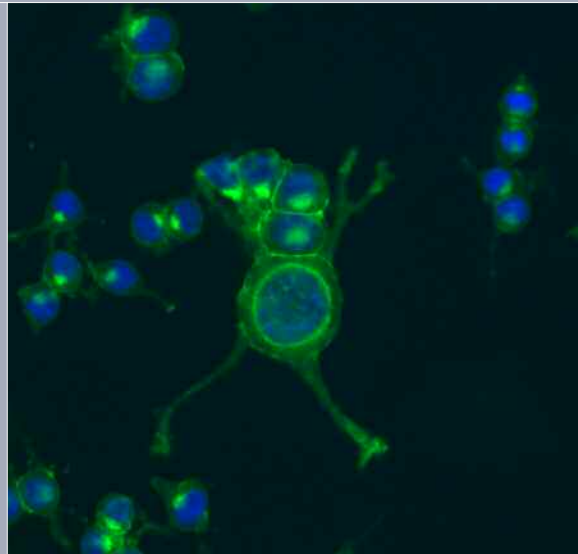


Infectology

The prion protein (green) is expressed at the surface and inside cultured neurons, near the nucleus of the cell (blue) but also, interestingly, at the extremity of the neurite. The latter is best seen on the large central cell but also on smaller bipolar cells. This observation suggests that the normal prion protein could be involved in synaptic transmission. In prion diseases, the prion protein is converted into a misfolded form and causes the death of the neurons. These cultured neuronal cells are not infected, but can be infected, with prions. The cells were labeled with DAPI for the



nuclei and with a specific antibody coupled to Alexa 488. They were then examined under objective x20 with a Zeiss microscope, and the images were analyzed by using Zeiss AxioView software. Work done by Nicole Sales, Ph.D., senior staff scientist, in the laboratory of Corinne Lasmézas, D.V.M., Ph.D., professor.



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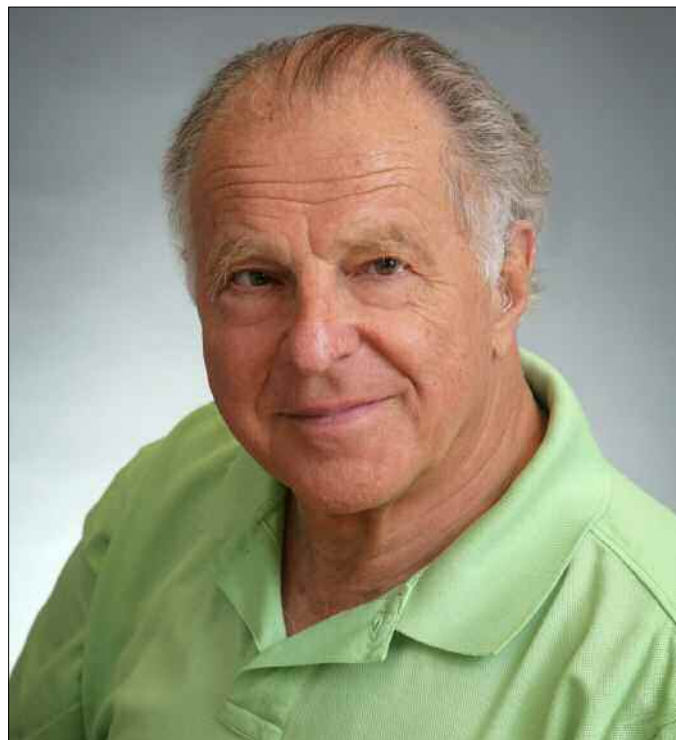
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Chairman's Overview

Researchers in the Department of Infectology currently focus on 2 main areas: prion diseases and hepatitis C. Three groups, those of Corinne Lasmézas and Joaquin Castilla and my own, study the

molecular biology of prions and the pathogenesis of prion diseases.

Prion diseases affect animals as well as humans and are of particular interest to us because of the unusual properties of the infectious agent. Prions consist of a multimeric assembly of PrP^{Sc}, a conformer of a normal monomeric host protein, PrP^C, and perhaps other as yet unidentified components. The seeding hypothesis posits that prion replication comes about by recruitment of host PrP^C into the PrP^{Sc} assembly, a process that entails conformational rearrangement of the PrP^C and occasionally cleavage of the assembly. Many strains of prions can be associated with the same protein sequence, and it is thought that different strains are encoded by different PrP^{Sc} conformations. Interestingly, the various strains not only target distinct brain areas but also differ in their capacity to infect disparate cell lines. The question of how cells can distinguish between different prion strains is being addressed by Shawn Browning, and the processes involved in maintaining prion infection are being investigated by Chris Baker and Jiali Li.

Important resources established in our laboratory by Sukhvir Mahal are the semiautomated, cell-based assay for prions (scrapie cell assay), which replaces the slow, expensive, and less accurate mouse-based bioassay, and the cell panel assay, which allows rapid distinction between different prion strains. Dr. Castilla has perfected the protein misfolding cyclic amplification procedure that allows the cell-free replication of prions. He

made the amazing discovery that prions that are infectious to animals can be generated spontaneously. He is also investigating the fidelity of prion replication *in vitro*. Dr. Lasmézas and her group are investigating the therapeutic possibilities of using antibodies to PrP and PrP-binding aptamers to curb prion diseases.

Our second field of endeavor is hepatitis C. Donny Strosberg and his group are studying interactions between hepatitis C virus proteins and are screening chemical libraries for compounds that disrupt such interactions,

in a search for drug candidates. Tim Tellinghuisen and his colleagues are using x-ray crystallography and site-directed mutagenesis to elucidate the role of the hepatitis C virus protein NS5A in viral replication and the interaction between NS5A and NS5B.

The project of screening for drugs against leishmaniasis, specifically against J-binding protein, has been terminated because the scientist involved moved to a different department at Scripps Florida.

INVESTIGATORS' REPORTS

Generation and Transmission of Prions

C. Weissmann, C.A. Baker, S.P. Mahal, S. Browning, J. Li, C. Demczyk, A. Sherman, E. Smith, D. Ercan

Prions, the agents that cause transmissible spongiform encephalopathies, consist mainly or entirely of PrP^{Sc}, an abnormal conformer of a normal host protein, PrP^C, and propagate by a PrP^{Sc}-catalyzed conversion of PrP^C. Intriguingly, distinct prion strains, which generate different disease phenotypes, are associated with the same PrP sequence, suggesting that the phenotypes are encoded by different PrP conformations. Our major interests are the mechanism of prion replication, the structural basis of strain specificity, and the mechanism of strain recognition by cells.

We have developed a cell panel assay (the strain discrimination panel) and a cell-based prion assay (the scrapie cell assay). Used in conjunction, these 2 assays allow rapid discrimination between prion strains in cell culture, largely obviating the mouse bioassay, which requires large numbers of animals and about a year to complete. The cell panel assay is based on the finding that some cell lines can be infected by one or a few prion strains but not by others. Thus, for example, the prion strain Me7 can infect the cell line LD9 but not the cell lines PK1 or R33, strain RML can infect LD9 and PK1 cells but not R33 cells, and strain 22L can infect all 3 cell lines. The cell panel assay has been expanded to discriminate between 5 prion strains.

We found that in some instances the characteristics of a prion strain change after passaging in cell lines. We are investigating whether this change is due to a "strain

switch" or to a modification imparted by the cell, such as different N-glycosylation, that is reversed when the prions are again passaged in mice (a phenomenon for which we propose the designation "type switch"). We are also determining, in collaboration with M.B.A. Oldstone, Molecular and Integrative Neurosciences, whether various prion strains passaged through transgenic mice that express PrP devoid of a glycosylphosphatidylinositol anchor retain strain specificity. Initial results suggested that strain specificity is not retained. In addition, in collaboration with J. Castilla, Department of Infectology, we are using protein misfolding cyclic amplification to determine whether prions propagated in a cell-free system maintain strain specificity.

N2a-PK1, a cell line highly susceptible to RML and 22L prions, was selected as a rare variant from an N2a neuroblastoma cell population. We have isolated a set of subclones from a cloned N2a-PK1 population. We found that the subclones are extremely variable in their susceptibility to RML and 22L prion strains. This variability was not correlated with either the PrP expression level (PrP expression is essential but not sufficient) or the doubling time of the cells and thus reflects some other property that remains to be identified. Array analysis of PK1 and its subclones revealed overexpression of 2 genes that was absolutely correlated with susceptibility to RML. The functional significance of these genes is currently being examined. We are also using comparative chromosome hybridization to correlate susceptibility with the genome-wide copy number of loci of these highly aneuploid cells. Our working hypothesis is that a mutation, such as a gene amplification event, on a single copy of a chromosome is responsible for susceptibility and that high variability in the population is due to unequal chromosome partitioning among daughter cells.

To test the hypothesis that a particular chromosome is required to maintain a high susceptibility, we devel-

oped subclones of RML-infected N2a-PK1 cells in which individual chromosomes were tagged with a lentiviral insert containing an antibiotic resistance gene as a selection marker and a coding sequence for green fluorescent protein. Currently, we are growing these clones in the presence and absence of antibiotic to select a clone that maintains infection under selection but not in the absence of selection. So far, the responses of the 22 clones tested have not been promising.

In other studies, we are investigating how cell lines persistently infected with prions maintain infection. Previously, we found that cloned RML-infected neuroblastoma cell populations are heterogeneous, consisting of infected and noninfected cells, probably because noninfected cells are continuously generated as cells replicate faster than RML prions do. The cell population remains persistently infected because noninfected cells are continually reinfected by prions secreted by the infected cells. However, neuroblastoma cell populations infected with strain 22L consist entirely of infected cells, presumably because the doubling rate of prions exceeds the doubling rate of the cells.

We also discovered that an inhibitor that abrogates maturation of N-linked glycans prevents infection of the neuroblastoma-derived cell line PK1 by certain prion strains, such as RML, but not by others, such as 22L. However, once infection is established, prion replication is not affected by the inhibitor. These results suggest that the inhibitor prevents uptake and/or transport of prions to an intracellular "replication compartment," that a glycosylated cell protein other than PrP is required for this process, and that improper glycosylation of this protein abrogates its function or prevents its formation.

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Therapy for Prion Diseases

C.I. Lasmézas, N. Salès, P. Saá Prieto, M. Zhou, A. Sturny, G. Sferrazza, Y. Karapetyan

Prions, the transmissible agents responsible for prion diseases, are thought to consist mainly or entirely of PrP^{Sc}, an abnormally folded isoform of the ubiquitous prion protein PrP. Prions are thought to replicate by an autocatalytic process of template-induced conformational change.

Determining targets for therapeutic intervention requires understanding the mechanisms of the neurotoxic effects elicited by the accumulation of PrP^{Sc} in the brain. We found that oligomeric assemblies of recombinant prion protein (provided by H. Rezaei, National Institute for Agricultural Research, Paris, France, and H. Schätzl, Technical University of Munich, Munich, Germany) are toxic to primary cultures of cortical neurons, whether or not the cells express PrP. This toxic effect could be prevented by antibodies that block the hydrophobic domain of the prion protein, located between amino acids 106 and 126. Antibodies to other PrP domains did not prevent oligomer-induced toxic effects.

Because the requirement of endogenous PrP^C expression for neuronal toxic effects has been a matter of unresolved debate, we verified in vivo the significance of our findings obtained with cultured neurons. Using stereotactic procedures, we injected oligomeric and fibrillar PrP preparations as well as monomeric PrP and an inert control medium alone subcortically into wild-type and PrP^{0/0} mice of the same genetic background. Histologic examination of brain samples obtained 24 hours after injection revealed that oligomeric PrP was highly toxic to the pyramidal neurons of the hippocampus adjacent to the injection site. Fibrillar PrP was moderately toxic; neither the control medium nor the monomeric PrP had any adverse effect on neuronal survival or morphology. These findings indicate that as in other amyloidotic neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease, systemic amyloidosis), oligomers of misfolded protein, rather than large molecular weight aggregates, are responsible for the toxic effects on neurons. On the basis of our data, new prion intervention strategies can be devised, aimed at blocking PrP oligomers, especially the hydrophobic domain responsible for toxic effects.

In an alternative approach to therapy of prion diseases, we are studying RNA aptamers that bind spe-

cifically to PrP. Aptamers are small nucleic acids (DNA or RNA) selected from a pool of random DNA or RNA sequences for the ability to bind to a given molecular target. The *in vitro* procedure involves several rounds of selection and amplification. We focused on RNA aptamers because RNAs adopt specific, sometimes complex, 3-dimensional structures. In collaboration with scientists at the Gene Center, Munich, Germany, we are assessing the capacity of 2 PrP-binding aptamers to inhibit prion replication and PrP^{Sc} formation in chronically infected cells. A major hurdle to effective therapy is delivering the aptamer into the proper cell compartment. We are evaluating various promoters in a lentiviral delivery system, which would enable *in vivo* therapeutical assays once the proper strategy has been determined in cell culture.

In collaboration with C. Weissmann, Department of Infectology, we are searching for factors that might play an important role in prion susceptibility and strain specificity. Because studies of the cellular pathogenesis of prion infection are hampered by the difficulty in distinguishing between PrP and PrP^{Sc}, we are working to improve current immunocytochemistry protocols and to develop strategies to detect PrP^{Sc} in infected cells.

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Protein-Protein Interactions in Hepatitis C

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INTERACTIONS BETWEEN HEPATITIS C VIRUS PROTEINS

The need to discover novel drugs for treatment of hepatitis C has not diminished. Although no vaccine has been developed, several inhibitors of a viral protease and the viral polymerase are in advanced clinical trials. However, because of its high mutability, hepatitis C virus (HCV) most likely will become resistant to these types of drugs. Therefore, identifying novel viral targets is desirable. For this purpose, we have continued our studies of protein interactions involving HCV

proteins. Our goals are to better understand the respective roles of the proteins and to identify small-molecule inhibitors that might affect HCV replication.

We have now prepared 4 pairs of interacting HCV protein domains that we identified by using a high-throughput protein-interaction mapping technology. cDNA constructs encoding these HCV protein domains tagged with oligonucleotides encoding epitopes such as the octapeptide Flag or glutathione-S-transferase (GST), were expressed in *Escherichia coli* and affinity-purified by using nickel-containing agarose, which recognizes the 6-histidine-containing epitope attached at the C terminus of the interacting domains.

Core-core interactions were first assayed by binding a GST-tagged core domain (the first 106 residues of the HCV core protein) to a glutathione-coated microtiter plate, exposing the plate to a Flag-tagged core domain, and using an antibody to Flag to determine the amount of bound Flag. We then developed a 384 well-based sensitive high-throughput homogenous time-resolved fluorescence assay with fluorescent antibodies to Flag and GST. We used the assay to screen a set of 18mer peptides covering the first 109 residues of the core domain and discovered 2 peptides that inhibit core 106 dimerization. We also screened a library of 1280 pharmacologically active compounds. We will now screen larger and chemically diversified collections of molecules in order to identify novel inhibitors of HCV.

A second pair of HCV protein domains tagged with GST and Flag, respectively, consisted of the protease part of the NS3 protein and the soluble part of its cofactor, NS4A. This cofactor is essential for the correct folding, membrane insertion, and activity of the protease. The NS4A dependence of NS3 has been confirmed with the proteins expressed in *E coli*, and we are now developing both the enzymatic screening assay and the homogenous time-resolved fluorescence assay to identify novel inhibitors of the NS3 protease.

INTERACTIONS BETWEEN HCV AND HOST PROTEINS IN HUMANS

Cyclosporine A, a well-known immunosuppressive agent, can block replication of both HIV and HCV. In HCV, cyclophilin B, one of the receptors for cyclosporine A, promotes the viral NS5B polymerase activity. Binding of cyclosporine A to cyclophilin B blocks the rotamase activity of cyclophilin B and thereby prevents its interaction with NS5B, resulting in inhibition of viral replication. Cyclosporine A thus is an example of a small-molecule inhibitor of a protein-protein interaction involving viral and host cell proteins. We will clone

and express the domains from NS5B, reported to be responsible for the interaction with cyclophilin B. We are currently developing a homogenous time-resolved fluorescence assay to identify compounds that would mimic cyclosporine A but would not have its immunosuppressive activity or, if possible, its effects on the rotamase activity of cyclophilin B.

HEPATOMA CELL CULTURE SYSTEM FOR HCV GENOTYPE 1B

To further evaluate potential inhibitors of interactions between HCV proteins or between HCV and human host proteins, we have developed a culture system in which HCV genotype 1b CG strain replicates at low levels in hepatoma cells (Fig. 1). The supernatant of the

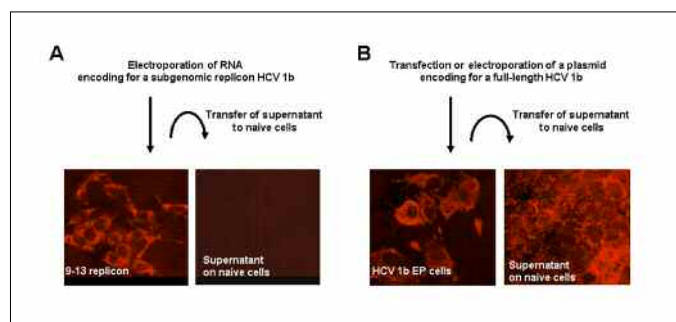


Fig. 1. Transfer of infectious HCV 1b. A and B, upper panels, Schema of the experiment. A and B, lower panels, Immunofluorescence detection of HCV NS5A in cells infected with subgenomic replicon HCV 1b or full-length HCV 1b. A, Supernatant of subgenomic replicon HCV 1b 9-13 was added to naive Huh7.5 cells. B, Supernatant of transfected or electroporated (EP) Huh7.5 by a plasmid that encoded a full-length HCV 1b was added to naive Huh7.5 cells. A replicon is a replicating part of the HCV genome devoid of the structural proteins and thus is noninfectious.

cells is infectious for naive cells. This system, which would still benefit from improvements, seems comparable to the genotype 2a JFH strain infectious system developed recently in part by our Scripps colleagues, F.V. Chisari, Department of Molecular and Experimental Medicine, and T.L. Tellinghuisen, Department of Infectology. We have confirmed the comparable inhibitory effect of cyclosporine A on the 2 infectious HCV culture systems.

The advantage of using a 1b HCV strain rather than the 2a strain is the wider prevalence of the 1b strain in the Western world. In addition, the 2a strain was isolated from a patient with a rare form of fulminant hepatitis C.

PUTATIVE TUMOR SUPPRESSOR GENE IN HEPATOCELLULAR CARCINOMA

The end-stage of HCV infection is hepatocellular carcinoma. Despite its rare occurrence, HCV infection is

nevertheless the leading cause of liver cancer in the United States. Recently, in collaboration with C. Nahmias, CNRS, Paris, France, we found that more than 10% of patients with hepatocellular carcinoma have mutant forms of the gene that encodes ATIP, a protein that specifically interacts with the C-terminal region of the AT2 receptor for angiotensin II. This putative tumor suppressor gene is underexpressed in other forms of cancer. A study of more than 90 primary hepatocellular carcinomas or cell lines indicated that the gene for ATIP is indeed consistently repressed more often in the most aggressive class of hepatocellular carcinoma than in the 3 other classes, thus supporting our previous findings for a specific role of ATIP in hepatocellular carcinoma.

Hepatitis C Virus RNA Replication

T.L. Tellinghuisen, J.C. Treadaway, K.L. Foss

Hepatitis C virus (HCV) is a human pathogen of global importance; according to some estimates, nearly 3% of the world's population is chronically infected with this virus. Long-term viral replication in these individuals leads to severe liver disease, including cirrhosis and often hepatocellular carcinoma. The current treatment regimen with agents nonspecific for HCV is poorly tolerated and is ineffective in about half of the patients, emphasizing the need for effective antiviral drugs specific for the virus.

The HCV replicase, the multicomponent machine that replicates the viral RNA, is an ideal drug target. The core replicase consists of 5 HCV proteins associated with well-characterized polymerase, protease, and helicase activities. Some HCV replicase proteins, such as NS5A, are essential for HCV replication; however, their specific functions remain enigmatic.

We have been characterizing NS5A. Our goal is to understand the role of this protein in replication and, more generally, the replicase itself. We have defined NS5A as an absolutely required, 3-domain metalloprotein component of the replicase. Our recent crystal structure of domain I of NS5A has provided a glimpse of the potential interactions of NS5A in the viral replicase and has suggested potential antiviral targets.

Using genetic methods, we have identified all of the amino acids in the poorly understood domains II and III that are required for HCV RNA replication. Additionally,

we recently discovered an interaction between the membrane anchor of NS5A and the protein NS4B, another component of the replicase. This interaction appears to localize NS5A to the replicase and is essential for RNA replication. We are identifying regions of NS5A whose functions are required for the production of infectious virus but not for RNA replication. Our findings suggest that NS5A may function as a regulator of the switch between RNA replication and virus production. We are conducting biochemical, genetic, and structural experiments to evaluate the potential interaction surfaces and activities of the NS5A observed in our previous structural and genetic research. We have also begun applying high-throughput genetic screens to identify required host components of the replicase.

Our ultimate goal is to understand, at the molecular level, the assembly, activity, and regulation of the HCV RNA replication machinery. Greater insight into the poorly understood replicase components, such as the NS5A protein, will provide a more complete view of the replicase complex and will fuel new drug design.

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Spontaneous Generation of Prions in a Cell-Free System

J. Castilla, N. Fernández-Borges, G.M. Cosseddu, J. de Castro, M. Márquez

Transmissible spongiform encephalopathies are a group of fatal neurodegenerative disorders that affect both humans and animals. The disorders can be classified as genetic, sporadic (putatively spontaneous), or infectious. The infectious agent associated with these encephalopathies, the prion, appears to consist of the single protein PrP^{Sc}, an abnormal conformer of the natural host protein PrP^C. PrP^{Sc} propagates by converting host PrP^C into PrP^{Sc}. Interestingly, prions occur in the form of different strains with distinct biological and physicochemical properties, even though all the strains are encoded by PrP with the same amino acid sequence, albeit in presumably different conformations.

The occurrence of spontaneous cases of prion diseases in humans (sporadic Creutzfeldt-Jakob disease) and in other species, such as bovine spongiform encephalopathy in cattle in Europe and the United States and the recent cases of atypical scrapie in sheep, suggest that de novo generation of prions may be taking place infrequently but ubiquitously. However, no cases of spontaneous prion disease in experimental wild-type rodent models have been reported. We have used a novel technique, protein misfolding cyclic amplification, with homogenates of normal brain as substrate, to rapidly propagate prions in the test tube. Prions propagated in vitro are infectious in vivo and maintain their prion strain specificity. Protein misfolding cyclic amplification has been used to efficiently amplify a variety of prion strains from mice, hamsters, bank voles, deer, cattle, sheep, and humans.

Because previously infectivity could not be generated de novo by starting with normal PrP^C, scientists thought that spontaneous generation of prions was an infrequent in vivo phenomenon that occurred in only a few species. Therefore, mimicking spontaneous generation of infectivity in vitro became one of the most important challenges in the study of prions. We recently, for the first time, generated infectious prions from 2 different rodent species (including wild animals) by starting with noninfectious brain homogenates. Several biochemically different prion strains have been generated, and we are characterizing their biological behavior in different rodent models. We will also determine if the in vitro spontaneous phenomenon takes place in other more relevant species.

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