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Viral neuropathogenesis/Prion disease

Chairpersons: T. Weber (Hamburg, D)
P. Kennedy (Glasgow, UK)
M. Oette (Düsseldorf, D)

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Circadian expression of PrPC in GT1-7 GnRH neuronal cells in culture

E. Nordström,[1] K. Luhr,[1] A. Taraboulos,[2] K. Kristensson[1]
1. Karolinska Institute (Stockholm, SE)
2. Hadassah (Jerusalem, IL)

The normal prion protein has been suggested to be involved in regulation of circadian rhythm, since PrPC knock-out mice show a disruption of both circadian activity rhythms and sleep patterns. Here we have used a cell culture system to study this hypothesis. Many clock-related transcripts have a circadian expression not only in the suprachiasmatic nuclei of the anterior hypothalamus, but also in other tissues. The treatment of certain cultured cells with high concentrations of serum induces a circadian expression of various genes whose transcription also oscillates *in vivo*. It has previously been shown that clock genes oscillate in GT1-7 GnRH neuronal cells (Chappell *et al.* Society of Neuroscience, 31st Annual Meeting, San Diego, 2001, 466.6). To investigate whether PrPC has a circadian expression in GT1-7 cells, total RNA was isolated every 4th h for 48 h from GT1-7 cells, which were pretreated with serum-rich medium for 2 h. RT-PCR analysis revealed that PrPC oscillate with a distinct expression pattern in GT1-7 cells. Recent studies suggest a circadian expression of PrPC in the rat brain and GT1-7 cells may serve as a useful model system to investigate the mechanism by which PrPC is involved in regulation of circadian rhythms in mammals.

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Clinical and genetic features of human transmissible spongiform encephalopaties in Catalonia

R. Sanchez-Valle,[1] C. Nos,[2] J. Yagüe,[1] T. Ribalta,[1] I. Ferrer,[3] F. Graus,[1] A. Dominguez,[2] A. Saiz[1]
1. CJD Unit-hospital clinic,
2. General Directorate of Public Health,
3. CSUB
on behalf of the Catalan Study Group for CJD (Barcelona, ES)

Objectives: To describe the clinical and genetic characteristics of Transmissible Spongiform Encephalopathies (TSEs) in Catalonia (an autonomous community of Spain, 6 million population).

Patients/Methods: We studied the 63 definite or probable TSEs patients dead from January 1993 to June 2001 in the context of a surveillance system.

Results: Fifty-five (87%) cases were sporadic Creutzfeld-Jakob disease (CJD) (34 definite and 21 probable CJD). Thirty-two were women and 23 men, and the median age of onset was 64 years (44–85). The presenting syndrome, when registered, was dementia in 20 patients, ataxia in 11 and Heideinhain syndrome in 4. The mean duration of the disease was 6 months (1–24). The 14-3-3 assay was positive in 95% cases and the EEG in 65%. Only in 7 cases (15%) the MRI was informed as typical. Thirty-three sCJD were studied for codon 129 polymorphism: 70% were methionine/methionine (M/M), 18% valine/valine (V/V), 12% M/V. Five out of 6 V/V cases did not present a typical EEG and the disease lasted more than 21 months in two. Seven cases (11%) were genetic: 3 fatal familial insomnia (D178N-M), 4 familial CJD(fCJD) associated to E200K. Three fCJD lacked familiar history of disease and 2 presented as status epilepticus. The only iatrogenic case was related to a dura mater graft. No case of variant CJD was identified.

Conclusions: The TSEs in Catalonia present similar characteristics to those reported in other european populations. Atypical features are seen more frequently in sporadic 129 V/V CJD and genetic cases.

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Biophysical studies on aggregation intermediates and fibril formation of the prion protein *in vitro*

K.W. Leffers, K. Jansen, L. Nagel-Steger, S. Metzger, T. Kaimann, E. Birkmann, H. Wille, D. Riesner
University of Düsseldorf (Düsseldorf, D)

The conversion of the soluble, alpha-helical, protease sensitive and not infectious form of the prion protein, PrP^C, into an insoluble, protease resistant, predominantly beta-sheeted, and infectious form, PrP^Sc, plays a key role in prion disease. We developed a system for simulating the involved structural transition *in vitro*: PrP 27–30 from hamster brain of the same amino acid sequence can be solubilized by sonication in low concentrations (0.2%) of the anionic detergent SDS. Under these conditions, the solubilized PrP (solPrP27–30) is monomeric and has a mainly a-helical structure. Removal of the SDS by mere dilution leads to a structural transition to a form rich in beta-sheet, accompanied by multimerization and an increased PK resistance. This system allows the detailed *in vitro* study of some of the features that are important in the conversion from PrP^C into PrP^Sc.

In order to separate the structural transition from the oligomerization process, equilibrium properties of recombinant PrP at intermediate SDS concentrations were analyzed.

Two different intermediate states were found and characterised with biochemical and biophysical methods: i) an a-helical PrP dimer, demonstrating the tendency of the molecule for self-interaction even in the alpha-helical state. ii) An oligomeric beta-sheeted structure as an intermediate on the pathway to larger aggregates. It shows that beta-sheet formation is unavoidably related to intermolecular interactions.

Attempts were carried out to refold and reaggregate sol PrP 27–30 by dilution of SDS down to 0.01% and long-time incubation up to six weeks at 37°C. In contrast to former, i.e. shorter incubation times, amyloidic fibres could be detected by electron microscopy besides the less structured aggregates described before.

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Non-proteinacious components of prions

C. Dumpitak, N. Weinmann, K.W. Leffers, E. Birkmann, D. Riesner
University of Düsseldorf (Düsseldorf, D)

Prions are the agents of transmissible spongiform encelopathies, composed of Prion-Protein (PrP) mainly. To understand their properties, knowledge about secondary components of prions is essential. Especially since no *in vitro* reconstitution of infectivity could be shown by today. Our research group is involved in studies about non-proteinacious components of prions. Nucleic acids longer than 80 nucleotides can be excluded as essential component of infectivity. (1) Small amounts of host sphingolipids are found to represent the environment during prion aggregation, but most probably not being necessary for prion infectivity as well. (2) An inert polysaccharide scaffold is found in prion rods (PrP 27–30) after intense PK-digestion. This scaffold consists of predominantly 1,4-linked glucose and makes up 5–15% of prion rods (3). To exclude the possibility of the scaffold being accumulated during the preparation of PrP 27–30, different prion preparations were analyzed. In all of them composition and methylation analysis showed glucose as dominant sugar component, predominantly 1,4-linked. The results give strong indication for the polysaccharide scaffold being a common secondary component of prions. Furthermore the influence of different saccharides on the prion protein aggregation was studied. While utilizing an *in vitro* transition system, in which PrP is soluble at medium Sodiumdodecylsulfate (SDS) concentrations and reaggregates at low SDS concentrations (4), two effects were observed: Under non-equilibrium conditions some of the saccharides accelerated PrP-aggregation. The same saccharides induced under equilibrium conditions, i.e. intermediate SDS concentrations, the transition to the aggregated structure rich in beta-sheet. Additional experiments concerning the PK-resistance and ultrastructure of the resulting PrP/saccharide-coaggregates were carried out.

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TRECs in the GRIV cohort of HIV-1 infected long-term non-progressors, and in the SHIV 89.6P macaque model of HIV-1 infection

M.W. Richardson,[1] A.E. Sverstiuk,[1] P. Silvera,[2] J. Greenhouse,[2] E.G. Gracely,[3] H. Hendel,[4] K. Khalili,[1] J.-F. Zagury,[4] M.G. Lewis,[2] J. Rappaport[1]
1. Temple University (Philadelphia, USA)
2. Southern Research Institute (Frederick, USA)
3. MCP-Hahnemann University (Philadelphia, USA)
4. Universite Pierre et Marie Curie (Paris, FR)

Objectives: Our main objective was to evaluate T-cell receptor excision circles (TRECs) in HIV-1 seropositive long-term non-progressors (NP) naíve to anti-retroviral therapy, and determine the value of TREC measurements in predicting maintenance of non-progression status. In addition, we wanted to evaluate TRECs in PBMC of rhesus macaques before and after viral challenge in the SHIV 89.6P model of HIV-1 infection.

Methods: TRECs in PBMC were evaluated at a single initial timepoint in the HIV-1 seropositive GRIV cohort of non-progressors (NP) and fast progressors (FP). Based on information obtained during a short follow-up period (median = 20 months), NP were sub-grouped as those exhibiting continued clinical stability (NP-NP), and those with signs of disease progression (NP-P). TREC measurements obtained prior to follow-up were compared in the NP sub-groups, and the predictive value of higher TRECs for maintenance of non-progression status was evaluated. TRECs in PMBC of rhesus macaques were quantitated pre and post intravenous 89.6P SHIV viral challenge.

Results: TRECs were lower in both non-progressors (NP) and fast-progressors (FP) compared to seronegative controls ($p < 0.0005$). TRECs were higher in NP who remained stable during follow-up (NP-NP), compared to both unstable NP (NP-P; $p = 0.002$) and FP ($p = 0.025$). Higher initial TRECs (>634 mol/mg) were 100% predictive of maintenance of non-progression status during subsequent follow-up. However, lower TRECs were only 25.5% predictive of progression, at least during the relatively short follow-up period. In rhesus macaques, TRECs were significantly lower following viral challenge in the SHIV 89.6P model.

Conclusions: Higher TREC levels are predictive of maintenance of long-term non-progression status. TRECs may be useful as a surrogate marker for the likelihood of continued clinical stability. Given the similar decrease in TRECs in SHIV 89.6P infected macaques compared to HIV-1 infected humans, TRECs may also be a useful surrogate marker for vaccine studies in SHIV models of HIV-1 infection.

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Functional interaction between JC virus late regulatory agnoprotein and cellular Y-box binding transcription factor, YB-1

M. Safak, B. Sadowska, R. Barrucco, K. Khalili
Temple University (Philadelphia, USA)

The human polyomavirus, JC virus (JCV) is a small DNA virus with a double-stranded circular genome and is a causative agent of Progressive Multifocal Leukoencephalopathy (PML). Although significant progress has been made in understanding the regulation of JCV gene transcription, the mechanism(s) underlying the viral lytic cycle

remains largely unknown. We have recently reported that the JCV late auxiliary agnoprotein may have a regulatory role in JCV gene transcription and replication. Here, we investigated its regulatory function in viral gene transcription through its physical and functional interaction with YB-1, a cellular transcription factor that contributes to JCV gene expression in glial cells. Time course studies revealed that agnoprotein is first detected at day three post-infection and its level increased during the late stage of the infection cycle. Agnoprotein is mainly localized to the cytoplasmic compartment of the infected cell with high concentrations found in the perinuclear region. While the position of agnoprotein throughout the infection cycle remained relatively unaltered, the subcellular distribution of YB-1 changed between cytoplasm and nucleus. Results from co-immunoprecipitation and GST pull-down experiments revealed that agnoprotein physically interacts with YB-1 and the amino terminal region of agnoprotein, between residues 1–36, is critical for this association. Further investigation of this interaction by functional assays demonstrated that agnoprotein negatively regulates YB-1 mediated gene transcription and the region corresponding to residues 1–36 of agnoprotein is important for the observed regulatory event. Taken together, these data demonstrate that the interaction of the viral late regulatory agnoprotein and the cellular Y-box binding factor, YB-1 modulates transcriptional activity of JCV promoters.

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Human polyomavirus JC control region variants in the cerebrospinal fluid of persistently infected individuals

C. Hindinger, K. Dörries
Julius-Maximilians-Universität (Würzburg, D)

A possible role for JCV rearrangements of the transcriptional control region (TCR) in the pathogenesis of progressive multifocal leukoencephalopathy (PML) was investigated by analysis of the nucleotide sequence and TCR structure of JCV DNA in cerebrospinal fluid (CSF) of persistently infected individuals. JCV-specific DNA sequences in the CSF of 4 individuals without polyomavirus associated disease were identified by PCR followed by Southern blotting. Further analysis of the viral DNA population and distribution of TCR subtypes was performed by PCR amplification of the JCV non-coding region followed by cloning and DNA sequencing. Whereas heterogeneous TCR populations with two, three and five different JCV variants, respectively, were detected in 3 individuals, the last carried a homogeneous JCV TCR population consisting of a new subtype, JCV-W8. Altogether, six different JCV subtypes were identified consisting of archetype, Type I, and Type II TCRs with prominent promoter rearrangements. Of all TCRs analyzed, the major subtype was JCV-W8, representing a PML-like variant of Type II. In detail, Type I TCRs were represented by JCV-W1, identical to the American prototype JCV-Mad1. As Type II JCV- GS/B, the Middle European prototype, the new type JCV-W8 and another new variant, JCV-W9, were identified. Single segment TCRs were JCV-W4 and the archetype GS/K. The results confirm earlier findings that rearrangements of the JCV TCR can be associated with the persistent state of infection. The findings suggest that a number of different JCV subtypes circulate among the human population and are not generated anew in each individual host during persistence of the virus. In addition, it appears unlikely that entry of virus into the central nervous system and existence of rearranged TCR sequences are the main factors of PML induction.

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Detection of human herpesvirus 6 (HHV-6) in human central nervous system tissue

D. Donati, C. Cermelli, A. Fogdell-Hahn, N. Akhyani, A. Vortmeyer, J. Heiss, W. Gaillard, W. Theodore, S. Jacobson
NINDS/NIH (Bethesda, USA)

Human herpesvirus 6 (HHV-6) is an ubiquitous virus acquired early in childhood and known to cause exanthem subitum. The virus can infect a wide spectrum of cells and has distinctive neurotropism. Several studies have reported HHV-6 in association with some CNS diseases such as encephalitis and multiple sclerosis (MS).

The present study is aimed to detect and characterize HHV-6 in brain tissue samples obtained during surgery to help in defining the range of viral tissue distribution. Quantitative PCR methodologies together with the immunohistochemical localization of virus will help in assessing the magnitude and extent of infection. In addition, primary explants of fresh brain biopsy material have been established and characterized to determine if HHV-6 can be detected in these cells. Preliminary results will be presented from fresh tissue samples and formalin-fixed paraffin embedded tissues obtained from patients with a variety of different CNS diseases including brain tumors, cavernous angiomas, epilepsy, etc. Cell lines phenotypically and morphologically characterized as astrocytes and oligodendrocytes have been established from which HHV-6 has been shown to be present. In addition, PCR analysis and immunohistochemical staining with HHV-6 specific monoclonal antibodies have detected this virus in a subset of brain specimens. Our preliminary results are consistent with previous observations that HHV-6 is present in human brain. Due to the ubiquity of HHV-6, it is tempting to hypothesize that in some susceptible individuals the presence of this virus as a commensal brain pathogen may contribute to development of some brain disorders.

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AP-1 family members, c-Jun and c-Fos, regulate transcription from JC virus promoters in glial cells

B. Sadowska, R. Barrucco, S. Radhakrishnan, J. Kim, K. Khalili, M. Safak
Temple University (Philadelphia, USA)

Progressive multifocal leukoencephalopathy (PML) is a subacute demyelinating disease of the central nervous system (CNS) that is observed primarily in patients with immunocompromised conditions and caused by the human polyomavirus, JC virus (JCV). The regulatory region of JCV contains several distinct DNA elements that serve as binding sites for a number of cellular transcription factors including activating transcription factor-1 (AP-1). Some of these factors have been characterized in detail for their role in regulation of JCV gene transcription and replication. In this respect, however, AP-1 family of transcription factors was poorly studied. AP-1 consists of a large family of

transcription factors and implicated in many important cellular and viral processes including cell proliferation, tumorigenesis and regulation of many viral genes; and is induced by a variety of exracellular stimuli including viral infection. Here, we investigated the role of AP-1 family members, c-Jun and c-Fos, in regulation of JCV gene transcription and replication. Our cotransfection assays showed that while c-Jun and c-Fos synergistically activate JCV gene transcription, they both suppress T-antigen mediated activation of JCV late promoter and that of the viral DNA replication. DNA binding studies demonstrated the specific interaction of c-Jun with its DNA binding site from JCV regulatory region. Further, our observations from transfection assays also indicated that JCV AP-1 binding site is sufficient to mediate c-Jun/c-Fos induced activation when transposed to a heterologous promoter. Moreover, analysis of c-Jun expression in infected cells by Western blotting indicated that c-Jun is posttranslationally modified by phosphorylation and its protein level is substantially upregulated during the late phases of viral infection cycle. Altogether, our findings indicate that AP-1 family members appear to be involved in regulation of JCV transcription and replication, and thereby may play a role in JCV-induced progression of PML.

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Transcriptional regulation of the human cdk9 and cyclin T1 genes by Sp1 and p53 in human astrocytes

H. Kim, E. Kheterpal, K. Khalili, S. Amini, B.E. Sawaya
Temple University (Philadelphia, USA)

The positive transcription elongation factor b (P-TEFb) controls transition from abortive into productive elongation through phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II. This p-TEFb complex was identified as a cyclin-dependent kinase (cdk9) paired with a cyclin subunit (cyclin T). The HIV-1 Tat protein regulates transcription elongation through binding to the viral TAR RNA stem-loop structure and through its interaction to Cyclin T1 and cdk9. In this study we investigated transcription and regulation of cdk9 promoter by a variety of cellular genes in astrocytic cells. Results from deletion and selected mutations suggest that multiple cis elements in both the proximal and distal regions of the promoter are important for the regulation of cdk9 gene transcription. The sequence spanning nucleotides −219 to −1 contain all the transcriptional regulatory elements needed for full promoter activity in transient expression assays. Using human cdk9 deletions mutant constructs, we determined that the GC-rich region at (−156/−136 bp) is sufficient for the basal promoter activity. Further, the proximal region encompassing nt. −64 to −1 was shown to be necessary for basal cdk9 gene activity by the tumor suppressor protein p53. Our binding studies demonstrated that Sp1 and p53 proteins are able to directly associate with cdk9 promoter. These data strongly suggest that the regulation of human cdk9 gene expression is a combinatorial process and involves multiple cis-acting regulatory sites.

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Interaction between TGFbeta signaling proteins and C/EBP controls basal and Tat-mediated transcription of HIV-1 LTR in astrocytes

T. Sweet,[1] J. Coyle-Rink,[1] S. Abraham,[1] B.E. Sawaya,[1] O. Batuman,[2] K. Khalili,[1] S. Amini[1]
1. Temple University (Philadelphia, USA)
2. SUNY (Brooklyn, USA)

Signal transduction pathways induced by cytokines can modulate the level of HIV-1 gene transcription and replication in a variety of cells including those from the central nervous system. Here, we investigated the effect of TGFb-1 signaling factors, including Smads, on transcription of the viral LTR in human astrocytic cells. Ectopic expression of Smad-3 increased activity of the viral promoter, while its partner protein, Smad-4, caused a slight decrease in viral gene transcription. Further, Smad-4 was able to suppress transcriptional activation of the LTR by Smad-3 as well as by C/EBPb, another activator of LTR transcription in these cells. Results from promoter deletion experiments identified the C/EBP binding site, which is positioned between nucleotides −114 to −102 as one of the targets for Smad-mediated regulation of the LTR. Band-shift studies showed inhibition of C/EBP binding to its target DNA in protein extract from cells overexpressing Smad-3 and Smad-4. Results from GST pull-down assay and combined immunoprecipitation/Western blot of protein extracts from human astrocytes verified association of Smad-3 and Smad-4 with C/EBPb, suggesting that interaction of C/EBPb with Smad-3 and Smad-4 may have a negative impact upon C/EBPb mediated activation of the LTR. Interestingly, Smad-4 showed no inhibitory effect on viral gene transcription in cells expressing Tat protein. However, in the presence of Smad-3, expression of Smad-4 exerted a negative effect on Tat-mediated activation of the LTR promoter. These observations pointed to the functional interplay between viral and cellular proteins in modulating LTR transcription.

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JCV DNA detection in cerebrospinal fluid: sequencing and analysis of rearrangements

V. Pietropaolo, M. Videtta, D. Fioriti, M. Mischitelli, N. Orsi, A.M. Degener
University of Rome (Rome, IT)

The human polyomavirus JC (JCV) infection is endemic in the world population and the infection rate by adulthood is greater than 70%. The virus reaches its target organs by the haematogenous route and becomes latent in kidney and brain where it can reactivate and replicate under conditions of immunological impairment. Although the primary infection and reactivation are asymptomatic, in immunocompromised patients JCV can cause a fatal demyelinating brain disease, the progressive multifocal leukoencephalopathy (PML). Once rare, PML is now an important complication in the HIV-1 associated CNS diseases. At present the amplification of viral DNA is used for PML diagnosis; in fact the PCR technique is used to search for JCV DNA in cerebrospinal fluid (CSF) drawn by lumbar puncture. The JCV non-coding control region (NCCR) is the part of the genome that shows the highest variability in the different isolates. Moreover this region carries signal sequences for viral interaction with cellular and viral factors directing virus-specific

RNA transcription and may play an important role in the viral pathogenesis. In our study, the presence of JCV genome was searched for by means of a specific and sensitive nested-PCR for the NCCR in CSF samples collected from HIV-1 positive and negative subjects (with or without PML).

The PCR fragments were sequenced and analysed comparing the JCV NCCR structure found in CSF with those collected in the Data Bank.

The archetypal NCCR can be divided into six regions which are consistently retained, duplicated or deleted in JCV genomes found in our patients. In particular, in HIV-positive and negative subjects without PML, the structure of the NCCR showed deletions or duplications by comparison with the archetype structure, whereas the sequences found in CSF of patients with PML show specific and characteristic rearrangements.

Our results suggest the presence of preferred target sequences for rearrangements that are generated in the host from a basic archetypal sequence during the infection.

During primary infection or reactivation new variants with higher brain tropism may be selected, inducing the development of PML.

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Differential susceptibility in sensory cultured neurons to rabies virus infection

M. Martínez-Gutiérrez, J.-E. Castellanos-Parra, M.-L. Velandia, N. Quiroga
Instituto Nacional de Salud (Bogotá, CO)

To determine if adult mouse dorsal root ganglia neuron subpopulations are differentially infected with rabies virus (RV), infected cultures were processed using a double immunodetection technique for virus and some neuron subpopulations or putative viral receptor markers. Calcitonine Gene Related Peptide (CGRP), Galanin (GAL), Substance P (SP), Neuropeptide Y (NPY) and Vasoactive Intestinal Peptide (VIP) were used as subpopulation markers. Double immunedetection for virus, low affinity neurotrophin receptor (p75NTR), Neural Cell Adhesion Molecule (NCAM) or nicotinic acetylcholine receptor (NAChR) alpha 4 subunit, was also carried out, as there is evidence of their participation as receptors for VR. The majority of neurons present in culture have small diameter ($<20\ \mu$m) but, in spite of this, more than 70% of the infected neurons have intermediate and large diameter ($>21\ \mu$m), which shows high susceptibility to infection of large neurons. A high association was found ($p < 0,05$) between infected neurons and large neuron marker expression (NPY, VIP, SP). In spite of most neurons in culture expressing the three putative rabies virus receptors, association was only found between infected neurons and p75NTR and alpha 4 subunit expression. Neuron subpopulations differential susceptibility could have in vivo implications, because the virus might be using large neurons from muscle to be transported to the central nervous system following a rabid incident. On the other hand, association with neurons bearing viral receptors provides additional evidence of p75NTR and NAChR participation in infection by rabies virus in the Peripheral Nervous System, but also it is rather doubtful NCAM participation in this sensory neuron model.

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Genetic identification of factors controlling JCV tropism

G.V. Gee, W.J. Atwood
Brown University (Providence, USA)

JC virus is a common human polyomavirus that infects 70% of the population worldwide. In immunosuppressed individuals, JCV causes a lytic infection of oligodendrocytes, resulting in the fatal, demyelinating disease PML. JCV has a cell tropism that is restricted to oligodendrocytes, astrocytes and B lymphocytes. The reason for this restricted tropism is not understood, but may be due to the presence of cell type specific factors such as a receptor or transcription factors. Our research with JCV involves the investigation of host factors that determine the cell specific tropism of JCV. Our current strategy to identify the factors necessary for JCV infection is to screen non-permissive cells with a commercially available human brain cDNA library in order to restore JCV susceptibility to these cells. One such non-permissive cell line being screened is the HeLa cell line. Since these cells are restricted at the level of viral entry, this approach may allow us to identify a receptor or co-receptor that is not normally expressed in non-permissive cells. A second approach is to develop resistant subclones using the permissive parental cell line, SVG. Multiple resistant subclones have been established of which SVGR2 is the best characterized. These cells appear to be resistant to JCV at a point following virus entry, most likely at the level of viral transcription. The SVGR2 cell line also has the added benefit of being more closely related to a permissive cell line, so it is more likely that only one factor is altered in the resistant clone. To improve the odds of isolating a clone, I am separating the library into testable pools. We are currently subdividing this library into testable pools in order to isolate a single positive clone. Once the clone is identified, we can determine if its presence is indeed necessary to support JCV replication.

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Effect of nicotinic agonists chronic exposure on adult mouse normal and rabies virus infected spinal ganglia culture

M. Martínez-Gutiérrez, M.-A. Benito, J.-E. Castellanos-Parra, C.-R. Ramírez, M. Avellaneda, E. Cepeda, H. Hurtado
Lab. Neurociencias. Inst. Nac. Salud (Bogotá, CO)

To determine the effect of some nicotinic agonists' chronic exposure on adult mouse normal spinal ganglia culture and on the same infected with rabies virus, such cultures were treated with Carbachol and Acetylcholine (0.1, 1 and 10 mM) and Nicotine (0.1, 1 and 2 mM). Non-infected cultures were processed for neurofilament to quantify the neurite networks and soma diameter present in them as regeneration and survival parameters. Infected neuron number and size were evaluated by immunoperoxidase technique using morphometric analysis. Drugs used produced partial inhibition of neuron infection. Image analysis also showed that Acetylcholine (1 and 10 mM), Carbachol (10 mM) and Nicotine (1 mM) caused changes in infected neurone distribution pattern, inhibiting infection in specific neuron subpopulations. On the other hand, the highest concentrations of drugs produced a decrease in the regenerated neurite area (also affecting neuron survival). This could partially explain the

infection's inhibitory effect, due to the lesser quantity of neurite networks for virus capture as well as the death of those sub-populations most susceptible to infection. The drugs showed a toxic effect on cultures for some of the concentrations and exposure times used. This work strengthens the hypothesis of nicotinic receptor participation as receptor for the rabies virus and also presents evidence of the effect of certain nicotinic agonists on specific neuronal subpopulations, be they modifying neurone survival or affecting distribution of those neurones which are infected by rabies virus.

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Distribution of the alpha (2-6)-linked sialic acid component of JCV receptor in normal human tissues

S. Dimitrova, W. Atwood
Brown University (Providence, USA)

JC Virus (JCV), a member of the polyomavirus family, causes a demyelinating disease of the central nervous system (CNS) known as progressive multifocal leukoencephalopathy (PML) in humans. Although the oligodendrocyte is the principal target cell of JCV infection and replication in PML patients, little is known regarding the site of JCV latency and the mechanisms by which the virus spreads to the CNS to cause disease. Previous studies in our lab have demonstrated that alpha (2-6)-linked sialic acid is a critical component of JCV receptor. The tissue specific distribution of the alpha (2-6) linkage of sialic acid is regulated by the expression of alpha (2-6) sialyltransferase. Using immunohistochemical analysis we are currently examining the binding pattern of biotinylated JCV and the alpha (2-6) sialic acid specific lectin, Sambucus nigra (SNA) to a panel of normal frozen human tissues. Cell type specific markers are also being used to define the cell type(s) to which virus and lectin bind.

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Coreceptor use of simian-immunodeficiency viruses isolated from the brains of macaques with neuroAIDS

E. Stephens, D. Singh, E. Pacyniak, D. Griffin
University of Kansas Medical Center (Kansas City, USA)

While the simian immunodeficiency virus infection of macaques (SIVmac) has provided valuable information about the neuropathogenesis of HIV-1 in humans, the recent development of chimeric simian-human immunodeficiency viruses containing the env of HIV-1 has provided a new nonhuman primate model of HIV-1 neurological disease. By expressing the envelope glycoprotein of HIV-1 (gp160), the SHIV system provides means to analyze the evolution of coreceptor use of an HIV-1 glycoprotein in macaque model. In this study, we investigated the sequence variability and co-receptor usage of viruses isolated from the CNS of macaques that developed neuroAIDS following inoculation with pathogenic simian-human immunodeficiency viruses (SHIV). Macaques were inoculated with CXCR4 utilizing SHIV viruses, developed severe CD4+ T cell loss within one month of inoculation and were euthanized at 20 weeks to 1.5 years post-inoculation. Env sequences were amplified from different regions of the CNS, viruses constructed with the amplified virus sequences and analyzed for coreceptor usage. Our results indicate that the viruses constructed with env genes isolated from different regions of the CNS maintained their use of the CXCR4 coreceptor. These results indicate that viruses that utilize CXCR4 are capable of invading the CNS and can maintain their CXCR4 coreceptor usage while causing encephalitis in macaques. This study further validates the use of the SHIV/macaque model of HIV-1 neuropathogenesis.

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PET imaging of HSV-1 amplicon vector-mediated gene expression

A.H. Jacobs, A. Winkeler, C. Dittmar, M. Hartung, G. Garlip, C. Kummer, S. Vollmar, R. Graf, K. Wienhard, W.D. Heiss
MPI for Neurological Research (Cologne, D)

Objective: To non-invasively assess HSV-1 amplicon vector-mediated gene expression *in vivo* by positron emission tomography (PET). Background: PET of herpes simplex virus type 1 thymidine kinase (HSV-1-tk) gene expression (1-4) mediated by replication-conditional HSV-1 vectors reveals TK-expression only in viable target tissue not yet been destroyed by vector replication (3).

Methods: To non-invasively assess the 'total' dose of HSV-1 vector-mediated TK-expression, non-toxic helper virus-free HSV-1 amplicon vectors were constructed carrying multi-functional imaging genes (5). 24 to 48 hours after HSV-1 amplicon vectors (2×10^7 t.u.) had been injected into s.c. human Gli36dEGFR gliomas in nude rats (n = 2) and nude mice (n = 4), the levels and locations of HSV-1-TK expression were assessed after i.v. administration of [18F]-FHBG (300 μCi/rat; 100 μCi/mouse) using new generation ECAT HRRT (Siemens, CTI) and microPET scanners.

Results: Various levels of HSV-1-tk gene expression ([18F]-FHBG % ID/g) could be assessed by FHBG-PET mostly located around injection sites. The highest levels of TK-expression were observed for tkgfp fusion (TG17; gfp green fluorescent protein gene) and tkIRESgfp (TIG; IRES internal ribosome entry site) constructs. In comparison to stabily expressing TG17-gliomas (positive control), the levels of FHBG-accumulation were comparatively low. No significant FHBG-accumulation was observed in non-transduced gliomas. Conclusions: HSV-1 amplicon vector-mediated gene expression can be monitored non-invasively by PET and subtle differences of HSV-1-TK expression can be differentiated by high-resolution PET imaging. With a therapeutic gene proportionally coexpressed with tk as PET marker gene, TK-imaging indirectly reveals the level and location of the 'total tissue dose' of any therapeutic gene expression mediated by helper virus-free HSV-1 amplicon vectors.

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JC virus (JCV) VP1 as tools for gene transfer and identification of cell surface molecules recognized by VP1

H. Iwata, C. Henmi, M. Satoh, S. Tanaka, H. Sawa, K. Nagashima
Hokkaido University School of Medicine (Sapporo, JP)

VP1 encoded by the late region of JC virus (JCV) is a major capsid protein forming the outer surface of the virion. Recombinant VP1 can form a virus-like particle (VLP) which can package exogenous DNA and enter the cells which originate from various tissues, suggesting that the VLP is a suitable vehicle of gene transfer. In this study, we have investigated the optimal conditions for DNA packaging into VLP and the transgene efficiency of VLP into various cells. JCV VLP was synthesized in E. coli and its morphology was confirmed by the negative staining method using electron microscopy. The VLP was recognized as 40 nm diameter particles at a density of 1.29 g/ml. In our study, the dissociation-reassociation cycle method had higher efficiency for packaging compared with the osmotic shock method. With this method, the VLP could package the plasmid at the maximum length of 4.7 kb. The molar ratio of exogenous gene to VLP was optimal at 1 to 5. To determine the efficiency of transfection of exogenous DNA, we have constructed JCV VLP packaging the plasmid encoding the marker gene, green fluorescence protein (GFP). The VLP harboring GFP plasmid had similar transgene efficiency as the lipofection method into human cells. Thus, JCV VLP seemed to be applicable for gene therapy. Next, in order to identify the target cell surface proteins recognized by JCV VP1, JCV VLP was applied into the ELISA assay. Initially, the neutralizing antibodies against JCV were made by immunization of the membrane fraction of human neuroblastoma cell to mice. The membrane fraction of the used cell was coated onto 96-well plate and the fluorescence intensity was measured after incubation of FITC-labeled JCV VLP which was pre-incubated with the acquired antibodies. Using this ELISA system, the antibodies which inhibit attachment of JCV VLP to membranous proteins were obtained, and the further limiting of the antibodies against the target molecules is performed for identification of cell surface proteins recognized by JCV VP1.

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High-avidity human serum antibodies recognizing linear epitopes of Borna disease virus proteins

C. Billich,[1] C. Sauder,[1] R. Frank,[2] S. Herzog,[3] K. Bechter,[4] K. Takahashi,[5] H. Peters,[6] P. Staeheli,[1] M. Schwemmle[1]
1. University of Freiburg (Freiburg, D)
2. GBF (Braunschweig, D)
3. University of Giessen (Giessen, D)
4. University of Ulm (Günzburg, D)
5. Fukushima Medical University (Fukushima, JP)
6. Dade Behring (Marburg, D)

The recent observation that Borna disease virus (BDV)-reactive antibodies from psychiatric patients exhibit only low avidity for BDV antigen questioned their diagnostic value and raised the possibility that antigenically related microorganisms or self antigens caused the production of these antibodies. To further characterize the specificity of these antibodies we established a peptide array-based screening test that allows the identification of antibodies directed against linear epitopes of the two major BDV proteins, the nucleoprotein (N) and the phosphoprotein (P). Initial tests employing sera of BDV-infected mice and rats or horses with Borna disease revealed a high specificity and sensitivity of this test. All sera recognized epitopes of either N and P or both. Sera of non-infected rats, mice and horses showed no signals on either peptide array. A fraction of human sera that recognized BDV antigen by indirect immunofluorescence (IFA) contained antibodies that recognized various linear epitopes of one or even both BDV proteins. Remarkably, antibodies purified from such human serum by matrix-immobilized peptides showed high-avidity binding to BDV antigens when assayed by IFA or western blotting. These data suggest that reactive antibodies found in psychiatric patients might indeed indicate infection with BDV or a BDV-like agent. However, the poor affinity maturation of BDV-specific human antibodies remains unexplained.

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Analysis of MHC I expression in cultured Borna disease virus-infected hippocampus slices in the presence and absence of interferon-gamma

I. Herpfer, C. Jehle, C. Sauder
Institute for Med. Microbiology and Hygiene (Freiburg, D)

Experimental intracerebral infection of adult rats with Borna disease virus (BDV) leads to immune-system mediated neurological disease. In brains of these rats, massive neuronal cell loss takes place. This has been suggested to be due to expression of MHC I on neurons, rendering these cells vulnerable to the action of cytotoxic T-cells. Although neurons have indeed been shown to be capable to express MHC I under certain conditions *in vitro*, the question of neuronal MHC I expression *in vivo* still is a matter of debate. To test the hypothesis whether IFN-gamma induces neuronal MHC I expression in the BDV-infected rat brain, we employed cultured hippocampus slices which were prepared 8 days post infection of newborn rats. In these slices, BDV upregulated beta-2-microglobulin mRNA which is consistent with the published observation of MHC I expression on activated microglia in brains of newborn BDV-infected rats. Infected and uninfected slices were double-stained with anti rat MHC I mAb Ox18 and neuron-, microglia-, or astrocyte-specific antibodies, respectively. Both in infected and uninfected slices, surface expression of MHC I could be observed on microglia. Interestingly, in the hippocampal pyramidal and granule cell layers, the observed MHC I staining pattern suggested neuronal MHC I expression. However, staining of slices with a microglia-specific antibody indicated that MHC I expression was rather mediated by microglia which were in very close contact to neurons. Incubation of slices with IFN-gamma induced massive upregulation of MHC I staining on microglia both in the presence and absence of BDV. Whereas IFN-gamma did not suppress spread of BDV in infected slices, no obvious differences in MHC I staining patterns were observed between IFN-gamma treated infected and uninfected slices, suggesting that even in the presence of IFN-gamma, BDV infection alone is not sufficient to render neurons capable of expressing MHC I.

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Borna disease virus infection of neurons interferes with Mapk signaling, neurotrophin responsiveness and neuroplasticity

A. Hans,[1] J.J. Bajramovic,[1] S. Syan,[1] I. Dunia,[2] M. Brahic,[1] D. Gonzalez-Dunia[1]
1. Institut Pasteur (Paris, FR)
2. Institut Jacques Monod (Paris, FR)

Borna disease virus (BDV), a non-segmented, negative-stranded RNA virus, causes central nervous system (CNS) diseases characterized by behavioral abnormalities in a wide range of animals species. Seroepidemiological studies indicate that BDV is also a human pathogen, possibly associated with neurospychiatric disorders such as schizophrenia. The persistence of BDV in the CNS is associated with neuronal dysfunction and damage to specific neuronal populations. Since the replication of BDV is non-cytolytic, the mechanisms underlying BDV neurotoxicity are not well understood. One hypothesis is that BDV infection alters the response of neurons to neurotrophic factors. Neurotrophins play instrumental roles in promoting neuronal survival and process outgrowth in the CNS. Neurotrophin signaling triggers a cascade of intracellular events, leading to adaptive biochemical responses. We have shown recently that PC12 cells infected with BDV become resistant to NGF-induced differentiation, a phenotype linked to defects in the MEK/ERK signaling cascade (Hans *et al*, J. Biol. Chem, 276, pp 7258–7265). We have now used primary rat neuronal cultures to examine the effects of BDV infection on neuronal physiology with a more relevant host cell. We found that cultures of hippocampal neurons were highly susceptible to BDV replication and spread. Although the viral infection was non-cytopathic and did not cause overt damage to the cells, we found that the expression of molecules involved in neuroplasticity, such as the growth-associated protein-43, synapsin, synaptophysin and VAMP-2 were selectively blocked after infection. Moreover, BDV-infected neurons responded only weakly to the stimulation by neurotrophins BDNF and NT3, not only in terms of ERK signaling cascade activation, but also in synaptic remodeling. These findings may be of importance to explain the molecular bases of BDV-induced cognitive and neurodevelopmental alterations.

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1-beta-D-Arabinofuranosylcytosine (Ara-C) inhibits Borna disease virus replication and spread

J. Bajramovic,[1] S. Syan,[1] M. Brahic,[1] J.C. de la Torre,[2] D. Gonzalez-Dunia[1]
1. Institut Pasteur (Paris, FR)
2.The Scripps Research Institute (La Jolla, USA)

Borna disease virus (BDV) is a negative-strand RNA virus that causes neurological disease in a variety of warm-blooded animal species. Experimental BDV infection is a well-described model for human neuropsychiatric diseases. Although the clinical consequences of human BDV infection are still controversial, there is consensus that BDV infects humans. To date, there is no effective treatment against BDV. Here we demonstrate that the nucleoside analogue 1-beta-D-arabinofuranosylcytosine (Ara-C), a known inhibitor of DNA polymerases, unexpectedly inhibits BDV replication. Ara-C treatment inhibits BDV RNA and protein synthesis and prevents BDV cell-to-cell spread *in vitro*. Replication of other (−)RNA viruses such as influenza or measles virus is not inhibited by Ara-C, underscoring the particularity of the replication machinery of BDV. We further show that Ara-C inhibits BDV replication *in vivo* in the brain of infected rats, preventing persistent infection of the CNS as well as the development of clinical disease. These findings open the way to the development of effective antiviral therapy against BDV.

P264

Borna disease virus induces fatal neurological disorders in Cyclosporine A-treated, immunosuppressed neonatal gerbils

K. Ikuta, M. Watanabe, B.-J. Lee, M. Yamashita, W. Kamitani, T. Kobayashi, K. Tomonaga
Osaka University (Osaka, JP)

Borna disease virus (BDV) is a noncytolytic, neurotropic RNA virus that causes neurological disturbances in various animal species in immune-dependent and -independent manners. In a previous study, we have shown that BDV infection in neonate gerbils induces acute fatal neurological disease, which might be associated with significant BDV replication in specific areas in the central nerve system (CNS). In this study, we investigated whether host immune responses and cytokines expression in the brain contribute for the fatal outcome of BDV-infection in newborn gerbils.

BDV-infected neonatal gerbils were injected with cyclosporine A (CsA) on a daily basis from neonatal day-15. The treated gerbils were examined for pathological and immunological changes.

Although CsA completely suppressed specific antibody production and brain inflammation in the infected gerbil brains, fatal neurological disorders were not inhibited by such treatment. Furthermore, we demonstrated that CsA-treatment significantly decreased brain cytokine levels in the infected gerbil, except for IL-1beta. These results suggested a role for IL-1beta in the fatal neurological disorders in BDV-infected gerbil brains. Thus, BDV shows a unique neuropathogenesis in neonatal gerbils that might be pathologically and immunologically different from that in rats and mice. This novel rodent model of BDV infection could be useful for studying the acute neurological disturbances without neuroanatomical and immunopathological alterations in the virus-infected CNS.

P265

Borna disease virus phosphoprotein interference with amphoterin inhibits p53 activity

K. Ikuta, G. Zhang, T. Kobayashi, W. Kamitani, M. Yamashita, S. Baba, S. Komoto, K. Tomonaga
Osaka University (Osaka, JP)

Borna disease virus (BDV) is characterized by low virus productivity, neurotropism, and nuclear localization for transcription and replication. Also, BDV shows noncytolytic replication in infected cells and therefore easily establishes persistent infection, which may be important for BDV-induced neuropathogenesis. Our recent study revealed that

BDV phosphoprotein (p24) directly binds a multifunctional protein, amphoterin (HMGB1), and inhibits its function in cultured neuronal cells. Previously, amphoterin has been reported to function in the nucleus as a coactivator of p53. In this study, we examined the possible effects of BDV p24 to inhibit the transactivation activity of p53 by interference with amphoterin.

We constructed a series of BDV p24 and amphoterin mutants to identify the binding regions between both. The ability of p24 to inhibit p53 activity was examined in the cells by co-transfection with plasmids expressing p24, amphoterin, p53, and several other p53-response element-containing gene constructs with luciferase as a reporter gene.

An A-box region in amphoterin has been identified as a domain to interact with p53. The same region was determined to be a target for binding with p24. By using mammalian two-hybrid analysis, we could demonstrate that p24 interferes with the specific binding between p53 and amphoterin in transiently transfected cells. We also showed that BDV infection reduced the levels of Bax and Apaf1 mRNAs, both of which are enhanced by p53 and induce the cell suicide response. Thus, BDV-p24 may inhibit p53 transactivity in infected cells through the binding to amphoterin, suggesting a mechanism for BDV persistence in the infected brain cells.

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Cortical cholinergic decline parallels the progression of BDV encephalitis

U. Gies,[1] T.J. Görcs,[2] J. Mulder,[3] O. Planz,[4] L. Stitz,[4] P.G.M. Luiten,[3] T. Harkany,[3] T. Bilzer[1]
1. Zentralkrankenhaus (Bremen-Ost, D)
2. University of Düsseldorf (Düsseldorf, D)
3. University of Groningen (Groningen, D)
4. Institute of Immunology-BFAV (Tübingen, D)

Borna disease virus (BDV)-induced meningoencephalitis is associated with the dysfunction of the cholinergic system. Temporal development of this cholinergic decline during pre-encephalitic and encephalitic stages of BDV infection remains however elusive. Changes in choline-acetyltransferase (ChAT) and acetylcholinesterase (AChE) activities were therefore determined in the cerebral cortex, limbic system and cholinergic basal forebrain nuclei (ChBFN) of rats infected with BDV. Immunocytochemistry for ChAT and vesicular acetylcholine transporter (VAChT) was employed to identify morphological consequences of BDV infection on cholinergic neurons. Whereas both ChAT and AChE activities changed only slightly under pre-encephalitic conditions, the encephalitic stage was characterized by a significant decrease of ChAT activity in the cerebral cortex, hippocampus and amygdala concomitant with a marked reduction of AChE activity in the cerebral cortex and hippocampus. The striatum and medial septum remained unaffected. ChAT and VAChT immunocytochemistry revealed axonal degeneration in affected cortical and limbic projection areas of ChBFN. In summary, our data indicate progressive deterioration of forebrain cholinergic systems that parallels the progression of BDV encephalitis.

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Transgenic mice expressing the nucleoprotein of Borna disease virus in either neurons or astrocytes

M. Rauer,[1] D. Schuppli,[2] J. Götz,[2] P. Stäheli,[1] J. Hausmann[1]
1. University of Freiburg (Freiburg, D)
2. University of Zürich (Zürich, CH)

The nucleoprotein (N) of Borna disease virus (BDV) is the major target of the disease-inducing antiviral CTL response in the CNS. We have established two transgenic mouse lines with CNS-specific expression of BDV-N. For neuron-specific expression (Neuro-N), the N gene was placed under the control of a modified thy-1.2 promoter. In Neuro-N mice, BDV replication was severely impaired and restricted to brain areas with low numbers of transgene-expressing cells. Especially cells of the hippocampus, a preferred site of BDV replication, showed a strongly reduced rate of infection in transgenic animals inoculated at 4–6 weeks of age. Thus, transgenic overexpression of BDV-N mediates resistance to BDV *in vivo*. Because expression of the transgene does not start before postnatal day 5-10, in transgenic mice infected as newborns BDV antigen was readily detectable in most of the transgene-expressing dentate gyrus neurons, suggesting that an established infection with BDV cannot be cured. The second line of transgenic animals was designed to express N in astrocytes under the control of the murine GFAP promoter (Astro-N). For efficient expression it was necessary to backcross the transgene at least two times to mice of the B10.BR background. N was mainly expressed in astrocytes in the hippocampus, cortex and cerebellum, but not in the thalamus. Despite strong transgene expression in both lines, neurological disease or behavioral abnormalities were never observed. Furthermore, none of the Astro-N mice exhibited overt astrocytosis. After peripheral challenge of Neuro-N mice with a recombinant vaccinia virus expressing BDV-N, no autoimmune-like disease or meningoencephalitis could be induced, suggesting either immunological tolerance or a requirement of additional factors to induce CNS inflammation in response to neuronal and astrocytic antigens.

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Immune priming induces perforin-independent, CD8+ T cell-mediated protection against Borna disease virus-induced neurological disorder

K. Schamel, R. Frank, P. Staeheli, J. Hausmann
Institut für Medizinische Mikrobiologie & Hygiene (Freiburg, D)

Borna disease virus (BDV) infection of MRL mice results in persistent CNS infection, a CD8 T-cell-mediated immunopathology, and severe neurological disease. Post-exposure vaccination of disease-resistant B10.BR mice with a recombinant vaccinia virus expressing the BDV p40 protein (VV-p40) harboring the immunodominant CTL epitope of BDV results in manifestation of neurological disease. To test the effect of p40-specific priming, we immunized MRL mice with VV-p40 prior to BDV infection and analyzed viral spread and disease expression. More than 80% of primed wild type animals were able to control infection and 97% were protected from disease expression. Priming of mutant MRL mice demonstrated that protection was dependent on CD8 T cells specific for the major epitope and involves perforin-independent effector functions. The data suggest that the balance between viral replication and the BDV-specific immune response determines whether CD8 T cells are pathogenic or beneficial in BDV infection.