The deposition of β-amyloid in the brain is the key pathological event in Alzheimer's disease. Among the various mechanisms proposed to explain the neurotoxicity of β-amyloid deposits, a new one, recently identified in our and other laboratories, suggests that β-amyloid is indirectly neurotoxic by activating microglia to produce toxic inflammatory mediators such as cytokines, nitric oxide, and oxygen free radicals. Three findings presented here support this mechanism, showing that β-amyloid peptides (25–35), (1–39), and (1–42) activated the classical NADPH oxidase in rat primary culture of microglial cells and human phagocytes: 1) The exposure of the cells to β-amyloid peptides stimulates the production of reactive oxygen intermediates; 2) the stimulation is associated with the assembly of the cytosolic components of NADPH oxidase on the plasma membrane, the process that corresponds to the activation of the enzyme; 3) neutrophils and monocytes of chronic granulomatous disease patients do not respond to β-amyloid peptides with the stimulation of reactive oxygen intermediate production. Data are also presented that the activation of NADPH oxidase requires that β-amyloid peptides be in fibrillar state, is inhibited by inhibitors of tyrosine kinases or phosphatidylinositol 3-kinase and by dibutyryl cyclic AMP, and is potentiated by interferon-γ or tumor necrosis factor-α.

The most common form of senile dementia, Alzheimer's disease (AD), is characterized by a progressive loss of neurons from particular regions of the brain, by the formation of neurofibrillar tangles in neurons, and by numerous senile plaques in affected brain regions. The major component of the senile plaques is the β-amyloid protein (Aβ), a 39–43-amino acid peptide, derived from a larger transmembrane glycoprotein called β-amyloid precursor protein (1). The Aβ of the senile plaques consists of fibrils that are 4–10 nm in diameter and exhibit green birefringence under polarized illumination when stained with Congo Red. According to spectroscopic and x-ray diffraction studies, the amyloid fibril assembly is correlated with the adoption of a β-sheet structure (2, 3).

According to the “β-amyloid cascade” hypothesis, the excessive deposition of Aβ is the key pathogenetic event in AD (4–8) and would be responsible for neurodegenerative changes of neurons. In the plaques the central deposit of extracellular amyloid fibrils (the core) is surrounded by dystrophic neurites (dendrites and axon terminals) and by activated microglia and reactive astrocytes (9, 10). Furthermore, it has been demonstrated that Aβ is neurotoxic in vitro (7, 11, 12) and that this toxicity correlates with β-sheet structure and fibrillar state (Ref. 13; Ref. 7 and references therein). Data have been also provided showing that it is the amphiphilic nature of the peptides rather than their β structure per se that causes toxicity (14).

Studies of Aβ cytotoxicity have resulted in some broad concepts of mechanism that are not mutually exclusive (7, 15). One proposes that Aβ can directly injure neurons by specifically or unspecifically interacting with molecules of cell surface (11). This direct toxicity could be due to free radicals oligopeptides derived from Aβ itself (16) or stimulation of intracellular production of reactive oxygen intermediates (ROI). Two groups of experimental data demonstrate this last mechanism. The first is that the interaction in vitro of Aβ (1–40) or Aβ (25–35) with PC12 cells, B12 cells, cultured rat cortical (14, 17), or hippocampal neurons (18) induces the stimulation of intracellular production of ROI. It has also been demonstrated that Aβ causes the appearance of markers of oxidative stress in neurons adjacent to senile plaque and in dystrophic neurites contiguous to Aβ deposits (19). The second group of experimental data is that antioxidants prevent the cytotoxicity by Aβ (14, 17, 18, 20, 21). Another proposal is that Aβ causes little cytotoxicity by itself but enhances the vulnerability of neurons to a variety of common insults, such as excitotoxicity, hypoglycemia, or peroxidative damage (22, 23). Finally, it has been recently suggested by us and others that Aβ can also be indirectly toxic to neurons by activating the production of toxic and inflammatory mediators such as NO and cytokines in microglia (24, 25) and in astrocytes (26) and ROI in monocytes and macrophages (27, 28) and in microglia (29–31). The mechanism by which Aβ stimulates the production of ROI is not known. Potential main sources of ROI are mitochondria, microsomal enzymes, xanthine oxidase, classical NADPH oxidase of phagocytes, some unidentified NADH-NADPH oxidases, lipooxygenase, and cyclooxygenase (Ref. 32 and references therein). Following previous work of our laboratory concerning the indirect
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Mechanism of neurotoxicity mediated by activated microglia (24, 26, 27) and on the basis of recent data that Aβ is able to stimulate the production of O$_2^-$ in microglia (29, 31), we have investigated the involvement of NADPH oxidase in this stimulation (33, 34). This oxidase is an enzymatic system, present in phagocytes and few other nonphagocytic cell types, which is dormant in resting cell and becomes active upon contact with appropriate receptor-dependent or -independent agonists. Microglial cells belong to the mononuclear phagocytic system and as monocytes and neutrophils present the classical burst of ROI production when stimulated by appropriate agonists, such as cytokines and PMA (35). In this paper we have investigated the effect of Aβ (25–35), Aβ (1–39), and Aβ (1–42) on rat primary cultures of microglia, human blood monocytes, and human neutrophils. The data presented support the concept that Aβ can be indirectly neurotoxic and clarify a further mechanism, in addition to the production of TNF-α and stimulation of inducible NO synthase (24–26), by which microglial cells are involved in the generation of toxic mediators. In fact, the results demonstrate that Aβ peptides cause the activation of the classical NADPH oxidase, which results in the stimulation of ROI production. This activation of the oxidase requires that Aβ peptides are in fibrillar form, is potentiated by IFN-γ and TNF-α, and is inhibited by inhibitors of tyrosine kinase or phosphatidylinositol 3-kinase and by dibutyryl cyclic AMP.

Materials and Methods

Reagents—The Aβ peptides Aβ (25–35) and Aβ (1–39) were synthesized, purified, and characterized by Prof. E. Peggio (Dipartimento di Chimica Organica, Università di Padova, Italy) or by Dr. R. Gennaro (Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, Italy) as described previously (24); Aβ (1–42) and Aβ (25–35) scrambled, IMLKGNGAG, were a gift by Dr. L. Otvoš (Wistar Institute, Philadelphia, PA). Lyophilized peptides were dissolved in sterile PBS or double-distilled H$_2$O at a concentration of 2–4 mg/ml, aged 5–7 days at 37 °C, aliquoted, and stored at 4 °C. Fibroblast formation was monitored by examining the birefringence after staining with Congo Red and quantified by measuring the fluorescence intensity after staining with Thioflavine T (36). Fibroblogenesis by Aβ (25–35) was rapid (minutes) in either PBS or H$_2$O at room temperature, whereas that by Aβ (1–39) and Aβ (1–42) was increased due to oxidation of H$_2$O$_2$ by Aβ at 37 °C. When the experimental protocol required Aβ peptides in nonfibrillar form, they were dissolved in Me$_2$SO or kept at 4 °C until solution. In these conditions the fluorescence by Thioflavine T was negative (see Fig. 2).

Recombinant rat IFN-γ was from BIOSOURCE International and mouse TNF-α was from PeproTech Inc. Wortmannin (WT) was kindly provided by Dr. M. Thelen (Theodor Rocher Institute, Bern), and glycyrrhizin (from bovine liver), trypsin, diphenyleneiodonium chloride (DPI), and horseradish peroxidase (HRP) (type 1) were purchased from Sigma Chemical Co. (St. Louis, MO). Lyophilized peptides were dissolved in sterile PBS or double-distilled H$_2$O at a concentration of 2–4 mg/ml, aged 5–7 days at 37 °C, aliquoted, and stored at 4 °C.

Antibodies—The anti-rat myeloid cell (CR3) was from Serotec. The anti-galactocerebroside and the anti-G. simplicifolia isolectin B4 and mouse anti-rat myeloid cell (CR3). The anti-galactocerebroside and the anti-G. simplicifolia isolectin B4 and mouse anti-rat myeloid cell (CR3) antibodies were kindly provided by Dr. D. Roos (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam), and Laboratory of Experimental and Clinical Immunology, University of Amsterdam). Solutions used throughout our experiments were prepared with endotoxin-free water for clinical use.

Cells—Neutrophils and monocytes (95–98% purity) were isolated from buffy coats of healthy donors as previously reported (37, 38). Primary microglial cell cultures were prepared from 2-day-old postnatal Wistar rat cerebral cortices as described previously (39) with some modifications. Briefly, the rats were decapitated, and the brains taken out and put into a Petri dish containing PBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The meninges were next removed, and the cortices were isolated and mechanically dissociated by chopping and triturating 20% PBS containing trypsin and antibiotics at room temperature. The mixed glial suspension was collected by filtration through gauze. Trypsin was next neutralized by adding an equal volume of Dulbecco’s modified Eagle’s medium containing 20% fetal bovine serum. The cell suspension was centrifuged, and the collected cells were seeded into 160 cm$^2$ flasks (Costar), in Dulbecco’s modified Eagle’s medium con- taining 10% fetal bovine serum. Twenty-four hours later the growth medium was replaced with fresh one. After 6–8 days microglial cells were separated from the astroglial cells monolayer by shaking the flask for 60 min at 120 rpm on a rotatory shaker. The released microglial cells were then suspended in Hanks’ balanced salt solution and used for the experiments. When the effect of IFN-γ or TNF-α was investigated, these cytokines were added to the cell culture 4–5 days before the detachment of microglial cells by shaking. The purity of microglial cell preparations (>98%) was confirmed by testing their positiveness for markers like G. simplicifolia isolectin B4 and mouse anti-rat myeloid cell (CR3). The anti-galactocerebroside and the anti-galactocerebroside acidic protein were used to confirm the concurrent absence of oligoglycans and astrocytes, respectively.

Threshold of ROI production—ROI production was measured by adding the agonists (Aβ peptides, PMA) to stirred cell suspension in Hanks’ balanced salt solution. In the first experiments the production of ROI was investigated with the procedures normally used in our laboratory, that is, as H$_2$O$_2$-HRP-dependent oxidation of homovanillic acid or as O$_2^-$-dependent SOD-sensitive reduction of cytochrome c (40, 41). However, because the respiratory burst by Aβ peptides in monocytes and microglia was very low and the above procedures required a large number of cells and consequently high concentrations of Aβ peptides, in most experiments we adopted the more sensitive method of H$_2$O$_2$ determination using DHR oxidation (42).

The procedure was substantially that previously reported (42), modified by the addition of HRP. The reason for this addition was the following. Henderson and Chappell (42) have demonstrated that in leukocytes stimulated by PMA, the oxidation of DHR occurred within the cells and was catalyzed by endogenous peroxidase and H$_2$O$_2$ generated outside the cell by O$_2^-$ from NADPH oxidase and crossing the plasma membrane of the cells. By adopting their procedure we have noted that upon stimulation of the cells with PMA the increase in fluorescence was delayed and in general the increase was increased by addition of exogenous HRP. This finding demonstrated that a correct estimation of the quantity of H$_2$O$_2$ produced by activated NADPH oxidase, which is located on the plasma membrane, required the trapping of all the peroxide released outside the cells. On this basis we have modified the procedure of Henderson and Chappell by adding HRP to cell suspension. HRP does not enter the cells but rather catalyzes the oxidation of DHR to DHR$_2^+$, the cells by trapping all the peroxide as it is generated by spontaneous dismutation of O$_2^-$, the first product of NADPH oxidase. The procedure that we have used was the following. Stirred cell suspension in Hanks’ balanced salt solution was pre-incubated with 200 units/ml of catalase for 2 min at 37 °C to degrade all the peroxide present in the solutions and cell suspension. After this time 1 μM Na$_3$VO$_4$ to inhibit the endogenous catalase, 0.5–2.0 μM DHR, and HRP (500 million units/ml) were added. One minute after HRP, the stimulus or the vehicle was added, and the changes in fluorescence at 534 nm after excitation at 505 nm were continuously recorded for the time required. Calibration was made by adding different amounts of exogenous H$_2$O$_2$. This highly sensitive procedure enabled the measurement of the respiratory burst using 0.5–3 × 10$^5$ cells/ml. In these conditions the relation- ship between free radicals production and number of cells was linear. When the effects of various drugs on the stimulation of the respiratory burst was studied, preliminary investigations were performed to calculate the eventual quenching of rhodamine 123 fluorescence.

Studies on the Activation State of NADPH Oxidase—The activation of NADPH oxidase was investigated by evaluating the translocation of Laboratory of the plasma membrane after cell stimulation as described previously (40). Briefly, neutrophils or monocytes (5 × 10$^5$) were incubated at 37 °C under stirring with or without the agonists. At the indicated time samples containing 1.5 × 10$^5$ cells were withdrawn and disrupted by sonication (two 15 s cycles at 200 W at 4 °C). The postnuclear supernatants were loaded on a discontinuous sucrose gradient of 1.5 ml of 15%
**RESULTS**

**Aβ Peptides Stimulate the Production of ROI**—The results of Table I and Fig. 1 show that Aβ (25–35), Aβ (1–39), and Aβ (1–42) were able to stimulate the production of ROI, measured as H2O2, in rat primary culture of microglial cells, human blood monocytes, and neutrophils. The data of Fig. 1 show that the stimulation of H2O2 production by Aβ (25–35), Aβ (1–39), and Aβ (1–42) was detectable already at 1 µM and was maximal between 10 and 20 µM. From the data of Table I and Fig. 1, it can be seen that the response of human neutrophils was much greater than that of human monocytes and rat microglia and that the latter cells were the less responsive. In all cell types the magnitude of the response to 10 µM Aβ peptides was smaller than that to 100 ng/ml of PMA, a dose that in the experimental conditions employed was the maximal one. The stimulation of H2O2 production was detectable within 1–2 min after the addition of the Aβ peptides and lasted for 30–60 min (not shown). Cells exposed for 30 min to 10 µM Aβ (25–35) scrambled peptide did not present any stimulation of H2O2 production (Table I).

**Aβ Peptides Are Active in Fibrillar State**—It is widely accepted that Aβ is active on neurons in fibrillar state (7, 12, 15, 43, 44). We present here two findings that demonstrate that this was the case also in our experiments. First of all, the preparations of Aβ (25–35), Aβ (1–39), and Aβ (1–42) that we have used were in fibrillar state as demonstrated by the birefringence under polarized illumination when stained with Congo Red and by the fluorescence when stained with Thioflavine T. A direct demonstration that only Aβ peptides in fibrillar state were able to stimulate the production of H2O2 is reported in Fig. 2. It has been shown that Thioflavine T associates with fibrils of Aβ peptides giving rise to an enhanced emission at 482 nm with excitation at 450 nm (36). Fig. 2A shows that this occurred when Thioflavine T was added with Aβ (1–39) dissolved in PBS and aged 5 days at 37 °C. On the contrary, when Thioflavine T was added with the peptide dissolved in PBS and kept at 4 °C, the fluorescence emission spectrum of Thioflavine T did not change, indicating that in this preparation Aβ (1–39) was not in fibrillar state. When these two preparations were tested for their agonistic activity in microglia, the results have shown that only the aged fibrillar Aβ peptide was able to stimulate the production of H2O2 (Fig. 2B). Similar results were obtained in one experiment made with Aβ (1–42). Furthermore, when Aβ (25–35), Aβ (1–39), and Aβ (1–42) were dissolved in MeSO they did not form fibrils and were unable to stimulate the production of H2O2 in all cell types. Fig. 2 reports the result obtained with Aβ (1–39).

The second finding was the effect of HS. On the basis of the previous demonstrations that sulfate or sulfonate compounds bound to the β-pleated sheet conformation of Aβ fibrils and inhibited the neurotoxicity of Aβ peptides (13, 43–45), we have...
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Fig. 3. Effect of HS (final concentration, 2 μg/ml) on the stimulation of H2O2 production by 10 μM Aβ peptides and by 2 ng/ml PMA. HS dissolved in double-distilled H2O was added to the solution of Aβ peptides. The mixture was kept at room temperature for 5–10 min and then added to the cell suspension. Results are shown as the means ± S.D. from four experiments. When PMA was the stimulant, the cells were preincubated with HS for 5 min, and the results are the means of two experiments.

investigated the effect of these compounds on the ability of Aβ (25–35), Aβ (1–39), and Aβ (1–42) to stimulate the production of H2O2. The results reported in Fig. 3 show that HS inhibited the stimulation of H2O2 production by Aβ peptides in microglia, monocytes, and neutrophils. An unspecific inhibitory effect of HS can be excluded by the finding that HS did not modify the response to PMA (Fig. 3).

Sources of ROI—Many sources could be responsible for the increase in the production of ROI by exposure to Aβ peptides. The most common cellular sources of ROI include mitochondrial respiratory chain, microsomal enzymes, xanthine oxidase, arachidonic acid cascade, the classical NADPH oxidase of phagocytes, and some unidentified NADH-NADPH-dependent enzymes (32). Three findings show that the exposure of the cells to Aβ peptides induced the stimulation of ROI production by activating the classical NADPH oxidase. The first was the direct demonstration of the activated state of the oxidase in cells treated with Aβ peptides. This oxidase is composed of several proteins, and when the cells are in resting state the oxidase is inactive, and some components are located on the plasma membrane (the two subunits gp91phox and p22phox of the flavocytochrome b558), whereas others are in the cytosol (the p47phox, p67phox, p40phox, and Rac1/Rac2). The activation of the NADPH oxidase involves multiple signal transduction pathways, all resulting in the translocation of the cytosolic components on the plasma membrane where, in association with the subunits of cytochrome b558, they allow the oxidation of cytosolic NADPH and the univalent reduction of oxygen with formation of O2− to take place (34). To identify the NADPH oxidase as a source of ROI, we investigated whether the stimulation of H2O2 production induced by Aβ peptides was associated with the translocation of its cytosolic components on the plasma membrane. For this purpose we fractionated neutrophils and monocytes in resting state and after stimulation with Aβ peptides or other stimulants and immunoblotted the light membrane fraction with antibodies raised against the cytosolic components p47phox, p67phox, and p40phox of NADPH oxidase. The results reported in Fig. 4 show that although the cytosolic components are practically absent or present in very low amounts in the plasma membrane of unstimulated neutrophils and monocytes, they were translocated on the plasma membrane upon stimulation with Aβ peptides, as occurred after the treatment of the cells with fMLP alone, fMLP plus cytochalasin B, or PMA. The stimulation of the production of H2O2 in the same experiment by various agonists is also reported in the legend of Fig. 4. It can be seen that despite the facts that the ECL system for detection of proteins is semiquantitative and a very precise correlation between translocation and activation of NADPH oxidase cannot be made, the entity of the translocations paralleled those of the stimulation of H2O2 production. The translocation of NADPH oxidase components was not feasible on rat microglial cells because antibodies against the components of the NADPH oxidase are species-specific and antibodies against components of rat oxidase are not available.

The second demonstration of the involvement of the NADPH oxidase was obtained by using neutrophils and monocytes of the CGD patients. CGD is an inherited condition wherein the absence of cytochrome b (X-linked form) or of p47phox or p67phox (autosomal forms) results in an inactive NADPH oxidase. Thus the leukocytes of CGD patients are unable to produce ROI in response to all the agonists of this enzyme. The disease is characterized clinically by recurrent and sometime life-threat-
ening bacterial and fungal infections due to the inability of leukocytes to produce ROI and efficiently kill microorganisms. We analyzed the effect of Aβ on leukocytes from two CGD patients (G. N. and Z. N.) affected by X-linked form lacking cytochrome b558 and one patient (A. M.) affected by an autosomal form lacking p47phox. As shown in Fig. 5, neutrophils and monocytes of all the CGD patients did not respond with an increase in H2O2 production to the exposure to Aβ peptides or PMA.

The last result concerning the involvement of the NADPH oxidase was the finding reported in Fig. 7 that 10 μM DPI markedly suppressed the stimulation of H2O2 production by Aβ peptides in all cell types. This result is not an absolute demonstration of the involvement of the NADPH oxidase because DPI is also an inhibitor of other flavoprotein dehydrogenases (46), but it may be a support to the demonstration that NADPH oxidase is the real source of H2O2 in cells stimulated with Aβ peptides.

**Mechanism of Activation of NADPH Oxidase by Aβ Peptides**—It is known that the activation of NADPH oxidase can result from many signal transduction pathways involving receptor-dependent and -independent agonists, different forms of G proteins, second messengers, protein kinase activation, and phospholipid turnover (33, 47). To understand the mechanisms of NADPH oxidase activation by Aβ peptides, we investigated the effects of PP1, a Src family-selective tyrosine kinase inhibitor (48), and of WT, an inhibitor of phosphatidylinositol 3-kinase (49). The results reported in Figs. 6 and 7 show that the stimulation of H2O2 production by Aβ (25–35), Aβ (1–39), and Aβ (1–42) was markedly sensitive to micromolar concentrations of PP1 and to nanomolar concentrations of WT. The results reported in Fig. 7 show also that the stimulation of NADPH oxidase by Aβ (25–35), Aβ (1–39), and Aβ (1–42) was inhibited by 1 mM dibutyryl cyclic AMP, indicating that protein kinase A could play a negative role in the transduction pathway of NADPH oxidase activation by Aβ peptides. All the inhibitors used were not toxic for the cells as shown by the lack of effect on the stimulation of H2O2 production by PMA (data not shown).

**Potentiation of the NADPH Oxidase Response to Aβ Peptides by IFN-γ or TNF-α**—Previous works in our (50) and other laboratories have shown that the stimulus-induced activation of the NADPH oxidase of macrophages can be potentiated by treatment with cytokines, mostly IFN-γ or TNF-α, and that this potentiation is due to the increased expression of oxidase components (50–54). It has been also shown that the maturation of monocytes to macrophages resulted in a progressive loss of the capability to produce ROI (51, 53) due to decreased expression of NADPH oxidase components (50, 51, 54). The expression of these components and the stimulus-induced activation of the oxidase were restored by treatment with IFN-γ (50, 54). Microglial cells are resident macrophages derived from blood monocytes, and their low responsiveness to Aβ peptides compared with that of monocytes probably reflects the down-regulation of NADPH oxidase components during the matura-

**Fig. 6.** Effects of different doses of PP1 and WT on the stimulation of H2O2 production by 5 μM Aβ (1–42) in microglia. Cells were preincubated with vehicle (Me2SO, 1 μM/ml), PP1, or WT for 5 min before stimulation. 100% value is 0.16 nmol H2O2/3 × 105 cells/30 min. Results are the means of two experiments.

**Fig. 7.** Effect of 5 μM PP1, 20 nM WT, 1 mM dibutyryl cAMP, and 10 μM DPI on the stimulation of H2O2 production by 10 μM Aβ (25–35), Aβ (1–39), or Aβ (1–42), in microglia, monocytes, and neutrophils. The cells were incubated with all the compounds 5 min prior to stimulation. Results are shown as the means ± S.D. from eight experiments with Aβ (25–35) and four with Aβ (1–39) and Aβ (1–42). With DPI the results are the means of two experiments. 100% values (expressed as nmol H2O2/50 min) in cells stimulated with Aβ (25–35), Aβ (1–39), and Aβ (1–42) respectively, are: 0.32 ± 0.06, 0.24 ± 0.07, and 0.21 ± 0.04 in 3 × 105 microglia; 0.70 ± 0.11, 0.60 ± 0.09, and 0.69 ± 0.13 in 3 × 10⁵ monocytes; and 1.48 ± 0.38, 1.02 ± 0.22, and 0.98 ± 0.14 in 5 × 10⁴ neutrophils.

We investigated here whether or not IFN-γ or TNF-α were able to potentiate the stimulation of H2O2 production by Aβ peptides in microglia. For this purpose we grew the primary culture of rat microglial cells for 4–5 days in presence of 50...
forming NADPH oxidase is involved in the stimulation and other ROI generation by activated 15498
produce inflammatory and cytotoxic mediators (24–28, 31, 55, 56).

In agreement with the data concerning the direct neurotoxicity (7, 12, 13, 43, 44), we have shown here that also for the NADPH oxidase.

We have previously presented a marked potentiation of the production of H2O2 when exposed to Aβ peptides.

**DISCUSSION**

According to the most accepted theory, the excessive formation of Aβ from β-amyloid precursor protein and its deposition in the brain is the key event in the progressive neuronal damage in AD (4, 5, 8). The studies performed on the neurotoxic effect of Aβ peptides (7, 11–26) have resulted in the concept that many mechanisms, direct or indirect, are responsible for neurotoxicity. In this paper we confirm the existence of indirect mechanisms involving the stimulation by Aβ peptides of microglia, a cell type of the mononuclear phagocyte system, to produce inflammatory and cytotoxic mediators (24–28, 31, 55, 56).

The results presented here indicate that the activation of classical O2-forming NADPH oxidase is involved in the stimulation of the production of ROI in microglia by Aβ peptides. Two experimental results supported this indication. One was the finding that the stimulation of H2O2 production was associated with the translocation of cytosolic factors p47phox, p67phox, and p40phox to the plasma membrane, a process that corresponds to the activation of the oxidase (34, 40). The second was the finding that neutrophils and monocytes of patients affected by CGD, a genetic condition lacking components of the NADPH oxidase, did not respond to the exposure to Aβ peptides with the stimulation of H2O2 production. The possibility that mechanisms other than NADPH oxidase are responsible for the stimulation of ROI production by Aβ peptides cannot be ruled out. However, the finding that leucocytes of CGD patients are unresponsive to Aβ suggests that the involvement of other mechanisms is unlikely. These data and those previously obtained in our (24, 26) and other (25) laboratories support the hypothesis that Aβ peptides activate two indirect mechanisms of oxidative damage involving microglia. One would be the induction of the NO synthase and production of NO, the other the stimulation of O2 and other ROI generation by activated NADPH oxidase.

In agreement with the data concerning the direct neurotoxicity (7, 12, 13, 43, 44), we have shown here that also for the stimulation of H2O2 production Aβ peptides were active in aggregated fibrillar form. The type of interaction of Aβ peptides with the cell surface is controversial. Data have been provided that scavenger receptors (29, 57–60), advanced glycation end product receptors (19), tachykinin receptors (11), and serpin-enzyme complex receptors (61) would be involved. In our experiments, the activation of NADPH oxidase due to the interaction of Aβ peptides with scavenger receptors is possible in microglia because in these cells these receptors are well expressed (29, 57–60). On the contrary the involvement of scavenger receptors in neutrophils or monocytes should be excluded because these receptors are absent in neutrophils and absent or minimally expressed in monocytes (59, 60). The participation of advanced glycation end product receptors in the activation of microglia by Aβ peptides has been denied (31), and our results that glycated human serum albumin was unable to stimulate the NADPH oxidase in microglia, monocytes, and neutrophils agree with this (Table I). The involvement of tachykinin receptors and serpin-enzyme complex receptors in the stimulation of NADPH oxidase remains to be investigated.

Data have been also provided showing that Aβ peptides interact with membranes to form cation-selective channels (62) or disrupt the integrity of plasma membrane by virtue of their lyophilic nature (63–65). Further studies are needed to understand whether the effect of Aβ peptides on NADPH oxidase may be attributable to their detergent properties (63) or to a receptor-mediated interaction.

It is known that various reactions participate in the signal transduction for NADPH oxidase activation. Some of these, i.e., the phosphorylation of cytosolic components, are constantly activated regardless of the agonist used, whereas others, i.e., the stimulation of phospholipase C, D, and A2, protein kinase C, mitogen-activated protein kinase, and trimeric G proteins, are differentially recruited depending on the agonist used (33, 34, 47). The inhibition by PP1, WT, and dibutryl cAMP supports the concept that the activation of NADPH oxidase by Aβ peptides involves the participation of Src family of tyrosine kinases (31) and of phosphatidylinositol 3-kinase and is counteracted by the activation of protein kinase A. Further investigations are needed to clarify the role of these enzymes.

The activation of NADPH oxidase occurred with Aβ peptides alone and did not require previous priming as, on the contrary, occurred for the induction of NO synthase that required a priming by IFN-γ (24–26). Previous findings in our (50) and other (51–54) laboratories have shown that in monocytes and macrophages IFN-γ or TNF-α increased the expression of components of NADPH oxidase and potentiated the production of ROI by various agonists. We have demonstrated here that microglial cells treated for some days with IFN-γ or TNF-α presented a response to Aβ peptides much greater than that of control cells (Table II). Microglial cells are resident macrophages, and their low response to Aβ peptides compared with that of monocytes reflects a down-regulation of the components of the oxidase during the maturation from monocytes to macrophages (50, 51, 54). Aβ peptides alone are able to stimulate the production of TNF-α in microglia (24), so that one may speculate that this process might, by priming microglial cells with autocrine or paracrine mechanisms, potentiate the production of ROI in response to Aβ peptides in AD.

Finally we discuss briefly the finding (Fig. 3) that the binding of heparan sulfate to Aβ fibrils inhibited the interaction with the cell surface and the stimulation of H2O2 production. This result agrees with those showing that sulfate or sulfonate compounds inhibited the direct toxicity of Aβ peptides on neurons in vitro (13, 43–45). Because in AD heparan sulfate is associated with Aβ fibrils in the neurite plaques (66, 67), one wonders if in vivo the fibrils covered by heparan sulfate are impeded in their interaction with the cell surface of neurons and microglial cells and consequently inhibited in their ability to express their direct neurotoxicity (43–45) and stimulate microglia to produce toxic compounds. If this were the case the amount of heparan sulfate in the plaques and its interaction with Aβ fibrils would play a regulatory role on the biological activity of Aβ fibrils.

In conclusion, the results presented in this paper agree with the view (56) that the pathogenesis of Alzheimer’s disease includes also a series of molecular events characteristic of an

**TABLE II**

| Effect of IFN-γ or TNF-α on the stimulation of H2O2 production by Aβ peptides in microglia |
|---------------------------------|---------------------------------|---------------------------------|
| Control | IFN-γ | TNF-α |
| Resting | <0.02 (5) | 0.34 ± 0.08 (6) | 0.30 ± 0.09 (4) |
| Aβ(25–35) 10 μM | 0.28 ± 0.06 (6) | 3.30 ± 0.32 (6) | 2.42 ± 0.25 (4) |
| Aβ(1–39) 10 μM | 0.32 ± 0.04 (4) | 3.68 ± 0.35 (5) | 3.44 ± 0.31 (4) |
| Aβ(1–42) 10 μM | 0.24 ± 0.06 (5) | 2.58 ± 0.28 (3) | 2.31 ± 0.48 (3) |

*The values are expressed as nmol H2O2/106 cells/30 min. Data are the means ± S.D. of the experiments indicated in parentheses.

*Microglial cells were cultivated for 4–5 days in the presence of 50 units/ml of IFN-γ or 50 units/ml of TNF-α as described under “Materials and Methods.”
inflammatory reaction. These events would be triggered by the interaction of β-amyloid fibrils with microglial cells resulting in the activation of NF-κB (68), production of cytokines such as TNF-α (24), interleukin-8 (27), MCP-1 (55), and interleukin-1 (69, 70), production of ROI (31), and induction of NO synthase with generation of NO (24). It is widely accepted that the control of the production of these inflammatory molecules could be one of the therapeutic strategies of Alzheimer’s disease. On this view, the finding that the O2 forming NADPH oxidase is involved adds new tools to this therapeutic design.

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