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COMPLEMENT INHIBITOR C4-BINDING PROTEIN IN AMYLOID DEPOSITS CONTAINING SERUM AMYLOID P IN ALZHEIMER'S DISEASE

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SUMMARY: Complement C4-binding protein (C4BP) is a plasma protein that complexes with serum amyloid P and is involved in inhibition of the complement cascade. We used immunochemical methods to demonstrate C4BP in brains of subjects with Alzheimer's disease and normal controls. C4BP antibodies stained cerebral amyloid deposits that were indistinguishable from those recognized by antibodies to serum amyloid P or by thioflavin S. Immunoblots of solubilized proteins from cerebral cortex, cerebral microvessels and cerebrospinal fluid showed specific antibody reactivity to 70-90 kDa bands. Our results suggest the involvement of complement regulatory proteins in the pathogenesis of Alzheimer's disease.

Current evidence suggests that cerebral amyloid deposits characteristic of Alzheimer's disease (AD) are sites of localized chronic inflammatory response which does not resemble acute inflammation. These extracellular deposits characterized by A4/ß protein have been found to contain a number of acute phase proteins (1) including complement proteins (2-4), the serpins (5-7), α_2-macroglobulin (8), inter-α-trypsin inhibitor (9) and serum amyloid P (SAP) (10-13). Moreover, the classical complement pathway which is largely confined to adaptive immune mechanisms is activated in the lesions (4). Thus far, it is unclear to what extent are the complement regulatory proteins involved in these deposits.

Complement C4-binding protein (C4BP) is a high molecular weight (570-590 kDa) heptameric molecule with a spider-like configuration (14). It belongs to a family of proteins characterized by short consensus repeats, SCRs (15) that are encoded by closely linked genes on chromosome 1. C4BP and the other members of the family (15) have in common the function of inhibiting the stable formation of C3 convertase, C4b2a, of the classical complement pathway. C4BP is thought inhibit the cascade by binding to C4b, promote dissociation of C2a and act as a cofactor for factor I, another serine protease, which degrades C4b into two components (16). Recent studies indicate that among other complement proteins C4BP specifically interacts with SAP now known to be localized in all amyloid deposits (17). C4BP also complexes with protein S (17) and is known to be deposited in glomeruli of patients with immune complex nephritis (16). Here, we present the first evidence for immunolocalization of C4BP in cerebral amyloid and compare its distribution with that of SAP in brains of subjects with AD.

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MATERIALS AND METHODS

Tissue: Brain tissue was obtained at autopsy from a total of 12 subjects. In addition, cerebrospinal fluid was obtained from 2 subjects diagnosed with AD. Table 1 gives details of subjects grouped by age and the neuropathological diagnoses. Tissue blocks of about 1 cm^3 were fixed by immersion in buffered formalin, transferred to sucrose and cut into coronal sections of 20-25 micron thickness, as described previously (12,13). In some cases, 12 micron flash frozen sections that were post-fixed in acetone were used (6).

Immunocytochemistry: Tissue sections were incubated with either a rabbit (Calbiochem; dilution 1:1,000) or sheep (The Binding Site; 1:500) polyclonal antiserum against purified human C4BP. Adjacent sections were incubated with antiserum to SAP (Dako; 1:1000) or amyloid A4/ß (1:100). Primary antibody bound antigens were then detected using the avidin-biotin complex (ABC) method (13). Previously immunostained or unstained adjacent tissue sections from each case were also stained with thioflavin S to reveal the amyloidotic pathology. To demonstrate specificity of staining, control sections were incubated with either irrelevant antibodies or preimmune serum and in the absence of primary antibody. Further specificity was ascertained by immunostaining sections after absorbing the primary antibody with normal human serum or SAP.

Immunoblotting: Immunoblots of solubilized proteins from the cerebral cortex, cerebrospinal fluid (CSF) and cerebral microvessels (Figure 1) were prepared as described previously (13). Immunoreactivity to these proteins transferred on to PVDF membranes (Millipore) was detected using the electrochemical luminescence (ECL) Western blotting system purchased from Amersham Inc. We found the ECL method to be highly sensitive in comparison to the more conventional peroxidase-diaminobenzidine method. To evaluate specificity of the antibodies, immunoblots were probed with antibodies absorbed with normal human serum (Figure 1) or incubated with preimmune serum. The antibodies were incubated (4°C for 11-16 hours) in ratio of 1:10 with human serum and used as primary antibody. Under reducing conditions, C4BP antibodies (1:2000, Calbiochem) reacted with approximately 80-90 kDa size proteins in homogenates of the cerebral cortex and the CSF and to about 70-75 kDa in cerebral microvessels (Figure 1). The ECL method also gave staining of lower molecular weight (MW) bands which were resolved to be non-specific (Figure 1). However, these immunoblotting results showing presence of C4BP in brain and CSF are in agreement with previous reports on C4BP purified from human plasma (14,15).

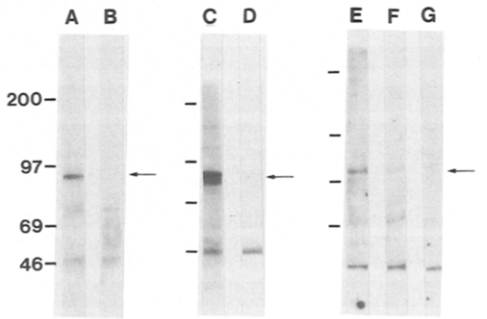

Figure 1. Immunoblots of proteins extracted from frontal cortex (lanes A,B), CSF (C,D) and cerebral microvessels (E,F,G). All samples analysed were from AD subjects. C4BP immunoreactivity (arrows) was detected in 80-90 kDa proteins in cortex and CSF (A and C) with a slightly lower MW of approx. 75 kDa in cerebral microvessels (E). Specificity of these bands was shown in blots immunoreacted with human serum absorbed antibody (lanes B, D and F) or preimmune serum (G). MW markers (shown in kDa) are same for all three sets of blots.

RESULTS AND DISCUSSION

Immunocytochemical staining revealed that C4BP reactivity was evident in amyloid plaques recognized by thioflavin S or antibodies to A4/ß protein (Table 1 and Figure 2). The distribution of immunoreactivity was remarkably similar to that of SAP in adjacent sections and the same amyloid lesions could be recognized in almost all cases (Figure 2a and b). In many samples extracellular tangles and cerebral capillaries were also stained (Figure 2d). However, the staining of intracellular tangles was rare. While the antibody from Calbiochem was clearly superior, the other antibody (sheep) often gave variable results. In contrast to the striking staining of amyloid deposits in AD, there was only diffuse vascular staining evident in cortical tissues from normal controls (Table 1). In these control brains, staining of all vessels was not evident but only those that presumably had residual plasma and bound protein. Sections incubated with preimmune rabbit serum or absorbed with human serum showed no positive reactivity. To provide further specificity, we tested the C4BP antibody against native SAP protein. Clear immunostaining of plaques and tangles was still evident after absorption of C4BP antibodies with SAP (not shown). This indicated that C4BP and SAP were distinct proteins co-localized in amyloid deposits.

Our results suggest the presence of C4BP in brain amyloid deposits and extracellular tangles that typically characterize AD. C4BP is a regulatory protein responsible for binding to C4b and impede C3 convertase, C4b2a, which cleaves C5 to initiate the lytic pathway resulting in insertion of the membrane attack complex, C5b-9 into cell membranes (15,16). A number of molecules associated with the classical complement pathway and C5b-9 are now recognized to be expressed in brain amyloid lesions associated with AD (2-4). The increased expression of C4BP evident here suggests the operation of inhibitory mechanisms which may interfere with establishment of the membrane attack complex during the pathological condition. Our findings may also have import to recent observations (18) showing expression of CD59 (or protectin), another complement inhibitor, in cerebral amyloid deposits.

Table 1. Subjects by age, Neuropathological diagnoses and C4BP expression

Case	Age (yrs)	Sex	PMI (hr)	Diagnosis	Amyloid deposition	C4BP immunostaining SP	NFT	Vessel
1	62	M	3	AD	+++	+++	+++	+
2	68	M	3	AD	+++	+++	++	++
3	69	F	4	C	-	+	-	+
4	72	F	6	AD	+++	+++	+	+++
5	72	F	10	AD	+++	+++	+++	+++
6	74	F	9	C	+	-	-	++
7	75	F	5	AD	+++	+++	+	++
8	78	F	4	C	-	-	-	+
9	79	F	5	AD	+++	+++	+	++
10	85	F	5	AD	+++	+++	-	++
11	86	F	5	AD	+++	+++	++	++
12	93	F	3	AD	++	++	-	+++

Abbreviations: PMI-postmortem interval; SP-senile plaques; NFT-neurofibrillary tangles; Vessel-vascular staining. Amyloid deposition was assessed by thioflavin S and A4/ß protein immunoreactivity. Causes of death included bronchopneumonia (90% of cases), congestive heart failure and internal hemorrhage. + to +++ denotes increasing frequency. Staining was assessed in frontal, temporal, entorhinal and occipital cortices. In addition, CSF was obtained from 2 AD subjects: a 69-year-old man and a 76-year-old woman.

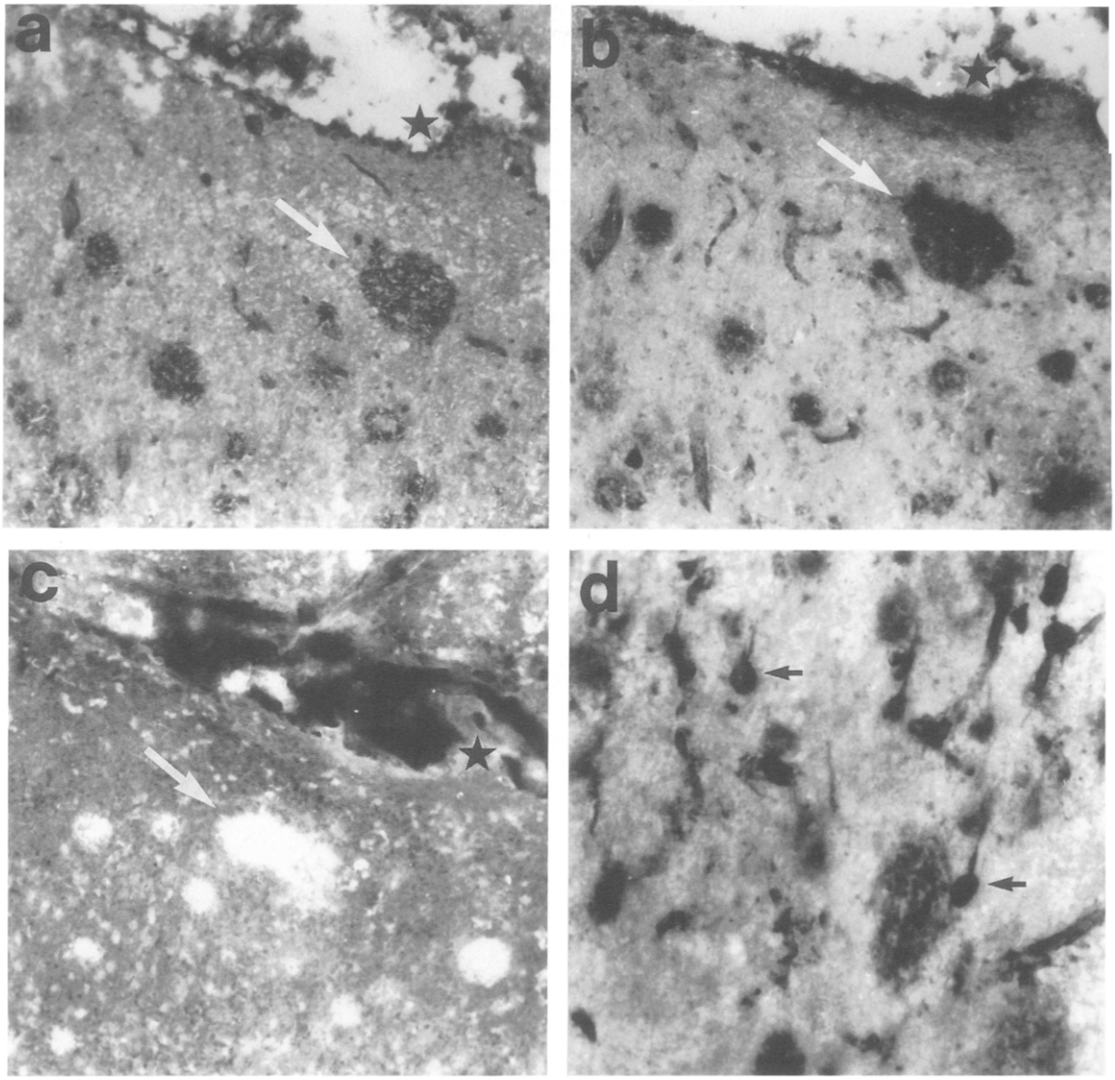

Figure 2. Immunocytochemical localization of C4BP in temporal cortex from a 68-year-old AD subject. Adjacent sections (★) show same amyloid deposits (arrows) stained by antibodies to C4BP (a) and SAP (b), and by thioflavin S (c). C4BP immunoreactivity (d) was also evident in extracellular tangles (small arrows). Magnification x105 (a-c) and x210 (d).

In light of previous evidence indicating strong binding between C4BP and SAP (17), and between C4BP and protein S (or vitronectin) these observations may implicate there is close interaction between the complement proteins and SAP during the pathogenesis. While its role in brain amyloidosis is not clear, it is possible that under certain conditions SAP is involved in complement fixation or some other related function. However, C4BP could also be interacting with protein S which by itself appears to inhibit the insertion of the membrane attack complex and is now evident to be highly expressed in amyloid deposits and vessels in brains of AD subjects (19). It is plausible that localization of complement regulatory proteins such as C4BP and others (18-20) including CD59 and protein S implies that various counteractive responses are increased in concert possibly to prevent further cortical cell membrane lysis (18).

The source of C4BP in brain is not yet clear. Although, like SAP (21), C4BP may originate in the liver via the circulation (16), it is likely that C4BP is produced locally by astrocytes or perhaps neurons. This latter possibility is consistent with recent findings in our laboratory (22) and by others (18,20) that mRNAs of complement proteins including C3 and C4 are expressed in human brain as detected by the polymerase chain reaction technique. Nevertheless, whatever the origin of C4BP, the released protein appears to be involved in the pathogenesis of cerebral amyloid deposition.

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