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Biochimica et Biophysica Acta 1622 (2003) 20–28
BIOCHIMICA ET BIOPHYSICA ACTA
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# Red cell perturbations by amyloid β-protein

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Received 3 March 2003; received in revised form 5 May 2003; accepted 9 May 2003

**Abstract**

Amyloid β-protein (Aβ) accumulation in brain is thought to be important in causing the neuropathology of Alzheimer's disease (AD). Aβ interactions with both neurons and microglial cells play key roles in AD. Since vascular deposition of Aβ is also implicated in AD, the interaction of red cells with these toxic aggregates gains importance. However, the effects of Aβ interactions with red blood cells are less well understood. Synthetic amyloid β-protein (1–40) was labeled with biotin and preincubated at 37 °C for 4, 14 and 72 h to produce fibrils. Flow cytometry was used to study the binding of these fibrils to red cells. The amyloid fibrils had a high affinity for the red cell with increased binding for the larger fibrils produced by longer preincubation. Bovine serum albumin (BSA) did not reverse the binding, but actually resulted in a more efficient binding of the Aβ fibrils to the red cells. The interaction of Aβ with red cells increased the mean cell volume and caused the cells to become more spherical. This effect was greater for the longer fibrils. At the same time the interaction of Aβ with red cells produced an increase in their fluorescence measured after 16-h incubation at 37 °C. This increase in fluorescence is attributed to the formation of fluorescent heme degradation products. The effect of prior hemoglobin oxidation, catalase inhibition and glutathione peroxidase inhibition indicated that the amyloid-induced oxidative damage to the red cell involved hydrogen peroxide-induced heme degradation. These results suggest that amyloid interactions with the red cell may contribute to the pathology of AD.
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*Keywords:* Amyloid fibril; Red cell; Oxidative stress; Biotin; Flow cytometry; Membrane

## 1. Introduction

Alzheimer's disease (AD) is characterized by neuronal degeneration, synaptic loss and excessive deposition of amyloid in the extracellular space of the brain [1]. The major component of amyloid plaques is amyloid β-peptide (Aβ) derived from amyloid precursor protein (APP) by proteolytic cleavage [2]. Increased Aβ production has been implicated in AD brain pathology with an emerging hypothesis for AD toxicity involving peptide mediated free radical generation. Aβ has thus been shown to contribute to neuro-degeneration by mechanisms involving lipid peroxidation [3], disruption of ion homeostasis [4], apoptosis [5] and oxidative stress [6,7]. However, the mechanism of Aβ accumulation and toxicity is poorly understood.

Alterations in oxidative processes also occur in early stages of AD in nonneuronal tissues including plasma, red cells, platelets and lymphocytes [8]. The changes in the RBCs of subjects with AD include increased levels of lipid peroxidation, perturbations in the physical state of membrane proteins [9] and irregular distortion of red cells [10] attributed to oxidative damage. In addition, abnormal cellular aging with increased IgG binding and breakdown of band 3 has been reported [11]. Can these changes be associated with Aβ interactions with the red cell?

APP is ubiquitously expressed with platelets as the major source of circulating APP [12]. APP is also expressed in heart, liver, pancreas, lymph nodes, spleen, skeletal muscle, skin, intestine and leukocytes [13–18].

Circulating blood cells are exposed to soluble Aβ (1–40)/Aβ (1–42) with nanomolar levels of Aβ (1–40/42) detected in blood [17]. Erythrocytes have also been reported

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0304-4165/03/$ - see front matter. Published by Elsevier B.V.
doi:10.1016/S0304-4165(03)00101-6

to sequester monomeric Aβ with binding detected even at physiological levels of 5 ng/ml [19].

Although Aβ levels in blood, plasma or urine in AD patients do not generally correlate with brain deposition [20], a possible relationship between blood Aβ and AD is suggested by results with Down syndrome patients. These patients are reported to have elevated Aβ in plasma and have a greater risk for developing AD [21].

In order to investigate a possible role for plasma Aβ, we have initiated detailed studies on the interaction of Aβ fibrils with red cells. Aβ accumulates as fibrillar plaques in blood vessels producing vascular endothelial damage [22], and the bioactivity and toxicity of Aβ have been reported to depend to a large extent on their aggregated state [23,24]. Blood cells are also exposed to fibrillar amyloid aggregates on the luminal surfaces of cerebral microvessels [25].

We have studied the interaction of Aβ fibrils with erythrocytes and found that these fibrils are very efficiently taken up by red blood cells. We have also investigated the damage to the red cell resulting from this interaction and the effect of Aβ fibrils on red cell oxidative stress coupled to hemoglobin autoxidation.

## 2. Materials and methods

Synthetic Aβ (1–40) was purchased from Biosource International (Camarillo, CA) and stored at −20 °C. *N*-Hydroxysuccinimido (NHS)-biotin was purchased from Pierce (Rockford, IL). Avidin-fluorescein isothiocyanate (avidin-FITC) was obtained from Becton Dickinson (San Jose, CA). Synthetic rat amylin was purchased from American Peptide Company, (USA). All other chemicals were of reagent grade.

### 2.1. Peptide biotinylation

Aβ (1–40) and amylin were biotinylated with sulfo-NHS-biotin by the reported procedure [26]. One milligram of Aβ (1–40) was dissolved in 1 ml of 50 mM sodium bicarbonate buffer, pH 8.5, containing 30% acetonitrile, and allowed to react with 0.32 mg of sulfo-NHS-biotin for 2 h at 4 °C. The unreacted biotin was removed by centrifugation at 1000 × *g* for 1 h using a Centricon-3 microconcentrator (Amicon, Beverly, MA). The sample was diluted in 30% acetonitrile, aliquoted and stored at −70 °C. The final concentration of the stored sample was 100 μM.

### 2.2. Preparation of amyloid fibrils

The stored Aβ (1–40) was lyophilized and dissolved using 4 °C milli-Q water, sodium chloride and sodium phosphate with the final reaction mixture containing 400 μM Aβ (1–40), 150 mM NaCl and 50 mM phosphate, pH 7.4. Polymerization reactions were performed in Eppendorf tubes (0.5 ml) at 37 °C. After incubation for 4, 14 or 72 h, the solution was used for erythrocyte binding studies. The Aβ fibril length was calculated by fitting the known values from earlier work [27] with the fibril length proportional to the time of incubation. The fibril lengths calculated for the incubation times used were ~ 170 nm after 4-h incubation, ~ 600 nm after 14-h incubation and ~ 3000 nm after 72-h incubation. Unbound peptide/fibrils were removed by centrifugation after incubation with red cells [28]. For the oxidation studies, fibrils incubated for 72 h were used and the Aβ fibrils were collected by centrifugation at 4 °C for 90 min at 25,000 × *g* to remove monomers.

### 2.3. Erythrocyte preparation and interaction with amyloid

Whole blood was collected by venipuncture from a healthy donor into a 20-ml $K_3$EDTA-vacutainer (Becton Dickinson, NJ). Blood samples were centrifuged at 1600 × *g* for 15 min at 4 °C to separate plasma from blood cells. After removal of plasma and buffy coat, blood cells were suspended in PBS and centrifuged three times with PBS. Amyloid fibrils ranging in concentration from 0.2 to 50 μM were incubated with a 2.5% hematocrit of red cells for 2 h. In some of these experiments, 2% bovine serum albumin (BSA) was added to the RBCs. After incubation the samples were centrifuged to remove unbound fibrils.

### 2.4. Flow cytometry

The samples were analyzed on an FACSCAN flow cytometer (Becton Dickinson Immunocytometry Systems; San Jose, CA) equipped with a 15-mW argon laser. Red blood cell fluorescence was measured at an emission wavelength of 530 nm with excitation at 488 nm. A total of 50 000 events was measured for each RBC sample. Both the fluorescence and light scattering properties of the treated cells were measured.

#### 2.4.1. Analysis of Aβ binding to RBCs

The pelleted cells were resuspended in PBS ($10^6$ cells in 50 μl); 4 μl of avidin-FITC was added and the cells were incubated at 5 °C for 15 min in the dark to label all the biotinylated Aβ on the red cell membrane. The cells were then washed three times with PBS to remove the unbound avidin-FITC and resuspended. The quantification of the number of molecules of biotinylated Aβ bound per erythrocyte was determined by flow cytometry [29]. Results were expressed in arbitrary units as the mean cell fluorescence intensity.

#### 2.4.2. Analysis of red cell autofluorescence

Washed RBCs (2.5% hematocrit) were incubated in PBS at 37 °C for 16 h with varying concentrations of Aβ fibrils, which were not biotinylated. In some cases, RBCs were pretreated with 1 mM sodium azide, 0.2 mM iodoacetamide (IAA) or 1 mM sodium nitrite for 5 min and washed

extensively to remove the excess reagent. Aβ was then added to these modified red cells. At the end of a 16-h incubation time, 10 μl of these cells was diluted into 0.5 ml of PBS and the autofluorescence determined by flow cytometry as described earlier [30].

### 2.5. Red cell volumes

The red cell volume was determined by a Coulter multisizer II (Coulter Electronics, Hialeah, FL). The volumes were determined both in isotonic media to provide the mean cell volume, and at a series of reduced salt concentrations so that the maximum swollen cell volume and the surface area could be determined [31].

## 3. Results

### 3.1. Binding of amyloid fibrils to red blood cells

Fig. 1 shows the flow cytometry results for RBCs treated with Aβ fibrils for 2 h. The fibrils used in this experiment were prepared by a 14-h preincubation at 37 °C. The increase in fluorescence, as a function of the concentration of Aβ, indicates that Aβ binds to the red cell. As a control, we used biotinylated rat amylin, which is not expected to bind to red cells and found no increase in fluorescence (data not shown).

The effect of the extent of fibril formation is shown in Fig. 2 where Aβ fibrils prepared after 4-, 14- and 72-h preincubation are compared. The longer incubation time produces larger fibrils, which are expected to bind to membranes differently [27]. Fig. 2 shows that, at higher fibril concentrations, membrane-associated Aβ (the level of fluorescence) approaches similar values irrespective of the size of the fibrils. However, there is a clear difference in the extent of binding for the 4–14-h fibrils and the 72-h fibrils. Aβ preincubated for 72 h reached saturating levels of binding at relatively low concentrations. However, for the Aβ preincubated for a shorter time, an initial sharp increase in binding is observed, followed by a more gradual increase in binding, which did not reach saturation even at 50 μM Aβ.

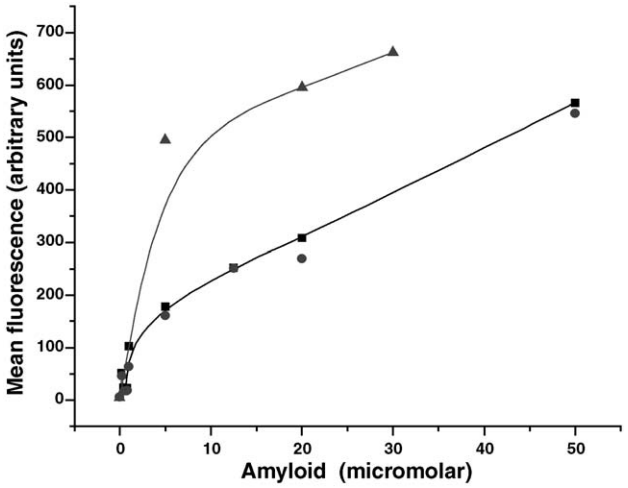


Fig. 2. Mean fluorescence versus Aβ concentration for different Aβ (1–40) fibrils. (■) 4-h fibrils; (●), 14-h fibrils; (▲), 72-h fibrils.

The fluorescence associated with the red cell does not appreciably decrease when an excess of nonlabeled fibrils was added (data not shown). This observation is consistent with nonspecific binding involving perhaps the cytoskeleton and not any specific receptor.

Fig. 3 shows the effect of 2% BSA on the fluorescence of RBCs treated with 72-h-preincubated Aβ fibrils. BSA did not lead to a decrease in fluorescence expected if BSA,

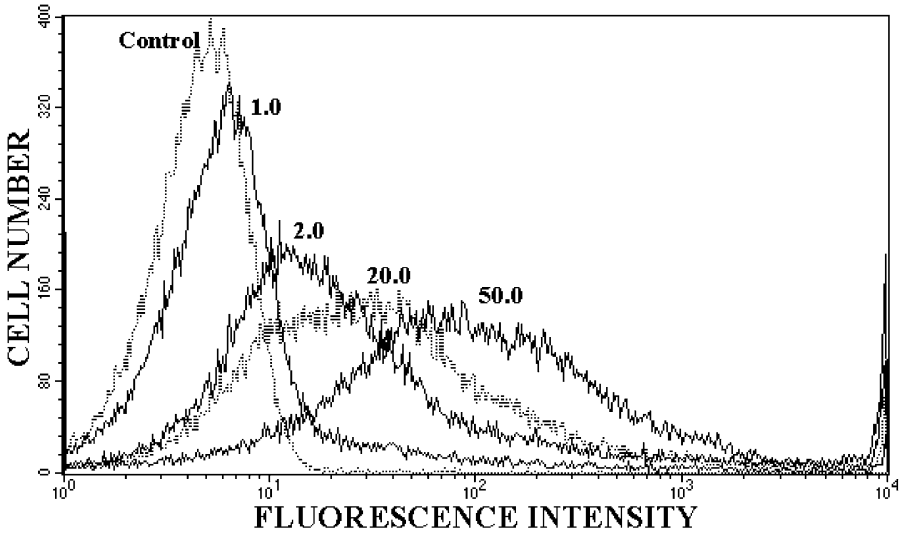


Fig. 1. Distribution of fluorescent intensity of RBCs reacted with different concentrations of biotin-labeled Aβ (1–40) fibrils formed by 14-h incubation at 37 °C. FITC-avidin was bound to the biotin group to provide a fluorescent tag. The fluorescence on each cell was determined by flow cytometry.

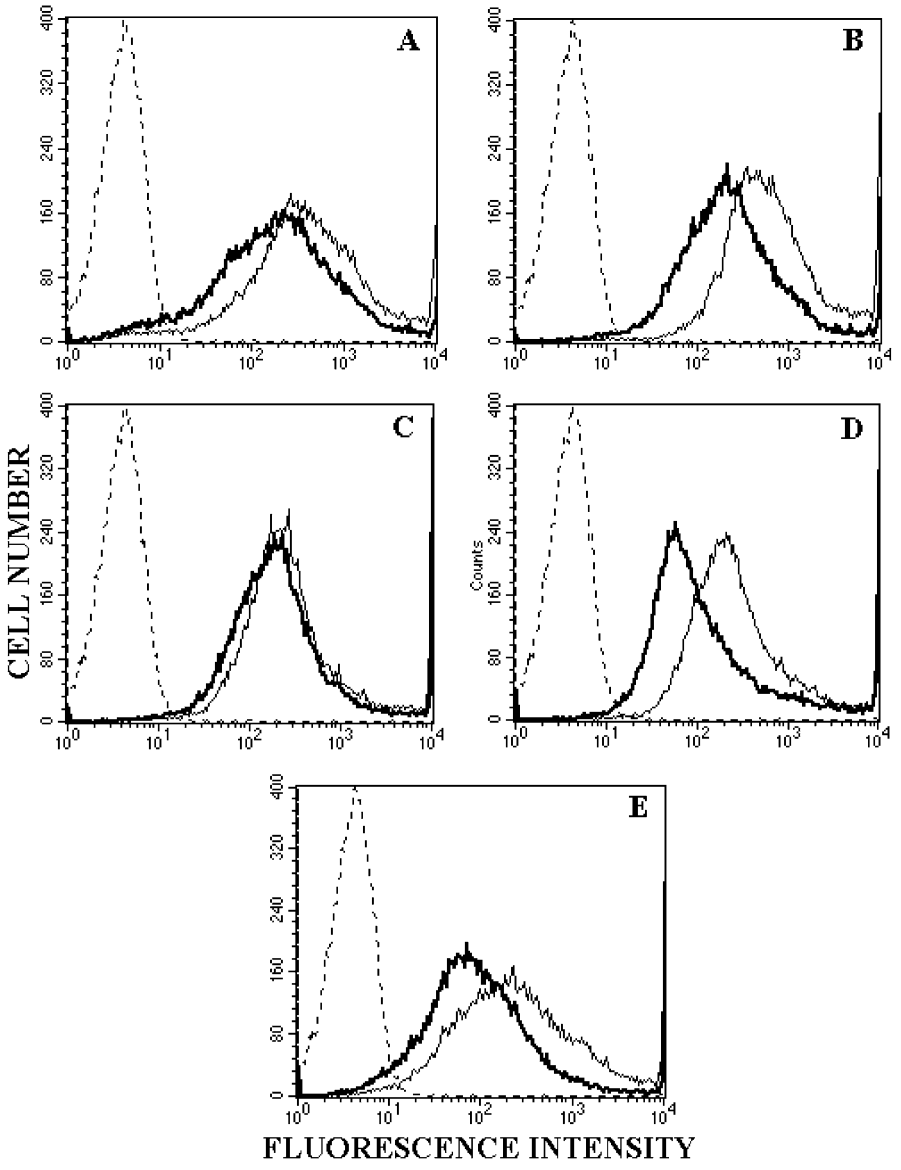


Fig. 3. Effect of bovine serum albumin on binding of Aβ (1–40) fibrils to RBCs. Fluorescence distribution of control with no Aβ (dotted line); Aβ-bound red cells (heavy solid line); and Aβ-bound red cells in the presence of bovine serum albumin (light solid line). (A) 5 μM Aβ; (B) 10 μM Aβ; (C) 20 μM Aβ; (D) 30 μM Aβ; (E) 50 μM Aβ.

known to bind monomeric Aβ [19], was able to remove the Aβ from the red cell. Instead, an increase in fluorescence was observed at most concentrations of Aβ.

### 3.2. Shape changes in the red cell produced by Aβ fibrils

Fig. 4 shows the increase in mean cell volume when Aβ fibrils prepared after 4- and 72-h preincubation react with RBCs. In both cases, there is a significant increase in the mean cell volume, indicative of swelling of the red cell with a much more pronounced effect with the larger fibrils prepared after 72-h preincubation. In fact, 30 μM of 72-h-preincubated Aβ produces red cells that are almost spherical as indicated by the maximum volume obtained in hypotonic solution.

### 3.3. Aβ-induced oxidative damage

Fluorescent heme degradation products are formed during autoxidation of oxyhemoglobin [32]. This reaction is initiated by the hydrogen peroxide formed during autoxidation. The hydrogen peroxide first reacts with hemoglobin to form ferrylhemoglobin, which then reacts with a second molecule of hydrogen peroxide producing superoxide, which goes on to degrade the heme-producing fluorescent products [33].

The formation of these degradation products in red cells can be monitored by incubating the red cells in vitro and measuring the increase in fluorescence by flow cytometry [30]. We have used this method as a measure of red cell oxidative processes. Fig. 5 shows the fluo-

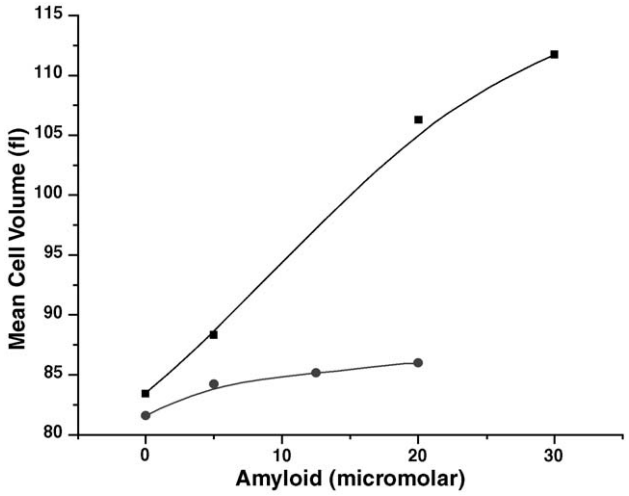


Fig. 4. Effect of Aβ (1–40) on the red cell mean cell volume. (●) 4-h fibrils; (■) 72-h fibrils.

rescent heme degradation products produced during a 16-h incubation at 37 °C as a function of added 72-h-preincubated Aβ fibrils.

Amyloid fibrils have been implicated in the production of reactive oxygen species [34]. In order to determine whether the enhanced fluorescence found in RBCs after Aβ fibril treatment is related to the hemoglobin oxidative processes, we have investigated the effect of reacting hemoglobin with nitrite. Fig. 6A shows that prior oxidation of hemoglobin with nitrite inhibits the formation of fluorescent products. This effect is consistent with the requirement of Fe(II) hemoglobin for the production of fluorescent products during the reaction with hydrogen peroxide [32].

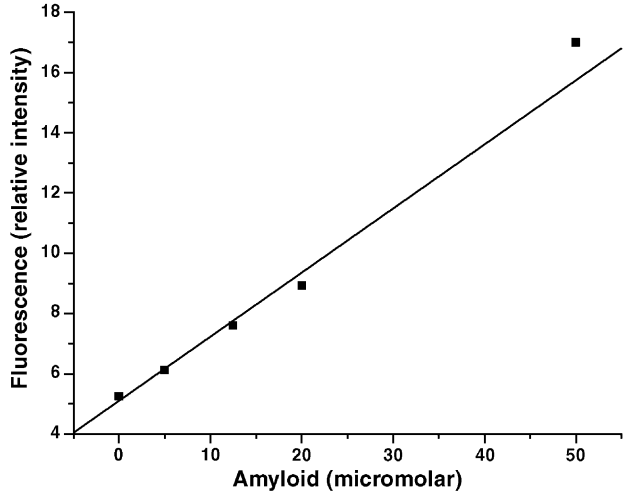


Fig. 5. Effect of Aβ (1–40) on red cell fluorescence after 16-h incubation of cells in phosphate buffered saline at 37 °C. The Aβ was unlabeled so the fluorescence was due to the formation of heme degradation products.

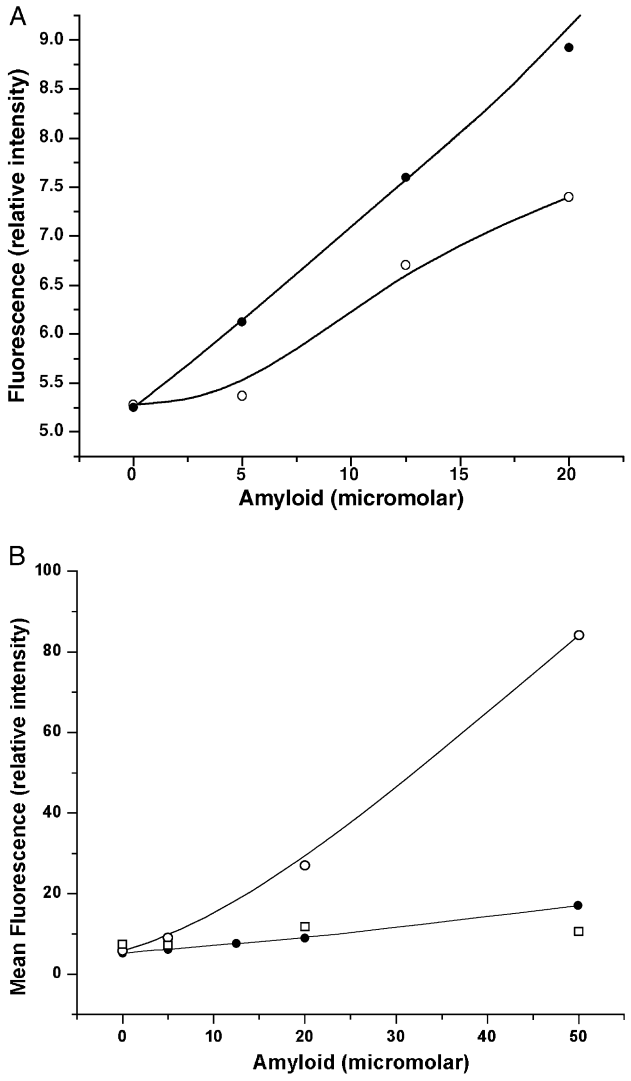


Fig. 6. (A) Effect of hemoglobin oxidation by nitrite on Aβ-induced red cell fluorescence. Solid symbols: control without added nitrite; open symbols: with 1 mM nitrite added. (B) Effect of inhibition of catalase by azide and inhibition of glutathione peroxidase by iodoacetamide on Aβ-induced red cell fluorescence. Solid symbols: control with no inhibitors added; open square: with 1 mM sodium azide added; open circle: with 0.2 mM iodoacetamide added.

The involvement of hydrogen peroxide in the formation of fluorescent products was investigated by inhibiting catalase and glutathione peroxidase, the two enzymes in the red cells, which react with hydrogen peroxide. In our previous studies [30], we have shown that the enhanced formation of fluorescent heme degradation products after the addition of azide and/or iodoacetamide is attributed to the inhibition of catalase and glutathione peroxidase, respectively.

Fig. 6B shows the effect of inhibiting these enzymes on the Aβ-induced increase in fluorescence. Interestingly, the effect of catalase inhibition is insignificant, while a clear increase in fluorescence is observed when glutathione peroxidase is inhibited by iodoacetamide.

## 4. Discussion

### 4.1. Aβ binding to red cells

Aβ toxicity, in part, correlates with distinct fiber morphology [35,36]. Thus, cellular toxicity in the low nanomolar range was only observed if a high proportion of mature fibers was observed by electron microscopy. Preparations consisting predominantly of monomers did not indicate toxicity in the MTT-based assay system even at high concentrations (100 μM). Furthermore, the greater toxicity of Aβ (1–42) than Aβ (1–40) has been attributed to a greater tendency to form fibrils [37].

Recent studies, however, have suggested that smaller assemblages of Aβ (protofibrils) elicit neurotoxic activity [34,38–40] and that aged polymerized Aβ may actually be nontoxic [41]. It is these smaller protofibrils, still in their formative stage [42], that may be transported in and out of the brain, perhaps via equilibrium with monomeric Aβ and/or via a mechanism whereby the protofibrils are directly transported across the blood–brain barrier (BBB).

Erythrocytes have been reported to sequester monomeric Aβ [19]. However, this is the first time that the uptake of the more toxic amyloid protofibrils by red cells has been studied. Aβ (1–40) protofibrils ranging in size from 170 to 3000 nm have been investigated, indicating a strong association with the red cell (Figs. 1 and 2), which is enhanced for the larger ~3000-nm fibrils (Fig. 2). The bovine serum albumin experiment (Fig. 3) further confirms that the red cell is able to take up fibrillar Aβ even in the presence of albumin. The increased fluorescence in the presence of BSA (Fig. 3) can be interpreted in terms of a more efficient distribution of fibrils on the red cell produced by the disruption of fibril clusters and less fluorescent quenching.

Recent data demonstrate a dynamic equilibrium between brain Aβ and plasma Aβ [43]. It has thus been shown in a transgenic mouse [44] that the injection of an anti-Aβ antibody (m266) increases the level of plasma Aβ a thousandfold, indicating that brain Aβ can be transferred across the BBB to the plasma. Considering such an equilibrium, the strong red cell–Aβ interactions demonstrated in this study should be a factor in the distribution of brain amyloids in AD.

### 4.2. Aβ induced oxidative processes on the red cell

Evidence for involvement of free radicals in AD includes the presence of elevated levels of protein oxidation [45], lipid peroxidation products [22,46] and oxidative damage to mitochondria [47]. The Aβ-associated free radical oxidative stress model for neuronal death in AD brain predicts that neuronal protein oxidation is a consequence of Aβ-associated free radicals [48,49]. Oxidative and structure-disrupting events induced by Aβ at the level of the plasma membrane appear to represent the initiating events in the cytotoxic cascade induced by this peptide [25]. Aβ inserts into the plasma membrane of neuronal or glial cells and oxygen-dependent reactive oxygen species are formed. These free radicals can attack the neuronal plasma membrane, induce $Ca^{2+}$ influx, disturb cell membrane functions, increase protein oxidation and induce lipid peroxidation [50]. The mechanism for Aβ-induced free radical formation is not fully understood although the involvement of the methionine residue at position 35 of Aβ has been implicated [45].

Red cell oxidative processes are associated with the autoxidation of hemoglobin, which continuously occurs at a low basel level. This process begins a cascade of oxidative reactions. Autoxidation produces superoxide, which dismutates to form hydrogen peroxide. Hydrogen peroxide reacts with Fe(II) hemoglobin to produce Fe(IV) ferrylhemoglobin. We have recently shown [33] that ferrylhemoglobin can react with an additional molecule of hydrogen peroxide to produce superoxide, which is ideally located to react with the heme. The superoxide damages the heme, producing a rhombic heme geometry, which finally results in the degradation of the heme and the formation of fluorescent products.

We have developed an assay for measuring these fluorescent heme degradation products in intact RBCs using flow cytometry [30]. Using this method, we have shown (Fig. 5) that Aβ fibrils dramatically enhance the formation of fluorescent products in the red cell. We wanted to determine whether the Aβ-induced increase in fluorescence is associated with hemoglobin-related oxidative processes. Oxidizing hemoglobin inhibits the secondary hemoglobin oxidative processes. The reaction of hydrogen peroxide with oxidized hemoglobin produces oxyferrylhemoglobin instead of ferrylhemoglobin and no fluorescent heme degradation products are formed [33]. The inhibition of the Aβ-induced fluorescence when the hemoglobin is pre-oxidized by nitrite (Fig. 6A) indicates that hemoglobin-induced oxidative processes account for at least part of the Aβ-induced increase in fluorescence. Aβ-induced oxidative processes not involving hemoglobin are indicated by the residual increase in fluorescence even after reacting with nitrite (Fig. 6A).

How is the Aβ–red cell interaction coupled to hemoglobin oxidative processes in the red cell?

The red cell has an adequate supply of enzymes to deal with superoxide and hydrogen peroxide generated as a result of autoxidation. We have therefore implicated the fraction of hemoglobin bound to the membrane in red cell oxidative processes [51]. This has recently been confirmed by the specific effect of glutathione peroxidase in protecting the red cell from hydrogen peroxide-induced heme degradation [52]. Glutathione peroxidase, unlike catalase, also reacts with membrane-associated substrates and would be expected to also react with hydrogen peroxide generated in the region of the membrane. We thus find that as long as glutathione is present as an essential substrate for glutathi-

one peroxidase, the accumulation of fluorescent products in the red cell is minimized even when catalase has been inhibited.

Amyloids bind to the membrane and perturb the structure of the red cell cytoskeleton [11]. The close proximity of membrane-bound hemoglobin and the amyloids, both of which are associated with oxidative stress, has a synergistic effect resulting in an appreciable increase in the production of fluorescent degradation products (Fig. 5). Here again, the enhanced heme degradation is limited (Fig. 6B) by the activity of glutathione peroxidase (it is accentuated by adding iodoacetamide to react with the glutathione) and not catalase (no affect of inhibition by azide).

### *4.3. Functional ramifications of Aβ-induced red cell oxidative stress*

Aβ-induced swelling (Fig. 4) can be attributed to oxidative damage to the membrane ATPases impairing cation homeostasis necessary to regulate cell volume. We have also found [53] that Aβ produces a decrease in red cell deformability as indicated by an increase in the cell transit time required to pass through 5-μm pores as well as a decrease in the rate at which cells successfully traverse these 5-μm pores. The decrease in deformability can be attributed to the swelling of the cells, which reduces the excess surface area necessary for deformation. However, oxidative damage to the membrane increases the membrane rigidity, further decreasing the deformability of the red cell. These changes in deformability impair the ability of the red cell to deliver oxygen to the tissues.

Changes in the RBCs of subjects with AD are consistent with Aβ-induced damage to the red cell. Thus, RBCs of AD patients have increased levels of lipid peroxidation and perturbations in the physical state of membrane proteins [9] attributed to oxidative damage. Bosman et al. [11] found in AD subjects abnormal cellular aging, increased IgG binding and breakdown of band 3. Goodall et al. [10] reported irregular distortion of erythrocytes in senile dementia. Solerte at al. [54] found that mild to moderate dementia of the Alzheimer type is associated with reduced erythrocyte deformability and increased red cell aggregation.

Even though plasma levels of Aβ in AD subjects are not appreciably elevated; these red cell perturbations could be attributed to elevated levels of Aβ associated with the red cell. To test this hypothesis, studies are being planned of the altered properties of red cells in AD subjects in conjunction with determinations of red cell Aβ.

These changes will result in impaired flow of blood through the microvascular system, which may affect the delivery of oxygen to the brain tissues. In addition, reduced blood flow and increased hypoxia activate the red cell oxidative processes [55] accentuating any damage.

The abnormal cellular aging and increased IgG binding suggest that RBCs may have a shorter life span in AD subjects [11]. Such an effect would minimize the actual increase in red cell Aβ, but would place a stress on the erythropoetic system producing larger cells, which are more susceptible to damage [56]. At the same time, the faster turnover of red cells suggests a possible role for red cells in removing Aβ from the circulation. The binding of Aβ to the red cell and its subsequent removal can decrease the brain amyloid burden [42]. Recent studies [57] have in fact reported that the erythrocyte, utilizing the complement receptor (CR1), can actually play a role in the clearance of certain pathogens without damaging the red cell. Such a mechanism, if applied to Aβ, would amplify the potential role of erythrocytes in this clearance.

For Aβ from the periphery to accumulate in the brain, it must be able to cross the BBB. Brain perfusion experiments in guinea pigs and IV bolus injection studies in mice indicate that radioactive iodinated human Aβ can be transported across the BBB [43,44]. There are reports that transport across the BBB is facilitated by binding to lipoproteins and albumin in human plasma. An alternative mechanism for transport across the BBB can involve the Aβ bound to RBCs. The amyloids can influence interactions of the red blood cells with the endothelial cells lining the blood vessels, thereby facilitating the transfer of Aβ produced in the peripheral circulation across the BBB into the brain.

It has been proposed that AD can originate as a vascular disorder [58], with cerebral hypoperfusion triggering the cognitive and neuro-degenerative changes associated with AD. Aβ bound to the red cell may play an important role in such a process. These amyloids may account for the vascular Aβ deposition found in AD patients. The impaired blood flow resulting from Aβ-induced damage to the red cell can account for hypoperfusion of the brain resulting in impaired oxygen delivery. Perhaps most important to consider in this process is the enhanced red cell oxidative processes triggered by Aβ binding. There is ample evidence that oxidative processes contribute to AD. However, this oxidative stress may, at least in part, originate from red cells, which have taken up Aβ. These oxidative processes will damage the vasculature resulting in further impairment in oxygen delivery beginning the cascade, which leads to AD pathology.

## 5. Conclusion

We have shown that Aβ can be bound by RBCs and that this process damages the red cell. Plasma and RBC levels of Aβ are very low in AD and there is therefore no direct evidence linking these processes to AD. However, a possible relationship is supported by the findings of RBC impairment in AD. These interactions can play a role in the transport of Aβ from or to the brain and may not result in any appreciable accumulation of Aβ in RBCs. At the same time, even low levels of RBC-associated Aβ can affect

the flow of blood through the cerebral microvasculature and can be a source of enhanced oxidative stress.

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