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PLATELETS ARE THE PRIMARY SOURCE OF AMYLOID β-PEPTIDE IN HUMAN BLOOD

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SUMMARY - The main component of Alzheimer's disease (AD) amyloid deposits is amyloid β-peptide (Aβ), a fragment of the larger amyloid precursor protein (APP). The cellular source of Aβ is not known, but a circulatory origin has been postulated. We studied human blood from healthy individuals and found that platelets account for almost 90% of the total anti-Aβ immunoreactivity detected in whole blood. Using reverse-phase HPLC, we identified a platelet peptide which corresponds to Aβ by three criteria: (a) it shares a retention time with the synthetic $A\beta_{1-40}$ peptide in two consecutive HPLC tests; (b) it interacts with two anti-Aβ antibodies in separate ELISAs; and, (c) its partial N-terminal amino acid sequence closely matches that of Aβ. The detection of this peptide in platelets indicates that, aside from the well-known non-amyloidogenic (secretory) pathway, the processing of APP in platelets from healthy individuals also involves an amyloidogenic pathway. These findings are consistent with the view that platelets are one of the major sources of Aβ in the circulation.

Amyloid deposits in the brain parenchyma and the walls of meningeal as well as cerebral blood vessels of Alzheimer's disease (AD) patients predominantly contain amyloid β-peptide (Aβ), a 4 kDa hydrophobic molecule which is part of the much larger amyloid precursor protein (APP) (1, 2). Current knowledge suggests that the proteolytic processing of APP comprises at least two pathways: (a) an extracellular non-amyloidogenic route whereby transmembrane APP is cleaved between residues 16 and 17, precluding the formation of intact Aβ (3); and, (b) an intracellular amyloidogenic route yielding C-terminal fragments which contain the Aβ sequence (4, 5). Although a number of cell types display the amyloidogenic Aβ-producing pathway (6, 7), the precise cellular origin of the Aβ deposited in the AD brain and cerebral blood vessels has not been identified (8). Neurons, astrocytes and microglia are known to express APP and to produce soluble Aβ; therefore, all of

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these cells are possible sources of Aβ in amyloid deposits. In addition, since various blood and endothelial cells express APP (9) and plasma exhibits Aβ immunoreactivity (7), the circulatory system has also been proposed as a potential source of Aβ (9). A prerequisite to eventual verification of this hypothesis, however, is to directly establish the existence of circulating Aβ and define which cellular constituent(s) of blood is capable of producing Aβ. Accordingly, this work was undertaken to determine which component(s) of human blood from healthy subjects contains Aβ. Our data further document the presence of Aβ in blood and point to platelets as one of the major sources of circulating Aβ.

MATERIALS AND METHODS

Human platelet packs were obtained from The Florida Central Blood Bank (Orlando, FL). A monoclonal antibody against $A\beta_{8-17}$ was purchased from Dako Corporation (Carpinteria, CA) and a polyclonal antibody against $A\beta_{1-28}$ from Boehringer Mannheim (Indianapolis, IN). Horseradish peroxidase-conjugated second antibodies were from Sigma (St. Louis, MO) and the synthetic peptides $A\beta_{1-40}$, $A\beta_{1-16}$ and $A\beta_{1-28}$ from Bachem (Torrance, CA).

Preparation of Blood Cells - Blood samples were collected from healthy donors in citric acid anticoagulant buffer. Erythrocytes, leukocytes and platelets were separated by means of differential centrifugation following standard methods (10). Cells were washed 3 times in phosphate-buffered saline, counted on a hematocytometer, and solubilized with 80 % ethanol. For the evaluation of Aβ immunoreactivity, cell extracts were centrifuged at 8,000 g for 10 min and 100 µl of each supernatant were coated in separate wells of an ELISA microtiter plate. In turn, plasma was further centrifuged at 25,000 g for 20 min prior to coating so as to avoid cell contamination.

Extraction of Aβ from Platelets - Aliquots of washed platelets were extracted with various solvents including acetone: 12 N HCl : H_2O (40 : 1 :5), 1% Triton X-100 followed by boiling, 5 M urea, acetonitrile : trifluoroacetic acid : H_2O (50 : 0.1 : 49.9), or 80% ethanol. The most effective extracting agent was ethanol in that it precipitated most of the large proteins while preserving the highest Aβ immunoreactivity. Acetonitrile/trifluoroacetic acid was almost as effective as ethanol, whereas the other solvents tested preserved many more proteins, resulting in low ELISA/protein ratios. Accordingly, all subsequent experiments were performed using 80% ethanol as solvent. The extracts were microcentrifuged and the supernatants normalized to equal protein concentration. The Aβ immunoreactivity in 100 µl of each extract was measured by ELISA.

Identification of Aβ by Reverse Phase-HPLC - Fresh platelet-enriched plasma samples (Florida Central Blood Bank) were centrifuged at 1200 g for 20 min and washed three times in 145 mM NaCl, 5 mM KCl, 3 mM EDTA, 20 mM Tris-HCl, pH 7.4 buffer (11). Washed platelets were extracted with 80 % ethanol and stored at -70°C for later use. Ethanol in the platelet extract was removed by exposing the extract to a stream of nitrogen at room temperature. Aβ-like peptide was progressively separated by two successive chromatographic runs. The procedures were modified from those employed for the purification of neuropeptide hormones (12). In the first run, the extract was cleaned by microcentrifugation and loaded on a C-4 reverse phase HPLC analytical column (4.5 x 250 mm, particle size 6.5). The mobile phase was: buffer A, consisting of 1% trifluoroacetic acid in H_2O; and buffer B, comprising 80% acetonitrile and 20% buffer A. The gradient was linear from 20 to 90% of buffer B over 50 min. The elution was carried out at a flow rate of 0.8 ml/min, and fractions were collected and analyzed by two independent ELISA analyses using anti-$A\beta_{1-28}$ and anti- $A\beta_{8-17}$, respectively. Fractions giving positive immunoreaction and eluted at a retention time similar to that of the synthetic $A\beta_{1-40}$ peptide were collected from separate chromatographic runs and pooled for later analyses. The $A\beta_{1-40}$ synthetic peptide was chromatographed separately under identical conditions. In the second HPLC run, pooled fractions from the first run were diluted five times with buffer A, and

loaded onto a C-18 analytical column (4.5 x 250 mm, particle size 6.5 mm) directly through the buffer pump (bypassing the injection loop). The column was extensively washed with 35% of buffer B and then developed in a gradient of 35-45% of buffer B over 60 minutes. Flow rate was 0.6 ml/min. Finally the fractions were analyzed by ELISA. The HPLC analysis was performed using a Gilson 45NC gradient HPLC system controlled by a 712 system controller software monitored on a DELL 386p computer.

Aβ Peptide Determinations by ELISA - Protein samples (cell extracts or HPLC fractions) were mixed with coating buffer (0.1 M carbonate buffer, pH 9.6) and coated on microtiter plate wells held overnight at 4^0C. The specificity of the antibodies is shown in Table 1. Bovine serum albumin, calcitonin gene related peptide, and nerve growth factor were used as negative controls to test the specificity of Aβ antibodies. Horseradish peroxidase-conjugated second antibodies were incubated with the samples, and the positive reaction color was developed using a "Single Component Substrate TEM (3,3',5,5'-tetramethylbenzidine) kit" (Bio-Rad, Hercules, CA). The reaction was stopped with the addition of 0.2 M H_2SO_4, and the absorbance was measured at OD_{450} in a BioKinetic EL312b automatic microtiter spectrophotometer.

N-Terminal Microsequence of Platelet Aβ - The fractions showing a positive reaction to both antibodies were lyophilized and N-terminally microsequenced by Edman degradation using the Applied Biosystems 477A Protein Sequencer of the "Protein and Molecular Biology Core Facility", University of Florida, Gainesville, Florida.

RESULTS

Determination of Aβ Immunoreactivity in Blood Cells - To study the distribution of Aβ-like proteins in blood, we determined the Aβ immunoreactivity to antibodies anti-$Aβ_{8-17}$ and anti-$Aβ_{1-28}$, in major blood components by ELISA. Table 2 shows that almost all the blood cells contain Aβ-immunoreactivity; however, platelets accounted for almost 90 % the total Aβ immunoreactivity. When the immunoreactive material was extracted with ethanol, plasma gave a relative 6.2 %, whereas leukocytes (which included lymphocytes, neutrophils, and monocytes) accounted for only 3.3% of total. In turn, erythrocytes contained no significant immunoreactivity (≤ 1 %). When the cells were extracted with acid/acetone or acetonitrile/trifluoroacetic acid, the data fluctuated somewhat and the relative amount contributed by platelets varied only by approximately 5 % (data not shown).

Identification of Platelet Aβ - The Aβ immunoreactive materials in the ethanol extracts were separated using two sequential reverse-phase HPLC runs. In the first run, the Aβ-immunoreactive

TABLE 1. SPECIFICITY OF ANTIBODIES

ANTIBODY	$Aβ_{1-40}$	$Aβ_{1-16}$	APP
Anti- $Aβ_{1-28}$	+	–	–
Anti- $Aβ_{8-17}$	+	+	+

Reactivity was evaluated by ELISA using 0.2 pmoles of peptide per well. APP secreted form was purified from platelets as previously described.

TABLE 2. RELATIVE Aβ IMMUNOREACTIVITY IN HUMAN BLOOD COMPONENTS

Blood Component	Cell Number per ml blood	Absorbance at 450 nm	Percent of total
Platelets	3.0×10^8	5240 ± 531	89.6 ± 9.1
Leukocytes	7.5×10^6	192 ± 29	3.3 ± 0.5
Erythrocytes	5.0×10^9	≤ 60	≤ 1.0
Plasma	------	362 ± 52	6.2 ± 0.9

Separation of blood components and cell counts per ml of whole blood were performed as described in Materials and Methods. Plasma content was 0.55 ml per ml of whole blood. Immunoreactivity was evaluated by ELISA using $A\beta_{1-28}$ antibody (1:100 dilution) and the relative absorbance units at 450 nm were normalized for cell counts per ml of whole blood. Values represent the mean ± SEM of 3 different experiments.

materials were separated on a C-4 column developed in a steep gradient (Fig. 1A); and to ensure high assay specificity, the fractions were analyzed by two independent ELISAs. As shown in Figure 1B, the profiles of the anti-$A\beta_{1-28}$ and anti- $A\beta_{8-17}$ reactivity did not exactly overlap but revealed several immunoreactive peaks. One fraction (#11) was recognized concomitantly by both antibodies. This peak was eluted with a retention time corresponding to that of synthetic $A\beta_{1-40}$ which was chromatographed separately under identical conditions (Fig. 1C). When this fraction was further analyzed on a C-18 column over a shallow gradient, the Aβ immunoreactive material was resolved into several protein peaks (Fig. 2A). One fraction (#14), eluted at the acetonitrile concentration of 34%, precisely at the position of the $A\beta_{1-40}$ peptide. Moreover, after all the fractions were analyzed by ELISA (Fig. 2B), this fraction was the only one which immunoreacted with both antibodies. Partial N-terminal sequencing of this peak revealed that the sequence obtained matched that of Aβ (5, 13) (Fig. 3). The peptide yield in the final sample was estimated by the sequencing data to be 80 pmoles.

DISCUSSION

The source of amyloid in intracerebral parenchyma and microvasculature of AD brains is still subject to debate. The postulated sites of origin include neurons, astrocytes, microglia and the systemic circulation (9). Regarding the latter, various cellular blood components have been found to express APP and thus may be potentially able to release Aβ into plasma. Our analyses show that the vast majority (about 90%) of the anti-Aβ immunoreactivity in whole blood is associated with platelets, pointing to these cells as one of the primary sources of Aβ in the bloodstream.

The platelet-associated peptide identified in the present study appears to be genuine Aβ by at least three criteria: (a) compared to synthetic $A\beta_{1-40}$, it displayed identical retention times in two consecutive

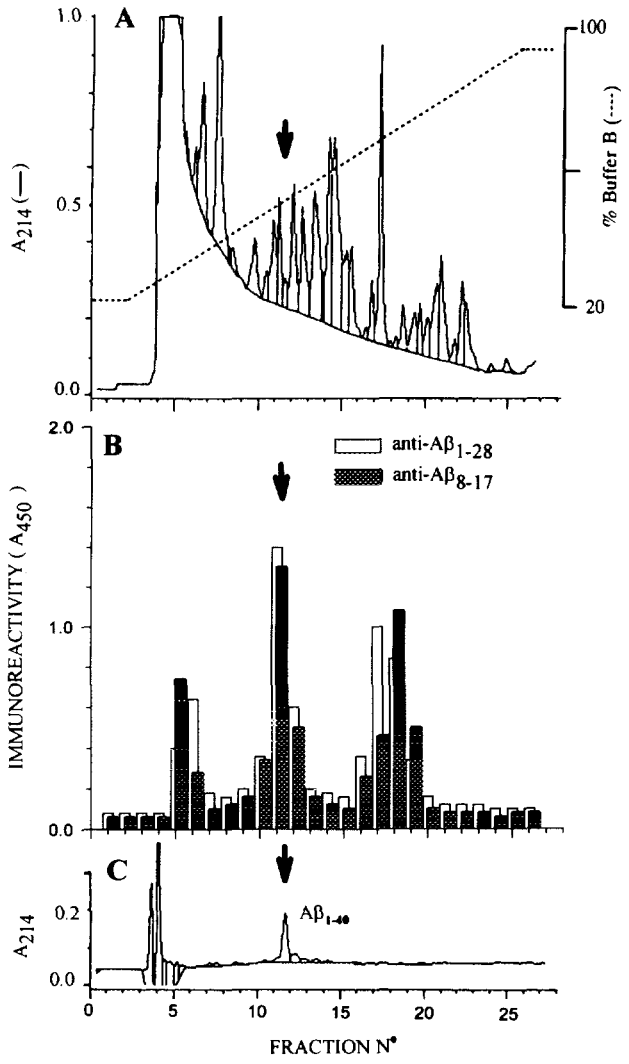

Figure 1. *Isolation of Platelet-Associated Aβ Immunoreactivity by HPLC.* **Platelet-associated Aβ was separated by two-step HPLC as described in** Materials and Methods. **(A) Shows a representative chromatogram of the platelet extract run through a C-4 HPLC column, buffer B as defined in** Materials and Methods. **(B) Immunoreactivity (OD_{450}) in each HPLC fraction obtained by using two distinct Aβ antibodies; i.e., anti-$Aβ_{1-28}$ (1:50 dilution) and anti-$Aβ_{8-17}$ (1:40 dilution). (C) Depicts the chromatographic profile of $Aβ_{1-40}$ synthetic peptide run under the same conditions as in (A). Arrows show fraction N° 11 which corresponds to the elution position of $Aβ_{1-40}$.**

HPLC determinations; (b) as determined by ELISAs, it was recognized by two different antibodies directed against specific domains of Aβ; and (c) its partial N-terminal sequence is consistent with that of Aβ. In addition to Aβ, platelets appear to contain other Aβ-related ELISA immunoreactive species. These may be shorter as well as longer fragments of the genuine Aβ, the exact identity of which awaits further characterization. Recently, a 16 kDa Aβ-encompassing APP fragment has been identified in platelets (14). The identification of platelet-associated Aβ indicates that the processing of APP in

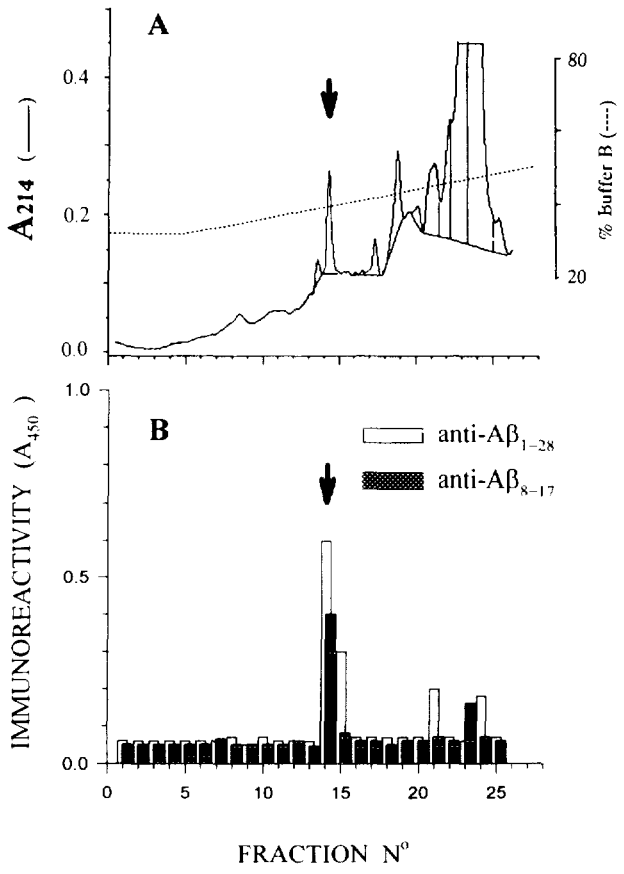

Figure 2. *Identification of Platelet-Associated* Aβ *Peptide.* **(A) Shows the chromatographic separation, through a C-18 HPLC column, of pooled fraction # 11 obtained from eight prior experiments using the C-4 HPLC column (see Fig. 1B). Fraction # 14 (arrow) contains a single protein peak which coincides with the chromatographic elution profile of the synthetic $A\beta_{1-40}$ peptide separated using a C-18 column under conditions identical to those described in (A). (B) ELISA analysis indicates that a single chromatographic fraction (#14) comprises most of the immunoreactivity against both anti-$A\beta_{1-28}$ and anti-$A\beta_{8-17}$ antibodies. Arrows show fraction N°14 which corresponds to the elution position of $A\beta_{1-40}$.**

platelets, aside from comprising the well-known non-amyloidogenic pathway, also involves an amyloidogenic pathway. Current knowledge indicates that platelets are the major circulating repository for APP (15, 16), and they have an effective mechanism to release large quantities of this protein at

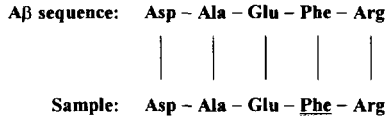

Figure 3. *Partial N-Terminal Amino Acid Sequence Determination of Platelet* **Aβ. Immunoreactive fraction #14 (see Fig. 2B) was lyophilized and the N-terminal sequence determined by the Edman degradation technique. The sequence obtained was compared with the known Aβ sequence (residues 1 through 5). The underlined residue was identified with less than full confidence.**

specific sites throughout the vasculature (15-18). Thus, platelets may be a potential source for the amyloid deposits in meningeal vessels and brain parenchyma. The present results showing that platelets produce soluble Aβ supports the hypothesis that the Aβ peptide in amyloid deposits of cerebral blood vessels and tissue is at least partly supplied by the circulation (19).

If soluble Aβ in blood contributes to amyloid deposits in the brain, the cerebrovasculature rather than brain parenchyma should be the favored site for blood-borne Aβ, because of the presence of the blood brain barrier. Such an amyloid deposition scheme is similar to other systemic amyloidoses in which amyloid is disseminated in the circulating blood (8, 20). It is plausible that when platelets release their contents as they senesce and are removed by reticulo-endothelial system, the platelet-associated Aβ could be released into the plasma. Over time and under certain circumstances, the plasma peptides would contribute, at least in part, to the amyloid deposits particularly in meningeal vessels, cortical arterioles, and capillaries, structures which are commonly damaged in AD (20). Inasmuch as synthetic amyloid has been shown to cross the blood brain barrier (21), it is reasonable to think that blood-borne amyloid reaches and can be deposited in brain parenchyma. Whatever the case may be, a delicate balance between the amyloidogenic/non-amyloidogenic pathways may be essential in maintaining a physiologically normal APP processing system. This balance could be eventually disturbed and may be an early triggering event in the pathogenesis of AD.

Previous studies have indicated that platelets from AD patients exhibit certain neurochemical alterations including increased membrane fluidity (22) and abnormal accumulation of serotonin (23). In this context, a sensitive measurement of soluble Aβ peptide levels could provide a potentially powerful systemic diagnostic marker for AD.

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