 1978-79 outbreak. Three out of 27 (11.1%) isolates studied were reassortants. Networking confirms these findings.

Conclusions: This study confirms, across all three segment regions, the presence of two major groups, which we propose calling groups A and B, with further division of group B into subgroups B1, B2, B3 and B4. It shows that all four subgroup B strains were represented among the 20 isolates during the 1978-79 outbreak, and notably, shows the absence of group A strains in this outbreak. Finally, it demonstrates, for the first time, presence of natural reassortants among RVFV strains from Zimbabwe in three of 27 (11.1%) strains studied.

CV 02 Universal hepatitis B vaccination of Glasgow adolescents: a feasibility study

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Background: The UK is one of only a few European countries which does not have a universal hepatitis B vaccine policy. Recent evidence indicates that its policy to vaccinate selected populations only, (particularly injecting drug users), is sub optimally effective in preventing the transmission of hepatitis B virus.

Aim: To assess the feasibility of introducing routine universal hepB immunisation among a population of Glasgow adolescents and to determine the factors which influence vaccine uptake.

Method: Hepatitis B vaccine, in the format of a 0,1 and 7 months schedule, was offered by the Schools Health Service to all first year secondary school pupils (approximately 10,800) attending 81 schools in the Greater Glasgow NHS Health Board area, during the academic year 2001/2002. Prior to the offer, a letter, information leaflet and consent form was posted to every parent/guardian.

Results: Consent was received from 92%. Vaccine uptake data indicate that 91.3%, 89.3% and 80.2% of the school roll have received one, two and three doses respectively. Absentee rates can be up to 10% on any school day. Determinants of uptake are being evaluated, there are early indications that vaccination rates vary across the city from 70% to 97%.

Conclusion: Glasgow is a city of diverse social, ethnic and religious backgrounds but the acceptability of hepatitis B vaccine has been demonstrated across this population. The study was positively received by public health organisations, the media, schools, Members of the Scottish parliament and religious leaders. Despite the considerable extra burden of work for staff involved, their commitment has resulted in the vaccine's successful delivery.

CV 03 Reliability of history in predicting susceptibility to varicella in staff at a London hospital (2001-2)

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The Oka varicella zoster virus (VZV) vaccine is now licensed in the UK for healthy seronegative adults. Guidance on vaccination of workers in the healthcare setting is imminent. Whereas a history of chickenpox has a high positive predictive value (PPV) for VZV serological status in Europe, its' reliability in individuals from tropical/sub-tropical countries, where the seroprevalence of infection in adults may be only 50%, is uncertain.

Staff and students attending pre-employment health assessment at Guy's & St Thomas' Hospital were interviewed by Occupational Health nurses. Country of birth and subsequent countries of residence were documented together with any previous history or household exposure to VZV infection. Age at the time of chickenpox, site and nature of the rash and any residual scarring were recorded.

Questionnaires were completed for 547 individuals, of whom 393 (72%) have been screened for VZV status. Of these 393, 275 (70%) gave a history of chicken pox and 30% either denied a past history (17.6%) or were unsure (10.7%). The reliability of history was also analysed for sub groups based on first country of residence. The PPV of history was less than 95% in both groups. The false positive rate was substantial with 17 (4.3%) seronegative individuals claiming a prior history of varicella. Although on average 10 years older, the seroprevalence of VZV among individuals in the "tropics" subgroup was 10% less than

in the other subgroup. Epidemiological data reflecting the susceptibility of the current work force to VZV should be considered in the formulation of pre-vaccination occupational health screening policies.

	All	U.K., Europe, Middle East, S.E. Asia, USA & Australia	Africa, Philippines, Caribbean & Central America
N	393	278	115
Average age (y)	27.6	24	34.5
Seroprevalence (VZV %)	89.8	92.8	82.6
Sensitivity	73.1	79.1	56.8
Specificity	57.5	40	75
PPV	93.8	94.4	91.5
NPV	19.5	12.9	26.8
% false positives	4.3	4.3	4.3

CV 04 A randomized, controlled trial comparing ganciclovir or ganciclovir plus foscarnet (each at half dose) for pre-emptive therapy of cytomegalovirus infection in transplant recipients

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¹Dept of Virology and ²Bone Marrow Transplant, ³Liver Transplant and ⁴Renal Transplant Units, Royal Free and University College Medical School & Royal Free Hampstead NHS Trust, London

Whole blood from bone marrow, liver and renal transplant patients was tested twice weekly by polymerase chain reaction (PCR) for cytomegalovirus (CMV) DNA. Patients with two consecutive PCR positive samples were randomized to receive either full dose ganciclovir (5 mg/kg iv twice daily) or half dose GCV (5 mg/kg iv once daily) plus half dose foscarnet (FOS) (90mg/kg iv once daily) for fourteen days. Separate randomisation codes were used for each patient group and the primary end point was the proportion of patients who became PCR negative within fourteen days (primary endpoint). Forty-eight patients were randomised. Their base line demographic characteristics were well matched as was the base line CMV viral load (4.28 log₁₀ in the ganciclovir (GCV) group, compared to 4.01 log₁₀ in the GCV plus foscarnet arm). In the GCV group, 71% (17/24) of patients became CMV PCR negative within 14 days compared to 52% (12/23) in the GCV plus FOS arm (p = 0.23). Eight patients required the study drug to become stopped or reduced; seven out of the eight patients were in the GCV plus FOS arm (p<0.05). Baseline viral load was measured in 42 patients, and the rate constant k for viral growth or decay computed. Median baseline viral load was 0.8 log₁₀ (p = 0.02) higher in patients who failed the primary endpoint. In addition, replication rate was faster (t_{1/2} = 1.5 versus 2.7 days; p < 0.001) and viral decay slower (t_{1/2} = 2.9 versus 1.1 days; p < 0.001) in patients who failed in primary endpoint.

This study does not support for ganciclovir plus foscarnet synergism in vivo and nearly all adverse side effects were seen in patients receiving this combination. Ganciclovir plus foscarnet treatment regimen may be of use in patients with impaired bone marrow function, where administration of full dose ganciclovir is precluded

Measuring baseline viral load and computation of viral replication identifies patients at risk of treatment failure who require a prolonged course of antiviral treatment.

CV 05 *In vitro* transformation of human mesothelial cells after polyomavirus infection

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Simian Virus 40 (SV40) is specifically associated with about 50% of human Malignant Mesotheliomas (MM). Growing experimental data suggest that SV40 may be a causative agent of MM. On the other hand, the common human polyomaviruses JCV and BKV are only sporadically detected in MM specimen. Here we investigated whether these viruses differ in their pattern of infection of Human Mesothelial cells (HM) *in vitro*. The results showed that both BKV and SV40 readily infected four different primary HM cultures, while JCV failed to productively infect HM. Both BKV and SV40 induced the highly pleiotropic Notch-1 pathway, *met* phosphorylation and telomerase activity early after HM infection. However, higher levels of BKV replication compared to SV40 in infected HM caused high cell mortality in the BKV-infected HM populations, and virtually no transformation (measured as focus formation). Instead, SV40 infection did not produce mayor cell mortality, and caused (on average) the formation of 1 focus out of 5000 infected HM (frequency of transformation of 0.2×10^{-3}). Overall, these data confirm the high susceptibility of HM to SV40-mediated cell transformation and immortalization. Furthermore, our results provide a rationale on why SV40 (not BKV or JCV) is specifically associated with MM.

CV 06 Archetypal and non-archetypal SV40 in human mesotheliomas

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SV40 is a DNA tumor virus able to transform human cells *in vitro* and induce tumor formation in humans. Recent studies have detected SV40 DNA in human tumors, mostly brain tumors and mesotheliomas. Molecular testing performed in our lab on human mesotheliomas has verified the presence of SV40 DNA with variable genomes. Genetic variations were previously observed in the SV40 strains found in brain tumors. However, there seems to be a characteristic difference among the SV40 strains found in mesotheliomas compared to those found in brain tumors. SV40 containing "wild-type" regulatory regions (2-72 base pair enhancer elements) was typically observed in mesotheliomas. "Archetypal" arrangement in which there is only a single 72-base pair enhancer was rarely found in mesotheliomas. Instead, archetypal SV40 is often found in brain tumors. The unusual ability of mesothelial cells, compared to other human cell types, to survive infection by 2-72 base pair "wild-type" SV40 may account for the different prevalence of SV40 strains among mesotheliomas and brain tumors.

CV 07 African Swine Fever virus (ASFV): microrarray analysis of differential gene expression in porcine macrophages

PAUL A. HOPWOOD¹, FUQUAN ZHANG², CHARLES ABRAMS², LINDA DIXON², ALAN ARCHIBALD³, RICHARD TALBOT³, DAVE BURT³ & STEWART LOWDEN¹

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A porcine cDNA microarray has been constructed to examine gene expression profiles in macrophages infected with African swine fever virus (ASFV). ASFV is a serious threat to the pork industry worldwide, causing rapidly fatal haemorrhagic fever in domestic pigs. However, although the virus infects African

species (e.g. warthog and bushpig), they are resistant to disease through unknown mechanisms. Using a microarray to investigate global gene expression in infected cells has provided insights into the virus-host interaction & immune-evasion strategy and to improved understanding of viral pathogenesis. The parallels with other haemorrhagic fevers such as Ebola and Marburg suggest ASFV as a valuable model for these diseases.

The microarray consists of 2500 cDNAs comprising selectively cloned target transcripts including cytokines, cell surface markers and signal transduction molecules, and clones derived from subtracted macrophage libraries. To establish the differential gene expression pattern associated with ASFV infection, the microarray has been interrogated with probes from *Sus scrofa* macrophages; both uninfected and infected *in vitro* with a virulent strain of ASFV. We will present this data and also describe ongoing experiments which aim to investigate gene expression in macrophages from ASFV-resistant species and cells infected with mutant virus. These studies will further elucidate the mechanisms of viral pathogenesis and host-resistance.

Thursday 10 April 2003

This half day meeting has been developed with our postgraduate members in mind – although supervisors and others are, of course, welcome! The aim of the session is to inform the audience, but we also seek to find out whether there are any particular needs which SGM might be able to fulfil.

Professor Howard Green, from the UK Council for Graduate Education will review the changing postgraduate environment, and highlight the impact for both institutions and students. He will consider two main themes: where we have come from, and current and future challenges. Key trends in postgraduate taught and postgraduate research over the past 10 years will be identified, leading to an overview of current initiatives and imperatives, many deriving from the Harris Review of Postgraduate Education. Their impact on provision and the overall postgraduate experience, will be described. **Tim Brown**, General Secretary of the National Postgraduate Committee, will address the student viewpoint, and their 'rights, wrongs and expectations'. Many new research students often start out not realizing the extent to which they have responsibility over their work, nor their empowerment to produce original work, for which they have ownership. This presentation will introduce research students to the challenges of a doctorate, and allow them to identify their rights, or otherwise, in this role.

Most Universities now run a Graduate School, where elements of skills training are provided for postgraduates. **Liz Sockett**, from the University of Nottingham will talk about a skill which is not always recognized...that is the ability of a student to manage their supervisor! In a sometimes light-hearted way she will explain how understanding the hopes, fears and job demands of your supervisor can enable you to get the best from them, and for them to think highly of you as a PhD student too. **Jo Verran** from the Manchester Metropolitan University will consider other skills aspects which might be addressed in such courses, and will present findings from a questionnaire recently circulated to our postgraduate membership.

These presentations will precede a forum, led by **Professor Hilary Lappin-Scott** from the University of Exeter, where broader issues relating to general postgraduate activities, and more specifically SGM postgraduate membership may be discussed. The audience is encouraged to bring information and experiences to contribute to the discussion, either as part of the discussion, or as a more free-standing item - preferably informing the Chair (J.verran@mmu.ac.uk) in advance, so that provision may be made for such presentations within the session.

A light lunch will be provided for those delegates who indicate attendance at this session on their registration form.

0940 The changing postgraduate experience

D.H. GREEN

UK Council for Graduate Education, Stoke-on-Trent

1000 Rights, wrongs and expectations

T. BROWN

National Postgraduate Committee, Guildford

1020 Managing your supervisor

R.E. SOCKETT

University of Nottingham

1040 PhD and what else? (Findings from questionnaire sent to SGM postgraduate members and skills acquisition)

J. VERRAN

Manchester Metropolitan University

1130 SGM and you

H.M. LAPPIN-SCOTT

University of Exeter

1200 Open forum/panel discussion

'Gripes, tips and what next'

To be chaired by H.M. LAPPIN-SCOTT (University of Exeter)

Wednesday 9 April 2003

0900 Recent developments in biocontrol and future avenues

J. DEACON
University of Edinburgh
Abstract not received

0940 Insecticidal toxins of *Bacillus thuringiensis*

DAVID J. ELLAR
Biochemistry Dept, Cambridge University, Tennis Court Road, Cambridge CB2 1GA

The spore-forming bacterium *Bacillus thuringiensis* (Bt) has been found to produce three groups of novel protein toxins that are specific for a large number insect crop pests, insect disease vectors and some nematodes. The Cry and Cyt toxins are produced as cytoplasmic inclusions during sporulation and the Vip toxins (Vegetative Insecticidal Proteins) are secreted from the cells during vegetative growth. In vivo the Cry and Cyt toxins bind to insect-specific receptors on the surface of gut epithelial cells and in a second, irreversible step insert into the cell membrane to form leakage channels that result in cell death by colloid osmotic lysis. The receptors for some Cry toxins have been shown to be aminopeptidase-N and cadherin-like proteins exposed on the gut epithelial cell surface. Much less is known about the mechanism of the secreted Vip proteins and their receptors, but a recent report claims that Vip3a (89kDa) binds to the epithelial cell surface protein Tenascin-X and causes cell lysis by triggering apoptosis. Vip1 and Vip2 are binary toxins. The protein nature of these toxins coupled with genetic engineering offers great potential for pesticide improvement and has allowed them to be expressed in plants as systemic biopesticides.

1020 Production of *Metarhizium anisopliae* blastospores and ecological factors influence quality

I. YPSILOS¹, D. CHANDLER² & N. MAGAN¹
Applied Mycology Group, Biotechnology Centre, Cranfield University, Silsoe, Bedford, MK45 4DT - email i.ypsilos.s00@cranfield.ac.uk, fax +44(0) 15225 863540
²HRI, Wellesbourne, Warwick CV35 9EF

For the successful development of a fungal biological control agent, high yields of good quality inocula must be produced using cheap raw materials. In this work we tested the effect of interaction of pH, a_w -modifying solute and nitrogen source on blastospore production and blastospore quality and germinability. Either cornsteep solids or cottonseed flour was used as nitrogen source in Adámek's medium which was modified to 0.98 a_w using either PEG 200, KCl or NaCl and adjusted to six different pH values in the range between 3.5 and 10. Blastospore production was greatly affected by all three factors tested with pH having the most important effect followed by the nitrogen source. Regardless of nitrogen source and a_w -modifying solute, maximum spore yield occurred between 6.8 and 8 pH values. Subsequent analysis of endogenous reserves showed that the amount of two types of sugar alcohols, mannitol and erythritol, were also greatly affected by the three aforementioned factors. Increased intracellular amounts of low molecular weight polyols, like erythritol, could enhance blastospore germination under low relative humidity levels, conditions under which many fungal biological control agents are rendered ineffective. Germinability studies using medium with imposed water-stress conditions showed a good correlation between increased amounts of erythritol and enhanced germination. This approach could improve quality of biocontrol agents by increasing the range of relative humidity over which they can germinate.

1040 Application of biocontrol agents to sterilised soil

AMANDA J. BENNETT
Horticulture Research International, Wellesbourne, Warwick
Steam sterilisation of soil destroys plant pathogenic microorganisms, weed seeds and nematodes. However, this process also creates a microbial vacuum in the soil as saprotrophic and beneficial microorganisms are also killed. Re-invasion of sterilised soil can occur naturally from propagules that subsequently land on the soil surface, or move up the soil profile from deeper unsterilised layers, and colonisation can quickly and easily occur due to the readily available nutrients and lack of competition from other microflora. Serious problems can arise when the primary colonisers of sterilised soil are plant pathogens, as this can lead to high levels of disease in the crop that is subsequently planted. To combat this, an integrated approach is being investigated, combining soil sterilisation with the specific addition of biocontrol agents to delay the re-invasion of the substrate by plant pathogenic microorganisms. *Coniothyrium minitans* (a mycoparasite of *Sclerotinia sclerotiorum*) and *Bacillus subtilis* MBI 600 (a bacterium antagonistic to pathogens causing damping-off diseases) are the two biocontrol agents used in this work, and experiments have been set up to investigate their survival and biocontrol activity in sterilised, pasteurised and non-sterile soil.

1330 Modelling baculoviruses for control of glasshouse pests

WOPKE VAN DER WERF¹, FELIX J.J.A. BIANCHI^{2,3} & JUST M. VLAK²
Wageningen University, Dept of Plant Sciences, Group Crop & Weed Ecology¹ & Laboratory of Virology²; ³Present address: Alterra Green World Research, Wageningen University & Research Center. Contact: wopke.vanderwerf@wur.nl

A simulation model was developed to investigate control options for the beet armyworm, *Spodoptera exigua*, using wild type and genetically modified baculoviruses. The model is process-based and includes descriptions of insect life cycle, insect clustering in patches, encounter processes affecting horizontal virus transmission, vertical virus transmission, virus inactivation, crop growth and damage. The model was parameterised with detailed laboratory and glasshouse experiments and validated in the latter. Scenario studies with the model highlight the effectiveness of spraying regimes, concentrations, UV protective agents and genetic improvements in the virus. They show the importance of an integrated approach when designing and evaluating crop protection approaches. For instance, fast killing genetically modified viruses provide greater reductions in crop loss than wildtype viruses if crop infestation by adult moths is a continuous process, but not necessarily if crop infestation by adult moths is an infrequent event. In the latter case, good monitoring methods, to accurately time virus application, are more important than fast killing agents. Further work is ongoing to model insect-virus population systems in cotton.

References: Bianchi, F.J.J.A., J.M. Vlak, R. Rabbinge & W. van der Werf, 2002. Biological control of beet armyworm, *Spodoptera exigua*, with baculoviruses in greenhouses: Development of a comprehensive process-based model. *Biological Control* 23, 35-46 / Bianchi, F.J.J.A., J.M. Vlak & W. van der Werf, 2002. Validation of a comprehensive process-based model for the biological control of beet armyworm, *Spodoptera exigua*, with baculoviruses in greenhouses. *Biological Control* 23, 47-55 / Bianchi, F.J.J.A., J.M. Vlak & W. van der Werf, 2002. Evaluation of the control of beet armyworm, *Spodoptera exigua*, with baculoviruses in greenhouses using a process-based simulation model. *Biological Control* 24, 277-284.

1410 **Trichoderma: traits and exploitation in biological control**

GARY E. HARMAN

Depts of Horticultural Science & Plant Pathology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA

Trichoderma spp. are becoming increasingly commercially both for biocontrol and to enhance plant growth and productivity. Strongly rhizosphere competent strains can exert significant biocontrol effects for at least several months. As recently as a few years ago, we viewed the primary modes of action of *Trichoderma* spp. as resulting from direct effects upon pathogens especially through antibiosis and mycoparasitism. These are indeed important abilities of *Trichoderma* spp., but direct effects upon plants are at least as important. Data from a number of labs on several strains have demonstrated that these fungi are potent inducers of systemic resistance in plants. This permits control of diseases by many classes of pathogens at sites temporally and spatially distant from the point of application. Moreover, they also substantially enhance plant root development, including deeper rooting. This permits plants to tolerate a variety of biotic and abiotic stresses. They also enhance nutrient uptake due to greater root volumes and via direct effects on plant nutrient uptake. Very recently, it has become apparent that these fungi are also going to be very valuable in remediation of polluted soils and waters. These fungi, especially strongly rhizosphere competent ones, should be viewed as opportunistic plant symbionts.

1450 **Biology of the *Pasteuria penetrans*-nematode interaction and the role of functional genomics**

KEITH G. DAVIES & CHARLES H. OPPERMAN

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Keith.Davies@bbsrc.ac.uk

Pasteuria penetrans is part of a group of Gram-positive endospore forming bacteria that are reported to infect nematodes and *Cladocera* (Water fleas). Research into these bacteria has been focused on them being parasites with potential for development into biological control agents of plant parasitic nematodes. The obligate nature of the bacterium and its host specificity has prohibited its commercial development but very recently *in vitro* culture has become possible and this development has led to the reassessment of the bacterium's life cycle. In host parasite interactions 'arms races' produce coding regions with high levels of sequence divergence. The key stages in the life cycle of this nematode - bacterial interaction where arms races may have occurred will be of paramount importance in the development of this bacterium as a control agent. These include endospore adhesion, germination and penetration of the nematode cuticle and avoidance of nematode immunity. Research has shown that the nematode cuticle and the surface of the various stages of the bacterium are immunologically distinct suggesting functional diversity. Experiments are described to exploit genomic data of both the bacterium and the nematode that is now becoming available.

1600 **ACC deaminase in bacteria and its role in the alleviation of plant stress**

BERNARD R. GLICK

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When they are bound to a plant surface such as a root or a seed, plant growth-promoting bacteria that contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase can lower the level of ACC within the plant and thereby decrease levels of the hormone ethylene within the plant. Plants that are treated with ACC deaminase-containing plant growth-promoting bacteria have: longer roots, at least at the seedling stage; a decreased rate of senescence of the petals of some flowers; *Rhizobia* spp. that form more nodules on the roots of their cognate legumes; and a significant level of protection against the deleterious effects of a variety of environmental stresses. Some

of the stresses where these bacteria have been shown to be effective at protecting the plant against damage include flooding, drought, phytopathogen attack, the presence of heavy metals, and the presence of high levels of salt. It is suggested that to protect plants from various environmental stresses, the use of ACC deaminase-containing plant growth-promoting bacteria may provide an effective alternative to genetically engineering plants.

1640 **Systemic resistance induced by rhizobacteria**

L.C. VAN LOON

Faculty of Biology, Section Phytopathology, Utrecht University, The Netherlands

Plant growth-promoting rhizobacteria can suppress diseases through antagonism between the bacteria and soilborne pathogens, as well as by inducing a systemic resistance in the plant against both root and foliar pathogens. Specific *Pseudomonas* strains induce systemic resistance in e.g. carnation, cucumber, radish, tobacco and *Arabidopsis*, as evidenced by an enhanced defensive capacity upon challenge inoculation. In carnation, radish and *Arabidopsis*, the O-antigenic side chain of the bacterial outer membrane lipopolysaccharide acts as an inducing determinant, but other bacterial traits are also involved. Pseudobactin siderophores have been implicated in the induction of resistance in tobacco and *Arabidopsis*, and another siderophore, fluorebactin, may explain induction of resistance associated with salicylic acid (SA) in radish. Although SA induces phenotypically similar systemic acquired resistance (SAR), it is not necessary for the systemic resistance induced by most rhizobacterial strains. Instead, rhizobacteria-mediated induced systemic resistance (ISR) is dependent on jasmonic acid (JA) and ethylene signaling in the plant. Upon challenge inoculation of induced *Arabidopsis* plants with a pathogen, leaves expressing SAR exhibit a primed expression of SA-, but not JA/ethylene-responsive defense-related genes, whereas leaves expressing ISR are primed to express JA/ethylene-, but not SA-responsive genes. Although some bacterial strains are equally effective in inducing resistance in different plant species, others show specificity, indicating specific recognition between bacteria and plants at the root surface.

Thursday 10 April 2003

0900 **Exploitation of functional genomics to enhance biocontrol traits in *Pseudomonas***

F. O'GARA

BIOMERIT Research Centre, Dept of Microbiology, National University of Ireland, Cork

Genotypic and phenotypic traits characteristic of the soil-borne fluorescent pseudomonads make this group of rhizobacteria ideal candidates for biocontrol applications. Utilization of recombinant DNA techniques has established direct links between anti-microbial metabolite production and biocontrol efficacy. Phytopathogen control is mediated in *P. fluorescens* F113 by the production of the metabolite 2,4-diacetylphloroglucinol (Phl). In *P. fluorescens* Phl production occurs in late log phase and is regulated at the transcriptional level and more recent evidence has indicated that post-transcriptional regulation also occurs. Repression of Phl production in early log phase is due to the interaction of a repressor, PhlF with a specific sequence *phO*, which is located, downstream of the σ^{70} RNA polymerase-dependent *phlA* promoter. A regulatory RNA molecule and a cognate repressor are involved in the regulation of Phl production at the post-transcriptional level. Advances in functional genomics have made it possible to reprogramme the regulation of transcriptional and post-transcriptional systems to enhance the biocontrol ability of *P. fluorescens* strains. Manipulation of signalling mechanisms that occur between microbial communities in the soil also has the potential to alter biocontrol traits of bacterial inoculants. Innovative polyphasic design systems based on genomic technologies can be adopted to enhance GM Pseudomonads. This integrated systems-based strategy can facilitate the biosafety assessment and subsequent registration process for the

commercialisation of microbial plant products used in environmental biotechnology applications such as biocontrol.

0940 Suppressive soils; concepts and principles

C. STEINBERG & C. ALABOUVETTE

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Soils suppressive to diseases induced by the most important soil-borne pathogens have been described; they include fungal and bacterial pathogens but also nematodes and they control root rot and wilt diseases induced by many different species. Every soil has some potential of disease suppression, leading to the concept of soil receptivity to a disease, its capacity to control the pathogenic activity. Various hypotheses have been proposed to explain mechanisms limiting the pathogenic activity in suppressive soils. They come within either biotic factors or abiotic factors or both. Each of the mechanisms proposed is relevant to a specific study case and cannot explain similarly all the situations encountered. Because of the physico-chemical properties of the soil and the biodiversity of the microbial community it harbors, the soil interferes in several ways with the relationships between and among micro-organisms, pathogens and plants, and it can modify the interactions among micro-organisms themselves.

Taking into account the suppressiveness of soils to diseases caused by two pathogens having different ecological requirements illustrates the diversity of mechanisms responsible for disease suppression, but conversely, this shows it should be possible to promote the soil health by altering cultural practices and their subsequent effects on the components and the activity of the soil biota.

1020 Improving ecological competence of the biocontrol yeast *Pichia anomala* and fluidised bed-drying formulations

STELLA MOKIOU & NARESH MAGAN

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For BCAs to be successfully used, several aspects of their development, including economic mass production of ecologically competent inocula and appropriate formulation, offering at least one year product shelf-life, need to be ensured. *Pichia anomala* is being considered as a BCA for commercial treatment of moist cereals to control *Penicillium roqueforti* and the mycotoxigenic *P. verrucosum*. Manipulation of the yeast physiology by modification of water stress [water activity (a_w), 0.98/0.96] of cane molasses media using different compatible solutes/sugars and NaCl markedly affected yield, endogenous water potential, sugar/sugar alcohol contents and thus, quality of cells. Intracellular accumulation of the desiccation protectant trehalose and those of glycerol and arabitol was found to be dependent on the type of solute used; in the former case it was pronounced when proline, glucose and sorbitol were added to media. Ecological competence of the yeast treatments was examined by plating on non-stressed (0.995 a_w) and water stressed media (0.96 a_w). Addition of proline or NaCl in molasses media resulted in improved viability. Subsequently, *P. anomala* cells were exposed to fluidised bed-drying using different regimes of drying temperature and time. Viability, water potential and water content of cells was measured and after having optimising drying conditions the best fresh cell treatments were dried as they were and after being washed with isotonic PEG 200 (0.98/0.96 a_w) solutions. Finally, several adjuvants were added to the cells prior to fluidised bed-drying and viability of the different cell formulations was checked.

1040 A biological control strategy for the bacterial wilt pathogen, *Ralstonia solanacearum*

J. SMITH¹, L. OFFORD¹, S. ELLIOT¹, Z.K. MURIMI² & G.S. SADDLER³

¹CABI Bioscience, Egham, Surrey TW20 9TY; ²Kenya Agricultural Research Institute, Nairobi, PO Box 14733, Kenya; ³Scottish Agricultural Science Agency, East Craigs, Edinburgh EH12 8NJ – email j.smith@cabi.org

Potato is a major food and cash crop in Kenya, yet production is highly variable and rarely achieves its potential. Bacterial wilt (*Ralstonia solanacearum*) in particular is a major disease constraint that has to date evaded effective control. Biological control using non-pathogenic mutants of the wild type bacterium may provide, at least in part, a solution. The population diversity of *R. solanacearum* in Kenya was assessed using rep-PCR and genomic macro-restriction digestion used in conjunction with pulsed-field gel electrophoresis (MR-PFGE). Transposon-induced mutagenesis was applied to representatives of the major groups and avirulence to potato confirmed by host testing. Efficacy testing of these putative biocontrol agents has been undertaken against a differential set of potato genotypes and wild type isolates and has consistently recorded levels of disease reduction. Additional assessments related to the use of the biocontrol agents are ongoing, addressing issues such as longevity, environmental impact and impact on other microbial communities (in conjunction with total soil 16S rDNA analysis with density gradient gel electrophoresis (DGGE)). In addition an on-farm nursery seed production systems is being developed to establish an appropriate delivery mechanism for the biocontrol agent.

1130 Physiological approaches to improving the ecological fitness of fungal biocontrol agents

NARESH MAGAN

Applied Mycology Group, Biotechnology Centre, Cranfield University, Silsoe, Bedford MK45 4DT

A prerequisite for the successful development and commercialisation of fungal biocontrol agents is the production and formulation of a product which has the necessary physiological quality, shelf-life and consistency of performance when used. Thus a major hurdle to success has been the production of quality inocula with the necessary ecological competence. We have examined the potential for physiological manipulation of the growth of fungal biocontrol agents to channel or synthesise useful endogenous reserves which are implicated in improved environmental stress tolerance combined with conserved biocontrol capacity. Increased accumulation of trehalose has implications for desiccation tolerance, while sugar alcohol accumulation can improve tolerance to water and temperature stress. Examples will be chosen from studies on biocontrol yeasts (*Candida sake*, *Pichia anomala*) and filamentous fungi (*Gliocladium* species, *Ulocladium atra*, entomogenous fungi). Recent physiological work has been coupled with examining formulation of characterised inocula using fluidised bed drying to conserve quality and examine shelf-life of produced inocula. These studies suggest that the physiological quality and biocontrol capacity of fungal biocontrol agents can be conserved using these approaches.

1210 Symbiotic relations between entomopathogenic nematodes and their bacterial symbionts

RALF-UDO EHLERS

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Entomopathogenic nematodes of the genera *Steinernema* and *Heterorhabditis* are used to control insect pests in horticulture and turf grass. They are symbiotically associated with bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively, which are the major food source for the nematodes and contribute to overcome the insect's defence mechanisms. The nematode's infective juvenile (IJ) carries between 200-2.000 bacterial cells in

the anterior part of its intestine. After invasion of the insect they exit from the developmentally arrested IJ stage and release the bacterial cells into the haemocoel. The bacteria produce toxins and other metabolites, which cause insect death within 2 days after infection. The bacteria proliferate and produce suitable conditions for nematode reproduction. Feeding on the symbiotic cells, the nematodes develop to adults and produce offspring. As long as abundant nutrients are available, additional adult generations develop. When the nutrients are consumed, the offspring develop to IJ that retain the symbiotic bacteria in the intestine and leave the insect cadaver in search for other hosts. The molecular biology of the symbiotic bacteria has gained considerable attention due to commercial interest in insecticidal metabolites active on ingestion and causing similar symptoms in the gut like the *Bacillus thuringiensis**-endotoxin.

1400 Systematics of *Bacillus thuringiensis*: is there a correlation with insect toxicity?

FERGUS G. PRIEST

Heriot-Watt University, Edinburgh

General characteristics of *B. thuringiensis* and their crystal proteins. Diversity of crystal protein genes and their toxicity range.

The phylogenetic placement of *B. thuringiensis* within the genus *Bacillus* using 16S rRNA. Relationships between *B. thuringiensis*, *B. anthracis* and *B. cereus* based on 16S rRNA sequences.

Subspecific classification of *B. thuringiensis* by serotyping and by PFGE of chromosomal DNA digests. Correlation between serotyping/PFGE typing and toxicity. Evidence for clonal population structure.

Multilocus sequence typing of *B. thuringiensis*, *B. anthracis* and *B. cereus* based on seven alleles. Evidence for recombination between *B. cereus* and *B. thuringiensis* and perhaps *B. anthracis*.

Conclusions: *B. thuringiensis* has a clonal population structure and can be considered as specific clones within a wider *B. cereus*/*B. thuringiensis* background.

1440 Fungi as plant disease control agents: status and prospects

JOHN M. WHIPPS

Horticulture Research International, Wellesbourne, Warwick CV35 9EF

There are now about 40 fungal products near to or on the market that have biological control activity against plant pathogens in soil, root, aerial and post-harvest environments. Most of these have been developed relatively recently in response to environmental concerns which have forced reductions of fungicide and fumigant use and availability. These are dominated by *Gliocladium* and *Trichoderma* species. With the exception of a products based on *Phlebiopsis gigantea* for control of stem and root rot of pine and some *Trichoderma*-based products, few have been on the market for 10-20 years or more. Indeed, several have come and gone.

Key features for achieving a successful biocontrol product must be cost-effective and reproducible disease control. In the last 15 years, the importance of understanding the ecological interactions between a biocontrol agent, its target pathogen and host plant before this can be achieved has gradually become appreciated. Against this background, several successful biocontrol agents have been selected, screened for activity, characterised (in terms of identification; mode of action; physiology; ecology; production, formulation, application and shelf life) and then undergone registration and marketing. Reassessment of the concepts and processes involved in natural disease suppression has also provided novel biological approaches to disease control.

ENVIRONMENTAL MICROBIOLOGY GROUP - POSTERS

EM 01 Bacterial seed treatments for the control of seedling blight of wheat

A.A. EL AGAEL, P. JENNINGS & G.D. CRAIG

Dept of Biological Sciences, Manchester Metropolitan University, John Dalton Building, Chester Street, Manchester M1 5GD

Fusarium and *Microdochium* species cause seedling blight and foot rot of wheat. There are no cultivars of wheat which are immune to these diseases and chemical control is inconsistent. The aim of this investigation was to isolate natural bacterial antagonists from the wheat rhizosphere and screen them as seed treatments for the control of seedling blight of wheat. *Bacillus*, *Pseudomonas* and *Streptomyces* species were isolated from rhizosphere soil using selective media and screened for their antagonism against *Microdochium nivale* using a dual plate bioassay. Antagonistic isolates of *Bacillus* and *Pseudomonas* were identified as *B. subtilis* and *P. fluorescens* using matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Plant trials were carried out using selected bacterial isolates as seed treatments. Several isolates increased percentage seed germination and plant fresh weight and reduced seedling blight symptoms in wheat. In conclusion, natural rhizosphere bacteria have been isolated which show potential for the biocontrol of seedling blight of wheat. Future studies will evaluate combined bacterial inocula as seed treatments.

EM 02 Microbial ecology of riverine biofilms

M.J. ANDERSON, J.B. GILLESPIE & N. CLIPSON

Dept of Industrial Microbiology, University College Dublin, Belfield, Dublin 4

The Cloghoge River is an episodically acidic, pristine upland river in Co. Wicklow, Ireland. It is surrounded by bogland in its upper reaches, is largely devoid of human disturbance, and provides an ideal site to study natural riverine biofilms in their pristine state. In flowing systems, microbial communities are concentrated within biofilms, which typically develop on surfaces associated with the stream bed, with the water flow carrying transient planktonic communities.

Rock biofilms were sampled along a 15km stretch of the Cloghoge River between July 2001 and September 2002. Terminal Restriction Fragment Length Polymorphism was used to profile the bacterial biofilm communities along the river. TRFLP is a rapid and sensitive molecular technique based on the variability of the 16S rRNA gene as determined by restriction endonucleases and the automated detection of the terminal fluorescent-labelled fragment. The biofilm communities varied down stream, mainly in relation to minor members of the community. In some cases however the similarity between sites at a certain time point was greater than 50%. Dominant species were observed at all the sites. These species accounted for 50-70% of the total number of cells within the biofilm community.

In order to investigate the phylogenetic diversity of this system, a clonal library was constructed from one of the sites. RFLP analysis of the library showed 19 OTUs, with 5 dominant OTUs accounting for 69.5% of the total clones analysed. 16S rDNA sequencing revealed that the Cyanobacteria and α -Proteobacteria groups dominate these biofilms but also include members of the CFB and β -Proteobacterial groups.

EM 03 *In vitro* and *in vivo* trials of a new virulent bacteriophage against *Escherichia coli* O157:H7

ANDREW D. BRABBAN¹, RAUL RAYA¹, PETER VAREY¹, MIKE DYEN¹, TODD R. CALLAWAY², TOM S. EDRINGTON² & ELIZABETH KUTTER¹

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Escherichia coli O157:H7 is an important food-borne pathogen, causing >70,000 illnesses, 2000 hospitalisations, and 60 deaths yearly in the U.S. alone. Of these outbreaks >75% are directly

linked to ruminants. At present 28% of U.S. cattle presented for slaughter harbour O157:H7. Reducing the population of O157:H7 in ruminant intestines could therefore significantly reduce the incidence of human infection. In this study we have isolated and characterised a new virulent bacteriophage CEV1 isolated from the faeces of sheep that were resistant to gastrointestinal colonisation by O157:H7 strains that had previously colonised sheep. EM of CEV1 revealed structures characteristic of T-even phages. Molecular analysis showed that its genome (180 kb) contained modified nucleotides, and the main capsid protein (gp23) was 94% homologous with the corresponding T4 protein. *In vitro* CEV1 was found to be highly virulent against a range of *E. coli* O157:H7 and other strains. Under conditions designed to reflect the ruminant intestine, CEV1 efficiently infected *E. coli* O157:H7 (ATCC 12900) with burst sizes of 50 pfu cell⁻¹, a latent period of 26 min and an eclipse period of 18 min. *In vivo* studies are currently underway to assess if this phage is effective as a protective/therapeutic agent in sheep and cattle.

EM 04 Persistent baculovirus infections: complications for viral biocontrol

JOHN P. BURDEN¹, CLARE P. NIXON² & ALISON E. HODGKINSON¹, ROBERT D. POSSEE¹, STEVEN M. SAIT³, LINDA A. KING² & ROSEMARY S. HAILS¹
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Baculoviruses are insect pathogens that are usually only found when their hosts reach outbreak densities, and it has been assumed that horizontal transmission is the principle mechanism by which these pathogens pass between hosts. The Cabbage Moth (*Mamestra brassicae*) has been shown to harbour a persistent baculovirus infection in laboratory culture. Here we demonstrate for the first time that such infections are also present in natural populations of the moth. In all populations sampled, between 88% and 100% of the individuals tested were found to be carrying the virus, which persisted within the populations for at least 5 generations as a transcriptionally active infection. The persistent infections were triggered into the lethal overt state by exposure to another baculovirus or fungal pathogen. Two different baculoviruses were found as persistent infections within the populations sampled. These results have broad ranging implications for our understanding of host pathogen interactions in the field, and in the use of pathogens as biocontrol agents.

EM 05 Quantitation of intestinal bifidobacteria, bacteroides and enterococcus populations using real-time PCR

M.J. HOPKINS, ELIZABETH FURRIE & G.T. MACFARLANE

MRC Microbiology and Gut Biology Group, University of Dundee, Dundee

A substantial part of the human colonic microbiota cannot be detected using existing methods of cultivation. This has stimulated the development of a number of molecular techniques for studying complex microbial communities, particularly those involving analysis of 16S rDNA sequences. Recent advances in real-time PCR methodologies offer increased sensitivity and sample throughput over techniques such as slot-blot analysis. Real-time PCR also has the advantage that it can be more easily related to bacterial cell numbers in an ecosystem. The aim of this study was to develop real-time PCR methods for quantitating bacterial populations in the large gut. Primers were designed and PCR conditions optimised for the detection of bifidobacteria, bacteroides and *Enterococcus faecalis*. Several DNA isolation procedures were tested, and a modified commercial kit was eventually chosen for the extraction procedure. Primer sets for each bacterial group gave linear standard curves in the range of 10² to 10⁶ copies for specific DNA sequences. Even when

extracted with large amounts of non-specific background DNA, DNA from the three bacterial groups could be accurately detected when 10⁵ or more cells were present in faecal samples. Results were shown to correlate well with cell numbers obtained using viable count procedures. This study demonstrates that real-time PCR is a sensitive technique that facilitates rapid bacterial cell population analysis in complex faecal communities.

EM 06 Isolation and characterisation of novel halotolerant iron-oxidising acidophiles from the marine environment

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The occurrence of chemoautotrophic, acidophilic bacteria in marine environments has been widely noted and they have been implicated in the biogeochemical cycling of iron and biodeterioration of iron-containing structures in the oceans. However, the isolation, molecular ecology, growth profiles and physiological responses of these bacteria at elevated salt levels have rarely been described, despite widespread interest in their unique metabolic capacity and potential application in the extraction of metals via bioleaching of salt contaminated ores. In this study, three novel strains of halotolerant gram-positive, rod shaped, acidophilic bacteria were isolated from estuarine and coastal areas. Enrichment cultures were set up using pyrite medium of different salinities with sediment and seawater samples from a variety of metal contaminated areas exposed to the sea or to brackish water. These enrichment cultures were then further purified using end-point dilution culture methods. The growth characteristics, morphology and growth profiles on metaliferous ore samples of the strains were characterised and the 16S rRNA genes were sequenced and phylogeny assessed. The strains exhibit autotrophic growth on a variety of iron and sulfur compounds, heterotrophic growth on yeast extract medium as well as mixotrophic growth on a combination of these substrates. The strains grew optimally at 30g sea salts l⁻¹ medium at a pH of 2 and a temperature of 37°C. The growth characteristics displayed by these strains highlight their potential application in high salinity bioleaching operations.

EM 07 Partial purification & characterization of a bacteriocin produced by *L. acidophilus* Razi786 isolated from a milk samples in Iran

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In last few decades, a growing interest has developed for studying bacteriocins and other antibacterial compounds produced by beneficial bacteria such as lactobacilli. In this study we isolated a bacteriocin from an indigenous *Lactobacillus* strain, *Lactobacillus acidophilus* RaziNM 786 isolated from a milk sample. The bacteriocin produced by this strain inhibited the growth of *S.agalactae*, *S.epidermidis*, *S.aureus*, *Ps.aeuroginosa* and *L.monocytogenes*. Attempts to partially purify this bacteriocin by 20% ammonium sulphate precipitation, DEAE-cellulose column chromatography, SDS-PAGE and non-denaturing gel electrophoresis resulted in separation of a 4.5 Kda protein with antibacterial activity. This partially purified bacteriocin showed maximum inhibitory activity against the indicator organisms as demonstrated by their AU/ml and was stable at 80°C and 100°C for 40 and 10 minutes respectively. The bacteriocin was designated as acidocinRaziNM786 and its role in controlling the growth of *L.monocytogenes* in cheese samples was also studied.

EM 08 Intra-protozoan growth of *Listeria monocytogenes* modifies antibiotic susceptibility

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Listeria monocytogenes, the causative agent of human listeriosis, is ubiquitous in nature but its ecology still remains unclear. *L. monocytogenes* is a common component of the intestinal microflora of many livestock. Subsequently, faecal contamination by livestock causes its prevalence in the environment, predominantly in soil and water. Soil and water are also important reservoirs of unicellular protozoa. Common habitats for *L. monocytogenes* and free-living protozoa suggest that in nature these organisms are able to freely interact. There is evidence that *L. monocytogenes* can survive and multiply within *Acanthamoeba* sp., but no studies into virulence and antimicrobial susceptibility of amoeba-grown *L. monocytogenes* have been presented. We established co-cultures of *L. monocytogenes* within Neff's amoeba saline, where *L. monocytogenes* survived and replicated within *Acanthamoeba castellanii*, but were predated and killed by the ciliate *Tetrahymena pyriformis*. Bacterial suspensions of *Acanthamoeba*-grown *Listeria*, and control cultures grown in LB broth, were exposed to bactericidal concentrations of the antibiotics Ampicillin (100, 250, and 500µg/ml), Co-trimoxazole (250, 500, and 750µg/ml), and Gentamicin (50, 250, and 500µg/ml). Survival of these cells was monitored at various times by performing plate counts on saline-washed cells. Amoeba-grown *L. monocytogenes* cells were significantly less sensitive ($p < 0.05$) to all of these antibiotics, than were the LB broth grown cells. These antibiotics are commonly used in the treatment of human listeriosis. Protozoa may therefore not only act as vehicles of infection but may also contribute to their antibiotic susceptibility profile.

EM 09 Self transmissible pesticide-degrading plasmid pHRIM620 from *Arthrobacter globiformis* D47 contains a sulphonamide resistance transposon

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Soil bacteria possess a vast array of degradative enzymes and play a key role in governing the environmental persistence of pesticides. *Arthrobacter globiformis* D47 degrades a range of substituted phenylurea herbicides, an important group of compounds used in agriculture and amenity horticulture that often appear as groundwater contaminants. Previous work characterised a phenylurea hydrolase gene (*puhA*) and demonstrated that the degradative genes were located on plasmid pHRIM620. This plasmid has now been sequenced and transposon-encoded sulphonamide resistance has been identified. We have demonstrated transfer of pHRIM620 into non-degrading strains of *A. globiformis* D47 and shown subsequent herbicide degradative ability in the recipients. We discuss the evolutionary significance of antibiotic resistance linked with pesticide degradation and the clinical implications of these functions being located on self-transmissible plasmids.

EM 10 Evolutionary relationships among *Vibrio cholerae* based on phylogenetic and genotypic analysis

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The genetic structure and molecular evolution of *Vibrio cholerae* populations are not well characterised, and the relationship between different pandemic isolates is unclear. In our study, we examined the evolutionary relationships among *V. cholerae* isolates by constructing a phylogenetic tree based on the house-keeping gene malate dehydrogenase (*mdh*), which previously was

shown to be an accurate indicator of overall genetic relationships. Additionally, we investigated the virulence-associated gene content of the *V. cholerae* strains analyzed in this study. Our phylogenetic analysis combined with PCR profiling suggests that closely related strains may not encode the same complement of virulence genes. Furthermore, epidemic *V. cholerae* (O1 and O139) strains do not contain identical virulence gene profiles. Interestingly *V. cholerae* possesses two chromosomes and in this study we investigated the molecular evolution of chromosome I and chromosome II by comparative sequence analysis of *groEL*, a copy of which is found on each chromosome. This study will better our understanding of the evolution and emergence of pathogenic *V. cholerae* isolates.

SYSTEMATICS & EVOLUTION GROUP - POSTER

SE 01 Genetic analysis of the mechanisms of genome plasticity among *Pseudomonas aeruginosa* isolates

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Pseudomonas aeruginosa is a common inhabitant of soil and water that causes a variety of human infections ranging from superficial skin infections to acute infections of damaged sites as well as the inherited fatal disease cystic fibrosis. *P. aeruginosa* is believed to be the most important bacterial pathogen responsible for chronic lung disease in cystic fibrosis patients.

The description of the full genomic sequence of *P. aeruginosa* strain PA01 (Stover *et al*, 2000) provides a reference sequence map with which to initiate studies of genetic diversity at the whole genome level. We explored the genetic diversity and mechanisms of genome plasticity within *P. aeruginosa*, by examining evolutionary relationships and the virulence genes encoded among *P. aeruginosa* isolates recovered from cystic fibrosis patients in Ireland. Evolutionary relationships were analysed using the house keeping gene malate dehydrogenase (*mdh*) and the virulence genes investigated include *Exo U*, *Exo T*, *Exo S*, *Las A*, *Las B*, *phz M*, *phz H* and *phz S*. Initial analysis indicates sporadic distribution of these genes among *P. aeruginosa* isolates.

Thursday 10 April 2003

1400 Importance of yeast genomics to brewing

S.G. OLIVER

Manchester University

Abstract not received

1435 Differences and similarities between yeasts for brewing and whisky fermentations

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Most beers and whiskies are produced by strains of *Saccharomyces cerevisiae* and related species. There are a number of differences between the processes, but the major differences that relate to yeast are:-

- In brewing, the yeast is recycled and used for a number of cycles (generations). In whisky production the yeast culture is used once.
- Brewing fermentations are largely aseptic because the wort is boiled prior to inoculation (pitching) with yeast. Distilling wort (wash) is unboiled and is contaminated with bacteria (usually *Lactobacilli*) and wild (unwanted) yeasts. Studies in this Centre have found that different species of *Lactobacilli* are characteristic to a particular distillery and specific parts of the process.
- Distilling yeast strains have the ability to utilize small wort dextrins due to the secretion of an extracellular glucoamylase.
- Intensification of the brewing process (for example, high gravity brewing, high adjunct wort, shorter fermentation and ageing times, use of large cylindro-conical vessels and dried yeast cultures) places significant additional stresses on yeast.

These stresses include ethanol, osmotic pressure, carbon dioxide, mechanical stress and desiccation. These stress effects will influence yeast viability and vitality, cell surface integrity and flocculation characteristics and will affect overall fermentation performance and beer quality and stability.

1600 Gene expression specificities of *S. cerevisiae* in enological fermentations

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During wine fermentations, the yeast is subject to multiple stress such as, high sugar, acidity, nutrient deprivation, starvation, high alcohol, etc. How wine yeasts regulate gene expression to cope with changing and stressing conditions is poorly understood. To obtain a comprehensive picture of yeast gene regulation during a wine fermentation, we monitored the whole genome expression throughout fermentation using DNA microarrays. Our data show that strong transcriptional reprogramming takes place during wine alcoholic fermentation which affect more than 2000 genes and are triggered by changes in nutritional, environmental and physiological conditions. Genes of many pathways are regulated in a highly coordinated manner and those involved in critical metabolisms - glycolysis, ergosterol uptake, thiamine - maintain high absolute expression levels throughout the entire fermentation period. We show that under enological conditions where growth arrest is triggered by nitrogen depletion nitrogen sensing and signalling pathways play a key role in gene expression remodelling. The entry into stationary phase is a key event which is followed by a general stress response. At these

stages the integration of various stress signals by carbon repressed cells under anaerobiosis gives rise to a unique stress response that involves large variety of cellular targets. In addition, many of the genes which respond at these stages and which may contribute to the industrial yeast properties are of unassigned function.

1635 Effects of manipulation strategies on performance of *S. cerevisiae* during wine fermentation
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Genetic manipulation of wine yeast strains is a valuable tool for biotechnological improvement of the wine making process. Many industrially relevant traits can be modified by combination of genetic engineering techniques and classical genetics. However, natural wine yeast strains have complex genetic properties which make manipulation difficult and sometimes detrimental for their performance or the organoleptic properties of the resulting wine. Growth and sugar consumption rates are critical parameters that may result diminished by strategies that affect the natural genomic content such as sporulation. Sporulation derivatives of wine yeast strains are often unable of performing complete wine fermentations. This fact limits the utility of sporulation for the obtention of null mutants.

Other strategies for genetic improvement of wine yeasts have been successfully carried out without negative effects on their performance. Overexpression of endogenous genes have allowed the obtention of wine yeast strains overproducing glycogen which display higher resistance to glucose deprivation and produce wines with normal organoleptic properties. Metabolic engineering of pathways involved in acetate esters biosynthesis is also being used for improvement of wine flavor.

1710 Contributions of lactic acid bacteria to the quality of sweet fortified wines

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It is well recognised that the activity of certain species of Lactic Acid Bacteria (LAB) can profoundly affect the quality of most styles of wine. The effects can be beneficial, as in the case of the deacidification and "complexing" effects of a malo-lactic fermentation as carried out by *Enococcus oeni* in dry red wines. The potential advantages of contributing to the sensorial "complexity" of a wine are such that considerable effort is being made in this field.

Many negative contributions of LAB are well documented in many styles of wine. These range from unacceptable turbidity and deposits, through the development of off flavours and aromas, to the production of toxic compounds such as biogenic amines and ethyl carbamate. Most of these defects can (occasionally) be encountered during the production and ageing processes of naturally sweet fortified wines, such as Port, Madeira and the *Vins Doux Naturelles* of southern France, despite these wines possessing ethanol concentrations of between 17 and 21% v/v. *Lactobacillus hilgardii* and *L.fructivorans* are the LABs most frequently cited as spoilers of this type of wine although these species show differences in their behaviour in this environment.

0900 Extracellular amino acid sensing by yeast

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Lager brewing yeast is a species hybrid belonging to the genus *Saccharomyces*. Like other *Saccharomyces* yeasts it can synthesise all 20 amino acids necessary for building up proteins, or it can take up the amino acids from the medium, depending on availability and needs. Side products of amino acid metabolism are important for flavour or shelf life of the beer. Important examples are higher alcohols, which curiously are side products of both synthesis and catabolism of amino acids, in particular isoleucine, leucine and valine. The higher alcohols can be acetylated to flavour-active esters.

Several environmental factors influence the amino acid uptake, which is controlled by a number of systems. Using *Saccharomyces cerevisiae* as a model organism, we study one of these, in which extracellular amino acids induce transcription of genes encoding several permeases, including those that import isoleucine, leucine and valine. A key protein in this sensing is Ssy1p, situated in the plasma membrane, which structurally belongs to the amino acid permease family, but does not detectably transport amino acids. Analysis of constitutive and hyper-responsive *SSY1* mutants suggests that amino acids interact with Ssy1 at the cell surface and that sensing is associated with a change of the conformation of Ssy1.

0935 Glycosidase activities of enological yeasts involved in aroma release

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In grape berries several potent aroma compounds, monoterpenes, C₁₃-norisoprenoids and shikimate derivatives are accumulated as nonvolatile and flavorless glycosides. In the usual winemaking conditions, major proportion of the glycosides remains unexploited after completion of grape juice fermentation. The relevant glycosidases involved in the release of volatiles from glycosides are now well established. Among the enzymes, β -glucosidase is the key enzyme as the aglycone moiety of glycosidic flavor precursors is always linked to β -D-glucopyranose. Low level of glycosidase activities were often detected in grape juice fermented with *Saccharomyces cerevisiae*. Consequently, several work concerned about the selection of non-*Saccharomyces* yeast strains able to hydrolyse efficiently the glycosides during grape juice fermentation. Alternatively exogenous glycosidases from yeast or filamentous fungi were used for aroma enhancement in wine. Recently we have screened several hundred of yeast colonies from grape berries and wines for those producing high levels of β -glucosidase activity. This work allowed to isolate *Debaryomyces vanriji* strain. β -glucosidase from this yeast was purified to homogeneity and partially characterised. When this yeast was co-cultured with *S. cerevisiae* during grape juice fermentation, the concentration of several volatiles was found to be significantly different compared to the juice fermented with *S. cerevisiae* (control wine). *D. vanriji* isolated musts showed higher levels of β -glucosidase activity through fermentation compared to the control wine. The increase in geraniol concentration in *D. vanriji* inoculated wines could be explained by the hydrolysis of the corresponding glucosides by β -glucosidase from *D. vanriji*. However the hydrolytic action of that enzyme was quite limited

since a large amount of the glycosides was still present in the wine.

1040 Ageing in industrial yeast

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Ageing in *Saccharomyces* species is the predetermined cessation of life as a result of the genetically controlled progression from youth to old age. The metric of the brewing yeast lifespan is not chronological, but relates to the number of divisions an individual cell has undertaken. This replicative lifespan potential is strain specific and genetically determined, but may be influenced by environmental factors such as media composition, pH and the occurrence of physiological stress.

Replicative lifespan culminates in a physiological state known as senescence as a consequence of the permanent termination of replication leading to a phase in which cells are still viable but cannot be cultured.

The transition from youthful and active individuals to those exhibiting ageing impaired physiological states is accompanied by several morphological and physiological biomarkers including an increased cell size, cell wall compositional and structural modifications and the increased propensity to retain daughters. Each of these characteristics influence yeast handling in a brewery causing selection of populations on the basis of replicative age. Furthermore populations of brewing yeast enriched for young or old cells exhibit significant differences in stress tolerance and the capacity to ferment beer with the required flavour characteristics.

1115 Importance of metal ions in beverage fermentations

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Metal ions play important roles in regulating yeast growth and metabolism. As a consequence, fermentation efficiency and fermented beverage quality are all be influenced by yeast inorganic nutrients. This presentation will highlight key roles of magnesium, calcium and zinc in fermentation performance and stress tolerance of industrial strains of the yeast, *Saccharomyces cerevisiae*. Specifically, high levels of calcium in industrial media will be shown to be detrimental to yeast fermentation performance, most likely due to antagonism of essential magnesium-dependent metabolic functions by calcium ions. One of these functions, the activity of pyruvate decarboxylase, was found to be dependent of levels of cellular magnesium in yeast. Increased yeast cell magnesium also confers a degree of stress-resistance on yeast cells in terms of improved abilities to withstand temperature extremes and toxic ethanol. The bioavailability of another divalent metal ion, zinc, has similarly been shown to strongly influence both fermentation progress and yeast cell responses to stress. For example, the specific activity of the terminal fermentative enzyme, alcohol dehydrogenase, was strongly dependent on levels of cellular zinc in *S. cerevisiae*. We conclude that the bioavailability of magnesium and zinc ions is very important in ensuring efficient ethanolic fermentation by yeast.

1150 Immobilized yeast fermentation and maturation in brewing

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Traditional beer production is based on batchwise fermentation and maturation processes, leading to relatively long production cycles and batchwise quality fluctuations. Yeast re-use and yeast management play a key role in traditional beer production process. Application of immobilized yeast systems on beer fermentation and maturation is one strategy 1) to shorten the total

production time 2) to enter into continuous production mode with more consistent quality and 3) to reduce total production costs.

Immobilized yeast systems are established technology in lager beer maturation and alcohol free beer production in industrial scale. Immobilized yeast primary fermentation has moved to pilot scale phase with promising results of good beer produced with several weeks of continuous operation.

However, immobilized yeast systems with practically no/low yeast growth taking place during the process is giving new challenges to understand yeast behaviour in new process conditions.

1225 Use of PCR in detection of beverage spoilage organisms

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The Polymerase Chain Reaction (PCR) is a well established tool for the fast and specific detection of microorganisms. By this procedure a known and for one single organism specific part of DNA is amplified (in vitro) and then detected in a second step. The selection of the DNA-sequence allows a very exact differentiation of the organisms.

The screening system based on the LightCycler™ of Roche allows for example the detection of all relevant beer spoilage bacteria in a brewery in one single reaction. In addition to the testing for presence of the beer spoilage bacteria the method enables identification of the germs without further laborious investigations. The detection by real-time PCR is more sensitive than conventional methods and leads to more specific results. The time to result is 24 to 48 h in comparison to 7 days or more. The easy-to-use method is suitable for the routine QA-lab of any brewery since it neither requires molecular biological skills from the user nor a reconstruction of the lab.

FERMENTATION & BIOPROCESSING GROUP – POSTERS

FB 01 Solution of the problems of malclarification, turbidity and separation in mango syrups by fungal pectinases

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The present investigation is concentrated mainly on the treatment of separation, turbidity, and malclarification problems occurring in mango syrup produced industrially for commercial use. Three pectinase(s) were used: a) Crude ST-pectinase produced by *Aspergillus niger*, S-48 TAT, while attacking the peels wastes of *Solanum tuberosum* (ST) under SSF conditions. b) Purified St-pectinase and c) A pure pectinase {Koch-Light Laboratories (KLL). The percentage (%) in reduction of viscosity (R.V.) of the fruit juice samples was the main evidence for solving the problems accompanied by clear cut clarification of treated juice sample. This study emphasized that: a) the optimum enzyme concentrations (units/ml) were 8721.44,4498.72 and 6748.08 for crude ST, purified ST and KLL-pectinases respectively, b) An optimum incubation period of 30,90 and 120 min. for crude, purified ST-and KLL-pectinases respectively, c) The best incubation temperatures were 20°C for crude ST-pectinase and 10°C for both purified ST-and KLL-pectinases, d) An optimum pH value (using citrate-buffer) was 3.2 (for all enzymes used), e) The optimum application conditions for the three enzymes used were detected after the shelf life of mango syrup samples (i.e. six months) were at both optimum pH and optimum temperatures

corresponding to final pH (2.97,2.91 and 2.89) for purified crude ST-, purified ST- and KLL-pectinase respectively.

FB 02 Free amino nitrogen (FAN) in wort and beer

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¹ICBD, Heriot Watt University, Riccarton, Edinburgh EH14 4AS; ²Scottish Courage Brewing Ltd, Edinburgh Amino acids constitute an important fraction of brewer's wort and the determination of FAN is of considerable interest in brewing industry. During fermentation the amino acids are taken up by the yeast, so providing the cells with nitrogen. The carbon skeletons of the absorbed amino acids are utilized by the yeast for the biosynthesis of new amino acids or they may be transformed in higher alcohols. However, there is a lack of knowledge as to how, these wort nitrogenous compounds affect different yeast strains and their fermentation performance and also what levels are needed so that high quality beer to be produced.

The EBC-ninhydrin and gradient elution HPLC methods were used for measuring FAN and amino acids, respectively. For ammonia determination a spectrophotometric assay was utilized. Differences in FAN and individual amino acid composition were examined between normal and various high gravity worts, with and without adjuncts. Furthermore, it was observed that different yeast strains exhibited different amino acid absorption rates and preferences. It was also noticed that in static fermentations, adequate amino acid consumption took place. In shake flask fermentations insufficient amino acid uptake occurred.

FAN affects a great range of other fermentation parameters such as cell growth, biomass, viability, pH, attenuation and flocculation rate. Yeast amino acid uptake depends on an immense variety of factors including: percentage of total assimilable nitrogen, individual amino acid concentration, quality and absorption rate, amino acid competitive inhibition, yeast strain and generation and yeast growth phase.

FB 03 Purification and characterization of amylase produced from thermotolerant, high-alcohol producing *Pichia ohmeri* CW 2.1

WIN AUNG & FUMIO HASHINAGA

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A highly amylytic strain *Pichia ohmeri* CW 2.1 was isolated from traditional alcohol-starter of Kampongcham, Cambodia. Identification of yeast was carried out according to the method described by Kurtzman, C.P. (Ed.) (1999, The Yeasts, A Taxonomic Study, Elsevier) It was also found that *Pichia ohmeri* CW 2.1 has temperature tolerance to 42. and high alcohol producing activity [11.5% (w/v)]. In amylase production, to select the optimum submerged culture medium, experiments were carried out with five different submerged culture media, which have been proposed by previous workers for production of amylase. Further, the enzyme was purified to electrophoretically homo-geneous form by various chromatographic techniques, its properties such as optimum pH, temperature and effect of metal ions, thermostability, molecular weight, and organic compounds were determined.

FB 04 Effects of dissolved carbon dioxide on filamentous fungi in batch cultures

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The inhibitory effects of dissolved carbon dioxide on microorganisms have been reported for many years. Many studies have described the adverse effects of dissolved carbon dioxide on bacteria. Meanwhile, few studies have been reported on the adverse effects of dissolved carbon dioxide on filamentous fungi despite their industrial relevance. The present study has focused on the impact of dissolved carbon dioxide on growth, morphology and product formation (antibiotics). Different levels

of carbon dioxide gas were sparged into the fermenter in a number of batch cultures such as 5, 10 and 15%. It was found that at 10% carbon dioxide the product formation decreased to about 50% and to about 70% at carbon dioxide level of 15%. In addition, the biomass formation was inhibited in comparison to the standard batch fermentation in which air with out carbon dioxide was sparged. The morphology was severely affected specially at carbon dioxide level of 15%.

Keywords: Filamentous fungi- carbon dioxide- growth-morphology - product formation.

FB 05 Regulation of cell wall mannoproteins in *Saccharomyces* species

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Flocculation may be defined as the phenomenon in which individual and discrete yeast cells within a population reversibly aggregate to form clumps which either sediment to the bottom of a cylindrical or dish-bottomed fermenter or rise to the surface of an open square fermenter.

Flocculation is dependent on the expression of lectins that permit reversible lectin-like interactions with sugar receptors on adjacent cell walls. Since this process involves the interaction between the outer walls of adjacent yeast cells cell surface composition is critical for the maintenance of flocculation performance. It has been suggested that the expression of the cell wall surface proteins Cwp1 and Cwp2 may indirectly influence flocculation performance due to their role in the regulation and expression of cell surface physical properties. To investigate this hypothesis, the occurrence of *CWP1* and *CWP2* in *Saccharomyces* species including industrial ale and lager brewing strains have been established using PCR. Deletion mutants for these cell wall proteins in the laboratory haploid strain *Saccharomyces cerevisiae* S288c have been analysed for phenotypic characteristics associated with flocculation. The potential role of *CWP1* and *CWP2* expression in the separation of brewing yeast biomass from beer is considered.

FB 06 Rapid detection and identification of wild yeast in brewery samples

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Production of beer is generally performed using a single yeast strain that is specific to a particular product. Contamination of beer, wort or yeast slurry with non-brewing yeast strains (wild yeast) during the brewing process is known to be difficult to detect and hard to control. Wild yeast present during the brewing process cause a variety of defects in the final product, ranging from superattenuation and poor separation of yeast from beer to flavour defects such as phenolic, acetic and solvent like characters. Traditional techniques used to achieve this are based on cultivation and growth requirements and as such often yield results several days or even weeks after beer packaging. Recent developments in PCR technologies provide alternative detection and identification methodologies which are rapid and reliable.

The ability of new PCR based techniques, such as simple sequence detection and PCR fingerprinting to accurately distinguish *Brettanomyces*, *Pichia*, *Hanseniaspora* and *Zygosaccharomyces* species from brewing *Saccharomyces* strains was assessed. The specificity, sensitivity and limits of detection of traditional methods and PCR based techniques were also investigated. It was observed that PCR techniques offered substantial improvements over traditional techniques in terms of species identification and specificity. However, the limits of detection for PCR based methods suggest that pre-enrichment may be required for routine use within the brewery.

Here we demonstrate that novel PCR techniques are a powerful tool for rapid detection of wild yeast contaminants. It is suggested that future improvements to PCR technology may allow the widespread implementation of PCR technology into routine brewery quality control.

FOOD & BEVERAGES GROUP – POSTERS

FdBev 01 Inactivation of pathogens on food and contact surfaces using ozone as a biocidal agent

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The effective decontamination of food surfaces and contact materials are important hygiene requirements in food preparation and processing. Traditional treatments involving chlorine-based compounds have shown limited effectiveness and have also been associated with the formation of potentially harmful by-products. Consequently there is considerable interest in alternative disinfection treatments.

One such alternative involves the use of ozone as a biocidal agent. Ozone is formed by the addition of an oxygen atom to diatomic oxygen. Ozone, in gaseous or aqueous form, can be used for sterilisation purposes with the main advantage that after a relatively short time period, it reverts to a stable form (O₂) leaving no chemical residues.

This study focuses on the inactivation of a range of food borne pathogens using ozone as a biocidal agent. Experiments were carried out using *Campylobacter jejuni*, *E. coli* and *Salmonella enteritidis* in which population size effects and different treatment temperatures were investigated. The results demonstrated that in suspension tests these foodborne pathogens are highly susceptible to ozone exposure with 6-7 log reductions achieved with contact times < 5 minutes. The effectiveness of ozone for the inactivation of organisms on surfaces was also examined using surface film preparations of *E. coli* and *S. enteritidis* as well as *Bacillus cereus* endospores and *Aspergillus niger* conidiospores.

FdBev 02 Inactivation of spoilage microorganisms in wines using pulsed electric fields

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Wine is a fermentation product that is relatively resistant to microbial spoilage due to its alcoholic content and high acidity. Nevertheless microbial spoilage can occur with production of off flavours. Current treatment methods for controlling microbial spoilage of wine include heating and the addition of sulphur dioxide. However, it has been reported recently by the FDA that approximately 1% of individuals may have some degree of sensitivity to sulphites. The use of Pulsed Electric Fields (PEF) may be a possible alternative method for treating wine.

This study will report on the susceptibility of a range of wine spoilage organisms (*Obesumbacterium proteus*, *Zygosaccharomyces bailii*, and *Lactobacillus brevis*) to PEF treatment. A range of dry and sweet wines were PEF treated. Microbial susceptibility varied significantly depending on the type of organism treated, e.g. a 7-log reduction of *Z. bailii* was achieved after 30 pulses whereas 200 pulses produced a 4 log reduction of *L. brevis*. The influence of various parameters on the effectiveness of PEF treatment was tested such as conductivity, pulse number, pulse duration repetition frequency, applied voltage and microbial density. The results of this suggest that PEF may offer a possible non-thermal alternative method for treating wines.

FdBev 03 Inactivation of *Campylobacter* species using pulsed light

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The growing numbers of gastroenteritis cases reported annually throughout the world indicates clearly that food and water-borne microorganisms continue to pose a major threat to public health. In developed countries, *Campylobacter* species account for most of the bacterial cases associated with gastro-intestinal illness.

Therefore methods which can reduce or eliminate these problematic organisms are therefore of high importance and warrant investigation.

The use of ultraviolet light is becoming an increasingly popular method for reducing the microbial loads on surfaces, packaging, water and air. However, the applicability of traditional UV delivery systems is limited due to long exposure times required with such low intensity emissions. Treatment with pulsed ultraviolet light (PUV), overcomes this problem by emitting highly intense light pulses, and these achieve high levels of microbial inactivation within short timescales.

Experiments using different *Campylobacter* strains have shown that these bacteria are highly sensitive to PUV exposure. Treatment with as few as 5 light pulses has been shown to reduce microbial numbers by ~ 8 log₁₀. Similar inactivation experiments with *Escherichia coli* and *Salmonella enteritidis* resulted in 5 and 6 log₁₀ reductions respectively. The latter results highlight the comparative sensitivity of *Campylobacter* spp to PUV treatment.

FdBev 04 Properties of potential probiotic strains isolated from the elderly: acid tolerance, bile tolerance and oligosaccharide fermentation profile

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The beneficial effect of lactic acid bacteria in the human gut has been shown by various studies. Beneficial bacteria are known as probiotics and can be consumed as live microbial food supplements. Probiotics can be ingested in conjunction with non-digestible food ingredients, such as oligosaccharides, which selectively stimulate their growth, this synergistic system is known as a synbiotic. An orally ingested probiotic has to reach, survive and colonise the gastrointestinal tract and therefore has to resist the gastric secretions of the stomach and the small intestinal bile secretions. Three lactobacilli and two previously uncultured *Bifidobacterium* strains isolated from the elderly were screened for survival at pH 3 and pH 2. Two *Lactobacillus* strains survived for 15min and 1h respectively at pH 2. The strain which exhibited better survival was then tested for bile tolerance at two different concentrations, 0.2% Oxgall and 0.4% Oxgall. The strain survived for 6h at both concentrations with bacterial numbers reaching between 10² - 10³ cfu/ml at the end of the assay following a 4-5 log reduction. The fermentation profile of this strain was then examined by measuring the growth (optical density at 620nm) on a range of commercially available oligosaccharides. The most suitable oligosaccharide for use in a synbiotic system was determined. Future work will include validation with *in vitro* models of a synbiotic specifically targeted for the elderly population.

FdBev 05 Osmotic stress responses in ale and lager brewing yeast

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Osmotic stress in brewing yeast elicits changes in morphology, vacuolar integrity and the accumulation of the intracellular compatible solute glycerol, which permits tolerance to hyper-osmolarity. Genes involved in glycerol biosynthesis, dissimilation

and export have been identified in haploid laboratory strains of *Saccharomyces cerevisiae*. However the occurrence, homology and regulation of expression of these genes has not been previously determined for *Saccharomyces cerevisiae* (ale) and *Saccharomyces cerevisiae* (syn *pastorianus*) production brewing strains. In this study the regulation and role of glycerol is demonstrated.

Glycerol accumulation in ale and lager yeast strains was solute and strain dependent, and reflected strain to strain rather than species to species polymorphism. For the strains utilised in this study atypical glycerol accumulation profiles during osmotic stress were observed possibly due to intracellular degradation or export of this compatible solute. The expression of key genes in the glycerol synthetic and export pathways have been assessed using Northern analysis and RT-PCR. The expression profile of these key genes during osmotic stress in brewing strains will be shown.

FdBev 06 The importance of *TIP1* during cold shock in *Saccharomyces* species

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Temperatures below 20°C initiate the cold shock response in haploid laboratory strains of *Saccharomyces cerevisiae*. This is of great significance in the brewery environment since the temperatures experienced by brewing yeast range from 18.5 to 2°C. During cold shock, strains of *Saccharomyces* species exhibit the global stress responses. Increased glycogen, trehalose and catalase activity has been demonstrated during low temperature storage of brewing yeast strains. Furthermore cold shock specific genes are up-regulated when temperatures are reduced. Those currently identified include genes involved in cell wall structure (*TIR1*, *TIR2*, *TIR4*) and ribosome biosynthesis (*NSR1*, *LOT1*, *LOT2*, *LOT3*). The temperature shock induced protein (encoded for by *TIP1*) has a putative function of an extracellular esterase. The occurrence of this gene in production brewing strains of *Saccharomyces cerevisiae* (ale strain) and *Saccharomyces pastorianus* (lager strain) has been confirmed using PCR. The deletion mutant for *TIP1* in the laboratory haploid strain *Saccharomyces cerevisiae* S288c has been analysed for phenotypic characteristics and a putative function during brewing fermentations has been assigned. The potential impact of up-regulation of *TIP1* in brewing yeast strains is demonstrated.

FdBev 07 Adaptive resistance to biocides in *E. coli* O157 and *Salmonella enterica* and cross-resistance to other antimicrobials

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The commonplace addition of antimicrobials and biocides to domestic disinfectants has raised concern about promoting microbial resistance and potential cross-resistance to therapeutic antibiotics. This study investigated adaptive resistance to commonly used biocides in *Salmonella* and *Escherichia coli* O157, to identify mechanisms underlying resistance and any cross-resistance to antibiotics. *Salmonella* and *E. coli* were serially passaged in sub-inhibitory concentrations of erythromycin, benzalkonium chloride, chlorohexidine and triclosan. Following each passage the MIC of the biocides and any adaptive resistance, or cross-resistance was noted. Adaptive resistance was obtained in all strains investigated. Erythromycin resistant *S. enteritidis* expressed cross-resistance to chloramphenicol, whereas erythromycin resistant *S. typhimurium* showed cross-resistance to chlorohexidine. Benzalkonium chloride resistant *S. virchow* showed cross-resistance to chlorohexidine, however chlorohexidine resistant *S. virchow* did not show cross-resistance to benzalkonium chloride suggesting specific rather than global mechanisms. *E. coli* triclosan-resistant strains repeatedly exerted decreased susceptibility to various antimicrobials, including chloramphenicol, erythromycin,

imipenem, tetracycline and trimethoprim, as well as to various biocides. The adaptive mechanisms underlying resistance were stable for up to 30 days when strains were passaged in antibiotic/biocide free media. These results support the concern that repeated sub-lethal exposure to biocides not only promotes adaptive resistance but also confers a decreased sensitivity to antibiotics.

FdBev 08 Bacterial quality of Ghanaian bottled and plastic bagged drinking water

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The past decade has seen a dramatic increase in the consumption of bottled and, more especially, plastic bagged drinking water in Ghana. The proliferation of such water products raises the question as to whether they are hygienically produced, especially when the poor sanitary environment in urban Ghana is taken into account.

The aim of this study has been to assess the microbiological quality of Ghanaian bottled and plastic bagged drinking water sold on the streets of metropolitan Kumasi.

Eight bottled, 88 factory plastic bagged and 40 hand-tied polythene drinking waters were purchased on the street and tested for total viable counts of heterotrophic bacteria (TVC), total coliforms, faecal coliforms and faecal streptococci. No faecal indicators were detected in bottled water, but 5% of the factory plastic bagged and 43% of the hand-tied polythene water were positive for coliforms and 5% of the hand-tied polythene water were positive for faecal streptococci. Heterotrophic bacteria were found in all three types of water TVCs 100 ml⁻¹ ranged from 1.00 x 10¹ – 4.60 x 10⁴ in bottled water, 1.76 x 10² – 6.33 x 10⁷ in factory bagged and 2.33 x 10⁵ – 7.33 x 10¹⁴ in hand-tied polythene water.

It is concluded that Ghanaian bottled water is of good quality but some factory plastic bagged and hand-tied polythene water are of doubtful quality. Regular monitoring and improved processing of these products is recommended.

FdBev 09 The impact of wastewater use on fresh vegetables sold in Kumasi (Ghana)

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There is increasing evidence that vegetables in both the developed and developing world can be contaminated with pathogenic micro-organisms. This is particularly true when contaminated water is used for irrigation.

In Kumasi, Ghana. 90% of vegetables consumed is produced in the city with the rest from rural areas. To sustain the city's requirement of fresh vegetables all year round in the absence of irrigation systems, most farmers impound streams, create channels or dig shallow wells in streams that receive the bulk of the city's wastewater to water their vegetables. There are frequently competing interests for the use of the water e.g., drinking, cooking, washing, laundry and, when close to a road, cleaning cars and taxis.

This study was to evaluate the effect of wastewater use on the microbiological quality of urban grown vegetables.

Sixty samples each of lettuce, cabbage and spring onions were bought from vegetable sellers in the main Kejetia market, Kumasi (Ghana). Samples of each vegetable were homogenised separately enumerated for total and faecal coliform. Total coliforms ranged from 1.5 x 10⁷ to 2.0 x 10⁹ per gram wet weight for lettuce, from 1.5 x 10⁷ to 1.5 x 10⁹ for cabbage and from 1.5 x 10⁶ to 4.00 x 10⁸ for spring onions. Faecal coliforms ranged from 3.9 x 10⁶ to 2.1 x 10⁸ for lettuce, from 1.50 x 10⁵ to 2.80 x 10⁷ for cabbage and from 1.5 x 10⁵ to 4.00 x 10⁶ for spring onions. Total coliforms in the 7 streams used in watering vegetables were as

high as 1.34 x 10¹⁰ 100ml⁻¹ and faecal coliforms 6.69 x 10⁸ 100ml⁻¹.

It is concluded that wastewater use is the main contributor to microbial contamination of city grown vegetables. Education on the use of salt, vinegar and potassium permanganate in washing vegetables before eating may be intensified by the Ghana Food and Drugs Board while alternative city vegetable farming methods are explored.

FdBev 10 Assessment of mutagenicity and carcinogenicity effects of plastic bags and disposable food containers in the salmonella/ microsome test

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Nowadays, plastic bags and disposable food containers made of high and low-density polyethylene have found increasing use in Iran. As there is a direct relation between, the safety of such a type a product with the society's health, in this research, the mutagenicity and carcinogenicity of these products have been investigated using Ames test and *Salmonella typhimurium* (TA100, TA104). As the result of the effects of mutation, these bacteria strains have no potential to produce histidine on culturing in a minimal glucose agar medium. In general, carcinogen materials will cause a reverse mutation of these bacteria and results in production of histidine by the bacteria in the MGA medium for the Ames test. To investigate the mutagenicity of the PE bags and containers, we faced the pieces of these products on a test plate *S. typhimurium* (TA100, TA104) and the results of the experiments were these compared with MGA medium containing sodium azide as the positive control and distilled water as the negative control. The comparison of the colonies in the negative control, which are produced spontaneously, with those of the investigating PE samples a way to prove the mutagenicity of the experimental samples. The result, showed that the HDPE grades as the raw material and their products made in form of (disposable glasses and containers, drinking bottles and milk bags don't possess the mutagenicity property. The thin film as plastics bags caused reverse mutation of *S. typhimurium* of these products. If these products coated by liquid or solid oils were found to have much more effects on mutagenicity of the films. Following the above tests, rat liver tissue microsomes it was later obtained under sterile conditions and was added to a MGA medium including the suspected PE samples. The results obtained from the latter experiments confirmed that the PE thin bags used for packaging of the food products in Iran suffer from carcinogenicity.

Key words: Mutagenicity and Carcinogenicity; Polyethylene (PE); *S. typhimurium*; Microsome

FdBev 11 The evaluation of microbial population of pizza cheese in refrigerator and freezer conditions

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As we know production of cheese in this result of lactic fermentation of milk. Mozzarella is a soft cheese that could be used freshly, but one type of this cheese which has more fat and less water is called pizza cheese. In this cheese amount of fat and water are 24 percent and 48 percent respectively, while ozzarella that is obtained from milk which is fatted that amount of fat and water are 18 percent and 52 percent respectively. Infact pizza cheese is an Italian cheese which was made of buffalo milk formerly, but nowadays it is made of cow milk.

In this research 280 samples of pizza cheese in temperature of +4 and -20 degrees of centigrade in this times of examination 0,1,3,5,7,10,14,30,60 days and 10⁻¹ 10⁻² dilutions according to current methods of microbiology labs were studied. In this worth mentioning that is this project microbial contamination of pizza cheese examined in 5 bacteria: *E. coli*, coliform, *S. aureus*,

Enterococcus spp., *Cl. perfringens*. According to the result of this research pizza cheese was not contaminated by *E. coli*. this indicates that milk was pasteurized.

The result of 0 time showed that there was no *E. coli* in pizza cheese, but coliforms (*Citrobacter freundii*, *Aerobacter aerogenes*), *Cl. perfringens*, *Enterococcus*, *S. aureus* was observed more than standard level. Thus, they were isolated. the result in the temperature of +4 degrees of centigrade in survival of bacteria showed that coliforms, *Cl. perfringens*, *Enterococcus* increased in temperature of refrigerator compared to 0 time but *S. aureus* decreased.

During the first days the amount of enterococcus and coliforms increased in the temperature of -20 degrees of centigrade. From fifth day bacteria decreased gradually, but from fourteenth day bacteria decreased strongly, but *S. aureus* at the first days decreased. according to the obtained results the following suggestion are offered that : 1) Pizza cheese should be kept 15 days of storage after production in freezer -20 degrees of centigrade then be contributed to the market; 2) Quality control producing factories should be sampled after 15 days of production storage in fridge -20 degrees of centigrade; 3) For home usage pizza cheese should be packed in small sizes, so that the whole package would be consumed after freezing; 4) Pizza cheese should be used cooked to kill micro-organisms (mentioned in this research) in this cheese.

FdBev 12 The effect of Glycyrrhiza glabra extract on the damages caused by *Helicobacter pylori* in a rabbit model

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In the last decade, it has been shown that plant drugs and spices possess antimicrobial and antioxidant effects. Glycyrrhiza glabra extract is one of the products that suppress acid secretion and has been employed for healing gastric disorders such as, gastritis and gastric ulcer. In this study we investigated on the effect of Glycyrrhiza glabra extract on controlling damages resulted from *H. pylori* in rabbit model. Isolates were confirmed as *H. pylori* using a standard urease as well as culture and biochemical methods. The bacterial suspension containing 10⁸ CFU/ml of *H. pylori* was prepared. Eight rabbits kept in animal house under new environmental condition for two weeks, were divided into four groups so that each group contained two rabbits. The rabbits group 1 were fed through sterile tube with fresh bacterial solution. The rabbits group 2 were fed only with plant extract (10ml). The rabbits group 3 were fed at first with bacterial solution, then with plant extract. The rabbits group 4 were fed just with physiological serum (10ml). This experiment was repeated under similar conditions for three times. Although we had demonstrated *in vitro* the susceptibility of *H. pylori* to glycyrrhiza glabra extract at a fairly moderate concentration but the data obtained in this study tends to show no improvement of damages resulted from *H. pylori* in rabbit stomach.

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0900 Microarray analysis as a novel approach to study host-pathogen interactions

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Functional genomics technology is highly amenable to the analysis of both host- and microbial response to infection, the roots of which lay in the increasing comprehensiveness of genome sequences of both pathogens and mammalian hosts. Robust datasets with response profiles of endothelial cells to a wide variety of environmental cues has allowed for a more insightful interpretation of transcription patterns in the infected host cell environment. While these studies have revealed a stereotyped proinflammatory response of endothelial cells following infection, they have also identified a complex host cell response to pathogens and commensal organisms, within which are pathways targeted by pathogen-specific determinants. Arrays to monitor pathogen gene expression inside host cells are now beginning to offer exciting opportunities to characterize the adaptive response of microorganisms following environmental perturbation, to define a diagnostic signature of infection and form hypothesis-driven predictions about putative virulence-associated genes and genes of unknown function. With microarray-based comparative genomics, one can now systematically and comprehensively compare genomes for conserved and unique gene sequences that may represent such pathogenic determinants. Our efforts to understand in more detail the intimate dialogue between pathogen and host have benefited from concurrent progress in genomics, bioinformatics and proteomic technologies.

0940 Bacterial pathogens and apoptosis of endothelial cells

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Bacteria and bacterial products can activate or damage endothelial cells, leading to vasculitis. *Haemophilus somnus* is a gram negative pathogen of cattle and other ruminant species that causes a wide array of disease problems, including pneumonia, reproductive disorders and a fulminant thrombotic meningoencephalitis (TME). Vasculitis is a common finding in systemic *H. somnus* infections. Our laboratory has demonstrated that *H. somnus* and its lipooligosaccharide (LOS) cause apoptosis in bovine pulmonary artery and brain microvascular endothelial cells in vitro. Endothelial cell apoptosis was dependant on caspase-8 activation of caspase-3. Addition of a caspase-8 specific inhibitor significantly reduced LOS mediated apoptosis; whereas, a caspase-9 specific inhibitor had less effect. The purinergic receptor P2X₇ is involved in the LOS-mediated signaling that results in endothelial cell apoptosis. Addition of selective inhibitors of P2X₇ (i.e. o-ATP or PPADS) significantly reduced LOS-mediated endothelial cell apoptosis and caspase activation. Conversely, addition of P2X₇ agonists (i.e. ATP or BzATP), significantly enhanced LOS-mediated caspase-3 activation. Caspase-1 and IL-1 β play protective roles in the response to LOS. Inhibition of caspase-1, or addition of recombinant IL-1 receptor antagonist, enhanced LOS-mediated apoptosis. Conversely, treatment of endothelial cells with recombinant IL-1 β diminished endothelial cell apoptosis in response to LOS. Intracellular oxygen and nitrogen intermediates are required for maximal endothelial cell apoptosis; addition of inhibitors of their production diminished the response. *H. somnus* and its LOS activate bovine platelet expression of Fas-

ligand and P-selectin. Furthermore, LOS activation of bovine platelets enhanced the subsequent apoptosis of endothelial cells *in vitro*. These studies provide new insights into how this gram negative pathogen causes endothelial cell apoptosis, which may be involved in the vasculitis that often accompanies *H. somnus* infections.

1115 Mechanisms contributing to *Acanthamoeba*-mediated cytotoxicity of human brain microvascular endothelial cells

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Background: Granulomatous amebic encephalitis due to *Acanthamoeba* is a serious human disease, leading almost always to death, however its pathogenesis and pathophysiology remains unclear. Aims of this study were to determine whether (1) *Acanthamoeba* produces cytotoxicity on human brain microvascular endothelial cells (HBMEC) and (2) factors responsible for its cytotoxicity. **Methods:** Two isolates of *Acanthamoeba* belonging to T4 (*A. divionensis*) and T7 (*A. astronyxis*) genotypes were incubated with HBMEC and cytotoxicity determined using LDH assays. To determine whether *Acanthamoeba*-mediated cytotoxicity is contact-dependent or independent, HBMEC were incubated with conditioned media of *Acanthamoeba*. To examine the role of extracellular serine proteases, conditioned media were treated with various protease inhibitors prior to cytotoxicity assays. In addition, zymography assays were performed to determine the nature and approximate molecular weights of the secretory proteases. **Results:** Up to 60 % cell death occurred with the T4 isolate (*A. divionensis*) or its conditioned media. These effects were not observed with the T7 isolate (*A. astronyxis*). Moreover, PMSF treatment of conditioned media abolished HBMEC cytotoxicity, suggesting that secretory serine proteases are essential in *Acanthamoeba*-mediated HBMEC cytotoxicity. Zymography assays revealed three serine proteases (approximate molecular weights, 130, 85 and 55 KD). **Conclusion:** These data have shown that *Acanthamoeba* isolate belonging to T4 genotype produced HBMEC cytotoxicity while T7 isolate did not and *Acanthamoeba*-mediated HBMEC cytotoxicity require secretory serine proteases.

1145 Verocytotoxins, endothelial cells and haemolytic uraemic syndrome

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Haemolytic uraemic syndrome (HUS) is the most extreme manifestation of human infection with verocytotoxin-producing *E.coli* (VTEC) infection. The epidemiology of HUS in children strongly implicates verocytotoxin (VT) in the pathogenesis, with VT2-producing strains occurring more often than VT1. How VT leads to microangiopathic haemolytic anaemia and the glomerular thrombosis that characterise HUS is unclear. In VTEC-induced HUS, abnormalities of coagulation appear prior to the decrease in renal function¹. It is therefore assumed that glomerular thrombosis leads directly to renal impairment, and that endothelial cells participate in local coagulation.

One hypothesis is that VT causes protein synthesis inhibition in endothelial cells directly leading to cell death, either necrotic or apoptotic, and loss of the normal thromboresistance in the

microvasculature. This concept arose from experiments in rabbits in which injected VT caused endothelial lesions in the spinal cord and paralysis. The site of the lesion correlated with the distribution of the toxin and its receptor, Gb3. Humans express Gb3 in various tissues and particularly the renal proximal tubular cells. However, glomerular endothelium has weak expression except perhaps in infancy. The ricin model of HUS in rats shows that glomerular thrombosis does not correlate with the tissue distribution of the toxin suggesting other mechanisms are involved².

VT does not circulate freely in plasma but it has been shown to bind to a surface receptor on human neutrophils with a lower affinity than Gb3. It has therefore been postulated that neutrophils deliver VT to target sites³.

There is evidence that at low dosage VT causes cellular effects other than protein synthesis inhibition and cell death. Epithelial cells and monocytes exhibit superinduction of pro-inflammatory cytokines and chemokines. This phenomenon involves cell signalling by p38MAP kinase and the transcription factor NFkappaB. Very recently sub-lytic doses of VT2 on human endothelial cells have been found to cause excretion of MCP-1 and IL-8, and up regulation of adhesion molecules and leukocyte endothelial interaction⁴. It is therefore possible that very low doses of VT, delivered by neutrophils to susceptible endothelium, may induce the recruitment of additional leukocytes and augment toxin delivery.

It remains to be seen whether VT at sub-lethal dosage induces procoagulant changes in endothelium, why certain vascular beds (colon and glomerulus) are particularly vulnerable, and whether leukocyte engagement with the endothelium itself contributes to vascular injury.

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1350 E. coli translocation of the blood-brain barrier KWANG SIK KIM

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Bacterial meningitis continues to be an important cause of mortality and morbidity despite advances of antimicrobial chemotherapy and supportive care. A major contributing factor to this high mortality and morbidity is our incomplete understanding of the pathogenesis of this disease. Most cases of bacterial meningitis occur as a result of hematogenous spread, but it is unclear how circulating bacteria cross the blood-brain barrier. Recent studies with *E. coli* K1 show that successful traversal of the blood-brain barrier requires a high degree of bacteremia, *E. coli* binding to brain microvascular endothelial cells (BMEC), *E. coli* invasion of BMEC, host cell actin cytoskeleton rearrangements and related signaling pathways, and traversal of the blood-brain barrier as live bacteria. Several microbial determinants contribute to BMEC binding and invasion, such as FimH and OmpA for binding and the K1 capsule, Ibe proteins and CNF1 for invasion. Of interest, *E. coli* trafficking mechanisms differ from those of other meningitis-causing bacteria such as *Listeria monocytogenes* and group B streptococcus. Complete understanding of bacteria-BMEC interactions contributing to translocation of the blood-brain barrier should help develop novel strategies to prevent bacterial meningitis

1430 Activity of microbial toxins on brain endothelial cells: breaching the blood-brain-barrier

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The brain is marked by its own unique internal milieu, the composition of which depends on the molecular screen of the

blood-brain barrier (BBB). The BBB is characterised by the presence of tight junctions between its cellular components. Among them, the cerebral microvascular endothelium is the main site at which the permeability of the BBB increases during various diseases of the central nervous system (CNS), including bacterial meningitis. Indeed, blood-borne bacteria invade the CNS through a variety of mechanisms by which they interact with, invade, or disrupt cells of the BBB. In addition, circulating microbial products, such as toxins, can induce loss of BBB function. For example, haemolysins of the streptococcal species *S. suis* and *S. pneumoniae* (named suilysin and pneumolysin, respectively) have been shown to be cytotoxic for human brain microvascular endothelial cells. Thus, they may contribute to breach the BBB allowing the entry of these pathogens into the CNS. In addition, the suilysin induces the release of pro-inflammatory cytokines by this type of cells. The local production of cytokines could also enhance permeability of the BBB. Suilysin, as well as pneumolysin, belong to the family of toxins known as antigenically related cholesterol-binding cytolytic toxins. Other members of this family of toxins, as well as several different toxins produced by other bacterial pathogens, such as *Escherichia coli*, *Clostridium perfringens*, and *Streptococcus agalactiae*, are able to induce alterations of the endothelial barrier and promote bacterial invasion and/or CNS dysfunctions.

1540 Cerebral malaria – endothelial cell/plasmodium interactions

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1620 Effect of N. meningitidis surface structures on endothelial cell surface expression

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Compromise of the normal functioning of the vascular endothelium explains many of the clinical and physiological manifestations of meningococcal shock. The vessel wall is not simply a passive conduit for blood in this process, but closely regulates the intimately linked inflammatory and haemostatic pathways at the endothelial cell surface. *Neisseria meningitidis* has a tropism for the vessel wall, rendering it an important target for the host inflammatory response to bacterial invasion. Intact meningococci, in concert with other host inflammatory cells, appear to induce a range of signals in the endothelium that are distinct from those seen with purified LPS alone and are modulated by the capsulation status and LPS structure of the meningococcus. These interactions occur in both a LPS-dependent and LPS-independent manner, acting as a focus for neutrophil mediated disruption of the endothelium. Therefore although LPS is the principal activator of the inflammatory response, other co-signals may be important, transduced through both conventional LPS receptors and/or a range of newly identified pattern recognition receptors. It remains unclear why compared with other forms of severe sepsis, the accelerated shock, coagulopathy and microvascular thrombosis that characterise meningococcal disease are so severe. A clearer understanding of the mechanisms that underlie meningococcal host-interactions will provide important clues to this process.

MICROBIAL INFECTION GROUP – POSTERS

MI 01 Identification and characterisation of an Fc binding protein in *Bartonella* species

AMANDA READ

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The genus *Bartonella* includes a number of arthropod-transmitted pathogens of humans and domestic animals that are naturally maintained by establishing chronic, asymptomatic infection in a range in mammalian reservoir hosts. In the course of infection of these hosts, the bacteria are thought to first infect a primary niche (probably the vascular endothelium) from where they are synchronously released in every few days into the bloodstream. Once in the blood, the bacteria associate with and invade erythrocytes, in which they multiply and establish a chronic infection. Infection of a reservoir appears to be ultimately limited by the host's humoral response, presumably initiated during the extracellular stages of infection either prior to primary niche invasion or during transfer to erythrocytes. That *Bartonella* may have evolved mechanisms for countering this humoral threat seems a reasonable hypothesis, thus the aim of this study was to determine if *Bartonella* possess one mechanism for doing so, namely the ability bind the F_c portion of antibodies. The binding of antibodies by their F_c portions excludes them from participating in an immune response, and an F_c binding ability has been previously demonstrated for numerous pathogens. Western blotting and ELISA were used to detect an F_c-binding capability in several *Bartonella* species and to localise this ability to a protein of approximately 65kD size, work is ongoing to further characterise this protein.

MI 02 The cytokine response to *Salmonella* infection in an *in vitro* avian model

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We recently used a chicken primary cell culture model (chick kidney cells – CKC) to show that invasion of the broad host range serotype *S. typhimurium* caused an increase in production of the pro-inflammatory cytokine IL-6, whilst invasion by the host-specific serotype *S. gallinarum* caused no increase. This finding correlated with the pathogenesis of *Salmonella* in poultry. *S. typhimurium* invasion produces a strong, pro-inflammatory response that may limit the spread of *Salmonella* largely to the gut, whilst *S. gallinarum* does not induce an inflammatory response and may not be limited by the immune system, leading to the severe systemic disease, fowl typhoid.

Our hypothesis is that this apparent evasion of the innate immune response by host-specific serotypes may be due to host adaptation by the bacterium. This could be due to the differences in the pathogen associated molecular patterns (PAMPs) on the surface of the bacterium, such that host-specific serotypes are not recognised by chicken innate immune receptors. Alternatively, components of the proteins complexes encoded by the *Salmonella* pathogenicity island (SPI) genes in host-specific serotypes may down-regulate aspects of the innate immune responses in the chicken.

To test this hypothesis, we measured cytokines responses following invasion of CKC *in vitro* with both wild-type strains and defined mutants of *S. typhimurium*, *S. gallinarum* and another host-specific serotype, *S. pullorum*, by bioassays and Taqman. SPI-1 mutants of *S. pullorum* show increased levels of the pro-inflammatory cytokines IL-1 β , IL-6 and IL-8, suggesting that wild-types *S. pullorum* may modulate innate immune reactions following invasion *in vivo*.

MI 03 Recombinant antibodies against bacterial virulence factors: isolation and characterisation of anti-K99 scFvs

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Enterotoxigenic strains of *Escherichia coli* (ETEC) are a frequent cause of diarrhoea in newborn calves. Infection can be fatal. The most important factor for adherence and colonisation of the bovine intestine are the K99 fimbriae expressed by ETEC strains. We have isolated and characterised recombinant single-chain (scFv) antibodies against this virulence factor by phage display. K99 fimbriae were precipitated from culture of a bovine ETEC isolate and purified by ion-exchange chromatography. The material was then coated to plastic and a large semi-synthetic scFv library (the Griffin.1 library, Centre for Protein Engineering, Cambridge) screened by iterative rounds of biopanning. After four round of selection, five scFv clones with high activity against K99 fimbriae were isolated and sequenced. These recombinant antibodies possessed high reactivity towards the fimbriae in ELISA tests when expressed in *E. coli* TG1 as fusions to the phage coat protein pIII. The recombinant antibodies were expressed in *E. coli* HB2151 as soluble scFv proteins for further characterisation of their immunological and biological properties.

MI 04 DNA Vaccines against type F botulinum toxin: a comparison of promoters

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DNA vaccination is a vaccination methodology in which genes encoding protein antigens are delivered into host cells enabling antigen production to occur *in vivo*. DNA vaccines consist of plasmid vectors that include heterologous genes inserted under the control of a eukaryotic promoter, allowing protein expression in mammalian cells. In this study a number of different eukaryotic promoters have been compared in their use in DNA vaccines against type F botulinum toxin. DNA vaccines which expressed the Hc fragment of the *Clostridium botulinum* type F neurotoxin (BoNTF/Hc) fused to a signal peptide downstream of 4 different eukaryotic promoters were prepared. Subsequently, the immunogenicity of the DNA vaccines and protection afforded in mice against challenge with 10⁴ MLD of type F botulinum toxin was evaluated. In this study, the DNA vaccine containing the human ubiquitin gene promoter induced the highest BoNTF/Hc-specific antibody concentration following two intramuscular immunisations and afforded 90 % protection against challenge. The results from this study indicate that the selection of promoter used in DNA vaccination studies may be of importance in designing optimised vaccines.

MI 05 Identification of ABC transporters homologues in *Burkholderia pseudomallei*, *Francisella tularensis* and *Yersinia pestis*

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ABC transporters are responsible for the import and export of a remarkably wide variety of molecules across cellular membranes and comprise one of largest protein superfamilies found in prokarya, eukarya and archaea. The evolution of ABC transporters is not linked to the evolution of host organisms, but is related to the type of molecule transported. As a result, ABC transporters of specific molecules have a high level of homology between

different species. ABC transporter proteins in some bacteria have been identified as virulence factors or immunodominant proteins. In this study ABC transporters in the dangerous pathogens *Burkholderia pseudomallei*, *Francisella tularensis* and *Yersinia pestis* have been identified using bioinformatics. Subsequently, ABC transporter proteins with homologues in all 3 pathogens have been identified. These proteins may be important in virulence of pathogenic bacteria.

MI 06 The relationship between the aging process and bacterial disease in the nematode *Caenorhabditis elegans*

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A short life span and genetic tractability has made the nematode *C.elegans* a useful model for the study of bacterial pathogenesis and the mammalian aging process. *C.elegans* mutants have been identified that confer resistance to bacterial pathogens. In addition, longevity mutants have also been discovered and recently, studies have demonstrated a correlation between these two groups of mutants. Survival of a strain possessing the *age-1* and *age-2* alleles was compared to the wild type strain, when exposed to Gram negative bacterial pathogens that have been shown previously to kill *C.elegans*. We have found that the *C.elegans* *age* mutants have enhanced resistance to *Pseudomonas aeruginosa* strain PA14, *Burkholderia cepacia* strain 25416, *Salmonella enterica* var. Typhimurium strain LT2 and *Yersinia pseudotuberculosis* strain YpIIIpIBI on modified nutrient agar. Enhanced resistance to *P.aeruginosa* strain PA14 and *B.cepacia* strain 25416 was also noted on high sorbitol media. The *C.elegans* *age* mutations did not confer resistance to *P.aeruginosa* strain PA01 when grown on BHI media. A comparison of the three nematode strains that possess *age* alleles showed that, resistance to *P.aeruginosa* strain PA14 is related to the life span of the nematode. Wild type nematodes have the least resistance and the shortest life span. The *age-2* mutation conferred less resistance than *age-1*, with the strain possessing both alleles demonstrating the most resistance.

MI 07 Sensitivity of cystic fibrosis pathogens to doxycycline, chloramphenicol and fusidic acid in the presence and absence of lactoferrin

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Burkholderia cepacia is a major threat to the management of patients with cystic fibrosis (CF) being particularly resistant to antibiotics. MICs of chloramphenicol for *B. cepacia* isolated from the respiratory tracts of CF patients were lower in the presence of human lactoferrin at the concentration found in CF sputum (0.9 mg/ml).

Effect of lactoferrin on MICs and MBCs of doxycycline and fusidic acid for *Stenotrophomonas maltophilia* and fusidic acid for *B. cepacia* also tested. There were no changes in the sensitivity when lactoferrin was added. MICs of doxycycline for *S. maltophilia* were surprisingly low. Doxycycline may have a role in the treatment of *S. maltophilia*.

The findings suggest that chloramphenicol might be used in combination with other drugs for the treatment of *B. cepacia* infections in CF patients. Addition of lactoferrin to sensitivity media for testing of CF isolates may give more useful results than conventional tests.

MI 8 Testing the function of FlhB and its C-terminal homologue HP1575 in *Helicobacter pylori* flagellar export

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Helicobacter pylori is a highly motile Gram-negative bacterium, and its flagella have been implicated as having an important function in the colonisation of the human stomach. Here we show that motility in *H.pylori* has been shown to be maximal at 37°C and pH 6 and enhanced by co-culture with AGS cells. FlhB (denoted HP0770 in *H.pylori* strain 26695) is an essential component in the basal body structure and flagellar export apparatus, mutations in HP0770 result in the production of elongated hooks or polyhooks giving a phenotype that is non-motile. In *Salmonella*, Macnab and co-workers have shown that FlhB contains a cleavage site within the cytoplasmic C-terminal domain that brings about a conformational change in the protein and in substrate specificity. *H.pylori* FlhB shows high sequence similarity with *Salmonella* FlhB in the region surrounding the proposed cleavage site, including the essential NPTH sequence, and Western analysis showed that HP0770 is processed in *E.coli* DH5 α into a truncated N-terminal region of 31kDa and a short C-terminal section of 11kDa. Sequence comparisons have shown a protein (HP1575) present in the *H.pylori* genome with high similarity to just the C-terminal domain of HP0770. Alignments of full-length FlhBs and HP1575 shows that its sequence begins one amino-acid before the position of the NPTH site in the FlhBs. A homologue of HP1575 does not appear to be present in *E.coli* and *Salmonella* genomes but is present in a range of other bacteria including *Pseudomonas* and *Campylobacter*. HP1575 function is, at present, unknown. We describe the effects of production of HP0770 and HP1575 in *E.coli* DH5 α and RP437 following HP0770 cleavage with C terminal tags. Creation of mutants in *H.pylori* deficient in HP0770 and HP1575 for phenotypic analysis and complementation testing with cognate *Helicobacter* genes, *Salmonella flhB* and chimaeric *Helicobacter-Salmonella flhB* constructs is underway. It is hoped that this will reveal the functions of these two gene products in *Helicobacter*.

MI 09 Two distinct binding sites on the tetanus toxin H_C fragment are essential for ganglioside binding

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Tetanus toxin (TeNT) from *Clostridium tetani* consists of an N-terminal enzymatic L chain (50 kDa) disulphide bonded to a C-terminal (100 kDa) H chain, responsible for binding, trafficking and cytosolic escape of the L chain. H_C, the 50kDa C-terminal fragment of the H chain, retains the binding and trafficking characteristics of whole toxin. Structural data suggests there are two distinct ganglioside-binding sites on H_C; one binds Gal-GalNAc, the other binds sialic acid.

Mutant H_C proteins containing amino acid substitutions within both sites were constructed and purified from *E. coli*. Proteins were assayed for binding to GT1b gangliosides and to N18 RE-105 neuroblastoma cells.

Our results confirm that both sites on H_C are essential for binding ganglioside. GT1b binding of Gal-GalNAc site mutants is reduced to below 13% of wild type and binding of one mutant in the sialic acid is reduced to 10%, though it still binds cells. Surface plasmon resonance analysis indicates that H_C does not bind ganglioside in a 1:1 stoichiometry and native PAGE suggests H_C migrates as a tetramer. Pre-incubation of mutant proteins with GT1b and analysis by native PAGE demonstrated retardation of mutants with near wild type ganglioside binding, but not of mutant proteins.

MI 10 Characterisation of a pneumococcal sortase

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Sortase enzymes are widely distributed in Gram-positive bacteria acting to anchor specific proteins to the cell wall. Recently they have been shown to contribute to the virulence of *Staphylococcus aureus* and *Listeria monocytogenes* in murine models. Here we have investigated a sortase, *srtA* from the human pathogen

Streptococcus pneumoniae (the pneumococcus). A null mutant was generated by allelic replacement in the strain D39 and compared to its wild type parent in murine models of pneumonia and bacteraemia. In both infection types, the *srtA* mutant showed reduced virulence. Sortases are seen as potential vaccine and antimicrobial targets. In this regard, we have cloned and over-expressed pneumococcal SrtA with a view to assessing its potential as a vaccine candidate. Additionally, we have examined, by PCR and sequencing the strain distribution and sequence diversity of *srtA* from a range of laboratory and clinical pneumococcal isolates. The gene could be detected in all strains examined and was highly conserved in sequence. These properties may enhance the exploitation of SrtA as a vaccine or therapeutic target.

MI 11 Identification and characterisation of five *Salmonella enterica* proteins secreted via the Type V autotransporter pathway

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Members of the *Enterobacteriaceae* are a remarkably versatile range of organisms that include pathogens of plants, animals and humans, one of which (*Salmonella enterica*) commonly contaminates the food chain. The mechanisms by which these organisms cause disease invariably involves proteins secreted to the cell surface or beyond. Autotransporter proteins play roles in virulence by acting as adhesins, toxins, mediators of motility and immunomodulatory proteins. By utilising molecular features common to autotransporter proteins, we recently identified five members of this family of proteins in *Salmonella enterica* (i.e. *yaiU*, *STM3691*, *apeE*, *shdA*, and *misL*). Sequence homology analyses suggests that all of these proteins are outer membrane associated and may be involved in infection by acting as enzymes or adhesins. Screening of these autotransporters throughout the SAR (SALmonella Reference) A, SAR B and SAR C collections demonstrated that they are wide spread among pathogenic *Salmonella* strains but all of them are not systematically present.

MI 12 Identification and characterisation of a Pic homologue, situated on a pathogenicity island in uropathogenic *Escherichia coli* CFT073

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Pic is a 109.8-kDa extracellular protease secreted by the type V secretion system. This protein was first characterised from *Shigella flexneri* 2457T and enteroaggregative *Escherichia coli* 042 and is a member of the serine protease autotransporters of the *Enterobacteriaceae* (SPATE). The precursor is a 146.5-kDa protein, constituting a signal peptide, mature protein (109.8 kDa) and integral membrane protein. Pic is a multifunctional protein, having been demonstrated to cleave mucin and to induce haemagglutination. These functions may be involved in enteric pathogenesis. A homologous protein to Pic (UpaB) has been identified in the uropathogenic *E. coli* strain CFT073. UpaB is encoded on a 102-kb pathogenicity island, which may infer a role in the pathogenicity for this organism. We have cloned the UpaB gene from *E. coli* CFT073 and expressed the gene product in *E. coli* HB101. The sites, at which the precursor protein is processed to form the mature secreted Pic and UpaB proteins, have been determined by N-terminal sequencing. Functional studies to determine the enzymatic properties of the mature UpaB protein are described. A comparison has been made between the Pic pathogenicity islands of *S. flexneri*, and the uropathogenic and enteroaggregative *E. coli*.

MI 13 *Salmonella* pathogenicity island 10 is a hyper-variable region of the *Salmonella enterica* genome, which contains three genes specific to subspecies I serovar typhi that are conserved between strains

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Salmonella enterica subspecies I contains serotypes that commonly infect warm-blooded animals. These include *Salmonella enterica* serovar Typhi (*S. Typhi*), which causes Typhoid fever, and serovars that cause gastroenteritis in humans, such as *S. typhimurium*. Comparison of *Salmonella enterica* genomes helps us to understand the evolution of the species and to identify genes that contribute to pathogenicity and host restriction. There are ten genomic regions that vary significantly between *E. coli* and *S. Typhi*, termed *Salmonella* Pathogenicity Islands (SPI's) 1 to 10. Using a combination of bioinformatics, microarrays, Southern blotting and PCR, we find that SPI-10, adjacent to tRNA^{LeuX}, is a hyper-variable region and a hot spot for horizontal gene transfer. In contrast, most of the other SPI's are similar between members of subspecies I. *E. coli*, *S. Typhi* and *S. Typhimurium* contain totally different tRNA^{LeuX}-adjacent genes. Although SPI-10 is a hyper-variable region, we find that three *S. Typhi* SPI-10 genes, that are related to eukaryotic serine-threonine kinases and a phosphatase and are inserted into a P4 phage found within SPI-10, are both unique to this serotype and are conserved between strains.

MI 14 Microbiological quality of air in university lecture theatres and the potential application of pulsed ultra violet light disinfection

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Airborne transmission of microorganisms is recognised as being a significant source of infection, with potentially fatal illnesses such as tuberculosis and meningitis spread via this route. Consequently, more research is required in this area to both establish the physical mechanisms of airborne transmission in specific environments and develop effective means of achieving air disinfection.

The present study is concerned with an investigation of air quality in university lecture theatres. Due to high student numbers and absence of air quality control within lecture rooms, there is potential for the build-up of airborne contamination and therefore cross-infection.

In this study a SAS-Super180 sampler was used to take microbial and fungal counts before, during and after a 1hour lecture in order to identify the variation in airborne contamination levels that occur, with results demonstrating a significant increase in airborne populations due to the presence of large congregations. Various environmental parameters were also monitored to examine their effect on the microbial air load. Also, a pulsed UV light system developed by the EPAST group (which has had success in surface and liquid applications) was used to disinfect the air, and results highlight the effectiveness of the PUV system for the inactivation of airborne microorganisms.

MI 15 The role of TspA in meningococcal association to human cell monolayers

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Introduction: The function of the *Neisseria meningitidis* immunogenic protein TspA has been investigated by comparing

the association patterns of TspA deficient and wild type bacteria to human cells.

Results and discussion: Recent work in our laboratory has suggested that TspA deficient meningococci have an altered morphology that appears to result in non-functional pili structures. Since pili are important in the association of meningococci to cells, the adherence of TspA mutant bacteria and their wild type counterparts was compared.

Two human cell lines were used, HEP-2 cells and primary meningeothelial cells derived from human meningitis tumours. Association was determined using three methods: conventional colony forming unit counts, fluorescence microscopy and environmental scanning electron microscopy. Results obtained from all three methods showed that the TspA mutant bacteria adhere to monolayers of both cell types at significantly lower levels than the wild type bacteria. Furthermore, the adherence patterns of a series of isogenic mutants carrying deletions in the amino and carboxy terminals of TspA was investigated. Mutants carrying a deletion in the N-terminus of TspA did not show reduced adherence.

Conclusion: Results have suggested that TspA may have a role in meningococcal pathogenesis. Further work is underway to determine any possible links with pili.

MI 16 The structure and function of the meningococcal T cell stimulating protein-A (TspA)

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Introduction: TspA is a surface exposed T-cell and B-cell stimulating protein of *Neisseria meningitidis*. It is a high molecular weight protein. It has a highly positively charged N-terminus, a hydrophobic putative trans-membrane region and a highly negatively charged C-terminus. In this study the molecular features and function of TspA were investigated.

Results and discussion: TspA was cloned, expressed, purified and used to raise rabbit polyclonal antiserum. An isogenic deletion mutant and truncated TspA mutants, which lacked part of the N- or C-terminus were produced. On examination under the electron microscope, the morphology of the Null and C-terminal mutants were abnormal with deformed pili, projections from the cell surface and disorganised microcolony structure. Furthermore, these results, and the homology to FimV, the *Pseudomonas* gene related to twitching motility, suggested a link between TspA and the type IV pilus. To aid confirmation that the function of TspA is related to the type IV pilus, transformation competence was examined and showed that there was a clear reduction in competency in the Null mutant.

Conclusion: These findings imply that TspA interacts with the meningococcal pilus. Further work on adhesion is underway to establish this link.

MI 17 Differentiation of *Francisella tularensis* strains LVS and Schu 4 using the polymerase chain reaction

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The Live Vaccine (LVS) strain of *Francisella tularensis* is the current vaccine for protection against Tularemia. There exists the possibility that LVS seedstocks are contaminated with Schu 4, a virulent strain of *F. tularensis*. For licensing and manufacture of LVS as a human vaccine, it must be established there is no Schu 4 present in the seedstocks. The *ilvB* gene of LVS contains a 102-bp deletion not present in Schu 4. On this basis PCR primers were designed to amplify a fragment of *ilvB* such that the Schu 4 PCR product was 102 bp larger than the LVS product and visible on an agarose gel. We have shown the ability of this PCR test to differentiate Schu 4 and LVS and have determined the limit of detection to be 10:1 ratio of LVS: Schu 4 DNA, above this ratio there is no detectable amplification of Schu 4 DNA. Additionally, a second PCR test has been developed that is Schu 4-specific and 500 times more sensitive in

detecting Schu 4 in the presence of an excess of LVS DNA compared to the original *ilvB* PCR.

MI 18 Post genomic analysis of *Listeria monocytogenes* using targeted plasmid integration

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The Gram positive foodborne pathogen *Listeria monocytogenes* must adapt to adverse environmental conditions encountered in the food processing environment and during host infection. We have analysed the contribution of putative regulatory loci to growth under sub-optimal environmental conditions and during murine infection. Disruption of two *in vivo* inducible loci encoding a type I topoisomerase and a putative DNA methylase revealed a significant role for the DNA methylase in growth at alkaline pH and in the presence of ethanol (5%). A MarR family regulator was required for growth at alkaline pH and in the presence of ethanol. Neither the topoisomerase, methylase, nor *marR* were involved in virulence in the murine model of infection. Disruption of the alternative sigma factor Sigma H resulted in a mutant which was mildly affected in virulence. Murine studies indicated a minor role for this sigma factor in the infectious process. Strikingly, disruption of both *perR* and *fur* genes resulted in mutants that are significantly affected in virulence, with the *fur* mutant demonstrating the greatest reduction in virulence potential. Both *perR* and *fur* mutants demonstrated significantly increased sensitivity to ethanol and alkaline conditions, and exhibited increased resistance to hydrogen peroxide. The *fur* mutant was sensitive to low iron conditions *in vitro*. The virulence defect of both *fur* and *perR* mutants was rescued by iron-overload following esculetin treatment of mice suggesting that the *in vivo* role of these loci is to procure iron for bacterial growth. Future work will concentrate on defining the roles of these genes and their associated regulons in the pathogenesis of *L. monocytogenes*.

MI 19 Analysis of the bile stress response in *Listeria monocytogenes* LO28

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Bile is one of the many barriers that pathogens must overcome in the human gastrointestinal tract in order to infect and cause disease. We have demonstrated that *Listeria monocytogenes* LO28 can tolerate the average concentration of bile/bile acids encountered *in vivo*. Data indicates that prior adaptation of the bacterium to sublethal levels of bile or other stresses encountered during food processing and sanitizing treatments (acid, heat, salt, SDS) may alter cellular physiology to enhance bile resistance. Present studies are focusing on the molecular mechanisms underlying bile tolerance. Three genes possibly involved in the degradation/transformation of bile salts were identified following analysis of the recently published *L. monocytogenes* EGDe genome. These genes are absent from the genome of the non-pathogenic strain *L. innocua*. Initial physiological analyses of deletion mutants suggest that all three genes contribute to listerial bile tolerance. In order to identify other genetic loci involved in bile stress, transposon and plasmid integration banks were screened for bile sensitive mutants. This revealed a role for a number of genes including *zurR*, *btlA* and *gadE* in bile resistance. Interestingly, all loci identified so far play putative roles in the maintenance of the cell envelope or in stress responses.

MI 20 The role of the human cationic peptide, LL-37, in bacterial infection

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Cationic peptides are being increasingly recognized as a form of host defense against bacterial infection. Some cationic peptides

are induced, in response to challenge by microbes or microbial signaling molecules like LPS. The principle aim of our research is to ascertain the role of LL-37 in clearance of bacterial infection. We have recently demonstrated that the human cationic peptide, LL-37 was able to selectively boost the innate immune response without initiating a general inflammatory response. Although LL-37 has weak antimicrobial activity, we found it was protective in several murine models of infectious disease including *Salmonella*, *S. aureus*, *Citrobacter* and *P. aeruginosa* models. We utilized large microarrays to study the global gene expression pattern changes in response to a range of peptide concentrations in the presence or absence of bacteria or bacterial components. Gene microarrays were performed on human macrophage and epithelial cells in the presence of LL-37 and in the presence of peptide and LPS. These results indicated the regulation by LL-37 of expression of genes with known or putative roles in innate immunity. Thus LL-37 appears to enhance the innate immune response against bacterial infection in ways other than acting as a direct antimicrobial agent.

MI 21 How does *Shigella flexneri* invade epithelial cells?

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Poster withdrawn

MI 22 Cholera case caused by nontoxigenic *Vibrio cholerae* O1 in an immunocompromised patient

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Poster withdrawn

MI 23 Rapid *in vivo* replication rates of *Salmonella enterica* correlate with enteropathogenesis, but not systemic infection and host specificity in pigs

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The host and bacterial factors that determine whether *Salmonella enterica* serovars cause intestinal or systemic disease within a given host remain largely undefined. Here the *in vivo* killing and growth rates of three serovars of *Salmonella*, with differing virulence for pigs, were compared by measuring the carriage and partitioning rates of the temperature-sensitive plasmid pHSG422 in bacterial populations isolated from orally inoculated pigs.

Serovar Choleraesuis caused pyrexia, diarrhoea and severe systemic infection. In contrast serovar Typhimurium caused a transient pyrexia and self-limiting diarrhoea. Serovar Dublin was avirulent. Enumeration of plasmid-bearing bacteria indicated that serovar Dublin was rapidly killed compared to the other serovars, demonstrating high sensitivity to porcine defence mechanisms and explaining the avirulence of this strain for pigs. The proportions of plasmid-bearing bacteria isolated from intestinal sites suggested that serovar Typhimurium replicated faster than serovar Choleraesuis, both within the intestinal lumen and mucosa. Despite this, serovar Choleraesuis was always isolated from mesenteric lymph nodes in higher numbers than Typhimurium. Thus, a rapid replication rate *per se* does not lead to systemic infection. This model provides a useful background for studying virulence gene expression in the context of enteric disease, systemic disease and host specificity.

MI 24 The role of Vi antigen in *S. Typhi* interactions with human monocyte derived macrophages

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Studies of *S. typhimurium* in the mouse have shown that the interaction between *Salmonella* and host macrophages is a key

event in the pathogenesis of systemic salmonellosis. Unlike *S. Typhimurium*, the human systemic pathogen *S. Typhi* can express a capsule, known as the Vi antigen. In this study we used isogenic Vi⁺ and Vi⁻ strains to investigate the affect of the capsule on the uptake and intracellular fate of *S. Typhi* Ty2 within human monocyte derived macrophages (MDMs). We found that the Vi⁻ mutant was more readily taken up by MDMs than the Vi⁺ parental strain indicating that the capsule interferes with phagocytosis of *S. Typhi*. However, the proportion of wild type bacteria positive for Vi surface expression declined dramatically following uptake. Concurrent with this decrease in expression, aggregates of the Vi antigen were observed within the cytoplasm of the infected MDMs. These results suggest that the intracellular role of the Vi antigen may be restricted to the initial stages of the *S. Typhi*-MDM interaction. Consistent with this hypothesis, gentamicin-protection assays showed no difference in the number of viable wild-type and Vi⁻ *S. Typhi* over an 8 hour infection period. Further studies are in progress to determine the nature of the Vi aggregates, and the potential interaction between this shed antigen and host-cell antigen-presenting molecules.

MI 25 Involvement of flagella in infection by *Salmonella enterica* serovar Enteritidis

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S. enterica serovar Enteritidis remains a major health problem. It generally causes a self-limiting gastroenteritis but can on occasion elicit a severe systemic infection. In this study, the roles of flagella in pathogenicity were investigated. Aflagellate mutant strains of *S. enteritidis* (fla⁻, produced by insertional inactivation of FliC) were less able than flagellate (fla⁺) parent strains to adhere to and invade rat ileal explants *in vitro*. They were also disadvantaged *in vivo*. Fla⁻ strains did not colonise or persist as effectively as fla⁺ strains in the upper gastrointestinal tract of rats. They induced less severe inflammatory responses in the gut. Furthermore, at 24-48 hours post-infection, the numbers of fla⁻ in the liver and spleen were much lower than for fla⁺. This systemic difference was however transient. From 72 hours, the numbers of fla⁻ in the liver and spleen were high and equivalent to those for fla⁺. Rats dosed with fla⁺ developed high titres of circulating antibodies against flagellin, the flagellar core protein.

Flagella are therefore important for the interactions of salmonella with both the gut and systemic tissues. They are not however a prerequisite for development of an infection. *S. enteritidis* may partially compensate for the loss of these components.

MI 26 Rat and mouse models of salmonellosis

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Salmonella species cause disease states ranging from asymptomatic carrier status through to severe systemic infection. This pathogenicity is often studied in animal models.

S. typhimurium causes a lethal infection in susceptible (ity^s) mouse strains. This is widely used a model of human typhoid-like disease. However, lethality in ity^s mice is primarily linked to the rapid systemic proliferation of the pathogen. The infection is thus atypical for this serovar.

Salmonellosis in the rat resembles self-limiting gastroenteritis. *S. enteritidis* colonises the gut, invades and spreads in moderate numbers to the liver and spleen. No proliferation occurs in systemic tissues and bacterial numbers decline between 6 and 12 days. Infiltration of inflammatory cells and disruption of the gut are evident.

Resistant (ity^r) mice, such as C3H/HeN, develop a persistent infection when exposed orally to high numbers of *S. enteritidis*. There is colonisation of the gastrointestinal tract. Moderate numbers are found in the liver and spleen but little or no proliferation occurs in these tissues. There is infiltration of inflammatory cells and disruption of the gut. The infection may not be self-limiting.

The rat and resistant (ity^r) mouse could be useful models for evaluating effects of host, bacterial and dietary factors on salmonellosis.

MI 27 The immunodominance of *Campylobacter jejuni* glycoproteins during human infection

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Recent work has identified a system of general protein glycosylation in the zoonotic pathogen *Campylobacter jejuni*. Many surface-exposed proteins appear to be glycosylated and highly immunogenic when blotted with rabbit serotyping sera. To determine the antigenicity of these proteins during human infection a number of western blotting studies were done. Serum from an infected co-worker, taken 1 day after *C.jejuni* isolation and 21 days later was used in blots of acid-glycine extracted surface proteins of *C.jejuni* strain 81116 separated by 2D SDS-PAGE. A number of proteins were recognised by antibodies in the second serum sample that weren't recognised by antibodies in the second sample. The corresponding spots were excised from silver-stained 2D gels and analysed by mass spectrometry following tryptic digestion. All of the proteins identified were part of a set of proteins identified by Young et al (2002) as glycoproteins containing an Asn-linked glycan. The immunogenic importance of the glycan was confirmed with blots of *C.jejuni* mutants with inactivated genes (*wla/pgl*) involved in protein glycosylation, which showed the non-glycosylated proteins in the mutant were no longer recognised. The identity of the proteins and possible biological significance of glycosylation will be presented.

MI 28 Characterisation of genomic differences between two *Campylobacter jejuni* strains with differing chicken colonisation potentials

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C. jejuni is a major cause of acute bacterial enteritis in man. A major vehicle of infection is the consumption of contaminated poultry meat. Identification of bacterial factors involved in chicken colonisation may lead to control measures to reduce and eventually eradicate *C.jejuni* from the food chain. We have used subtractive hybridisation to compare two strains of *C.jejuni* with differing colonisation potentials. Strain NCTC11168 has been genome sequenced and is a poor coloniser in the 1-day chick model. In contrast strain 81116 is able to colonise the chicken gut in a dose dependent manner. Twenty-three DNA fragments were found to be present in strain 81116 yet absent from strain NCTC11168 (Ahmed *et al.*, 2002). At the amino acid level, similarities to restriction modification enzymes (6 fragments) and genes involved in arsenic resistance (2 fragments) were identified. Some fragments had partial homologies with hypothetical proteins or proteins with assigned functions in strain NCTC11168. Several fragments had no database matches. We isolated and sequenced the genes from two of the fragments. One had similarity to gamma-glutamyltranspeptidase (*ggt*) and the other to a di-tripeptide transporter (*dtpT*). Mutants in each of these genes were generated by insertional inactivation with an antibiotic cassette. Preliminary characterisation of these mutants, using both *in vitro* and *in vivo* methods, has suggested a potential

role in both motility and colonisation for the *dtpT* orthologue mutant. We have also determined the genomic location of an additional fragment, which has similarity to a gene essential for DNA transfer in bacterial conjugation and also type IV secretion systems (*traG*). The fragment appears to be part of a previously unidentified 50kb island which encodes an integrase, contains inverted repeat sequences and is flanked by 18 bp direct repeat sequences.

MI 29 Difference of susceptibility to *Salmonella typhimurium* infection in C3- and FcR γ chain-deficient mice

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Mice infected with *Salmonella typhimurium* are commonly used as a model to study *S. typhi* infections in humans (typhoid fever). Our aim in the present study is to determine the role of complement and antibody binding to FcR γ in host resistance to *Salmonella* in the mouse model. In the early stages of *S. typhimurium* infection, C3^{-/-} C57BL/6 mice showed higher bacterial loads in the spleen and liver as compared to FcR γ ^{-/-} C57BL/6 or control C57BL/6 mice. Additionally, passive transfer of *Salmonella*-immune serum in C57BL/6 and C3^{-/-} C57BL/6 mice prior to *Salmonella* infection was sufficient to significantly reduce the bacterial load in the tissues. In the presence of immune serum FcR γ ^{-/-} C57BL/6 mice showed however a less pronounced protection to *S. typhimurium*. All together these results indicate a different role for the C3 component of the complement cascade and FcR γ receptors in immunity to *Salmonella*.

Monday 7 April 2003

0900 Gene manipulation in *Clostridium perfringens*

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C. perfringens genetics is the most advanced of the pathogenic clostridia. Several strains can be electroporated, there are numerous shuttle vectors, and chromosomal mutants can be constructed by allelic exchange. The genome sequence of one strain is published and that of the type strain has been completed. We have used these genetic tools to study the role of extracellular toxins in disease and the role of the two-component VirSR system in the regulation of toxin gene expression. This system directly activates the perfringolysin O gene, *pfoA*, and indirectly regulates other genes, including the α -toxin gene. Random mutagenesis studies have led to the identification of a putative transmembrane region that is essential for the function of the VirS sensor kinase. Bioinformatic analysis followed by site-directed mutagenesis has shown the C-terminal domain of the VirR response regulator contains a conserved FxRxHrS motif that is essential for the functional integrity of the VirR protein and is probably involved in DNA binding. Finally, we have shown that VirR binds independently to two directly repeated VirR boxes located immediately upstream of the *pfoA* promoter. Transcriptional activation requires VirR binding to both VirR boxes, which must be located on the same face of the DNA helix.

0945 Gene manipulation in *Bacteroides* species

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Great progress has been made over the past two decades toward developing an advanced genetic system for investigating the biology of *Bacteroides* species. It is now possible to create targeted gene disruptions, to do transposon mutagenesis and to introduce genes on multicopy plasmids. Moreover, the recent availability of genome sequences for 2 strains of *Bacteroides fragilis* and 1 strain of *Bacteroides thetaiotaomicron* will aid considerably in finding genes and generating hypotheses for further testing. Using the *B. thetaiotaomicron* sequence database, we have already found some possible conjugative transposons whose existence we had not previously suspected. In addition, it appears that some of the vectors developed for use in *Bacteroides* species will be useful in studies of other members of the *Bacteroides*-*Cytophaga*-Flavobacterium group. Some problems remain, however. Not all strains of *Bacteroides* can be genetically manipulated, and the reason for this is not clear. Providing a heterologous promoter for a gene has also proved problematic; sometimes the construct functions to give high levels of gene expression and sometimes it does not. Spacing between the promoter and the start codon seems to be important. A third problem is that the construction of deletions in the chromosome is still very difficult. Finally, indicators like X-gal do not work in the reduced environment in which anaerobes are cultivated. Despite problems, however, it is now possible to do a great deal of genetic work using *Bacteroides* strains.

1100 Gene transfer in the gastro-intestinal tract

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There is huge potential for genetic exchange to occur within the dense, diverse anaerobic microbial population inhabiting the gastro-intestinal tracts (GIT) of humans and animals. The resident commensal bacteria form a reservoir of genetic

information that can be accessed by them and any incoming bacteria, including pathogens. Any gene acquisition, either by transformation or conjugation, contributes to gene flow and bacterial evolution. The natural incidence of mobile genetic elements such as conjugative plasmids and transposons has not been well studied among the commensal GIT anaerobic bacterial population. These genetic elements often contain non-essential genes, for instance encoding antibiotic resistance, heavy metal resistance and even virulence determinants. Recent evidence has shown that commensal anaerobic bacteria native to the rumen or human GIT harbour both novel antibiotic resistance genes and novel conjugative transposons. Transfer of conjugative plasmids and transposons has been demonstrated between a wide range of commensal bacteria under laboratory and simulated *in vivo* conditions. The presence of identical resistance genes in diverse bacterial species from different hosts provides evidence that gene transfer has occurred in nature between GIT bacteria, and between GIT bacteria and pathogenic bacteria.

1145 The problems and possibilities of conjugative transposons in the anaerobes

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Conjugative transposons are discrete, non-replicative DNA elements, usually integrated into the bacterial genome, which can transfer from a donor to a recipient by conjugation. These elements are extremely common in anaerobes where they frequently mediate the spread of antibiotic resistance genes. The fact that most conjugative transposons have an exceedingly broad host range makes them a serious clinical problem as they can readily transfer their antibiotic resistance to other, often unrelated bacteria. There is also evidence that these elements can exchange modules with other mobile genetic elements to further increase their host range. It is also these characteristics, which cause problems in the nosocomial environment, that enable the use of conjugative transposons as tools for the genetic manipulation of anaerobes, both for insertional mutagenesis and as cloning vectors. Furthermore various modules of these elements have been used in the construction of novel vectors for the genetic manipulation of anaerobes. This talk will review our current understanding of the biology and exploitation of conjugative transposons in anaerobes.

1400 Phosphate limitation in *clostridium acetobutylicum*

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Limitation of growth by the amount of phosphate supplied in the medium favours the onset of sporulation in clostridia. In addition, in *Clostridium acetobutylicum* phosphate-limitation in combination with pH values below 5 results in a metabolic shift from acid to solvent formation. To elucidate the role of phosphate limitation within the complex regulatory network of sporulation and solventogenesis, we have started to analyse the phosphate regulon of *C. acetobutylicum*. The mRNA levels of two operons, encoding a two-component regulatory system and an ABC-transport system and tentatively designated *phoPR* and *pstSCABphoU*, respectively, were found to be up-regulated under phosphate limitation. Interestingly, Northern blot analysis revealed that in a phosphate-limited chemostat at a pH-value of 4.5 (conditions optimal for solvent production) the level of the

pstS-specific mRNA in *C. acetobutylicum* is decreased by a factor of eight and of the total *pst* operon by a factor of two. Under the assumption that no alternate high-affinity phosphate uptake system becomes active under these conditions, it might be that the impaired supply of cells with phosphate is crucial for the initiation of solvent formation (and sporulation). In agreement with our hypothesis we observed faster phosphate consumption in cultures of *C. acetobutylicum* at higher pH values.

1445 Post-translational modifications to the proteases of *Porphyromonas gingivalis*

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The presence of glycoproteins in prokaryotes has been demonstrated only recently. Examples include cell wall proteins in the archaea, secreted or cell wall-associated enzymes of clostridia and flavobacteria and proteins from human pathogens involved in interactions with the host such as the pilin proteins of pathogenic neisseriae and *Pseudomonas aeruginosa* and surface antigens of *Mycobacterium tuberculosis*. Despite increasing evidence that these post-translational additions are of major significance to the biological and chemical properties of the resultant glycoconjugates, the biochemistry and genetics of glycoprotein biosynthesis in bacteria are poorly characterised. We recently demonstrated that the extracellular cysteine proteases specific for arginyl peptide bonds of the oral anaerobic pathogen *Porphyromonas gingivalis* are members of this growing family of prokaryotic glycoproteins. The Arg-gingipains, Rgps, of *P. gingivalis* W50 are encoded by two homologous genes, *rgpA* and *rgpB*, which give rise to five isoforms: HRgpA, RgpA_{cat}, mt-RgpA_{cat} (derived from *rgpA*), RgpB and mt-RgpB (derived from *rgpB*). HRgpA is a non-covalently linked heterodimer of the α -catalytic chain and a β adhesin chain derived from the C-terminus of the initial translation product. Conversely RgpA_{cat} and RgpB (and their membrane type forms: mt-RgpA_{cat} and mt-RgpB) are composed of an α -catalytic chain which is covalently modified with different amounts of carbohydrate up to 30% by weight. Immunochemical analyses have demonstrated that the glycan additions to RgpA_{cat} and both membrane type enzymes are cross reactive with a capsular polysaccharide of this organism suggesting that there may be a link between the synthesis of this cell surface macromolecule and the post-translational maturation pathway of these enzyme isoforms. Conversely the glycan additions to RgpB appear to be immunologically unrelated to this cell surface polymer. We are currently examining the genetics of protease glycosylation in *P. gingivalis* by a targeted mutagenesis approach utilising the genome sequence of this bacterium. A candidate locus on the chromosome was identified which contains genes homologous to those putatively involved in glycosylation in *Neisseria meningitidis*, *Caulobacter crescentus*, *P. aeruginosa* and *Campylobacter jejuni*. Mutagenesis of a *porR* gene at this locus leads to loss of those enzyme isoforms which are immunologically related to the surface polymer and also influences the immune recognition of this macromolecule. In contrast, the post-translational additions to RgpB were unaffected in this *porR* mutant. Consequently it appears that the *porR* locus in *P. gingivalis* is required for glycosylation of the Rgps whose glycans share structural similarity to capsule polysaccharide whereas RgpB is glycosylated via a *porR* independent mechanism.

1600 Signalling and gene regulation in *P. gingivalis*

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Porphyromonas gingivalis is an aggressive pathogen in severe forms of periodontal disease. In the oral cavity, *P. gingivalis* exists both within the complex multispecies plaque biofilm and in close association with the epithelial cells that line the gingival crevice. Colonization of dental plaque involves an initial adherence event followed by communication with antecedent plaque bacteria and accumulation of additional *P. gingivalis* cells into a biofilm. Accretion of *P. gingivalis* into a biofilm on a

substratum of *S. gordonii* involves cross-species LuxS-based signalling. Differential display RT-PCR has identified groups of *P. gingivalis* genes that are regulated by the initial adherence event and by autoinducer-2, produced through the action of LuxS. Association with gingival epithelial cells results in internalization of *P. gingivalis*. Multidimensional proteomics was utilized to investigate the pattern of protein expression in *P. gingivalis* as it adapts to this environment. *P. gingivalis* whole cell proteins were separated by 2D capillary HPLC and analyzed by ion trap mass spectrometry. Approximately 70% of the theoretical proteome of the organism was identified by this method. Several hundred proteins were differentially expressed by *P. gingivalis* in association with epithelial cells. *P. gingivalis* is thus capable of sensing and adapting to distinct oral environments through orchestrated regulation of gene expression.

1645 Toxin regulation in *Clostridium difficile*

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Clostridium difficile, the major source of antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (PMC), is also the most frequently identified source of hospital-acquired infectious diarrhea in man. Virulent strains of *C. difficile* produce two toxins ToxA and ToxB. Both toxins belong to a class of proteins named large clostridial cytotoxin that share the same general structure and enzymatic function. They monoglucosylate mammalian Ras-related, small GTP-binding proteins which leads to the depolymerization of the actin filaments and cytotoxicity. The transition from mild diarrhea to potential lethal PMC, seems to be due to differential expression of colonization and virulence factors, especially genes for production of toxins A and B. However, the molecular mechanism(s) that triggers this transition was unknown. The transcription of the *tox* genes was found to be growth phase dependent and carbon source regulated and we have shown that temperature controlled toxin synthesis at the level of transcription, with an optimum at 37°C. Expression of the *tox* genes under physiological conditions tested, is strongly dependent on TxeR, the product of the gene located upstream of the *toxB-toxA* cluster. By *in vivo* and *in vitro* transcription assays we demonstrated that TxeR is a novel RNA polymerase sigma factor that permits core RNA polymerase to recognize the promoters of the *toxA* and *toxB* and initiate their transcription. Several other positive regulators of toxin and bacteriocin genes, including *C. tetani* TetR, *C. botulinum* BotR and *C. perfringens* UviA are related to TxeR and we showed recently that UviA also functions as a sigma factor, activating core RNA polymerase to bind to and transcribe from the *C. perfringens* *bcn* promoter. Because sequences of these proteins bear little discernible resemblance to other members of the σ^{70} family, we suggest that TxeR and UviA are the first members of another distantly related group (herein designated group 5) of the σ^{70} family.

Tuesday 8 April 2003

0900 Bacterial diversity in the human large intestine

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The large intestine is principal site of permanent bacterial colonisation of the human gastrointestinal tract. The adult colon contains approximately 200 g of digestive material, which is equivalent to a total bacterial cell mass of about 90 g. Bacteria are therefore a major component of faecal material, where viable cell counts are usually in excess of 10^{12} per gram of gut contents. However, many intestinal bacteria do not appear in culture using traditional methods, and molecular analyses (DGGE, TRFLP) of the microbiota indicate that considerably more species are present in the colon than was originally thought. Several hundred different types of bacteria exist in the large bowel, most of which are anaerobes. Microscopic observations show that cell numbers increase distally through the gut. There is some evidence for changes in bacterial community structure in the proximal and

distal colons, while distinct differences occur in microbial metabolism, that are related, in part, to substrate availability and bowel function. Overall, the large intestinal microbiota exhibits great metabolic diversity, rivalling that of the liver. Intestinal contents are not homogeneous, and biofilm communities colonising the surfaces of food residues in the gut lumen are metabolically distinct to non-adherent populations. Other biofilms exist on the colonic mucosa, although little is known of their metabolism or how they interact with the host.

0945 S-layer proteins provide new insight on *Clostridium difficile* adhesion

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Clostridium difficile is the well known etiological agent of most cases of pseudomembranous colitis and antibiotic-associated diarrhoea. Besides the studies on toxins A and B, recent approaches focus more and more on surface structures with adhesive properties that, facilitating colonization of the gut, could promote toxin delivery. We described the presence of two superimposed paracrystalline surface layers on different *C. difficile* strains, each composed of a glycoprotein subunit which slightly varies in molecular mass in the different strains examined. Both subunits were encoded, as a precursor protein, by a gene (*slpA*) located in a cluster carrying 17 ORFs in the same orientation. The N-terminal domain of the S-layer protein precursor of *C. difficile* 253 appears to be located on the exterior surface of the bacteria and shows adhesive properties in Caco-2 cell monolayers. Another protein codified by one of the ORFs in the cluster is the Cwp66 of *C. difficile* 79-685 with the C-terminal domain sharing 77% of identity with *C. difficile* 253 *slpA*. This Cwp66 protein has also been characterised as a surface protein with adhesive properties. The isolation of this cluster is an important step in the characterisation of the process of colonization by *C. difficile*, suggesting the presence of multiple surface-exposed proteins, including S-layer proteins, acting as adhesins.

1120 The genome sequence of *Bacteroides fragilis* answers a 20-year old question

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Bacteroides fragilis is a member of the normal resident intestinal microbiota of humans and an important opportunistic pathogen. It is the obligately anaerobic Gram negative bacterium most frequently isolated from clinical infections. These include intra-abdominal, vaginal, pilonidal, perianal and brain abscesses. It is also the most common cause of anaerobic bacteraemia, with a potential mortality of up to 19%. Such infections are generally considered to arise from faecal contamination of normally uncolonised body sites. The risk of *B. fragilis* infection after surgical operations involving the lower intestinal tract and hysterectomy requires prophylactic antibiotic cover. Prior to the introduction of antibiotics, mortality from infection arising from colonic perforation, for example as a result of a ruptured appendix, was between 30 to 100%. Even in the 1970s intra-abdominal abscesses were associated with up to 30% mortality, largely due to a lack of appreciation of the role of anaerobes in these infections and therefore lack of treatment. Post-operative anaerobic infections and peritonitis following appendicitis and bowel perforation can prolong hospitalisation time of patients and involve intensive and costly antibiotic therapy. Potential virulence determinants include resistance to complement mediated killing and phagocytic killing by human neutrophils in vitro. Phase and antigenic variation of the surface components of *B. fragilis* has been long recognised. Bacteria with different sizes of encapsulating structures can be observed within a population

of an individual strain by both light and electron microscopy. In addition, labelling with monoclonal antibodies reveals extensive antigenic variation within populations enriched for one capsular type. The underlying mechanism generating the variation was unknown until analysis of the complete genome sequence of *B. fragilis* (http://www.sanger.ac.uk/Projects/B_fragilis/); multiple inverted sequences that potentially control the variation of a range of surface components, including polysaccharides and proteins have been identified. In addition, some of the invertible regions are between divergent coding regions, while some include, and invert, large coding sequences, generating complex inversions. A Salmonella H-flagellar antigen invertase homologue, Fin2, which binds to the invertible regions associated with polysaccharide variation, has been identified and is encoded on a 36.5 kbp plasmid.

B. fragilis appears to be capable of an unprecedented number of potential DNA inversion events.

1205 Comparative analysis of clostridial genomes

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Genomes from ongoing and completed clostridial genome sequencing projects have been compared using MUMMER and BLAST. Detailed comparisons of genes encoding surface and secreted proteins and virulence factors have been performed and interesting differences found in sortase-sorted proteins and WXG100 protein secretion systems. We will also create a new online resource for comparative clostridial genomics, ClostridiaBase (<http://clostridiabase.bham.ac.uk>), which falls within the umbrella of our ViruloGenome site (<http://www.vge.ac.uk>).

1400 Polymer breakdown and the manipulation of gut metabolism

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Plant polysaccharides that cannot be degraded by mammalian digestive enzymes provide major energy sources for microbial growth in the rumen and large intestine of mammals. Anaerobic gut micro-organisms use a variety of mechanisms to compete for these substrates. These range from cellulosome-type enzyme complexes found on the cell surface in cellulolytic gram-positive ruminococci, to binding proteins and periplasmic hydrolases found in starch and xylan-utilizing members of the gram-negative CFB phylum. In the rumen, eukaryotic micro-organisms also make a significant contribution to polysaccharide breakdown. Understanding the enzymatic mechanisms of microbial degradation can help to optimise the diverse uses of plant products in food, feed, and elsewhere. In addition, there is increasing interest in using particular poly/oligo-saccharides to promote desired fermentation patterns and microbial balance in the large intestine of humans and of farm animals. It is important to develop a better understanding of microbial competition for these substrates in the GI tract, which must consider population dynamics and cross-feeding within the gut ecosystem, as well as the enzyme systems of individual species.

1445 Clostridia in cancer therapy

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The possibility of using gene therapy to treat cancer is actively being pursued through the use of various viral (retroviral, adenoviral, adeno-associated virus) and a number of non-viral delivery systems. However, fundamental problems exist with regard to an inability of these systems to specifically target tumours and their poor distribution throughout the tumour mass. To circumvent these deficiencies, we have proposed the use of a harmless clostridial-based delivery system. It is based on the fact that intravenously injected clostridial spores exclusively germinate and grow in the hypoxic regions of solid tumours. They cannot grow in healthy normal tissue because the required anaerobic conditions are not available. Thus, clostridial spores may be employed to deliver therapeutic agents to tumours. Specifically, it may be employed to deliver recombinant prodrug-converting enzymes, able to bring about the localised conversion of innocuous circulatory prodrugs into cytotoxic drugs agents. Here we discuss progress on the exploitation of this novel property for the treatment of solid tumours.

1600 Novel therapeutics from neurotoxins

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The clinical utility of botulinum neurotoxin derives from its potent and specific inhibition of neuronal acetylcholine release, particularly from motorneurons. An important clinical feature of the neurotoxins is their prolonged duration of action. Given the unique clinical value of neurotoxin, we have sought to develop novel therapeutic agents derived from the neurotoxin enabling their potency and duration to be more widely applied to therapeutic use. The neuronal specificity of clostridial neurotoxins derives from the carboxy terminal half of the heavy chain. Removal of the C-terminal domain of the heavy chain of neurotoxin produces an endopeptidase fragment (termed LH_N) deficient in cell binding. Conjugation of LH_N to a ligand creates novel agents in which the cell binding function is provided by the ligand. Coupling the LH_N fragment of serotype A (LH_N/A) to wheatgerm agglutinin (WGA) provided a conjugate able to inhibit secretion in a range of cell types, including non-neuronal, demonstrating the feasibility of retargeting neurotoxin endopeptidase activity. Using a different ligand, *Erythrina cristagalli* lectin (ECL), coupled to LH_N fragments resulted in conjugates with selectivity for nociceptive primary sensory afferents compared to anatomically adjacent spinal neurons. LH_N/A-ECL potently inhibits release of neurotransmitters in *in vitro* primary nociceptor neuronal culture systems for many weeks. LH_N/A-ECL also displays long lasting analgesic properties in a range of *in vivo* models of pain. LH_N/A-ECL therefore provides proof of principle for the concept of targeted clostridial endopeptidases as agents with therapeutic (analgesic) potential.

1645 Clostridial vaccines

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The toxigenic Clostridia produce a diverse and important group of toxins, many of which are essential for a successful infection or intoxication. However, for over 100 years, man has been able to harness the power of the immune system to design vaccines to counter the effects of these toxins. Conventional toxoid vaccines are based on formaldehyde inactivation of toxins, and provide variable levels of protection depending on toxin, method of detoxification, dose of vaccine, and route of immunisation. We have explored the immunogenicity of tetanus toxin and have shown that the 50 kDa carboxy terminal fragment (termed

fragment C or Hc) can provide solid protective immunity against tetanus. The Hc fragment functions to target the toxin to neuronal cells and contains determinants for binding to gangliosides and protein receptors. Tetanus Hc vaccines can confer protection in animals when given as pure protein, as DNA vaccines and when expressed in attenuated bacteria and given orally. We have recently expanded our studies to the related toxin, botulinum type F. We will discuss our results in context of the structure of the clostridial neurotoxins and will discuss how our approach may influence vaccine design.

PHYSIOLOGY, BIOCHEMISTRY & MOLECULAR GENETICS GROUP - POSTERS

PBMG 01 Cloning of a putative membrane-anchored α -amylase from a human colonic Gram-positive anaerobe

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Resistant starch makes up a large proportion of the dietary substrate reaching the human colon, where it is fermented by human colonic bacteria to produce short-chain fatty acids (SCFA) as major end-products. Resistant starch is a potential prebiotic, but knowledge at a molecular level of the enzymes involved in starch degradation by colonic commensal bacteria is limited to the gram negative *Bacteroides* spp.. Recent studies have shown that low % G+C gram-positive bacteria are among the most abundant components of the adult faecal flora. Amylases of several new isolates belonging to the Clostridial cluster XIVa were visualised using zymogram analysis and found to be of high molecular weight.

An amylase gene from the human colonic strain *Butyrivibrio fibrisolvens* 16.4 was sequenced following polymerase chain reaction (PCR) amplification with degenerate oligonucleotides and chromosome walking. The putative amylase consists of an open reading frame of 4038 nt encoding a protein of 1333 amino acids with a calculated M_r of 144,470. The catalytic domain shows high sequence homology with a previously characterised α -amylase from a ruminal *B. fibrisolvens* strain and shares the conserved active centres in the N-terminal half of the protein. The highly charged C-terminus contains two repeat regions of unknown function that may be involved in binding to resistant starch. The C-terminus also contains several domain types that may be involved in cell wall and membrane binding of the enzyme. Functional studies should help elucidate the function of this novel C-terminus and provide insights in how extracellular enzymes are organised in this group of bacteria.

PBMG 02 Construction of recombinant conjugative transposons to deliver antisense RNA into the nosocomial pathogen *Clostridium difficile*

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Clostridium difficile is the causative agent of most (90%) of the reported cases of pseudo-membranous colitis and almost a third of cases of post antibiotic-associated colitis. The pathogenesis of *C. difficile* is mediated by a variety of virulence factors, the most studied of which are the enterotoxin (toxin A) and the cytotoxin (toxin B). Other virulence factors include proteins that mediate adhesion, such as Cwp66. One factor that has severely limited research into the virulence of *C. difficile* is the inability to transform the organism making genetic manipulation difficult. Until recently the only way to introduce heterologous DNA into the organism was to use conjugative transposons. We have

developed a recombinant conjugative transposon, derived from Tn916, which enables delivery of antisense constructs that can be used to regulate gene expression. This system was used to introduce an antisense *cwp66* construct into *C. difficile*. Expression of the asRNA was observed, by RT-PCR, however there was no significant alteration in Cwp66 expression or cell adherence. Nevertheless the use of recombinant conjugative transposons has now proven to be a useful tool for the introduction and expression of heterologous DNA into *C. difficile*.

PBMG 03 Alternative fermentation pathways for butyrate formation by human colonic bacteria

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Butyrate, which is formed in the human colon as a bacterial fermentation product, serves as the prime energy source for the colonic epithelium and is believed to have beneficial effects on human colonic health. Butyrate generating bacteria are strictly anaerobic Gram positives related to the low mol% G+C clostridia. Two alternative metabolic pathways exist for the final step of butyrate formation from butyryl-CoA: the butyrate kinase and the butyryl-CoA:acetate CoA transferase pathway. We screened a wide range of butyrate producing human faecal isolates for the presence of these pathways employing degenerate PCR as well as enzymatic studies. The butyrate kinase pathway was only found in a relatively small number of the isolates, whereas the CoA transferase pathway was widespread among human gut isolates. It seems therefore, that the CoA transferase is the major route for butyrate generation in this ecosystem. Acetate is required as a co-substrate for this reaction and is usually present in high amounts in the human large intestine (averaging approximately 60 mM). Thus, this suggests that the colonic environment may select for groups of bacteria employing this pathway.

PBMG 04 Cellulosome of *Clostridium cellulolyticum* analysed by antisense strategy

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The anaerobic bacterium *Clostridium cellulolyticum* secretes many cellulases, the major of them being organised into large complexes called cellulosomes. Enzymatic component of this macromolecular machine are assembled on a scaffolding protein which is devoid of enzymatic activity and ensures anchorage on cellulose. To date the genes coding for 12 enzymes, almost all cellulases, which can be incorporated in the cellulosomes have been sequenced and many of the corresponding proteins extensively studied. Among them, the cellulases Cel48F and Cel9E are represented in large amount compared to other enzymes. The influence of the enzymatic composition of the system on its activity towards cellulosic substrates has been analysed by *in vivo* inhibition of gene expression using an antisense RNA (AsRNA) strategy. In a first step, we constructed an *E. coli*-*C. cellulolyticum* shuttle vector containing an AsRNA gene in order to knock down *cel48F* mRNA. The cellulolytic system purified from the transformant showed a remarkable decrease in Cel48F content compared to the wild-type system, accompanied by a lower activity on crystalline cellulose. Analysis of other AsRNA expressing strains are in progress.

PBMG 05 Co-haemolytic activity of *Propionibacterium acnes*

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The Gram positive anaerobic coryneform bacterium, *Propionibacterium acnes*, is a member of the normal human microbiota. *P. acnes* is postulated to be pathogenic in the skin condition *acne vulgaris* and is also associated with dental, ocular and implant associated infections. In a study of failed prosthetic hip joints that required revision surgery, *P. acnes* was detected as frequently as coagulase-negative *Staphylococcus* spp.

A 30-kDa surface associated and secreted protein antigen was identified in abundance in *P. acnes* prosthetic hip isolates, but not in culture collection strains or skin isolates, by labelling with specific monoclonal antibodies. The antigen was purified and the N-terminal sequence obtained. Based on this sequence the antigen was identified as a co-haemolysin, or CAMP (Christie Atkins Munch-Petersen) factor, from the *P. acnes* genome sequence. A further four CAMP-factor homologues were identified in the *P. acnes* genome. The classical CAMP reaction is synergistic haemolysis of sheep erythrocytes by a co-haemolysin of *Streptococcus agalactiae* and the beta-toxin of *Staphylococcus aureus*, first described by Christie *et al.* in 1944. A similar co-haemolytic reaction of sheep erythrocytes with *S. aureus* beta-toxin was demonstrated in all *P. acnes* isolates studied. CAMP-factor activity could be blocked with specific antiserum. Polymerase chain reaction (PCR) amplification confirmed the presence of all CAMP homologue genes in all *P. acnes* isolates studied, although some of these strains did not produce all five CAMP factors. The relevance of CAMP factor production to prosthetic joint infection remains to be determined.

PBMG 06 The influence of external factors on *recA* activation and phage lambda repressor in *E. coli*

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recA is a major recombinase in *E. coli*. In addition to its capacity to catalyse DNA recombination it also has the ability to function as a protease, specifically cleaving the phage lambda repressor when it is activated as part of the SOS cascade in a lysogenised *E. coli* that has incurred DNA damage.

Cleavage of the phage lambda repressor allows transcription of phage lambda genes resulting in induction of a phage from a lysogenic state to a lytic cycle and the subsequent lysis of the host *E. coli*.

Chemostat culture and a range of molecular techniques are being employed to investigate *recA* activation and the possible *in vivo* autolysis of phage lambda repressor.

The aim is to elucidate conditions under which non-mutagenic induction of a bacteriophage can be achieved, thus providing a novel consumer friendly method for the control of food-borne pathogens.

PBMG 07 Polysaccharide variation in *Bacteroides fragilis*

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Bacteroides fragilis is an important opportunistic pathogen present in the normal human resident microbiota. This Gram negative, obligately anaerobic microbe has been isolated from intra-abdominal, brain and lung abscesses either alone or as part of a mixed infection. A number of characteristics have been implicated in the microbes virulence including extracellular polysaccharide production, release of extracellular enzymes, iron scavenging mechanisms and enterotoxin production. Phase variation is evident by light microscopy whereby bacteria can be seen with a large or small polysaccharide encapsulating structure, as well as non-capsulate bacteria within one population. Electron microscopy reveals that the bacteria which appear to be non-capsulate by light microscopy possess an encapsulating electron dense layer. Antigenic variation in these surface structures has been demonstrated using monoclonal antibodies specific for the high molecular mass polysaccharide as detected by PAGE and immunoblotting profiles. Analysis of the whole genome sequence of NCTC 9343 has revealed 7 potential polysaccharide biosynthesis operons upstream of which are invertible regions (fin 1-7) flanked by repeated sequences (*fixL* and *fixR*) homologous to those found in *Salmonella typhimurium* (*hixL* and *hixR*). In addition, two Hin-like recombinases, (Fin1 and Fin2) have been identified and have been shown to bind to the invertible repeat sequences. We also present data comparing within-strain phase and antigenic variation in recent faecal and clinical isolates from a variety of anatomical and geographical sites which indicate that this mechanism is not confined to the *B. fragilis* type strain.

PBMG 08 Analysis of the *Clostridium difficile luxS* signalling system

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¹Institute of Infections and Immunity, University of Nottingham, NG7 2RD; ²Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wilts SP4 0JG *Clostridium difficile* is a multiply antibiotic resistant human pathogen, which is becoming increasingly difficult to treat. As a consequence, novel therapeutic agents are urgently needed. The attenuation of virulence through the blockade of bacterial cell-to-cell communication (quorum sensing) is one potential therapeutic target. To begin our exploration of quorum sensing and the pathogenicity of *C. difficile*, we focused on *luxS*/autoinducer-2 (AI-2) signalling.

Cell-free culture supernatants of *C. difficile* induced bioluminescence in a *Vibrio harveyi* AI-2 biosensor indicating that this pathogen produces AI-2 or a related signal molecule. The cloned *C. difficile luxS* gene complemented the AI-2 negative *E. coli* DH5 α *in trans* and restored AI-2 production. Genome sequencing suggests that the *C. difficile luxS* homologue forms part of an operon which includes two other genes (a *methH* homologue and a gene of unknown function) and an adjacent two-component system. To inhibit expression of the *luxS* operon genes and putative response regulator, we have employed an antisense RNA approach. While we have so far been unable to block AI-2 production, AI-2 levels could be modulated by controlling expression of the putative transcriptional regulator. To our knowledge, this is the first reported use of antisense technology for the modulation of gene expression in *C. difficile*.

PBMG 09 Sequence analysis of TnB1230 and other genetic elements found to harbour *tet(W)* in bacteria from diverse habitats

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The world-wide use of antibiotics in human and veterinary therapeutics and as prophylactics and growth promoters, has resulted in rapid emergence and spread of antibiotic resistance. Whilst previous studies have concentrated on resistance in pathogens, commensal bacteria are now recognised as important reservoirs for these genes. We identified a novel tetracycline resistance gene, *tet(W)* amongst important rumen, porcine and human commensal bacteria. The remarkable sequence conservation between different copies of *tet(W)* found in environmental and phylogenetically distinct bacteria implies rapid transfer in nature.

Previous work showed that *tet(W)* could be transferred *in vitro* between strains of the rumen anaerobe *Butyrivibrio fibrisolvens*. However, very little is known about transfer of *tet(W)* in *B. fibrisolvens* or in other Gram-positive anaerobes known to harbor *tet(W)*.

This work provides the first evidence *in vivo* for the presence of *tet(W)* on a conjugative transposon and the first identification of a conjugative transposon from a rumen obligate anaerobe. Analysis of 20 kb of sequence flanking *tet(W)* on TnB1230 in *B. fibrisolvens* reveals many open reading frames with close similarity to the transfer region of a van^R conjugative transposon from *Enterococcus faecalis*, Tn1549. The region flanking *tet(W)* in other rumen and human bacteria has also been compared.

PBMG 10 Development of host vector systems for *Clostridium sporogenes*/ *Clostridium botulinum* based on an indigenous clostridial plasmid, pBP1

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Members of the bacterial species *Clostridium botulinum* are not a homogenous assemblage and may be divided into at least four different species, or groupings. Group I, proteolytic *C. botulinum* strains may simplistically be considered strains of *C. sporogenes* that have acquired the genes encoding botulinum neurotoxin and associated complex proteins. Paradoxically, strains of *C. sporogenes* are being investigated as possible tumour delivery systems for prodrug-converting enzymes in cancer therapy. Effective host vector systems are therefore required, both to progress this applied aim and to assist in the analysis of the soon to be completed genome sequence of *C. botulinum* ATCC 3502 (http://www.sanger.ac.uk/Projects/C_botulinum/). Towards this aim we have isolated a cryptic plasmid, pBP1, from a Group I strain of *C. botulinum*, identified its replication functions, and used the information to construct a number of clostridial/ shuttle vectors. These have been introduced into various clostridial strains (including *C. botulinum* and *C. sporogenes*) by either electroporation or conjugative mobilisation. Once introduced, these vectors have proven remarkably stable in terms of segregational stability in the absence of antibiotic selection.

PBMG 11 The introduction of autonomous plasmid vectors into the *Clostridium difficile* genome strain CD630 via conjugative transfer from *Escherichia coli* donors

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Despite the increasing importance of *Clostridium difficile* in human disease, the organism remains extremely poorly characterised, particularly with regard to virulence mechanisms. Significant insight is likely to accrue from the determination of the genome sequence, to be completed at the Sanger Institute during 2002. However, to maximise the benefits of this data, systems of genetic manipulation and transfer are urgently required.

Recently, we were able to show that plasmids based on an indigenous clostridial replicon could be introduced into two toxigenic strains of *C. difficile* (CD3 and CD6) via *oriT*-based conjugative transfer from *Escherichia coli* donors. Transfer was dependent on the vectors being appropriately methylated, due to the presence of formidable restriction barriers. We have shown by a combination of *in silico* analysis and experimental evaluation of cloned DNA, that the CD630 genome strain possesses five distinct methylase genes. However, there are no cognate restriction enzyme genes in the vicinity of the methylase genes and, furthermore, no respective restriction activity may be detected in appropriately prepared lysates. Accordingly we observed that non-methylated *oriT*-based plasmids are readily transferred to CD630 from standard *E.coli* donors using our previously developed conjugative procedure. These observations, and evidence from elsewhere, suggest that the majority of clostridial methylases play no role in restriction/modification.

PBMG 12 The cyanide insensitive oxidase protects the opportunistic pathogen *Pseudomonas aeruginosa* against cyanide

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Pseudomonas aeruginosa synthesises the potent respiratory inhibitor hydrogen cyanide at concentrations of up to 300µM. In addition it also possesses a branched electron transport chain, terminated by up to five terminal oxidases, one of which is the cyanide insensitive terminal oxidase (CIO). This oxidase is proposed to have a role in allowing aerobic respiration during cyanogenic growth conditions. Furthermore, it has been proposed that the electrons derived from oxidative HCN synthesis are accepted by oxygen via the CIO terminated pathway. Therefore, we have investigated the relationship of the CIO to cyanide synthesis and cyanide sensitivity in *P. aeruginosa*.

We demonstrate that the presence or absence of a CIO has no impact on cyanogenesis in liquid cultures, however minimum inhibitory concentrations for cyanide suggest the CIO has a protective role in the growth of *P. aeruginosa* under cyanogenic conditions. A number of further growth studies confirmed the importance of the protective role of the CIO to the growth of *P. aeruginosa* in the presence of physiologically relevant concentrations of cyanide. These data suggest that a functional CIO is required for active growth under cyanogenic conditions but that its presence is not required for cyanide synthesis *per se*.

PBMG 13 The identification of attachment genes in lactobacilli and their potential in the development of probiotics

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Lactobacilli present in the intestinal tract of chickens are considered to be beneficial organisms. Growth and colonization of potentially pathogenic organisms is prevented by conditions within the crop. Lactobacilli present produce lactic and acetic acids, this lowers the local pH and the growth of many organisms is inhibited. The EU ban on many antibiotics as growth promoters in poultry production has renewed interest in probiotics. These live microbial feed supplements are thought to benefit the host by improving the intestinal microbial balance. Lactobacilli in the chicken are thought of as potential probiotic organisms as they are part of the normal intestinal flora and are known to attach to intestinal epithelial cells.

Lactobacilli isolated from the crop of broiler chickens have been identified by 16s rRNA sequencing. The adhesion ability of these isolates has been investigated by haemagglutination and adhesion to an intestinal epithelial cell line. The varying attachment of these isolates has prompted investigations to determine the microbial genes involved. Differential display technology is currently being employed to identify genes involved in the attachment of lactobacilli to mucus components. Further studies will identify genes involved in attachment to an intestinal epithelial cell line.

PBMG14 Bacterial acetone-butanol production in the former Soviet Union

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A major part of the technically important acetone, butanol and ethanol (ABE) in the first half of the 20th century was produced by bacteria from biomass – a process gaining a lot of interest again in the light of the Kyoto protocol. Unfortunately, most of the information on the production processes and most of the laboriously selected industrial strains (*Clostridium acetobutylicum*, *C. beijerinckii* and others) seem to be lost. Unlike in the Western countries, in Russia this production, the Weizmann process, was run up to the 60ths. After the Perestroika the archives in the Soviet Union became accessible and the details on the Russian Weizmann process can now be studied as it was actually run. The Russian archives promise to have conserved more of the previously confidential material, because the production plants were state-run.

A preliminary summary of the study done so far in Russian archives is presented, including data on the scale of the process, and on the fermentation technology and microbiology used. More archival studies have to be done, especially on the strains and the technical processes used. We will undertake to revive the strains and to investigate their identity and their production potential in the light of the present-day needs for modern biotechnological processes.

PBMG 15 Cellulolysis mutants of *Clostridium cellulolyticum*: identification of two insertion elements

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¹Laboratoire de Bioénergétique et Ingénierie des Protéines, CNRS, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France; ²Université de Provence, Marseille, France *Clostridium cellulolyticum* secretes multi-enzymatic complexes (cellulosomes) which degrade crystalline cellulose. Many genes coding for subunits of the cellulosomes were found organised in a large cluster. The gene *cipC* encoding the scaffolding subunit

of the cellulosomes is followed by *cel* genes encoding various cellulases.

Spontaneous mutants, less efficient in cellulose degradation than the wild type-strain, have been selected. A truncated CipC protein has been found in the culture supernatant of the mutants. However, none of the major cellulases, usually found in the case of the wild-type strain, could be detected. This phenotype resulted from the insertion of either one or the combination of two endogenous insertion sequence elements (ISCce1 and ISCce2) into the *cipC* gene. ISCce1 is 1292 bp long and has imperfect 23-bp inverted repeats (IRs) near its extremities. ISCce2 is 1359 bp long and has imperfect 35-bp IRs. Both ISCce1 and ISCce2 generate 8-bp long direct repeats (DRs) of the target sequence upon transposition, and carry only one gene encoding a transposase. Southern blot analysis has revealed the presence of at least twenty copies of ISCce1 and seven copies of ISCce2 in the *C. cellulolyticum* genome. The two insertion elements were found to be specific of *C. cellulolyticum*.

PBMG 16 Identification of a differentially-expressed oligopeptide binding protein (OppA) in *Streptococcus uberis* by cDNA RDA

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Streptococcus uberis is an increasingly significant cause of intramammary infection in the dairy cow, currently responsible for approximately 33% of all cases in the UK. Following experimentally induced infection of the lactating mammary gland *S. uberis* is found predominantly in the luminal areas of secretory alveoli and ductular tissue indicating that much of the bacterial growth occurs in residual and newly synthesised milk. In this present study we have used cDNA RDA to identify genes in a clinical isolate of *S. uberis*, whose expression is modified in response to growth in milk, with the objective of characterising potential virulence determinants. We have identified a number of differentially-expressed genes that may contribute to the overall pathogenicity of the organism. Of these, a transcript encoding an oligopeptide binding protein was isolated and partially characterised. To directly analyse the induction of this transcript during growth in milk, a quantitative PCR assay was developed. Analysis of the Q-PCR data by $\Delta\Delta$ Ct analysis showed that the expression of the transcript was increased up to 100 fold in 4 independent cDNA samples. This induction was not observed in cultures grown in tryptic soy broth using lactose as a sole carbon source (46g/L - equivalent to that of milk), suggesting that the differential regulation occurs not in response to carbon source or osmolality, but to a factor in milk.

PBMG 17 Use of MHF to study AI-2 degradation

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4-hydroxy-5-methyl-3(2H)-furanone (MHF) is suggested to be a major product of spontaneous cyclisation of the LuxS reaction product 4,5-dihydroxy-2,3-pentanedione. MHF is structurally related to the signalling molecule Autoinducer 2 (AI-2) and can induce bioluminescence in *Vibrio harveyi*. MHF has also been shown to be DNA damaging and mutagenic. Here we describe the inoculation of a minimal medium containing 5 mM MHF with samples from a range of domestic and environmental sources. Growth of a variety of Gram-negative bacteria and fungi was observed, demonstrating that organisms capable of growth using MHF as the sole source of carbon exist. These organisms must therefore contain pathways for MHF uptake and degradation. Given the chemical similarity of AI-2 and MHF, it is possible that pathways capable of degrading MHF will also be involved in AI-2 degradation. Characterization of these pathways

will determine whether AI-2 enters cells and is converted to common intermediates of central metabolism.

PBMG 18 Preliminary analysis of the genome sequence of proteolytic *Clostridium botulinum* ATCC 3502 (Hall A)

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Clostridium botulinum comprises a large and diverse group of obligately anaerobic, Gram-positive, spore forming bacteria, which are divided into four groups. *C. botulinum* strains have one common determinant, namely, the production of highly toxic botulinum neurotoxins (BoNTs) which cause the paralytic disease botulism. Three forms of botulism occur in humans: wound botulism, foodborne botulism and intestinal (infant) botulism. The latter two forms are directly related to ingestion of the toxin, or ingestion of bacterial spores that germinate and produce toxin in the gut. The capability to produce highly resistant spores may allow *C. botulinum* to survive harsh environmental conditions, including processing and preservation methods used in the food industry. Under favourable conditions, spores may germinate leading to vegetative cells that produce the neurotoxins and lead to disease. The genome of a representative strain of Group I, proteolytic *Clostridium botulinum* ATCC 3502 (Hall A), has been sequenced at the Sanger Institute in Cambridge (http://www.sanger.ac.uk/Projects/C_botulinum/). We will present preliminary findings from the genome sequence related to sporulation/germination and neurotoxin production. The genome sequence furthermore revealed the presence of a plasmid that is dedicated to the production of a bacteriocin, boticin.

PBMG 19 Analysis of iron-regulated gene expression in *Streptococcus uberis*

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The prevalence of bovine mastitis caused by bacteria which shows a contagious route of transmission has declined over the past 25 years as a result of a control plan aimed at reduced exposure, duration and transmission of intra-mammary infections by bacteria. However, this has had little impact on the incidence of disease due to bacteria infecting the udder from an environmental reservoir, particularly, *Streptococcus uberis*. Failure to control *S. uberis* infection is largely due to a lack of knowledge of the mechanisms by which it causes disease and there is accordingly an urgent need to improve our understanding of *S. uberis* pathogenesis.

Growth and survival in a host requires a number of adaptive responses on behalf of bacteria, and the acquisition of iron is an important response for pathogenesis, with iron-limitation often serving as a key cue that an organism has entered a host.

Following experimental infection, *S. uberis* is found predominantly in residual and newly synthesised milk. This environment provides a nutritionally challenging, iron-limited environment for the infecting bacteria. Accordingly, we are studying the role of iron-regulated gene expression in pathogenesis through analysis of differential gene expression in *S. uberis* using iron-regulatory mutants.

PBMG 20 The protective effect of the arginine deiminase system against acid stress in *Listeria monocytogenes*

S. RYAN, C.G.M. GAHAN & C. HILL

Dept of Microbiology, University College Cork

Listeria monocytogenes is a Gram positive facultative intracellular pathogen, which is an important agent of food-borne infection. This is partially due to its ability to tolerate highly acidic environments which enhances the potential for growth in low pH foods and survival in gastric fluid. One method by which *L. monocytogenes* may survive at potentially lethal pH values is by utilising the arginine deiminase system, a 3-enzyme pathway whose major function in bacteria is to protect against the effects of acid stress. The system functions at low pH and produces NH₃ within the cell, raising the cytoplasmic pH and providing protection against acid damage. A gene encoding the arginine deiminase gene (*arcA*) and its putative regulator (*argR*) were identified in the *L. monocytogenes* EGDe genome. Deletion mutants in these genes were created using the Splicing by Overlap Extension procedure. Both $\Delta arcA$ and $\Delta argR$ were found to be exquisitely acid sensitive when analysed in both complex broth and gastric juice. This demonstrates that *arcA* plays a major role in facilitating low pH survival of *L. monocytogenes* and suggests a role for *argR* in the regulation of this pathway. Analysis of the role of *argR* in regulation of *arcA* is ongoing in our laboratory.

PBMG 21 Expression of non-toxic fragments of *Clostridium botulinum* neurotoxins; characterisation of functional domains and their application as novel therapeutic agents

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Centre for Applied Microbiology and Research (CAMR), Porton Down, Salisbury SP4 0JG

The clostridial neurotoxin family, consisting of tetanus toxin from *Clostridium tetani* (TeNT) and 7 antigenically distinct toxins from *C. botulinum* (BoNT/A-G), have attracted considerable interest as therapeutic agents and as tools for the analysis of exocytosis. Purified BoNT/A and BoNT/B have entered the clinic as treatments for a variety of neuromuscular disorders. Although produced as a single chain polypeptide, the active form of the toxin consists of a Zn²⁺-dependent endopeptidase light chain (LC) covalently linked to a cell targeting heavy chain (HC) via a disulfide bond. The LCs specifically cleaves one of the SNARE proteins involved in the docking and fusion of secretory vesicles with the plasma membrane. The heavy chain mediates high affinity binding and internalisation into neuronal cells. The clinical features of botulism and tetanus result from the blockade of vesicle docking with the plasma membrane at nerve terminals resulting in flaccid and spastic paralysis respectively. Therapeutically the injection of minute amounts of the toxin allow selective and localised control of muscle contraction. At CAMR we are interested in the properties of both the enzymatic LC and neuronal-cell targeting HC as the basis of novel therapies. The native neurotoxin genes have been isolated from clostridia and their codon usage shows a high AT bias. Here we describe the expression, purification and characterisation of non-toxic derivatives of BoNTs and their potential application as novel therapeutic agents.

PBMG 22 Physiological comparison of *Azospirillum brasilense* Sp245 and its *scrP* mutant

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Morphological plant root changes have been observed repeatedly upon *Azospirillum* inoculation and have been attributed to the production of plant growth promoting substances by this

bacterium. The auxin, indole-3-acetic acid (IAA) is responsible for the changes observed in root morphology after bacterial inoculation. The key enzyme of this pathway is the indole-3-pyruvate decarboxylase (encoded by the *ipdC* gene). The figure below shows the *ipdC-scrP* operon. The *scrP* gene is co-transcribed with the *ipdC* gene. An *scrP* mutant was compared with the wild type in a Bioreactor and greenhouse experiment. Results show glaring differences between the two strains. The role of the *scrP* gene seems to be in the negative regulation of the expression of the *ipdC* gene and/or *ipdC* activity. At set points, the mutant survives longer and produces significantly higher levels of IAA at pH 6.3. The wild type on the other hand survives longer at pH 6.8 and at uncontrolled pH values. This does not, however, result in higher levels of IAA synthesis. These results point clearly to a possible application of the mutant in acidic soils.

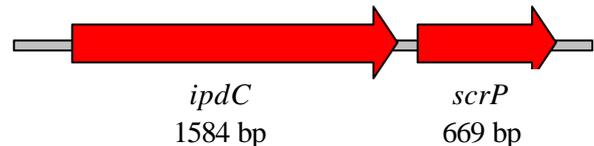


Figure. Genomic organization of the *A. brasilense ipdC-scrP* operon.

PBMG 23 Molecular, biochemical and immunological characterisation of a peptostreptococcal serine protease

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Dept of Oral & Dental Science, Dental School, Lower Maudlin St, Bristol BS1 2LY

Peptostreptococcus micros is an anaerobic Gram positive coccoid bacterium associated with periodontal disease, and particularly with oral and non-oral abscess formation. This microorganism is highly proteolytic, expressing a range of exo- and endopeptidases. To initiate an understanding of the potential role of proteases in peptostreptococcal virulence we have cloned and characterised an extracellular serine protease, EppS, with capacity to hydrolyse gelatin, casein and fibrinogen. The molecular mass of the protease is around 175 kDa, and the predicted peptide sequence is most similar to C5a peptidase, a complement factor-degrading protease recently shown to play a role in cell invasion processes and binding of fibronectin, expressed by Group A and B streptococci. The N-terminus and central portion of these proteases have similar domain structures responsible for catalytic function, although the respective substrate specificities are different. In comparison with the C5a peptidase EppS contains an additional C-terminal domain. Deletion of this domain does not affect capacity to hydrolyse gelatin, casein or fibrinogen, although zymography indicates the recombinant protein to be degraded to several smaller catalytic peptides. This domain may, therefore, play a role in stabilisation of the protease. IgG antibodies specific for EppS are detected in sera from patients with periodontal disease or with dentoalveolar abscesses. EppS, therefore, may be expressed by *P. micros in vivo*, and contribute to initiation and progression of various oral diseases to which this organism has been linked.

PBMG 24 Bacterial infection as a potential cause of back pain?

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The exact pathophysiology of back pain and sciatica is not fully understood. Evidence of an inflammatory or possibly

autoimmune response around the intervertebral disc has been reported.

In a recent study of 36 sciatica patients by Stirling and colleagues (2001), bacteria were cultured from intervertebral disc using a broth enrichment technique in 53% of cases which suggests a possible bacterial aetiology in the pathogenesis of sciatica. The anaerobic bacterium *Propionibacterium acnes* was identified in 84% of these. In addition bacteria were observed directly in samples by microscopy. Previous work in our laboratory has demonstrated that *P. acnes* is present as frequently as *Staphylococcus epidermidis* in failed hip prostheses. These data were obtained by sampling directly from retrieved prostheses, followed by culture without enrichment. Immunofluorescence microscopy with specific monoclonal antibodies was also used. We have applied these techniques to disc and tissue samples removed during sciatica surgery and a control group undergoing other spinal operations. We have detected bacteria in both disc material and associated muscle from sciatica patients, but not in the controls. Whether this represents contamination at the time of surgery or infection will be considered along with the relationship to lipid S-specific antibody levels in patient sera.

Monday 7 April 2003

0900 The producers' perspective

J.W. ALMOND

Aventis Pasteur, Lyon, France

Abstract not received

0945 Eradication of polio

PHILIP MINOR

NIBSC Potters Bar

Poliomyelitis caused by wild type virus is all but eradicated. The last wild type 2 virus was isolated in October 1999. There are pockets of circulating virus in India and Nigeria in particular and the global number of cases in 2002 is greater than in 2001, but there is no technical or practical reason why the wild type virus could not be eradicated in 6 to 12 months. As the vaccine itself causes poliomyelitis in rare instances the strategy for stopping immunisation must be thought out. Among the real, likely and hypothetical problems to be faced are the occurrence of circulating paralytic strains of virus derived from the vaccine, chronic long term excretors of the virus, containment of laboratory and manufacturing stocks, bioterrorism and the re-emergence of the virus by adaptation of another C species enterovirus to become a poliovirus.

1100 HIV vaccines

A. McMICHAEL

John Radcliffe Hospital, Oxford

Abstract not received

1145 Development of a plant-based system for the production of edible vaccines

G.P. LOMONOSSOFF

John Innes Centre, Norwich

Abstract not received

1400 Epidemiology of measles and measles vaccination

B.T. GRENFELL

University of Cambridge

With their relatively simple natural history and rich data sources, viral and bacterial childhood infections such as measles and pertussis offer an unusual opportunity for dissecting the interactions between noise and nonlinearity in spatio-temporal population dynamics. Here, we review recent work on the spatio-temporal dynamics of childhood infections. This work reveals hierarchical waves in measles dynamics and a markedly different dynamical response of measles and pertussis to the perturbation of vaccination. We then discuss the dynamical forces which drive these pre-vaccination and vaccine era patterns and outline how they could be captured with detailed metapopulation extensions of current models. We also briefly consider analogies with the dynamics of other human and animal diseases.

1445 Smallpox vaccines

D.A. HENDERSON, MD, MPH

Johns Hopkins University Distinguished Service Professor
Office of Public Health Preparedness, US Dept of Health & Human Services, Washington, USA

Shortly following the 1980 declaration of the World Health Assembly that eradication of smallpox had been achieved, countries throughout the world ceased vaccinating and, as well, stopped production of smallpox vaccine. Today, with the threat of variola virus possibly being used as a biological weapon, a number of countries are seeking to produce and/or to procure supplies of the vaccine. A number of stocks of vaccine dating back to the 1970s and preserved at low temperature, have been

found to be potent today and studies in the US have shown that the responses in humans with the US material in storage are comparable to what they were at the time they were produced.

In the US, new supplies of vaccine are being produced in Vero cell cultures using the New York City Board of Health strain. Early trials in human subjects indicate the new vaccines to have comparable levels of reactogenicity and immunogenicity (as measured by neutralizing antibody) as the calf-lymph produced product. Trials are continuing with the expectation that the new vaccine may be able to be licensed early in 2004.

Meanwhile, vaccination programs have begun to vaccinate those at greatest risk of smallpox should the virus be released, utilizing a Wyeth-produced product from the 1970s

1600 Structurally designed VLP vaccine for emerging bluetongue virus disease

POLLY ROY

Dept of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London

My laboratory over the last few years has been involved in defining the biochemical and molecular basis of arthropod-borne orbiviruses and to rationally design suitable vaccines. Orbiviruses have similar morphological and biochemical characteristics, with distinctive pathobiological properties and host ranges. The prototype orbivirus, bluetongue virus (BTV) causes economically important disease in sheep and cattle in many parts of the world. Recently BTV caused a considerable loss of farm animals in Southern Europe. BTV is architecturally complex with a particle that is made up of two capsids. The outer capsid is composed of two proteins, VP2 and VP5, while the inner capsid, or 'core', which is transcriptionally active has a surface layer made up of VP7 and an inner shell of VP3 that surrounds a dsRNA genome and enzymatic minor proteins. We have developed baculovirus multigene expression vectors to synthesise BTV core-like and viral-like multiprotein structures (VLPs, CLPs) at high level. These engineered particles essentially mimic the virus particles, but do not contain any genetic materials. The immune responses of these empty particles (non-replicating) have been tested both *in vitro* virus neutralising tests and *in vivo* animals (BTV susceptible sheep). Based on this initial data, a series of clinical trials have been undertaken using Marino sheep (from 50 to 200 sheep in each trial). When one unpurified protein (virus neutralisation protein VP2) was used for vaccination of sheep, 100 µg/dose could afford protection in sheep against virulent virus challenges, while much less of this same protein was needed for complete protection when mixed with the second virus outer capsid protein, VP5. In contrast, vaccination with 10 µg VLPs in several trials (each including 100-200 sheep) gave long lasting protection (at least 15 months, maybe longer) against homologous BTV challenge. Cross-protection was also achieved depending on the challenge virus and the amounts of antigen used for vaccination. The data clearly demonstrate that VLPs are highly immunogenic even at low doses. It can also be anticipated from the results obtained that this technology has much to offer for development of vaccines for other veterinary and human diseases.

1615 Serological responses against recombinant HIV-1 gp120 envelope protect against challenge in model systems

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N. BERRY¹, R. HULL¹, J. COWIE¹, J. LINES¹, W.
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A safe, effective, affordable vaccine is desperately needed to curb the AIDS pandemic. Recombinant envelope vaccines have progressed furthest in clinical trials in Thailand and N America. However, little is known about the responses that these vaccines need to elicit to protect.

We have used the SHIV model to characterise this protection. Groups of 4 vaccinates received 3 or 8 immunisations with 100µg HIV-1_{W61D} rgp120 from a GMP batch and administered in a proprietary adjuvant ASO2. Four weeks after the final boost all vaccinates were challenged with 10³ TCD₅₀ SHIV_{W61D} that expresses an envelope that is homologous with the vaccine.

Superior protection was observed amongst vaccinates that received 8 immunisations. Analysis of serological prior to virus challenge revealed significant differences in the titre of virus neutralising activity between the two groups of vaccinates. Furthermore, transfer of 22.5ml/kg immune serum collected from the second group of vaccinates between the 4th and 8th immunisation to a group of 3 naïve recipients conferred protection against subsequent challenge with SHIV_{W61D}.

These data demonstrate that envelope based vaccines can protect against virus challenge. The demonstration of the importance of antibody responses in protection provides a framework to develop HIV vaccines that are broadly protective.

1630 Recombinant HBsAg preparations differ in their LBP and CD14 dependent attachment to monocytes, immune suppressive potential and T-cell immunogenicity

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¹Center for Vaccinology, Dept of Clinical Biology, Microbiology and Immunology, Ghent University Hospital, Ghent, Belgium; ²Dept of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands

We reported recently, that Hepatitis B surface antigen which is a non-infectious viral-like particle, normally produced by infected hepatocytes, binds to monocytes. This interaction requires the expression of the LPS receptor CD14 and is catalysed by the LPS binding protein (LBP). We demonstrated that HBsAg suppresses the LPS induced production of TNF α and IL-1 β , while upregulating expression of IL-10. Based on these observations we concluded that HBsAg acts like an apoptotic-like lipoprotein. Unfortunately, this was only observed with recombinant HBsAg (rHBsAg), produced in *Saccharomyces cerevisiae* and not with the natural HBsAg purified from plasma (pHBsAg). The absence of phosphatidylserine and phosphatidylinositol in pHBsAg was suggested to be responsible for this lack of binding. Here we report that different yeast-expressed rHBsAg preparations can differ in their capacity to bind to CD14 positive cells. A correlation with a reduced potential to inhibit the LPS-induced activation of monocytes and an increased potential to stimulate T-cell proliferation is suggested. Surprisingly, no differences in phospholipid content are found between these rHBsAg preparations that might explain these observations. It remains to be determined if the suppressive interaction of yeast-expressed HBsAg with monocytes affects the anti-HBsAg levels after immunization with pure rHBsAg and if this immunosuppressive effect is retained after adsorption of rHBsAg on to aluminium hydroxide.

1645 Attenuating mutations in the influenza virus genome which may increase the safety of vaccine production

ALISON WHITELEY, MICHAEL TRIKIC & WENDY BARCLAY

School of Animal and Microbial Sciences, University of Reading, Whiteknights

Influenza virus epidemics occur on an annual basis and cause severe disease in the very young and the old. The vaccine administered to high-risk groups is generated by amplifying reassortant viruses, with chronologically relevant viral surface antigens, in eggs. Every twenty years or so, influenza pandemics

occur causing widespread fatality in all age groups. These viruses display novel viral surface antigens acquired from a zoonotic source and vaccination against them poses new issues since production of large amounts of a respiratory virus containing novel surface antigens could be dangerous for those involved in manufacture. To minimise risks, it is advisable to use a virus whose genetic backbone is highly attenuated in man. Traditionally the A/PR/8/34 strain of virus is used, however the genetic basis of its attenuation is unclear. Cold-adapted strains of influenza virus are all based on the H2N2 subtype, itself a virus with pandemic potential, and again the genetic basis of temperature sensitivity is not yet established. Reverse genetics technology allows us to engineer designer influenza viruses to order. Using this technology, we have been investigating mutations in several different gene segments to effectively attenuate potential vaccine strains allowing the safe production of vaccine to protect against the next pandemic.

1700 Recombinant bovine respiratory syncytial viruses (RSV) that do not express the non-structural proteins are attenuated and immunogenic in calves J.-F. VALARCHER¹, J. FURZE¹, S.G. WYLD¹, R. COOK¹, K.K. CONZELMANN² & G. TAYLOR¹

¹Institute for Animal Health, Compton, Newbury, Berkshire RG20 7NN; ²Max von Pettenkofer Institute & Gene Center, Ludwig-Maximilians-University Munich, D-81377 Munich, Germany

Human (H) and bovine (B)RSV are a major cause of respiratory disease in young children and calves, respectively, and the epidemiology and pathogenesis of infections with these viruses are similar. Previous studies have shown that BRSV and HRSV induce little or no type I IFN compared to other respiratory viruses and that the non-structural proteins (NS) act co-operatively to counteract the anti-viral effects of type I interferons (IFN).

The function(s) of the NS genes were studied further by comparing the ability of recombinant (r)BRSV lacking these genes to induce type I IFN, *in vitro*. The replication of viruses lacking NS2 or NS1/2 proteins was highly restricted in bovine nasal fibroblasts and bronchoalveolar macrophages and these viruses induced higher levels of type I IFN than wild type (WT) BRSV. Induction of type I IFN was dependent upon replication of the viruses.

BRSVs lacking either NS1, NS2 or both proteins were highly attenuated in the respiratory tract of young calves. Despite their poor replication *in vivo*, prior infection with the single deletion mutants induced serum antibodies and protection against challenge with virulent BRSV almost as good as that induced following infection with WT rBRSV. The greater immunogenicity of rBRSV Δ NS2 may be associated with the ability of this virus to induce type I IFN.

1715 Development of marker vaccine for rinderpest SATYA PARIDA, SAI KUMAR, MANDY CORTEYN, SUBASH C. DAS, EDMUND P. WALSH, MICHAEL D. BARON & THOMAS BARRETT

Institute for Animal Health, Pirbright Laboratory, Pirbright, Surrey GU24 0NF

Rinderpest is a severe disease of wild and domestic ruminants and a global eradication campaign is currently underway with a target date of 2010 to achieve this goal. Vaccination has now ceased in most countries but a vaccine will still be required in this transition phase for use to prevent virus spread if isolated outbreaks of the disease occur. For this eventuality it is desirable to have a marker vaccine, which would make it possible to identify the vaccinated animals serologically. Using reverse genetics techniques we have introduced positive and negative markers into the vaccine strain of the virus via a DNA copy of the RNA genome. As positive markers we have introduced variants of the green fluorescent protein (GFP) and a mutated form of the influenza haemagglutinin protein which is defective in receptor binding (Walsh et al., 2000, Journal of Virology, 74, 10165-10175). Since there is no immunogenic gene which can

be deleted from the virus to produce a negative marker, replacement of one of the virus genes (the nucleocapsid protein gene) with a functionally equivalent gene from a related virus (peste des petits ruminants virus) has resulted in a chimeric virus which can be used as a vaccine to enable vaccinated animals to be serologically differentiated from those which have recovered from natural infection. The differentiation is achieved using two specific competitive ELISA tests, based on the antibody response generated to the nucleocapsid and haemagglutinin proteins, respectively.

Tuesday 8 April 2003

0900 Standardization and control of viral vaccines: some recent scientific developments

G. SCHILD

National Institute for Biological Standards & Control, Potters Bar

Efforts to ensure high standards of quality, safety and efficacy for biological medicinal products are of major importance to public health. This is particularly the case for vaccines, the most widely used of all biologicals, which are unique in that they are administered routinely to almost all healthy children in the world and play a critically important role in the control, and potential elimination, of previously devastating infectious diseases, in rich and poor countries alike. The challenge of quality control of vaccines is particularly complex in that they are manufactured from biological source materials by complex processes and their testing requires biological assays which are often difficult to standardize. Maintaining, and indeed raising the standards of quality for vaccines is thus of paramount importance and a major priority for both manufacturers and regulatory institutions. Knowledge that vaccines are independently evaluated by regulatory authorities, often involving batch by batch testing, is important for the public confidence in these products; concerns on safety clearly can have a serious impact on vaccine uptake and can result in the re-emergence of outbreaks, as is currently a risk for measles.

The talk will review the salient principles and procedures applied in vaccine manufacture to secure quality, safety and batch to batch consistency. It is of critical importance that the laboratory methods used in the quality control of vaccines keep up with the state of art science and technology. Several examples of vaccine 'regulatory research' aimed specifically at the development, evaluation and application of new and improved methods for standardization and control testing will be presented. These involve influenza vaccines, polio vaccines and MMR. The new approaches frequently use molecular biological and physico-chemical methods to complement conventional biological assays.

A further key aspect of vaccine standardization, which will be addressed, is the production and evaluation of stabilised biological reference materials (WHO International Standard Preparations). These provide the basis for standardized measurements of biological activity, often in terms of International Units of biological activity and serve a valuable basis for establishing internationally agreed criteria for measurement of vaccine parameters such as antigenic content, infectivity, immunological potency and purity.

0945 Attenuated RSV vaccines

P. COLLINS

NIH, Bethesda, USA

Abstract not received

1100 PrP antibody therapy and vaccination

J. COLLINGE

University College London

Abstract not received

1145 Commercial production of antibody-based therapeutics in plants

LARRY GRILL

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Plant RNA viral vectors have been developed for large-scale production of commercially-relevant proteins, which include single-chain antibodies (scFv) and larger immunoglobulin fragments (FAB). The process requires that gene sequences of the proteins be cloned into DNA plasmids that encode a modified Tobacco Mosaic Virus genome. Approximately 2 weeks after growing plants are inoculated with the transcribed vectors, new proteins encoded by the gene sequences are purified utilizing small or large-scale extraction methods. These plant viral vectors have been used to produce vaccine antigens, enzymes, scFv's and FAB's in large quantities and processes have been developed to extract the proteins from plants. Outdoor scale-up has been accomplished by extracting viral-vector proteins from hundreds of tons of field-produced plants. Several advantages of this viral expression system include rapid production, high expression levels, post-translational processing, and scalability. Many of the proteins that have been produced have had complex post-translational modifications, which includes glycosylation, and have been shown to have the desired *in vitro* or *in vivo* activity. In particular, multiple plant-produced, patient-specific scFv vaccines against non-Hodgkin's lymphoma have demonstrated promising results in Phase I Clinical Trials.

1400 Herpesvirus vaccines

JULIAN HICKLING

Xenova Research Ltd, Cambridge Science Park, Cambridge CB4 0WG

Herpesvirus infections are characterised by the fact that following infection the virus establishes a life-long persistent or latent infection which may also involve periods of reactivation. The complex nature of herpesvirus infection has made it difficult to design effective vaccines to prevent herpesvirus infection and disease. However progress has been made, and a live attenuated vaccine for the prevention of chicken-pox has been used in several nations for some years.

This presentation will focus on the development of vaccines against the other neurotropic herpesviruses, HSV-1 and -2. Despite the success of the live attenuated VZV vaccine, this strategy has not been successful for HSV. A number of alternative vaccine strategies are currently being evaluated in clinical trials, the most advanced being a protein sub-unit vaccine consisting of HSV-2 glycoprotein D formulated with an alum plus MPL adjuvant. A variation on traditional attenuated virus vaccines is the development of a genetically disabled infectious single cycle (DISC) HSV-2. By constructing a virus which is infectious but only able to undergo a single round of replication following immunisation, the aim is to produce a vaccine which combines the safety features of inactivated vaccines with the immunological advantages of live whole virus vaccines.

1445 Cross-neutralisation of Bat Lyssaviruses by human sera following classical rabies vaccination

S.M. BROOKES, G. PARSONS, D. SELDEN,

N. JOHNSON, L.M. McELHINNEY & A.R. FOOKS

Rabies Research and Diagnostic Group, Veterinary Laboratories Agency, New Haw, Surrey KT15 3NB

The human diploid cell vaccine (HDCV) is widely used for protection against classical rabies (RABV); lyssavirus genotype 1 (genotype 1). Other genotypes include, European and Australian bat lyssaviruses (EBLV-1 & 2, ABLV, genotypes 5, 6 & 7 respectively). All are capable of producing clinically indistinguishable and fatal disease in terrestrial mammals. Britain is free of RABV, but EBL 2 strains have been isolated from Daubenton's bats (1996 & 2002) and a human case (Nov. 2002).

Using virus neutralisation assays we assessed the ability of antibodies in serum from HDCV vaccinated humans to neutralise

RABVs, EBLV-1 & 2 and ABLV *in vitro*. Most of the sera, except some low titre samples, were capable of neutralising the viruses tested. Does cross-neutralisation *in vitro* imply that the vaccine is capable of cross-protecting recipients against virus challenge? Mice vaccinated (ip) with HDCV are protected against intra-cerebral challenge with RABV and an EBLV-2, but only partial protection was observed with a wildtype RABV (40%), EBLV-1 (20%) and ABLV (50%). Using a peripheral challenge (footpad) model HDCV protected mice against RABV, EBLV-1 & 2 but only partially against ABLV (80%). It is highly recommended that those at risk of exposure to lyssaviruses receive and maintain pre-exposure RABV vaccination.

1500 Evasion of innate immunity mediated by the V proteins of paramyxoviruses

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The earliest phase of a viral infection is characterised by the need to replicate in the presence of the innate immune system, a collection of non-antigen-restricted responses that restricts virus replication, thereby buying time for the development of a more specific acquired immune response. A major component of innate immunity is interferon (IFN), and it is becoming clear that to replicate efficiently *in vivo* all viruses must, at least to a degree, have some strategy for circumventing the IFN response either by limiting IFN production or blocking IFN actions. We have been studying the evasion of innate immunity using SV5 and related paramyxoviruses. The product of the SV5 V gene is able to inactivate the signalling response to type I and type II IFNs. Furthermore, SV5 pathogenesis and host range is influenced by the ability of V to function correctly in cells from different species. Inactivation of IFN signalling is mediated by the specific targeting of STAT1 for degradation by proteasomes in a process that involves the host UV-DNA damage repair protein, DDB1. The SV5 V protein also severely limits the induction of IFN- β by SV5 infection, with the activation of both NF- κ B and IRF-3 being impaired. This property of the SV5 V protein is mechanistically distinct from the block to IFN signalling, and is a property that is shared by the V proteins of other paramyxoviruses examined; thus, limiting the yield of IFN- β during infection may be a general property of paramyxoviruses.

1515 A role for BVDV E^{rns} in the control of the activation of beta interferon by double-stranded RNA

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BVDV is a major cattle pathogen, which is widespread and infects 60-70% of cattle worldwide. Virus may be present as a persistent infection in 1-2 % of calves, which occurs as a result of *in utero* infection. The virus present in these persistent infected calves is a non-cytopathogenic biotype (ncp BVDV). We and others have shown that cells infected with ncp BVDV are refractory to the interferon (IFN) inducing effects of infection with heterologous virus or addition of double-stranded RNA (dsRNA). Further experiments were carried out in which cells constitutively expressing E^{rns} or addition of exogenous E^{rns} modulate the IFN-induction by dsRNA. As the induction of IFN- α/β by the host cells represents one of the first lines of defence against virus infection, therefore we adduce that E^{rns} may be an interferon antagonistic factor to counteract host antiviral responses for persistence of BVDV infection.

1600 The Yaba-like disease virus protein 134R induces STAT 3 phosphorylation via the IL-20/IL-24 heterodimeric receptor complexes

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Yaba-like disease virus (YLDV) is a member of the family *Poxviridae*, a group of complex DNA viruses that replicate in the cytoplasm. Analysis of the predicted amino acid sequence and secondary structure of the 134R protein encoded by this virus indicated that it is similar to the IL-10 family of cytokines, particularly IL-19, IL-20 and IL-24. To investigate if YLDV 134R mimicked or inhibited the activity of these cytokines, the protein was expressed from mammalian cells and found to be a secreted 22 kDa glycoprotein. This protein bound to the IL-20/IL-24 receptors on keratinocytes and BHK cells inducing phosphorylation and nuclear translocation of STAT3 in a manner similar to that observed for IL-20 and IL-24. In contrast, 134R was unable to signal via the related IL-22 receptor complex. Taken together these results indicate that 134R is a new viral member of the IL-10-like cytokine family, most closely related to IL-20 and IL-24 with respect to receptor binding and STAT activation. In order to understand the role 134R plays in the virus life cycle we are investigating the effect of this protein (and related cellular ligands) on the growth properties and protein expression of cells *in vitro* and on virus virulence *in vivo*.

1615 The vaccinia virus proteins A46R and A52R are intracellular inhibitors of Toll-like receptor signalling and contribute to virulence

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Vaccinia virus (VV) is a poxvirus, a group of large, complex DNA viruses. VV genes A46R and A52R encode proteins with amino acid similarity to signalling molecules and expression of these genes in mammalian cells interfered with IL-1 and Toll-like receptor signal transduction. To characterise these proteins in the context of VV infection and investigate their functions *in vitro* and *in vivo*, mutant viruses lacking either or both genes were constructed. Deletion of either or both genes did not affect VV growth properties or plaque phenotype *in vitro*. Using specific antisera, each protein was detected within cells early during infection, but whereas A46R was predominantly cytosolic, A52R was predominantly membrane associated.

Deletion of either or both genes caused virus attenuation in mice infected intranasally compared to animals infected with control viruses containing the intact genes. Thus both proteins contribute to virus virulence in this model. We are investigating the mechanisms by which these proteins contribute to virulence.

1630 Novel genes in human cytomegalovirus with potential roles in host modulation

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Re-evaluation of the human cytomegalovirus sequence has identified 11 novel genes. One, RL5A, encodes a domain that is the hallmark of proteins encoded by the viral RL11 gene family, most of which are predicted glycoproteins. This domain is also present in a subset of human adenovirus E3 glycoproteins and

may represent a divergent form of the immunoglobulin domain. Evolution of genes specifying the domain is characterized in both virus families by extensive divergence, gene duplication and selective sequence loss. These features indicate that the encoded proteins may modulate a family of variable host proteins.

Two novel genes (UL131A and UL128), both spliced, flank UL130. Compared with virus in infected human tissues, three of six isolates passaged in human fibroblasts contain disruptions of UL128, one is frameshifted in UL130 and one in UL131A, and one has a deletion affecting UL130 and UL131A. Two other primate cytomegaloviruses, which have been also been passaged in human fibroblasts, exhibit disruptions of UL128. Expression of any one of UL128, UL130 and UL131A therefore appears disadvantageous to growth of primate cytomegaloviruses in cell culture. These genes are predicted to specify small secreted proteins, and sequence comparisons suggest that UL128 encodes a β -chemokine.

1645 The epidemic keratoconjunctivitis causing adenovirus 19a secretes a novel leukocyte-binding E3 protein

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The early transcription unit 3 (E3) of human adenoviruses (Ads) encodes proteins that subvert host defence mechanisms. We recently discovered a novel ORF of 49K in the E3 region of Ad19a, an Ad that causes epidemic keratoconjunctivitis. 49K is present in all subgenus D Ads examined (1) and encodes an 80-100 kDa, highly glycosylated, type I transmembrane protein. Synthesis of 49K begins in the early phase of infection but continues throughout the infection cycle. 49K is primarily localized in the Golgi/TGN but also in early endosomes and at the plasma membrane (2). Pulse-chase analysis indicated that 49K is cleaved close to the C-terminus.

We now demonstrate that the large N-terminal portion of 49K is not degraded but secreted. This is the first E3 protein and actually the first Ad protein known to be secreted. In contrast to all known E3 proteins, which act on the infected cell, 49K is likely to affect surrounding cells. Supporting this notion, 49K was found to bind to lymphocytes. We hypothesize, that secreted 49K functions as a regulatory factor for infiltrating cells of the immune system.

References: (1) Blusch et al. (2002) *Virology*, 296, 94-106. (2) Windheim and Burgert (2002), *J. Virol.*, 76, 755-766.

1700 Apoptosis in bluetongue virus infection

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Bluetongue virus is transmitted by *Culicoides* insect to livestock causing severe disease in sheep. It has been recently reported that programmed cell death in BTV infected sheep may play a direct role in animal pathogenesis. In view of the fact that BTV infection causes a haemorrhagic disease in sheep, but is asymptomatic in insects, we have investigated apoptosis in insect and mammalian cells *in vitro*. We tested the ability of BTV to trigger apoptosis in three different mammalian cell lines and in three different insect cell lines including *Culicoides* (KC) cells (the natural vector for BTV). Cell morphology, fragmentation of chromosomal DNA, and caspase-3 dependent cleavage of the virus have been used as markers for apoptosis. BTV infection triggers apoptosis in all mammalian cell lines expressing caspase-3 signalling pathway. We examined the role of individual BTV proteins in the development of cell apoptosis, and have found that binding of viral outer capsid proteins is required for the induction of apoptosis. In contrast, no signs of apoptosis were detected in any of the three insect cell lines as a response to BTV infection. However, it is still unclear how the insect cells are protected from apoptosis. The mechanisms used by the virus to

produce cytopathic effect (CPE) and to determine whether BTV-induced CPE is associated with apoptosis in its host cells are still not well understood. Such knowledge might be essential to an understanding of viral pathogenesis and may lead to develop novel drugs that target the apoptotic pathway.

1715 The CD2v protein of African swine fever virus interacts with the actin-binding adapter protein SH3P7

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The predicted extracellular domain of the CD2v protein of African swine fever virus shares significant similarity with that of the CD2 protein in T cells but has a unique cytoplasmic domain of unknown function. Here we show that CD2v is expressed as a glycoprotein of about 105 kDa in ASFV infected cells. In the absence of an extracellular ligand the majority of CD2v appears to localize to perinuclear membrane compartments. Further we show using the yeast two-hybrid system and by direct binding studies that the cytoplasmic tail of CD2v binds to the cytoplasmic adaptor protein SH3P7 (mAbp1, HIP55), which has been reported to be involved in diverse cellular functions such vesicle transport and signal transduction. A cDNA clone encoding a variant form of SH3P7 could be also identified and was found to be expressed in a wide range of porcine tissues. Deletion mutagenesis identified proline rich repeats of sequence PPPKPC in the ASFV CD2v protein to be necessary and sufficient for binding to the SH3 domain of SH3P7. In ASFV infected cells CD2v and SH3P7 co-localised in areas surrounding the perinuclear virus factories. These areas were also stained with an antibody which recognises a Golgi network protein, indicating that they contain membranes derived from the Golgi network. Our data provide a first molecular basis for the understanding of the immunomodulatory functions of CD2v in ASFV-infected animals.

Thursday 10 April 2003

0900 Tumorviruses and cell cycle regulation

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Viruses may contribute to carcinogenesis by different mechanisms. Part of them interact directly, by coding for specific oncogenes stimulating cellular DNA synthesis, others contribute by indirect modes, e.g. immunosuppression. Epstein-Barr virus, high risk human papillomaviruses (HPV) and some others represent examples for directly transforming viruses. Their interference with cell cycle regulation will be briefly discussed.

The HPV E6/E7 oncogenes are required for cell immortalization and malignant transformation. The long latency period between primary infection and cancer occurrence indicates already that viral oncogene expression is not sufficient for the modification of the proliferative phenotype of infected cells. We analysed specifically the host cell regulation of HPV oncogene expression and characterized steps essential for the transition of immortalized cells to malignant transformation. As prime model the HeLa cell/human fibroblast fusion system was used, as initially developed by E. Stanbridge. Our studies show that the transition is frequently accompanied by an interruption of TNF- α regulated signaling pathways in malignant cells. Whereas in immortalized cells TNF- α reduces HPV transcription, malignant cervical carcinoma cells fail to show this effect. In addition, TNF- α induces selectively interferon- β synthesis in non-malignant cells, but fails to do so in their malignant derivatives. The latter effect is apparently due to a dysregulation of IRF-1 and p48.

Cervical carcinoma cells are still able to induce interferon- β synthesis upon viral infection, thus specifically the TNF- α mediated pathway seems to be affected. In non-malignant cell hybrids, obtained by fusing cells from two different cervical carcinoma cell lines (e.g. CaSki and HeLa cells), as well as in non-malignant HeLa revertants, inducibility of interferon- β by TNF- α is restored, suggesting an important role for the interruption of TNF- α driven interferon- β synthesis in malignant transition of previously non-malignant cells.

0945 Oncogenesis by retroviruses

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A review of oncogenesis by retroviruses will focus on two retroviruses. Two mechanisms for oncogenesis are known: *acute transforming retroviruses* carry viral oncogenes, typically transduced from normal cell proto-oncogenes; *non-acute retroviruses* insertionaly activate host cell proto-oncogenes. Moloney murine leukemia virus (M-MuLV) is a prototypical non-acute retrovirus that induces T-lymphoma in mice. Insertional activation of proto-oncogenes by M-MuLV will be reviewed. In addition, M-MuLV induces preleukemic events in infected mice, including changes in hematopoiesis, enhanced apoptosis in the thymus, and generation of MCF recombinant viruses. The relationships of these changes to leukemogenesis will be discussed. Jaagsiekte sheep retrovirus (JSRV) is the etiologic agent of ovine pulmonary adenocarcinoma (OPA). We recently isolated an infectious and oncogenic molecular clone of JSRV, which has opened the way for detailed studies of infection and oncogenesis. JSRV appears to carry an oncogene, as measured by transformation of murine NIH3T3 fibroblasts. Interestingly, the oncogene is the viral envelope protein.

Multiple domains of the JSRV envelope protein appear to be necessary for transformation. Potential mechanisms for transformation will be discussed.

1100 Studies of the polyoma virus-mouse system with possible implications for non-virus-induced cancers

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The murine polyoma virus is perhaps the most rapidly and broadly acting oncogenic agent known. The major viral capsid protein binds sialic acids which are widely and abundantly expressed on cell surfaces. Regulatory sequences in the viral DNA contain binding sites for multiple host transcription factors enabling the virus to be expressed in many different cells. The virus encodes three T (tumor) antigens which bind host factors and intervene in multiple pathways regulating cell growth and survival (Benjamin, 2001: *Virology* Minireview). Despite these features, the virus is able to induce tumors in some inbred strains of mice and not in others.

To expand the search for cellular targets of the polyoma T antigens, host range mutants of the virus have been isolated. These mutants were selected to grow in cells derived from spontaneous or carcinogen-induced tumors but to be unable to grow in normal mouse cells. The 'tumor host range' (THR) selection procedure was undertaken in attempts to identify cellular proteins whose functions must be overcome to allow virus replication and cell transformation (Li et al, PNAS 2001). These targets might possibly function as tumor suppressors, cell cycle regulators, effectors of apoptosis, or other factors whose expression are commonly lost or altered in non-virus-induced cancers. Results regarding two such targets will be discussed.

1145 Oncogenic papillomaviruses-the T cell perspective

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Virtually all cervical cancers, and some skin, head and neck cancers are linked to human papillomavirus (HPV) infection. The increased prevalence of HPV associated lesions (pre-malignant and malignant) in immunosuppressed patients suggests a protective role for cell-mediated immunity. CD8⁺ cytotoxic T cells are known to protect against persistent viruses, but importantly can also mediate tumour regression in animal models and in a limited number of clinical studies in man. For HPV associated cervical cancer, the persistent intracellular expression of HPV E6 and E7 oncoproteins provides tumour-specific targets for T cell based immunotherapy. However until recently it has been difficult to detect T cells specific for HPV E6 and E7 oncoproteins. In this talk, I will review what is known about human T cell responses to oncogenic HPVs. I will also describe the possible mechanisms by which cervical carcinoma cells can avoid recognition by HPV specific cytotoxic T cells. Understanding these mechanisms, in particular the defects in MHC class I antigen processing, will provide a rational basis for the development of anti-cancer vaccines with greater therapeutic potential.

1400 Structural Constraints imposed on the Vif protein of HIV-1 in the development of resistance to inhibitors of viral protease

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The HIV-1 accessory protein Vif has been shown to be required for the production of infectious virus in the natural host cells of the virus. Definition of its precise role in virus replication has remained elusive but it appears to act late in the morphogenesis pathway of the virus particle possibly by affecting the kinetics of Pr55^{gag} cleavage by the virion encoded protease. As part of studies to try and more precisely define the possible role of Vif in these events we have examined the extent of variation within the viral protease, Pr55^{gag} and Vif in a cohort of HIV-1 infected patients receiving protease inhibitors (PIs) as part of their HAART treatment and showing signs of PI resistance. In addition to the classical mutations in the viral protease associated with the development of PI resistance a number of distinctive changes in the Vif sequence were observed. The most striking changes found were T47P, H37N and Q83H in Vif from HAART patients showing PI resistance compared to the sequence from a treatment naïve control cohort. The possible significance of these and the other changes found will be discussed.

1415 New roles for structural elements in the HIV-1 packaging signal: A Rev binding site in the leader RNA

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The packaging signal (Ψ) of the human immunodeficiency virus type 1 (HIV-1) enables encapsidation of the full-length genomic RNA against a background of a vast excess of cellular mRNAs. The core HIV-1 Ψ is approximately 109 nucleotides and contains sequences critical for viral genomic dimerisation and splicing, in addition to the packaging signal. As such it is a potential target for anti-viral strategies. Ψ consists of a series of stem loops (termed SL-1 – 4). Using 1D NMR, 2D NMR, and UV melting experiments, we have elucidated the tertiary structure of two conserved internal loops within SL-1¹. The structures of these loops are novel, and both this and the fact that they are highly conserved amongst all HIV-1 isolates suggests that they may play critical roles in the virus lifecycle. Using a combination of *in vitro* functional assays and viral mutants, the potential role(s) of these structures has been investigated. Amongst new findings that will be discussed is a binding site for Rev, one of the viral regulatory proteins, the potential significance of which will be considered.

1430 Epstein-Barr Virus (EBV) disrupts a genotoxin-induced G1 checkpoint by preventing the inactivation of cyclin E/cdk2 by p21^{WAF1}

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It is often proposed that when EBV drives B cell proliferation it mimics physiological B cell activation induced by specific antigen binding and cognate T cell help rather than by bypassing checkpoints in the cell cycle like the small DNA viruses. Our recent results suggest this is not the case by showing that EBV overrides the p53/pRb-mediated G1 checkpoint activated by genotoxic stress in normal B cells.

Primary human B cells that had been induced to proliferate by either infection with EBV or treatment with CD40 ligand plus IL4 were exposed to cisplatin. We show that in normal cycling B cells, following genotoxic damage by cisplatin, cyclin D2 is

down-regulated, p21^{WAF1} is released from cyclin D2/Cdk6 complexes and binds cyclin E/Cdk2, concomitantly p53 induces *de novo* synthesis of p21^{WAF1}. This results in de-phosphorylation of pRb, a sustained block to DNA synthesis and arrest in G1. However, in similar B cells driven to proliferate by latent infection with EBV, degradation of cyclin D2 and therefore the transfer of p21^{WAF1} from cyclin D2 to cyclin E containing complexes does not occur. Furthermore, although p53 is stabilised and induces the transcription of the p21^{WAF1} gene, newly synthesised p21^{WAF1} protein does not accumulate. Consequently, in the EBV-infected cells cyclin E/Cdk2 activity is not inhibited, hyperphosphorylation of pRb is maintained and DNA synthesis proceeds. The observation that many of the cells subsequently undergo apoptosis is consistent with reports that in the absence of functional p21^{WAF1} the result of sustained DNA damage is programmed cell death. This comprehensive disruption of a key cell cycle checkpoint and replication of damaged DNA can result in genomic instability and may partly explain the pathogenesis of several EBV-associated B cell malignancies. It also suggests that EBV-driven tumour cells may be more likely to undergo apoptosis than normal B cells in response to anti-cancer drugs.

1445 Suppression of a cell cycle checkpoint at the G2-M transition by Epstein-Barr Virus (EBV)

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We have recently shown that EBV disrupts at least two cell cycle checkpoints that normally prevent the replication of damaged DNA or chromosomes in G1 and at G2/M. Here the G2/M checkpoint is further characterised. We show that in EBV-negative Burkitt's lymphoma-derived cell lines, genotoxic drugs activate a p53-independent checkpoint that leads to arrest in the G2/M phase of the cell cycle accompanied by default apoptosis. This checkpoint – that is suppressed by EBV latent gene expression – is activated when cyclinB/Cdc2 is already active in the nucleus. This suggests the checkpoint is being triggered at, or after, the G2 to M transition but before cytokinesis. The B cells expressing all of the EBV latent genes (or a subset encoded by the P3HR1 strain of virus) fail to arrest or undergo apoptosis when treated with the genotoxic drug cisplatin. They continue to divide before developing an extremely enlarged nucleus. Although some polyploidy develops, the DNA content of the surviving cells is largely 4N and the nuclear membrane is intact. This unusual phenotype is then stable for many days. In contrast, EBV has no effect on the G2-arrest induced by the inactivation of Cdc2 in response to γ -irradiation. It seems that EBV acts specifically downstream of Cdc2. This ability of EBV to suppress a G2/M checkpoint (in addition to the G1 checkpoint) suggests that it will increase the likelihood of damaged/mutated DNA replicating and genomic instability. In this way EBV probably contributes to the development of a variety of B cell malignancies.

1500 Novel genes regulated by Epstein-Barr Virus:

The transcription factor RUNX3 is induced by EBNA-2

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The gammaherpes virus Epstein-Barr virus (EBV) infects epithelial and B cells and is associated with several cancers and lymphoproliferative diseases including glandular fever, Burkitt's lymphoma and nasopharyngeal carcinoma.

Experimental infection of primary human B cells induces continuous cell proliferation leading to the outgrowth of immortalised lymphoblastoid cell lines (LCLs). EBNA2 is an essential viral transcription factor required for this process. To identify novel cell genes regulated during immortalisation, gene expression profiling was carried out following activation of

EBNA2 in a cell line containing a conditional estrogen receptor-EBNA2 fusion protein.

The transcription factor RUNX3 (AML-2) was identified as being a direct target of EBNA2 and was induced upon infection of primary B cells with EBV. Freshly isolated quiescent B cells expressed high levels of another member of the RUNX family, RUNX1, but RUNX1 was rapidly downregulated after EBV infection.

RUNX protein expression was also analysed in a panel of Burkitt Lymphoma (BL) cell lines. BL cells exhibit two main types of EBV latency (Group I and Group III) depending on which viral genes are expressed. RUNX3 was associated predominantly with a Group III phenotype (EBNA-2 expressing) while RUNX1 was associated with Group I cells (EBNA-2 negative). The RUNX factors were rarely expressed simultaneously suggesting that they are co-ordinately regulated.

To investigate whether RUNX3 expression is responsible for regulating RUNX1 levels in B cells, BL cell lines expressing endogenous RUNX1 were stably transfected with a plasmid encoding inducible RUNX3. After RUNX3 induction, RUNX1 protein expression decreased and this correlated with a decrease in the abundance of RUNX1 mRNA transcripts.

This is the first example of transcription factors that distinguish between different BL phenotypes, and it appears that EBV induced RUNX3 regulates expression of other members of the RUNX family. We are currently investigating whether these factors contribute towards EBV induced immortalisation or phenotypic differences seen in BL cell lines.

1515 Identification of the elements required for episomal persistence of herpesvirus saimiri, the prototype gamma-2 herpesvirus

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Herpesvirus saimiri (HVS) is closely related to other herpesviruses with oncogenic potential, such as Kaposi's sarcoma associated herpesvirus (KSHV) and Epstein Barr virus (EBV). In common with other herpesviruses, HVS has been shown to establish a latent episomal state in infected cells in which the viral episome is efficiently segregated into progeny upon cell division. It is believed that this viral segregation is achieved by tethering the viral genome to the host cell mitotic chromosomes

To identify the minimal viral requirements for HVS episomal maintenance, and to further elucidate the mechanisms involved, a systematic deletion of the complete HVS genome has been produced utilising a HVS Bacterial artificial chromosome (BAC) clone generated in our laboratory.

This approach has enabled us to identify two regions of the genome essential for episomal maintenance. A *trans*-acting region encoding the ORF73 gene product and a *cis*-acting region encompassing the terminal repeat region. Furthermore, we have demonstrated that the ORF 73 gene product can associate with host cell mitotic chromosomes and this interaction is via two distinct chromosomal binding domains.

1600 Functional activity of the complement regulator encoded by Kaposi's sarcoma associated herpesvirus

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Kaposi's sarcoma-associated herpesvirus (KSHV) is closely associated with Kaposi's sarcoma and certain B-cell lymphomas. The fourth open reading frame of the KSHV genome encodes a protein (KSHV complement control protein: KCP) predicted to

have complement regulating activity. Here, we show that soluble KCP strongly enhanced the decay of classical C3 convertase but not the alternative pathway C3 convertase, when compared with the host complement regulators: factor H, C4b-binding protein and decay accelerating factor. The equilibrium affinity constant (K_D) of KCP for C3b and C4b was determined by surface plasmon resonance analysis to range between 0.47-10 μ M and 0.025-6.1 μ M, respectively, depending on NaCl concentration and cation presence. Soluble and cell-associated KCP acted as a cofactor for factor I- mediated cleavage of both C4b and C3b and induced the cleavage products C4d and iC3b, respectively. In the presence of KCP, factor I further cleaved iC3b to C3d, which has never been described before, as complement receptor 1 only mediates the production of C3dg by factor I. KCP would enhance virus pathogenesis through evading complement attack, opsonisation, and anaphylaxis, but may also aid in targeting KSHV to one of its host reservoirs, since C3d is a ligand for complement receptor 2 on B-cells.

1615 The hepatitis C virus NS5A protein activates a phosphatidylinositol 3-kinase-dependent survival signalling cascade

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Hepatitis C virus (HCV) establishes a chronic infection in up to 80% of patients resulting in liver cirrhosis and hepatocellular carcinoma. The non-structural protein NS5A has been implicated in modulation of host mitogenic signalling. We previously described that NS5A interacts with members of the Src family of tyrosine kinases and inhibits AP-1 transcription factor activity in hepatocyte derived cell lines (Macdonald *et al.* submitted 2002). In this study we have extended our analysis of the interplay between NS5A and mitogenic signalling pathways. We report that NS5A binds directly to the SH3 domain of the p85 regulatory sub-unit of PI3-kinase and that this may involve a novel (non-proline rich) sequence within NS5A. Co-immunoprecipitation analysis revealed a heterotrimeric protein complex consisting of NS5A bound by p85 and active p110 catalytic sub-unit. Furthermore, we demonstrate that NS5A enhances p110 phosphotransferase activity in a titratable manner both *in vitro* and using HCV replicon expressing hepatoma cell lines. The down-stream effects of an NS5A-PI3-kinase interaction included increased phosphorylation of protein kinase B (AKT), which plays a role in regulating host cell apoptosis. Moreover, these data suggest that modulation of PI3-kinase may represent a mechanism for HCV induced carcinogenesis and a potential therapeutic strategy to counteract the occurrence of human hepatocellular carcinoma.

1630 The hepatitis C virus p7 protein is an ion channel target for anti-viral therapy

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Hepatitis C virus (HCV) is a major cause of Hepatocellular Carcinoma and is now the leading cause for liver transplantation in the developed world. Current therapy for HCV infection is subject to only modest efficacy, severe side effects and increasing viral resistance. Recently, inclusion of Amantadine has been shown to improve the effectiveness of current drug regimes in clinical trials, though its mode of action is unclear. We have shown that the p7 protein of HCV oligomerises both in cells and *in vitro* to form an ion channel structure as determined by biochemical and biophysical methods. Ion channel activity was demonstrable both in artificial lipid bilayers and in a cell-

based functional assay. Amantadine was shown to specifically inhibit the ion channel activity of p7 at concentrations in the micromolar range, which also inhibits the M2 ion channel of Influenza A virus. Furthermore, mutation of a conserved region of charged amino acids abrogated ion channel activity and this protein displayed a dominant negative phenotype. Given the absolute requirement for a functional p7 protein in the related Pestivirus, Bovine Viral Diarrhoea virus for the production of infectious progeny, HCV p7 presents a novel candidate drug target for anti-viral therapy.

1645 Therapeutic immunisation with codon-optimised genes prevents papilloma wart disease in dogs

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Canine Oral Papilloma Virus (COPV) infects canine mucosal epithelia. Both the viral genome and the infectious life cycle are similar to that of the low risk mucosal human papillomaviruses. Approximately 6 weeks after infection at mucosal epithelial sites papillomatous warts begin to develop. Warts grow to maximum size by week 8-9 before beginning to regress spontaneously. They have usually fully regressed by week 14-16 leaving no scarring (reviewed in 1). Spontaneous wart regression is coincident with the infiltration of canine CD4 and CD8 T-cells which begins before regression and becomes maximal during the period of rapid wart regression in weeks 9-16 (2). Seroconversion to the major COPV late capsid antigen L1 also occurs during this regression period and animals that have recovered from disease are protected from subsequent re-challenge with infectious virus. Just as in humans, in COPV-induced wart disease the immunological effector mechanisms and in particular the antigen-specific responses that drive disease resolution are still poorly defined.

The mucosal COPV disease model has been used as a key model in the development of papillomavirus vaccines, including those based upon heterologous warts extracts, and L1 virus-like particles (VLP's). Previously, we showed that intra-epithelial immunisation by particle mediated immunotherapeutic delivery (PMID) of plasmid DNA encoding the late COPV L1 gene could completely protect dogs from challenge with infectious virus (3). We extended these findings to show that immunisation with plasmid DNA encoding codon optimised early genes could completely protect animals from disease after challenge with virus (4). In these studies we show that codon-optimised (but not wild-type genes) also prevent development of disease when dogs are immunised therapeutically - after challenge with infectious virus. The route of immunisation is critically important in providing optimal vaccine efficacy.

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1700 A bacterial toxin subunit greatly enhances LMP-specific CTL killing of EBV-transformed cells

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Epstein-Barr virus (EBV) is associated with a number of important human cancers including nasopharyngeal carcinoma, gastric carcinoma and Hodgkin's lymphoma. These tumours express a viral nuclear antigen, EBNA1, that cannot be presented to T-cells in an MHC class I context, and the viral latent membrane proteins (LMPs). Although the LMPs are expressed in these tumours, no effective immune response is made. Here we report that exposure to the cholera-like enterotoxin B-subunit (EtxB) in EBV-infected lymphoblastoid cell lines (LCLs) enhances their susceptibility to killing by LMP-specific CD8+ CTL in an HLA class I-restricted manner. Brief exposure of LCLs to EtxB results in the co-localisation, capping, and internalisation of LMPs. CTL killing of LCLs is dramatically increased through both TAP-dependent and independent epitopes following EtxB treatment. The use of mutant B-subunits revealed that the enhanced susceptibility of LCLs to CTL killing is dependent on the B-subunit's interaction with GM₁ but not its signalling properties. These important findings could underpin the development of novel approaches to treating EBV-associated malignancies, and may offer a general approach to increasing the presentation of other tumour and viral antigens.

1715 Expression of LFA-3 and ICAM-1 in adenovirus-transformed human cells and their sensitivity to nk-mediated cytotoxicity

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We aim to investigate the susceptibility of Adenovirus 12 and Adenovirus 5 transformed human cells to NK cell lysis *in vitro* using a human NK cell-line, YT. The YT cell-line is not inhibited by target cell expression of MHC class I. This allows the effect of molecules other than MHC class I to be investigated for their role in determining the susceptibility to NK cell lysis.

We investigated the expression of adhesion molecules on the Ad5 and Ad12 transformed cells using flow cytometry. Despite their susceptibility to NK cytotoxicity, the Ad12-transformed cells expressed significantly lower levels of cell surface LFA-3 than either the Ad5- or control transformed cell lines. ICAM-1 could not be detected on any of the Ad-transformed cells.

The results indicate that Ad12 and Ad5 transformed cells are differentially sensitive to NK cells and that these differences are not due to altered levels of MHC class I. Furthermore, the expression of LFA-3 and ICAM-1 on the target cells does not correlate with their sensitivity to NK cells. Current experiments are aimed at identifying the mechanisms that determine the susceptibility of Ad-transformed cells to NK cells.

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0900 HTLV and leukaemia

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0945 Epstein-Barr virus and oncogenesis: From latent genes to cancer

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Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus associated with the development of both lymphoid and epithelial tumours. As a common virus infection, EBV appears to have

evolved to exploit the process of B cell development to persist as a life-long asymptomatic infection. However, the virus can contribute to oncogenesis as evidenced by its frequent detection in certain tumours, namely Burkitt's lymphoma (BL), post-transplant B cell lymphomas, Hodgkin's disease (HD) and nasopharyngeal carcinoma (NPC), and by its unique ability to efficiently transform resting B cells *in vitro* into permanently growing lymphoblastoid cell lines (LCLs). These transforming effects are associated with the restricted expression of EBV genes such that only a subset of so-called latent virus proteins (EBNAs, LMP1, LMP2) are expressed in virus infected tumours and in LCLs. Distinct forms of EBV latency are manifest in the different tumours and these appear to be a vestige of the pattern of latent gene expression used by the virus during the establishment of persistent infection within the B cell pool. Key to the ability of EBV to efficiently colonise memory B cells and to contribute to oncogenesis is the expression of LMP1 and LMP2 both of which provide essential survival signals by mimicking the activity of cell surface receptors.

1100 Hepatitis viruses

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Hepatitis B virus (HBV) and hepatitis C virus (HCV) induce chronic liver disease affecting 3- 5% of the population worldwide, and they have been causally related to the development of liver cirrhosis and hepatocellular carcinoma. These two hepatotropic viruses belong to different viral families (Hepadnaviridae and Flaviviridae), and differ largely in genome structure and mode of replication.

The role of hepatitis B and C viruses in tumor formation appears to be complex and may involve direct and indirect mechanisms. Chronic liver inflammation and hepatic regeneration induced by cellular immune responses may favor the occurrence of genetic alterations. Prolonged expression of viral gene products, such as the regulatory protein X and the large envelope protein of HBV, and the core protein of HCV, may contribute in deregulating cell transcription and proliferation. Evidence implicating these viral proteins in liver carcinogenesis has been provided in transgenic mouse models. A distinct mechanism in HBV-related tumorigenesis relies on the integration of viral DNA into the host genome, which induces direct insertional mutagenesis of diverse cancer-related genes in a number of cases, and might also play a role in inducing chromosomal instability. Furthermore, recent genome-wide studies have revealed that different oncogenic pathways might be activated in HBV- and HCV-associated tumors. This search might permit an accurate evaluation of major targets for early diagnostic of liver cancer and therapeutic intervention.

1145 Replication-selective oncolytic virotherapy for cancer: a novel therapeutic platform

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The field of oncolytic virotherapy has exploded over the last ten years. This approach is based on the clinical application of viruses that replicate selectively in cancer cells while leaving normal tissues unharmed. Four main strategies have been explored (Kirn D. *et al. Nature Med* 2001; 7: 781-7): 1) the use of inherently cancer-selective viruses (e.g. RNA viruses), 2) deletion of viral genes necessary for replication in normal cells but expendable in cancer cells (e.g. adenovirus, poxviruses, HSV), 3) use of tissue-specific promoters to drive expression of viral replication machinery only in cancer cells (e.g. adenovirus, HSV), 4) coat protein retargeting to ablate native receptor recognition and engineer tumor-specific ligands into coat proteins for selective uptake (e.g. adenovirus). The optimal features for a virus species and/or strain include the following: 1) rapid replication and spread in tumor mass; 2) feasibility of intravenous delivery to metastatic tumors; 3) large carrying

capacity for therapeutic genes; 4) immune clearance avoidance; 5) selectivity mechanism(s) available. In addition to these viral factors, the role of the immune response is still being elucidated. The optimal approach will be to avoid immune-mediated clearance of the virus (at least not until after efficacy achieved) while at the same time stimulating antitumoral immune mechanisms. Basic virologic mechanisms, preclinical pharmacology and clinical research results to date will be presented and discussed.

1400 SV40 and human cancer

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SV40 DNA, RNA and proteins have been found in some human mesotheliomas and brain tumors. Because SV40 is a strong oncogenic virus, these findings suggested that SV40 might contribute to some malignancies. Conflicting epidemiological studies and occasional negative reports led to a controversy about the presence and role of SV40 in some human tumors. In 2002 three reports from three independent review panels, led respectively by Drs. G. Klein and C. Croce; Drs. M. Wong and J. Pagano; and by the Institute of Medicine, National Academy of Sciences, USA, all agreed that SV40 was present in some human tumors. The epidemiological studies that had stirred so much controversy were found "flawed" because it was not possible to reliably identify cohorts exposed to SV40 from non-exposed cohorts (reviewed in A. Gazdar, J. Butel, and M. Carbone, *Nature Reviews Cancer*, 2002). In my laboratory we have studied the biological effects caused by SV40 in human mesothelial cells. We found that these cells are unusually susceptible to SV40 infection and transformation. We demonstrated that SV40 remains episomal in transformed mesothelial cells, and we elucidated the mechanisms that allow SV40 to remain episomal without causing cell lysis. SV40 was shown to cooperate with asbestos – a fiber that causes mesothelioma – in the process of mesothelial cell transformation. In addition to inactivating p53 and Rb, SV40 specifically induced telomerase activity and Notch-1 in infected human mesothelial cells. These activities mediated by both the large T and the small t antigen were required for immortalization and transformation.

1445 KSHV biology

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Kaposi's sarcoma-associated herpesvirus (KSHV), also called Human herpesvirus 8 (HHV-8), is associated with three types of human tumours: Kaposi's sarcoma (KS), multicentric Castlemann's Disease and primary effusion lymphoma (PEL). Our current work is focussed on understanding mechanisms by which the virus evades the immune response. KSHV is unique in that it encodes four homologues of cellular interferon regulatory factors (IRFs), referred to as viral IRFs (vIRF-1, -2, -3 and -4). Since induction of cellular IRFs forms part of a complex pathway by which interferons exert their pleiotropic effects, it is probable that vIRFs make an important contribution to KSHV biology. Our work on vIRF-2 indicates that this protein subverts the type I interferon signalling cascade by inhibiting transcription from interferon stimulated promoters, specifically during activation by cellular IRF-1 or IRF-3. The vIRF-2 protein therefore participates in blocking this aspect of the innate immune response. KSHV also encodes a protein with homology to cellular regulators of complement activation that we refer to as KSHV complement control protein (KCP). Three KCP isoforms are predicted from differential splicing events. All three inhibit complement activation, as determined by reduced C3 deposition at the surface of cells expressing each KCP isoform, following activation of the classical complement cascade. KCP therefore provides a strategy for evading complement attack, opsonisation, and anaphylaxis. These are two examples of a sophisticated array of immune evasion genes encoded by KSHV that presumably enhance virus infection, persistence and pathogenesis.

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Clinical Microbiology Group - Poster:

CM 19 Genomic diversity of cryptic type III secretion system in *Escherichia coli* strains using long-range PCR

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The availability of the complete genome sequences of various *Escherichia coli* strains provides a tool to assess the genomic diversity within *E. coli* strains and isolates. We have developed long range PCR to amplify large segments of DNA based on the complete genome sequences of *E. coli*. Using long PCR the diversity of the recently discovered cryptic type III secretion system (TTSS) in *E. coli* were investigated in 79 *E. coli* strains, including the reference collection of 72 natural isolates (ECOR strains), *E. coli* RS218, *E. coli* 042, ETEC H10407, EAEC25, *E. coli* CFT073, *E. coli* K12 and *E. coli* 0157:H sakai strain. We mapped the PCR data onto the dendrogram generated by Herzer et al., 1990 and showed that all of the TTSS genes were lost in group B2 including the *E. coli* RS218 and *E. coli* CFT073 strains. Most of group A and B1 strains including *E. coli* K12, EAEC25 and ETEC have lost genes that encode components of the type III secretion apparatus. In addition, all the stains in group D including *E. coli* 042 have lost the genes that encode the translocases or chaperones. Furthermore, strains in group E only have sporadic losses in the TTSS region. We therefore conclude that long PCR is a useful tool for assessing genomic diversity and for detecting large-scale chromosomal insertions or deletions.

Environmental Microbiology Group - Poster:

EM 11 Biohazards of landspreading form of disposal of spent compost wastes in Northern Ireland

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Spent mushroom compost (SMC) is a major waste of the mushroom industry with low economic value. SMC arises after mushroom production in phase II compost (pIIC), predominantly comprising straw and chicken litter as principal raw ingredients. Although the majority of SMC waste is disposed off by application to agricultural land and it is as an attractive proposition for soil inorganic fertiliser supplementation, there is limited data available as to its consequences, in terms of microbiologically loading of food-borne pathogens, those of significance to mushroom industry itself and the resulting imbalance of the natural flora of the agricultural land. Therefore this study aims to (i) examine the SMC for prevalence of faecal bacterial pathogens that may arise from chicken litter and (ii) to ascertain the pathogenic bacteria (*Pseudomonas syringae*, pv *phaseolicola* or *tolasii*) and fungal populations (*Trichoderma*, *Verticillium* species) originating mainly from the straw component of the SMC, which are also of relevance to disease management and for mushroom production. The qualitative diversity of bacterial populations within SMC was ascertained through rDNA, PCR and direct sequencing techniques on the culturable microflora. Conventional microbiological analyses of SMC material (n=30) from six commercial operations in both Northern Ireland and the Republic of Ireland, failed to detect *Salmonella* spp, *Listeria* spp. or *Campylobacter* spp. in any of the SMC material examined. The main morphotypes were identified and were further examined by employment of partial 16S rRNA gene amplification and sequencing techniques, yielding several genera and species. This is the first preliminary report on the microbial diversity of SMC waste and demonstrating data upon the presence of several species which have not been previously described in SMC or pIIC, in addition to two potentially novel species within the genera *Microbacterium* and *Stenotrophomonas*. The DNA relatedness of *Pseudomonas syringae* pv *phaseolicola* or *tolasii* wild type isolated from composts to those of race-specific strains of UK and corresponding phage sensitivities using dsRNA phage psi6 are also reported. The implications of dsRNA mycoviruses and other viral particles found in mushrooms or compost samples are also discussed. It is thus important to examine the ecological microbe-microbe and plant-microbe interactions that are occurring between the native bacterial soil flora and those added annually (approximately 10¹⁸ cells) through the application of SMC. Such studies would be beneficial in helping to ascertain the ecological consequences involved in the disposal of SMC waste on agricultural land overlooking the issues concerning plant, animal and human health.

Main Symposium - Poster:

MS 22 The chemical genetic approach in cellular microbiology

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The pharmaceutical industry has utilised high throughput screening (HTS) of small molecule libraries as a tool for drug discovery. More recently academic researchers have used HTS to identify small molecules that selectively inhibit cellular processes. This approach aims to enhance our understanding of these processes at the molecular level using small molecules: a technique termed chemical genetics.¹ Unlike classical mutational studies, the chemical genetic approach has the advantage of being readily reversible and allows the modification of the activity of essential proteins (whose mutation would result in a lethal phenotype). The chemical genetic approach is readily accessible due to advances in small molecule library synthesis and the advent of commercially available small molecule libraies.²

Forward chemical genetic approaches frequently culminate in target identification studies. Hits can be chemically attached to a solid phase resin via a synthetic linker allowing the use of affinity chromatography or pull down assays to identify the protein target(s) of the small molecule (chemical proteomics).³

A HTS screen has been used to identify several small molecules that perturb the invasion of BSC-1 cells by *Toxoplasma gondii*.⁴ The invasion of this obligate, intracellular protozoan parasite is poorly understood at the molecular level. Interestingly small molecules that both inhibit and enhance invasion were identified. Selection of a small molecule that enhanced invasion has directed synthetic towards a solid supported molecule with the aim of carrying out affinity chromatography studies.

1) G. E. Ward, K. L. Carey & N. J. Westwood, *Cellular Microbiology*, 2002, **4**; 471-482 / 2)

http://iccb.med.harvard.edu/screening/chemical_libraries/index.htm / 3) N. S. Gray, *Current Opinion in Neurobiology*, 2001, **11**; 608-614 / 4) Ward, Westwood, Mitchison, unpublished results.

Virus Group

Tuesday 8 April 2003

0945: Attenuated RSV and PIV vaccines

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Acute respiratory tract infections are the leading cause of mortality worldwide due to infectious diseases. Respiratory syncytial virus (RSV) subgroups A and B and parainfluenza virus (PIV) serotypes 1, 2 and 3 account for nearly half of pediatric hospitalizations for respiratory tract disease. We are developing live attenuated mutants of RSV and the PIVs for intranasal vaccination of very young infants. One challenge is to achieve a satisfactory level of attenuation while retaining satisfactory immunogenicity in the very young infant, where immune responses are reduced due to immunologic immaturity and the immunosuppressive effects of maternally-derived virus-specific IgG. The need to fine-tune the attenuation of each virus can be met using reverse genetics to produce virus from cloned DNAs: this provides a way to systematically identify and evaluate attenuating mutations and to insert desired combinations into vaccine candidates. An array of methods of attenuation have been identified, including point mutations in proteins and nucleotide signals, gene deletions, and replacing “internal” genes with their counterparts from related animal strains that are naturally attenuated in primates. In addition, it is possible to make modifications to the viral backbone that improve vaccine immunogenicity, such as moving the major protective antigens to be promoter-proximal for increased expression. Other strategies will be discussed, such as the use of one vaccine virus to vector the protective antigens of a second one, thereby reducing the number of separate viruses to be administered. Some of these vaccine candidates are in clinical trials.

VIRUS Workshop – Virus entry, morphogenesis and exit

Wednesday 9 April 2003 – 1400-1800

Early events in infection by foot-and-mouth disease virus

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Foot-and-mouth disease virus (FMDV) uses a number of RGD dependent integrins ($\alpha\beta 1$, $\alpha\beta 3$ and $\alpha\beta 6$) as a receptor to initiate infection. Following integrin binding, FMDV is believed to enter the cell by uptake into the endosomal/lysosomal pathway where the prevailing low pH triggers capsid disassembly and release of the vRNA. The main evidence to support this infectious route comes from studies that show that reagents that raise endosomal pH, such as monensin and concanamycin-A inhibit infection by FMDV.

We have shown previously that SW480 cells are made permissive for FMDV by transfection with the $\beta 6$ subunit and expression of $\alpha\beta 6$ (SW480- $\alpha\beta 6$). Furthermore, $\alpha\beta 6$ serves as the major receptor for virus attachment on these cells since virus binding and infection are both inhibited by a function blocking Mab to $\alpha\beta 6$. In this current study we have used the confocal microscope to follow the intracellular trafficking of FMDV at early times post-infection. For these studies we used SW480- $\alpha\beta 6$ and a $\beta 6$ transfected, heparan sulphate deficient CHO cells line, CHO-677. Virus was absorbed to cells at 4°C and infection initiated by warming to 37°C. At time 0, FMDV was seen to co-localize with $\alpha\beta 6$ at the cell surface but not with another RGD-dependent integrin $\alpha\beta 5$, thus confirming our previous observation that $\alpha\beta 6$ serves as the major receptor for virus attachment. At early time post-infection (~5 to 10 mins), virus co-localized with a specific marker for early endosomes. At 0.5 h post-infection, the majority of the virus was located in the cytoplasm. In CHO-677 $\beta 6$ -transfected cells, virus was seen to accumulate to a region on one side of the nucleus whereas in SW480- $\alpha\beta 6$ cells, virus accumulated in a number of discrete regions that were dispersed through the cytoplasm. Currently, we are using Mabs specific for distinct compartments of the endosomal/lysosomal pathway to more precisely define the early trafficking events during infection.

Identification of Morbillivirus Cell Receptors

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Morbilliviruses are highly contagious pathogens that cause some of the most devastating viral diseases of humans and animals worldwide. They include measles virus (MV), canine distemper virus (CDV), rinderpest virus (RPV) and peste des petits ruminants virus (PPRV). New morbilliviruses causing disease in marine animals have also been identified. These include dolphin morbillivirus (DMV), porpoise morbillivirus (PMV) and phocine distemper virus (PDV).

Cellular receptors have been identified as major determinants of host range and tissue tropism of a virus. CD46 and SLAM have been identified as cellular receptors for MV. CDV and RPV also use SLAM as a receptor.

We investigated the use of CD46 and SLAM by DMV and PDV. Immunocytochemistry studies have revealed that these viruses infect normal Chinese hamster ovary

(CHO) cells and CHO cells expressing CD46 and SLAM. Greater infection was observed with the latter. Further studies showed that infection of Vero cells with DMV and PDV was not inhibited in the presence of an anti-CD46 monoclonal antibody. This result suggests that CD46 is not a receptor for DMV and PDV. Therefore, Vero cells must express another receptor for DMV and PDV. In addition, both viruses appear to use an unidentified receptor on CHO cells.

Infection of B95a cells, with both viruses, was inhibited in the presence of an anti-SLAM monoclonal antibody, indicating the use of SLAM as a cellular receptor on B95a cells.

Inhibition of Semliki Forest virus entry into cells

Joanne Cusack & Martina Scallan

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Tyrphostins are a group of synthetic protein kinase inhibitors that were developed as selective inhibitors of tyrosine kinase receptor autophosphorylation. AG1024 has been reported to be a specific inhibitor of the insulin-like growth factor-1 receptor (IGF-1R) and the insulin receptor, with a significantly lower IC₅₀ for the IGF-1R (Parrizas, M. *et al.*, 1997). . We have observed that in a range of cell-types, AG1024 inhibits Semliki forest virus (SFV) replication. No extracellular virus can be detected 5 hours after infection, in contrast to 3.2 x 10³ PFU/ml in the supernatant of non-inhibitor treated controls. Virus-binding assays, internalisation assays, and western blot analysis of the time-course of translation of virus non-structural proteins were performed to determine the point of action of the inhibitor. Binding of SFV to cells is unaffected by AG1024, however internalisation of the virus is reduced in the presence of the inhibitor. AG1024 exerts this antiviral effect at a point other than the IGF-1 receptor, and does not impose a general block to receptor-mediated endocytosis, as uptake of FITC-labelled transferrin is unaffected by this tyrphostin.

The HSV-1 gHgL heterodimer binds to integrins.

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Glycoprotein H (gH) homologues have been found in all herpes viruses and all gH homologues form a heterodimer with a small non-membrane anchored protein termed gL. The herpes simplex type I (HSV-1) gH:gL heterodimer is essential for virus entry, cell to cell spread and along with gB and gD is necessary and sufficient for fusion. It is thought that the gH:gL heterodimer plays an essential role in the entry of all herpesviruses.

A potential receptor for the HSV-1 gH:gL heterodimer has been identified by expression cloning using a vero cell cDNA retrovirus library. Vero cells panned over a soluble recombinant gH:gL protein bind and are resistant to gentle washing. CHOK1 cells fail to bind in such a manner. CHOK1 cells were transduced with a vero cell cDNA retrovirus library and cells that could bind the soluble recombinant gH:gL protein were selected. Analysis of the integrated library cDNA's indicates that an interaction with integrins is responsible for the vero cell binding which suggests that the HSV-1 gHgL heterodimer binds to integrins.

MHV-8 interaction with target cells: roles for GAGs and the viral glycoprotein M7

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The murine herpesvirus-68 (MHV-68) major envelope glycoprotein M7 has positional homologues in the highly related human gamma-herpesviruses Epstein Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV), gp350/220 and K8.1, respectively. Both gp350 and K8.1 have been shown to bind to cell surface heparan sulfate. gp350 also binds the complement receptor 2 (CR2) on B cells.

In order to determine the function of the M7 gene product in the context of viral infection, we used the recently developed BAC technology to create a recombinant MHV-68 virus in which we mutated the M7 ORF (M7-K-virus). The truncated M7 gene product expressed from this virus lacks most of the C-terminus, including the transmembrane domain and should therefore be absent from the viral envelope. Experiments done so far suggest that the M7 protein is dispensable both for viral replication *in vitro* and more importantly, *in vivo*. In fact, titres of M7-K-virus at peak time-points for both lytic and latent infection in C57BL/6 mice are comparable to wild-type titres. However, when assayed *in vitro*, the M7-K-virus has a subtle growth deficit in that total virus titre reaches its maximum after approximately 48h and remains at least one log below wild type titre. Since the phenotype is more pronounced for supernatant virus than for cell associated-virus, we predict a cell to cell spread deficit. Interestingly, the M7-K-virus is less dependent on cell surface heparan sulfate for infection and is also less inhibited by soluble heparin than wild type virus. We propose that the function of the M7 glycoprotein on the viral envelope is to bind to heparan sulfate on the cell surface of target cells and in consequence to facilitate the interaction of a different viral protein to its cellular receptor.

Barriers to morbillivirus cross species infection

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A recombinant clone of the Onderstepoort vaccine strain of canine distemper virus (CDV) encoding unique restriction sites has been generated and rescued. These unique restriction sites allow glycoprotein exchanges to be made between CDV and the recombinant RBOK rinderpest virus vaccine (RPV) clone. Recombinant viruses expressing the haemagglutinin (H), or both the fusion (F) and haemagglutinin glycoproteins of CDV in a RPV backbone have been rescued. Immunocytochemistry and confocal microscopy were used to indirectly visualise the location of the glycoproteins in Vero cells infected with the recombinant viruses. Growth analysis in Vero cells demonstrated that the recombinant viruses replicated at rates comparable to the parental RPV and CDV at titres of approximately 10^6 TCID₅₀ ml⁻¹. Bovine skin fibroblast (BSF) cells support RPV replication with titres reaching 10^6 TCID₅₀ ml⁻¹. CDV was unable to replicate in the cells. However, recombinant viruses expressing the glycoprotein(s) of CDV were able to enter and replicate in the BSF cells with titres of approximately 10^5 TCID₅₀ ml⁻¹. The block to productive CDV infection in BSF cells is currently being investigated.

BTV ENTRY MECHANISM

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The entry mechanism of non-enveloped viruses, such as Bluetongue virus (BTV) is not well understood. BTV consists of two capsids, an inner capsid or 'core' and an outer shell that is composed of two proteins, VP2 and VP5. We have previously shown that the larger VP2 protein is a trimer and binds to a cellular receptor. In this study we have undertaken a detailed investigation on the outer capsid proteins, in particular, on VP5 to understand the mechanism of BTV entry. We have prepared a novel recombinant baculovirus designed to express VP5 on the cell surface. In this construct VP5 is fused to the VSV G protein transmembrane domain and with baculovirus gp64 signal sequence. To determine whether VP5-VSV (in presence or in absence of VP2-VSV), was sufficient to induce membrane fusion, insect cells were infected and then exposed to low pH for a short time. Cells infected with VP5-VSV have shown fusion-like activity and syncytium formation in a pH dependent manner. However, when VP5 was co-expressed with VP2 on the cell surface its fusion activity was inhibited. A series of deletion mutants were generated in order to localize the VP5 domain responsible for its fusion-like activity. Such activity was confirmed in mammalian cells by constructing similar constructs using a mammalian vector system. *Our accumulating data demonstrate that the non-glycosylated surface protein of a non-enveloped virus uses a similar strategy to the fusion proteins of many enveloped viruses.*

Analysis of the N-linked Glycosylation of Hantaan Virus (HTNV) Glycoproteins (G1 and G2) and the Roles of Oligosaccharide on Protein Folding, Intracellular Transport and Immunoreactivity

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The membrane glycoproteins G1 and G2 of Hantaan virus (HTNV, strain 76-118, family *Bunyaviridae*) are modified by N-linked glycosylation. The glycoproteins contain six potential sites for the attachment of N-linked oligosaccharides, five sites (at residue N134, 235, 347, 399 and 609) on G1 and one (N928) on G2. The property of the N-linked oligosaccharide chains was analyzed with treatments of Endo H, PNGase F, tunicamycin and deoxynojirimycin (DNJ) and was confirmed to be a completely high mannose type. The role of individual N-glycan was assessed with respect to its effect on the folding, intracellular transport and immunoreactivity. Ten glycoprotein precursor mutants were constructed by site-directed mutagenesis, including six single N-glycosylation-site mutants and four double site mutants. We determined that four sites (N134, 235, 347 and 399) on G1 and the only site (N928) on G2 in their ectodomains are utilized, whereas the fifth site (N609) that faces the cytoplasm is not glycosylated. The importance of individual N-oligosaccharide chains varies with respect to the folding and intracellular transport. The first N-oligosaccharide chain (at residue N134) was found to be crucial to protein folding, whereas single mutation at other glycosylation sites was much tolerable. Mutation at two N-glycosylation sites (N235 and N399) resulted in G1 misfolding. Our data demonstrated that N-linked glycosylation of HTNV glycoproteins plays important and differential roles in the protein folding, intracellular transport and maintaining the antigenic epitope conformation of the glycoproteins.

Subcellular targeting of Adenovirus core polypeptides Mu and VII

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Adenovirus DNA is non-covalently bound to the viral core proteins Mu, V, and VII. Nuclear and nucleolar targeting sequences have previously been demonstrated in protein V, and since proteins Mu and VII have similar arginine rich regions, their putative targeting sequences were investigated.

Regions of the open reading frame of preMu and preVII protein were amplified from adenovirus DNA by polymerase chain reaction, and further oligonucleotide mutants of Mu and VII were designed with selected arginine codons replaced by alanine or lysine codons. Fragments were cloned into a mammalian expression plasmid to express the amino acid sequences as N-terminal fusions to enhanced green fluorescent protein. Full-length preMu and preVII demonstrated exclusive nucleolar targeting. Mu protein, and a similar sequence within protein VII, demonstrated nucleolar targeting with a background distribution within the cytoplasm. Site-directed mutation of arginine to alanine residues reduced the intensity of nucleolar targeting. Our data suggests that, as with protein V, Mu protein and protein VII demonstrate nucleolar and/or nuclear targeting sequences, rich in basic amino acids.

Nuclear shuttling of α -herpesvirus UL47 tegument proteins

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The UL47 genes of the γ -herpesviruses herpes simplex virus type-1 (HSV-1), bovine herpesvirus type 1 (BHV-1) and pseudorabies virus (PrV) encode for the tegument proteins VP13/14, VP8 and PrV UL47 respectively. Although these proteins are major virion components, little is known about their role in virus entry and assembly. In spite of a lack of significant sequence homology, we show here that these three UL47 proteins all localise to dots within the nucleus. We further show that these proteins contain discrete, transferable, arginine-rich nuclear targeting signals in their N-termini. Moreover, using heterokaryon and actinomycin D assays we demonstrate that these proteins are capable of shuttling between the nucleus and the cytoplasm and we have localised the nuclear export signal for VP8 to the N-terminal half of the protein. Taken together with previous data showing RNA binding by VP13/14, we suggest that the nucleocytoplasmic shuttling properties of this group of proteins may indicate a role in virus-specific RNA transport from the nucleus. Such a function could be relevant at virus entry, late virus infection, or both.

Characterisation Of The Herpes Simplex Virus-Type 1 DNA Packaging Protein Ul17

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In herpes simplex virus-type 1 (HSV-1) infected cells two types of enveloped particles, the infectious virion and non-infectious structures termed L-particles (light-particles), are produced. The HSV-1 virion has three structural components: the capsid containing the linear double stranded DNA genome, which is surrounded by an amorphous layer of protein, referred to as the tegument, and an outer envelope. L-particles contain the envelope and tegument components of the virion, but lack the capsid and viral DNA. The translocation of the HSV-1 genome into the capsid requires seven packaging proteins, three of which, UL6, UL25 and UL17, have been described as minor capsid proteins. However, in a separate report the UL17 protein was found to be associated with L-particles, in the tegument layer. We have used a monoclonal antibody specific for UL17 to determine which components of the virion UL17 is associated with and at which stage of

capsid assembly. Our results suggest that UL17 is both a tegument and a minor capsid protein and that it is probably incorporated into the virion at two different stages.

Influenza A Virus filaments: the predominant phenotype

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Influenza viruses can exist in two forms; as 100nm spheres and also aa filaments which can extend for several microns in length. We have mapped the genetic determinants of morphology to the matrix protein, M1. In MDCK cells viruses with a filamentous genotype induce protrusions from some of the cells but still form spheres in other cells. We have found that the filamentous genotype is the predominant one in viruses which have not been passaged in mice or egg adapted. We also note large differences in the propensity of different cell lines to support filament production.

These studies may give insights into the intricate interactions between virus and host cell components during the assembly process.

Vaccinia virus F12L protein interacts with microtubules

Brendan J. Murphy, David C. Carpentier & Geoffrey L. Smith

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Vaccinia virus (VV) is a large DNA virus that replicates in the cytoplasm. VV produces four types of virion: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell associated enveloped virus (CEV) and extracellular enveloped virus (EEV). During morphogenesis, microtubules are used both to transport IMV away from the virus factories to the site of wrapping by intracellular membranes to form IEV and to transport IEV from the site of wrapping to the cell surface. Actin tails drive CEV particles away from the cell.

The role of the VV F12L protein in IEV transport is being investigated. F12L is conserved in chordopoxviruses but shows no other match against protein databases. A virus deletion mutant (Δ F12L) lacking F12L is highly attenuated *in vivo*. In infected cells virus morphogenesis is blocked after IEV formation, such that the production of CEV and EEV are reduced and actin tails are not formed. Confocal microscopy showed that F12L co-localises with IEV particles and microtubules and co-immunoprecipitation experiments showed an association of F12L with γ - and β -tubulin. In addition, during infection the heavy and light-chains of kinesin become redistributed to the cell periphery and co-localise with F12L. The roles of specific domains of the F12L protein are being investigated by expression of mutated forms of F12L within cells infected with Δ F12L.

Targetting of the influenza virus nucleoprotein to the apical plasma membrane

Marlene Carrasco, Debra Elton & Paul Digard
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Influenza virus budding is polarised, with new particles only forming from the apical plasma membrane (p.m.) of infected cells. This process requires the assembly of three viral integral membrane proteins (HA, NA and M2) the matrix (M1) protein and the viral ribonucleoprotein (RNP) particles at the correct cellular membrane. The glycoproteins contain well characterised targetting signals that direct them to the apical surface and this has generally been assumed to underpin the vectorial mechanism behind virion assembly. However, we have found that NP (the primary protein component of the RNP) is also targetted specifically to the apical p.m. At late times in virus infected cells, NP imaged by confocal microscopy was not found throughout the cytoplasm but was largely localised at the

apical but not basolateral membrane surfaces. This was in contrast to the M1 and NS2 proteins which were found throughout the cell, including at all p.m. surfaces. Furthermore, NP expressed in the absence of other viral proteins showed a similar ability to localise specifically at the apical p.m., suggesting the existence of an intrinsic targeting mechanism. This vectorial behaviour of NP may contribute to the polarity of influenza virus budding.

VIRUS Workshop 2 – Pathogenesis Wednesday 9 April 2003 – 0900-1230

Session 1: Chairman Stuart MacFarlane

1. 9.00-9.13

Ashley Banyard, Michael Baron & Thomas Barrett
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The roles of the leader and trailer regions of rinderpest virus in determining pathogenicity

Rinderpest virus (RPV) is the cause of a major economic disease of bovids in Asia and Africa. The most highly pathogenic form of the virus is the Saudi/81 strain. Its genome contains a 3' genome promoter followed by the coding regions for the six genes and a 5' antigenome promoter. These promoters have roles in directing replication and transcription as well as RNA encapsidation and packaging. Work in our laboratory using a CAT minigenome system has identified several nucleotides in the genome promoter region of the virus that affect its replicative ability. It is possible that minor changes in the virus genome and antigenome promoters may have significant effects on the ability of the virus to replicate and cause disease in cattle and this might explain the emergence of highly pathogenic strains, such as Saudi/81, or highly attenuated strains, such as Kenya/kudu/96. Here, we describe the effect(s) that changes to the genome and antigenome promoters of different strains of RPV have on the ability of the virus to replicate in different types of host cell. Further, we detail how swapping the genome and antigenome promoters affect virus pathogenesis in the host species.

2. 9.14-9.27

Paul Britton, Rosa Casais, Brian Dove, Teri Hodgson, Sharon Evans & Dave Cavanagh
Division of Molecular Biology, Institute for Animal Health,
Compton Laboratory, Newbury,
Berks RG20 7NN

Biological properties of a recombinant infectious bronchitis virus expressing an altered spike protein

The avian coronavirus infectious bronchitis virus (IBV) is the major respiratory virus of the domestic fowl. In order to ascertain unequivocally the role of the viral proteins in replication, pathogenicity, host range and immunity one needs a reverse genetics system whereby one can precisely modify the virus. We have such a system (Casais *et al.*, 2001, *Journal of Virology*, 75, 12359-12369), based on a full length DNA copy (cDNA) of the IBV RNA genome (27.6 kb) cloned into the genome of vaccinia virus under the control of a T7 DNA-dependent RNA polymerase promoter. T7 polymerase provided by a recombinant fowl poxvirus results in the transcription of infectious RNA for the rescue of IBV. We have modified the spike protein gene of our cloned IBV and have completed the biological characterisation of the recombinant IBV *in vitro* and *in vivo*.

3. 9.28-9.41

Richard B. Hitchman^{1,2}, L.A. King² & R.D. Possee¹
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BAC cloning whole genomes; a novel tool for investigating baculovirus pathogenesis

The baculovirus, *Panolis flammea* multi-nucleopolyhedrovirus (PafINPV) is an insect-specific pathogen, containing a 145 kb double-stranded DNA genome. It was originally isolated from *P. flammea* (pine beauty moth) larvae, during a natural epizootic in Scotland.

In the environment *P. flammea* can harbour a number of closely related PafINPV genotypes, which are regarded as variants of a single virus. When PafINPV is amplified within different insect host species, different genotypes within the population become more dominant as determined by Southern hybridisation. However, after several passages through the host the less dominant genotypes are also still present. *In vivo* cloning of a virus population purified from a single infected *P. flammea* isolated twenty-five different PafINPV genotypes. Genotypic variation was observed when the virus genomes were digested with restriction enzymes and fractionated using agarose gel electrophoresis. Most of the variation appeared to be located in specific areas on the virus genome. The pathogenicity of seven of the 25 variants was also studied and found to differ within and between different host species. The role of these variants and why so many are maintained in the virus population is unknown. It is tempting to speculate that the differences in pathogenicity between the variants is a consequence of the genetic heterogeneity observed in the variable regions of the virus genome. Sequencing of these variable regions has identified genes which may be important in this process.

The unusually high genetic diversity of PafINPV populations is unique among DNA viruses and provides a useful system for studying baculovirus pathogenesis. However, there are difficulties in cloning pure virus genotypes. The host is difficult to rear in the laboratory and there is no cell culture system available. Isolating PafINPV genotypes by *in vivo* cloning is time consuming and requires an alternative host, usually *Mamestra brassicae*. This in turn often leads to the activation of a latent MbNPV-like virus. When an individual genotype is finally isolated it may still contain closely related genotypes which are almost undetectable using the techniques currently available. Therefore, an alternative system for cloning PafINPV genotypes from mixed populations is presented using a modified bacterial artificial chromosome.

4. 9.42-9.55

Lyda Neeleman & **John F. Bo**
Institute of Molecular Plant Sciences, Leiden University,
Gorlaeus Laboratories, Einsteinweg 55, 2333 CC Leiden, The Netherlands

Translation of alfamovirus RNAs requires binding of coat protein dimers to the 3' end of the viral RNAs

The family *Bromoviridae* contains the genera *Bromovirus*, *Cucumovirus*, *Oleavirus*, *Alfamovirus* and *Iilarvirus*. A mixture of the three genomic RNAs of bromo- and cucumoviruses is infectious to plants and the RNAs contain a 3' terminal tRNA-like structure (TLS) that can be charged with tyrosine. In contrast, a mixture of the three genomic RNAs of alfamo- and ilarviruses is not infectious unless viral coat protein (CP) or the subgenomic CP messenger, RNA 4, is added to the inoculum. The 3' untranslated region (3' UTR) of these RNAs can adopt two alternative conformations: one representing a strong CP binding site and the other resembling the TLS of bromo- and cucumoviruses. This TLS is required for minus-strand promoter activity. Recently, we showed that CP of *Alfalfa mosaic virus* (AMV, alfamovirus) autocatalytically stimulates translation of its messenger, RNA 4, in tobacco protoplasts by binding to the viral RNA. This phenomenon was further investigated. The 3' UTR of RNA 4 contains two CP binding sites: a 3' terminal site that is present in all AMV RNAs and an upstream site that is unique to RNAs 3 and 4. Only the 3' binding site was essential for translation of the RNAs. Replacement of the 3' UTR of RNA 4 by a plasmid derived sequence reduced translation in protoplasts to undetectable levels. However, replacement of the 3' UTR of AMV RNA 4 by the 3' UTR of *Brome mosaic virus* (BMV, bromovirus) resulted in translation of RNA 4 at wild-type (wt) levels, irrespective whether the RNA encoded wt CP or CP with

N-terminal mutations which interfered with RNA binding. The N-terminus of AMV CP is required for RNA binding whereas the C-terminus is required for dimerization of the CP. RNA 4 encoding CP with a C-terminal deletion of 21 amino acids was not translated in protoplasts at detectable levels but translation of this mutant was efficiently complemented *in trans* by wt CP. Probably, stimulation of translation requires the binding of CP dimers to the viral RNAs.

5. 9.56-10.09

Sang Hvon Kim, Eugene V. Ryabov, John Brown & Michael E. Taliany

Scottish Crop Research Institute, Dundee DD2 5DA

Is the nucleolus a gateway for umbravirus systemic infection?

The ORF3 protein encoded by Groundnut rosette virus (GRV, an umbravirus) is a multifunctional RNA-binding protein. It has been shown that this protein protects viral RNA from RNA attack (possibly including RNA silencing-directed degradation) and facilitates its long-distance movement through the phloem. Recently it has been also demonstrated that this protein targets the nucleoli in infected cells. Possible involvement of the nucleolus and nuclear factors in umbravirus systemic infection will be discussed.

6. 10.00-10.14

Gang Zhang & John W. McCauley

Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire RG20 7NN

Different sub-cellular distribution between NS2/3 and NS3 in relation with cytopathogenicity of bovine viral diarrhoea virus

Both NS3 and NS2/3 polypeptides were membrane-associated proteins in cells infected with bovine viral diarrhoea virus (BVDV). However, only NS2/3 is present in cells infected with non-cytopathogenic (NCP) strains of BVDV, and NS3 is suspected to be associated with cell death in cells infected with cytopathogenic (CP) strains of BVDV and the pathogenesis of mucosal disease. The sub-cellular location of NS3 and NS2/3 in cells infected with BVDV has been examined. In BVDV infected cells, NS3 and/or NS2/3 were shown to co-localise, using antibodies specific to cellular marker proteins and confocal microscopy, with the endoplasmic reticulum (ER) protein, ERP60, but not Golgi or lysosomal proteins. Sub-cellular fractionation analysis showed that NS2/3 was almost exclusively associated with the rough ER membrane. In addition to the association with the rough ER membrane, a significant amount of NS3 was detectable in the smooth ER membrane fractions. The differences of distribution of NS2/3 and NS3 on ER membranes in cells infected with CP strains of BVDV has also been observed using confocal microscopy and antibodies that are specific to NS2 and NS3 respectively. Our results on distribution of NS3 and NS2/3 on the ER membrane have revealed a further distinct biological property between CP and NCP strains of BVDV and may provide a link between NS3 and cytopathogenicity of BVDV and by extension pathogenesis of mucosal disease.

7. 10.15-10.28

Debbie Ferguson, Neil Berry¹, Jenny Cowie¹, Gail Davis¹, Ricardo Davis¹, William Elsley¹, Sue Harron¹, Sharim Karim¹, Jenny Lines¹, Sarah MacManus¹, J.Clements², M.Murphy-Corb³, Richard Stebbings¹, Alison Wade-Evans¹ & Neil Almond¹
NIBSC, Retrovirology Dept, South Mimms, ²Johns Hopkins University School of Medicine, Baltimore, USA, ³University of Pittsburgh, USA

Examination of the lymphoid tissue pathology induced during infection with delta NEF attenuated simian immunodeficiency viruses

Live attenuated simian immunodeficiency virus (SIV) has the ability to protect animals against super infection by a range of SIV challenges in the SIV/maaque model of human immunodeficiency (HIV). The use of such a vaccine strategy in humans however is prevented due to serious safety concerns but

a greater understanding of the mechanisms underlying this protection may enable them to be replicated by safer means.

As part of studies to examine the breadth and persistence of protection conferred by live attenuated SIV and the lymphoid tissue pathogenesis associated with these viruses, cynomolgus macaques were infected with one of a range of viral isolates that contain nef disruptions and result in the expression of an attenuated disease phenotype. Groups of animals infected with SIVmacC8, a virus that contains a twelve base pair deletion in its nef gene, were subsequently challenged with one of a range of SIV or SIV/HIV chimeras. *In situ* hybridisation (ISH) and immunohistochemistry (IHC) have been used to examine lymphoid tissues from both protected and super infected animals in conjunction with animals receiving a single viral challenge. This has enabled us to compare the distribution and frequency of virus infected cells and monitor changes in host responses.

Examining animals infected with one of a range of nef attenuated viruses showed that elevated levels of DC-SIGN⁺ cells were present in lymphoid tissues, especially the spleen, when compared to tissues from both SIV naïve animals and animals infected with a nef intact virus. Clear differences were observed from 21 days post infection. Lymphoid tissue from animals protected against wild type superinfection by SIVmacC8 'vaccination' also showed increased levels of DC-SIGN⁺ cells when compared to both dual infected and wild type infected animals.

In conclusion, a distinct lymphoid tissue distribution pattern of DC-SIGN⁺ cells has been observed in animals infected with a nef attenuated virus. This pattern develops shortly after inoculation and persists to at least 6 months post challenge. Animals protected against superinfection have maintained this elevated pattern of DC-SIGN⁺ cells. Studies are underway to further characterise the functional capabilities of these DC-SIGN⁺ cells.

Combining results from a range of viral challenges and superinfected/protected animals enables common elements to be identified, assisting our understanding of the mechanism by which live attenuated vaccines work.

Session 2: Chairman Antonio Alcami

8. 11.00-11.13

Nicola Stock¹, J.Andrejeva¹, D.F.Young¹, S.Goodbourn² & R.E.Randall¹

¹Centre for Biomolecular Sciences, University of St Andrews, ²Dept of Biochemistry and Immunology, St George's Hospital Medical School, University of London

Paramyxoviruses and Interferon evasion

Paramyxoviruses are small, enveloped viruses with a negative sense, single-stranded RNA genome. The sub-family Paramyxovirinae includes four genera, each of which contain viruses which have been shown to evade the Interferon (IFN) system.

Interferon is part of the innate anti-viral defence system in mammalian cells. In response to viral infection (stimuli such as the presence of double-stranded RNA molecules in the cell) small, soluble molecules of Interferon are secreted which activate signalling pathways by binding to receptors on the cell surface. These signalling events lead to the activation of various anti-viral genes such as PKR and 3-5' OAS and positive feedback to produce more IFN. The IFN system is highly efficient at eliminating viral infections and as such, most successful viruses have mechanisms of IFN evasion.

In recent years, our group and others have demonstrated such evasion strategies in Paramyxoviruses. Although varied, most involve the cellular STAT proteins which act as signal transducers and transcriptional activators in the IFN signalling cascade. The strategies range from degradation (Simian virus 5, Human Parainfluenzavirus 2), to destabilisation (Sendai virus) and relocalisation (Nipah virus) of STAT proteins. SV5, hPIV2 and Sendai virus have also been shown to block the production of IFN, although the mechanisms for this are still under

investigation. These various strategies all rely on the product(s) of a single viral gene, P/V (or P/V/C).

Current work in our group focuses on the V proteins of various "exotic" Paramyxoviruses from a range of animal hosts and aims to discover mechanisms whereby these viruses are able to evade the IFN response.

9. 11.14-11.27

Polly Fowler & Stacey Efstathiou

Division of Virology, University of Cambridge

The role of ORF73 in MHV-68 pathogenesis

In vitro studies have established that the Latency associated Nuclear Antigen encoded by the human Kaposi's sarcoma associated Herpesvirus (KSHV) and the related ORF73 gene product of Herpesvirus Saimiri interact with virus origins of replication to facilitate episomal maintenance of DNA. Such a function implies a critical role for ORF73 in the establishment and maintenance of latency *in vivo*. In order to directly determine the role of ORF73 in virus pathogenesis we have disrupted the equivalent ORF73 gene product encoded by Murine Herpesvirus-68 and examined the affect of such a disruption *in vivo* using a well characterised murine model system. Our studies have revealed that ORF73 is not required for efficient lytic phase replication either *in vitro* or *in vivo*. In contrast a severe latency deficit is observed in splenocytes of animals infected with an ORF73 mutant as assessed by infectious centre reactivation assay or by *in situ* hybridisation detection of latent virus. These data indicate a crucial role for ORF73 in the establishment of latency and for virus persistence in the host.

10. 11.28-11.41

Alan Townsley, B.M. Dutia & A.A. Nash

Laboratory for Clinical and Molecular Virology, Royal Dick School of Veterinary Studies, Summerhall, The University of Edinburgh, EH9 1QH

The role of the M4 gene product in the acute phase of murine gammaherpesvirus infection

MHV-68 infection of mice represents a flexible and viable small-animal model for the study of gammaherpesvirus pathogenesis. MHV-76, an additional murid herpesvirus isolated at the same time as MHV-68, is a deletion-mutant of MHV-68, and lacks 4 MHV-68-specific genes (M1-M4) and 8 viral tRNA-like sequences at the 5'-end of the genome, speculated to encode latency-associated and/or immune evasion proteins. As a consequence, MHV-76 is attenuated in the acute phase of *in vivo* infection with respect to MHV-68. M4 is an MHV-68 specific gene with no obvious homologues, and is absent in MHV-76. M4 was detected as an immediate-early/early transcript and was abundantly expressed during lytic replication of MHV-68. To elucidate the contribution M4 makes to *in vivo* pathogenesis, we created a novel MHV-76 mutant (MHV-76inM4), inserting the region of MHV-68 coding for M4 and accompanying putative promoter elements into the 5'-region of the MHV-76 genome. *In vivo* data from this recombinant virus implies a significant role for M4 during the acute phase of infection; viral titres from the lungs of MHV-76inM4 infected mice are significantly increased with respect to both MHV-76 and MHV-68 at early times in the lung. Additionally, between days 14 and 21 post-infection, approximately 10-fold more latently-infected splenocytes are detected in MHV-76inM4 infected mice compared to MHV-76 infected mice, without any evidence of the splenomegaly characteristic of MHV-68 infection at this time. However, by day 31 post-infection, and extended till at least day 100 p.i., the number of latently-infected splenocytes detectable from MHV-68, MHV-76 and MHV-76inM4 infected mice are equivalent, predicting little or no role for M4 in the long-term establishment of latency by MHV-68. *In vivo* replication of a rescue virus is indistinguishable from MHV-76, both in acute infection of the lung and spleen and in the long-term establishment of latency.

11. 11.42-11.55

Jessica Boname

Division of Virology, University of Cambridge

Viral degradation of the MHC class I peptide loading complex

The murine gamma-herpesvirus-68 MK3 gene product degrades MHC class I heavy chains by ubiquitinating their cytoplasmic tails. However, in A20 and RMA cells, MK3 also down-regulates the GPI-linked, non-classical MHC class I glycoprotein Qa-2. This reflects reduced peptide transport into the endoplasmic reticulum (ER) and impaired MHC class I complex stability. There is a loss of tapasin, TAP1 and TAP2 in cells expressing MK3 that depends on the integrity of its PHD/LAP finger, a domain previously shown to be essential for substrate ubiquitination.

In TAP2-deficient RMA/S cells, MK3 was unstable and failed to degrade MHC class I, tapasin or TAP1. An overall model emerges of a tapasin-dependent stabilisation of MK3 by TAP2, and the degradation of multiple components of the peptide loading complex by MK3 that limits its levels in the cell but broadens the scope of its immune evasion. Thus, forced degradation of the class I peptide loading complex represents a new strategy of viral immune evasion.

12. 11.56-12.09

Neil Bryant, Nick Davies-Poynter, Alain Vanderplassen and A. Alcami. Department of Medicine, University of Cambridge

Glycoprotein G from alphaherpesviruses function as broad-spectrum chemokine binding proteins

Mimicry of host chemokines and chemokine receptors to modulate chemokine activity is a strategy encoded by beta- and gammaherpesviruses, but very limited information is available on the anti-chemokine strategies encoded by alphaherpesviruses. The secretion of chemokine binding proteins (vCKBPs) has hitherto been considered a unique strategy encoded by poxviruses and gammaherpesviruses. We describe a family of novel vCKBPs in equine herpesvirus 1, bovine herpesvirus 1 and 5, and related alphaherpesviruses with no sequence similarity to chemokine receptors or other vCKBPs. We show that glycoprotein g (gG) is secreted from infected cells, binds a broad range of chemokines with high affinity and blocks chemokine activity by preventing their interaction with specific receptors. Moreover, gG also blocks chemokine binding to glycosaminoglycans, an interaction required for correct presentation and function of chemokines *in vivo*. In contrast to other vCKBPs, gG may also be membrane-anchored and, consistently, we show chemokine binding activity at the surface of cells expressing full-length protein. These alphaherpesvirus vCKBPs represent a novel family of proteins that bind chemokines both at the membrane and in solution.

13. 12.10-12.14

James Stewart, Anja Kipar & John P. Quinn
University of Liverpool, Liverpool

Tachykinins and the host response to virus infection

Tachykinins not only function as neurotransmitters but also as immunological mediators. Many cells involved in host defence (e.g. T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, and mast cells) produce SP also express the high affinity receptor NK1. This has led to the hypothesis that SP not only acts as a mediator of the cross talk between the nervous and immune systems but is also biologically involved in the direct interaction between immune cells in a paracrine and/or autocrine fashion, independently of sensory nerves or neurogenic inflammation.

A number of studies have shown that viruses (e.g. respiratory syncytial virus) can induce SP and neurogenic inflammation, particularly in lungs in the context of a respiratory challenge. However, there was no data on the direct influence of tachykinins in combating viral infection. The aim of this study was to assess the role of tachykinins in the host response to virus infection using murine gammaherpesvirus (MHV-68) infection of mice. This virus is related to the Epstein-Barr virus and the Kaposi sarcoma-associated herpesvirus that cause disease in humans. We have been using MHV-68 infection of mice to model related diseases in the mouse.

We utilized infection of tachykinin-deficient (*PPT-A*^{-/-}) mice and wild-type controls with MHV-68 to assess their role in the host response to a virus infection. Although infection was ultimately controlled in *PPT-A*^{-/-} mice, there were higher titers of infectious virus in lungs accompanied by a more rapid influx of inflammatory cells. Clearance of latently-infected cells from the spleen was also delayed.

To study the organs and cell types involved in the production of tachykinins during infection we utilised transgenic mice containing the human PPT-A locus with the Laz gene inserted within exon 7. These mice are therefore a convenient tool for localising tachykinin production within tissues. At early times post-infection (1-3 days) we have observed tachykinin gene and peptide expression induced in lymphocytes and epithelial cells within the trachea, mediastinal lymph nodes and alveoli. These studies point to the tachykinins as being important in the early host responses to viral infection.

14. 12.15-12.28

Eva S. Gramoustianou, Nicola Cattini, Richard Wooster, Paul D. Griffiths, Paul Kellam & Vince C. Emery
Royal Free and University College Medical School, London

Viral and host gene expression during human cytomegalovirus infection of fibroblasts and endothelial cells using DNA microarrays

Clinical data have shown that human cytomegalovirus strains exhibit different levels of virulence depending on their passage history in cell culture. Approximately 13kb of novel DNA sequences located near the right edge of the unique long component of the genome have been discovered in Toledo strain and clinical isolates. This region (UL/b') encodes several interesting proteins, is missing from the prototypic laboratory variants of Towne and AD169 and is thought to play an important role in disease pathogenesis. In this study, a glass-based DNA array has been constructed which allows the expression of the 19 novel ORFs to be monitored simultaneously. The viral DNAs were deposited along with 5,500 human genes in order to study host responses to viral infection. Gene expression was monitored by hybridisation to the glass microarrays with fluorescently labelled cDNAs prepared from mock infected and human fibroblasts and endothelial cells during a 96-h time course after infection with HCMV Toledo. This analysis reveals for the first time the expression pattern of the UL/b' region in fibroblasts and endothelial cells, highlights common and cell type-specific transcriptional pathways and pinpoints several host genes which may be implicated in the development of CMV disease.

Virus Group – Workshop Nucleic Acid Replication

DNA VIRUSES

14.00 Recruitment of HSV-1 transcriptional regulatory protein ICP4 into foci juxtaposed to ND10 in live infected cells

Roger D. Everett*, George Sourvinos & Anne Orr
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At the early stages of HSV-1 infection, parental viral genomes frequently become juxtaposed to cellular nuclear structures known as PML nuclear bodies or ND10, while Immediate-Early protein ICP0 precisely co-localises with these structures. Previous indirect immunofluorescence studies observed that HSV-1 transcriptional regulator ICP4 has a mainly diffuse nuclear distribution early in infection, and is later recruited into viral replication compartments. We have constructed HSV-1 viruses expressing ICP4 and ICP0 linked to ECFP and EYFP respectively, both singly and in combination. Coupled with an efficient method of expressing autofluorescent PML in ND10, we have studied the dynamics of ICP0, ICP4 and ND10 in live infected cells. We found that early in infection ICP4 forms discrete foci, some of which are juxtaposed to ND10, while ICP0 co-localised precisely with PML. As expected from these results, we observed that foci of ICP0 and ICP4 were also juxtaposed but not co-localised early in infection. Some of the ICP4 foci must

contain parental viral genomes because they later developed into replication compartments. We propose that a proportion of the ND10-associated ICP4 foci represent ICP4 molecules being recruited onto parental viral genomes, a process likely to be a critical step early in lytic infection. These results may be analogous to the localisation of ie1 and ie2 during HCMV infection, thus suggesting a principle common to the alpha and beta herpesviruses.

14.15 Strain variation in the region of the HCMV genome between genes UL105 (helicase) and UL111A (vIL-10)

Ralph Hector* & Andrew Davison

MRC Virology Unit, Church Street, Glasgow G11 5JR

In the original analysis of the HCMV strain AD169 genome, the 5.8 kbp region between genes UL105 (encoding a helicase) and UL112 (encoding a nuclear protein) was characterized as containing seven small ORFs (UL106-UL111A) potentially encoding proteins. UL111A was found subsequently to be a spliced gene encoding vIL-10. Also, a 5 kb immediate early RNA was mapped to the region between UL105 and UL111A, but was assessed as unlikely to encode protein. The corresponding sequence in chimpanzee cytomegalovirus, which is the closest known relative of HCMV, is similar in size (5.7 kbp), but is substantially diverged and lacks homologues of UL106-UL111A. The corresponding regions in the more distantly related genomes of tupaia, mouse and rat cytomegalovirus are 6.7, 9.2 and 11.5 kbp in size, respectively, and again lack conserved ORFs.

The implication -is that the region of the HCMV genome between UL105 and UL111A may not encode proteins. This is supported by our analysis of six additional viral strains, including two analysed directly from human tissues. Although the sequence is well conserved and the coding regions of UL105, UL111A and UL112 are maintained, the coding potential of each of UL106-UL111 is disrupted in certain strains. Moreover, in contrast with flanking protein-coding sequences, this region in HCMV and other cytomegaloviruses contains unusually A+T-rich sequences. We conclude that the region between UL105 and UL111A is unlikely to encode proteins, and speculate that it may function via a non-translated RNA or, more probably, as an important cis-acting sequence.

RNA VIRUSES – Negative sense genome

14.30 Identification and functional characterisation of the promoter sequences for transcription and RNA replication in respiratory syncytial virus, a non-segmented, negative-sense RNA virus

Rachel Fearn*, David R. McGivern & Vanessa M. Cowton
University of Dundee

Respiratory syncytial virus (RSV) is a member of the order *Mononegavirales*. In these viruses transcription and RNA replication are both initiated from promoter(s) at the genome 3' end. Transcription involves synthesis of subgenomic, polyadenylated mRNAs, whereas RNA replication involves synthesis of a complete complement of the genome. Currently it is unclear what the initial steps in these processes are and whether or not they are controlled by the same promoter. In RSV the essential sequences for initiation of transcription and replication are located within the 44 nt leader (Le) region and the start sequence of the first gene. Previous studies using a mini-genome assay identified a sequence within the first 11 nts at the 3' terminus of the Le region that is required for both transcription and replication, suggesting that this might be a common promoter element. Nts 1, 2, 6, and 7 were shown to be required specifically for replication and the start sequence of the first gene was shown to be required specifically for transcription. Further mapping of the Le region has now been performed. These analyses demonstrated that the remainder of the Le contains additional replication and transcription specific sequences, which do not overlap. Analysis of mutants in which the relative spacing of the cis-acting sequences is altered suggests that these elements must be distanced appropriately to be functional. Sequence analysis of the RNAs produced from mutant mini-genomes demonstrated

that some of the sequences are important for determining the initiation sites for RNA synthesis.

14.45 Cellular factors affecting the balance between transcription and replication of the influenza A virus genome

Anne Mullin*, Debra Elton & Paul Digard

Division of Virology, Dept of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP

The negative-sense RNA (vRNA) genome of influenza A virus is the template for the production of two types of positive-sense RNA. During transcription a capped, poly-adenylated mRNA is produced in a primer-dependent reaction that uses host cap structures. Replication of vRNA is a primer-independent reaction that yields cRNA, the full length copy of the genome. The mechanism governing the balance between transcription and replication has not yet been clearly described. To further understand this process we used a plasmid based system for the expression of model v- or cRNA segments containing the CAT gene; that, coupled with primer extension analysis of RNA offers a direct measure of each viral RNA species. In this system we find that at 31°C, RNA synthesis is strongly biased towards genome replication as compared to 37°C or 39°C. This observation is neither virus strain specific, nor specific to the sense of the input CAT reporter RNA. Titrating NP did not recapitulate this bias towards v- and cRNA synthesis. The shift towards genome replication is not due to intrinsic temperature sensitivity of RNPs since it was observed only in virus-infected cells that were pre-incubated at 31°C for two days and not in those initially maintained at 37°C or 39°C. A lower abundance of priming PolIII transcripts can not account for the shift away from transcription at 31°C, since cells incubated at all temperatures contained equivalent amounts of translatable mRNA. We propose that a cellular factor that has an altered activity profile at 31°C influences the balance between influenza transcription and replication.

15.00 Functional analysis of influenza temperature sensitive (ts) PA mutants

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The RNA-dependant RNA polymerase of influenza A virus is essential for transcription and replication of the viral genome. The polymerase complex consists of three subunits PB1, PB2 and PA. The first two subunits of the polymerase complex are relatively well characterised; PB1 is the polymerase itself and PB2 is the cap binding protein. The function of PA remains poorly defined although studies of temperature sensitive virus mutants suggest it may play a role in genome replication. In this study the PA genes from four temperature sensitive (ts) mutants with lesions in segment 3 were cloned and sequenced. All four mutants were found to contain unique point mutations, although one isolate of ts C30 contained an apparent extra suppressor mutation. In order to identify the nature of the ts defects, the phenotype of the PA mutants was first studied in a recombinant influenza virus gene expression assay based on plasmid transfection and a synthetic v- or cRNA molecule containing a reporter chloramphenicol acetyltransferase gene. PA ts C28 showed essentially normal transcription function at the non permissive temperature while ts mN4 and ts C45 showed ts gene expression activity with both senses of reporter RNA. However, PA ts C30 showed normal transcription activity when supplied with vRNA but not cRNA, suggestive of a defect in vRNA synthesis. Further analysis of the molecular activities of the mutants including direct measurement of c- and vRNA synthesis are ongoing.

RNA Viruses dsRNA genome

15.15: Studies of purified Bluetongue Virus polymerase

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Bluetongue virus (BTV) genome segment L1 encodes a 150 kDa minor virion protein VP1, which is believed to be the viral RNA-dependent RNA polymerase (RdRP). The L1 coding region has been cloned and expressed using the Baculovirus recombinant system. Recombinant VP1 has been purified to homogeneity and its polymerase activity assayed *in vitro*. We present evidence that VP1 alone is sufficient to synthesize the complementary strand using a single-stranded RNA (ssRNA) template when supplied with rNTPs and suitable buffer conditions. Subsequently BTV '+' strand, '-' strand, truncated '+' strand, extended '+' strand, and double-stranded RNA (dsRNA) templates have been used to investigate the specificity of VP1 polymerase activity *in vitro*. The presence of BTV sequence at the 3' end of '+' strand template is not necessary for synthesis of the complementary strand, as has been shown for the polymerase of the dsRNA genome bacteriophage phi6. The data show that the RdRP of BTV can synthesize dsRNA outside the context of the viral core.

RNA Viruses POSITIVE SENSE GENOME

16.00 Studies on the replication of a bovine Norovirus

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Jena virus (JV), a member of the *Caliciviridae*, is a bovine enteric virus associated with diarrhea in calves. The similar tropism and pathogenicity between JV and the human Noroviruses indicates that JV may be an appropriate animal model of Norovirus replication and pathogenicity. It is not possible to propagate Noroviruses in cell culture, nor is it known where the block to replication occurs. Positive strand RNA viruses must synthesise a negative strand copy of the genome in order to synthesise more positive strand templates. RNA-dependent RNA Polymerase (RdRP) activity and negative strand synthesis of the Hepatitis C virus has been demonstrated in a cell culture system by co-transfecting a plasmid expressing the RdRP under the control of the T7 promoter with a DNA fragment expressing the RNA template for the RdRP into HeLa cells, pre-infected with vTF7-3 recombinant vaccinia virus. The template consisted of the 5'-UTR of HCV, including a section of the capsid coding sequence, followed by a Polivirus IRES-CAT fusion sequence in antisense orientation. Successful expression of CAT was detected by ELISA, demonstrating the activity of the RdRP in synthesising a negative RNA strand copy of the template and thus allowing cap-independent translation of the CAT via the IRES (Goobar-Larsson, L. *et al.*, 2001).

A similar experimental principle was applied to a bovine Norovirus using Fowlpox/T7 pre-infected cells. An EMCV IRES-EGFP DNA sequence was cloned into the capsid encoding region of JV in both sense and inverse orientations. Cells transfected with the JV IRES-EGFP clone were positive for fluorescence, as expected. However, transfection with JV IRES-EGFP_{INV} did not result in fluorescence.

These results indicate that JV does not synthesise negative strand RNA in cell culture and that the block to replication lies at an earlier stage in the replicative cycle.

16.15 Mutagenesis of Dengue virus type 2 NS5: characterisation of a temperature sensitive mutant and compensatory second site mutations

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The NS5 protein of *Dengue virus type-2* contains a N-terminal methyltransferase domain linked to a C-terminal RNA dependent RNA polymerase domain by a stretch of amino acids containing a nuclear localisation sequence. To identify amino acids that are either essential for NS5 function or involved in interaction with other proteins, seven regions in the methyltransferase domain

were mutated and their effects on viral replication examined using a genomic length cDNA clone. RNA transcripts containing the mutations were electroporated into BHK-21 cells subsequently incubated at 33 or 37 °C. Virus was routinely recovered for only one of the mutations at 33 °C. Temperature shift experiments (33 to 37 °C) using Vero cells demonstrated a 4.5 fold log₁₀ reduction in the titre of the mutant virus compared to wild type, corresponding with a severe reduction in viral RNA levels. Sporadically, virus was recovered after electroporation of RNA transcripts containing two other sets of mutations. The genome of a representative of each of the aberrant viruses was completely sequenced. In each case the original mutations were retained and a second site mutation had arisen. The second site mutations were engineered into the wild type and corresponding mutant viruses and their effects on virus replication examined.

16.30 Initiation of translation by caliciviruses

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Caliciviruses, of which Norwalk virus (NV) is the prototype, contain single-stranded, positive-sense RNA genomes. The genomes are organised into a short 5' untranslated region (UTR), overlapping open reading frames and a 3' UTR followed by a poly A tail. A viral-encoded protein, VPg, is attached to the 5' end of the genomic RNA, though its function is unknown. Details of translation and replication of the Norwalk virus genomic RNA are poorly understood as the virus does not replicate in cell culture. However feline calicivirus (FCV) virions can be obtained after transfection of a full-length cDNA clone into cells in culture.

A feature of all calicivirus genomes is the presence of an extremely short 5' UTR and in Norwalk virus this region is only four nucleotides long (GUGA) and precedes three in-frame AUG codons. An RNA encoding the first 110 nucleotides of the Norwalk virus genome has been shown to bind a number of cellular proteins involved in the function of HCV and poliovirus IRES elements and this region is predicted to form a double stem-loop structure.

When assayed in a bicistronic reporter construct we were unable to demonstrate IRES activity in the 5' end of the NV genome. When the 5' 199 nucleotides were cloned upstream of the Firefly luciferase gene and the effect on protein expression determined enzymatically, a positive influence on translation was observed. Characterisation of this translational enhancer and the analysis of other calicivirus 5' sequences will be described. The presence or absence of a translation enhancer element within calicivirus genomes may contribute to the differences in the replication of different caliciviruses.

16.45 Production of replication competent HCV replicon transcripts from a polII promoter

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Production of a replication competent genome by pol II has been demonstrated for a number of +ve strand RNA viruses, but not HCV. We recently described a baculovirus delivery system for efficient expression of an infectious HCV 1a genome under the control of a tetracycline regulable promoter in hepatocyte-derived cell lines (McCormick *et al.* 2002). To confirm that pol II-derived HCV transcripts are replication competent, we have constructed baculovirus transfer vectors containing a culture-adapted HCV replicon cDNA (Krieger *et al.* 2001) flanked by the tetracycline promoter and Hepatitis Delta Virus ribozyme at the 5' and 3' ends respectively. Transfection of HuH-7 cells with these plasmids leads to the production of replicon containing cell lines, although the replicon transcripts produced require the correct 5' terminal nucleotides (either by precise positioning of the 5' UTR with respect to the transcription start site or by use of a hammerhead ribozyme) for efficient replication. The use of

baculoviral transduction further increases the efficiency with which the replicon is introduced into HuH7 cells (approximately 10-fold), confirming the advantage of this delivery system over DNA transfection. These same baculovirus constructs have now been used to transduce HepG2 cells in order to study the consequences of the presence of a replication-competent HCV transcript in a cell line that is not permissive for stable maintenance of the replicon. Initial results show that the presence of a replication-competent, but not a polymerase-knockout replicon, induces the production of IFN- β in HepG2 cells but not HuH7 cells.

17.00 The role of decay accelerating factor in enterovirus entry and uncoating

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A cellular receptor for the haemagglutinating human enteroviruses (huENV), and the protein that mediates haemagglutination, is the membrane complement regulatory protein decay accelerating factor (DAF; CD55). In binding studies using both mutagenised forms of soluble DAF and a panel of primate cells expressing variant forms of DAF and representing "natural mutant proteins", we have identified several distinct DAF-binding phenotypes of the huENVs. These studies complement our recent determination of the crystal structure of SCR₃₄ of human DAF and, together, have enabled us to better map the regions of DAF with which enteroviruses interact and, in certain cases, predict specific virus-receptor contacts. We have also investigated the role of DAF in enterovirus uncoating, which is required for cellular infection. Until recently, evidence had indicated that DAF acts as an initial attachment receptor, with subsequent uncoating and internalisation requiring the presence of additional cell surface components or proteins. However, using lipid vesicles loaded with human DAF, we have demonstrated that binding of echovirus 12 leads to uncoating, suggesting DAF alone may be sufficient to mediate infection of certain enteroviruses.

17.15 The Poliovirus 2C Cis-acting replication element-mediated uridylylation of VPg is not required for synthesis of negative-sense genomes

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The nucleotides in the terminal loop of the poliovirus 2C cis-acting replication element (2CCRE), a 61 nucleotide (nt) structured RNA functions as the template for the addition of two U nts to the viral protein VPg. This uridylylation reaction leads to the formation of VPgpUpU, which is subsequently used by the viral RNA polymerase as a nucleotide-peptide primer for genome replication. Although VPg primes negative and positive-sense strand replication, the requirement for 2CCRE-mediated uridylylation is not known. The analysis of genome replication using a cell free replication system allowed us to determine the role of the 2CCRE in genome replication. We demonstrate that the VPgpUpU primer formed using the 2CCRE as a template is not required for the initiation of negative sense strands. We therefore propose that the 3 poly (A) tail could serve as the template for the formation of a VPg-poly(U) primer that functions for the initiation of negative-sense strands.