Instituto Juan March
de Estudios e Investigaciones

30 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

# Resistance to Viral Infection

Organized by

L. Enjuanes and M. M. C. Lai

M. Ackermann
R. Blasco
L. Enjuanes
M. Esteban
P. A. Furth
F. L. Graham
L. Hennighausen
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*The lectures summarized in this publication were presented by their authors at a workshop held on the 20th through the 22nd of June, 1994, at the Instituto Juan March.*

Depósito legal: M. 25200/1994
I. S. B. N.: 84-7919-530-4
Impresión: Ediciones Peninsular. Tomelloso, 37. 28026 Madrid.

# INDEX

PAGE

# PROGRAMME

## RESISTANCE TO VIRAL INFECTION

**MONDAY, June 20th**

Registration.

Introduction.

**I. Vectors Based on DNA Viruses**
**Chairperson: E. Paoletti**

M. Esteban - Combined Live Virus Immunization Against Pathogens.

E. Paoletti - Recombinant Poxvirus Vaccines.

R. Blasco - Vaccinia Virus Mutants with Reduced Transmissibility as a Novel Approach to Safer Vaccines.

F.L. Graham - Human Adenovirus Vectors for High Level Expression, Recombinant Viral Vaccines, and Gene Therapy.

P. Roy - Assembly of a Multicomponent Animal Virus Using Baculovirus Expression System and Development of a Novel Vaccine.

**II. Interference with Virus Replication**
**Chairperson: B. Roizman**

B. Roizman - What is Host Resistance to Herpes Simplex Virus Infection, and How Does the Virus Respond?

M. Ackermann - Tuning Gene Expression of Recombinant Bovine Herpesvirus 1.

J.F.Rodríguez- Searching for African Swine Fever Virus Genes Involved in Immune Evasion Mechanisms.

J.A. Melero - What Have We Learned about Resistance to Human Respiratory Syncytial Virus Infections?

M. Del Val - Efficient Processing of an Antigenic Sequence for Presentation by MHC Class I Molecules Depends on its Neighboring Residues in the Protein.

**TUESDAY, June 21st**

**III. Replication of RNA Viruses and Targets in Protection**
**Chairperson: M.M.C. Lai**

S. Makino - Coronavirus Defective Interfering RNA: A Useful Tool for the Understanding of Coronavirus Multiplication Mechanisms.

W.J.M. Spaan - Towards the Development of a Coronavirus Expression Vector.

J. Ortín - The Influenza Virus NS1 Protein: A Key Factor in the Viral Gene Expression.

S.G. Siddell - Immunological Prevention of Coronavirus Infection.

L. Enjuanes - Induction of Mucosal Immunity to Transmissible Gastroenteritis Coronavirus.

**IV. Replication of RNA Viruses and Vector Development**
**Chairperson: W.J.M. Spaan**

M.M.C. Lai - The Use of Coronavirus DI RNA As an Expression Vector.

C.M. Rice - Alphavirus-Based Expression Systems.

**Round Table: Trends in *in Vivo* Expression Vectors**
**Chairpersons: B. Roizman and M.M.C. Lai**

**V. Transgenic Animals Resistant to Viral Infections**
**Chairperson: P. Staeheli**

P. Staeheli - Virus Resistance Mediated by Interferon-Induced Mx Proteins.

J.J. Rossi - Ribozyme Based Therapeutic Strategies for the Treatment of HIV Infection.

G. Keil - A Structural Component of Alpha-Herpesvirus Virions Induces Resistance Against Superinfection.

**WEDNESDAY, June 22nd**

**VI. Gene Expression and Resistance to Infection**
**Chairperson: L. Hennighausen**

H.L.Robinson - Gene Vaccines, a New Approach to Immunization.

P.A. Furth - Jet Injection As a Tool to Transfer Genes Into Somatic Tissues.

L. Hennighausen - Control of Temporal and Tissue Specific Gene Expression in Transgenic Animals Using Tissue-Specific and Tetracycline Responsive Promoters.

K. Olson - Intracellular Immunization of Mosquito Cells to Dengue and Yellow Fever Viral Infection Using Double Subgenomic Sindbis Expression Viruses.

**Round Table: Trends in Transgenesis and Resistance to Viral Infections.**
**Chairpersons: P. Staeheli and L. Hennighausen.**

Visit to the Centro Nacional de Biotecnología.

# INTRODUCTION

L. Enjuanes

The aim of the meeting has been to review current knowledge on the use of vaccines to induce systemic and secretory immunity, and the development of transgenic animals with resistance to viral infection. Recent advances in the use of virus to fight virus have been discussed. Topics have included the development of new vectors such as fowlpoxvirus, adenovirus, alphavirus, and coronavirus. The strategies of replication, latency establishment, and defense in herpesvirus have also been discussed. Relevant model systems as African swine fever virus, syncytial respiratory viruses, and influenza virus, were presented. A new trend in protection is DNA immunization. DNA delivery systems may replace viral vectors in the near future, and were discussed.

Two round tables, which are commented below, considered new tendencies in vector development and transgenesis in providing resistance to virus infection. The regulatory elements used for gene expression could have a viral, bacterial, or eukaryotic origin. The two first ones are widely used today, but increasing recommendations for the use of regulatory elements with eukaryotic origin is observed, both in vector development and gene therapy. The advantages and disadvantages of fully infectious or defective vectors, and of amplicons in antigen delivery systems were studied. Defective viruses are safer but their antigenic potential is smaller than that of fully infectious vectors. Suicide vectors have been designed using Semliki forest virus replicons with no encapsidation signal, to replicate defective helper genomes coding for virus structural proteins. Adenovirus and herpesvirus, increasingly used in vaccine development and gene therapy, have the advantage of their organ tropism and good expression levels. In contrast, these viruses have the disadvantage of carrying genes involved in cell transformation. Herpesviruses seem promising vehicles for gene therapy.

Immunization by jet injection with microgram quantities of DNA induces the expression of protein nanograms which elicited protective immune responses. DNA is integrated in one percent of the cells, what might bring some clues on the use of this very promising technology. Progress in the construction of transgenic animals allows today to switch on and off transgene expression using tetracycline operon combined with p16 protein transcription enhancer from human cytomegalovirus.

A variety of avenues in protection have been addressed. Future efforts might be concentrated on specific subjects which experience will discover as the most realistic approaches.

# I. Vectors Based on DNA Viruses

COMBINED LIVE VIRUS IMMUNIZATION AGAINST PATHOGENS.

**Mariano Esteban**. Centro Nacional de Biotecnologia, CSIC, Campus Universidad Autónoma, Cantoblanco, 28049 Madrid, Spain.

Clearance of a pathogen from an infected host usually involves activation of both arms of the immune system, humoral and cellular. For a vaccine to be most effective it should be able to trigger high titre neutralizing antibodies and adequate production of cytotoxic T-lymphocytes (CTL). Although for safety reasons purified antigens are desirable over live viruses, it is not yet clear if purified proteins migh have wider utility as vaccines.The strong immune response of live viruses and our ability to generate highly attenuated recombinant vectors expressing multiple antigens suggest that live vaccines might provide an effective strategy to control infectious diseases. The identification of B and T cell epitopes on clinically relevant antigenic proteins provides a rationale for the desing of improved live vaccines. We have shown that experimental animals immunized with a combination of live recombinants of influenza and vaccinia viruses expressing B and T cell epitopes of Plasmodium yoelii circumsporozoite (CS) protein, are markedly protected when challenged with sporozoite-induced malaria. The protocol of immunization was critical. Immunization of mice with a recombinant influenza virus expressing a CD8+ T cell epitope of P. yoelii, followed by a recombinant vaccinia virus expressing the CS protein, was an effective way to induce protective immunity against sporozoite-induced malaria (1). When an attenuated recombinant of vaccinia virus was used to boost mice which have been immunized with recombinant influenza viruses, 81% of mice were fully resistant to malaria challenge (2). Protection against malaria was largely due to activation of CD8+ lymphocytes. Our findings indicate that it is possible to develop a highly efficient antimalaria vaccine based on the generation of non-pathogenic live viruses expressing important plasmodial antigens and underscore the considerable potential of live carriers for the development of vaccines against multiple pathogens.

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# RECOMBINANT POXVIRUS VACCINES

Enzo Paoletti, Ph.D.

Three highly attenuated poxvirus-based vectors are being investigated for their potential utility as recombinant vaccines.
The TROVAC vector is based on Fowlpox virus and is designed primarily for use in the poultry industry. Protection against Avian Influenza and Newcastle disease has been obtained by vaccination of day old birds with the respective TROVAC recombinant.
The ALVAC vector is based on the host restricted Avipox virus, canarypox. The safety and immunogenicity of ALVAC-based recombinant vaccine candidates has been demonstrated in numerous species including man. A single inoculation of an ALVAC rabies recombinant has provided protection against rabies virus challenge for a period of three years. In human clinical trials this recombinant was shown to be well tolerated and immunogenic. ALVAC-based recombinants for Canine distemper and Canine parvovirus were shown to be efficacious in dogs while an Equine Influenza recombinant was shown to protect horses against a natural influenza epizootic. ALVAC based recombinants for HIV and Japanese encephalitis are currently in clinical trials.
The NYVAC-based vector is a highly attenuated vaccinia derivative engineered by the precise deletion of 18 open reading frames associated with virulence or host range. NYVAC based recombinants have been shown to be safe and to provide protection against Japanese encephalitis and Pseudorabies in swine and Influenza virus in horses. Several NYVAC-based vaccine candidates are currently in human clinical testing. Demonstrating the power of the poxvirus vector system for incorporating large quantities of foreign DNA, a NYVAC construct expressing 7 antigens from the P. falciparum malaria parasite has been prepared for clinical evaluation.

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VACCINIA VIRUS MUTANTS WITH REDUCED TRANSMISSIBILITY AS A NOVEL APPROACH TO SAFER VACCINES. Rafael Blasco. Centro de Investigación en Sanidad Animal, INIA, 28130 Madrid, Spain.

Vaccinia virus morphogenesis is a complex process, taking place in the cytoplasm of the cell, which leads to the formation of two forms of infectious virions. The first one, termed Intracellular Mature Virus (IMV) is assembled in the cytosol, and acquires its membranes by budding through the intermediate compartment. Subsequently, some IMV become wrapped by cisternae derived from the trans-Golgi network, acquiring a two-membrane shell. Those virions are transported to the cell membrane, where fusion of the outermost membrane with the plasma membrane occurs, releasing to the extracellular milieu virions which contain an additional membrane with respect to IMV, and are termed Extracellular Enveloped Virus, or EEV. Characterization of the IMV and EEV has indicated that those two forms are biochemically, antigenically and functionally different. Although most of the infectious virus remains as IMV, several studies indicate that EEV are important in an animal infection.

The fact that the virus is infectious without the outer EEV envelope has opened the possibility of obtaining mutated viruses in which the genes coding for different components of the outer envelope have been altered. In several cases, those mutant viruses have unexpected phenotypes, with characteristics such as EEV formation, EEV infectivity, liberation of viruses from the cell membrane or ability to cause cell-to-cell fusion dramatically altered. A virus mutant, in which the gene coding for the major protein of the EEV envelope was deleted, showed a decreased cell to cell transmissibility, concomitant with a decrease in EEV formation. Animal studies have shown a high degree of attenuation of that virus mutant.

Therefore, it seems likely that the biological characteristics of the virus can be modulated by introducing mutations in genes coding for EEV envelope proteins, and that specific mutations in those genes could substantially increase the safety of vaccine strains.

# Human adenovirus vectors for high level expression, recombinant viral vaccines, and gene therapy

Andrew Bett, Ludvik Prevec, and Frank L. Graham
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Human adenoviruses (Ads) are currently attracting considerable attention because of their potential utility for three different applications: for gene transfer and gene therapy, for development of live viral vectored vaccines, and for protein expression in mammalian cells. Engineering of Ad vectors designed for these applications requires a variety of different reagents in the form of Ad viruses and bacterial plasmids containing viral DNA sequences, and requires different strategies for construction of vectors for different purposes. To simplify Ad vector construction and to develop a procedure, with maximum flexibility, efficiency and cloning capacity we have developed a new vector system based on use of Ad5 DNA sequences cloned in bacterial plasmids. Expanded deletions in early region 1 (E1) (3180 bp) and E3 (2690 or 3132 bp) can be combined in a single vector which should have a capacity for inserts of up to 8 kb, enough to accommodate the majority of cDNAs encoding proteins, along with control elements to regulate expression. Genes can be inserted into either or both of the E1 or E3 regions and mutations or deletions elsewhere in the viral genome can be readily introduced into the vector.

Vectors with substitutions in E3 are nondefective, can therefore replicate in any normally permissive human cell, and are able to infect and induce high level expression of the foreign gene insert in a variety of different cell types and *in vivo* in virtually all mammals. Vectors with substitutions in E1 are defective and replicate only in 293 cells but are nonetheless able to deliver and express cloned genes in cell culture and *in vivo*. Both defective and nondefective recombinant adenoviral vectors are therefore capable of inducing an immune response in inoculated animals and could be highly effective live viral vaccines. We have constructed a large number of vectors expressing genes from various pathogens but will focus on vectors expressing the rabies glycoprotein to illustrate the potential of ad vectors as vaccines.

## Assembly of a multicomponent animal virus using baculovirus expression system and development of a novel vaccine.

Polly Roy[1,2]

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The Orbivirus genus within the family Reoviridae consists of architecturally complex viruses. The viruses are 810Å diameter in size and are comprised of two protein shells containing seven proteins, surrounding a genome of ten double-stranded RNA segments. The prototype virus, bluetongue virus (BTV) is the etiological agent of a disease that can reach epidemic proportions among sheep and cattle. Like reoviruses and rotaviruses, BTV is a non-enveloped virus with ten double-stranded (ds) RNA segments, enclosed by an icosahedral capsid of a complex nature of seven proteins. To understand the assembly process of BTV, we have utilized novel baculovirus multigene expression vector system, by employing suitably constructed plasmid transfer vectors several genes can be introduced into the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) genome and expressed simultaneously. So far, two-five BTV genes have been co-expressed from single recombinant vectors. The resultant particulate structure resembles BTV virus-like and sub-virus-like particles by lack detectable amounts of RNA or DNA. The assembly studies have been complemented by the cryoelectron microscopy and image processing analysis of the 3-D structure of the particles as well as X-ray crystallography of some protein components. Their properties as vaccines and opportunities as foreign immunogen delivery systems will be discussed.

# II. Interference with Virus Replication

# What is host resistance to herpes simplex virus infection, and how does the virus respond ?

**Bernard Roizman and Joany Chou**

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The herpes simplex virus genome encodes 75 different genes. Of this number, 36 genes are required for viral replication and 38 genes are dispensable for viral growth in cell culture (1). Of these genes one plays a particularly significant role in infection in that it enables to overcome a very important defensive mechanism on the part of the host.

The $\gamma_1$34.5 gene of herpes simplex virus 1 (HSV-1) maps in the inverted repeats flanking the long unique sequences of HSV-1 DNA. Its promoter is the terminal a sequence involved in packaging of viral DNA into preformed capsids and in the isomerization of the viral DNA. The gene is dispensable in some cell lines (e.g. Vero) and the ability of the virus to replicate cannot be differentiated from that of the wild type virus (2-4). Preliminary studies suggest that this promoter is induced by stress. The significant finding was that the deletion mutant lacking the $\gamma_1$34.5 gene is unable to replicate in the central nervous system of mice and therefore is totally devoid of the capacity to cause pathology (5). $\gamma_1$34.5 gene appears to be required for the growth of the virus in CNS and lead us to investigate the capacity of the virus to multiply in cells of neuronal origin.

Studies in human neuroblastoma cell line SK-N-SH and in other cell lines of neuronal origin led to the discovery that the $\gamma_1$34.5$^-$ deletion mutant triggers a premature total shut off of all protein synthesis thereby rendering the cell non viable and reducing drastically viral yields (6). The termination of synthesis of proteins is triggered by the initiation of viral DNA synthesis inasmuch as exposure of cells to phosphonoacetate, an inhibitor of viral DNA synthesis precludes the premature termination of translation (6).

The 263 amino acid $\gamma_1$34.5 protein consists of an amino terminal domain, a variable linker sequence consisting of three amino acids repeated 5 to 10 times, and a carboxyl terminal domain homologous to the corresponding domain of MyD116, a 657 amino acid protein expressed in myeloid leukemia cells induced to differentiate by interleukin 6 and GADD34, a protein induced by growth arrest and DNA damage. We have constructed several viral mutants from which various domains of the $\gamma_1$34.5 gene had been deleted or rendered mute by the insertion of a stop codon. Studies on those mutants show that the domain of the $\gamma_1$34.5 gene necessary to preclude the total shut off of protein synthesis corresponds to the carboxyl terminal domain of the $\gamma_1$34.5 gene homologous to the corresponding coding domain of the MyD116 gene (7).

It would appear that most primary human cell lines and cell lines of neuronal origin express a stress response to HSV infection. This response results in complete shut off of protein synthesis and, invariably, a premature death of the infected cell. It is an important response inasmuch as it curtails the ability of the cell to support viral replication and spread throughout the body. The inability to prevent the cellular stress response which causes the infected cell to die may be responsible for the inability of the deletion mutant to multiply and cause pathology in the central nervous system of mice. The virus has evolved a gene, possibly borrowed in part from eukaryotic cells, to preclude this response in order to replicate in the infected cells.

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## Tuning gene expression of recombinant bovine herpesvirus 1

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Bovine herpesvirus 1 (BHV-1) is the causative agent of infectious bovine rhinotracheitis and infectious pustular vulvovaginitis (IBR/IPV) in cattle. Similar to other α-herpesviruses, BHV-1 replicates productively in peripheral tissues of infected cattle as well as in permissive cell cultures. The infected cells are efficiently destroyed. Three temporal phases of replication are distinguished, following each other consecutively in a cascade fashion. These phases are tightly regulated and termed *alpha* or immediate-early (IE), *beta* or early, and *gamma* or late.

In contrast, latency is established primarily in neuronal cells. The cascade of replication which is seen in the productively infected cells, seems to be out of order. Apparently, the IE genes are silenced during latency. The IE proteins, the major regulators of the consecutive viral gene expression, are not synthesized and consequently, the *beta* and *gamma* genes are not expressed either.

Our working hypothesis is based on the assumption that forced expression of IE protein(s) in neuronal tissue may prevent the establishment of latency of BHV-1. In order to test this hypothesis we have identified and started to functionally analyze the IE genes and IE gene products of BHV-1. Our results are as follows.

(i) Two IE transcriptional units running in opposite directions and specifying four IE translation products have been detected and were termed, as far as possible, according to their counterparts in herpes simplex virus 1 (HSV-1). The BHV-1 infected cell protein (bICP) bICP4 and bICP22 genes are located within the internal (IR) and terminal repeat (TR) sequences of the BHV-1 genome and are therefore present in duplicate. The genes for bICP0 and *circ* exist only as single copies because they map at the right and left termini of the genomic unique long sequence, respectively.

(ii) The corresponding IE proteins, which have been identified by anti peptide sera and expression of the corresponding genes in recombinant baculoviruses, migrate with the following relative mobility (Mr) in polyacrylamide gels: bICP4 (Mr 180/140 K), bICP0 (97 K), bICP22 (50 K), and *circ* (34 K). With the exception of the *circ* protein, which is a myristylated virion component with unknown function, the IE proteins of BHV-1 have been shown to influence the viral gene expression. bICP0 is a very potent transactivator, bICP4 has the capacity to both up and down regulate the expression of specific viral genes, and bICP22 appears to have mostly down regulating capacity.

(iii) A series of recombinant BHV-1 (rBHV) with alterations concerning the IE genes has been constructed. Thus far, deletion of the bICP0 gene has not been tolerated, whereas deletion of the *circ* gene did not affect viral replication or gene expression of rBHV in cell cultures. Duplication of the bICP0 gene resulted in the generation of viable progeny rBHV with specific modifications in the amount of synthesis of IE gene products. The expression of late genes remained unaffected. The mutant as compared to wild type BHV-1, however, was able to grow to higher titers in infected Vero cells.

We conclude that rBHV-1 with specific alterations in the IE genes are potential tools to study the biology of bovine herpesviruses. The behaviour of these viruses in their natural hosts remains to be tested.

## Searching for African swine fever virus genes involved in immune evasion mechanisms.

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African swine fever virus (ASFV) is a large enveloped deoxyvirus with icosahedral symmetry that belongs to an, as yet, unnamed family of animal viruses. ASFV infects several members of the Suidae family as well as soft ticks of the *Ornithodoros* genus (reviewed in 1, 2).

In its natural swine hosts, warthogs and bushpigs, the infection results in a mild disease with little detectable clinical signs. ASFV infection of susceptible ticks causes an increase in their mortality rate but most of the specimens survive the infection and are able to transmit the virus for very long periods of time (3). In contrast to this, ASFV was first described as the causative agent of the African swine fever (ASF), a devastating hemorrhagic disease of domestic pigs (4). However, data collected during the last 40 years show that ASF occurs in a wide variety of clinical forms ranging from the peracute to the chronic asymptomatic infection (reviewed in 5).

The lack of an effective vaccine has frustrated all attempts to eradicate the virus from enzootic areas. A vaccine is also needed to prevent the eventual spreading of ASF to, as yet, unaffected countries. Unfortunately, although a number of strategies have been explored including immunization of domestic pigs with inactivated ASFV particles, attenuated virus strains and purified virus polypeptides, reliable protective immune responses have never been achieved (6, 7).

The difficulties encountered in the search for an ASFV vaccine along with the proficiency of the virus to establish persistent infections have led to speculation that ASFV might use mechanisms to counteract the host's immune defenses. The genome of ASFV is formed by a single molecule of double stranded DNA with a size of 170-190 kb and a coding capacity of more than 150 genes (2, 8). Thus, it seems feasible that, similar to what has been observed with other large DNA viruses (9, 10), some of the ASFV genes might be devoted to interfere with the host's immune system.

We have analyzed the complete nucleotide sequence of the BA71V strain of ASFV and all the detected virus-encoded proteins have been

subjected to an extensive computer analysis. This has led to the identification of several ASFV genes encoding proteins with a strong sequence similarity to immunologically relevant polypeptides such us the adhesion receptor of natural killer cells and T lymphocytes CD2 (11), C-type lectins and the protooncogen bcl-2. These ASFV genes are likely to be involved in strategies to block or modulate important aspects the host's defense mechanisms. The functional role of some of these genes is currently under investigation.

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**WHAT HAVE WE LEARNED ABOUT RESISTANCE TO HUMAN RESPIRATORY SYNCYTIAL VIRUS INFECTIONS?**

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Human respiratory syncytial (RS) virus, a member of the Pneumovirus genus of the family Paramyxoviridae, is the major cause of lower respiratory tract infections in infants and young children (1). Early attempts of vaccination with formalin-inactivated virus failed to protect young vaccinees against natural infection. In addition, vaccinated children experienced more frequently an exacerbated disease after a natural infection than control children. Retrospectively, it was found that the vaccine failure to induce protection was related to modification of epitopes onto the two major glycoproteins (F and G) recognized by neutralizing antibodies (2). Disease enhancement was related to alterations of the cell-mediated immune response.

Several evidences support the notion that neutralizing antibodies play a major role in resistance to RS virus infection: 1) High levels of maternally acquired antibodies correlate with less severe infection or complete protection. 2) Administration of high titre human antisera have prophylactic and therapeutic effects against RS virus infection. 3) Animals inoculated with either purified G and F glycoproteins or vaccinia recombinants that express these antigens produce neutralizing antibodies which correlate with resistance to a RS virus challenge. 4) Transfer of anti-G or anti-F monoclonal antibodies to laboratory animals confer passive protection to viral challenge (3, and references therein).

Results obtained recently in our laboratory will be presented to illustrate the importance of antigen presentation upon the induction of both neutralizing antibodies and a protective immune response.

Another relevant aspect of RS virus immunobiology is the antigenic variation of RS virus isolates that has been related to the virus ability to reinfect the same individual (4). There are at least two antigenic subgroups of human RS virus. Within each subgroup there are multiple cocirculating lineages. Extensive antigenic and genetic analysis of RS viruses isolated during consecutive epidemics in distant geographical locations have demonstrated the capacity of this virus to disseminate worldwide and the influence of immune selection in RS virus evolution (5).

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**Efficient Processing of an Antigenic Sequence for Presentation by MHC Class I Molecules Depends on its Neighboring Residues in the Protein.**

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Processing of endogenously synthesized proteins generates short peptides that are presented by MHC class I molecules to CD8 T lymphocytes. Here it is documented that not only the sequence of the presented peptide but also the residues by which it is flanked in the protein determine the efficiency of processing and presentation. This became evident when a viral sequence of proven antigenicity was inserted at different positions into an unrelated carrier protein. Not different peptides, but different amounts of the antigenic insert itself were retrieved by isolation of naturally processed peptides from cells expressing the different chimeric proteins. Low yield of antigenic peptide from a disfavorable integration site could be overcome by flanking the insert with oligo-alanine to space it from disturbing neighboring sequences. Notably, the degree of protection against lethal virus disease directly related to the amount of naturally processed antigenic peptide.

# III. Replication of RNA Viruses and Targets in Protection

Coronavirus defective interfering RNA: A useful tool for the understanding of coronavirus multiplication mechanisms.

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Understanding how a virus expresses its genes is essential for controlling infection by that virus and for developing an expression vector system based on that virus. We are interested in understanding how coronavirus, a disease causing neurotropic virus, regulates its gene expression. In place of the elusive cloned coronavirus cDNA, cloned coronavirus defective interfering (DI) RNAs have proven to be very effective genetically flexible tools for studying coronavirus multiplication. With DI RNAs we identified how coronavirus gene expression is regulated by sequences that flank the coronavirus transcription consensus sequence, and determined that expression of genes 1 and 7 is sufficient for coronavirus RNA synthesis.

We found that the sequences flanking the intergenic region preceding gene 7 do not regulate subgenomic DI RNA transcription (J. Virol. 67, 3304-3311, 1993). Through construction of artificial flanking sequences we studied the effects of flanking sequences on subgenomic transcription in more detail. We prepared a series of 0.4 kb DNA fragments with identical transcription consensus sequences sandwiched between different flanking regions, which were derived from various regions of MHV. We inserted the fragments from this series, singly, at the same fixed location in a DI cDNA. We found that the efficiencies of subgenomic DI RNA transcription varied among these DI RNAs. This data indicated that the sequence flanking the transcription consensus sequence affected MHV transcription.

We studied how proximal insertion of two intergenic regions affected subgenomic DI RNA synthesis. DI RNAs with two intergenic regions separated by more than 100 nt synthesized two subgenomic DI RNAs that were virtually equal in quantity. When those same two intergenic regions were separated by less than about 35 nucleotides, a significantly lower amount of the larger subgenomic DI RNA (synthesized from the upstream intergenic region) was made. Deletion and site-directed mutagenic analyses demonstrated that a downstream intergenic sequence suppresses subgenomic DI RNA synthesis from an upstream intergenic region.

We identified the MHV genes required for MHV RNA synthesis using an MHV-JHM-derived DI RNA, DIssA. DIssA is a naturally occurring self-replicating DI RNA with nearly intact genes 1 and 7. DIssA interferes with most MHV-JHM-specific RNA synthesis, except for synthesis of mRNA 7, which encodes N protein; mRNA 7 synthesis is not inhibited by DIssA. Coinfection of MHV-JHM containing DIssA DI particles and an MHV-A59 RNA- ts mutant followed by subsequent passage of virus at the permissive temperature resulted in elimination of most of the MHV-JHM. Analysis of intracellular RNAs at nonpermissive temperature demonstrated efficient synthesis of DIssA and mRNA 7, but not of the helper virus mRNAs. By oligonucleotide fingerprinting analysis, we determined that the structure of mRNA 7 was JHM-specific, and therefore, must have been synthesized from the DIssA template RNA. Sequence analysis revealed that DIssA lacks a sequence, found in wild type MHV from the 3' half of gene 2-1 to the 3' end of gene 6. Replication and transcription of another MHV DI RNA was supported in DIssA-replicating cells. Because the gene 2 and 2-1 products are not essential for MHV replication, we concluded that expression of gene 1 proteins and N protein was sufficient for MHV RNA replication and transcription.

TOWARDS THE DEVELOPMENT OF A CORONAVIRUS EXPRESSION VECTOR

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Our research is aimed towards the development of coronavirus expression vectors. Coronaviruses are used since these viruses replicate in the cytoplasm without integration into the host genome, since they naturally express their genes from multiple internal promotors, since they have a large theoretical capacity for incorporation of foreign genetic information, since these viruses have a broad host range spectrum and since each coronavirus has a restricted host and species specificity.

We propose to develop coronavirus carriers by exploiting the properties of defective RNA viruses to express foreign genetic information in a controllable fashion through a dead-end, abortive infection.
Coronaviruses are a family of plus-stranded RNA viruses generally causing respiratory and enteric infections in man and animals. The organization of the viral genome is such that the replicating functions are clustered roughly within the 5'-terminal 2/3 part of the RNA while the structural functions are encoded by the remaining 3'-region. Defective genomes lacking various parts of the genome can still replicate and be assembled into (defective) viral particles provided the missing functions are complemented in trans by wild-type helper virus. The general picture of defective coronaviral genomes analyzed sofar by others and ourselves is characterized by the conserved presence of a large part of the 5' region and a small part of the 3'-terminus. Apparently, from the intervening domain deletions wildly varying in size and location can be tolerated.
Structurally, coronaviruses are quite simple. The helical nucleocapsid is surrounded by a lipid membrane in which three viral membrane proteins are integrated. One, called M is a small glycoprotein that spans the bilayer three times and whose prime function involves the assembly of the viral particle in the infected cell. The small membrane protein (sM) has only recently been identified in purified virus and its function is not known yet. The third protein (S) is a large glycoprotein that constitutes the spike of the virion. Recent studies have shown that a virion spike actually constitutes a trimer of S which is preassembled before virus particle formation. The S protein has several essential functions the most important ones being the recognition of and binding to the target cell receptor and the induction of a fusion reaction between the viral and cellular membranes. By way of these functions the virus expresses its species and cell type tropism and introduces its nucleocapsid into the cytoplasm of the host cell to initiate virus replication. It is interesting to note that tropism with these viruses appears to be governed at the level of virus entry: species barriers can be overcome and replication can be achieved in cells not naturally affected by some coronavirus if only one manages to experimentally introduce the "foreign" viral RNA into the cell. An important practical implication of this is that it should be possible to infect cells with a heterologous coronavirus by incorporating in it the homologous spike protein. A coronavirus DI-based expression vector could thus be endowed at will with different cell/species specificities.

The ultimate goal is to develop a replication competent coronavirus RNA vector and a set of packaging systems. As a first step we have developed an RNA vector based upon MHV defective interfering (DI) particles (**J. Virol., 1991,65, 3219-3226**). The DI replication studies have also revealed that the MHV DI RNAs require a functional open reading frame (ORF)

for efficient propagation (**J. Virol., 1992, *66*, 5898-5905**). To determine whether the DI-polypeptide or translation perse is involved we have analyzed additional DIs containing deletions, insertions and 3' non-coding regions of different lengths. From the data we obtained we conclude that the relative portion of the DI RNA that is translated determines its fitness. It was subsequently shown that the DI-vector could be used for mutagenesis of the helpervirus genome by homologous recombination (**Nucl. Acids Res., 1992, *20*, 3375-3381**). Recombination vectors containing the 3'-8 kb of the genome, fused to different parts of the 5' polymerase gene have been constructed which will be used for inserting heterologous genes into the HE pseudogene of the coronavirus genome. The minimal promoter sequence required for the expression of inserted genes has recently been determined (**J. Virol., 1994, *68*, 3656-3666**). In addition we will explore the possibilities to introduce mutant spike genes in the coronavirus genome by means of homologous recombination.

In order to be able to express foreign genes using a replication competent but defective genome we aim towards a system which obviates the need of a helper virus. To this end a vector containing the gene encoding the RNA dependent RNA polymerase, and the replication signals is required. Currently, we are mapping the replication signals and are trying to reconstruct the polymerase gene. Such a vector can only be rescued into virus particles in the presence of the structural proteins. We have analyzed the assembly of DI particles in the absence of helpervirus using transient expression of the structural proteins and the synthetic DI RNA. Preliminary data will be presented.

**THE INFLUENZA VIRUS NS1 PROTEIN: A KEY FACTOR IN THE VIRAL GENE EXPRESSION**

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The influenza virus RNA segment 8 encodes two proteins, NS1 and NS2, by differential splicing. The collinear transcript acts as mRNA for NS1 protein while the spliced mRNA encodes the NS2 protein. The splicing of NS1 mRNA was studied in cells transfected with recombinant plasmids in which the cDNA of RNA segment 8 was under the control of the SV40 late promoter and polyadenylation signals. The NS1 mRNA was poorly spliced to yield NS2 mRNA. However, inactivation of the NS1 gene, but not NS2 gene led to a large increase in the splicing efficiency. This effect was not specific for NS1 mRNA, since the splicing of the endogenous SV40 early transcript was altered in such a way that t-Ag mRNA was almost eliminated. These changes in the splicing pattern coincided with a strong inhibition of the mRNA nucleocytoplasmic transport. Both NS1 and NS2 mRNAs were retained in the nucleus of cells expressing NS1 protein, but no effect was observed when only NS2 protein was expressed. Furthermore, other mRNAs tested, as T-Ag mRNA and the non-spliceable NP transcript, were also retained in the nucleus. In spite of the almost complete retention of mRNAs, the observation was made that the level of the corresponding proteins did not diminish. Experiments will be presented to show that expression of NS1 protein from cloned DNA leads to a large enhancement in the expression of other viral genes. This effect is mediated at the translational level, most probably by increasing the rate of initiation of protein synthesis. Since we show that the NS1 protein binds specifically to the p220 component of the eIF-4F factor, it is proposed that it acts as a viral mRNA-specific protein synthesis initiation factor.

## Immunological Prevention of Coronavirus Infection

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Viruses are intracellular parasites and, at least in the case of enveloped RNA viruses, infection depends on the fusion of viral and cellular membranes. This process is mediated by viral glycoproteins and it is an obvious target for the development of prophylactic and therapeutic strategies to prevent or limit viral infection.

In the first series of experiments, we will describe the construction, expression and characterisation of a single-chain antibody molecule specific for the surface glycoprotein of the murine hepatitis coronavirus, MHV. The single chain antibody, A1scFv, was derived from a murine monoclonal antibody that has a high binding affinity and is able to neutralise virus infectivity. The heavy and light chain mRNAs of Mab A1 were isolated and degenerate primers were used for cDNA synthesis and PCR amplification of the VDJ and VJ regions, respectively. Extension PCR was then used to join the variable regions with a $(GGGGS)_3$-linker and overlap PCR was used for cloning the scFv into eucaryotic expression vectors. Additionally, an epitope "tag" sequence was cloned to the carboxyl terminus of the molecule. Using eucaryotic expression vectors with human cytomegalovirus or metallothionein promoters, we have expressed the A1scFv molecule in CRFK and DBT cells and assayed the resistance of these cells to virus infection and S protein-mediated membrane fusion.

In a second series of experiments, we have analysed the structure-function relationships of the MHV S protein, with particular emphasis on its fusogenic activity. The approach taken has been to analyse interactions between different domains and subunits of the MHV S protein by isolating and characterising monoclonal antibody resistant (MAR) variants that carry amino acid substitutions outside the antibody binding site. The location and nature of these substitutions suggest that there are specific interactions between defined regions of the S1 and S2 subunits of the MHV S protein and that these interactions are relevant to its fusogenic activity. These data provide new insights into the function of the S protein and may be useful in designing single-chain antibody molecules that prevent the fusion process.

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## INDUCTION OF MUCOSAL IMMUNITY TO TRANSMISSIBLE GASTRO-ENTERITIS CORONAVIRUS

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Transmissible gastroenteritis coronavirus (TGEV) infects enterocytes of the intestine leading to death when animals are infected at birth. Protection against TGEV can be provided by lactogenic immunity. The antigenic structure of TGEV has been defined both at the level of B and T cells, and this structure has been correlated with the physical one (Correa et al., 1988; Gebauer et al., 1992; Antón et al., 1994). The antigenic and genetic variability of the virus has also been documented (Sánchez et al., 1990; Sánchez et al., 1992). Animals are infected at birth and killed in three days, leaving little time to set an efficient immune response. To interfere with virus replication three types of strategies are proposed: induction of lactogenic immunity in foster mothers by using vectors with tropism for mucose associated lymphoid tissues (MALT), virus interference, and development of transgenic animals providing protective antibodies during lactation. Recombinant adenoviruses expressing the whole spike (S) protein or truncated forms of this protein have been developed. This recombinants induced TGEV neutralizing antibodies in the milk of female hamsters during lactation, providing that the recombinants included either sites B or A.

To develop vectors based in coronaviruses, TGEV defective genomes have been identified. A cDNA corresponding to a 9.5 kb defective interfering (DI) genome has been cloned and partially sequenced. The cDNA codes for 5' and 3' ends, and ORF 1b sequences of the parental virus. The defective genomes did encapsidate and were stable upon passage on ST cells. Nevertheless, some heterogeneity has been detected at the 3' end of cDNA from cloned DI genomes. We have shown that recombination takes place between coronavirus of the TGEV group, although at low frequency, indicating that modification of the *wt* genome, by recombination with genetically engineered cDNAs coding for defective genomes, is in principle possible. TGEV chimeras with S proteins from enteric and respiratory viruses are being constructed to determine the role of S protein in tropism.

Oral administration of purified immunoglobulins from immune sows provided full protection against virulent TGEV. A monoclonal antibody (MAb) recognizing a highly conserved epitope, critical in TGEV neutralization, has been identified. Immunoglobulin genes coding for the variable modules of the light and heavy chains of this MAb have been sequenced, cloned, and fused to constant modules of human immunoglobulins. Recombinant antibodies expressed in COS or SP2-0 myeloma cell lines efficiently neutralized TGEV. These genes are being expressed under the control of *wap* promoter, responsible for the expression of whey acidic protein, an abundant milk component (Hennighausen, 1992). This promoter is activated in the mammary gland during lactation. To optimize expression under the control of *wap* promoter, luciferase gene has been cloned into the first *wap* exon, between the first and third exon, and at the forth exon. Higher luciferase activity was obtained when luciferase gene was cloned into the first exon. A system to analyze the expression of immunoglobulin genes under the control of *wap* promoter is being set up comparing the efficiency of the immunoglobulin expression in several *in vitro* systems, using plasmids containing cytomegalovirus enhancer. Selected constructs are being used to produce transgenic mice secreting TGEV neutralizing antibodies in the milk.

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# IV. Replication of RNA Viruses and Vector Development

THE USE OF CORONAVIRUS DI RNA AS AN EXPRESSION VECTOR.

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Coronavirus mouse hepatitis virus (MHV) defective-interfering (DI) RNA has been used as a tool for studying the recognition signals for RNA replication, transcription and recombination. We have now demonstrated that this DI RNA can be used as a vector for expressing foreign or viral genes. The genes of interest are inserted behind an intergenic sequence in the middle of the DI RNA, and is expressed only in MHV-infected cells. We have successfully expressed chloramphenicol acetyltransferase (CAT), luciferase and MHV hemagglutinin-esterase (HE) proteins. For the HE protein, the expressed protein can be incorporated into virus particles, and the recombinant DI RNA can be passaged for at least four passages. Both the wild-type and recombinant HE proteins are incorporated, thus generating a pseudo-recombinant virus. The signal for the incorporation of HE protein into MHV virions is currently being studied. This DI RNA is thus an effective expression vector for delivering foreign genes into virus-infected cells.

The DI RNA carrying a CAT gene can be used for studying the cis- and trans-acting signals of viral RNA synthesis. This is particularly useful for studying negative-strand RNA synthesis. By RNase protection assay of the CAT gene, we have identified the cis-acting signal for MHV (-) strand RNA synthesis. We have demonstrated that the (-) stand RNA primarily exists as a double-stranded RNA form and requires only the 3'-end sequence of the genomic RNA. Thus, the (-) strand RNA synthesis is regulated by a significantly less complex mechanism than (+) strand RNA synthesis. This DI vector has thus provided a complete picture of cis-acting signals for (+) and (-) strand RNA replication and transcription.

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# ALPHAVIRUS-BASED EXPRESSION SYSTEMS

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Alphaviruses are enveloped positive-strand RNA viruses transmitted to vertebrate hosts via mosquito vectors. The genome RNA of the prototype virus, Sindbis virus, is a capped and polyadenylated mRNA of 11.7 kb. RNA replication occurs via synthesis of a full-length minus-strand intermediate which is used as the template for synthesis of additional genome-length RNAs and for transcription of a 4.1 kb subgenomic RNA from an internal promoter. This subgenomic RNA is the mRNA for translation of the structural proteins. In the course of studying Sindbis virus, we have explored the use of this viral RNA replication and packaging machinery for cytoplasmic expression of heterologous RNAs and proteins in animal cells. As a transient expression system, Sindbis virus offers several potential advantages: 1) a broad range of susceptible host cells including those of insect, avian, and mammalian origin, 2) high levels of cytoplasmic RNA and protein expression without splicing and, 3) the facile construction and manipulation of recombinant RNA molecules using a full-length Sindbis virus cDNA clone from which infectious RNA transcripts can be generated by *in vitro* transcription. Several strategies have been tried and will be reviewed. These include engineering recombinant viruses to express a second subgenomic RNA or replacement of the structural genes to produce self replicating RNA "replicons" which can be packaged into infectious particles using defective helper RNAs. In addition, random insertion mutagenesis of the viral glycoproteins has been used to select viable chimeras which display heterologous peptides on the virion surface. This technology may be useful for restricting tissue tropism and targeting infection to specific cell types. Demonstrated and potential applications of these vector systems will be highlighted.

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# ROUND TABLE

## TRENDS IN *IN VIVO* EXPRESSION VECTORS

M. M. C. Lai

Several issues regarding the development and use of expression vectors were discussed:

## I. The comparative merits and selection of different expression vectors.

Many expression vector systems are currently available, and many more will be developed in the near future. What is the guideline for choosing a particular vector? It appears that each vector system offers particular advantages and disadvantages. Consideration of various factors, such as immunogenicity (strength and duration), host range of virus vectors, duration of foreign gene expression, tissue specificity of expression, presence or absence of integration of vectors into chromosome, ability to cause persistent infection, cytopathogenicity of virus vectors, and capacity for foreign gene insertion, should aid in choosing the most appropriate vector system. The participants of this workshop were reluctant to make any specific recommendation regarding the selection of vector systems.

Retrovirus vectors are commonly used in human gene therapy, but are largely ignored in the animal vaccine field. No retrovirus vectors were discussed in this workshop. It was recognized that this avoidance of retrovirus vectors is due to the restriction imposed by the governmental regulatory agencies.

## II. Tissue targeting of virus vectors.

One of the major difficulties in vector development is the specific expression of foreign genes in tissues of interest. This has largely been overcome by the use of specific promoters. However, the specificity of promoters has its limitations. An alternative approach may be the development of virus vectors which can specifically infect certain target cells. This has been accomplished in herpes simplex virus vectors which target neurons. Viruses that have natural target specificity (such as adenovirus for respiratory systems or coronavirus for gastrointestinal tracts) may serve the same purpose. Genetic engineering into vectors of gene products which recognize surface proteins on certain target cells may be a potential means of either restricting or expanding the target cell specificity of virus vectors.

## III. The potential of using defective-interfering (DI) viruses as expression vectors.

Many viral genomes, particularly RNA genomes, are too large for easy genetic engineering. DI RNA offers an alternative approach. The utility of this approach is becoming evident with the development of Sindbis virus, influenza virus and coronavirus vectors. This is particularly important for RNA viruses with large or unwieldy RNA genomes.

# V. Transgenic Animals Resistant to Viral Infections

## VIRUS RESISTANCE MEDIATED BY INTERFERON-INDUCED Mx PROTEINS

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The Mx1 gene was originally defined as a dominant autosomal trait that mediates selective resistance of mice to experimental infections with influenza A viruses. Mouse strains lacking a functional Mx1 gene ($Mx^-$ mice) show an incomplete response to virus-induced interferon: cells from $Mx^-$ animals fail to synthesize the nuclear 72-kDa Mx1 protein and fail to become resistant to influenza A virus after treatment with type I interferon. Transfected $Mx^-$ cells expressing Mx1 cDNA show a high degree of resistance to influenza A virus but remain susceptible to unrelated viruses. Furthermore, transgenic mice expressing Mx1 cDNA show enhanced resistance to influenza A virus infections, demonstrating that Mx1 is necessary and sufficient for influenza virus inhibition in cell culture and in the animal. Mx1 protein inhibits viral mRNA synthesis.

Interferon-induced human cells accumulate an abundant cytoplasmic 76-kDa protein, designated MxA. Transfected cell lines expressing MxA protein show enhanced resistance to influenza A virus, vesicular stomatitis virus (VSV) and measles virus. MxA-expressing cells further show a very high degree of resistance to infection with Thogoto virus, a poorly characterized tick-born orthomyxovirus. Transgenic mice failed to accumulate significant amounts of MxA in some organs, although MxA cDNA expression was driven by the promoter of a ubiquitously expressed gene. The MxA-transgenic mice resisted infections with high doses of Thogoto virus, whereas non-transgenic littermates did not. However, the MxA-transgenic mice were not significantly more resistant to infections with VSV or influenza A viruses than control animals. The mechanism of Thogoto virus inhibition by MxA is unknown. Purified MxA protein has GTPase activity. However, this biochemical activity is dispensable for inhibition of VSV mRNA synthesis in vitro, arguing against the possibility that Mx proteins block viral RNA polymerases by depleting the cells for GTP. Mx proteins seem to interact physically with the RNA polymerase complexes of susceptible viruses and thereby inhibit their activities.

# RIBOZYME BASED THERAPEUTIC STRATEGIES FOR THE TREATMENT OF HIV INFECTION

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Ribozymes are receiving considerable attention for use as therapeutic reagents. A number of different catalytic motifs have been engineered for application as trans-acting ribozymes capable of cleaving any targeted RNA. The hammerhead ribozyme contains the simplest catalytic center, and has therefore been adapted for a wide variety of potential therapeutic applications. We have been investigating various parameters for improving the intracellular efficiency of these ribozymes, with the ultimate goal of utilizing hammerhead ribozyme based strategies for the treatment of HIV-1 infection. These parameters include protein facilitation of ribozyme turnover, targeting and co-localization, transcription and delivery strategies. The ubiquitous cellular protein hnRNP A1 and the HIV-1 encoded NCp7 nucleocapsid protein have been found to stimulate ribozyme turnover several fold *in vitro*, but there are strict limits to the hybrid arm lengths and base composition which allow this facilitation. These results will be discussed in light of their potential for facilitating the intracellular functioning of ribozymes. A unique targeting strategy which takes advantage of the specific binding of the cellular $tRNA^{Lys3}$ by HIV reverse transcriptase during virion formation. We have constructed $tRNA^{Lys3}$ variants which have an anti-HIV ribozyme appended adjacent to the mature tRNA sequence. These molecules are selectively bound by reverse transcriptase, positioning the ribozyme for site directed cleavage of the HIV-1 genomic RNAs. Results from anti-viral testing of this strategy will be discussed. Finally, experiments testing various promoter constructs in both AAV and retroviral vector delivery systems have been carried out. The results of ribozyme intracellular localization and anti-viral effectiveness as a function of the various promoter constructs will be described.

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## A STRUCTURAL COMPONENT OF ALPHA-HERPESVIRUS VIRIONS INDUCES RESISTANCE AGAINST SUPERINFECTION.

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Resistance of virus infected permissive cells against superinfection with homologous or heterologous viruses, named viral interference, is widespread in nature and it is hypothesized that one of the functions is to prevent eclipse of viral progeny by already infected cells.

For herpesviruses, viral gene expression dependent inhibitory effects of herpes simplex virus 1 (HSV-1) on HSV-2 and vice-versa and limited replication of HSV-1 in human cytomegalovirus replicating cells have been described. Large quantities of HSV - viable or UV inactivated - prevent entry of challenge HSV into host cells apparently by blocking cellular receptor(s) involved in penetration. Inhibi-tion of virus entry can also be achieved by incubation of cells with soluble forms of HSV-1 glycoprotein D (gD). Analogous results were obtained with cells expressing the gD homologs of bovine herpesvirus 1 (BHV-1) or pseudorabievirus (PRV).

To analyse superinfection exclusion under less artificial conditions, permissive cells were infected with BHV-1, HSV-1 or PRV with 1 to 10 pfu per cell and either coinfected or superinfected at increasing times p.i. with genetically or biochemically distinguishable homologous and heterologous virus strains. These experiments demonstrated that alpha-herpesvirus infected cells develop resistance against superinfection by other alphaherpesviruses and also by vacciniavirus whereas foot and mouth disease virus is still able to enter these cells. This process, which can be induced by one infectious particle per cell, confers complete inhibition of secondary infections at 6 hours p.i.. This type of interference is caused by molecules associated with virions, because its establishment does not require de novo N-glycosylation, transport of membrane proteins or synthesis of viral or cellular proteins. Superinfecting virions still attach to completely resistant cells but are unable to penetrate. The expression of transfected genes is not affected. This type of interference is different from the gD mediated inhibition of infection because gD deleted BHV-1 mutants are also to induce resistance.

Characterization of the molecular viral and cellular mechanisms involved in generation of this type of interference might open new possibilities for the characterization of cellular molecules involved in penetration of herpesviruses and allow to develop alternative strategies to control herpesvirus infections.

# VI. Gene Expression and Resistance to Infection

GENE VACCINES, A NEW APPROACH TO IMMUNIZATION. Harriet L. Robinson, University of Massachusetts Medical Center, Worcester, MA 01655, USA

Direct DNA inoculations are being developed as a method of subunit vaccination. In this method, plasmid DNAs are constructed that are eukaryotic expression vectors for the proteins to be used as immunogens. The constructed DNAs are directly administered to the animal to be vaccinated. The take up and expression of the vaccine DNA by cells in the host produces the protein that raises the immune response. Early studies using DNA vaccines have demonstrated the raising of long lasting humoral, cell mediated, and protective responses.

DNA vaccine trials in my laboratory have used influenza and immunodeficiency virus models. Vaccinated animals have included chickens, mice, ferrets, rabbits, and macaques. Vaccine DNAs have been administered by injections in saline as well as gene gun delivery of DNA-coated gold beads. These trials have demonstrated successful immunizations following intramuscular, intradermal, and intravenous inoculations of purified DNA in saline. They have also demonstrated that gene gun delivery is 100 to 1000 times more efficient than direct injections.

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## Jet injection as a tool to transfer genes into somatic tissues

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Jet injection of a plasmid or episomal DNA solution can be used to transfect skin (1-5), muscle, fat and mammary tissue of living animals (1). In this method a jet injector, such as the Ped-o-jet (Stirn Industries, Dayton, NJ), is used to form approximately 100 - 300 $\mu$l of a DNA solution into a jet. This jet has sufficient force to travel into and through differentiated tissues of living animals. Cells which lie around the path of the jet injection are transfected with the plasmid or episomal DNA.

We are interested in determining which variables influence the efficiency of gene transfer by jet injection. Specifically, we have compared levels of gene transfer in different tissues of mice and sheep by the same injection force. We have evaluated the influence of different injection solutions. Finally, we have investigated how varying the force of the injection affects the efficiency of gene transfer in the same tissue. The goal of these investigations is to establish optimal injection parameters for each selected tissue.

At the present time jet injection has been used most extensively as a means of introducing papillomavirus genomes into the skin of rabbits (2-5). We are currently exploring its utility in effecting genetic immunization in animals (6). Cellular transfection by both needle injection of plasmid DNA into muscle (7) and particle bombardment into skin (8),(9) have been successful in eliciting an immunological response. As there is evidence that transfection into skin can produce a more vigorous immunological response, we are interested in determining whether the combined transfection of both skin and underlying muscle by jet injection is an efficient and practical means of genetic immunization.

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# Control of temporal and tissue specific gene expression in transgenic animals using tissue-specific and tetracycline responsive promoters

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The roles of mammalian gene products in development and oncogenesis have been defined by their actions in dominant gain of function experiments in transgenic animals. The transgenes in these experiments are controlled by either tissue specific or ubiquitously active promoters. Their temporal and spatial expression patterns are dependent upon the characteristics of the promoters employed. There are, however, experimental questions which require a precise control over the timing and tissue specific expression of a transgene. Two examples which require temporally regulated gene expression will be discussed. First, questions related to mammary oncogenesis can only be tackled if the temporal expression of oncogenes during development can be controlled. Secondly, the large scale production of human pharmaceutical proteins in milk of transgenic lifestock can only be successful if the synthesis is confined to the mammary gland of the lactating animal.

## Background

Although several inducible promoter systems have been established in transgenic mice, they all have limitations. In binary systems, the target gene by itself is silent, and it is activated in double transgenic animals which contain a second transgene encoding either a transactivator or a recombinase. In those systems that have been reported, the temporal activity of the target gene is dependent on the expression pattern of the transactivator gene, and it cannot be directly regulated by changing experimental conditions. In systems based on environmental signals such as steroid hormones or heavy metal ions, gene expression can be modulated. However, generalized physiologic or toxic effects from the inducing chemicals and high basal transcriptional activity from the promoters limit their utility. Finally, tissue specific and hormone inducible promoters such as the MMTV-LTR or the whey acidic protein (WAP) gene promoter, direct gene expression to only a few selected tissues; the timing of gene expression is primarily controlled by endogenous hormone levels.

Previous work was directed toward the transfer of well characterized genetic regulatory systems from *Escherichia coli* into mammalian cells, and the adaptation of these systems to deliberately control expression of eukaryotic genes. Transgenic systems based on the *lac* operon have proven inadequate because of inefficient induction levels and kinetics. The successful development of a novel regulatory circuit in tissue culture cells based on the tetracycline-resistance operon, opened a new approach to controlling transgene expression. In this system, a fusion protein composed of the *tet*R and the C-terminal domain of the HSV-VP16 strongly activates transcription from a basal promoter containing *tet*O sequences. The hybrid *tet*R-VP16 transcription factor binds to the *tet*O sequences in the absence of tetracycline but not in its presence. The result is that transcription ceases upon the introduction of tetracycline.

We will discuss the *tet*R-VP16/*tet*O system as a means to temporally regulate transgene expression. The luciferase reporter gene was used as a sensitive measure of expression levels in whole tissues, and the β-galactosidase gene was used to monitor expression at the single cell level.

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# Intracellular Immunization of Mosquito Cells to Dengue and Yellow Fever Viral Infection Using Double Subgenomic Sindbis Expression Viruses.

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A novel expression system based on infectious cDNA clones of mosquito-borne Sindbis (Togaviridae) virus has been used to express heterologous genes in C6/36 (*Aedes albopictus*) cells and *Aedes* species of adult female mosquitoes. The double subgenomic Sindbis (dsSIN) virus expression system generates recombinant SIN viruses which contain a heterologous RNA sequence downstream of a duplicated SIN internal initiation site; the heterologous sequence is transcribed as a subgenomic mRNA within an infected cell. dsSIN viruses replicate in the cytoplasm of infected mosquito cells and produce no apparent cytopathology. Mosquito cells as well as adult mosquitoes infected with dsSIN viruses become "transiently" transformed since mosquito cells become persistently infected with the viruses. Previous work has shown that 100% of C6/36 cells can be infected with a dsSIN virus expressing chloramphenicol acetyltransferase (CAT). Each infected cell expresses approximately $1X10^6$ CAT polypeptides. Furthermore, female *Aedes* mosquitoes that are intrathoracically injected with the dsSIN/CAT virus express CAT in neural, midgut, salivary gland, and ovary tissues up to 40 days post-infection. [**Olson, K.E.**, Carlson, J.O., and Beaty, B.J. 1992. Expression of the chloramphenicol acetyltransferase gene in Aedes albopictus (C6/36) cells using a noninfectious Sindbis virus expression vector. Insect Mol. Biol. 1:49-52. Higgs, S., Powers, A.M., & **Olson, K.E.** (1993). Alphavirus expression systems: applications to mosquito vector studies. Parasitology Today, **9**, 444-452. **Olson, K.E.**, Higgs, S., Hahn, C.S., Rice, C.M., Carlson, J.O., & Beaty, B.J. (1994). Expression of chloramphenicol acetyltransferase in Aedes albopictus (C6/36) cells and Aedes triseriatus mosquitoes using double subgenomic recombinant Sindbis virus vectors. Insect Biochem. Mol. Biol. 24, 39-48.]

We have previously demonstrated that dsSIN viruses that transcribe the small segment RNA of Lacrosse virus (LAC; Bunyaviridae) in an antisense orientation significantly inhibited LAC replication in C6/36 cells [Powers, A.M., **Olson, K.E.**, Higgs, S., Carlson, J.O., & Beaty, B.J. (1994). Antiviral intracellular immunization using a recombinant Sindbis virus vector. Virus Research. (in press)]. dsSIN viruses have now been engineered to express specific dengue type 2 (DEN-2) and yellow fever (YFV) viral coding regions or RNA sequences. dsSIN viruses were generated which express the PrM protein of YFV and the capsid and PrM proteins of DEN-2; dsSIN viruses were also produced that transcribe these genes in an antisense orientation. C6/36 cells have been infected with these viruses and challenged at a multiplicity of infection of 0.1 with either DEN-2 or YFV 48 hours later. C6/36 cells infected with the dsSIN viruses that express DEN-2 and YFV proteins or antisense RNAs show significant interference to replication of the challenge virus; cells previously infected with control dsSIN viruses were not resistant to replication of the challenge virus. We are currently using these dsSIN viruses to induce interference to DEN-2 and YFV in female *Aedes aegypti* mosquitoes. The dsSIN viral expression system should allow us to identify molecular strategies for interfering with arboviral replication in mosquitoes.

# ROUND TABLE

## TRENDS IN TRANSGENESIS AND RESISTANCE TO VIRAL INFECTIONS

L. Hennighausen

**New technologies**

New technologies are frequently the driving force for major scientific advances. Two emerging technologies were discussed and their impact on our experimental approaches to gene regulation, viral pathogenesis and immunization were evaluated.

**DNA vaccines**

The demonstration that DNA can be used to generate a protective antibody response in animals has introduced a new approach to immunization. Developments in this area focus on identifying appropiate antigens to express, generating optimal plasmid vectors and establishing efficient, practical and safe means to introduce the DNA into animals and people.

**Inducible gene expression systems**

The physiological role of viral and cellular gene products can be defined by their actions in dominant gain of function or loss of function experiments. However, to fully understand the role of a protein in the context of the dividing cell and the growing organism, it is necessary to precisely control the timing of the expression of the gene under investigation. Extensive work on well characterized genetic regulatory systems from *Escherichia coli* paved the way for an inducible gene system in mammalian cells. The successful development of a novel regulatory circuit in tissue culture cells based on the tetracycline-resistance operon, opened a new approach to controlling transgene expression. In this particular system, transcription ceases upon the introduction of tetracycline, and can be reactivated upon removal of the drug.

Using such a system it will be possible to study in detail the function of genes at particular times of cell propagation and at specific times of the life of an animal. Specifically, and most importantly for gene products whose continued expression is incompatible with life, it will be possible to introduce genes into cells and organisms and keep them silent until analysis is required.

Inducible expression systems might be specially useful to control transgenes providing resistance to viral infections. Several gene therapeutic approaches may be utilized to inhibit virus production. "Intracellular immunization" has followed various schemes: ribozymes, antisense oligonucleotides, trans-dominant negative mutant viral proteins, and molecular decoys. Inducible expression of these transgenes might help to avoid secondary effects, while keeping optimal the intracellular immunity.

The role of different genes in development or in protection (i.e., interleukin 2, tumor necrosis factor, etc.) can be studied using the gene knockout technology. This approach, which has been very informative, can be misleading, since in some cases (IL 2) has been dependent of the genetic background on the species on which the experiments were performed.

# POSTERS

# IDENTIFICATION OF DOMINANT T HELPER EPITOPES IN THE IMMUNE RESPONSE TO TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS (TGEV)

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Transmissible gastroenteritis (TGE) is an enteric viral disease of swine with high mortality in piglets under 2 weeks of age. Passive immunity is of primary importance in providing newborn piglets with protection against TGEV infection. Lactogenic immunity can be induced by vaccination of pregnant sows. The design of effective vaccines requires the identification of T helper epitopes involved on the synthesis of neutralizing antibodies. Cellular responses from TGEV immune mini-swine of $SLA^{d/d}$ haplotype have been studied to identify dominant T helper epitopes. Lymphocytes from mesenteric lymph nodes (MLN) gave blastogenic responses with an stimulation index higher than 100. These responses were antigen dose dependent. Infectious TGEV did not affect the proliferative response. TGEV-specific blaastogenic reponse was inhibited by monoclonal antibodies specific for porcine CD4. The three major structural proteins of TGEV (S, M and N) were expressed as fusion products with maltose-binding protein and purified by affinity chromatography. A specific, dose dependent response to the recombinant viral proteins was detected, which followed a kinetics similar to the response to the whole virus. Predictive algorithms have been used to select sequences in the structural proteins with higher probability to contain T sites. The ability of sixty 15-mer oligopeptides to induce proliferation of immune cells was evaluated. Peptides from the half carboxiterminus of N protein induced the main response, followed by the response to overlapping peptides of the M protein. Peptide N7, derived from the nucleoprotein, induced the maximum T cell response detected and was not recognized by polyvalent antisera from TGEV immune animals, suggesting that it does contain a T cell epitope but no dominant B cell epitopes. The universality of this epitope is being determined using immune lymphocytes from mini-swine with $SLA^{a/a}$ or $SLA^{c/c}$ haplotypes. T lymphocytes from N7 immune mini-swine were able to reconstitute *in vitro* the synthesis of neutralizing TGEV-specific antibodies when added to TGEV-immune B cell. These results show that N7 defines a true T helper epitope involved on the synthesis of TGEV-specific neutralizing antibodies. The relevance of the defined epitope will be tested in T-B recombinant antigens expressed using Adenovirus 5-based vectors.

# THE INTERACTION OF THE HIV-1 RT WITH DNA

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Human immunodeficiency virus (HIV), the causitive agent of AIDS, was found to exhibit rapid and extensive genetic diversity. The highly variable and continuously evolving nature of HIV within individuals accounts for the rapid emergence of viral variants resistant to neutralizing antibodies and antiviral drugs. The inaccurate DNA synthesis by virus-encoded enzyme-reverse transcriptase (RT) was implicated as a major factor that contributes to the genetic variability of the virus. Extension of mismatched 3' termini of the primer DNA was shown to be a major determinant of the infidelity of HIV RT. The RT exhibits a variabion in its comperative efficiencies to extend mismatched base pairs. Factors that influence the efficiency of mismatch extension are: the nature of the mispair itself and the template nucleotide sequences. It was important to determine the relative contributions of the interaction of RT with mismatched DNA and whether they affect the extent of extension. The interaction of oligonucleotide DNA with HIV RT was investigated by using a gel retardation assay. First we have studied the DNA-binding properties of HIV-1 RT. The enzyme binds single-stranded DNA as well as double-stranded DNA (both, blunt-ended and recessed). HIV-1 RT binds DNA with no apparent sequence specificity. Therefore, this enzyme belongs to sequence non-specific DNA binding proteins. The formation of the RT-DNA complex was not influenced by the presence of either template-complementary or non-complementary dNTPs, indicating that both polymerization and binding of the RT to the dNTPs do not affect the stability of the complex.Furthermore, HIV-1 RT can interact and form stable complexes with template-primers containing preformed

mismatches. HIV-1 RT binds purine-pyrimidine (e.g. A:C) and purine-purine (e.g. A:A or A:G) mispairs.Hence, HIV-1 RT may refer to as a general mismatch recognition protein. A general mismatch binding activity was found to be sequence independent.Thus, differences in the efficiencies of mismatch extension by the enzyme probably reflects the intrinsic efficiency of nucleotide addition onto a base mispair compared to a correct base pair, instead of weaker binding of the polymerase to DNA containing terminal mismatch.

The gel retardation assay was utilized to examine the effect of various HIV-1 RT inhibitors (i.e. AZT-TP, TIBO and 3,5,8-trihydroxy-4-quinolone) on the enzyme-DNA interaction. The results indicate differences in the mode of action of these compounds. Most effective anti-HIV compounds are inhibitors of HIV RT. Consecuently, the interaction of the enzyme with DNA might constitute a discrete step which can serve as a target for interference by novel specific anti-HIV RT drugs.

# VARIABLE ANTIGENIC BEHAVIOUR OF FOOT-AND-MOUTH DISEASE VIRUS CONTINUOUS EPITOPES DEPENDING ON THEIR POSITION IN A RECOMBINANT CHIMERIC PROTEIN

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A DNA fragment encoding the immunodominant site A of foot-and-mouth disease virus (amino acids 134 to 156 of protein VP1) has been introduced in different regions of the *lacZ* gene. The insertion sites were selected using both structural predictions and comparative analysis of β-galactosidases, to situate the viral peptide at the molecule surface. The resulting chimeric proteins have been produced in *E. coli* under the control of a temperature-inducible system, and some of them are enzymatically inactive. All these recombinant antigens react to different extent with several monoclonal antibodies elicited with the whole virus and directed against different continuous, overlapping epitopes within site A. By competitive ELISA we have detected preferences of each one of these antibodies for specific antigens, resulting in a specific pattern of reactivity for each chimeric protein. This observation provides further evidence to the suggestion that site A presents a number of different conformations in intact virus. Each of the MAbs tested was probably elicited by and recognizes a different conformation of site A in the virus, which is better represented in a particular recombinant antigen. The overall antigenic behaviour of site A seems to be closer in some presentations than in others to that found in the VP1 at the virus surface Therefore, this antigenic resemblance could be modulated by selecting appropriate, surrounding sequences in the carrier proteins. This observation could of great relevance regarding the design and construction of recombinant subunit vaccines against viral diseases.

**SOLID PROTECTION OF DOGS AGAINST CANINE PARVOVIRUS WITH A SYNTHETIC VACCINE**

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Fourteen synthetic peptides corresponding to previously mapped antigenic sites in VP2 of canine parvovirus (CPV) were used for immunization of rabbits to look for antiviral properties favourable for inclusion into a vaccine. Most antipeptide antisera obtained were reactive with viral protein, and with one of them it was possible to locate the hypothetical amino terminus of VP3 within positions 15-31 of VP2. Virus neutralizing antibodies were reproducibly obtained with two overlapping 15-mer peptides corresponding in sequence to the amino terminus of VP2. Antibodies in the neutralizing sera bound most strongly to amino acids of the sequence DGGQPAV within the N-terminus of VP2, indicating that this stretch should be present in a peptide for vaccination against CPV. The two peptides induced long lasting immunity (at least 8 months) either using Freund´s adjuvant or aluminium hydroxide plus Quil A. Thus as a synthetic peptide the amino terminal region of VP2 appeared to be an attractive candidate for eliciting virus neutralizing antibodies in experimental animals.

These findings have led to the development of an effective synthetic vaccine against canine parvovirus , which yields solid protection in dogs when challenged with virulent canine parvovirus and therefore qualifies for application in common practice. Like in deletion vaccines, it is possible to discriminate betweeen vaccinated dogs not yet exposed to the virus and animals that had undergone some viral replication. This is the first example of a synthetic peptide vaccine that induces real protection in target animals.

## EXPRESSION OF IMMUNOGLOBULIN GENES IN THE MAMMARY GLAND

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Transmissible gastroenteritis virus (TGEV) and related coronavirus infect enteric and respiratory mucosal areas. 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.

Transgenic animals secreting TGEV neutralizing monoclonal antibodies (MAbs) in 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 modules from a murine MAb and conserved modules of human origin (gamma 1 and kappa) were constructed using RT-PCR. The chimeric immunoglobulin genes were initially cloned into pING plasmids, under the control of an immunoglobulin enhancer, SV-40 promoter and polyadenilation coding sequences. These plasmids have been used to express the chimeric immunoglobulins in murine myelomas, to test for secretion of TGEV neutralizing MAb. The chimeric immunoglobulin genes have being cloned into different exons of 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 mamamry gland of lactating swine. Transgenic mice are being generated by microinjection of these constructs together with *mar* sequences (matrix attachment region), which flank expression units incresing genetic their transcription.

TRANSFORMING GROWTH FACTOR B (TGF B) MEDIATED MECHANISM OF T CELL IMMUNOSUPPRESSION IN EQUIDE HERPESVIRUS-1 (EHV-1)

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Immunity to EHV-1 in horses is poorly understood. The evidence from natural and experimental studies suggest that T cell responses are more relevant in protection than antibodies. However a precise correlation has not been made and several authors have suggested that responses are complicated by a poorly defined immunosuppression during EHV-1 infection.

T cell responses employing lymphoproliferation (SI) and limiting dilution assay (LDA) in ponies exposed to UV inactivated EHV-1 with a latex adjuvant are currently under investigation. When autologous serum from upto 3 wk. p.i. was employed in T cell proliferation assays it was found to have a immunosuppressive effect. The suppressive factor in the sera of ponies exposed to EHV-1 also inhibited T cell responses to PHA. The T cell suppressive action of sera from ponies exposed to EHV-1 could be nullified with anti-TGF B whereas anti-TNF or anti- interferon failed to restore the suppressive effect. A definition of these immunosuppressive mechanism and the way in which they are induced must be central to the design of vaccines and to an understanding of the pathogenesis of EHV-1.

# Characterization of the IgA and subclass IgG responses to neutralizing epitopes after infection of pregnant sows with the Transmissible gastroenteritis virus or the antigenically related porcine respiratory coronavirus.

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In this study we have investigated the characteristics of secretory IgA (S-IgA) and other classes of immunoglobulins induced after vaccination of sows with the Transmissible gastroenteritis virus (TGEV) and the highly antigenically related Porcine respiratory coronavirus (PRCV). Both viruses induced neutralizing antibodies of different classes secreted in milk, but protected their suckling piglets against TGEV in a different degree. Quantitative differences in the induction of S-IgA by both viruses were found among the different viral antigenic sites and subsites. In TGEV-vaccinated sows, the antigenic subsite A was the best inducer of S-IgA followed by antigenic site D. After vaccination with PRCV, lower levels of S-IgA than in TGEV-vaccinated sows were detected in colostrum and milk, being antigenic site D and subsite Ab the immunodominant sites. This quantitative differences in epitope recognition could justify the differences in newborn piglet protection rates found using immunoglobulin classes purified from milks of sows immunized with both viruses and presenting similar neutralizing activities *in vitro*. Apparently, only S-IgA recognizing at least antigenic sites A and D would confer a good *in vivo* protection, while any immunoglobulin class recognizing only one antigenic site may neutralize the virus in cell culture. This could indicate that the formulation of a subunit vaccine against TGEV has to consider the inclusion of more than one antigenic site involved in virus neutralization.

PLASMID VECTORS FOR THE GENETIC MANIPULATION OF AFRICAN SWINE FEVER VIRUS.

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The demostration that the genome of African swine fever virus (ASFV) can be manipulated by conventional techniques has provided the possibility of applying new strategies for studying different aspects of ASFV biology. In order to facilitate those studies we have constructed and tested a series of plasmid vectors specifically designed for: i) the generation of ASFV recombinants expressing foreign genes; ii) the construction of ASFV deletion mutants; and iii) the inducible expression of either foreign or endogenous genes during the replicative cycle of the virus.

## Dynamics of antigenic variation in viral quasispecies

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The high mutation rates operating during RNA virus replication or retrotranscription dictate that virus populations consist of complex distributions of variants termed quasispecies. In foot-and-mouth disease virus (FMDV) we have previously documented that fluctuations in quasipecies distributions lead to antigenic variation in the absence of immune selection (Díez *et al.* 1989; Domingo *et al.* 1993). We have now expanded these observations to compare in multiply passaged virus populations the frequency of amino acid replacements within FMDV antigenic sites *A*, *C* and *D* (Lea and Hernández *et al.* 1994). Amino acid changes in the absence of immune pressure were fixed at each of the sites examined. However their frequency within site *A* appears to be significantly larger than within site *D*.

Site *A* is located within the highly flexible G-H loop of VP1. In contrast, the discontinuous site *D* occupies an area of contact between VPI, VP2 and VP3 that form the viral capsid. Thus, stronger structural constraints appear to operate at site *D* than at site *A* (Lea and Hernández *et al.* 1994). The results suggest that tolerance to replacements is the most likely mechanism to explain antigenic variation in the absence of immune selection.

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# Construction and Expression of a Single-Chain Antibody Molecule Directed Against the Murine Hepatitis Virus MHV-JHM

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A single-chain Fv fragment directed against the JHM-strain of the Murine Hepatitis Virus was developed and characterized in vitro. The scFv encompasses the light and heavy chain variable regions of the neutralizing antibody "A1" [Wege et al., 1984, Journal of General Virology, 65, 1931-1942]. The variable regions of the antibody were RT-PCR amplified from mRNA isolated from the respective hybridoma cell-line. The PCR products were cloned and sequenced. 8 independent clones were analysed and yielded the consensus of the heavy and light chain sequences displayed in figure 1. The heavy and light chains were then combined into a single chain molecule by overlap PCR. The scFv-clone used for *in vitro* studies was analysed by an in vitro transcription-translation system to verify that the open reading frame of the protein was uninterrupted. The scFv was linked to a peptide-tag containing a 14 amino-acid stretch of the MHV-JHM surface protein that is specifically recognized by the monoclonal antibody 30B [Routledge et al., 1991, Journal of Virology, 65, 254-262]. The scFv coding region was fused in frame to the signal peptide of the human growth hormone and linked to 150bp of the murine metallothionein promoter (containing the CAAT- and TATA-box). Downstream of the scFv stop-codon, a polyadenylation signal of the human growth hormone was inserted. The scFv expression-construct was introduced into feline CFRK and murine DBT cells. Expression was analysed by Western blotting. Unexpectedly the protein could not be detected in the cell culture supernatant of these cells but only in the cytoplasmic protein fraction, suggesting that the signal peptide portion of the scFv was not sufficient for secretion of the molecule from the cell.

Clones of DBT cells expressing the scFv intracellularly are currently being analyzed for their ability to block replication of MHV-JHM in vivo.

## OPTIMIZATION OF CITOPLASMIC INJECTION OF ANTISENSE OLIGONUCLEOTIDES AS A MODEL FOR THE STUDY OF TRANSIENT INHIBITION OF MOUTH-AND-FOOT DISEASE VIRUS.

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The inhibition of the viral life cycles is an important tool for the study of viral life cycles and for the development of treatmets that induce resistance to viral infection. One important aproach is the use of antisense DNA and RNA which is able to target specific steps of the viral activity. The inclusion of the foreing DNA can be achieved by different methods, but microinjection provides some advantages, being less lessive for the cell and allowing the inclusion in the same cell of different compounds of various characteristics, simoultaneously or consecutively. Our aim was to optimize the method of microinjection of DNA in BRL cells and, afterwards, to study the effect of some antisense oligonucleotides (asODNs) in the transient inhibition of mouth-and-foot disease virus.

Three different factors were evaluated in the microinjection protocol, presure of microinjection, the effect of coinjection of FITC-dextran to label the microinjected cells and the toxicity of the DNA concentration. No significant cell inhibition was observed with injection presures between 50 and 200 hPa, FICT-dextran concentrations below 2% and concentrations of oligonucleotides lower than 5$\mu g/\mu l$. Among the 6 antisense oligonucleotides tested, a transient positive inhibition of the VP1 expression with the oligonucleotide complementary to the second AUG region of the Mouth-and-foot desease virus.

## EFFICACY OF LIVE ATTENUATED RECOMBINANTS OF INFLUENZA AND VACCINIA VIRUSES EXPRESSING B AND T-CELL EPITOPES OF THE CIRCUNSPOROZOITE PROTEIN IN INDUCING SPECIFIC CD8+ T CELLS, ANTIBODIES AND PROTECTION AGAINST MALARIA.

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Recombinant live viruses expressing foreign antigens have been used successfully to elicit protective immunity to a variety of pathogens. Based on these findings, it has been suggested that recombinant viruses expressing foreign microbial antigens could serve as vaccines against infectious agents in humans and animals. We recently investigated whether animal viruses expressing a plasmodial antigen could induce protective immunity against the pre-erythrocytic stages of malaria parasites known to be mediated by humoral and T cell mediated mechanisms.

In an earlier study we described the immunogenic properties of a recombinant influenza virus and of a recombinant vaccinia virus, both expressing sequences of a key plasmodial antigen, the circumsporozoite (CS) protein[1]. We found that mice immunized with these recombinant viruses developed a protective immune response directed against sporozoites and liver stages of the rodent malaria parasite Plasmodium yoelii. Priming the mice with the recombinant influenza virus, followed by a booster with the recombinant CS-expressing vaccinia virus, resulted in a CD8+ T cell mediated inhibition of development of the liver stages of the malaria parasites[2]. The sequence of administration of these recombinant viruses to mice was crucial, since protective immunity failed to be generated when the two recombinant viruses were administered in the reverse order, i.e., priming with the recombinant vaccinia virus, followed by exposure to the influenza virus.

In view of these encouraging results, it was of interest to determine the relative effectiveness of several recombinant influenza and vaccinia viruses engineered according to different approaches. We consider the comparison of the efficacy of different constructs to be important, since earlier studies, in other systems, had revealed serious limitations in the efficient expression of foreign epitopes and the induction of the respective immune responses. These limitations were mainly attributed to the restricted availability of sites amenable to the insertion of foreign sequences, and to constraints imposed by residues flanking the antigenic determinant. Recent advances in the genetic manipulation of live carriers, particularly of viruses, have made it feasible to engineer recombinant viruses of diverse degrees of attenuation expressing foreign epitopes in different structural arrangements.

In the present study we present experiments in which sequences corresponding to malaria parasite-derived epitopes were inserted into different sites of influenza viruses, to determine the possible effect of the insertion site on the induction and level of the antimalaria antibody and T cel responses. In addition, we compared the immunogenicity of recombinant vaccinia viruses displaying different degrees of virulence[3,4]. The immune response to these constructs was evaluated, quantitatively

with regard to epitope-specific humoral and CD8+ T cell responses and their capacity to confer protection against malaria.

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## INDUCTION OF MUCOSAL IMMUNITY AGAINST TRANSMISSIBLE GASTROENTERITIS CORONAVIRUS USING VECTORS WITH ENTERIC TROPISM

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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*, and were able to induce TGEV neutralizing antibodies in rabbits. Currently, the potentiality of these vectors to induce lactogenic immunity to TGEV in swine is being tested.

Adenovirus 5 (Ad5) has been used as an eukaryotic vector to express TGEV antigenic determinants which are glycosylation dependent. Several truncated forms of S gene from TGEV coding for the aminoterminus half were cloned into Ad5 and expressed *in vitro*. The recombinant Ad-5-TGEV viruses selected were stable and expressed the recombinant antigen. These recombinants induced both serum and milk neutralizing antibodies in hamsters. The induction of lactogenic immunity against TGEV in swine with the Ad5 recombinant vectors is also under study. The tropism of Ad5 in swine has been analyzed by using a recombinant adenovirus vector carrying the luciferase gene as a reporter gene. This recombinant vector has been inoculated in swine by different routes and both the expression of luciferase and virus titer have been determined in different tissues.

# SPECIFIC INHIBITION OF APHTHOVIRUS INFECTION BY RNAs TRANSCRIBED FROM BOTH 5' AND 3' NONCODING REGION

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RNA molecules containing the 3' terminal region of FMDV RNA both in antisense and sense orientation were able to inhibit viral FMDV translation and infective particle formation in BHK-21 cells, following co-microinjection or co-transfection with infective viral RNA. Antisense, but not sense, transcripts from the 5'NCR including the proximal element of the internal ribosome entry site (IRES) and the two functional initiation AUGs were also inhibitory, both in in vitro translation and in vivo. The inhibitions found were permanent, sequence specific and dose-dependent; an inverse correlation between length of the transcript and extent of the antiviral effect was noticed. In all cases, the extent of inhibition increased when viral RNAs and transcripts were allowed to reaneal before transfection, concomitant with a decrease in the doses required. The antiviral effect was specific of FMDV since trancripts failed to inhibit infective particle formation of other picornavirus as EMCV. These results indicate that the capability of RNA transcripts to inhibit viral multiplication depends on their efficient hybridization with target regions on the viral genome. RNA-5'1as, complementary to the 5'NCR, was able to inhibit in FMDV RNA vitro translation, suggesting that the inhibitions observed are mediated by a blockage of the viral translation initiation. Conversely, hybridization of short sequences both of sense and antisense transcripts from the 3' end induces distortion of predicted highly ordered structural motifs, which could be required for the synthesis of negative stranded viral RNA, and correlates with inhibition of viral propagation.

## Pathogenesis of Mucosal Disease - Characterization of Cytopathic Pestiviruses

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Bovine viral diarrhea virus (BVDV), a small enveloped RNA virus, belongs to the pestiviruses. This genus forms together with the genera flavivirus and hepatitis C virus group the family Flaviviridae. The pestiviral genome consists of a single stranded RNA of positive polarity with a length of about 12.3 kb. The viral RNA is translated into one polyprotein.
Concerning replication in tissue culture, cytopathic (cp) and non cytopathic (noncp) isolates can be differentiated. Antigenetically closely related cp and noncp BVD viruses can be isolated from cattle suffering from fatal mucosal disease (MD) and are called a BVDV pair.
The genomes of several cpBVDV strains contain insertions highly homologous to cellular sequences. For five of these the cellular homologue was shown to be a ubiquitin-coding sequence. The host cell derived inserts are either integrated between formerly neighbouring nucleotides or are flanked by large duplications of viral sequences. The insertions are always located within the genomic region coding for the nonstructural protein p125.
Analyses of additional BVDV pairs revealed that the respective cp strains do not contain insertions of cellular sequences. However, two of such genomes show dramatic rearrangements of viral sequences including duplication of the p80 coding sequence, the C-terminal region of p125. In spite of the genomic variations, all these viruses are replicating autonomously. Only recently, we have characterized a completely novel kind of cytopathic agent, which is an internal deletion type defective interfering BVD particle (DI). While the helper virus alone was noncytopathic, presence of the DI conferred cytopathogenicity. p80, the marker protein of cpBVDV, is expressed from the genome of the DI. Insertions, duplications, rearrangements and deletions of sequences are missing in the genomes of the noncp counterparts even though the genomic sequences from the members of each pair are almost 100% identical. Our data show that cpBVDV develops from noncp BVDV by RNA recombination.
Generation of the cpBVDV strain in an animal persistently infected with a noncp BVD virus is regarded as causative for development of MD. Our results strongly suggest, that there is a linkage between RNA recombination, cytopathogenicity and lethal disease.

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Workshop on

RESISTANCE TO VIRAL INFECTION

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*Texts published in the*
*SERIE UNIVERSITARIA*
*by the*
*FUNDACIÓN JUAN MARCH*
*concerning workshops and courses organized within the*
*Plan for International Meetings on Biology (1989-1991)*

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Organized by V. Conejero and L. C. Van Loon. Lectures by L. C. Van Loon, R. Fraser, J. F. Antoniw, M. Legrand, Y. Ohashi, F. Meins, T. Boller, V. Conejero, C. A. Ryan, D. F. Klessig, J. F. Bol, A. Leyva and F. García-Olmedo.

248 Beato, M.:
**Course on DNA - Protein Interaction.**

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Organizado por Juan F. Santarén. Seminarios por Julio E. Celis, James I. Garrels, Joël Vandekerckhove, Juan F. Santarén y Rosa Assiego.

253 **Workshop on Genome Expression and Pathogenesis of Plant RNA Viruses.**
Organized by F. García-Arenal and P. Palukaitis. Lectures by D. Baulcome, R. N. Beachy, G. Boccardo, J. Bol, G. Bruening, J. Burgyan, J. R. Díaz Ruiz, W. G. Dougherty, F. García-Arenal, W. L. Gerlach, A. L. Haenni, E. M. J. Jaspars, D. L. Nuss, P. Palukaitis, Y. Watanabe and M. Zaitlin.

254 **Advanced Course on Biochemistry and Genetics of Yeast.**
Organized by C. Gancedo, J. M. Gancedo, M. A. Delgado and I. L Calderón.

255 **Workshop on The Reference Points in Evolution.**
Organized by P. Alberch and G. A. Dover. Lectures by P. Alberch, P. Bateson, R. J. Britten, B. C. Clarke, S. Conway Morris, G. A. Dover, G. M. Edelman, R. Flavell, A. Fontdevila, A. García-Bellido, G. L. G. Miklos, C. Milstein, A. Moya, G. B. Müller, G. Oster, M. De Renzi, A. Seilacher, S. Stearns, E. S. Vrba, G. P Wagner, D. B. Wake and A. Wilson.

256 **Workshop on Chromatin Structure and Gene Expression.**
Organized by F. Azorin, M. Beato and A. A. Travers. Lectures by F. Azorin, M. Beato, H. Cedar, R. Chalkley, M. E. A. Churchill, D. Clark, C. Crane-Robinson, J. A. Dabán, S. C. R. Elgin, M. Grunstein, G. L. Hager, W. Hörz, T. Koller, U. K. Laemmli, E. Di Mauro, D. Rhodes, T. J. Richmond, A. Ruiz-Carrillo, R. T. Simpson, A. E. Sippel, J. M. Sogo, F. Thoma, A. A. Travers, J. Workman, O. Wrange and C. Wu.

257 **Lecture Course on Polyamines as modulators of Plant Development.**
Organized by A. W. Galston and A. F. Tiburcio. Lectures by N. Bagni, J. A. Creus, E. B. Dumbroff, H. E. Flores, A. W. Galston, J. Martin-Tanguy, D. Serafini-Fracassini, R. D. Slocum, T. A. Smith and A. F. Tiburcio.

258 **Workshop on Flower Development.**
Organized by H. Saedler, J. P. Beltrán and J. Paz Ares. Lectures by P. Albersheim, J. P. Beltrán, E. Coen, G. W. Haughn, J. Leemans, E. Lifschitz, C. Martin, J. M. Martínez-Zapater, E. M. Meyerowitz, J. Paz-Ares, H. Saedler, C. P. Scutt, H. Sommer, R. D. Thompson and K. Tran Thahn Van.

259 **Workshop on Transcription and Replication of Negative Strand RNA Viruses.**
Organized by D. Kolakofsky and J. Ortín. Lectures by A. K. Banerjee, M. A. Billeter, P. Collins, M. T. Franze-Fernández, A. J. Hay, A. Ishihama, D. Kolakofsky, R. M. Krug, J. A. Melero, S. A. Moyer, J. Ortín, P. Palese, R. G. Paterson, A. Portela, M. Schubert, D. F. Summers, N. Tordo and G. W. Wertz.

260 **Lecture Course Molecular Biology of the Rhizobium-Legume Symbiosis.**
Organized by T. Ruiz-Argüeso. Lectures by T. Bisseling, P. Boistard, J. A. Downie, D. W. Emerich, J. Kijne, J. Olivares, T. Ruiz-Argüeso, F. Sánchez and H. P. Spaink.

261 **Workshop The Regulation of Translation in Animal Virus-Infected Cells.**
Organized by N. Sonenberg and L. Carrasco. Lectures by V. Agol, R. Bablanian, L. Carrasco , M. J. Clemens, E. Ehrenfeld, D. Etchison, R. F. Garry, J. W. B. Hershey, A. G. Hovanessian, R. J. Jackson, M. G. Katze, M. B. Mathews, W. C. Merrick, D. J. Rowlands, P. Sarnow, R. J. Schneider, A. J. Shatkin, N. Sonenberg, H. O. Voorma and E. Wimmer.

263 **Lecture Course on the Polymerase Chain Reaction.**
Organized by M. Perucho and E. Martínez-Salas. Lectures by D. Gelfand, K. Hayashi, H. H. Kazazian, E. Martínez-Salas, M. Mc Clelland, K. B. Mullis, C. Oste, M. Perucho and J. Sninsky.

264 **Workshop on Yeast Transport and Energetics.**
Organized by A. Rodríguez-Navarro and R. Lagunas. Lectures by M. R. Chevallier, A. A. Eddy, Y. Eilam, G. F. Fuhrmann, A. Goffeau, M. Höfer, A. Kotyk, D. Kuschmitz, R. Lagunas, C. Leão, L. A. Okorokov, A. Peña, J. Ramos, A. Rodríguez-Navarro, W. A. Scheffers and J. M. Thevelein

265 **Workshop on Adhesion Receptors in the Immune System.**
Organized by T. A. Springer and F. Sánchez-Madrid. Lectures by S. J. Burakoff, A. L. Corbi-López, C. Figdor, B. Furie, J. C. Gutiérrez-Ramos, A. Hamann, N. Hogg, L. Lasky, R. R. Lobb, J. A. López de Castro, B. Malissen, P. Moingeon, K. Okumura, J. C. Paulson, F. Sánchez-Madrid, S. Shaw, T. A. Springer, T. F. Tedder and A. F. Williams.

266 **Workshop on Innovations on Proteases and their Inhibitors: Fundamental and Applied Aspects.**
Organized by F. X. Avilés. Lectures by T. L. Blundell, W. Bode, P. Carbonero, R. W.Carrell, C. S. Craik, T. E. Creighton, E. W. Davie, L. D. Fricker, H. Fritz, R. Huber, J. Kenny, H. Neurath, A. Puigserver, C. A. Ryan, J. J. Sánchez-Serrano, S. Shaltiel, R. L. Stevens, K. Suzuki, V. Turk, J. Vendrell and K. Wüthrich.

267 **Workshop on Role of Glycosyl-Phosphatidylinositol in Cell Signalling.**
Organized by J. M. Mato and J. Larner. Lectures by M. V. Chao, R. V. Farese, J. E. Felíu, G. N. Gaulton, H. U. Häring, C. Jacquemin, J. Larner, M. G. Low, M. Martín Lomas, J. M. Mato, E. Rodríguez-Boulan, G. Romero, G. Rougon, A. R. Saltiel, P. Strålfors and I. Varela-Nieto.

268 **Workshop on Salt Tolerance in Microorganisms and Plants: Physiological and Molecular Aspects.**
Organized by R. Serrano and J. A. Pintor-

Toro. Lectures by L. Adler, E. Blumwald, V. Conejero, W. Epstein, R. F. Gaber, P. M. Hasegawa, C. F. Higgins, C. J. Lamb, A. Läuchli, U. Lüttge, E. Padan, M. Pagès, U. Pick, J. A. Pintor-Toro, R. S. Quatrano, L. Reinhold, A. Rodríguez-Navarro, R. Serrano and R. G. Wyn Jones.

269 **Workshop on Neural Control of Movement in Vertebrates.**
Organized by R. Baker and J. M. Delgado-García. Lectures by C. Acuña, R. Baker, A. H. Bass, A. Berthoz, A. L. Bianchi, J. R. Bloedel, W. Buño, R. E. Burke, R. Caminiti, G. Cheron, J. M. Delgado-García, E. E. Fetz, R. Gallego, S. Grillner, D. Guitton, S. M. Highstein, F. Mora, F. J. Rubia Vila, Y. Shinoda, M. Steriade and P. L. Strick.

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5 **Workshop on Structure of the Major Histocompatibility complex.**
Organized by A. Arnaiz-Villena and P. Parham. Lectures by A. Arnaiz-Villena, R. E. Bontrop, F. M. Brodsky, R. D. Campbell, E. J. Collins, P. Cresswell, M. Edidin, H. Erlich, L. Flaherty, F. Garrido, R. Germain, T. H. Hansen, G. J. Hämmerling, J. Klein, J. A. López de Castro, A. McMichael, P. Parham, P. Stastny, P. Travers and J. Trowsdale.

6 **Workshop on Behavioural Mechanisms in Evolutionary Perspective.**
Organized by P. Bateson and M. Gomendio. Lectures by J. R. Alberts, G. W. Barlow, P. Bateson, T. R. Birkhead, J. Carranza, C. ten Cate, F. Colmenares, N. B. Davies, R. I. M. Dunbar, J. A. Endler, M. Gomendio, T. Guilford, F. Huntingford, A. Kacelnik, J. Krebs, J. Maynard Smith, A. P. Møller, J. Moreno, G. A. Parker, T. Redondo, D. I. Rubenstein, M. J. Ryan, F. Trillmich and J. C. Wingfield.

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Organized by M. Salas and L. B. Rothman-Denes. Lectures by S. Adhya, H. Bujard, S. Busby, M. J. Chamberlin, R. H. Ebright, M. Espinosa, E. P. Geiduschek, R. L. Gourse, C. A. Gross, S. Kustu, J. Roberts, L. B. Rothman-Denes, M. Salas, R. Schleif, P. Stragier and W. C. Suh.

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9 **Workshop on Control of Gene Expression in Yeast.**
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14 **Workshop on Frontiers of Alzheimer Disease.**
Organized by B. Frangione and J. Ávila. Lectures by J. Avila, K. Beyreuther, D. D. Cunningham, A. Delacourte, B. Frangione, C. Gajdusek, M. Goedert, Y. Ihara, K. Iqbal, K. S. Kosik, C. Milstein, D. L. Price, A. Probst, N. K. Robakis, A. D. Roses, S. S. Sisodia, C. J. Smith, W. G. Turnell and H. M. Wisniewski.

15 **Workshop on Signal Transduction by Growth Factor Receptors with Tyrosine Kinase Activity.**
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16 **Workshop on Intra- and Extra-Cellular Signalling in Hematopoiesis.**
Organized by E. Donnall Thomas and A. Grañena. Lectures by M. A. Brach, D. Cantrell, L. Coulombel, E. Donnall Thomas, M. Hernández-Bronchud, T. Hirano, L. H. Hoefsloot, N. N. Iscove, P. M. Lansdorp, J. J. Nemunaitis, N. A. Nicola, R. J. O'Reilly, D. Orlic, L. S. Park, R. M. Perlmutter, P. J. Quesenberry, R. D. Schreiber, J. W. Singer and B. Torok-Storb.

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**19 Workshop on Viral Evasion of Host Defense Mechanisms.**
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**20 Workshop on Genomic Fingerprinting.**
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**21 Workshop on DNA-Drug Interactions.**
Organized by K. R. Fox and J. Portugal. Lectures by J. B. Chaires, W. A. Denny, R. E. Dickerson, K. R. Fox, F. Gago, T. Garestier, I. H. Goldberg, T. R. Krugh, J. W. Lown, L. A. Marky, S. Neidle, D. J. Patel, J. Portugal, A. Rich, L. P. G. Wakelin, M. J. Waring and C. Zimmer.

**22 Workshop on Molecular Bases of Ion Channel Function.**
Organized by R. W. Aldrich and J. López-Barneo. Lectures by R. W. Aldrich, C. M. Armstrong, P. Ascher, K. G. Beam, F. Bezanilla, S. C. Cannon, A. Castellano, D. Clapham, A. Ferrús, T. Hoshi, A. Konnerth, R. Latorre, J. Lerma, J. López-Barneo, R. MacKinnon, G. Mandel, A. Marty, C. Miller, B. Sakmann, B. Soria, W. Stühmer and W. N. Zagotta.

**23 Workshop on Molecular Biology and Ecology of Gene Transfer and Propagation Promoted by Plasmids.**
Organized by C. M. Thomas, E. M. H. Wellington, M. Espinosa and R. Díaz Orejas. Lectures by J. C. Alonso, F. Baquero, P. A. Battaglia, P. Courvalin, F. de la Cruz, D. K. Chattoraj, E. Díaz, R. Díaz Orejas, M. Espinosa, J. C. Fry, K. Gerdes, D. R. Helinski, B. Hohn, E. Lanka, V. de Lorenzo, S. Molin, K. Nordström, R. W. Pickup, C. M. Thomas, J. D. van Elsas, E. M. H. Wellington and B. Wilkins.

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**25 Workshop on Genetic Recombination and Defective Interfering Particles in RNA Viruses.**
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Organized by J. Downward, E. Santos and D. Martín-Zanca. Lectures by A. Balmain, G. Bollag, J. Downward, J. Erickson, L. A. Feig, F. Giráldez, F. Karim, K. Kornfeld, J. C. Lacal, A. Levitzki, D. R. Lowy, C. J. Marshall, J. Martín-Pérez, D. Martín-Zanca, J. Moscat, A. Pellicer, M. Perucho, E. Santos, J. Settleman, E. J. Taparowsky and M. Yamamoto.

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Becker, J. L. Bos, G. T. Bowden, D. E. Brash, A. Cano, C. J. Conti, G. P. Dotto, N. E. Fusenig, J. L. Jorcano, A. J. P. Klein-Szanto, E. B. Lane, H. Nakazawa, G. Orth, M. Quintanilla, D. R. Roop, J. Schweizer, T. J. Slaga and S. H. Yuspa.

29 **Workshop on the Biochemistry and Regulation of Programmed Cell Death.** Organized by J. A. Cidlowski, H. R. Horvitz, A. López-Rivas and C. Martínez-A. Lectures by J. C. Ameisen, R. Buttyan, J. A. Cidlowski, J. J. Cohen, M. K. L. Collins, T. G. Cotter, A. Eastman, D. R. Green, E. A. Harrington, T. Honjo, H. R. Horvitz, J. T. Isaacs, M. D. Jacobson, P. H. Krammer, A. López-Rivas, C. Martínez-A. S. Nagata, G. Núñez, R. W. Oppenheim, S. Orrenius, J. J. T. Owen, M. C. Raff, L. M. Schwartz and J. Yuan.

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*The lectures summarized in this publication were presented by their authors at a workshop held on the 20th through the 22nd of June, 1994, at the Instituto Juan March.*

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