Instituto Juan March
de Estudios e Investigaciones

19 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Viral Evasion of Host Defense Mechanisms

Organized by

M. B. Mathews and M. Esteban

E. Domingo
L. Enjuanes
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G. McFadden - Strategies for Immune Evasion by Poxviruses: the Myxoma Model.

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OVERVIEW OF THE WORKSHOP

M. Esteban

Animal viruses have acquired the ability to multiply in mammalian cells and maintain a selective advantage over the host, either through lytic or latent stages. Viruses assure their multiplication by the utilization of multiple strategies. It is through a combination of strategies that viruses gain entry into cells, take over the host cell translational machinery, control transcription and scape immune surveillance. How animal viruses manage to scape the different mechanisms that the host cell uses to eliminate an infection was the subject of the workshop entitled "Viral Evasion of Host Defense Mechanisms" sponsored by Fundación Juan March. As Bernie Fields put it, this topic has its own right to be a chapter on a virology text book.

After the 26 communications, it became clear that viruses encode a number of genes that subvert the host defenses using a variety of ways. The interference mechanisms used for viral evasion and the viral genes involved are outlined:

1. **Interference with inflammatory responses.** This has been best characterized in poxviruses. A number of genes have been identified in vaccinia virus: a 38KDa protein, A44L, with homology to human 38-hydroxysteroid dehydrogenase which affects infiltration of neutrophils in tissues; a 55KDa protein, B15R, that shares homology with the typeII IL-1 receptor and suppresses the IL-1 mediated local and systemic effects (Smith); serine protease inhibitor(Serp-1) of myxoma prevents an early inflammatory response and might have clinical benefits, as preliminary results by McFadden indicate a reduction in artheroma formation when this protein was infused in blood vessels.

2. **Interference with the complement cascade.** A well characterized protein of 35KDa encoded by vaccinia virus gene C21L was presented by Moss. This protein binds to complement components C4b and C3b and interferes with both the classical and alternative pathways. Poxviruses and herpesvirus might scape the complement-mediated virus neutralization thought interference by the virus complement control protein.

3. **Interference with the action of cytokines.** Because cytokines are produced by infected cells, the virus has to counteract their action. This effect was described at two levels: interference with the receptor and inactivation of cytokine-mediated cellular responses. Two genes were described in myxoma virus (McFadden) that encode an homolog of the tumor necrosis factor(TNF) receptor and of interferon(IFN)-gamma receptor. Adenovirus encode four proteins, the products of the E3A(14.7, 14.5 and 10.4 KDa) and of E1B (19 KDa) regions that are involved in preventing TNF-mediated cell destruction (apoptosis); the E1A region is necessary to induce cellular sensitivity

to TNF (Gooding, Wold). Animal studies using vaccinia recombinants revealed that indeed, coexpression of adenovirus E3 product(14.7 KDa) and of TNF results in increased pathogenicity(Horwitz).
Various presentations concerned the signal transduction pathways in response to IFN and the mode of action of the IFN-induced ds-RNA dependent protein kinase(PKR) and of 2-5A-dependent RNase. Gene products with roles in the activation pathways by IFN-alpha-beta (tyrosine kinase Tyk2) and by IFN-gamma (tyrosine kinase of the JAK family) were described (Kerr). The signal transduction pathway triggered by IFN is being uncovered gradually. PKR has antiviral and tumor repressor function(Hovanessian). These various effects are likely to be the consequence of inhibition of protein synthesis by phosphorylation of initiation factor eIF-2 and by induction of apoptosis (Esteban). As shown in vitro,the transcriptional factor IKB is a target of PKR and, this in turn, might release NFkB from the complex and activate transcription of cellular genes(Williams). Adenovirus evades the action of PKR by production of the short VA RNA1 with a central domain responsible for inhibition of the kinase (Mathews).The transactivator protein Tat of HIV-1 could be responsible for resistance of the virus to IFN by inactivation of PKR, while the IFN-induced 9-27 gene product could be responsible for inhibition of HIV replication(Pitha). Influenza virus has also developed a strategy to overcome PKR by the induction of p58, a cellular protein that binds and inactivates the kinase; the gene maps in human chromosome 2p21 (Katze). Another protein with antiviral action is the 2-5A-dependent RNase whose gene has been isolated and shown to confer resistance to picornavirus when overexpresed in cells(Silverman); as yet no viral gene products have been identified that interfere with this enzyme.

4. **Repression of MHC class I synthesis.** Another way to evade immune surveillance is by preventing presentation of MHC class I antigens at the cell surface and avoid killing by cytotoxic T lymphocytes. This process has been well characterized in the case of adenovirus type 12. Production of E1A protein by Ad12-infected cells turns off expression of MHC class I genes. The enhancer domains(-205 to -159) for class I expression and the binding sites were characterized in detail (Ricciardi, van der Eb).These studies help to understand the long-term persistence and tumoregenicity exhibited by Ad12.

5. **Interference with cell adhesion molecules.**These type of molecules play important roles in the activation of immune cells, natural killer, helper and cytotoxic T cells. Virus interference with cell adhesion molecules should reduce the extent of immune surveillance. A gene, EP402R, with extensive homology to CD2, an adhesion receptor present in the cell membrane of immature thymocytes, mature T cells and natural killer cells has been identified in African swine fever virus (Rodriguez). Understanding how the virus CD2 homolog interferes with the natural receptor will help in the development of strategies that could control ASFV infection.

6. **Interference with virus entry.** The host uses several mechanisms to prevent virus entry into cells, like B-cell activation with production of neutralizing antibodies. Detail analysis on the three dimensional structure of the influenza virus

haemagglutinin (HA) revealed important contact sites for neutralizing monoclonal antibodies on the most distal part of the HA1 molecule. Recent crystallographic data on fragments of HA indicate dramatic changes in protein conformation from the inactive state(at neutral pH) to the active and fusogenic state (acid pH) of the molecule.(Skehel). Strategies to prevent mucosal infection through expression in the milk of neutralizing antibodies might provide the means to control a severe animal disease caused by Transmissible gastroenteritis virus (Enjuanes).

7. **Genetic diversity.** Viruses scape immune surveillance through high rates of mutations in viral structural genes during replication cycles. A remarkable example was presented for HIV(Wain-Hobson). It was shown that pulps taken from the same tissue(spleen) of an individual with HIV-1 infection have high rates of mutations in the envelope gene(V1 and V2 regions). The error rates of the reverse transcriptase of the virus is a strategic advantage for the virus, since dominant mutants will be selected in vivo. Another example is foot-and-mouth disease virus, where a detail analysis of the mutations in VP1 during virus persistence in culture has been determined and found to be comparable with mutations introduced in natural isolates (Domingo).

8. **Interference with non-specific host responses.** This area of research was best illustrated with reovirus.This virus has various strategies to resist inactivation by acid pH, to bind to the receptor, during interaction with membranes, virulence and during conversion of mature particles to ISVP, a form responsible for cromium release of infected cells(Fields).

9. **Transformation of lymphocytes.** Through the process of transformation a virus can perpetuate its own genetic information. An example was presented with herpes saimiri that transforms primary human T-lymphocytes to permanent cells lines.The left terminal stp-C gene is required for transformation(Fleckenstein). In the case of Epstein-Barr virus(EBV) the gene LMP2 expressed during latency in B lymphocytes provide a selective advantage since it prevents activation of lytic EBV infection(Kieff).

10. **The Mx gene family.** The gene products of these family have GTPase activity and inhibit the replication of several RNA viruses. Studies by Staheli and Paulovic have defined protein domains of human MxA and mouse Mx1 important for biological functions. In vitro systems have been developed that allow measuments of transcription of VSV and influenza virus in the presence of purified Mx proteins. These studies suggest that Mx1 interacts with the polymerase subunit PB2 of influenza and blocks initiation and elongation of transcription.

I. Virus Defense Mechanism During Acute Infections

Evasion by poxviruses of complement mediated host defense mechanisms

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The complement system contributes to host defense against a wide variety of microorganisms. Although most studies have focused on the role of complement in the opsinization of bacteria, there is renewed interest in the effects of these proteins on virus infections. One stimulus for this interest is the finding that members of the herpesvirus and poxvirus families encode complement regulatory proteins. Putative complement regulatory proteins of vaccinia virus were recognized by the presence of 60 to 70 amino acid short consensus repeats (SCR). The vaccinia virus complement control protein (VCP), has an apparent Mr of 35,000 and is secreted in large amounts early during vaccinia virus infection. The open reading frame, C21L, is predicted to encode a 28.6-kDa protein with 4 SCR that have a 38% identity with those of the human C4-binding protein. Initial experiments indicated that VCP inhibited complement-mediated hemolysis of sensitized sheep erythrocytes. Further studies demonstrated that VCP binds to both C4b and C3b and serves as a co-factor with Factor I in cleaving these two molecules. In addition, VCP inhibited the formation and accelerated the decay of the classical complement pathway convertase. The viral protein also accelerated decay of the alternative pathway convertase, although higher concentrations were needed. Thus, VCP interferes with several steps in both the classical and alternative pathways of complement activation. Several approaches have been taken to determine possible roles of VCP in evasion of host defense mechanisms. *In vitro* studies demonstrated that VCP, at concentrations present in the medium of infected cells, can prevent antibody-dependent complement-enhanced neutralization of vaccinia virus infectivity. By contrast, no such activity was present in the medium of cells infected with a mutant virus lacking the VCP gene. Although such mutants replicate normally in tissue culture cells, they are greatly attenuated when injected into animals; skin lesions were smaller and healed more rapidly. The resolution of the lesions coincided with the appearance of virus neutralizing antibody in the infected animals.

Glycoprotein gp42, with an Mr of 42,000, is encoded by the 317-amino acid B5R open reading and also has 4 SCR. Unlike VCP, gp42 is a class I integral membrane protein and is present on the surface of extracellular virions. Deletion of the B5R gene inhibits extracellular virus envelope formation and dissemination. The severe attenuation of B5R deletion mutants, therefore, can be attributed to the effect on transmission. A

truncated form of gp42, that is secreted from infected cells, has been engineered and efforts to detect binding to C4b and C3b are in progress.

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VACCINIA VIRUS EXPRESSES 3β-HYDROXYSTEROID DEHYDROGENASE AND AN INTERLEUKIN-1β RECEPTOR.

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Vaccinia virus displays many strategies to interfere with the host response to infection. Two of these will be described: one is the expression of an enzyme that synthesizes steroid hormones; the second is the secretion of a soluble receptor for interleukin-1β.

Open reading frame SalF7L (A44L in vaccinia Copenhagen) encodes a 38 kDa protein with 31% amino acid identity to human 3β-hydroxysteroid dehydrogenase (3β-HSD). This is a essential enzyme in the synthesis of steroid hormones. The vaccinia gene is expressed early during infection and the protein is an active 3β-HSD that can convert pregnenolone to progesterone *in vitro*. A virus deletion mutant lacking the gene grows normally in cell culture but is attenuated in a murine intranasal model compared to the WT virus and a revertant virus in which the 3β-HSD gene is re-inserted into the mutant genome. Murine nasal infection by the deletion mutant is characterised by an increased inflammation of lung tissue and lower amounts of virus in lungs and other organs.

Gene B15R of vaccinia virus strain WR encodes a 55 kDa glycoprotein that is secreted from infected cells late during infection. The protein contains three immunoglobulin domains that collectively share 32% amino acid identity with the external domain of the type II IL-1 receptor (IL-1R). The B15R protein binds IL-1β with high affinity (Kd 234 pM) but, surprisingly, does not bind IL-1α or the IL-1 receptor antagonist protein. Deletion of the gene did not alter virus replication in cell culture but influenced virus replication *in vivo*. Although the number of mortalities of mice infected by the intranasal route by WT or mutant virus were indistinguishable, those infected with the deletion mutant showed greater signs of illness and lost weight more rapidly that WT-infected animals. Not all vaccinia virus strains express the IL-1β receptor. Recent data indicate that animals infected with strains of virus that naturally lack the IL-1β receptor or strains engineered not to express this protein, develop fever, while viruses expressing the receptor suppress the febrile response.

Genes related to the vaccinia 3β-HSD and IL-1β receptor genes are present in variola major virus (the cause of smallpox), but these are fragmented by frameshift and non-sense mutations and are presumed to be non-functional.

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Strategies for immune evasion by poxviruses: the myxoma model

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Myxoma virus is the agent of a virulent systemic disease of domestic rabbits, myxomatosis. Originally described by G. Sanarelli in the last century, myxoma was the first virus pathogen discovered for a laboratory animal and was the first viral agent ever deliberately introduced into the environment for the explicit purpose of pest eradication. Since its release into the Australian and European feral rabbit populations more than 40 years ago, the field strains of both the rabbit and virus have been subjected to mutual evolutionary and selective pressures that have resulted in a steady-state enzootic in the inoculated areas (reviewed in 1,2).

A member of the leporipoxvirus genus, myxoma shares many of the features associated with other poxviruses, namely cytoplasmic location of replication and a large double stranded DNA genome (160 kilobases). Multiple lines of evidence indicate that myxoma encodes multiple gene products where function is to permit the spread and propagation of the virus in a variety of rabbit tissues. Some of these viral proteins specifically counteract or subvert the development of the host inflammatory response and acquired cellular immunity.

Examples of such modulatory gene products currently under study in our lab include:

(1) Myxoma growth factor (MGF), which stimulates neighboring cells in a paracrine-like fashion via the cellular epidermal growth factor receptor (3-6).

(2) Serp 1, a secreted glycoprotein with serine protease inhibitor activity, that prevents development of the early inflammatory response (7-9).

(3) T2, a secreted viral homologue of the cellular tumor necrosis factor (TNF) receptor superfamily, that binds and inhibits rabbit TNF (10,11).

(4) T7, a secreted viral homologue of the cellular interferon-γ receptor, that binds and inhibits rabbit interferon-γ (12).

(5) M11L, a surface receptor-like protein that interferes with the inflammatory response by an unknown mechanism (5, 13).

(6) Unmapped late gene product(s) that disrupt cellular class I major histocompatibility complex proteins and are believed to interfere with class I mediated antigen presentation (14).

It is highly likely that more immunomodulatory viral genes remain to be discovered within the myxoma genome. Not only will these and related gene products provide useful tools to dissect out the different arms of the host antiviral defense mechanisms, but they may also provide new probes to identify novel elements of the cellular immune repertoire.

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AFRICAN SWINE FEVER VIRUS ENCODES A PROTEIN HOMOLOGOUS TO THE CD2 ADHESION MOLECULE.

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African swine fever virus (ASFV) is a large icosahedral deoxivirus capable to infect soft ticks as well as different species of swine. While the infection of domestic pigs (*Sus scropha*) with unattenuated ASFV strains leads to an accute disease with a very high mortality rate, in its natural swine host, the warthog (*Phacochoerus aethiopicus*), ASFV causes only a mild disease with little clinical signs. However, the presence of infectious ASFV particles in the blood of warthogs long after their apparent recovery, suggests that the virus is able to establish persistent infections in this host (9). This, along with the repeated failure of conventional approaches to generate an ASFV vaccine has led to speculation that the virus might possess mechanisms to avoid the host's immune surveillance. In an attempt to identify ASFV genes that might be involved in such mechanisms, we have analyzed the complete nucleotide sequence of the genome of BA71V, an attenuated strain of ASFV (manuscript in preparation). One of the identified genes, EP402R, encodes a polypeptide of 402 amino acid residues with an extensive sequence homology to CD2, an adhesion receptor present in the cell membrane of immature thymocytes, mature T cells, and natural killer cells (2, 3). CD2 molecules are known to interact with two different ligands: i) CD58, a 26 kDa glycoprotein present in the cell membrane of a wide variety of cell lineages including target cells for both cytotoxic T cells and natural killer cells, and antigen-presenting cells for T helper cells (8); ii) CD59, a widely distributed, 18-20 kDa membrane glycoprotein that restricts the lysis of human erythrocytes and leukocytes by human complement (4). The CD2/CD59 interaction is thought to be involved in T cell adhesion and activation. (6). The CD2/CD58 interaction plays an important role in T cell activation and natural killer cell activity (1). The CD2/CD58 interaction is also responsible for

the formation of rosettes between human T cells and both autologous and heterologous erythrocytes (5).

We have shown that the disruption of EP402R, achieved through the replacement of a 354 bp-long fragment from within EP402R by the marker gene LacZ, abrogates the ability of the virus to induce the adsorption of pig erythrocytes to the surface of infected cells (7), and that the reintroduction of the gene in a different genomic location restores the normal hemadsorption phenotype of the virus. These results demonstrate that the protein encoded by EP402R is a functional adhesion molecule capable of interacting with a specific ligand on the surface of the erythrocyte. The role of this protein during the ASFV infection is under investigation.

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STRATEGIES TO PREVENT MUCOSAL INFECTIONS BY CORONAVIRUSES

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Transmissible gastroenteritis virus (TGEV) and related coronaviruses infect enteric and respiratory mucosal areas, even in the presence of circulating antibody of high titer. In addition, animals are infected at birth, when their immune system is unmature, producing high mortality rates. At this time protection can only be provided by immune mothers through lactation. New strategies to provide passive protection to newborn animals against these coronaviruses have to be developed. The antigenic structure of TGEV has been defined. The spike (S) protein has been identified as the major antigen involved in protection and in the binding to host cells. The antigenic structure of S protein and the correlation with its physical structure has been defined (1). Highly conserved antigenic sites involved in the induction of TGEV neutralizing antibodies have been identified. Using this information, two approaches are being used to protect against TGEV: antigen presentation in gut associated lymphoid tissues (GALT) to induce mucosal immunity and development of transgenic animals secreting virus neutralizing monoclonal antibodies (MAbs). Two vectors with tropism for GALT are being used, one prokaryotic (*Salmonella typhimurium*) and another one eukaryotic (human adenovirus 5) (2). In addition, the use of coronaviruses as vectors is proposed.

Coronaviruses which cause no apparent disease have been isolated and could be used to develop vectors to induce mucosal immunity. Since the genome of coronaviruses is larger than 30 kb, the selection of defective viruses with a shorter genome will help the construction of cDNAs that might be the basis for a vector. Defective interfering particles (DI) were selected by passage of TGEV at high moi. Northern blot analysis indicated that these DI genomes have both the 5' and 3'-end of *wt* virus, but not the genes coding for the structural proteins of the virion. The genome (12 kb) of one of these DI viruses is encapsidated and has been cloned. In order to construct vectors which deliver the antigen to the enteric or respiratory track, the molecular basis of TGEV tropism were studied. An epidemiological tree has been proposed for TGEV. The virus has evolved by accumulation of point mutations. In addition, the introduction of a deletion in the 5'-end of the spike gene yielded the porcine respiratory coronaviruses (PRCVs) which lost the enteric tropism. Nucleotide differences in the sequences of S genes from enteric and respiratory isolates with a full length S gene were determined. Precise mutations in residues mapping in the deletion, have been proposed as responsible for the lost of enteric tropism (3,4). To determine other areas of the genome that might be involved in the tropism of these viruses a collection of recombinants between enteric and respiratory TGEVs were selected. One third of the characterized recombinants had the crossing-over at the 5'-end of S gene, 3' down-stream of the area involved in the lost of enteric tropism. These recombinants have the half 5'-end from the enteric virus, and the half 3'-end from the respiratory isolates. Preliminary experiments indicated that these recombinants have enteric tropism. These data suggest that the ability of TGEV to infect the gut is controlled by sequences mapping around nt 655 of S gene. Changes in the tropism of PRCV, by taking the S protein conferring enteric tropism from engineered DI particles, are being used to study the molecular basis of TGEV virulence, tropism and induction of protection.

Transgenic swine secreting TGEV neutralizing monoclonal antibodies (MAb) in the milk during lactation are being constructed. Immunoglobulin gene fragments coding for the VH and VL modules of a TGEV neutralizing MAb have been cloned and sequenced. Chimeric immunoglobulin genes with the variable module from the MAb and a conserved module of human origin (gamma 1) were constructed. The chimeric immunoglobulin genes were initially cloned into pING plasmids, under the control of an immunoglobulin enhancer, and SV-40 promoter and polyadenylation coding sequences. These plasmids have been used to express the chimeric immunoglobulins in

murine myelomas, to test for the secretion of TGEV neutralizing MAb. The immunoglobulin genes have been cloned into the whey acid protein (*wap*) gene. This gene is regulated by peptide and steroid hormones which promote tissue specific expression in the mammary gland. These constructs are being tested for transient expression using gene gun technology on tissue explants from the mammary gland of lactating swine.

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II. Interferon Related Mechanisms

Mutants and protein tyrosine kinases in the signal transduction pathways controlling interferon-inducible gene expression

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IFN-inducible genes have been isolated and characterised. They have in their 5'flanking DNA interferon-stimulable response elements (ISREs) which can confer IFN-responsiveness on marker genes. A number of transcription factors that interact with these response elements have been cloned and characterised. The Type I ($\alpha\beta$) and II (γ) IFNs interact with distinct cell membrane receptors, components of which have also been cloned. The detailed structure of the receptors is not, however, known.

In collaboration with George Stark's group IFN-inducible promoters driving drug-selectable or cell-surface markers have been used to obtain and complement mutants in the IFN-$\alpha\beta$ and IFN-γ signal transduction pathways. To date recessive mutants in a number of complementation groups which affect one or other, or both, of these pathways have been isolated. Five have now been complemented. Three of the complementing genes correspond to non-receptor protein tyrosine kinases of the JAK family. Of these Tyk2 is uniquely involved in the IFN-$\alpha\beta$ and JAK2 in the IFN-γ pathways, respectively. JAK1 is essential for both pathways. The other two complementing genes correspond to p48 and p91 transcription factor components involved in the IFN responses. Additional mutants may be defective in a signal transduction component(s) of the IFN receptors, an additional kinase(s), a protein tyrosine phosphatase(s) or other novel component(s).

Characterisation and complementation of the mutants has, therefore, provided definitive evidence for a role for the JAK family kinases in the IFN response pathways and a central role for transcription factor p91 in both the IFN-$\alpha\beta$ and the IFN-γ responses. In more general terms the model currently emerging is of IFN-$\alpha\beta$ or -γ membrane receptor complexes involving, in addition to the trans-membrane receptors, the different combinations of JAK kinases which interact to activate, in response to the different IFNs, unique and common transcription factors which, on release from the membrane and migration to the nucleus, activate the appropriate transcriptional responses. Exactly how many components are involved and precisely how they fit together, remain, however, to be established.

MOLECULAR, CELLULAR, AND VIRAL STUDIES ON 2-5A-DEPENDENT RNase

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2-5A-dependent RNase is a uniquely-regulated enzyme that functions in the response of cells to interferon treatment and virus infections. Recently we reported the molecular cloning of the human and murine forms of 2-5A-dependent RNase (Zhou et al., *Cell* 72, 753, 1993). Mutagenesis studies and sequence analysis of 2-5A-dependent RNase revealed an intriguing structure. The amino terminal half of the RNase contains nine ankyrin-repeats implicated in mediating protein-protein interactions. The 2-5A binding domain was localized by site-directed mutagenesis to a duplicated phosphate-binding loop motif present in the seventh and eighth ankyrin repeats. On the carboxy terminal side of the ankyrin repeats is an apparent zinc finger and a region with homology to protein kinases. The carboxy terminus is required for RNase function. Structural models describing the mechanism of 2-5A-dependent RNase activation by 2',5'-oligoadenylates (2-5A) will be presented. To study the intrinsic activities of 2-5A-dependent RNase, the human form of the enzyme was expressed in insect cells from a baculovirus vector. The purified, recombinant RNase has potent and highly specific, 2-5A-dependent RNase activity. To study the biological role of 2-5A-dependent RNase, a truncated form of the murine RNase that retains 2-5A binding activity while lacking RNase activity was stably expressed in murine cells (Hassel et al., *EMBO J.*, **12**, 3297,1993). The truncated RNase functioned as a dominant negative mutant suppressing endogenous 2-5A-dependent RNase activity and greatly reducing the anti-encephalomyocarditis virus and anti-proliferative activities of interferon α/β. A method that directs 2-5A-dependent RNase to specific RNA targets in cells will be described (Torrence et al.,*Proc. Natl. Acad. Sci. U.S.A.* 90, 1300, 1993). Remarkably, chimeric oligonucleotides with 2-5A linked to antisense against PKR mRNA selectively ablated mRNA to PKR in HeLa cells as determined by reverse transcriptase-coupled PCR.

Neutralization of the Interferon-Induced eIF-2 Kinase by Viral Products

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The protein kinase DAI (also known as P1, p68, PK-ds, PKR, etc.) is induced by interferon and activated by double-stranded RNA (dsRNA), presumably of viral origin. Activation is accompanied by auto-phosphorylation and leads to the phosphorylation of protein synthesis initiation factor eIF-2, the entrapment of a recycling factor (GEF or eIF-2B), and the consequent blockade of translation. Activation requires the interaction of two motifs in the N-terminus of DAI with perfectly duplexed dsRNA of suitable length (at least 30 and preferably 85bp), and seems to involve a conformational change in the complex. The resultant shut-down of protein synthesis limits viral multiplication.

Many viruses evade this cellular defense strategy by elaborating gene products that interfere with DAI accumulation, activation, or activity. Among the best-studied of these counteracting products are the adenovirus VA RNAs. As exemplified by Ad2 VA RNA_I, they are short (~160nt), highly structured RNAs that are produced abundantly at late times of infection and bind DAI using the same two motifs as dsRNA. Instead of causing activation of the enzyme, however, VA RNA_I prevents its activation by dsRNA. Binding of VA RNA_I to DAI involves a base-paired stem (the apical stem) while the region of the molecule responsible for its inhibitory function is a complex stem-loop (the central domain) containing conserved sequences and structures. Comparable activities have also been reported for small RNAs generated by Epstein-Barr virus and HIV-1. On the other hand, a second VA RNA species, VA RNA_{II}, that is produced by several adenovirus serotypes is only weakly effective against DAI. Its sequence conservation suggests that it may play a different role in the viral infectious cycle.

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Opportunistic infection and cytokines upregulate HIV-1 replication; molecular mechanisms.

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Transcription of HIV-1 provirus is regulated by a virus encoded RNA binding protein Tat as well as by cellular factors that interact with the enhancer and promoter regions of the HIV-1 LTR. Since the tat defective mutant is transcriptionaly inactive Tat mediated transactivation represents a limiting step in HIV-1 replication . Tat transactivation requires the interaction between Tat and cellular factors binding to the NFkB and Sp-1 binding sites as well as to the TATA box in the HIV-1 promoter region.While in acute infection, Tat is the major transcriptional activator, the activation of HIV-LTR and the latent HIV-1 provirus by different extracellular stimuli such as cytokines and HSV-1 is associated with the enhancement of binding of NFkB specific proteins.

Our goal in the present study was to determine whether Tat mediated transactivation is the irreplaceable bottle neck in the HIV-1 replication cycle or whether the virus develops an alternative pathway allowing it to replicate in the absence of Tat or other accessory cellular proteins. Since both HSV-1 and TNFa can effectively upregulate transcription of HIV-1 provirus in vitro, we examined whether such transactivators can function in the absence of Tat protein and whether TNFa and HSV-1 are able to support replication of the Tat defective HIV-1 provirus.

Previously we have shown(1)that TNFa mediated upregulation of HIV-1 expression in T cells is associated with the induction of p50,p55,p65,p75 and p85 NFkB binding proteins.Transactivation is abolished by mutation or deletion of the NFkB binding region .In the present studies we have used the CEM R7 cells that contain integrated Tat defective HIV-1 provirus, expressed only at very low levels.TNFa treatment significantly increased relative levels of HIV-1 transcripts and HIV-1 specific proteins.In order to determine the molecular mechanism of TNF mediated enhancement of HIV-1 expression, we compared the rate of HIV transcription in Tat transfected and TNFa induced CEM R7 cells.While Tat increased the initiation of transcription and eliminated the polarity of transcription, TNFa more effectively enhanced the transcription of the gag region then the env region.Thus, the mechanisms by which Tat and TNFa transactivate the transcription of the HIV-1 provirus are not identical.However, in spite of this difference, TNFa was able to induce infectious, Tat defective provirus and support its replication and spread through the culture.This data indicates that HIV-1 developed a mechanism which permitted the virus to replicate even in the absence of virus encoded Tat.(2) This alternative pathway is, however, much less efficient than Tat mediated transactivation.

The activation of HIV-1 provirus by HSV-1 infection suggested an existence of another interesting mechanism which is able to facilitate the Tat transactivation.We have previously shown(3) that both HSV-1 encoded and induced proteins participate

in the HSV-1 mediated transactivation of HIV-1 provirus. HSV-1 infection in T cells induces binding of two NFkB specific proteins(p50 and c rel) to the enhancer region as well as 50 kDa protein (HLP-1)that binds to the LBP-1 sites in HIV-LTR.Furthermore, two HSV-1 encoded "immediate early" proteins contribute to the transactivation of HIV LTR (4). One (TAR 150) binds to the TAR region of HIV-LTR DNA and may be related to the HSV-1 encoded transactivator ICP4.However,its role in the transactivation of HIV-LTR is unclear,as neither overexpression nor deletion of the TAR DNA region affects the expression of HIV-LTR. In contrast, the overexpression of HSV-1 encoded transactivator ICP 0 very efficiently stimulates HIV-LTR and shows a high cooparativity with Tat .This cooperative effect illustrates two interesting features. First transactivation is independant of the presence of NFkB and Sp-1 sites required for the Tat transactivation in the absence of ICP 0, and second ICP 0 can activate mutated HIV-LTR which does not contain the TAR binding region.Thus, ICP 0 can recruit Tat to the vicinity of the transcription initiation complex, as well as, substitute for the transactivators binding to the enhancer region of HIV LTR normally required for the efficient transactivation by Tat.These results suggest that infection with herpes viruses may facilitate transcription of TAR defective HIV mutants. Weather it able to support full virus replication remains to be seen.

In summary this data indicates that both cytokines and heterologous viral infection can not only upregulate expression of `HIV-1 provirus but also facilitate expression of HIV-1 proviruses that show a defect in the tat transactivation mechanism.

Tat mediated transactivation not only enhances significantly HIV-1 replication but also evades the antiviral effect induced by the interferon system.We have shown (6,7) that during a single replication cycle, interferon effectivaly inhibits HIV-1 replication by blocking the reverse transcription and formation of the provirus.In contrast, no significant inhibition of HIV-1 replication by interferon was observed after completion of proviral synthesis and integration into the cellular genome.Similarly, in cells transfected with proviral DNA the relative levels of HIV-1 RNAs, proteins, or their processing, were identical in the presence and absence of interferon.Interferon, however, induced in these cells the expression of several interferon responsive genes including the 9-27 gene, which encodes a RNA binding protein. Overexpression of 9-27 protein inhibits HIV-1 replication. Interestingly, IFN treatment significantly reduced the HIV-1 mRNA levels encoded only by the Tat defective HIV-1 provirus but not by the wt HIV-1.This inhibition could be overcome by transfection with the Tat and Rev expressing plasmids.Overexpression of another IFN induced protein 2,5 OAS which activates RNAase L did not inhibit HIV-1 expression even in the absence of functional Tat protein. These results suggest that IFN mediated inhibition of HIV-1 expression, seen in the absence of Tat, is mediated by the 9-27 protein. The mechanism by which Tat enables HIV-1 to evade the IFN effect remains elusive. It may simply provide high levels of rev protein that can compete effectively with 9-27 for binding to the Rev responsive element

,or, as suggested by others,binding of Tat to the TAR element may interfare with the induction of the other antiviral mechanisms such as 2,5 OAS or PKR pathways. Thus, the HIV-1 encoded regulatory proteins Tat and Rev not only allow HIV-1 to replicate efficiently, but may also renders it insensitive to antiviral mechanisms induced by IFN.

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III. Virus Defense Mechanisms in Persistence and Latency

Role of cell variation in the initiation of a persistent foot-and-mouth disease virus infection

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RNA viruses mutate at rates averaging 10^{-3} to 10^{-5} substitutions per nucleotide site per round of copying. As a result, RNA virus populations consist of complex distributions of related but nonidentical genomes termed viral quasispecies (reviews in Eigen and Biebricher 1988; Domingo *et al.*, 1994). Foot-and-mouth disease virus (FMDV) is an important animal pathogen which conforms to a quasispecies genetic organization (Domingo *et al.*, 1992). FMDV normally causes acute infections but occasionally it persists in animals by unknown mechanisms. Persistence of FMDV in cell culture (de la Torre *et al.*, 1985, 1988) offers a model system to explore parameters involved in persistence. We have developed a virulence assay to examine whether it is the cell or the virus the component which triggers the establishment of persistence. Cells which survived a cytolytic infection were cured of any detectable FMDV by ribavirin treatment. These early survivors showed increased resistance to FMDV. The virus which survived in these cells was as virulent as the parental FMDV. In spite of its inability to produce attenuated variants, the FMDV quasispecies showed high mutant frequencies at the onset of persistence. Viral genetic lesions (ts, small plaque phenotypes)—generally associated to persistence of lytic viruses— were observed only after persistence had already been initiated. Thus, in this system, cell variation is determinant of the establishment of a persistent infection (Martín Hernández *et al.*, submitted for publication).

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Invasion and Use of Non-Specific Host Defenses by the Mammalian Reoviruses

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The first host defenses that regulate the initiation of viral infection are nonspecific and occur shortly after the virus has entered a eukaryotic host. For enteric viruses these include gastric acidity and proteases present in the lumen of the small intestine as well as macrophages and their products. The reoviruses have developed ways to resist inactivation by acid pH, to utilize pancreatic proteases, to change from a stable inactive form to an activated form, and to enter cells for primary replication, the cells being macrophages. In this talk I will first discuss the mammalian reoviruses and then illustrate how the three forms of reoviruses are built to evade these earliest host defense mechanisms. Reoviruses are double capsid naked icosahedral virions whose genomes consist of segmented double-stranded RNAs. There are three serotypes distinguished by hemagglutination inhibition and neutralization assays. The segmented genome has allowed us and others to readily reassort genome segments between different isolates of virus following mixed infection in cell culture. These reassorted virions have allowed us to identify the role of various gene products in their interaction with hosts and cells. Of particular note are three specific gene products that are located in the outer capsid shell.

1. One gene is the S1, encoding the σ1 protein, the viral hemagglutinin. This protein functions as the cell attachment protein binding to sialic acid and sialylated proteins at cell surfaces.
2. The second gene is S4, encoding the σ3 protein which is a zinc containing protein that sits on the outer shell of the virion and plays a critical role in stabilizing the virion in its intact form.
3. The third gene is M2, encoding the μ1 protein. This protein undergoes cleavages during its activation steps and plays a critical role in allowing reovirus to interact with cell membranes.

During the early interactions of reovirus with the host there are three structural forms of reovirus produced by a highly orchestrated series of events which allow the virus to undergo stages of controlled disassembly, involving the transformation of the intact virion to a second form termed the infectious subviral particle (ISVP) and ultimately to a third form, the so-called viral core. The functions of these three forms of reovirus are: the virion is built for maximum stability; the ISVP has undergone changes that cause its receptor interacting protein σ1 to extend, and to expose an active form of the μ1 allowing the ISVP to interact with cell membranes; the third form, the viral core, undergoes transcription once the virus has come into direct contact with the cytoplasm.

Recent studies using cryoelectron microscopy and image reconstruction have allowed us to examine at moderate resolution (27 to 32 Å) features of the three structural forms of reovirus. The three particle forms thus provide "snapshot" views of the virus at discrete early stages of infection. Of particular note is that by comparing virions, ISVPs and cores we were able to detect dramatic changes in supramolecular structure and protein confirmation related to the early steps of reovirus infection. The intact virion, about 850 Å diameter, is designed for environmental stability and thus allows the virus to pass through harsh environments such as that found in the highly acidic pH of the stomach. In part, this stability is mediated by tight σ3/μ1, λ2/σ3, and λ2/μ1 interactions in the outer capsid. In addition, the core shell is densely packed. Following exposure to proteases in the small intestine, ISVPs are generated that are approximately 800 Å diameter. This transition involves the release of 600 σ3 subunits and a striking change in the vertex associated viral hemagglutinin. Particularly noteworthy in the image reconstructions are the organization of the μl and its cleaved products on the surface of the ISVP into multiple trimers extending through the surface of the icosahedral structure. The third form of reovirus, the core particle of approximately 600 Å diameter, is the form through which viral RNA is transcribed from a particle-associated transcriptase. The transition from ISVP to core involves release of the twelve σ1 fibers and μ1 subunits (arranged as 200 trimers). The surface of the core displays 150 nodules that merge with a solid shell centered at a radius of about 275 Å. In the virion and ISVP, flower shaped pentamers of λ2 are centered at the vertices. In the ISVP to core transition domains of the λ2 subunits rotate upward and outward to form structures extending from radii 305-400 Å with an outer diameter of 140 Å and a central channel 84 Å wide. This conformational change allows a potential diffusion of substrates for transcription and exit of newly synthesized mRNA segments.

The remainder of this talk will involve features of the M2 gene product, the μ1 protein. The μ1 protein encoded by the M2 gene segment has a molecular mass of approximately 76.3 kilodalton. A potential myristoylation sequence at the amino terminus allowed us to show that the μ1 protein is modified with an amide linked myristoyl group. The amino terminal end undergoes a cleavage that generates a 4,000 molecular mass peptide that is found in both virions and ISVP. This proteolytic fragment is termed μ1N. During the conversion of virions to ISVP proteolytic cleavage takes place in the μ1 product near the carboxy-terminal end. This cleavage yields a 13 kilodalton carboxyl terminal fragment named ϕ. When trypsin is the protease causing the cleavage it occurs between arginine 584 and isoleucine 585. Chymotrypsin cleaves between tyrosine 581 and glycine 582. The sequences in the ϕ portion of the cleaved μ1 protein may participate in the unique functions attributed to ISVP, mainly membrane interaction. In particular the δ-ϕ cleavage junction is predicted to be flanked by a pair of long amphipathic α-helices. These together with the myristoyl group of the extreme amino terminus of the μ1 protein are proposed to interact directly with the lipid bilayer of a cell membrane during penetration by mammalian reoviruses.

To demonstrate that the μ1 product is involved in membrane interactions two assays were performed. One is a chromium release assay whereby cells prelabeled with chromium are exposed to virus, ISVP or core. We were able to show that only the ISVP form causes membrane leakage. Genetic analysis allowed us to show that the M2 gene is linked to this property. A second assay involved study of the interaction of various reovirus forms with lipid bilayers to study electric current passing across the membrane. In these studies we were able to show that the ISVP form alone (at a neutral pH) is able to induce ion channels in these membranes. These studies taken together strongly suggest that the M2 gene product found in its exposed form on the ISVP is the membrane interacting form of the mammalian reoviruses.

Thus, what I have presented is the fact that reovirus has three distinct viral forms that play distinct roles in their interaction with the eukaryotic host allowing it to bypass the earliest host defenses. Recent studies have suggested a biochemical strategy whereby this naked non-enveloped virus is able to interact with membranes through features of the M2 gene product, the μ1 protein.

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Epstein-Barr virus latency in B lymphoctes requires regulated interactions with host defenses. Elliott Kieff, Mark Birkenbach, Ken Kaye, Ken Izume, Cheryl Miller, Richard Longnecker, Mike Kurilla, Erle Robertson and Sankar Swaminathan. Departments of Medicine and Microbiology and Virology Program, Harvard University, 75 Francis St. Boston Ma, 02115.

Epstein-Barr Virus replicates in the oro pharyngeal epithelium, establishes latency in B lymphocytes and reactivates from B cells to reinfect the epithelium for spread to non immune humans. In order to maintain stable latent infection in B lymphocytes, the virus expresses a set of genes which encode nuclear proteins (EBNAs), integral membrane proteins (LMPs) and small RNAs (EBERs). Most EBNAs and LMPs are targets of the host immune response. Several EBNAs and LMP1 are critical for the perpetuation of EBV episomes, for cell proliferation or for survival of the latently infected cells. Others appear to be non critical for any aspect of latent or lytic infection, in vitro, but must be critical in vivo because they are targets of the host immune response and therefore subject to negative selection. One gene expressed in latent infection, LMP2, interacts with B lyphocyte src family tyrosine kinases and with syk and blocks transmembrane signal transduction following sIg, CD19 or class II crosslinking. Surprisingly, virus recombinants with specifically mutated LMP2 genes are fully competent for establishing latent infection or for transforming primary B lymphocytes. In contrast to B lyphocytes transformed by wild type virus recombinants, crosslinking of sIg on cells transformed by the mutated recombinants results in normal B lymphocyte signal transduction and activation of lytic EBV infection in the mutant recombinant infected cells. These experiments indicate that LMP2 can prevent fortuitous activation of lytic EBV infection which would otherwise occur in response to activation of latently infected B cells in the peripheral circulation. Lytic infection in this setting would result in death of the infected cells and neutralization of virus produced from any cell which escaped immune cytolysis. The EBER genes also have no apparent effect on latent or lytic infection, in vitro. Because of their similarity to the adeno VA RNAs the EBERs are likely to protect infected cells against interferon effects. Although primary B lymphocyte transformation is interferon sensitive, wild type and EBER deleted virus are similarly sensitive to interferon and the infected cells could support similar levels of VSV replication in the face of interferon treatment. Another virus gene expressed in lytic infection is nearly identical to the human IL10 gene and does affect the interferon response to virus infection. Although, Il10 has effects on macrophages, B lymphoctyte growth and T lymphocyte and NK cell functions, including gamma interferon blockade (Kevin Moore and Timothy Mosman et. al.), the dominant effect of the EBV homologue is likely to be on cytotoxic T lymphocyte and NK cell functions including gamma interferon secretion. This conclusion is based on the results of experimental murine infection with a recombinant vaccinia virus which expresses the murile IL10 gene and on the inhibition of gamma interferon secretion from human peripheral blood mononuclear cells which were incubated with B lymphocytes infected with wild type EBV. In contrast, cells incubated with B lymphocytes infected with EBV recombinants carrying specific mutation in the EBV IL10 homologue secreted high levels of gamma interferon.

Influenza virus infection and its inhibition by antibodies

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The first steps in influenza virus infection, receptor recognition and virus membrane-cell membrane fusion are mediated by the haemagglutinin (HA) glycoprotein. Antibodies which bind to the membrane distal region of the haemagglutinin neutralize virus infectivity. The sites at which they interact are known from electron microscopy of HA-antibody complexes and X-ray crystallography of variant HAs to correspond to the locations of amino acid substitutions in the HAs of the antigenic mutants which they select. These antibodies block receptor-HA interactions and more rarely prevent the structural changes in HA required for membrane fusion. The receptors for HAs are sialic acid residues of cell surface glyconjugates. Identification of the receptor binding sites and details of the interaction of sialic acid substituents with conserved amino acids in the sites have been deduced from studies of HA-receptor analogue complexes (Sauter et al 1992). The surface area of the binding sites and their location are consistent with the inhibition of binding by infectivity neutralizing antibodies. Membrane fusion mediated by HA is induced at endosomal pH and involves structural reorganization of the molecule. Evidence has been obtained that this reorganization is required for membrane fusion activity (Godley et al 1992) and the recently determined structure of a fragment of the HA in its fusion-pH conformation shows the extensive nature of the structural changes.

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Genetic variation among human retroviruses: important or merely a reflection of life style?

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The human immunodeficiency viruses (HIV) and human T-cell leukemia viruses (HTLV) infect predominantly CD4 cells in vivo. The former is characterized by unparalled genetic and antigenic variation, so much so that an infected individual may harbour between 10^6-10^{10} variants at any one time, depending upon the disease stage. The latter is estimated to diverge on a time scale of decades to centuries.

HIV is a perpetual mutation machine whereby variants are a remorseless consequence of unprecedented numbers of replication cycles. This is almost certainly due to the fact that the virus can integrate and lay dorment thus escaping immune surveillance. As will be demonstrated there is little or no evidence in favour of strong positive or Darwinian selection acting on HIV or the related simian immunodeficiency viruses, SIV. In vivo analyses of HIV and T-cells would suggest that HIV production results from cellular activation by antigen. Thus a particular variant would be "selected" over and above fellow kin by chance rather than for reasons of fitness. The bottom line remains replication competance. For 3% variation in situ to occur, possible in as little as 14 months, at least 300 rounds of replication must occur. Even if the burst size was 2 this would lead to more virus than atoms in the universe. Consequently while massive replication may occur massive destruction of infected cells must occur in order to limit the viral load to ~10^6-10^{10}. In fact the average burst size can be calculated to be ~0.01-0.05 per cycle. Yet massive and continual CD4 destruction would lead to immune defiency?

By contrst HTLV-1 replicates to very low titres in comparison to HIV. By a PCR analysis of the integration sites from asymptomatic carriers and those presenting with tropical spastic paraparesis it has been possible to show that HTLV preferentially replicates by mitosis resulting in oligoclonal expansion of between 5-25 cellular clones. Thus reverse transcription is limited due to the positive effect of the tax protein on cell proliferation.

The differences perhaps lie in the relative genetic organization of the two viruses. For HIV/SIV the powerful transcription amplifier, tat, is produced from rev (regulator of viral gene expression) independent and dependent transcripts meaning that replication lacks negative feedback. For HTLV, tax (the transcription amplifier) and rex (regulator of viral gene expression) are derived from the same mRNA. Hence rex exerts a negative effect on HTLV replication. Furthermore tax is diluted by it effects on specific cellular genes.

Thus the two extremes in terms of retroviral genetic variation reflect different strategies in replication and, behind this, genome organization.

IV. Roles of the Interferon-Induced Kinase DAI/PKR

Viral and cellular targets of the interferon induced, dsRNA activated, kinase, PKR.

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The interferon (IFN)-induced protein kinase of Mr 68,000 (PKR) has been shown to specifically phosphorylate the inititation factor eIF-2α *in vitro* when activated by double-stranded RNA. This kinase mediates in part the antiviral activities of interferons and as consequently different viruses have devised means of inhibiting the kinase. We have cloned and characterized both human and mouse interferon regulated PKR cDNAs (1-3) and expressed these in mammalian cells, the budding yeast *Saccaharomyces cerevisciae*, and *E. coli*. Expression in yeast results in a slow growth phenotype and has allowed us the opportunity to exploit this to identify potential substrates or inhibitors of the kinase. The human immunodeficiency virus (HIV) Tat (1-72 amino acid) protein is one such inhibitor we have identified using the yeast expression assay. The inhibition by Tat 72 is the result of a direct interaction between Tat 72 and PKR. Tat inhibited autophosphorylation and phosphorylation of exogenous substrates by PKR. Both Tat protein binding and the Tat-mediated inhibition of PKR activation were reversed by the addition of eIF-2α. Interestingly, the 86 amino acid version of Tat, which is produced early in HIV infection, had no effect on kinase activity *in vitro* or *in vivo*. These results suggest HIV is capable of directly targeting PKR for inhibition late in infection thus providing a mechanism by which HIV could escape the action of IFN.

When PKR is used as a matrix in affinity chromatograpy experiments, a number of proteins bind suggesting there exists different substrates for PKR in the cell. One of these substrates is IκB, the cytoplasmic inhibitor of the transcription factor, NFκB. PKR is able to phosphorylate purified IkB *in vitro* and regulate NFκB activity through IκB phosphorylation in cell extracts. In cells transfected with a mutant (lys^{296}-arg) PKR, dsRNA induction of a κB-dependent promoter construct is blocked suggesting dsRNA is able to induce gene expression through PKR. These data support a role for PKR in regulating transcription of NFκB dependent promoters which include the IFN-β gene and the HIV LTR.

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THE INTERFERON-INDUCED ds-RNA ACTIVATED PROTEIN KINASE AS AN INDUCER OF APOPTOSIS. Mariano Esteban, Centro Nacional de Biotecnología, Campus Universidad Autónoma, Cantoblanco, Madrid, Spain.

Apoptosis, a process of programmed cell death, is characterized by cell shrinkage, chromatin condensation, membrane blebbing, and DNA fragmentation into nucleosome sizes (1). Apoptosis has been implicated in oncogenesis (2). Only few genes, like the tumor-suppresor p53 (3), have been identified with roles in apoptosis . Since interferons (IFN) exert antiviral, antitumor and immunomodulatory functions and some of these effects occur toghether with cell death (4), it is possible that IFN triggers apoptosis through induction and activation of one or more of the IFN-induced gene products. The IFN-induced ds-RNA activated p68 protein kinase has been shown to exert growth control arrest (5), tumor- suppressor activity (6 ,7) and antiviral action (8, 9). To analyze the biological function of p68 kinase, we have used an inducible system where expression of the kinase can be regulated by the lacI repressor/operator controlling elements. Vaccinia virus was used as a vector for the expression of p68 kinase in cultured cell. In this virus-cell system, p68 is autoregulated and is activated with time, most likely by ds-RNA produced as a result of virus infection. By 24 hpi, both viral and cellular protein synthesis ceases and cells died with apoptotic-like characteristics. Cell death was not observed in cells which expressed a mutant form(lys296–arg) indicating that cell death observed is the result of p68 kinase expression and activation. We propose that human p68 kinase fuctions as a tumor-supressor gene by actively participating in apoptosis.

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REGULATION OF THE INTERFERON-INDUCED, DS RNA ACTIVATED PROTEIN KINASE (PKR) IN VIRUS-INFECTED AND UNINFECTED CELLS

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The PKR protein kinase is one of greater than 30 genes induced by interferon and is one of two interferon induced gene products activated by dsRNA. As a result of activation by dsRNA, PKR, a serine/threonine kinase, undergoes autophosphorylation and catalyzes the phosphorylation of the alpha subunit of protein synthesis eukaryotic initiation factor 2 (eIF-2). These events can lead to limitations in functional eIF-2 and thus to dramatic decreases in protein synthetic rates inside the cell. Recently we directly demonstrated that PKR can regulate translation in vivo and that PKR action is effected by mutations in either the kinase catalytic or N-terminal regulatory domains. The majority of our work, however, has focused on the mechanisms which viruses have evolved to avoid the negative effects of PKR on virus replication. Examples to be discussed include influenza virus which downregulates PKR by activating a cellular PKR inhibitor termed P58, based on its Mr of 58,000. The gene encoding P58 has now been cloned, sequenced, and expressed as a functional protein in E. coli. These data will be presented as will a description of the complex regulatory pathways underlying P58 control of PKR. Poliovirus also utilizes a cellular gene product to regulate PKR: it is likely that a cellular protease, acting together with viral specific dsRNAs, are responsibly for PKR degradation in poliovirus-infected cells. Models of PKR regulation in both the influenza and poliovirus systems will be presented. Recent evidence now suggests that PKR may play an important role in the antiproliferative properties of interferon as well as its antiviral properties. For example, due to its translational inhibitory properties, the wild-type PKR is growth suppressive in yeast and likely in mammalian cells as well. We also have evidence that PKR may function as a tumor suppressor gene since introduction of catalytically inactive PKR mutants into NIH3T3 cells induce their malignant transformation.

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Interferon-Induced dsRNA-activated Protein Kinase (PKR) : Antiviral and Antitumoral Functions.

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The double-stranded RNA (dsRNA)-activated protein kinase, recently called PKR for protein kinase RNA-dependent, is induced in mouse and human cells upon treatment with interferon. In general, PKR is activated by binding to dsRNAs ; however, some single-stranded RNAs, by virtue of their stem-loop structure, can bind and thus trigger activation. Besides RNA molecules, the purified PKR has been shown to be also activated by heparin. Once activated, the known function of this serine/threonine protein kinase is the phosphorylation of the protein synthesis initiation factor eIF2, thus mediating inhibition of protein synthesis. Through this latter mechanism, PKR is implicated in the antiviral and antiproliferative effects of interferon. Direct evidence for this was provided by the expression of the recombinant human kinase in murine cells (Meurs et al., J. Virol. 66: 5805-8514, 1992) and in *Saccharomyces cerevisiae* (Chong et al., EMBO J., 11: 1553-1562, 1992).

By transfection of the cDNA encoding the human PKR in murine NIH 3T3 cells, we have established several stable clones expressing either the wild-type kinase or an inactive mutant possessing a single amino acid substitution in the invariant lysine 296 in the catalytic domain II. Upon infection with encephalomyocarditis virus (EMCV), wild-type but not mutant expressing clones were found to partially resist virus growth. Under these experimental conditions, the wild-type kinase and the small subunit of eIF2 were phosphorylated.

Besides its antiviral and antiproliferative functions, PKR has been shown to manifest a potential tumor suppressor function. Indeed, expression of a mutant inactive form of PKR in NIH/3T3 cells was correlated with a malignant transformation phenotype, giving rise to the production of large tumors in all inoculated mice. This suggested that functional PKR, by a still undefined mechanism, may also act as a tumor suppressor (Meurs et al., PNAS, 90:232-236, 1993). The observation that the inactive mutant transfected PKR behaves as a dominant-negative inhibitor of the endogenous functional PKR (murine), suggests that the largely expressed mutant protein may perhaps sequester a phosphorylatable substrate implicated in the mechanism of tumor suppressor.

V. Virus Infection and the Major Histocompatibility Complex

HUMAN ADENOVIRUSES ENCODE FOUR UNIQUE PROTEINS THAT PREVENT TNF-MEDIATED CELL DEATH AND TNF-INDUCED ACTIVATION OF PLA_2. L. Gooding[1], S. Laster[2], and W. Wold[3]. [1]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA; [2]Department of Microbiology, North Carolina State University, Raleigh, NC; [3]Department of Molecular Microbiology and Immunology, St. Louis University School of Medicine, St. Louis, MO.

TNF, a potent pro-inflammatory cytokine, is produced by macrophages in response to a variety of stimuli including virus infection. Antiviral activities attributed to TNF include inhibition of virus replication and direct cytolysis of virus-infected cells. TNF mediates these effects by binding to high affinity cell surface receptors. Most cells express these receptors but are not sensitive to the lytic effects of TNF. The mechanism underlying TNF mediated cell death is incompletely understood but it appears to involve activation of the large cytoplasmic, arachidonate selective phospholipase A_2 ($cPLA_2$). Arachidonic acid metabolism in turn leads to other mediators of the inflammatory response, e.g. prostaglandins and leukotrienes, and to active oxygen species that are probably the proximal mediators of cell death.

Human adenoviruses encode several proteins that modify cellular signals transduced following encounter with TNF. E1A expression converts most normal, TNF-resistant cells to TNF sensitivity. Mutational analysis indicates that the capacity of E1A to alter cell cycle regulation via binding to the retinoblastoma protein, thereby releasing the cellular transcription factor E2F, is also responsible for altering TNF susceptibility. The adenovirus genome encodes four unique proteins that protect the virus-infected cell from E1A-induced sensitivity to TNF. The function of these proteins is cell-type dependent. E1B-19K, a protein associated with the nuclear membrane, protects adenovirus-infected human cell lines from TNF lysis. However E1B-19K does not function in any of the mouse cell lines tested. E3-14.7K, an abundant cytoplasmic protein, suppresses TNF lysis in nearly all mouse cell lines tested. A complex of two plasma membrane proteins, E3-10.4K/14.5K, also protects most mouse cell lines from TNF. This redundant expression of viral proteins, all apparently inhibiting the same host anti-viral response, suggests that TNF is an important mediator, suppression of which is critical to the reproductive strategy of the virus.

The reported involvement of $cPLA_2$ in TNF-mediated killing led us to investigate the effect of the adenovirus proteins on TNF-induced $cPLA_2$ activation, measured by arachidonic acid release. TNF activates $cPLA_2$ in adenovirus infected mouse cells, and expression of either E3-14.7K or E3-10.4K/14.5K prevents this activation. Furthermore, E3-14.7K expressed in a stably transfected cell line prevents TNF-induced arachidonate release. In preliminary experiments, E1B-19K likewise prevents $cPLA_2$ activation in human cells. In each case, the adenovirus inhibitors functioned to prevent $cPLA_2$ activation only under the circumstances in which they also inhibit TNF-mediated cytolysis. This coincidence of function supports the idea that $cPLA_2$ activity is essential for TNF-induced cell killing. The different sub-cellular locations of the inhibitor proteins suggests that each blocks a different step in the sequence leading from TNF-receptor binding to $cPLA_2$ activation.

To assess the impact of expression of the TNF inhibitor proteins on adenovirus-induced disease, a mouse model system was employed in which wild type or mutant virus was inoculated

intranasally and lungs examined by histology seven days later. All viruses tested caused a moderate degree of histopathology characterized by a perivascular and peribronchiolar mononuclear cell infiltrate. However, a marked increase, notably in the interstitium, was elicited by virus lacking both E3-14.7K and E3-10.4K/14.5K function. The pathology elicited by this virus is similar to the interstitial pneumonia seen in adenovirus infections in children. Thus, the pathogenesis of pulmonary adenovirus infection may arise from TNF-induced activation of $cPLA_2$ leading to production of inflammatory mediators.

ADENOVIRUS PROTEINS THAT INHIBIT AND PROMOTE CELL DEATH. W. Wold[1], A. Tollefson[1], A. Scaria[1], T. Hermiston[1], J. Ryerse[2], R. Tripp[3], T. Sparer[3], and L. Gooding[3]. [1]Department of Molecular Microbiology and Immunology, [2]Department of Pathology, St. Louis University School of Medicine, St. Louis, MO; [3]Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA.

The human adenovirus (Ad) E1A proteins alter the activities of pre-existing cellular transcription factors and thereby affect expression of viral and cellular genes. Among other functions, E1A induces apoptosis as well as susceptibility to TNF-induced cytolysis; these properties map to a specific domain in E1A termed conserved region 1 (CR1). The Ad E1B-19K protein inhibits both E1A- and TNF-induced cell death, thereby allowing virus replication to proceed. Two "sets" of Ad region E3 proteins, the E3-14.7K protein and the E3-10.4K/14.5K complex of proteins, also independently inhibit TNF-induced cytolysis. The E3 proteins do not inhibit E1A-induced cytolysis. Linda Gooding will discuss collaborative studies which address the roles that the Ad proteins play in TNF-induced cytolysis *in vitro* and inflammation *in vivo*.

The Ad E3-gp19K protein is a transmembrane glycoprotein, localized in the endoplasmic reticulum (ER), which forms a complex with MHC class I antigens and blocks their transport to the cell surface. In collaboration with Linda Gooding, we have shown that gp19K inhibits killing of Ad-infected cells by Ad-specific CTL. Using a series of in-frame deletions and Cys-to-Ser mutations in gp19K, we now show that virtually the entire ER lumenal domain (residues 1-107) of gp19K is important for binding to class I antigens. We propose that the ER lumenal domain consists of two subdomains: (i) residues 1 to ca. 80, a region that is variable in sequence among Ad serotypes, may bind to the variable α1 and α2 domains of class I α chain; and (ii) residues ca. 80 to 107, a region that is conserved among Ad serotypes, may interact with a conserved cellular protein.

The role that gp19K may play in pathogenesis has been investigated in a mouse ($H\text{-}2^b$) model. Seven days following intranasal infection, moderate perivascular and peribronchiolar infiltration of mononuclear cells was observed. Unexpectedly, similar pathology was observed with wild-type and mutants that lack gp19K. *In vitro*, γ-interferon (IFN) abrogates the ability of gp19K to prevent killing of Ad-infected syngeneic target cells by Ad-specific CTL, apparently because γ-IFN up-regulates class I antigens. *In vivo*, we postulate that increased γ-IFN synthesis leads to increased class I expression, and this overwhelms the ability of gp19K to block transport of class I antigens to the cell surface.

The mechanism by which Ad is released from infected cells in unknown. We report a new function for the E3-11.6K protein from Ad2 and Ad5 (subgroup C human Ads) that may relate to Ad release from infected cells as well as the more general issue of cell viability. We have shown that 11.6K is synthesized in small amounts from E3 mRNAs at early stages of infection, but in very large amounts from novel major late mRNAs at late stages of infection. Also, 11.6K is an N- and O-linked integral membrane glycoprotein that initially localizes to the ER and Golgi but ultimately localizes to the nuclear membrane. Ad mutants lacking 11.6K have very small plaques that are slow to develop. Mutants lacking 11.6K replicate as well as wild-type, but mutant viruses are released more slowly than wild-type from infected cells. Cell

viability assays (morphology, release of lactic dehydrogenase, trypan blue exclusion, MTT assay for mitochondrial activity) indicate that cells infected with 11.6K mutants stay alive much longer than cells infected with wild-type virus. Also, cellular DNA is degraded more slowly with 11.6K mutants than with wild-type virus. Thus, 11.6K appears to promote cell death.

The Ad2 version of 11.6K is 101 amino acid residues. There is an internal (aa 41-60) uncleaved signal-anchor domain for membrane insertion, and the protein is oriented in the membrane with the N-terminus in the lumen and the C-terminus in the cytoplasm (or nucleoplasm). Mutants with extensive deletions on the N- and C-terminal sides of the 11.6K signal-anchor domain are near wild-type in viability assays, implying that the signal-anchor domain is the key region that promotes cell death. Sequences within aa 61-78 are important in targeting 11.6K to the nuclear membrane.

The E1B-19K protein localizes to the nuclear membrane and to structures on the nuclear periphery. Interestingly, double-label immunofluorescence indicates that 11.6K co-localizes with E1B-19K at very late stages of infection. Also, with mutants that lack E1B-19K, 11.6K localizes to all cellular membranes rather than the nuclear membrane and the structures on the nuclear periphery. We propose that 11.6K interacts with E1B-19K at very late stages of infection and abrogates the ability of E1B-19K to inhibit cell lysis. In this way, 11.6K promotes cell lysis and the subsequent release of virus particles.

The mechanism by which Ad12 E1A mediated repression of class I synthesis in transformed cells contributes to escape from immunosurveillance

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General background: All adenovirus seratypes can transform cells *in vitro*, but only some can generate tumors in rodents. An explanation for this difference in tumorigenic potential was originally made by van der Eb's lab, where they showed that expression of the major histocompatibility complex (MHC) class I antigens are diminished in rat cells transformed by Ad12 (highly oncogenic) but not by Ad5 (non-oncogenic) (1, 2). Since class I and foreign (e.g., viral) antigens must be co-recognized by cytotoxic T lymphocytes (CTLs) for lysis of target cells to occur, the reduced levels of class I antigens on Ad12-transformed cells may favor their escape from immunosurveillance and act as an important step in tumorigenesis.

Ad12 E1A represses each of the class I genes of the MHC in transformed cells of different species: We have shown that class I repression by Ad12 extends to both mouse and human transformed cells and that Ad12 E1A is responsible for this effect. By using genetically matched sets of Ad5- and Ad12-transformed mouse cell lines, we demonstrated that there is diminished expression of class I antigens and mRNAs encoded by each of the class I genes (H-2 K, D, and L) of the MHC locus (3). Stimulation of class I expression in Ad12-transformed cells by interferon-g revealed that there is not a deficiency of the H-2 genes (3). Transformation of human cells with Ad12 E1A (and a non-adenovirus oncogene used to obtain complete transformation), resulted in greatly reduced levels of all class I antigens and mRNAs (HLA-A, -B, and -C) (4). This provided compelling evidence that the Ad12 E1A protein products mediate class I repression in the absence of other adenovirus genes.

Tumors correlate with low class I expression: We found that tumors generated by injection of Ad12-transformed cells into syngeneic mice express low levels of the H-2 antigens, indicating that this phenotype is maintained *in vivo* (3). We also found that Ad12-transformed cells infected with influenza virus *in vitro* were resistant to lysis by syngeneic, influenza virus-specific CTLs (5). The relevance of diminished class I expression to oncogenicity was made apparent by a reversal in tumorigenicity following treatment of Ad12-transformed cells with IFN-g or by introduction of an exogenous class I gene under the control of a non-H-2 promoter (6).

Class I reduction is at the level of transcription: Using nuclear run-on assays, we (7) and others (8, 9) demonstrated that the low levels of class I mRNA in Ad12-transformed cells results from a decrease in the rate of transcription.

Class I enhancer activity is repressed in Ad12-transformed cells: We and others have recently demonstrated that expression of an exogenous H-2K promoter (driving the CAT reporter gene) was dramatically lower in Ad12- compared to Ad5-transformed cells (10, 11). By mutational analysis we have shown that the class I enhancer (-205 to -159) is much less active in Ad12-

transformed cells; this was observed with constructs in which the enhancer was juxtaposed either to its own basal promoter or to heterologous promoters (11).

R1 and R2 factor binding sites within the class I enhancer are each repressed in Ad12-transformed cells:

The R1 site : In most cells, the R1 site contributes the major transcriptional stimulation by the class I enhancer. Although factor binding to the R1 site was seen to be generally the same between Ad5- and Ad12-transformed cells, constructs containing different multimers of the R1 site joined to the H2 basal promoter were less active in Ad12- than in Ad5-transformed cells (12).

The R2 site: Generally, the R2 element provides a moderate enhancer activity compared to the R1 element (13). In Ad12 transformed cells, we found that the ability of R1 alone to stimulate the H2 basal promoter, was blocked by juxtaposing R2 next to R1 (12). Binding activity to the R2 site of the class I enhancer is significantly higher in Ad12- compared to Ad5-transformed cells (11, 12, 14) . Both the binding activity (12, 15, 14) and repression of the R1 element (12) is lost when the R2 site of the enhancer was mutated at nucleotides known to be critical for binding RXRb, a retinoid receptor protein (16). Indeed, treatment with retinoic acid was shown to be capable of relieving the repression. Most recently, a COUP-like hormone receptor has now been identified as the nuclear receptor protein which binds to the R2 site of the class I enhancer in Ad12 transformed cells (15).

Potential significance for immuno-escape: A model for the mechanism for MHC class I repression in transformed cells and the possible significance of this mechanism to the establishment of latency by infectious Ad12 in humans will be discussed.

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Oncogenicity of adenovirus-transformed cells; relationship with expresion of class I MHC antigens

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The human adenoviruses are divided into 2 groups on the basis of their oncogenic potential in hamsters: the oncogenic adenoviruses, e.g. adenovirus 12 (Ad12) and the non-oncogenic adenoviruses, e.g. Ad5. Both groups of viruses, or their DNA, can transform cultured cells, but only cells transformed by oncogenic viruses form tumors in immunocompetent syngeneic animals, whereas cells transformed by non-oncogenic viruses can not. Both types of transformed cells, however, can induce tumors in immunodeficient nude mice. A possible explanation for this phenomenon is the observation that the E1A region of oncogenic Ad12 inhibits expression of the class I MHC genes in the transformed cells (P.I. Schrier et al. Nature, 305, 771-775, 1983). Reduced expression of class I MHC antigens could result in evasion of the T-cell immune defence. In contrast, cells transformed by non-oncogenic Ad5 can be efficiently recognized and killed by cytotoxic T-lymphocytes (CTL). Previous work has shown that these CTLs recognize a single immunodominant epitope of the Ad5 E1A protein of 10 amino acids long (SGPSNTPPEI). Studies were under taken to generate E1A mutants which are no longer recognizable by the wild type E1A-specific CTLs. We then investigated whether cells transformed by these mutant-E1As elicit CTLs that recognize a minor T-cell epitope of the E1A protein, or whether they escape detection by T-cells. It will be shown that the cells transformed by the mutant-E1A induce strong CTL activity. However, the epitope recognized by the CTLs were not derived from any of the viral proteins.

Our studies on the mechanism of inhibition of class I MHC gene expression by Ad12 E1A have shown that the class I-regulatory element (CRE), located between -160 and -200 of the mouse H-2 promoter, plays a role in this repression, and that the H_2TF1 element in the CRE is particularly important. In Ad12-transformed cells the H_2TF1 levels are considerably lower than in Ad5-transformed cells. The effects of re-expression of H_2TF1 on MHC class I expression and oncogenicity will be discussed.

IN VIVO EXPRESSION AND FUNCTION OF THE ADENOVIRUS E3 GENES THAT AFFECT VIRULENCE M.S. Horwitz, J. Tufariello, G. Fejer and A. Grunhaus, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, New York 10461

The E3 region of the human adenoviruses (Ads) contains genes that code for proteins with the potential for modulating the immune response to infection. Among these proteins are the gp19K that binds to the class I major histocompatibility complex (MHC) to prevent its transport to the cell surface, and the 14.7K that inhibits cytolysis by tumor necrosis factor (TNF-α). Because the human Ads do not replicate in the mouse, we have expressed the Ad gp19K and 14.7K genes either in vaccinia virus (VV) constructs or in transgenic mice in order to understand the function of these Ad genes *in vivo*.

These experiments have determined that the Ad 14.7K protein can increase the virulence of VV in a murine pneumonia model induced after intranasal inoculation of the virus. For these studies, a VV construct containing the TNF-α gene inserted into the HindIIIF region was further modified to contain the 14.7K gene in either the expressing (VV/14.7+/TNF) or nonexpressing orientation (VV/14.7-/TNF). These VV's allowed us to deliver the pathogen to Balb/c mice together with the agonist (TNF-α) and the antagonist (14.7K) in various combinations. TNF-α had been previously shown to decrease the mortality after VV infection. However, the 14.7K gene product made the VV pneumonia more severe as evidenced by increased mortality, enhanced VV growth in the lungs and more severe pulmonary histopathology. The 14.7K cloned into VV in the absence of the cytokine did not aggravate disease, further arguing that the effect of the 14.7K is mediated through its antagonism of the TNF-α.

Comparable experiments were also performed in SCID mice to determine if T or B cells participated in the 14.7K effect. In contrast to immunocompetent animals, SCID mice infected with VV containing TNF survived acute infection with little or no disease. They did not succumb within the first 2 weeks as did the immunocompetent animals. However, over a period of 1 to 3 months, the animals developed evidence of viremic spread of VV from the lungs to the skin, manifest by vesicles on non-hairy surfaces such as tail, ears and paws. The animals infected with VV/14.7+/TNF became ill and viremic 2 to 4 weeks before animals with VV/14.7-/TNF. These studies indicate that T or B cells are not necessary for the 14.7K effect which can still be demonstrated in SCID animals.

From studies of the Ad gp19K cloned into VV we have determined that down regulation of class I MHC is not sufficient to alter VV growth or disease *in vivo*. This confirms another recent study of VV infection in ß2-microglobulin knockout mice, where it was also demonstrated that the elimination of class I MHC on the cell surface did not affect mortality.

Transgenic animals containing the entire E3 region behind its own single promoter have been constructed, and expression of the promoter and 5 of the mRNAs within this complex transcription unit have been determined. These analyses included both the amounts of transcripts in various organs and in the cells of primary tissue culture derived from E3-transgenic lung and kidney. These studies were undertaken to determine the activity of the E3 promoter and measure some of the Ad protein products synthesized in transgenic mice tissue before determining their effects on pathogenesis. The E3 promoter is activated by Ad E1A during viral infection and has also been shown to be induced directly by NFkB or indirectly by TNF in plasmid constructs. In primary tissue culture of transgenic lung and kidney, the E3 promoter was inactive until induced by infection with Ad dl 7001 virus which produced E1A but does not contain any endogenous E3 genes. The pattern of mRNA production as measured by PCR indicated that gp19, the major E3 transcript following Ad infection in HeLa cells, is decreased in lung cultures compared to other E3 transcripts, even those made from the same PCR primers. However, the pattern of gp19 predominance was demonstrated in transgenic kidney cell cultures, suggesting that there is differential splicing of the E3 transcript in lung compared to kidney cells. In direct confirmation of some of the transcriptional results, the gp19K protein has been detected in kidney cells but not in lung cell cultures derived from the transgenic animals after trans-activation with E1A.

Analysis of E3 transcripts directly in various organs from transgenic mice showed that the E3 promoter was very active in kidney and thymus but much less active in liver, lung, spleen and brain. After induction for 5 hours with LPS, there was activation of the E3 promoter in liver, lung and brain but not in spleen. The effect of the E3 genes on disease induced by a variety of viruses, including the mouse adenovirus that does not contain an E3 region comparable to the human Ads, will be studied in this model transgenic system.

VI. Mx and Other Host Defense Mechanisms

GENERATION OF HUMAN T-CELL CLONES BY USING A T-LYMPHOTROPIC HERPESVIRUS

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A T-lymphotropic tumour virus of New World monkeys, Herpesvirus saimiri strain C-488, transforms primary human T-lymphocytes to permanent growth in a reproducible and efficient way. Stably proliferating cell-lines have been derived from adult PBMC, from cord blood lymphocytes, from thymocytes, and from isolated T-cell clones. The resulting (oligo-) clonal T-cell lines proliferate in presence of IL2 independently of antigen or mitogen, and show the phenotype of activated mature peripheral T-lymphocytes. The cells carry the CD4 or CD8 molecule on the surface, and harbour viral episomes in high copy number. The transforming activity of Herpesvirus saimiri C-488 was assigned to the *stp-C* oncogene (saimiri transforming protein of virus strain C-488). In transformed lymphocyte cultures, viral transcripts have not been detected except from the left terminal *stp-C* gene, which is translated into a cytoplasmatic protein. The oncogene seems to be expressed independently from other viral factors, but is subjected to T-cellular regulation.

Transformed T-cell clones resemble the parental cultures in many features. Tyrosine phosphorylation patterns and calcium mobilization levels after CD3- and/or CD4-ligation, as well as abundance and activity of the lymphocyte specific tyrosine kinase $p56^{lck}$ are not altered. Infection of antigen specific human T-cell clones directed against myelin basic protein or tetanus toxoid led to continuous growth of the cells without need for antigen restimulation. Nevertheless, the resulting growth transformed clonal T-cell lines retained their antigen specificity and responded with enhanced proliferation and IFN-γ production upon exposure to the specific antigen.

Transformation with Herpesvirus saimiri is therefore becoming an efficient tool for the expansion of antigen specific T-cell clones without the necessity of restimulation.

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Control of virus replication by Mx proteins: Inhibition of vesicular stomatitis virus in vitro mRNA synthesis by purified human MxA protein

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The interferon-induced human MxA protein is a cytoplasmic 76-kDa GTPase that can inhibit the multiplication of influenza A virus, vesicular stomatitis virus (VSV) and some other RNA viruses. The related mouse Mx1 protein is a GTPase that accumulates in the nucleus. The gene that codes for Mx1 determines susceptibility of mice to experimental infections with influenza A virus. Otto Haller's group recently showed that Mx1 also controls susceptibility of mice to thogoto virus, a still poorly characterized tick-born orthomyxovirus.
Mutant analysis indicates that a functional GTP-binding motif is of critical importance for GTPase and antiviral activity of Mx proteins. It remains unclear, however, whether GTP hydrolysis is indeed required for antiviral activity of Mx proteins or whether GTP binding (rather than hydrolysis) is of crucial importance. For example, the GTP hydrolyzing activity of the MxA variant T247N was about 10-fold lower than that of wild-type MxA, nevertheless, T247N blocked virus replication in transfected 3T3 cells with high efficiency. Mutants showing normal GTP-binding activity but lacking GTPase activity will be required to clarify this point. The MxA variant T103A has such properties. Unfortunately, assessing its antiviral activity is complicated by the fact that T103A shows aberrant distribution in transfected cells. Since VSV mRNA synthesis has been identified as the target of MxA action in vivo, we tested whether purified MxA can inhibit this viral multiplication step in vitro. A protocol for easy production of large quantities of GTPase-active MxA was established in collaboration with Jovan Pavlovic's group: MxA cDNA is cloned into the bacterial expression vector pQE9 which permits the production in E. coli of recombinant protein with six extra histidine residues at the amino terminus, and histidine-tagged MxA is purified to near homogeneity from the soluble fraction of bacterial lysates by nickel agarose affinity chromatography followed by chromatography on a MonoQ ion exchange column. We found that highly purified MxA indeed inhibited VSV transcription in vitro, regardless of whether ribonucleoprotein complexes were prepared from VSV-infected cells or from purified virions. Transcription was blocked almost completely when MxA was used at concentrations higher than 0.5 µg/µl and when the specific GTPase activity of the MxA preparation was at least 50 nmol/min/mg. MxA had no effect on the stability of preformed VSV mRNAs. Control experiments indicate that degradation of GTP in the reaction mixture cannot account for the observed inhibitory effect of MxA on VSV mRNA synthesis. Curiously, VSV transcripts synthesized in the presence of sub-optimal concentrations of MxA migrated on polyacrylamide/urea gels as fuzzy bands. The simplicity of this in vitro system should now allow to characterize the interaction of MxA with the VSV polymerase complex in great detail.

Inhibition of Influenza Virus RNA Synthesis by Mx1 Protein

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The Mx proteins form a small family of GTPases which are induced in response to interferon (IFN) type I. Human MxA and murine Mx1 are closely related proteins with intrinsic antiviral activities. The specificity of Mx1 appears to be restricted to members of the orthomyxovirus family. In contrast, MxA has a broader activity inhibiting the multiplication of influenza viruses (orthomyxoviridae), vesicular stomatitis virus (rhabdoviridae) and measles virus (paramyxoviridae). Mx1 is a nuclear protein whereas MxA accumulates in the cytoplasm. Mx1 and MxA inhibit influenza A virus multiplication at different steps of its replication cycle. Mx1 protein inhibits the transcription of influenza virus genes, whereas MxA protein interferes with a later replication step taking place in the cytoplasm. Interestingly, when recombinant MxA containing a SV40 nuclear translocation signal at its N-terminus is expressed in the nucleus of transfected cells it blocks influenza virus mRNA synthesis like Mx1.

The observed inhibition of influenza virus RNA synthesis by the mouse Mx1 protein could be due to a specific interaction of Mx1 with the viral transcriptase complex formed by the polymerase subunits PB1, PB2, PA and the nucleoprotein NP. We tested this possibility in collaboration with Mark Krystal by measuring the antiviral activity of Mx1 protein in transfected mouse cells overexpressing the polymerase proteins or NP. Indeed, efficient neutralization of Mx1 was observed in influenza virus RNP-transfected cells when the three polymerase subunits were overexpressed simultanously via recombinant vaccinia viruses. Expression of PB2 alone partially neutralized the activity of Mx1. In contrast, overexpression of PB1, PA or NP had no influence on the inhibitory activity of Mx1. Similar results were obtained when the individual viral polymerase proteins were expressed from a constitutive cellular promoter in stably transfected mouse cells. Only expression of PB2 diminished the inhibitory effect of Mx1 on influenza virus multiplication. So far we were not able to obtain biochemical evidence for a physical interaction of Mx1 with the viral polymerase subunits or complex.

To further elucidate the molecular mechanism of influenza virus inhibition by Mx proteins, we examined the function of purified Mx proteins in a *in vitro* transcription system of influenza virus. First, we expressed recombinant Mx1 and MxA proteins, carrying a histidine-tag at their N-terminus, in *E. coli* and purified them under nondenaturing conditions by Ni-chelate affinity chromatography. As expected, the purified Mx proteins showed a nucleotide hydrolysing activity specific for GTP. Interestingly, both Mx1 and MxA inhibited the β-globin mRNA or ApG-dinucleotide primed influenza virus RNA synthesis in vitro, while a mutant Mx1 protein with wild type GTPase activity but lacking antiviral activity *in vivo* was inactive *in vitro* also. The availability of recombinant Mx proteins allows us now to examine which PB2 dependent step of influenza virus mRNA synthesis (cap-stealing, initiation or elongation) is targeted by Mx1 protein.

POSTERS

CONTROL OF FEVER BY THE VACCINIA VIRUS IL-1β RECEPTOR

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The induction of fever by the host in response to infection is an important defense mechanism that enhances aspects of the immune response and restricts the replication of some microorganisms. Here we show that vaccinia virus has devised a mechanism to prevent the febrile response induced in infected animals.

Gene B15R of vaccinia virus strain WR encodes a soluble receptor for interleukin-1β (IL-1β) that is expressed late during infection and binds IL-1β with high affinity. Deletion of the gene does not affect virus replication in cell culture but alters the virus virulence. Although the number of mortalities resulting from infection of mice by the intranasal route with either the wild type (WR) or deletion mutant (vΔB15R) viruses were indistinguishable, animals infected with vΔB15R suffered a more severe infection with stronger signs of illness and accelerated weight loss. We show here that vΔB15R-infected animals develop fever in contrast with WR-infected animals. This fever is directly controlled by the B15R gene product since re-insertion of the gene into the vΔB15R deletion mutant prevented fever induction. Fever was also induced in animals infected with other vaccinia virus strains that do not express the IL-1β receptor (Copenhagen and Tashkent), but not with strains that express the receptor (Tian-Tan and IHD-J). The role of the IL-1β receptor in control of fever was confirmed by preventing the febrile response in mice infected with a Copenhagen virus strain engineered to express this protein. These results suggest that soluble IL-1β and not other pyrogens such as IL-1α, tumour necrosis factor (TNF) or IL-6, is the main mediator of fever in the context of a poxvirus infection.

The severity of the infection, measured by signs of illness and weight loss, was also reduced when the B15R gene was reinserted into the vΔB15R deletion mutant or restored in the Copenhagen strain. Despite this there was enhanced virus replication in the lungs of animals infected with WR virus compared with vΔB15R suggesting that a local inflammatory response mediated by IL-1β can restrict virus replication.

Translational Regulation and Functional Analysis of the Interferon-Induced, dsRNA Activated Protein Kinase (PKR).

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The double-stranded RNA activated protein kinase (known as PKR but also referred to as P68 and DAI) is an interferon-inducible enzyme thought to play a key role in the cellular defense against viral infection. PKR may also participate in the anti-proliferative effects mediated by interferon since enhanced expression of functional PKR is growth suppressive and constitutive expression of catalytically defective PKR mutants can lead to malignant transformation. To further define PKR's role in the control of cell growth in the absence of other interferon-induced gene products, we analyzed the transient expression of mutant and wild-type kinase molecules in transfected COS cells. We found that functional PKR was expressed inefficiently compared to a catalytically inactive PKR mutant which was expressed at levels 30-40-fold higher. Following protein stability and mRNA measurements we confirmed that PKR expression was regulated at the level of mRNA translation. Cotransfection experiments revealed that the catalytically inactive PKR mutant could stimulate reporter gene protein synthesis in a transdominant manner. In addition to studying the function of catalytically inactive PKR mutants, we have analysed the properties of PKR molecules possessing mutations in the regulatory domains. We found that certain mutants were incapable of interaction with both dsRNA activators or viral RNA inhibitors. We will report on the functional analysis of these dsRNA binding mutants including their effects on cell growth and proliferation. Finally, we have developed in vitro transdominant assays utilizing purified functional and defective PKR proteins in an attempt to elucidate mechanisms underlying PKRs role in tumorogenesis.

QUASISPECIES AND EVASION OF THE IMMUNE RESPONSES : ROLE OF POSITIVE SELECTION AND OF RANDOM CHANGE

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The high mutation rates operating during RNA virus replication or retrotranscription (averaging 10^{-3} to 10^{-5} substitution per nucleotide and round of copying) dictates that RNA viruses consist of complex distributions of non-identical but related genomes termed quasispecies. We previously showed that antigenic variation of foot-and-mouth disease virus (FMDV) need not be the result of immune selection (reviewed in Domingo *et al.*, J.Gen.Virol., 1993, in press). We have passaged eight samples of the same FMDV clone in the presence of a limited amount of neutralizing polyclonal antibodies directed to the major antigenic site (site A) of capsid protein VP1. Complex populations of variants showing increased resistance to polyclonal sera and to monoclonal antibodies were selected. Some populations exhibited marked decreases in viral fitness (overall multiplication and survival ability in the considered enviroment). Multiple amino acid replacements within site A - and also elsewhere in VP1 - accumulated upon passage of the virus either in the absence or presence of neutralizing antibodies. However, antigenically critical replacements at one position in site A occurred repeatedly in FMDV passaged under antibody selection but they were never observed in many passages carried out either in the absence of anti-viral antibodies or in the presence of irrelevant sera. Thus, even though antigenic variation of FMDV can occur in the absence or presence of immune selection, critical replacements which lead to important changes in antigenic specifity were observed only as a result of selection by neutralizing antibodies. (Borrego *et al.*, J.Virol. 1993, in press).

Thus, the antigenic evolution of FMDV may be the result of random fixation of tolerated substitutions at antigenic sites as well as of positive selection events. In the latter case, minority components of the quasispecies appear to be driven to dominance by antibody pressure at the expense of considerable fitness loss.

TUMOR NECROSIS FACTOR α STIMULATES EXPRESSION OF ADENOVIRUS EARLY REGION 3 PROTEINS IMPLICATED IN VIRAL PERSISTENCE

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Adenoviruses (Ads) can cause persistent infections in man. Implicated in this phenomenon appears to be the early transcription unit 3 (E3) of the virus which is not essential for virus growth in tissue culture cells. Its relative conservation in most Ad serotypes together with the biologic activity of some proteins encoded in this transcription unit argues for an important role in adenovirus pathogenesis and persistence (6).

Our studies focus on the function of the most abundant protein from this region, the E3/19K protein. Previously, we demonstrated that this protein interacts specifically with class I histocompatibility (class I MHC) antigens in the rough endoplasmatic reticulum (ER), thereby inhibiting transport of MHC molecules to the cell surface (1). As a consequence, MHC-restricted T cell recognition of E3/19K$^+$ target cells is drastically reduced (2). Subsequent studies from our group showed that the structure of MHC antigens responsible for binding E3/19K is contained within the two N- terminal domains, α1 and α2, comprising the peptide binding pocket of MHC antigens (3, 4). At present, we are using site directed mutagenesis to identify the amino acids that contribute to the complex formation.

Recently, Ginsberg and colleagues developed a mouse in vivo model for Ad-induced pneumonia (7). They showed that Ad-induced lesions were infiltrated, first, by macrophages/monocytes and subsequently by T cells. This was accompanied by a increased level of tumor necrosis factor-α (TNF-α) production. TNF-α and other cytokines are known to induce MHC expression. We therefore examined in our transfection system whether TNF-α and interferon-γ (IFN-γ) may overcome the E3/19K induced block of MHC class I expression. Surprisingly, TNF-α treatment caused a further reduction of MHC antigens. Subsequent studies on the mechanism of this effect showed an increased production of E3/19K mRNA and protein, indicating that this cytokine directly or indirectly stimulates the E3 promoter. Supporting this idea we also find an upregulation of other E3 proteins and a further reduction of the EGF-R. We now examine whether the effect is mediated by the NFk-B transcription factor. The significance of this phenomenon was confirmed during infection where a dramatic increase in the amount of E3/19K after TNF-α treatment was demonstrated. The study provides evidence for a novel interaction between the immune system and Ad in which the virus takes advantage of an immune mediator to escape immunosurveillance of the host (5).

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TACARIBE ARENAVIRUS GENE EXPRESSION IN CYTOPATHIC AND NONCYTOPATHIC INFECTIONS.

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Tacaribe virus(TV) replication was compared in vero cells infected under conditions leading either to cell death (cpe (+) infection) or to the establishment of persistence (cpe (-) infection).To this end, two virus preparations were employed: one containing a ratio of standard (plaque forming) viruses to interfering particles (IP) that would induce a distinct lytic response in Vero cells infected at multiplicities giving synchronous infection and another virus stock enriched in IP that would block the cell killing potential of the cytolitic virus stock. The following results were obtained : 1). Deleted viral RNAs could not be detected either at the establishment or during the early stages of persistence. 2). Levels of viral RNAs were severely reduced when the cells were infected with IP in addition standard viruses, the RNA accumulation being inversely proportional to the ratio of IP to standard viruses used in the infections. 3).Accumulation of the three measurable mRNAs , those corresponding to the glycoprotein precursor,(GPC), to the nucleoprotein (N), and to the p11Z proteins ended earlier in the cpe (-) infections (around 18 hr.p.i.) than in the cpe(+) infection (45-68 hr. p.i.). 4).The rate of synthesis of the GPC, N and p11Z proteins were largely determined in both, the cpe (+) and the cpe (-) infections by the amounts of their corresponding m RNAs.5). The kinetics of accumulation of the S genomes and also ,the ratios of the S genome to S antigenome were similar in the different infections (accumulation ending at 45- 68 hr.p.i.). 6). L genome accumulation proceeded for longer time (until 92 hr.p.i.) than that corresponding to the S genome in the cpé (+) infection, while in the cpe(-) infections, L genome accumulation ended around 45 hr.p.i. Until this time ratios of L genome to L antigenome were similar in the different infections. It is concluded that IP affect virus mRNA synthesis early after infection reducing in this way the rate of viral protein synthesis. Low levels of viral proteins might then limit virus replication.In addition the results support the idea that in TV infections transcription and replication are under temporal control. The implications of these results with regards to the nature and mode of action of TV IP will be discussed.

GENETIC MECHANISMS THAT GENERATE ANTIGENIC DIVERSITY OF THE HUMAN RESPIRATORY SYNCYTIAL VIRUS G GLYCOPROTEIN

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The phenomenom of antigenic variation has been related to the capacity of certain animal and human viruses to reinfect the same individual. Reinfections by respiratory syncytial (RS) virus occur frequently among the human population. Epidemiological data suggest that the reinfecting strain is antigenically different to the one that caused the previous infection. Thus, evasion of an immune barrier may be one of the factors determining natural evolution of human RS virus.

We have focused our study in the G glycoprotein (attachment) of human RS virus, since this is the viral polypeptide that shows the highest degree of antigenic variation among human isolates. To understand the genetic basis of that antigenic variation, escape mutants of the RS virus Lang strain were selected by passaging the virus in the presence of anti-G monoclonal antibodies. Analysis of the antigenic and genetic changes selected in the escape mutants indicated that: 1) Variable epitopes, not present in all the human isolates, are determined by the amino acid sequence of the G protein C-terminal third. 2) The antigenic variants contained different types of sequence alterations, including: i) nucleotide substitutions leading to amino acid replacements, ii) nucleotide substitutions that introduced premature in-frame stop codons (1), iii) frame-shift mutations generated by delections or insertions of a single adenosine (A) in oligo-A tracts of the G protein gene (2), and iv) reiterative U-C substitutions (hypermutations) in the G mRNA (3). The genetic mechanisms that generated these changes and their relevance to RS virus evolution will be discussed.

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INHIBITION OF MCMV IE GENE EXPRESSION IN NIH 3T3 CELLS BY IFN-ALPHA AND IFN-GAMMA.

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The replication of murine cytomegalovirus (MCMV) in embryo fibroblasts from different mouse strains or in NIH 3T3 cells was found to be very sensitive to the antiviral activity of IFN-alpha or gamma. Analysis of virus specified RNAs by Northern blot technique revealed that IFNs significantly reduced the expression of the major immediate early (IE1, IE2 and IE3) as well as of the early and late transcripts. An evaluation of the early steps after MCMV entry showed that uncoating and transport of viral DNA to the nucleus were not affected by IFN treatment. Since transcription of MCMV IE region is regulated by a strong enhancer element, a series of constructs containing the reporter gene CAT driven by segments of different lenght of the MCMV IE enhancer were prepared. After transfection in NIH 3T3 cells, CAT activity in transient assays was strongly inhibited by IFNs. When the transfected cells were infected with MCMV the viral transactivation of the reporter gene under the control of MCMV IE sequences was inhibited by both IFNs. Taken as a whole, these results suggest that IFN-alpha or IFN-gamma impair MCMV replication in NIH 3T3 cells at an early step during IE gene transcription.

Class I MHC molecules are retained in the endoplasmic reticulum in cells infected with herpes simplex virus.

Ann B. Hill, Duncan McGeoch, Barbara Barnett and Andrew McMichael.

We have studied the assembly and transport of MHC class I molecules in human fibroblasts (fb) infected with herpes simplex viruses types 1 and 2 (HSV1 and HSV2). Using a mutant strain of HSV2 G with the gene responsible for host protein synthesis shutoff deleted, we infected fb derived from donors of known MHC class I types. 2-4 hours after infection the cells were metabolically labelled with ^{35}S-methionine for 2 hours. MHC Class I was immunoprecipiated with W6/32 (recognizing ß2m associated class I) or HC10 (recognizing free class I heavy chain). The immunoprecipitates were analysed by isoelectric focusing, allowing both the separation of individual class I alleles, and the monitoring of transport through the Golgi complex by the aquisition of acidic charge due to sialation. In HSV-infected cells, little or no class I MHC acheived sialation, implying that assembled class I was retained in the endoplasmic reticulum. In addition, more class I was found as free heavy chain- ie it had not stably associated with beta-2 microglobulin and peptide. In contrast, Transferrin receptor was normally sialated in HSV-infected cells. The effect was also seen with HSV1- here the host protein synthesis shutoff gene (UL41) is weaker and both wild type and UL41- mutant viruses caused retention of class I. The effect is clearly seen within 2 hours of HSV infection. The effect was not observed under conditions in which only alpha (immediate early) proteins were expressed, and it was not diminished by Phosphonoacetic acid which inhibits late gene (gamma gene) expression, suggesting the the gene reponsible is an early (beta or early gamma) gene.

Ablation of PKR mRNA in intact cells induced by 2-5A-antisense chimeras

A. Maran[1], R.K. Maitra[1], B. Dong[1], K. Lesiak[2], W. Xiao[3], P.F. Torrence[3] & R.H. Silverman[1]

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"Antisense" refers to the concept that nucleic acids which are complementary in sequence may anneal in cells and inhibit gene expression. We have developed a novel antisense strategy in which 2-5A-dependent RNase is directed to specific RNA targets. Chimeric molecules were synthesized in which the 2-5A species, pA(2'p5'A)$_3$, was covalently linked to antisense oligodeoxyribonucleotides. The antisense cassette of the chimera binds to its complementary sequence in RNA while the accompanying 2-5A component attracts and activates 2-5A-dependent RNase which then cleaves the target RNA. In a cell-free system, we showed that 2-5A-antisense directed 2-5A-dependent RNase to specifically cleave a modified HIV mRNA (Torrence et al., *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1300-1304, 1993). To extend this work to a naturally-occurring RNA sequence, we selected mRNA to PKR, the dsRNA-dependent protein kinase. Accordingly, purified recombinant 2-5A-dependent RNase degraded PKR mRNA in the presence of 50 nM of 2-5A-antiPKR but not with 150 nM of 2-5A linked to an unrelated oligonucleotide. Furthermore, after incubating HeLa cells with 2-5A-antiPKR for 4 hrs, there was no intact PKR mRNA remaining as determined using reverse transcriptase-coupled PCR. 2-5A linked to irrelevant oligonucleotide sequences and antiPKR alone had no effect on PKR mRNA levels and 2-5A-antiPKR did not reduce levels of actin mRNA; thus demonstrating selectivity of action. In addition, 2-5A-antisense did not reduce cell growth rates and therefore it is not cytotoxic. This new approach to targeted RNA degradation promises to control gene expression and to act as a therapeutic agent.

The E3 region of C-type adenovirus interferes with the expression of the CD8+ T cell immunodominant E1A antigen and averts immunopathology.

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The E3 region of c-type adenoviruses is pleiotropic in facilitating viral escape from immune recognition. E3 region genes and their products have been shown to down regulate MHC-class I, the recognition structure for $CD8^+$ cytotoxic T (Tc) lymphocytes, in a molecule specific manner. We have shown that genes within the E3 region down regulate the E1A products, the immunodominant antigen for Tc cells. This down regulation occurs at the translational level and is specific for viral products. Using E3 deletion mutants the genes involved have tentatively been identified as E3 14.5kD and or the E3 10.4kD product(s). Furthermore the interaction between the E3 product(s) and E1A region products resulting in decreased translation of E1A and consequently in reduced adenovirus infected target susceptibility to lysis by adenovirus immune Tc cells, requires subtype homology between the E3 and E1A region.

E3 deletion mutants exhibit increased "virulence" in mice and preliminary data indicate this to be due to $CD8^+$T cell mediated immunopathology.

Immunological recognition of FMDV and its proteins in the bovine

Parkhouse R.M.E., Baron J., Collen T.C., Doel T.,
Flint M., Foster M., Garcia-Valcarcel M. & Ryan M.

AFRC Institute for Animal Health, Pirbright Laboratory, Woking, Surrey, UK.

1. RECOGNITION OF INTACT VIRUS

a) Humoral:

Unlike classical neutralising antibodies, total anti-FMDV antibodies, as measured in an ELISA capture assay, are highly cross reactive.

b) Cellular:

Proliferative T cell responses of peripheral blood mononuclear cells (PBMC) are barely detectable during primary responses to vaccine or virus, and frequently low during secondary responses. For good T cell proliferation *in vitro*, multiple immunisation is required.

This may reflect preferential stimulation of the Th2 ("B cell helping") CD4 T cell subset.

2. RECOGNITION OF INDIVIDUAL VIRAL PROTEINS

a) Expression Cloning:

Structural and non-structural proteins pseudogenes were cloned from cDNA by PCR, expressed in pGEX-3XUC, and purified by SDS-PAGE.

b) Humoral:

Structural and non-structural proteins were recognised by infected animals.

c) Cellular:

Some structural and non-structural proteins were recognised and were cross reactive.

Interestingly, VP_1 was strain specific, and the polymerase (3D) was the most immunogenic and cross reactive.

MUCOSAL IMMUNITY AS A WAY TO CONTROL HOST EVASION MECHANISMS OF TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS.

C. Smerdou and L. Enjuanes

Transmissible gastroenteritis virus (TGEV) is a coronavirus that infects pigs, producing a transitory enteritis in adult animals, and a mortality close to 100% in piglets younger than 10 days. TGEV escapes the immune system by infecting piglets, which are too young to develop an immune response against the virus in a short time. The infection affects the gastrointestinal tract, producing a severe diarrhoea leading to dehydratation and subsequent death. Piglets can be protected only by receiving passive protection through the milk. This protection can be obtained by inducing lactogenic immunity in the pregnant sow two weeks before delivery. The induction of lactogenic immunity can be achieved by antigenic stimulation at the gut associated lymphoid tissues (GALT), such as Peyer´s patches, or mesentheric lymph nodes. One of the strategies for inducing lactogenic immunity against TGE is based on the expression of selected viral antigens in prokaryotic or eukaryotic vectors with an enteric tropism. Attenuated forms of *Salmonella typhimurium* and adenovirus have been chosen as prokaryotic and eukaryotic enteric vectors, respectively, to express TGEV antigens involved in protection.

S. typhimurium Δ*cya* Δ*crp* is an attenuated mutant that is avirulent and keeps its immunogenicity and tropism for the GALT, being able to colonize and persist in this tissue for up to 3 weeks before being eliminated. The whole S glycoprotein of TGEV, as well as three overlapping fragments of this protein, containing both conformational and linear antigenic sites, have been expressed as fusion products with β-galactosidase in *salmonella,* using *asd* expression plasmids. These constructions have shown different levels of antigenicity and stability *in vitro* and *in vivo*. A second type of construction in *salmonella* was based on the expression of a linear epitope from the S protein which is glycosilation independent and able to induce neutralizing antibodies. This linear epitope was expressed as a fusion product with the B subunit of the heat labile toxin from *E. coli* in monomeric and polimeric forms. These constructions showed good antigenicity and very high levels of stability *in vitro*. The potentiality of all these recombinants to induce a protective immune response *in vivo* is being evaluated.

Adenovirus 5 (Ad5) has been used as an eukaryotic vector to express TGEV antigenic determinants which are glycosilation dependent. Several truncated forms of S gene from TGEV coding for the aminoterminus half were cloned into an SV-40 based expression cassette in Ad5 and expressed *in vitro*. The recombinant Ad-5-TGEV viruses selected were stable and expressed the recombinant antigen. These recombinants were able to induce TGEV neutralizing antibodies in hamsters.

Another approach to get lactogenic immunity is the development of transgenic animals able to secrete specific immunoglobulins in milk. A neutralizing antibody specific for TGEV recognizing a very highly conserved epitope has been selected. The genes coding for the heavy and light chains of this immunoglobulin have been cloned and will be incorporated in transgenic pigs under the control of the whey acid protein (*wap*) promotor, which is specifically activated in mammary glands during lactation.

Cytomegalovirus Prevents Antigen Presentation by Blocking the Transport of Peptide-Loaded MHC Class I Molecules into the Medial-Golgi.

Margarita del Val[1,2], Hartmut Hengel[1], Hans Häcker[1], Udo Hartlaub[1], Thomas Ruppert[1], Pero Lucin[1], and Ulrich H. Koszinowski[1].

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Selective expression of murine cytomegalovirus (MCMV) immediate early (IE) genes leads to the presentation by the MHC class I molecule L^d of a peptide derived from MCMV IE protein pp89. Characterization of endogenous antigenic peptides identified the pp89 peptide as the nonapeptide ^{168}YPHFMPTNL^{176}. Subsequent expression of MCMV early genes prevents presentation of pp89. We report on the mechanism by which MCMV early genes interfere with antigen presentation. Expression of the IE promoter driven bacterial gene lacZ by recombinant MCMV subjected antigen presentation of ß-galactosidase to the same control and excluded antigen specificity. The L^d dependent presence of naturally processed antigenic peptides also in non-presenting cells located the inhibitory function subsequent to the step of antigen processing. The finding that during the E phase of MCMV gene expression the MHC class I heavy chain glycosylation remained in an endo H sensitive form located the block to the endoplasmatic reticulum/cis-Golgi compartment. The failure to present antigenic peptides was explained by a retention of nascent assembled trimolecular MHC class I complexes. At later stages of infection a significant decrease of surface MHC class I expression was seen, whereas other membrane glycoproteins remained unaffected. Thus, MCMV E genes endow this virus with an effective immune evasion potential. These results also demonstrate that the formation of the trimolecular complex of MHC class I heavy chain, $ß_2$-microglobulin and the finally trimmed peptide is completed before entering the medial-Golgi.

Del Val, M., Hengel, H., Häcker, H., Hartlaub, U,. Ruppert, T., Lucin, P., and Koszinowski, U. H. (1992). J. Exp. Med., **176**, 729-738.

List of Invited Speakers

Workshop on

VIRAL EVASION OF HOST DEFENSE MECHANISMS

List of Invited Speakers

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VIRAL EVASION OF HOST DEFENSE MECHANISMS

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265 **Workshop on Adhesion Receptors in the Immune System.**
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