

Chronic Inflammation and Amyloidogenesis in Alzheimer's Disease: Putative Role of Bacterial Peptidoglycan, a Potent Inflammatory and Amyloidogenic Factor

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Summary

Glycosaminoglycans (GAGs), which occur in most organisms from bacteria to vertebrates, appear to be present in all amyloid deposits, and may play an essential role in the pathogenesis of amyloidosis. It is well established that senile plaques are the sites of chronic inflammation, but the factor activating the complement remain unknown. Here, we analyzed whether the amyloidogenic bacterial peptidoglycan, a potent activator of complement and of the GAG metabolic system is present in senile plaques. Neuropathologically analyzed 54 autopsied brains were investigated. The 54 cases consisted of 32 Alzheimer's disease (AD) cases with severe AD-type changes, 12 cases with low number of senile plaques and 10 cases without any AD-type changes. We have found that in the 32 AD cases with high number of plaques and in the 12 cases with low number of plaques, bacterial peptidoglycan was immunolocalized to senile plaques and on serial sections co-localized with beta-amyloid protein.

Bacterial peptidoglycan has a variety of biological actions in mammals. It is an inflammatory cytokine inducer, activates complement of the classic pathway, affects vascular permeability, generates nitric oxide, induces proteoglycan synthesis and apoptosis, in addition is amyloidogenic. It is well established that all these processes are implicated in AD, suggesting that bacteria or bacterial debris may be one among probably several factors which may trigger the cascade of events leading to chronic inflammation and amyloid deposition in AD.

Key words: Alzheimer's disease, bacteria, bacterial peptidoglycan, beta-amyloid, chronic inflammation, glycosaminoglycans, proteoglycans, spirochetes.

Glycosaminoglycans may play an essential role in amyloidogenesis.

Glycosaminoglycans (GAGs), which occur in most organisms from bacteria to vertebrates, appear to be present in all amyloid deposits regardless of the protein involved. Their early appearance, and their likely interaction with specific proteins imply that they may play an essential role in the pathogenesis of amyloidosis [Snow and Kisilevsky, 1985]. Snow and his co-workers [1990] using immunocytochemistry have shown, that it is the heparan sulphate proteoglycan (HSPG) known as perlecan which co-localizes to β -amyloid in the brain of patients with Alzheimer's disease (AD).

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The question is still open whether the increased accumulation of GAGs in amyloid plaques in AD may be due to a parallel response, together with β -amyloid, to an unknown amyloidogenic stimulus, most likely inflammation [Kisilevsky, 1983], or is secondary to the primary deposition of unusual proteins [Linker, 1989].

Alzheimer's disease and chronic inflammation: The factor or factors that activate complement in AD remain unknown.

Until recently, immune mechanisms in the pathogenesis of Alzheimer's disease have been largely overlooked. Cellular and molecular components of immune system reactions are both associated with AD [McGeer and Rogers, 1992]. Using traditional pathological techniques, it has not proven possible to separate small number of T-cells from the similar-sized oligodendroglial cells. Therefore, it has been assumed that lymphocytic infiltration does not occur in AD. However, using specific immunohistochemical markers, both T-helper/inducer and T-cytotoxic/suppressor lymphocytes have been observed [McGeer and Rogers, 1992]. There is evidence

that in AD there are reactive microglia which express high level of MHC class I and II glycoproteins and immunoglobulin receptors. Antibodies to cytokines and their receptors, as well as to the classical pathway complement proteins densely label pathologic elements in AD tissues. The terminal membrane attack complex, C5b-9, intended to lyse foreign cells, such bacteria, is also present in the cortex. Finally, in order to protect host tissue, protectin and clusterin are synthesized by the brain to inhibit insertion of the membrane attack complex [McGeer and Rogers, 1992].

The factor or factors that in vivo activate complement in AD remain unknown. Identifying the source of complement activation might open the possibility of an effective method of therapeutic intervention.

The amyloidogenic bacterial cell wall peptidoglycan is a potent activator of complement.

In bacteria, the cell wall consists of peptidoglycan, a complex polysaccharide composed of two sugar derivatives, N-acetylglucosamine and N-acetylmuramic acid and a small group of amino acids. Peptidoglycan is present only in bacteria, and is found in the wall of virtually all Eubacteria. It is absent in the evolutionary higher plant and animal cells (Eucaryotes). Bacterial cell walls are highly resistant to degradation by mammalian enzymes and thus may provide a persisting inflammatory stimulus [Ohanian and Schwab 1967].

Specific bacterial pathogens may infect a distant site, which on interaction with the immune-system, leads to a chronic inflammation [Lehman et al., 1983; Fox, 1990] As is proposed for Lyme disease, non viable, poorly degradable "bacterial remnants" or alternatively, "dormant" fastidious bacteria may persist indefinitely in the affected organs acting as a chronic antigenic stimulus inducing chronic inflammation [Fox, 1990]. It has been shown that, human intestinal bowel contains soluble bacterial cell wall components that are arthropathic in an animal model [Stimpson et al., 1986]. In these models it was the bacterial cell wall peptidoglycan component which was found to be the arthritogenic factor in experimentally induced arthritides [Fleming et al., 1986].

Both the clinical and the histological hallmarks of AD are present in the long standing form of general paresis.

The possibility, that a slow acting unconventional infectious agent, acquired at an early age and requiring decades to become active, may be involved in AD, has been proposed by several authors [Wisniewsky, 1978; Khachaturian, 1985].

Certain chronic infectious diseases, including rheumatoid arthritis, leprosy, and osteomyelitis, all lead to or are associated with amyloid formation [Khachaturian, 1985]. In addition, the successful experimental transmission of cerebral β -amyloidosis to the primate was recently reported by Baker et al., [1993].

A possible role of bacteria in AD was first evoked by Fischer [1907] and recently by several other authors [MacDonald and Miranda, 1987; MacDonald, 1988; Howard and Pilkington, 1992; Miklossy, 1993; Miklossy et al., 1994a,b; 1995; 1996].

The similarities between the clinical manifestations of AD and general paresis have long been observed. Alois Alzheimer himself has evoked the possibility of the clinical diagnosis of general paresis in his second famous case described in 1911. "The diagnosis of an atypical Lissauer's paralysis could not, however, be dismissed until the death, largely because of an experience which we had only few months before. A patient, who had shown quite a similar picture in many respects (profound mental impairment, sensory aphasia, agnosia, and apraxia), and who, just like case F., had no abnormality of complement in either blood or cerebrospinal fluid on repeated examination, had on microscopic examination turned out to have a progressive general paralysis with atypical localization." [Alzheimer, 1911].

With respect to the histopathological changes, multiple authors have described *Treponema pallida* colonies confined to the cerebral cortex in patients with general paresis [Schlossberger and Brandis, 1958; Pacheco e Silva, 1926-27]. The morphology and distribution of these colonies are identical to those of the senile plaques in AD (Figures 1 and 2). The occurrence of neurofibrillary tangles and deposition of amyloid in the brain in general paresis has been repeatedly reported.

It is well known that dementia associated with cortical atrophy and microgliosis occur in late stages of another chronic neurospirochetosis, Lyme disease, caused by *Borrelia burgdorferi*. The striking similarities between neurosyphilis and neuroborreliosis with respect to the clinical and pathological aspects are well established [Fallon et al., 1994].

Therefore, we expected to analyze whether bacterial peptidoglycan, this potent inflammatory and amyloidogenic factor is present or not in senile plaques in AD. If senile plaques contain bacterial peptidoglycan, this would suggest that bacteria or persisting bacterial remnants, may be a factor in the generation of chronic inflammation, GAG accumulation and amyloid deposition in AD.

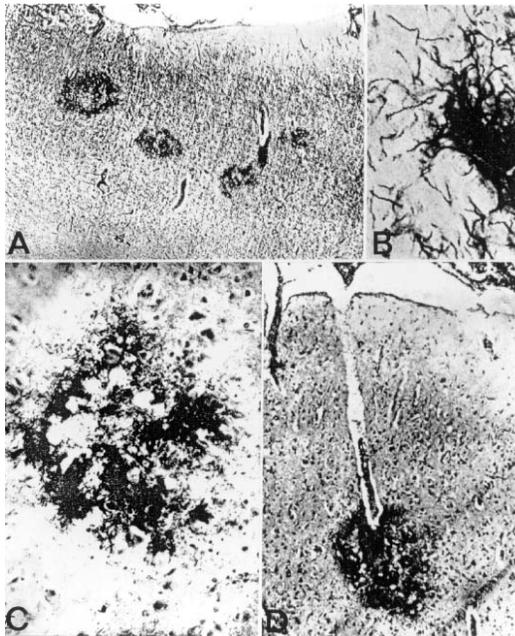


FIGURE 1: A-D: Reproduction of the illustrations of Pacheco e Silva [1926-27] showing spirochetal colonies in the form of plaques in the cortex of a general paretic case.

A: The legend used by the autor himself: "Colonias de espiroquetas em torno dos capillares periphericos do cerebro. Lobo parietal. Caso de paralyisia geral. Meth. Jahnel. Pequeno augmento." See, that the morphology and the distribution of spirochetal colonies are identical with those of senile plaques in Alzheimer's disease. B: With a higher magnification the typical spiral appearance of several spirochetes clearly indicates that the plaques are indeed made up by spirochetes and excludes the possibility of a concurrent AD and general paresis. C and D: Spirochetal colonies in the cerebral cortex morphologically identical with immature (C) and perivascular plaques (D).

MATERIAL AND METHODS

Autopsied brains of 54 patients were investigated. Blocks from at least three different cortical regions, - frontal, parietal and temporal - were taken and were frozen in liquid nitrogen, and were stored at -80°C until processing. After three weeks of formaldehyde fixation, another set of blocks were taken from the same brain regions, and embedded in paraffin. Seven μm thick frozen and paraffin sections were stained with the Gallyas silver technique, Thioflavin S and Congo

Red. For the immunohistochemical demonstration of the β -amyloid a monoclonal antibody to synthetic β -amyloid protein was used. The sections were treated with 80% formic acid for 15 minutes before immunostaining to β -amyloid. The frozen sections were fixed in 4% paraformaldehyde for 24 hours before the formic acid treatment. From the 54 cases in 32 the histopathological investigation revealed the presence of severe AD-type changes, with a high number of senile plaques. For the neuropathological diagnosis of AD we used the criteria proposed by Khachaturian [1985]. From

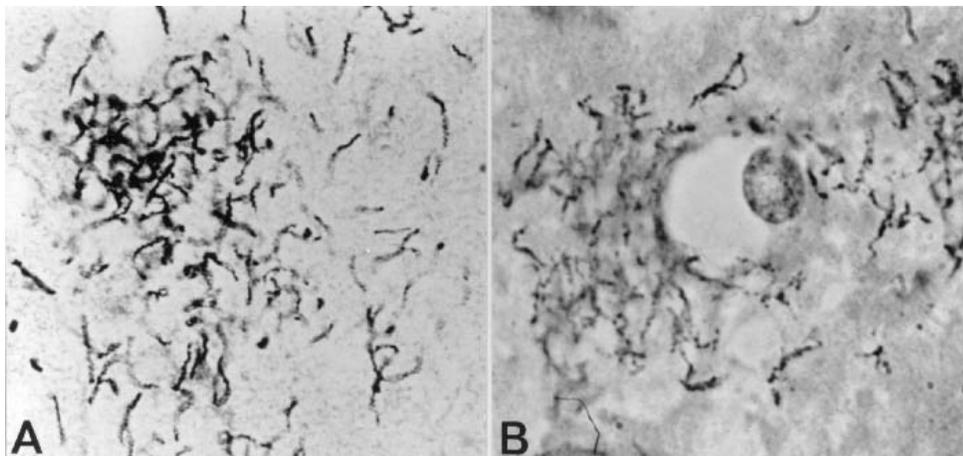


FIGURE 2: Illustration of the striking morphological similarity of a cortical spirochetal colony in a general paretic case and of a senile plaque in a sporadic Alzheimer's disease case when using the same magnification.

A: Illustration of Jahnel, showing accumulation of *Treponema pallida* in the cortex of a general paretic case [Schlossberger and Brandis, 1958]. B shows an amorphous senile plaque from a sporadic Alzheimer's disease case. Paraffin section taken from the temporal cortex was stained with the Bosma Steiner silver technique for spirochetes. The permission for the reproduction of Jahnel's illustration (A), reported by Schlossberger and Brandis in 1958, was kindly provided by Springer Verlag Publisher.

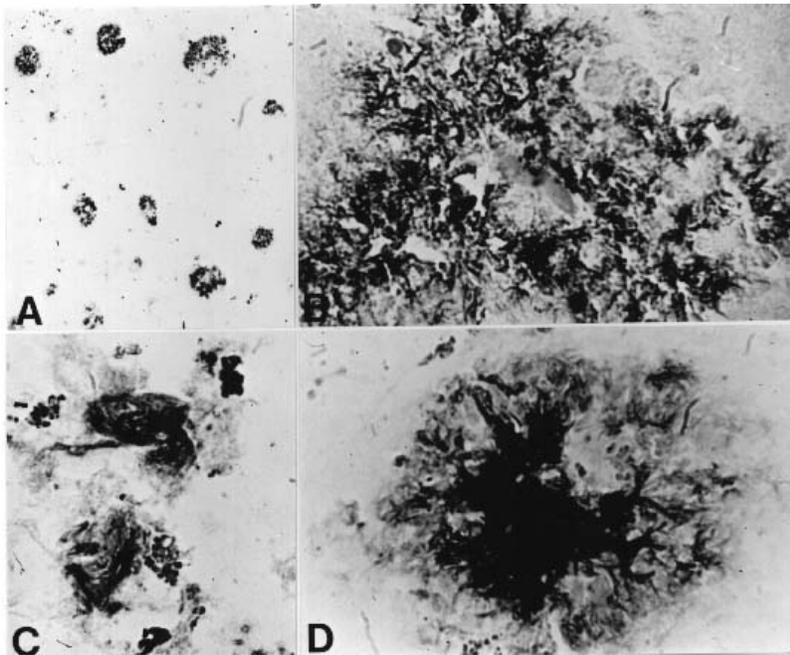


FIGURE 3: A-D: Photomicrographs illustrating the immunohistochemical localization of bacterial peptidoglycan in senile plaques of a familial AD and in neurofibrillary tangles of a sporadic Alzheimer's disease case.

A: Immunoreactivity of bacterial peptidoglycan in immature senile plaques with low magnification: X120. The photomicrograph was taken from a temporal frozen section of a familial Alzheimer's disease case, immunostained to bacterial peptidoglycan. B: Photomicrograph of an immature plaque showing immunoreaction to bacterial peptidoglycan. It was taken from the same case and from the same cortical region as A. Magnification: X1500. C: Immunostaining of neurofibrillary tangles of the temporal cortex in a case of sporadic AD. Magnification: X1200. D: shows the positive immunoreaction to bacterial peptidoglycan of a mature plaque. Same cortical region of the same familial Alzheimer's disease case as A and B. Magnification: X1200.

the 32 AD cases 28 cases were sporadic and 4 were familial. In 12 cases we have found only discrete AD-type changes, with low number of plaques, and there were 10 cases without any AD-type histological changes. The age of the AD cases varied between 28 to 81 years, those with discrete to mild AD type changes between 55 to 83 years and, of those without any AD-type changes between 34 to 77 years. Part of the material included in this study (related to 17 AD cases and 7 control cases) was the subject of a previous report [Miklossy et al., 1996].

Immunohistochemical analysis of bacterial cell wall peptidoglycan.

Two monoclonal antibodies, recognizing the 3D polymer complex structure of bacterial peptidoglycan were used for this study (Chemicon; dil:1:400 and Biogenesis; dil:1:800). For the production of the antibody TCA/heat and ethanol extracted peptidoglycan, obtained from *Streptococcus mutans* BHT cells, was used as immunogen [Jackson et al., 1983]. Before immunostaining to bacterial peptidoglycan the frozen sections were fixed in acetone for 10 minutes and were then treated with formic acid and sodium-dodecyl sulfate (SDS). The use of strong acid or detergent is necessary to expose the specific epitopes. The use of amylase (1%) treatment for 2-5 minutes at 37°C, enhanced immunostaining. For the immunohistochemical procedure, the avidin-biotin-peroxidase technique and as a secondary antibody the biotinylated F(ab)'s fragment of affinity

isolated rabbit anti-mouse immunoglobulins (DAKO, E413) were applied.

In order to analyze if the β -amyloid protein and the bacterial cell wall peptidoglycan is co-localized or not in senile plaques in AD, consecutive serial sections, were immunostained with monoclonal antibodies to β -amyloid protein and to bacterial peptidoglycan, respectively [Miklossy et al., 1996]. The smears of two Gram negative bacteria prepared from cultured *Escherichia coli* and *Borrelia spirochetes* (B31 strain of *Borrelia burgdorferi*) were used as positive controls for the immunohistochemical demonstration of bacterial peptidoglycan. Control negative sections, like immunostaining in omitting the primary antibody, or using an irrelevant monoclonal antibody, were also performed.

Immunoabsorbtion assay

To further test the specificity of the antibodies to bind bacterial peptidoglycan before immunostaining the monoclonal antibodies were immunoabsorbed using homogenized and sonicated bacteria (cultured *Escherichia coli* and B31 strain of *Borrelia burgdorferi*), but also TCA/heat and alcohol extracted bacterial peptidoglycan. The preparation of these antigens for immunoabsorbtion were performed as described previously [Miklossy et al., 1996]. Adjacent brain sections were immunostained at the same time without immunoabsorbtion.

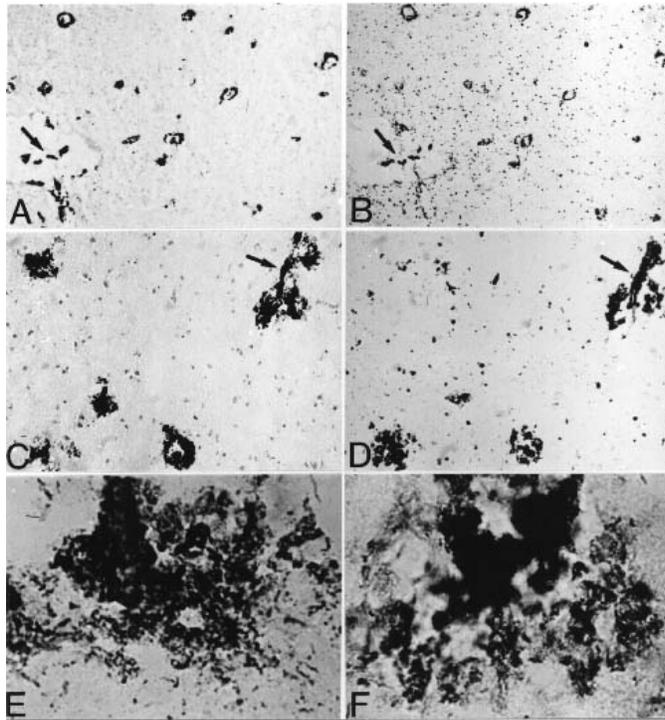


FIGURE 4: Illustration of the immuno-localization of bacterial peptidoglycan in senile plaques and intracortical and leptomeningeal vessel walls containing β -amyloid in the brains of a sporadic and a familial AD case. A: Photomicrograph taken from a frozen section of the temporal cortex of a sporadic Alzheimer's disease case immunostained with a monoclonal antibody to the β -amyloid protein. B: Same field as in A, but, from an adjacent frozen section spaced at 14 μm with respect to A, immunostained with a monoclonal antibody to bacterial peptidoglycan. Note de co-localization, in the same senile plaques and leptomeningeal vessel walls, of the β -amyloid and bacterial peptidoglycan. In A and B, the arrows point to leptomeningeal vessels showing positive immunoreaction. Magnification of A and B is the same and corresponds to X30. C and D: Photomicrographs taken from adjacent serial sections and from the same sporadic AD case as in A and B, but here with a higher magnification. C is spaced at 14 μm with respect to D. The β -amyloid protein C and bacterial peptidoglycan D are co-localized in the same senile plaques but also in the wall of the same small intracortical vessel. The arrows in C and D point to an intracortical vessel surrounded by perivascular senile plaques. Magnification of C and D: X120. E and F: show at higher magnification the immuno-localization of β -amyloid, E and bacterial peptidoglycan F in a senile plaque in the temporal cortex of a familial AD case. The photomicrographs E and F were not taken from consecutive serial sections but from the same region of the temporal cortex. Magnification of E and F: X1200. The permission for the reproduction of Figure 4 was kindly provided by the Rapid Science Publisher, London [J. Miklossy et al., 1996].

RESULTS

Bacterial cell wall peptidoglycan is immuno-localized to senile plaques and is co-localized with β -amyloid.

Positive immunoreaction of senile plaques to bacterial peptidoglycan was found in all the 32 AD cases including the four familial cases (Figure 3) but, also in the 12 cases with low number of senile plaques. Not only the mature (Figure 3D), immature (Figure 3B), but, also the diffuse plaques [Miklossy et al., 1996] exhibited positive immunoreaction to bacterial peptidoglycan. In addition, bacterial peptidoglycan immunostaining was also observed in the walls of some intracortical and leptomeningeal vessels, and in large part of the neurofibrillary tangles (Figure 3C). Positive bacterial peptidoglycan staining of small globular structures in some neurons without tangles was also observed. In agreement with our previous observation (see Figure 4) [Miklossy et al., 1996] on immunostained serial sections the β -amyloid protein and bacterial cell wall peptidoglycan were co-localized in senile plaques. In the cerebral cortex of the 10 cases, without AD-type histological changes, we did not find bacterial peptidoglycan accumulations. Smears of bacteria of cultured *Escherichia coli* and *Borrelia burgdorferi* showed a positive immunoreaction with antibodies to bacterial peptidoglycan. The negative control sections, where the primary antibody

was omitted, or an irrelevant monoclonal antibody was used during immunostaining were all negative. After immunoabsorption with homogenized and sonicated bacterial debris or with TCA/heat and alcohol extracted cell wall peptidoglycan, the bacterial peptidoglycan antibodies did not label senile plaques, tangles or vessel walls. Adjacent sections proceeded at the same time, without immunoabsorption, were always positive.

DISCUSSION

Similarly to host derived GAGs, bacterial peptidoglycan is immuno-expressed in senile plaques at an early stage of amyloid formation.

Positive immunoreaction of senile plaques to bacterial peptidoglycan was found in all sporadic and familial AD cases and also in 12 cases with low number of senile plaques. The brains of 12 cases without any AD-type changes were free of plaque-like bacterial peptidoglycan accumulations. As previously reported, on serial sections bacterial peptidoglycan is co-localized with β -amyloid in senile

plaques and in vessel walls in cases with severe AD-type changes [Miklossy et al., 1996], but also in cases with low number of plaques. This would suggest that the process of β -amyloid formation is similar in both conditions.

The immuno-localization of bacterial peptidoglycan in immature, mature, and also in diffuse plaques suggests, that similarly to the GAGs, it is present at an early stage of amyloid formation, before glial reaction, cytokine expression and fibrillar, Congo Red positive, amyloid formation.

Some recent reports claim that bacteria contain amyloidogenic proteins or their precursors [Jarrett and Lansbury, 1992; Miklossy, 1993]. A periplasmic outer membrane associated lipoprotein of the *Escherichia coli* resembles that of the C-terminal region of the β -amyloid protein of AD. This peptide called OsmB was shown to form Congo red positive amyloid fibrils in vitro [Jarrett and Lansbury, 1992].

Specificity of the antibodies.

The monoclonal antibodies against bacterial peptidoglycan we used for this study recognize the three-dimensional polymer complex structure of peptidoglycan. The epitopes appear to consist of discontinuous glycan and/or amino acid residues [Jackson et al., 1983]. The muramic acid and the amino acid sequences of the peptide core, containing D-Alanine is found only in bacterial cells. In addition, the three dimensional structure of bacterial peptidoglycan is a highly specific configuration for bacteria. The antibodies to bacterial peptidoglycan after immunoabsorption by TCA/heat and alcohol extracted bacterial peptidoglycan did not label neuritic plaques. These findings and previous observations, that in a competitive immuno-assay the N-acetyl-glycosamine was found to be ineffective as inhibitor strongly suggest, that a cross reactivity between the bacterial peptidoglycan and host derived GAGs is improbable. To further analyze the presence of the biologically active bacterial peptidoglycan in senile plaques, molecular biological studies were undertaken.

A possible relationship between the accumulation of bacterial peptidoglycan and host derived GAGs in senile plaques.

A role for highly sulfated proteoglycans (HSPG) in the Major Histocompatibility Complex (MHC) mediated infections, like bacterial infections is well established [Love et al., 1993; Price et al., 1995]. Bacteria possess high affinity receptors to bind proteoglycans and the degree of their virulence depends on their ability to bind sulfated proteoglycans [Parsons et al., 1988; Winters et al., 1993]. The in vitro and in vivo synthesis of sulfated proteoglycans

by host cells in response to bacterial infections has been repeatedly reported [e.g. Strugnell et al., 1988]. The reactive, soluble extracellular matrix (ECM) proteoglycans, via competitive inhibition, are very effective to prevent bacterial binding to membrane proteoglycans. Soluble proteoglycans act as nonspecific antiadherence factors blocking access of bacteria to host cells [Parson and Hurst, 1990].

Recent reports claim that secreted truncated beta-amyloid precursor protein (β -APP) and 50% of the mature cell-associated full length β -APP is in a proteoglycan form [Schubert et al., 1988, 1989] and that one of the biological activities of the soluble form of β -APP is its neuroprotective function.

Biological activities of bacterial peptidoglycan

In addition to the induction of synthesis of proteoglycan by host tissue, it is well established that the synthetic and natural components of bacterial peptidoglycan have a variety of biological actions in mammals. They activate complement of the classic pathway, affect vascular permeability, generate nitric oxide, induce apoptosis and inflammatory cytokines, in addition, they are amyloidogenic. All these processes are implicated in AD. This suggests that bacteria even in the form of nonviable bacterial remnants may well trigger the cascade of events leading to amyloid deposition in AD. If further investigations reinforce the observations that bacteria or their debris are present in senile plaques, the question would arise whether the interaction of biologically active bacterial components and of host derived soluble β -APP may lead to amyloid deposition in AD.

In conclusion, the present and previous findings show that bacterial peptidoglycan is immuno-localized to senile plaques in AD and is co-localized with β -amyloid protein. This suggests, that β -amyloid and GAG accumulation in AD may be related to a chronic inflammatory stimulus. This would be in agreement with the observations made on the immune-system responses in AD, indicating that senile plaques are the sites of a chronic inflammation. Even if bacteria or bacterial remnants may only play a partial role in the pathogenesis of AD, this would open the possibility of additional therapeutic approaches.

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