Journal of Alzheimer's Disease 7 (2005) 355–360
IOS Press

Discussion

Alzheimer Research Forum Live Discussion: The Pathogen Hypothesis[1]

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June Kinoshita: Hello, everyone, and thank you for being here today. Brian, could you give everyone here a quick summary of your mouse findings?

Brian Balin: The mouse findings were that infection through the intranasal route by the brain strain of *Chlamydophila pneumoniae* (Cpn) resulted in amyloid plaques appearing in the mouse hippocampus and other cortical areas.

Joseph Lyons: Brian, have you included a non-Cpn strain of *Chlamydia* as a control?

Brian Balin: The brain strain was isolated from an Alzheimer disease (AD) brain a number of years ago. No, we have not used a non-Cpn strain yet, but hope to do this some time in the near future.

June Kinoshita: Brian, in addition to plaques, do you see neurodegenerative changes or inflammation?

Brian Balin: We saw some inflammation with regard to astroglial responses, as determined by labeling for activated astrocytes. These appeared around areas of amyloid deposition, including around some blood vessels. We have not observed neurofibrillary tangles at this time. We believe that longer infections and/or infection in older animals may result in tangle formation.

June Kinoshita: Brian, any ideas as to what Cpn does once it enters the brains of the mice? Does it infect specific cells?

Brian Balin: We have found that Cpn was present in olfactory bulbs in the animals. In humans, Cpn shows up in glial cells, endothelial cells, and more recently we have found Cpn in neuronal cells. Alan Hudson at Wayne State University, Michigan, has also found this to be the case in a separate analysis. Both the olfactory pathways and the vascular route appear to be involved with brain infection. Probably the monocytes are taking the organism from lung to blood to the brain; a recent publication by Gieffers et al. [1] showed this.

Joseph Lyons: Brian, have you cultured the organism at later time points when elementary bodies (which are the mature and infectious form of this agent and a form not often seen in chronic/persistent infection) are histologically present, and if not, what do you think is going on?

Brian Balin: Joe, we have cultured the organism from mouse olfactory bulbs after four months post-infection. The organism does appear at times to change antigenic expression. This may also reflect some change in gene expression during persistent versus acute in-

[1] Note: The transcript has been edited for clarity and accuracy.

ISSN 1387-2877/05/$17.00

fection. Joe, we also have been doing much *in vitro* infection with Cpn and have been finding some really interesting results in neuroblastoma cells. Apoptosis is affected (i.e., delayed and inhibited by infection); also, some changes in cytoskeleton (tau protein) have been noted at 10 days post-infection.

Ruth Itzhaki: Is that published yet, Brian? I would be very interested to have details.

Brian Balin: Ruth, the in vitro data has not been published yet. Ruth, you may want to say something about familial AD and the recent herpes reactivation observations. This seems to tie infection to familial disease as well, something that we also have been considering but have not done at this point in time.

Ruth Itzhaki: It is rather a small number, so we cannot tie it in with apolipoprotein E (ApoE), but it is still very intriguing; also, Mori et al. [2] detected viral proteins, so maybe there is a persistent very low-level infection, at least in some brains, rather than true latency.

Brian Balin: Ruth, this is very interesting. Do you think that ApoE is a more general feature of many infections in both brain and elsewhere, because we also have evidence that ApoE could be involved with Cpn infection?

Ruth Itzhaki: Yes, it seems to affect the response in several diseases caused by very diverse pathogens including cold sores (which are definitely caused by HSV1), and liver disease caused by hepatitis C virus and malaria. All of these pathogens bind, like ApoE, to heparan sulfate proteoglycans (HSPG) and some enter cells via ApoE receptors. Regarding herpes simplex keratitis (HSK), there was an effect but not statistically significant, and we attribute these differences to differences in cell type involved. This would take several sentences to explain: See our Trends in Microbiology review [3].

June Kinoshita: Ruth, is that in humans who had HSK (I presume this occurs in people and not just mice . . .)?

Ruth Itzhaki: Yes, we studied humans with herpes simplex keratitis.

June Kinoshita: Marie, is ApoE genotype something you looked at with HSK?

Marie Sanchirico: No, until we started some work on atherosclerosis and AD in the laboratory, we never worried about ApoE.

June Kinoshita: Brian, do you think that all types of cells in the brain are equally vulnerable to Cpn, or is there selective vulnerability?

Brian Balin: June, that is a really good question. The mode of infection or uptake of the organism may differ from one cell type to another. Interestingly, and this crosses with Ruth's herpes work, Cpn seems to use heparan sulfate proteoglycans as a mechanism for uptake in some cells. We also think there may be more specific receptor-mediated uptake.

June Kinoshita: I am also curious about the anatomical distribution of plaques in the mice. Are plaques produced only by some cell populations in response to Cpn infection? Or is the anatomical pattern related more to the route of infection and the presence of Cpn?

Brian Balin: June, plaques in the mice seem to arise in areas that are connected to olfaction, but not exclusively. We see plaques also in areas not directly related to olfaction, but at times in areas such as cerebellum, midbrain, etc.

Joseph Lyons: Everyone, I am attracted to any model of AD initiation and progression that includes the nose, as it is both a common portal of entry and is constantly exposed to all of life's many insults, and the olfactory bulb and associated nervous system connections may play a role in amyloid-β clearance. Thus, Brian's model is significant as an infectious cause of disruption of normal activity in this area, but might be nothing more than an example of temporary bulbectomy, which was recently shown to result in amyloid-β deposits and associated mental impairment in mice [4]. Thus, the need for many more controls to establish specificity in the murine model. Comments, anyone?

June Kinoshita: Joe, that is interesting about the bulbectomy. So one possibility is that amyloid-β plaques are a generalized response to injury/infection.

Joseph Lyons: June, the authors found that six weeks after bilateral olfactory bulbectomy, a peptide with molecular weight of 4 kD was revealed in extracts of the neocortex and hippocampus from mice. Using monoclonal antibodies 4 G8, this peptide was identified as

amyloid-β. Its level was significantly higher in the bulbectomized animals than in sham-operated mice. The bulbectomized mice displayed sharp impairment in spatial memory when tested in the Morris water maze. The results suggest that bulbectomy initiates in the brain a pathological process similar to human AD in location, biochemistry, and behavioral manifestations.

Brian Balin: The infection seems to be isolated to the olfactory bulbs in the mice, but the pathology is found in the brain proper. Also, in other studies of toxin delivery to the olfactory system of dogs, neurodegenerative changes have been observed in deeper brain structures.

June Kinoshita: I think an important question is whether in the pathogen hypothesis, the development of AD is pathogen-specific or part of a more general response to insults and stresses. Marie has worked on a model where the neurodegeneration results from a pathogen-specific mechanism. Is everyone familiar with the work on HSK, or would it be helpful to have Marie provide a summary?

Brian Balin: The issue of generalized response may have some validity because, as we all know, amyloid can develop in the brain following different types of insult, such as brain injury, and with other mediators triggering an inflammatory response. Marie, could you please provide a synopsis?

Marie Sanchirico: HSK is a viral-induced autoimmune disease in humans. There is a terrific mouse model for this disease (HSV1 strain-specific) in which you can infect the eye with HSV1 and within two weeks mice develop HSK, very similar to humans. We have shown that it is CD4+-mediated, and also that a specific viral epitope makes the response more aggressive (mimicry). In HSK, we focused very much on the immune response rather than the infection. Infection is not persistent in mice, and is gone by seven days.

Brian Balin: I think that one of the key issues with regard to the pathogen hypothesis is that different pathogens, depending on their ability to enter the brain and/or trigger inflammatory responses, can result in activation of pathways that coincide with neurodegenerative responses such as amyloid formation and tangle development.

Keith Crutcher: In support of a suggested role for pathogens in amyloidosis beyond AD, there is a recent review of familial amyloidotic polyneuropathy (FAP) [5] that reports that transgenic mice carrying the relevant mutation show a lower incidence of the phenotype when raised in a pathogen-free environment [6].

Brian Balin: Marie, in your system, do the viruses become latent?

Marie Sanchirico: I am not sure with KOS (a designation for one of the available infective lab strains) if it does become latent.

Ruth Itzhaki: Marie, is there any evidence of changes in AβPP or amyloid in the mice?

Marie Sanchirico: I never checked AβPP or amyloid in these mice. Along those lines, one experiment I talked about with Ruth was to infect PDAPP and TgCRND8 mice with HSV1 and look to see if the pathology more resembles human AD. Unfortunately, I never got to do those experiments before leaving Harvey Cantor's Harvard University laboratory.

June Kinoshita: What Marie says about the infection being gone before the neurodegeneration sets in is so interesting, i.e., even in the absence of acute infection, and possibly even after the pathogen has been cleared, it leaves an immune system memory that leads to a neurodegenerative response. So the fact that there are inconsistent findings regarding the presence of HSV1 or Cpn in AD tissue may be irrelevant.

Ruth Itzhaki: That makes it even more difficult to show that a pathogen is involved, though.

Brian Balin: With any chronic disease process, there will be difficulty establishing gold standards for detection as well as sampling issues. In addition, the hit-and-run hypothesis of infection [suggests that a pathogen] could trigger [a disease process] without having to remain around, for example, the initiation of an autoimmune response due to antigenic mimicry, etc.

June Kinoshita: I would love to see the results of that experiment, HSV1 in an AβPP-overexpressing mouse. Is it possible to infect the mice without killing them outright?

Marie Sanchirico: It should be easy. Yes, try to give them mild non-lethal encephalitis and see if the AβPP transgenic (Tg) mice develop a human-like AD. Ruth

was starting to advise me on this. I do not remember who you said was the expert in this type of experiment.

Ruth Itzhaki: Mike Pappolla of the University of South Alabama suggested our collaborating on HSV-infected AβPP Tg mice but he moved his laboratory before we could start anything.

Brian Balin: Ruth, do you think that the mice need to be double transgenics or have a specific ApoE genotype?

Ruth Itzhaki: Brian, I would expect more relevant effects in doubly Tg or triply Tg mice. Would you?

Brian Balin: Yes, Ruth, the multiple transgenics would seem to be more relevant.

June Kinoshita: Ruth, I am not sure. It might be more important to know whether the amyloid-β produced in the model mimics the pathogenic epitope.

Ruth Itzhaki: June, you may be right if such a change in amyloid occurs.

Allen Bain: Is there a model where aggregated amyloid-β can be tested for its ability to activate a latent HSV or Cpn infection? Or is this already known?

Keith Crutcher: I tried to test the hypothesis that AD is a disease of response to infection by looking at antiviral activity against HSV1 using brain homogenates. Interestingly, there was greater antiviral activity in the AD tissue compared to control.

Joseph Lyons: At the City of Hope, an investigator named Ed Canton uses a similar model and has not observed any neurological signs following recovery. In his hands, a latent infection that can be stress reactivated is established, and he has shown sex and mouse strain differences in susceptibility and course of disease. I have attempted to get him interested in this connection without success. Marie, is there any olfactory involvement in your model?

Marie Sanchirico: We infected the eye and never looked further. One other important consideration is the strain of HSV1 used. Especially in mice, different viral strains are significantly more or less encephalitic in the same line of mice.

Ruth Itzhaki: Seems also to vary amongst mice of the same strain, i.e., some mice get herpes simplex encephalitis (HSE) and die, but others survive after suffering from a much milder disease.

Brian Balin: Ruth, is there an age effect on who gets HSE?

Ruth Itzhaki: In humans, I think it affects the middle aged, but I think there are two peaks; sorry, I cannot remember the details. Some say bimodal – with peaks in those less than 20 and in those over 50, and others say in middle aged and the elderly – while others say merely in those in middle age groups. Does Marie know?

Marie Sanchirico: I was hoping you did – no.

June Kinoshita: Marie, is there published information on which viral strains produce encephalitis in which mouse strains?

Marie Sanchirico: Not sure. I know RE (another HSV1 strain designation) is much more encephalitic than KOS. I am sure David Knipe or other HSV1 biologists would have a good idea of this.

June Kinoshita: Speaking of which, would anyone like to address the molecular mimicry question, that is, whether Cpn and HSV1 proteins have molecular mimics in the nervous system, for example, amyloid-β or some other proteins.

Brian Balin: The molecular mimicry issue is big in the Cpn field in that Hsp60, for one, has been shown to be highly homologous to eukaryotic Hsp60. Thus, presentation of the prokaryotic form may elicit responses that cross with the eukaryotic form. Also, Bachmaier et al. [7], showed some autoimmune phenomenon in mice following *Chlamydia infection* – cardiac myosin was attacked.

Marie Sanchirico: I think mimicry would only be an issue in AD if we start to think autoimmunity is involved or there is mimicry on the innate level (signaling through the Toll pathway). As Brian mentioned, in the cardiac model mimicry is suggested, but that is looking at an autoimmune reaction.

June Kinoshita: Marie, there is a relevant paper that just appeared in PubMed [8].

Marie Sanchirico: Also, June, I believe they have found autoimmunity in the patients that developed encephalomyelitis in the Elan trials – possibly boosted already existing immunity.

June Kinoshita: Ruth has been following the Elan study with great interest . . .

Joseph Lyons: We concluded our article with a suggestion that multiple infections over the course of a lifetime might be a better way of understanding the chronic processes of AD, hence, might I suggest merging two models of disease such as HSV and Cpn and throw in an episode or two of endotoxemia, this latter being perhaps a valuable tool to assess reactivity at an established site of deposition and microglial accumulation?

Marie Sanchirico: Joe, this definitely could be true. One infection early in life predisposes you immunologically and a second central nervous system (CNS) localized infection initiates the chronicity.

Brian Balin: Joe, your suggestion is one that we also have been considering. This would fit with a polymicrobial approach to this disease as well as with the influence of inflammation (i.e., body's response) in contributing to AD.

Joseph Lyons: Brian, you have the model for initial deposition that could certainly be exploited in this way.

Allen Bain: Joseph, given that many postmortem brains from patients without AD symptoms show lots of plaques, could it be that a latent infection *and* a presence of plaques due to previous acute infections may be the two hits necessary for rapid decline seen in AD, i.e., not necessarily two infections?

Joseph Lyons: That is the hypothesis I most favor for the role of infection in progression.

June Kinoshita: Regarding the two-hit model: Is there some way to analyze stored blood samples from a large epidemiological study, such as the Framingham Study, to see whether exposure to Cpn or HSV1 earlier in life increases the risk of later developing AD?

Ruth Itzhaki: June, at least in those at a low socioeconomic level, HSV1 infection occurs in infancy. I think the same is true for Cpn, is it not, Brian?

June Kinoshita: Ruth, are you saying these infections occur in 100 percent of these populations?

Brian Balin: Blood analysis will probably not help in determining later risk because with late age, most of the population already shows exposure. Cpn seems to occur more across the board as community acquired.

Ruth Itzhaki: Yes, eventually, by early adulthood, in such people, the infection level is almost 100 percent, in the peripheral nervous system (PNS), of course.

Joseph Lyons: June, Cpn epidemics do occur in institutional settings and ultimately, as Brian mentioned, no one escapes exposure, thus making it the interesting candidate agent to which Brian is so endeared.

Joseph Lyons: Brian, do you see any vascular involvement/damage in your model?

Brian Balin: Yes, Joe, we do see some vascular involvement in our model. Thus, we believe this route also is vulnerable in this infection.

Allen Bain: Brian, are you planning pharmacologic interference in upcoming studies with your model, such as antiinflammatories, myeloperoxidase (MPO) blockers, other immunomodulatory drugs, or even anti-*Chlamydia drugs*?

Brian Balin: Yes, Allen, we want to try antibiotics at various times following infection, or prior to infection, to determine how they affect plaque initiation.

Joseph Lyons: The problem with many intervention strategies is that they could in themselves "chase" Cpn into a persistent state which in the end may be the most pathologically destructive form of infection.

Keith Crutcher: Joe/Brian, do you think it is just coincidence that most AD patients officially die of pneumonia?

Joseph Lyons: Keith, the inclusion of that remark was made somewhat tongue-in-cheek, but given the almost pathognomic association of Cpn with death revealed by the two studies used to establish the relationship between AD and Cpn, it begs the question: During the throes of terminal degeneration, does a reactivation of Cpn infection at a distant site seed the brain via already compromised vascular lesions and act as the final death-inducing insult?

Keith Crutcher: No tongue-in-cheek here. It was a serious question and I think your answer is intriguing.

Ruth Itzhaki: The HSV1 idea needs careful repeat studies by others of human brain, plus investigation of mechanisms of HSV1-ApoE interactions, ApoE Tg mice studies, plus (what a hope!), clinical trials of antivirals.

Brian Balin: We need to focus on aging as a susceptibility factor with these infections, immunosenescence, influence of infection on the cells following persistency, etc.

June Kinoshita: It seems animal models could play an important role in advancing this hypothesis. What needs to be done in this area? Brian has made a great beginning with his Cpn mice. In addition to carrying out more control experiments, what do you want to do?

Brian Balin: Did you happen to see the Loeb at al. study with antibiotics in AD? Doxycycline and rifampin led to some improvement in some cognitive tests and memory for AD patients. With comparison to cholinesterase inhibitors, the data was just as strong on the delay of progression issue [9].

Ruth Itzhaki: It might be interesting to see whether HSV1 infected mice have serum that cross-reacts with human amyloid. This could support June's autoimmune hypothesis. (Remember that the HSV1 protein gB shows homology with amyloid.)

Brian Balin: We would also like to proceed by using different animal models, including transgenics, to try to understand cofactors in the infection process, and the result of infection.

June Kinoshita: Ruth and I have exchanged some e-mails about carrying out multi-lab studies using a reference set of tissue. Does that seem like a high-priority next step?

Ruth Itzhaki: That is, polymerase chain reaction (PCR) of tissue and of extracted DNA.

Brian Balin: Do we really have a gold standard for detection and how should we proceed? Use real-time PCR or culturing of samples?

Ruth Itzhaki: We do not have a real-time PCR machine.

June Kinoshita: Brian, regarding a gold standard, it might be worth some effort to establish a consensus.

Brian Balin: This is now in the works for Cpn, looking at genes for both active acute and persistent infections.

June Kinoshita: We are at the end of our hour. Thank you all for taking part in today's discussion.

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