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Alzheimer's β-amyloid peptides can activate the early components of complement classical pathway in a C1q-independent manner

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SUMMARY

β-Amyloid (β-A) accumulates in the brain of patients with Alzheimer's disease (AD) and is presumably involved in the pathogenesis of this disease, on account of its neurotoxicity and complement-activating ability. Although assembly of β-A in particular aggregates seems to be crucial, soluble non-fibrillar β-A may also be involved. Non-fibrillar β-A does not bind C1q, so we investigated alternative mechanisms of β-A-dependent complement activation *in vitro*. On incubation with normal human plasma, non-fibrillar β-A 1-42, and truncated peptide 1–28, induced dose-dependent activation of C1s and C4, sparing C3, as assessed by densitometric analysis of immunostained membrane after SDS–PAGE and Western blotting. The mechanism of C4 activation was not dependent on C1q, because non-fibrillar β-A can still activate C1s and C4 in plasma genetically deficient in C1q (C1qd). In Factor XII-deficient plasma (F.XIId) the amount of cleaved C4 was about 5–10% less that in C1qd and in normal EDTA plasma; the reconstitution of F.XIId plasma with physiologic concentrations of F.XII resulted in an increased (8–15%) β-A-dependent cleavage of C4. Thus our results indicate that the C1q-independent activation of C1 and C4 can be partially mediated by the activation products of contact system. Since the activation of contact system and of C4 leads to generation of several humoral inflammatory peptides, non-fibrillar β-A might play a role in initiating the early inflammatory reactions leading to a multistep cascade contributing to neuronal and clinical dysfunction of AD brain.

Keywords β-amyloid peptide complement C1q contact system

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly, accounting for > 50% of all cases. Several risk factors have been suggested in this neurodegeneration, including genetic, environmental and metabolic factors, but the aetiology is still unknown. The neuropathological hallmarks are neurofibrillary tangles and senile plaques. The major protein component of the plaques is a 39–42 amino acid peptide (β-amyloid (β-A)) proteolytically derived from the transmembrane amyloid precursor protein (APP) [1]. β-A was considered inert until recent studies clearly showed that it is biologically active, leading to neuronal degeneration and microglial activation *in vitro* [2]. Although it is now clear that neuritic plaques, reactive microglia and several inflammatory mediators are linked to neuronal degeneration in the AD brain, the mechanism underlying these links is still uncertain. During the past decade evidence has been provided suggesting that the complement system (C) may be involved in the inflammatory process. In the AD brain several factors of the classical pathway [3,4] and less consistently the complement membrane attack complex (MAC) [5,6] are commonly found on neuritic plaques, low levels of C1q in cerebrospinal fluid (CSF) correlate with the disease state [7], and *in vitro* β-A can activate the C classical pathway through an ionic interaction with C1q [8]. Although only the region between residues 4 and 11 of the β-A peptide is critical for binding to a collagen-like domain of the C1q A chain [9,10], a particular assembly state of β-A seems to be needed for C activation [11] since monomeric β-A does not bind C1q [12]. However, the finding that monomeric β-A 1–42 activates the classical pathway *in vitro* [13] raised the question of alternative mechanisms.

During activation of the contact system and fibrinolysis several peptides are generated, i.e. factor XIIf, kallikrein and plasmin, which, by acting on C1s, can activate the classical pathway in a C1q-independent manner, both *in vitro* [14,15] and *in vivo* [16]. The possibility that these systems may be involved in C activation in the AD brain is suggested by the *in vivo* finding that the contact

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system may be activated in these patients [17,18], and that soluble β-A has a stimulatory effect on plasminogen activation by tissue-type plasminogen activator *in vitro* [19].

The present study was undertaken to define the mechanisms through which non-fibrillar β-A activates C, by evaluating the generation of activation products of C1s, C4, C3 and factor B. We also checked whether non-fibrillar β-A peptide 1–42, and the truncate peptides 1–28, can substitute for C1q in classical pathway activation *in vitro*, and whether activation of the contact system and fibrinolysis are also involved.

MATERIALS AND METHODS

Chemicals and reagents

Synthetic analogues of β-A peptides 1–42 and 1–28, Tween-20, soybean trypsin inhibitor, b-Dellin, biotinylated rabbit anti-goat and goat anti-rabbit IgG, peroxidase-conjugated goat anti-rabbit, and avidin-alkaline phosphatase substrate were obtained from Sigma Chemical Co. (St Louis, MO). Goat polyclonal anti-high molecular weight kininogen (light chain) and goat anti-factor XII were from Nordic (Tilburg, The Netherlands). Rabbit polyclonal anti-C4/C4c, anti-C3/C3c, anti-C1INH, and anti-factor B were from Dako (Glostrup, Denmark). Sheep polyclonal anti-C1s was from The Binding Site (Birmingham, UK). Purified C4, Factor XII and Phe-Phe-Arg-chloromethyl ketone dihydrochloride (PPACK) were from Calbiochem Co. (San Diego, CA). Polyvinylidene-difluoride membrane (Immobilon) was from Millipore Co. (Bedford, MA). The ECL Western blotting detection reagents were from Amersham (Aylesbury, UK). The high-performance scanner and Image Master software were from Pharmacia (Uppsala, Sweden). Pure C1INH was a kind gift from Dr A. E. Davis III (Department of Nephrology, The Children's Hospital Research Foundation, Cincinnati, OH). Pure C1s and $C1r_2$–$C1s_2$ complex were a kind gift from Dr U. Martensson (Department of Medical Microbiology, Lund University, Sweden).

Plasma samples

Blood was collected from three healthy volunteers and from one subject genetically deficient in factor XII (F.XIId) in polypropylene tubes containing Na-citrate 0·13 mol/*l* (9:1). The mixture was handled at room temperature, centrifuged at 1300 ***g*** for 10 min, and used for the experiments within 30 min from bleeding. Spontaneous activation of C4, C3 and factor B ranged between 2% and 6%, and plasma was suitable for assessing activation of the classical and alternative C pathways by heat-aggregated IgG (HAG) and Zymosan, respectively. Na-citrate prevents activation of the coagulation system but *in vitro* activation of the contact system by negatively charged substances (Kaolin) and plasmin generation by plasminogen activators (tissue-type plasminogen activator in presence of fibrinogen fragments) can be tested. Human plasma genetically deficient in C1q (C1qd) was a kind gift from Dr P. Spaeth (ZLB Central Laboratory, Blood Transfusion Service SRC, Bern, Switzerland). C1qd and F.XIId plasma do not contain substances precipitable with polyclonal anti-human C1q or anti-human factor XII as assessed by single radial immunodiffusion and double immunodiffusion analyses in agarose. Fresh samples were used in each experiment.

Characterization of β-A peptides

β-A peptides were prepared by dissolving lyophilized β-A 1–42 or 1–28 in high quality distilled H_2O at a concentration of 200 μg/ml (50 mM) and immediately diluting to 25 μM with PBS pH 7·4. Within a few minutes of dissolution, β-A preparations were centrifuged (10 000 ***g***) for 10 min at room temperature and the state of aggregation was assessed by separating supernatants and pellets by 15% non-reducing SDS–PAGE. Freshly solubilized β-A peptides were morphologically characterized by light and electron microscopy. Thioflavin T stains were used for light microscopy of the peptides, in supernatants and pellets. For negatively stained specimens, samples (20 μl) were adsorbed to 200 mesh Formvar carbon-coated grids, air-dried, and negatively stained with 2% uranyl acetate in water (w/v) for 2 min. The grids were examined and photographed in a Jeol JEM 1010 electron microscope operating at 80–100 kV.

Complement system activation

C activation was assessed by measuring the degree of C4, C3, and factor B cleavage using densitometric analysis of immunostained blotting membranes after SDS–PAGE. This method simultaneously evaluates the native protein and its activation fragments. Plasma samples were loaded on 8% acrylamide gel and separated by SDS under non-reducing conditions. Proteins were transferred to polyvinylidene-difluoride (PVDF) membranes by electroblotting, blocked for 12 h at 4°C with 5% skimmed milk in Tris-buffered saline–0·1% Tween-20, washed and incubated for 2 h at room temperature with polyclonal rabbit anti-C4, C3 or factor B. The C factor bands were visualized with biotin-conjugated goat anti-rabbit IgG and an avidin-alkaline phosphatase substrate. The blotting membranes were analysed with a high-performance scanner and Image Master software to establish the level of protein activation, the cleaved protein being expressed as a percentage of total protein (band II *versus* bands I plus II). The interassay variation was 10%. C1s activation in plasma was assessed by evaluating the generation of C1 s–C1INH complexes. Briefly, the IgG fraction of polyclonal sheep anti-C1s was conjugated to cyanogen bromide-activated Sepharose 4B in sodium bicarbonate buffer pH 8·3. The residual active groups were blocked using Tris–HCl pH 8·0. Anti-C1s-conjugated Sepharose beads (50 μl) were incubated with samples of activated plasma (see above) for 15 min at room temperature under gentle agitation. The Sepharose beads were washed three times to remove non-adsorbed plasma proteins, resuspended in SDS-sample buffer (non-reducing) and incubated for 5 min in boiling water. The Sepharose beads were pelleted, and the protein-containing supernatants were subjected to SDS–PAGE, and transferred to PVDF membranes as described above. The bands were visualized on blotting membranes with polyclonal rabbit anti-C1INH, horseradish peroxidase-labelled rabbit anti-goat IgG and ECL detection reagents. The amount of stable C1s–C1INH complexes was evaluated by densitometry, and the values expressed as a percentage of total protein (200-kD band *versus* 200-kD plus 110-kD band).

Contact system activation

Contact system activation was assessed by measuring the degree of cleavage of high molecular weight kininogen (HK) [20,21], essentially as described for C factors. The HK bands were visualized on blotting membranes with polyclonal goat anti-light chain HK, biotin-conjugated rabbit anti-goat IgG and an avidin-alkaline phosphatase substrate. The apparent masses of native HK and its activation fragments were mol. wt 130 000 (band I), 107 000 (band II) and 98 000 (band III). HK activation was expressed as the percentage of total protein (band II plus III *versus* bands I plus II and III).

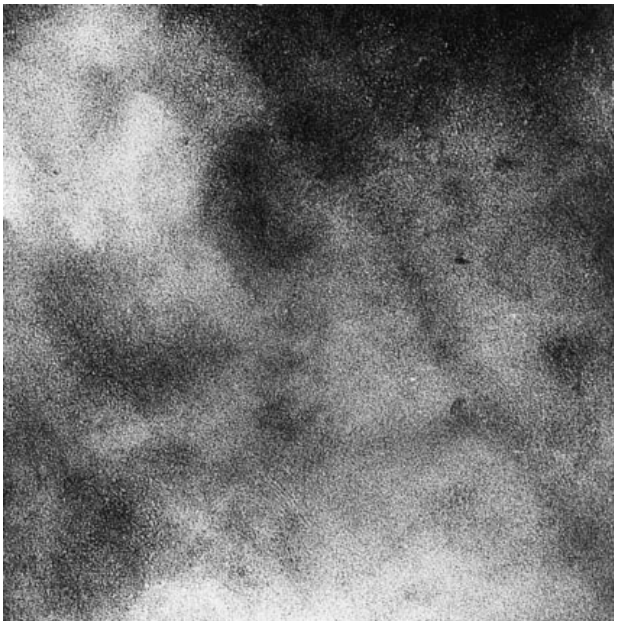

Fig. 1. Electron microscopic analysis of β-amyloid (β-A) preparation. β-A1–42 (20 μl) was adsorbed on Formvar-coated grids, negatively stained with 2% uranyl acetate in water, and observed in a Jeol JEM 1010 electron microscope operating at 80–100 kV. Freshly solubilized β-A1–42 adsorbed on the grids showed no fibrillar structure. The amorphous precipitate in the figure is an artefact of the drying process due to salt precipitation.

Complement activation by β-A peptides

C activation by β-A peptides was assessed as follows. Human Na-citrate plasma (100 μl) was incubated at 37°C for 30 min with 100 μl of freshly resuspended β-A1–42, or 1–28, at various concentrations (5–100 μg/ml). The reaction was quenched by adding 200 μl of PBS containing soybean trypsin inhibitor 100 μg/ml, EDTA 10 mM, Polybrene 0·05% (w/v). C4, C3 and factor B activation was assessed as described above. To investigate the mechanism of β-A-mediated C activation, β-A1–42 or β-A1–28 were incubated in separate experiments with normal human plasma containing EDTA (up to 20 mM), with C1qd plasma, with pure C4, C1s, or C1s–C1r complex.

Contact system activation by β-A peptides

Activation of the contact system by β-A was assessed by incubating Na-citrate plasma samples with β-A1–42 or 1–28 at various concentrations for 30 min at 37°C, essentially as for C activation. To investigate the importance of contact system activation in β-A-dependent activation of the classical pathway, β-A1–42 or 1–28 were incubated with a human F.XIId plasma or with F.XIId plasma containing the specific kallikrein inhibitor Phe-Phe-Arg-chloromethyl ketone dihydrochloride (10^{-6} M) [22]. To prevent classical pathway activation EDTA (up to 20 mM) was added to plasma samples before incubation. In additional experiments β-A was incubated with F.XIId plasma reconstituted with physiologic concentrations of purified F.XII (30 μg/ml); this concentration of F.XII normalized coagulation F.XII activity assessed by coagulometric assay.

Plasmin generation by β-A peptides

The capacity of β-A to induce plasminogen activation *in vitro* was evaluated by assessing the generation of plasmin–anti-plasmin (PAP) complex. Briefly, soluble β-A1–42 or 1–28 were incubated for 30 min at 37°C in normal plasma or F.XIId plasma, with or without limited amounts of tissue-type plasminogen activator (t-PA) [23]. After SDS–PAGE in non-reducing conditions and Western blotting, the activation bands were visualized by polyclonal goat anti-plasminogen, biotin-conjugated rabbit anti-goat IgG and an avidin-alkaline phosphatase substrate. The apparent masses of PAP complexes and native plasminogen were, respectively, 150 kD and 90 kD. As a control, plasma samples were incubated with t-PA and fibrin(ogen) peptides to induce PAP complex generation.

Effect of the plasmin inhibitor (b-Dellin) on C1s activity

The method described by Lennick *et al*. [24] was used to measure the activity of C1s, determined by conversion of the substrate NZLBz. This highly sensitive assay was adapted for microtitre plates and used to determine the effect of the plasmin inhibitor b-Dellin on the activity of pure C1s. The amounts of C1s giving the best linear increase in absorbance (5 min at 340 nm in a Titertek Twinreader) were chosen. Before exposure to the substrate C1s was incubated for 30 min at 37°C with serial amounts of b-Dellin (100–500 μg/ml) or buffer as control.

Data presentation

Each experiment was run in duplicate and repeated three times using different batches of β-A. Representative experiments are reported in Figs 2, 3 and 4.

RESULTS

Light microscopy analysis showed that freshly solubilized peptides β-A1–42 and 1–28 were Thioflavin T-negative and gave a single band at the expected molecular weight (5–6 kD) in coomassie blue-stained SDS–PAGE (data not shown). Electron microscopy examination revealed no discernible ultrastructure in β-A1–42 preparations, or when structure was present (Fig. 1), it was represented by amorphous precipitate due to salt precipitation during the drying process. The same results were obtained with β-A1–28.

Incubation of these freshly solubilized β-A1–42 or 1–28 with normal human plasma resulted in dose-dependent activation of C4 with no evidence of C3 activation, even at the maximal concentration (100 μg/ml) of β-A. The ability to activate C4 was not prevented by adding EDTA (up to 20 mM) to plasma before incubation (Fig. 2a). Both peptides had similar C4-activating capacity (Fig. 2b). Since the β-A preparations used in the current experiments had no direct cleaving activity on pure C4 (Fig. 2c), nor on C1s or $C1r_2$–$C1s_2$ complex, we determined whether the interaction with C1q was critical for C4 activation by incubating β-A1–42 or 1–28 with human C1qd plasma. β-A peptides retained their ability to induce C4 cleavage in the absence of C1q, whereas HAG did not (Fig. 3a). The cleavage of C4 was associated with the generation of high mol. wt complexes containing C1s and C1INH both in C1qd plasma and in normal plasma containing EDTA (Fig. 3b). By densitometric analysis we found that the levels of stable C1s–C1INH complexes (mean + s.d. of three experiments in duplicate) were three to four times higher in C1qd plasma incubated with β-A (56 + 9%) than with HAG (12 + 4%) or buffer (12 + 5%). Similar values were measured in normal plasma treated with EDTA (β-A, 54 + 8%; HAG, 14 + 6%; buffer, 13 + 4%).

To explore alternative mechanisms of classical pathway activation, we investigated whether activation of the contact system,

leading to factor XIIf and kallikrein generation [25,26], and of fibrinolysis were essential for C4 activation. Normal Na-citrate plasma and F.XIId plasma were incubated with non-fibrillar β-A1–42 at different concentrations. To prevent activation of the classical pathway through assembly of C1 complex, EDTA (up to 20 mM) was added to the plasma samples before incubation. Whereas F.XII deficiency prevented the β-A-dependent cleavage of HK, a reliable marker of contact system activation [21], β-A retained its capacity to activate C4, even in the presence of EDTA (Fig. 4a). Reconstitution of deficient plasma with physiologic concentrations of F.XII (30 μg/ml) normalized coagulation F.XII activity, restored the β-A-cleaving activity to HK, and increased (8–15%) the degree of C4 cleavage. The cleavage of C4 was not prevented in FXIId-EDTA plasma to which the specific kallikrein inhibitor (PPACK) was added before incubation with β-A1–42 or 1–28.

Increased fibrinolytic activity *in vivo* and *in vitro* may be associated with activation of the classical C pathway by plasmin-dependent conversion of native C1s to its active form [15,16]. To assess the importance of plasmin generation in β-A-dependent C4 activation, soluble β-A1–42 was incubated with F.XIId plasma containing EDTA to prevent activation of, respectively, the contact system and C1. Although non-fibrillar β-A1–42 did not induce plasmin generation in the absence of t-PA, in additional experiments we incubated β-A with F.XIId-EDTA to which the plasmin inhibitor b-Dellin was added before incubation. The plasmin inhibitor did not significantly reduce the level of C4 activation (Figs 4b and 5). Among the plasmin inhibitors available commercially, we used b-Dellin, a potent inhibitor with no effect on C1s activity, as assessed by measuring conversion of the substrate NZLBz.

DISCUSSION

The results of this study indicate that freshly solubilized, apparently non-fibrillar, β-A peptides can induce activation of early factors of C classical pathway *in vitro*, through a mechanism that is

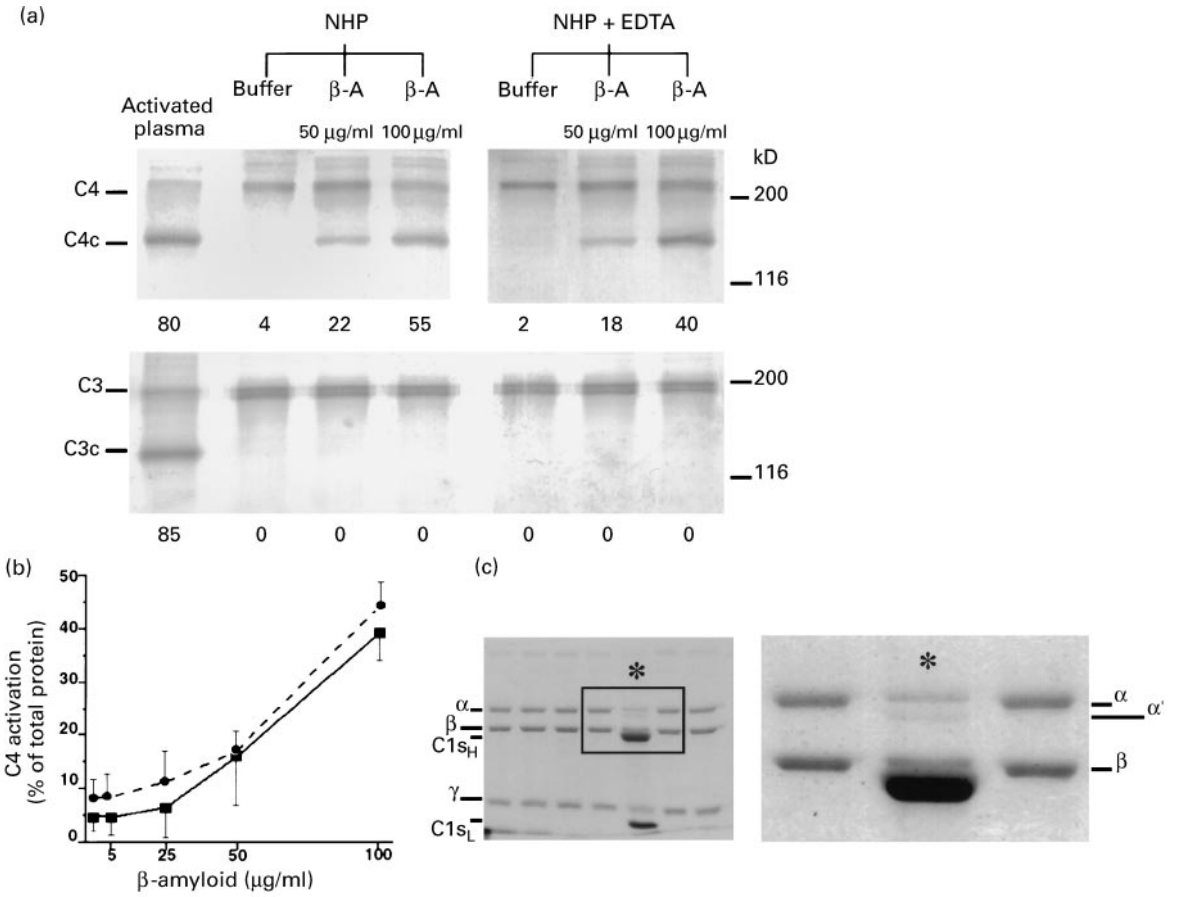

Fig. 2. Complement activation by non-fibrillar β-amyloid (β-A) *in vitro*. (a) Non-fibrillar β-A1–42 was incubated with fresh human Na-citrate plasma (NHP), or Na-citrate plasma to which EDTA (20 mM) was added before incubation (NHP + EDTA). C4 and C3 activation was assessed by SDS–PAGE in non-reducing conditions by Western blotting and densitometric evaluation of immunostained membranes. Non-fibrillar β-A peptides induced dose-dependent activation of C4, sparing C3, that was not prevented by EDTA. The first lane (activated plasma) refers to normal plasma massively activated with heat-aggregated IgG (HAG). The degree of C4 and C3 activation is reported at the bottom of each lane. (b) β-A1–42 (■) and 1–28 (●) have similar capacity to activate C4 in fresh human Na- citrate plasma. Values are the average of C4 activation measured in three experiments in duplicate (mean + s.d.). (c) SDS–PAGE analysis of the β-A cleaving activity on pure C4. C4 was mixed with β-A (100 μg/ml) from different batches or with purified C1s (*) and incubated for 30 min at 37°C. Specimens from each sample were run in SDS–PAGE under reducing conditions and stained with coomassie blue. β-A preparations did not induced the cleavage of C4 chains (α, β, γ), whereas C1s cleaved the α-chain (α′). C1s$_H$, C1s$_L$, heavy and light chains of C1s.

independent of the binding to C1q, and that might involve activation of contact system.

Freshly solubilized preparations of β-A, both 1–42 and 1–28, did not bind Thioflavin T, gave a single band in 15% SDS–PAGE, and had no fibrillar aggregates on electron microscope examination (Fig. 1), indicating that they were predominantly non-aggregated and not assembled in amyloid-like fibrils. Incubation of these β-A peptides in normal human plasma resulted in dose-dependent activation of C1s and C4, without any effect on C3 (Figs 2 and 3) and Factor B (not shown). The absence of any β-A cleaving activity on pure C4, C1s or on $C1r_2$–$C1s_2$ complex argues against the possibility that C activation could be due to contaminating proteases in the β-A preparations. Thus the morphological changes in β-A structure, due to fibrillation and aggregation, might therefore result in enhancement of β-A's ability to activate the classical pathway [8,11] more than being vital for starting C activation *in vitro* [13].

β-A1–42 and 1–28 at 25–50 μM retained their ability to lead to the C4 cleavage (Fig. 2) and to the generation of C1s–C1INH complexes (Fig. 3) after incubation in human plasma genetically deficient in C1q. This observation seems to rule out the possibility that the activity associated with freshly solubilized β-A might derive from the binding of C1q to dispersed fibrils present in our starting preparations [27–29], or from the conversion of some amount of the peptide into fibrillar configuration during the 30-min incubation in plasma [30].

A C1q-independent mechanism of classical pathway activation can be triggered by contact system and kallikrein activation, the second leading to generation of kinins [14]. Upon interaction with negatively charged substances, F.XII is converted to the catabolic peptides F.XIIf and F.XIIa which activate the classical pathway by acting at the site of C1r and C1s, the first directly [25] and the second through kallikrein generation [26]. Alternatively, increased fibrinolytic activity may result in activation of early C factors, since plasmin can activate C1s *in vitro* [15,25] and *in vivo* [16]. The involvement of the contact system and fibrinolysis in the inflammatory phenomena in the AD brain remains to be demonstrated, although several lines of evidence support it. Hageman factor is associated with β-A deposits [17], HK is massively cleaved in CSF of AD patients [18], and plasminogen [31], plasminogen activator and inhibitors are produced in brain [32,33]. *In vitro*, soluble β-A has the capacity to induce cleavage of HK [21], in a F.XII- and kallikrein-dependent manner (Fig. 4a), and enhances the t-PA-dependent conversion of plasminogen to plasmin (Fig. 4b) as effectively as fibrin(ogen) fragments [19]. Although further studies

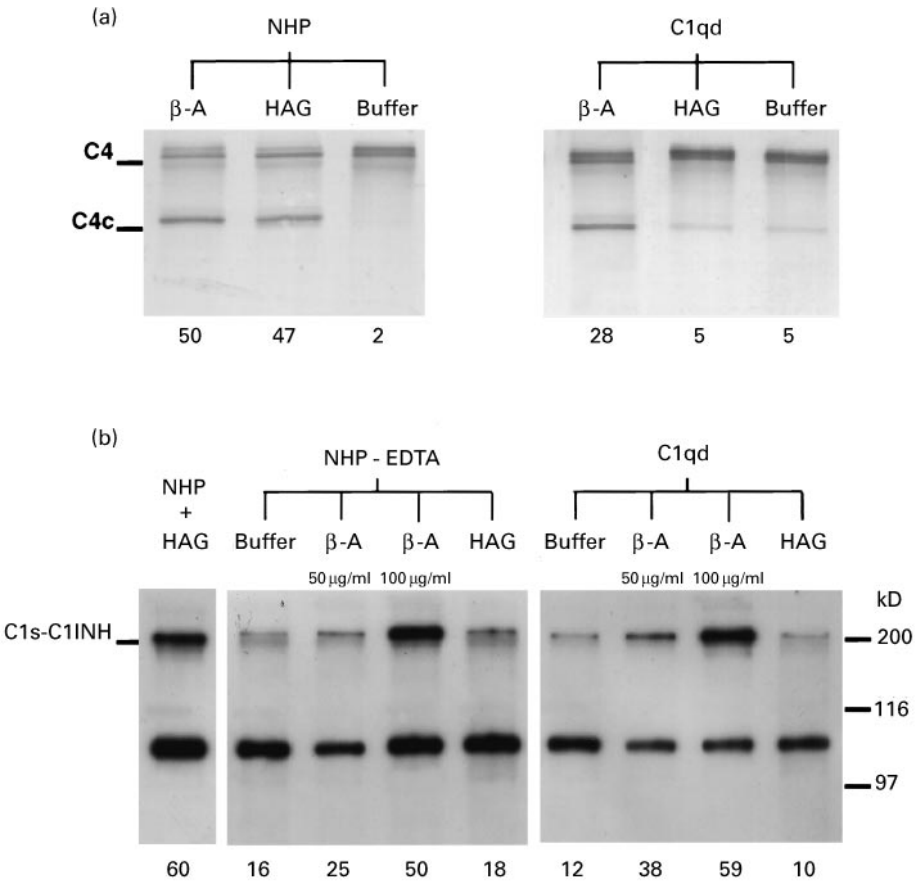

Fig. 3. C1q-independent complement activation by non-fibrillar β-amyloid (β-A)1–42. (a) Non-fibrillar β-A1–42, heat-aggregated IgG (HAG) (about 200 mg/ml, 100 μl) or buffer were incubated with human plasma congenitally deficient in C1q (C1qd) or with intact normal plasma (NHP). In NHP both β-A and HAG exhibited similar C4-activating ability, whereas in the absence of C1q, C4 was activated by β-A only. The degree of C4 activation is shown at the bottom of each lane. (b) β-A1–42 induced C1s–C1INH complex generation both in C1qd plasma and in normal plasma treated with EDTA, whereas HAG did not. The first lane refers to C1s–C1INH complex generated by HAG in normal plasma. The results of densitometric analysis of stable C1s–C1INH complex are shown at the bottom of each lane.

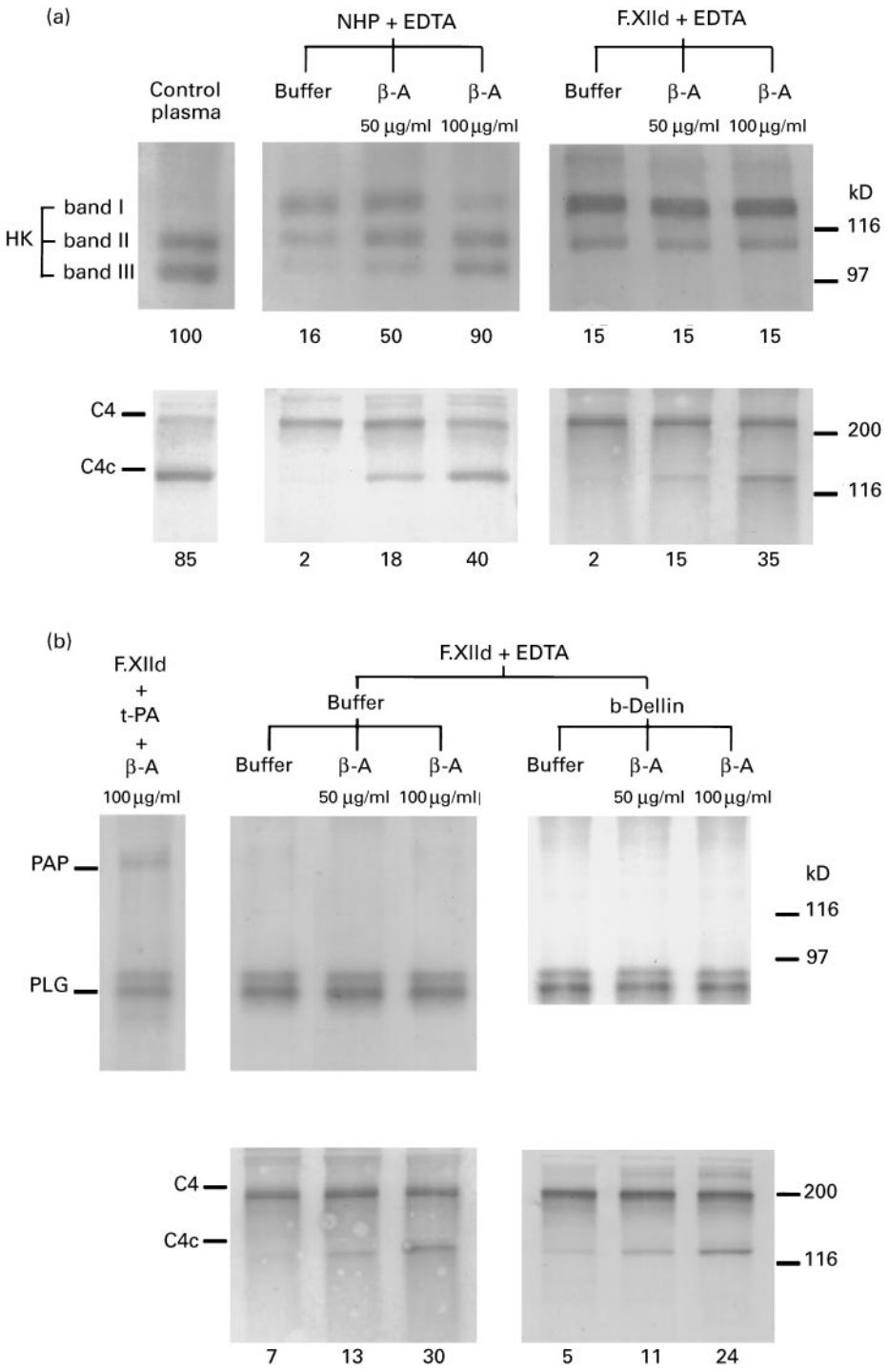

Fig. 4. Involvement of contact fibrinolysis system on C4 activation by β-amyloid (β-A) *in vitro*. (a) Normal human plasma (NHP) and plasma congenitally deficient in factor XII (F.XIId) were incubated with non-fibrillar β-A1–42 for 30 min at 37°C. To prevent C1q-dependent classical pathway activation, EDTA (20 mM) was added to the plasma before the incubation. Contact system activation was assessed by evaluating the degree of cleavage of high mol. wt kininogen (HK). Factor XII deficiency prevented the β-A-dependent activation of contact system, whereas the cleavage of C4 was only slightly reduced. The percentages of HK and C4 activation are shown at the bottom of each lane. Control plasmas refer to NHP incubated with kaolin or heat-aggregated IgG (HAG) for HK or C4 activation, respectively. (b) β-A1–42 was incubated with F.XIId plasma to which the plasmin inhibitor b-Dellin and EDTA were added before incubation. Plasminogen (PLG) activation was assessed by evaluating generation of plasmin–anti-plasmin (PAP) complexes. Non-fibrillar β-A, with apparently no effects on plasminogen activation, retained its ability to activate C4 even in presence of the plasmin inhibitor (b-Dellin). The first lane shows the pattern of plasminogen activation induced by β-A in the presence of tissue-type plasminogen activator (t-PA).

are needed to characterize the molecular events, they are likely to be due to reaction of the negatively charged residues of β-A peptides on F.XII [34] and t-PA. That activation of contact system might be involved in the C4 cleavage seen in our experiments is suggested by the finding that in F.XIId-EDTA plasma the amount of cleaved C4 was about 5% and 10% less than in C1qd and normal EDTA-plasma, respectively (Fig. 5), and that reconstitution of deficient plasma with physiologic concentrations of F.XII increased the level (8–15%) of C4 cleavage. Thus the finding that soluble β-A1–42 and 1–28 induced similar amounts of cleaved C4 after incubation in normal plasma (Fig. 2) might be only apparently in contrast with Jiang and co-workers' report [9] of a lack of significant C4 activation by the shorter peptide. The source of C factors might be responsible for this difference. In citrate plasma we could assess the effect of β-A on both the C and fibrinolysis-contact systems, whereas using serum Jiang *et al*. could only evaluate the effect on C, since fibrinolysis and contact systems were already activated by blood coagulation.

The pattern of β-A-dependent activation of early components of classical pathway seen in our experiments is unusual, in that C1s and C4 showed some degree of activation, but C3 was apparently normal. A similar C profile has been previously observed *in vitro* when certain polyanions [35] or cryoglobulins [36] are incubated with normal serum, and it has been attributed to the chemical composition of those reactants that allows for a greater accessibility of C4b or C4b2a to the control proteins (C4BP, H, I) of classical pathway C3 convertase. In addition, a partial classical pathway activation with the characteristic of sparing C3 may be due to a defective control on C1 activation as observed in patients with C1INH deficiency. Complement and contact systems share C1INH as a major inhibitor [37,38], thus activation of F.XII leading to C1 activation may result in C1INH consumption, and the deficiency of functional C1INH may enhance the susceptibility of C1 and F.XII to the activation. Because C1INH binds to activated C1 it is possible that such a consumption of C1INH may facilitate F.XII activation by β-A in our experiments. In addition, *in vitro* studies have shown [14] that the rate of inhibition of activated F.XII by C1INH is considerably reduced in the presence of negatively charged surfaces (β-A in our experiments). There is evidence that a local consumption or functional deficiency of C1INH may occur *in vivo*; in fact in AD neuritic plaques this protein has been detected by a MoAb that recognizes the complexed form [34]. Although the absence of C1s or C1r molecules at the site of C1INH immunoreactivity [4] could be due to the reduced antigenicity of the C1 subunits after their inhibition [39], the expression of a neo-epitope in the C1INH molecule might also be the result of binding to other proteases, such as F.XIIa, kallikrein, or plasmin. Recently, *in situ* hybridization revealed that in brain areas with neuritic plaques and activated glial cells, only neurones express C1INH mRNA. Thus a defective synthesis of C1INH combined with an increased rate of consumption may allow ongoing contact system and complement activation in the areas of β-A deposition [40].

Whatever the mechanism, the cleavage of C4 results in generation of proinflammatory peptides, although with a lower specific activity than those from C3 and C5. The small peptide C4a can enhance vascular permeability, and can diffuse away from the activation site chemotactically signalling to inflammatory cells; C4b through the binding to cell membrane can serve as opsonin.

From the summary Fig. 5 it appears that there was a consistent amount of uninhibited cleaved C4. Since it was about four to five times higher than in buffer-plasma control, it is unlikely that this could be due to an unspecific C4 proteolysis occurred during the 30-min incubation. Concerning the remaining uninhibited C4 cleavage, using genetically deficient plasmas we could ascertain reasonably the relevance of the defective factor, i.e. C1q or F.XII, whereas it is likely that some degree of activation could occur in plasma despite the inhibition of complement and/or contact-fibrinolytic proteases with chemicals or Ca-chelating agent.

It is apparent from our data that the ability of β-A to activate C1s and C4 does not necessarily imply the binding of C1q to fully fibrillar peptides. Under our experimental conditions we could not define the exact mechanism of such an activation, but we provide evidence that activation of contact system seems to play a role. This may be relevant in AD brain, where contact system has been found to be activated [8]. Since non-fibrillar β-A does not bind C1q, our finding could provide one possible explanation for the C-activating ability by monomeric β-A *in vitro* [13], and for the amount of activated C4 found in diffuse deposits in AD brain [3]. The fact that non-fibrillar β-A deposits may be found in the brain of non-demented elderly subjects indicates that *per se* non-fibrillar amyloid is not enough to explain the pathogenesis of AD. However, the AD brain shows increased production of β-A peptide, and it has been recently shown that β-A without the need of pre-aggregation may induce vascular endothelial damage and microglia activation [41,42]. Thus by showing that non-fibrillar β-A can induce generation of humoral inflammatory peptides, both from contact system and complement, our data could support the hypothesis that non-fibrillar β-A also may play a role in initiation of the inflammatory reactions associated with the progression of plaques and probably of the clinical disorder in AD.

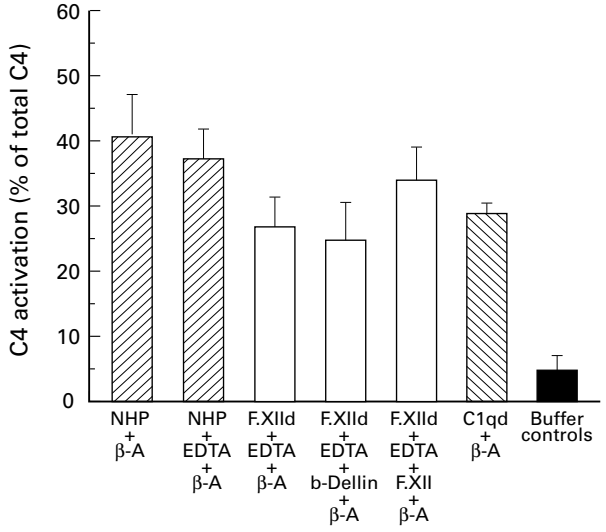

Fig. 5. Influence of complement, contact system and fibrinolysis inhibition on non-fibrillar β-amyloid (β-A)-dependent activation of C4. Bars represent the percentage of C4 activation in three separate experiments in duplicate (mean + s.d.).

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