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Antibodies to the β-amyloid peptide cross-react with conformational epitopes in human fibrinogen subunits from peripheral blood

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Antibodies to the Alzheimer disease (AD) β-amyloid peptide (βAP) were used to identify βAP precursor fragments in blood. The antibodies detected 3 major polypeptides with apparent molecular weights (MW) of 47–64000 in Western blots of plasma derived clot proteins, but these proteins corresponded to human A-α, B-β and γ-fibrinogen since they reacted with 2 different anti-fibrinogen antisera, and the anti-βAP and anti-fibrinogen antibodies recognized purified fibrinogen and fibrin. These data are significant for efforts to develop immunochemical assays to diagnose and monitor the progression of AD.

Alzheimer's disease; A4 protein; β-Amyloid precursor; Fibrin; Plasma

1. INTRODUCTION

Cerebral amyloid deposits in the core and corona of senile plaques (SPs) and in blood vessels (BVs) are prominent findings in Alzheimer's disease (AD) and a number of other neurodegenerative disorders [1–3]. The major peptide in SPs and BVs is a recently described amyloidogenic protein fragment with an apparent molecular weight (MW) of 4000–5000 called the β-amyloid peptide (βAP) or A4 protein [4–6]. The βAP appears to be derived from one or more of at least 3 distinct precursor proteins with predicted amino acid (aa) lengths of 695, 751 and 770 aa, respectively [7–10]. The predicted aa sequence of each of these β-amyloid precursor proteins (βAPP), i.e. βAPP_{695}, βAPP_{751} and βAPP_{770}, is consistent with that of a membrane-associated glycoprotein resembling a cell surface receptor protein, and/or several different protease inhibitors [7–13]. The mRNAs encoding each βAPP are present in both neural and non-neural cells, however the smallest βAPP is much more abundant in brain than in other tissues [1,2,7–15]. Thus, it is conceivable that one or more of the βAPPs in peripheral tissues might undergo fragmentation to release the βAP into peripheral blood. Since the excess deposition of β-amyloid fibrils in AD might reflect altered proteolysis of these βAPPs outside as well as within the brain, efforts have been made to identify and characterize βAPP fragments that might circulate in peripheral blood and subsequently enter the brain [16]. We report here on the characterization of low MW polypeptides, initially detected with anti-βAP antibodies in normal human peripheral blood, which we then identified as human fibrinogen subunits. Specifically, a polyclonal antiserum and a monoclonal antibody (MAb) previously raised to a peptide based on the first 28 aa of the βAP (AMY^{1-28} peptide), and shown to bind SPs and BV amyloid [17], recognized circulating human fibrinogen in peripheral blood and in the clots formed from plasma and purified fibrinogen. The immunobands detected by these 2 anti-βAP antibodies were identical to the triad of fibrinogen proteins recognized by 2 different anti-fibrinogen antisera, and the latter antisera also were shown to bind the AMY^{1-28} peptide.

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2. MATERIALS AND METHODS

2.1. *Generation of monoclonal and polyclonal antibodies*

Rabbit and mouse anti-βAP antisera and a panel of mouse anti-βAP MAbs were generated and screened using a synthetic βAP peptide designated here as AMY^{1-28}. The immunochemical and immunohistochemical properties of these antibodies have been described in detail elsewhere [17]. Purified human fibrinogen and an antiserum specific for human fibrinogen were purchased from Sigma Chemical Company. Another antiserum to human fibrinogen was purchased from Accurate Chemicals.

2.2. *ELISA, gel electrophoresis and immunoblotting*

Procedures for ELISA, gel electrophoresis and Western blotting have been described extensively in past publications [17–21].

2.3. *Preparation of plasma, serum and blood clot proteins*

Blood was collected in blue top Vacutainer tubes (Becton Dickinson) containing sodium citrate. These tubes are used to harvest

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samples for studies of the coagulation properties of human blood. The samples of human blood were then spun in a clinical centrifuge to separate plasma from cellular components, and EDTA was added to the plasma fraction to achieve a 1 mM final concentration of EDTA. Serum was prepared from aliquots of the plasma fraction by the addition of bovine thrombin (Sigma Chemical Company) in a dropwise manner until a clot formed, and the serum was separated from the clot by centrifugation in a microfuge for 3 min. To obtain purified clot proteins, EDTA was added to an aliquot of plasma to a final concentration of 5 mM, followed by the addition of approximately 100 μl of bovine thrombin to 750 μl aliquots of plasma. Alternatively, thrombin was added in a dropwise manner to a solution of purified fibrinogen (2 mg/ml) to induce clot formation. The formed clot was placed on a fine nylon mesh overlaid on a filter paper to withdraw residual serum, and the clot was washed in 20 ml of PBS. The clot was then gently lifted from the mesh with a forceps and homogenized in 750 μl of sample buffer and boiled for 10 min prior to electrophoresis. Sample buffer also was separately added to equal volumes of serum and plasma aliquots, and each was boiled for 10 min prior to electrophoresis. Typically, 6–10 μl of these samples were run in each lane of a 15 well comb.

2.4. *Analysis of human brain tissue and rat pheochromocytoma (PC12) cells*

Samples of normal human, AD and Down's syndrome brain tissue as well as PC12 cells [21] also were examined with the anti-βAP and anti-fibrinogen antibodies in Western blots using methods similar to those reported elsewhere from this laboratory (see [17–21] and citations therein). The diagnostic assessment of the human brain tissues has been described [17–20].

3. RESULTS AND DISCUSSION

The anti-AMY$^{1-28}$ antisera and 12 MAbs raised to AMY$^{1-28}$ were screened for reactivity against normal human plasma by Western blot analysis. One of the antisera and 4 of the MAbs recognized a complex of intensely labeled immunobands in plasma. The antiserum and one of the MAbs (an IgG1 designated AMY 33) were selected for the further characterization of these immunobands. As described previously, both this antiserum and the AMY 33 MAb strongly stained AD and control SPs and BV amyloid deposits, and both were specific for AMY$^{1-28}$ by ELISA, but failed to bind two unrelated peptides [17]. By Western blot analysis, several immunoreactive bands were demonstrated in normal human plasma from volunteers in the 3rd through 6th decades of life. In clot prepared from the plasma, the βAP antibodies detected 3 prominent immunobands with a MW of 45000–64000 that were not present in serum generated from the same plasma fraction. In Coomassie blue stained gels of the clot, 3 discrete protein bands with apparent MWs of 45000–64000 were among the most abundant species noted (Fig. 1A, lanes 1 and 2), and all 3 of these polypeptides were immunoreactive with the anti-βAP MAb (lanes 1 and 2 in Fig. 1B) and the antiserum, although the antiserum recognized these proteins to a lesser extent than the anti-βAP MAb (data not shown). These findings led us to conclude that the immunoreactive species identified here were involved in clot formation, especially since they were removed completely

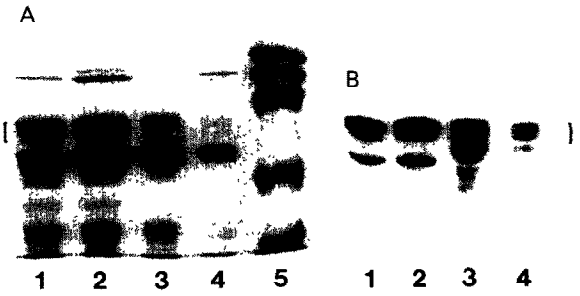

Fig. 1. Photographs of plasma derived clot samples (lanes 1 and 2 in A and B), purified human fibrinogen (lanes 3 in A and B) and clot proteins derived from purified human fibrinogen (lanes 4 in A and B) that were separated by SDS-PAGE and stained with Coomassie blue (A) or transferred to nitrocellulose and probed with AMY 33 (B). The MW standards shown in lane 5 of panel A include, from top to bottom, β-galactosidase, phosphorylase B, bovine serum albumin, egg albumin and carbonic anhydrase.

from serum during the clotting process. Notably, only a few plasma proteins exhibit these properties, among which fibrinogen is the most abundant plasma clotting factor (200–400 mg/100 ml of plasma) (see [22–24] and additional references therein). Further, fibrin, which is derived from plasma fibrinogen as a result of thrombin mediated cleavage of small fibrinopeptides from intact fibrinogen, is the principal clot protein. Accordingly, we then compared the immunobands produced by the anti-βAP antibodies with those detected by two different anti-fibrinogen antisera in similar Western blots of human plasma derived blood clots. Not surprisingly, the anti-fibrinogen antibodies produced a triplet of immunobands that were identical to those produced by the anti-βAP antibodies when they were used to probe the same clot proteins (data not shown). A commercial preparation of human fibrinogen was then used as a test antigen in Western blots, and both the anti-AMY$^{1-28}$ antiserum and the MAb labeled three prominent immunobands with an apparent MW of 47000, 56000 and 64000 (see lane 3 in Fig. 1B) exactly like both of the anti-fibrinogen antisera. Finally, clots prepared from the purified human fibrinogen by the addition of the serine protease thrombin also contained 3 similar bands that were labeled by both anti-βAP antibodies (see lane 4 in Fig. 1B).

Human fibrinogen polypeptides are among the most thoroughly characterized of human proteins, and they are known to be composed of 3 subunits referred to as the A-α, B-β and γ subunits. By SDS-PAGE, they have apparent MWs of 47000, 56000 and 63500, respectively [22–24]. Thus, the MWs of the bands detected in Western blots of the clot proteins and in the fibrinogen blots with the anti-βAP and anti-fibrinogen antibodies were identical to those ascribed to each of the 3 human fibrinogen subunits. Although the aa sequences of these fibrinogen subunits differ, they are highly homologous [22–24]. Thus, it is not surprising that the

AMY^{1-28} antiserum and the AMY 33 MAb recognized all 3 of these fibrinogen subunits rather than only one or two of them.

In order to quantitate the relative affinity of these cross-reactions, ELISA was performed using serial dilutions of the AMY^{1-28} peptide and purified fibrinogen. In these studies, dilutions of test antigen ranging from 0.005 to 5.0 μg/well were applied to different wells of 96 well ELISA plates. Subsequently, various dilutions of the AMY 33 MAb (ranging from undiluted supernatant to dilutions of 1:100) as well as of the anti-βAP and anti-fibrinogen antisera (i.e. 1:100, 1:1000, 1:10000) were applied to the ELISA plates. ELISA was recorded as positive if an optical density higher than background (i.e. >0.10) was obtained at a wavelength of 450 nm as described elsewhere [17–21]. The undiluted AMY 33 supernatant produced a positive ELISA against 0.02 μg of the AMY^{1-28} peptide, whereas >60-fold more (i.e. at least 1.25 μg) of the purified fibrinogen sample was needed to achieve a positive ELISA with the same MAb used under the same conditions. Further, at a dilution of 1:100, the AMY 33 MAb was able to detect as little as 0.75 μg of the AMY^{1-28} peptide, but it failed to yield a positive ELISA result even when tested using 5.0 μg of the purified fibrinogen sample. The anti-βAP antiserum did not recognize human fibrinogen at the dilutions used here, e.g., at a dilution of 1:1000 the antiserum detected 0.02 μg of the AMY^{1-28} peptide, but did not bind any amount of fibrinogen. As might be anticipated, the anti-fibrinogen antisera exhibited a higher affinity for purified fibrinogen than for the βAP peptide. For example, at a dilution of 1:100 the anti-fibrinogen antisera detected 0.625 μg of the AMY^{1-28} peptide, but the same amount of fibrinogen yielded a positive ELISA when the same antisera were used at a dilution of 1:10000. These results are summarized in Table I.

Additional immunoblot studies were conducted with the AMY^{1-28} antibodies and the anti-fibrinogen antisera to determine if similar immunobands were detected by both types of antibodies in homogenates of normal human, AD or Down's syndrome brain tissues, or in the rat PC12 cells. The AMY 33 MAb and all 3 antisera produced a highly complex but similar profile of immunobands, and at least 4 roughly equivalent immunobands were detected by these antibodies in the preparations described above (compare Fig. 2A and 2B).

The specificity of the cross-reaction between AMY 33 and fibrinogen was further substantiated in control experiments wherein we absorbed out the reactivity of AMY 33 for the βAP and senile plaque amyloid with the AMY^{1-28} peptide, as described earlier [17], and showed that the absorbed MAb failed to recognize fibrinogen (data not shown). In addition, 3 other MAbs (RMO93, RMS12, RMO55) with an IgG1 isotype that were raised to and shown to be specific for neurofilament proteins [18,19] did not bind to fibrinogen (data not shown). Finally, other polyclona antisera specific for components of senile plaques (i.e. paired helical filament antigens, α-1-anti-chymotrypsin) also did not demonstrate any affinity for fibrinogen (data not shown).

Table I

Test antigen	AMY 33 MAb		AMY AS 1:1000	Anti-fibrinogen AS	
	Undiluted	1:100		1:100	1:10000
AMY^{1-28}	0.02	0.75	0.02	0.625	5.0
Fibrinogen	1.25	NR	NR	0.01	0.625

This table summarizes the quantitative ELISA data on the relative affinity of the anti-βAP and anti-fibrinogen antibodies for the AMY^{1-28} peptide or purified human fibrinogen as described in the text. The lowest amount (in μg) of AMY^{1-28} or fibrinogen detected by the AMY 33 MAb, the AMY^{1-28} antiserum (AS) or the anti-fibrinogen antiserum at the dilutions listed below each antibody are shown here. NR, no reactivity with as much as 5.0 μg of the purified fibrinogen

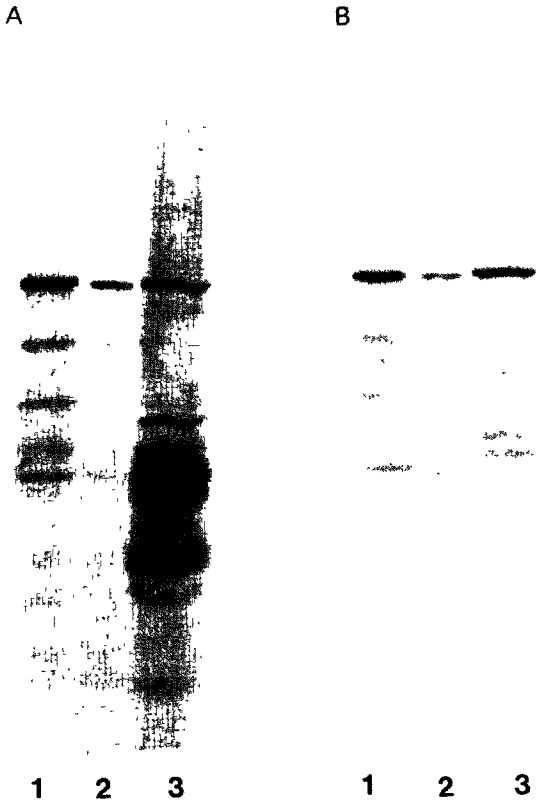

Fig. 2. Western blots of Triton X-100 soluble (lanes 1 in A and B) and insoluble (lanes 2 in A and B) homogenates of PC12 cells, as well as a Triton X-100 insoluble fraction from a Down's syndrome brain homogenate. The blots were probed with an anti-fibrinogen antiserum diluted 1:1000 (A) or with the anti-βAP antiserum at a dilution of 1:100 (B). Note that the banding pattern is not identical in both blots, but a number of immunobands in each lane display the same electrophoretic mobility.

The studies reported here provide evidence for a significant cross-reaction between anti-βAP antibodies and intact fibrinogen, as well as for the relatively large fibrinogen-derived cleavage products (i.e. fibrin proteins) in blood clots. In addition, we demonstrated a reiprocal cross-reaction between anti-fibrinogen antibodies and a βAP peptide, i.e. the AMY^{1-28} peptide. Although the AMY33 MAb recognized purified fibrinogen and crude fibrin prepared from human plasma in both ELISA and Western blot analyses, the anti-AMY^{1-28} polyclonal antiserum recognized fibrinogen in plasma and clots far less intensely in Western blots and it failed to detect fibrinogen in ELISA. In contrast, the anti-fibrinogen antiserum recognized the AMY^{1-28} peptide by ELISA, and, like the anti-AMY^{1-28} antiserum and MAb, it produced similar immunobands in homogenates of human brain tissues as well as in homogenates of PC12 cells.

Although 'molecular mimicry' among unrelated polypeptides with homologous or identical aa sequences is well known [25], the cross reactions described here do not reflect aa sequence identity between the βAP and fibrinogen. They also are unlikely to reflect homologies between epitopes that result from similar posttranslation modifications such as glycosylation and/or phosphorylation because the putative sites at which the βAPPs are glycosylated and phosphorylated lie outside the βAP or A4 region of these precursor proteins [7–10,26]. Further, the synthetic AMY^{1-28} peptide is neither a glycopeptide nor a phosphopeptide. Therefore, we ascribe these cross-reactions to conformational homologies between the βAP and fibrinogen. Since the secondary structure of the βAP peptide studied here has been well characterized by circular dichroism [27], and a similar peptide has been shown by electron microscopy to form amyloid-like fibrils [28], the underlying structure recognized by the antibodies described here may closely approximate the conformation that the AMY^{1-28} peptide has been proposed to assume in a microenvironment which promotes hydrogen bond formation (i.e. α-helix/β-turn/unordered/β-sheet/unordered) [27]. Regardless of the molecular basis for these cross-reactions, the observations reported here are especially significant for future efforts to develop immunological assays to diagnose or monitor the progression of AD using peripheral blood as well as neural and non-neural tissues and fluids [16,29]. Such efforts could be impeded if anti-βAP and/or anti-βAPP antibodies cross-react not only with fibrinogen, but also with other circulating human peripheral blood proteins because such cross-reactions would obscure potential differences in the levels of circulating βAPPs or βAPP peptides when control and AD subjects are screened. Indeed, such cross-reactions may account for the absence of differences in circulating plasma levels of βAPPs in a previous study of controls and patients with AD and Down's syndrome using radioimmunoassay and antisera raised to a carboxy terminal βAPP peptide [16].

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