Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4147–4152, April 1996
Neurobiology

Neurocytopathic effects of β-amyloid-stimulated monocytes: A potential mechanism for central nervous system damage in Alzheimer disease

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Communicated by William T. Greenough, December 22, 1995 (received for review August 14, 1995)

ABSTRACT **Growing evidence indicates that cells of the mononuclear phagocyte lineage, which includes peripheral blood monocytes (PBM) and tissue macrophages, participate in a variety of neurodestructive events and may play a pivotal role in neurodegenerative conditions such as Alzheimer disease. The present study sought to determine whether exposure of PBM to β-amyloid peptide (Aβ), the major protein of the amyloid fibrils that accumulate in the brain in Alzheimer disease, could induce cytopathic activity in these cells upon their subsequent incubation with neural tissue. PBM were incubated with Aβ for 3 days, centrifuged and washed to remove traces of cell-free Aβ, and then applied to organotypic cultures of rat brain for varying periods of time. By using a cell-viability assay to quantitate neurocytopathic effect, an increase in the ratio of dead to live cells was detected in cultures containing Aβ-stimulated PBM versus control PBM (stimulated with either bovine serum albumin or reverse Aβ peptide) as early as 3 days after coculture. The ratio of dead to live cells increased further by 10 days of coculture. By 30 days of coculture, the dead to live cell ratio remained elevated, and the intensity of neurocytopathic effect was such that large areas of brain mass dissociated from the cultures. These results indicate that stimulation of PBM with Aβ significantly heightens their neurocytopathic activity and highlight the possibility that inflammatory reactions in the brain play a role in the neurodegeneration that accompanies Alzheimer disease.**

Considerable attention has been focused on the deposition of insoluble β-amyloid peptide (Aβ) within the brain as a major etiologic factor in the pathogenesis of Alzheimer disease (AD). In fact, recent evidence from transgenic mice (1, 2) indicates that this event alone may be sufficient for the development of AD. What are still unresolved, however, are the questions of the mechanism by which overexpression of Aβ ultimately leads to the peculiar neuropathology and dementia pathognomonic for this disorder, and why AD follows a chronic and degenerative course.

There is indication that mononuclear phagocytes (mO) and the products they secrete may be involved in the destruction of central nervous system (CNS) tissue in a variety of neurodegenerative disorders, including AD (3, 4). These cells, which include peripheral blood monocytes (PBM) and tissue macrophages, possess cytotoxic potential and may be activated to a cytotoxic state by a variety of stimuli (5). In particular, phagocytic-dependent stimuli, such as fixed *Staphylococcus aureus* and yeast wall particles, have been shown to be potent activators of neurotoxin release from mO (6). When such findings are viewed in the context of electron microscopic images showing both cultured microglia, the resident macrophages of the brain, and perivascular brain macrophages *in situ* engaged in apparent phagocytosis of amyloid deposits (7, 8), they raise the interesting possibility that brain mO may be induced to neurocytopathic behavior by the presence and/or phagocytosis of insoluble Aβ in AD brain. Highlighting this possibility are several recent reports documenting that mO can be stimulated by Aβ to engage in varied activities. For example, Klegeris *et al.* (9) noted that exposure of rat peritoneal macrophages to Aβ fostered nitric oxide production, respiratory burst-associated oxygen consumption, and homotypic aggregation by these cells. Aβ has further been shown to be chemotactic for rat peritoneal macrophages and microglia (10), as well as to stimulate microglial secretion of growth factors associated with senile plaques (11), and to cause proliferation and morphological transformation of microglia (11).

In light of these considerations, the present study sought to determine whether exposure to Aβ could induce PBM to exert a neurocytopathic effect. Using a recently developed organotypic culture system (12) as a source of CNS tissue, combined with a fluorescence assay for quantifying cytopathic effect, our results indicate that stimulation of PBM with Aβ significantly heightens these cells' neurocytopathic activity. These results are discussed with respect to the role of immune mechanisms in AD, and with regard to possible targets for therapeutic intervention.

MATERIALS AND METHODS

Isolation/Characterization of PBM. PBM were isolated from blood samples of healthy volunteers at the University of Connecticut Health Center by the Percoll gradient method of Denholm and Wolber (13). Monocyte purity was routinely gauged to be between 85 and 90% by three independent criteria: (*i*) differential counting of Wright's stained cytocentrifuged preparations, (*ii*) nonspecific esterase staining, and (*iii*) direct immunofluorescence staining with a fluorescein-conjugated monoclonal antibody to CD14 (Sigma), a monocytic marker (13). Monocyte viability, as determined by trypan blue dye exclusion, was judged to be >95% at all times.

PBM Incubation with Aβ. Freshly isolated PBM were resuspended in RPMI 1640 medium (GIBCO/BRL) containing 5% calf serum and placed in a 15-ml sterile blue-capped tube (Becton Dickinson). Aβ peptide (amino acids 1–40; Sigma) was added directly to the tube, at varying concentrations, and allowed to "age" in the presence of the PBM for 3 days at 37°C in an atmosphere of 5% CO_2. Such aging has previously been shown to enhance the formation of Aβ aggregates (14), which are efficiently phagocytosed by mO (15). For controls, "reverse" Aβ peptide (amino acids 40–1; Sigma) or bovine serum albumin (BSA) was added at equivalent concentrations. Throughout the incubation period, PBM were rocked slowly to facilitate maximal interaction with the added

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Abbreviations: PBM, peripheral blood monocytes; AD, Alzheimer disease; Aβ, β-amyloid peptide; mO, mononuclear phagocytes; CNS, central nervous system; BSA, bovine serum albumin; EthD-1, ethidium homodimer; D/L ratio, ratio of dead to live cells.

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peptides. After incubation, the cells were pelleted at 200 × *g* and washed three times with fresh RPMI 1640 medium to remove traces of soluble Aβ. PBM were then immediately resuspended in rat cortex culture medium (see below). An aliquot of cells was taken for viability determination by trypan blue dye exclusion.

Organotypic Culture of Rat Cortex. Five- to six-day-old Long Evans hooded rat pups (Charles River Breeding Laboratories) were anesthetized by hypothermia; then they were decapitated and their brains were removed. Transverse brain sections (200 μm thick) were cut with a McIlwain tissue chopper and slices were immediately placed in Gey's salt solution (GIBCO/BRL). Individual slices were then transferred to 35-mm tissue culture membrane inserts (0.45 μm porosity; Millipore) that, in turn, lay inside a 6-well culture dish (Costar). A volume of 1 ml of medium, consisting of 50% minimal essential medium, 25% Hanks' balanced salt solution, and 25% horse serum (all from GIBCO/BRL), and supplemented with D-glucose, was placed under each tissue culture insert so that the tissue section was covered with a thin film of media at the media–air interface. This methodology is based on that initially described by Gahwiler (16) and Stoppini *et al.* (17), with modifications as recently described (12). Cultures were maintained in an atmosphere of 5% CO_2 in a humidified incubator at 34°C, and allowed to recover for 2 weeks before experimentation.

Coculture of PBM and Rat Cortex. Approximately 10^6 PBM (Aβ-stimulated or control), suspended in 200 μl of the same culture medium bathing the brain tissue, were placed directly on top of each section and the cultures were placed back at 34°C. Medium was exchanged every 3 days. At various times after the application of PBM, cultures were assessed for neurotoxic effects.

Assay of Neurotoxic Effects of Aβ-Stimulated PBM. Quantitation of the toxicity of Aβ-stimulated PBM on brain slices was determined using the live/dead Eukolight Viability/cytotoxicity Kit supplied by Molecular Probes. The Live/dead assay is a two-color fluorescence viability assay based on the simultaneous determination of live and dead cells with two probes, calcein AM and ethidium homodimer (EthD-1), which measure two distinct parameters of cell viability. For the assay, neural cultures were first rinsed free of media with phosphate-buffered saline (PBS) and then incubated with EthD-1/calcein AM solution for 45 min. Cells were visualized using an epifluorescent microscope (Leitz Orthoplan 2) with either a rhodamine filter (Leitz N2.1; to view dead cells only) or a wide-band fluorescein filter (Leitz I3; to view live and dead cells simultaneously). Cultures were examined under ×6.3 and ×10 objectives, and counts of the number of live and dead cells were made in designated areas using a reticle. Specifically, a 10 × 10 ocular grid, covering >95% of the field of view, was used. A culture was placed on the microscope stage and the total number of dead cells that appeared within the grid were counted. The culture was then repositioned so that a new region could be imaged under the grid and the number of dead cells in that region counted. This procedure was repeated until all regions of the culture were evaluated. The number of regions counted was used to calculate the area of the entire culture. Assurance that all regions were counted, and that no region was counted more than once, was facilitated by the fact that the background in regions where cells were already counted appeared darker due to photobleaching. After fluorescence counting, cultures were Nissl-stained and the total number of cells in the culture tallied. For photomicroscopic presentation of the data, 35-mm negatives of fluorescent images were scanned with a Polaroid Sprint-Scan 35 scanner at 2025 pixels/inch.

Statistical Analysis. Mean values for the ratios of dead to live cells (×100) in cocultures of neural tissue and Aβ-stimulated PBM were contrasted with corresponding ratios obtained from cocultures of neural tissue and reverse peptide-stimulated PBM or unstimulated PBM (controls), by a one-tailed Student's *t* test. Results were considered significant at levels of $P \leq 0.05$.

Confocal Immunofluorescence Microscopy. Brain sections cocultured with Aβ-stimulated PBM were rinsed with PBS and fixed in 3.7% formaldehyde/PBS for 2 h at room temperature. Sections were then permeabilized/blocked by incubation for 1 h in PBS containing 0.5% BSA, 0.5% casein, and 0.1% Nonidet P-40. Subsequently, sections were stained with a fluorescein-conjugated antibody that specifically recognizes human Mo-2 (anti-CD14; Sigma), a marker of cells of the mononuclear phagocyte lineage, which does not crossreact with analogous murine or rat antigens. Sections were analyzed on an inverted fluorescence microscope (Zeiss Axiovert) equipped with a confocal scanning laser microscope imaging system (Zeiss). Pictures were taken with a ×63, 1.4 N.A. Apochromat lens (Zeiss) using a 488 excitation filter and a 514–540 band-pass emission filter.

RESULTS

Fig. 1 shows the neurotoxic effects of coculturing PBM, previously stimulated with Aβ, with brain slices. To normalize the data between experiments, results are portrayed as a ratio of the numbers of dead to live cells (D/L ratio) detected in each culture. It is important to emphasize here that these experiments were performed with PBM that were exposed to Aβ before their coincubation with neural tissue, and that at no time was free Aβ directly added to the cultures. Thus, any effects may be considered due to a PBM response to Aβ. Controls for each experiment included brain slices incubated with PBM that had previously been stimulated with either BSA or reverse (40–1) Aβ peptide at equimolar concentrations, with no significant difference between these two treatments. After only 3 days of coculture, the D/L ratio was elevated in

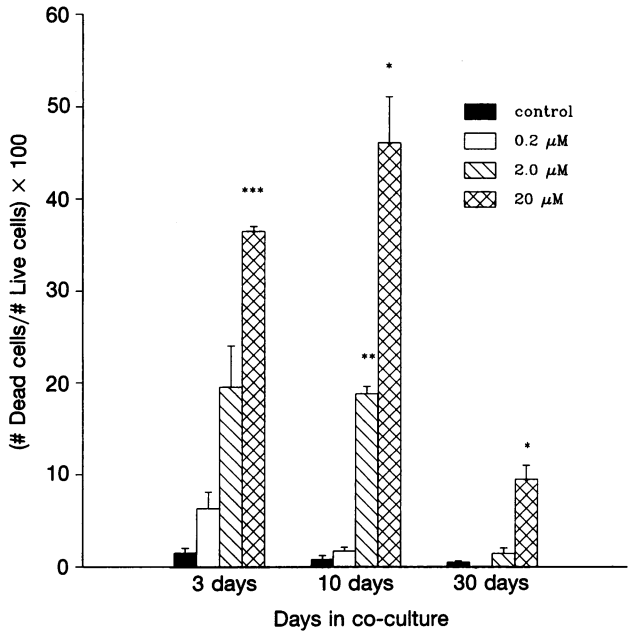

FIG. 1. Quantitation of the neurocytopathic effects of Aβ-stimulated PBM. Freshly isolated PBM, previously incubated for 3 days with either varying concentrations of Aβ or BSA at the highest equivalent Aβ concentration (control), were cocultured with rat brain sections for 3, 10, or 30 days. After these times, cultures were analyzed for the numbers of dead and live cells as described in *Materials and Methods.* Additional experiments performed using the reverse peptide as the control yielded virtually identical results (data not shown). ***, $P < 0.001$; **, $P < 0.01$; and *, $P < 0.05$.

all cultures containing Aβ-stimulated PBM when contrasted with controls. Increasing the concentration of Aβ to which the PBM were exposed resulted in a greater D/L ratio. The effect achieved with 20 μM Aβ, resulting in a D/L ratio of 37 (contrasted with a ratio of 1 in controls), was statistically significant. Upon coculture for 10 days, a more drastic neurotoxic effect was noted, with significantly heightened neural cell death occurring in cultures containing PBM stimulated with either 2.0 μM or 20 μM Aβ. Specifically, Aβ-stimulated PBM caused an increase in the D/L ratio from 0.8 in control cultures to 19 and 46 in cultures containing PBM exposed to 2.0 and 20 μM Aβ, respectively. Stimulation of PBM with only 0.2 μM, however, was again ineffectual. At 30 days of coculture, PBM stimulated with 20 μM Aβ yielded a significant increase in D/L ratio, 9.5 (20 μM Aβ) versus 0.5 (control). The reduction in D/L ratio seen at this time was not, however, due to any lessening of neurotoxic effect but, instead appeared to be the result of extensive areas of neural tissue becoming detached from brain slices exposed to Aβ-stimulated PBM for the extended coculture period. This was indicated by large cellular masses floating in the culture supernatant. Thus, after 1 month of coculture with Aβ-stimulated PBM, neural tissue had become so necrotic that it virtually began to disintegrate. The consequence of this was that there were actually a lesser number of dead cells to quantitate, resulting in an artifactually lowered D/L ratio. Photomicroscopic evidence of the heightened cell death caused by Aβ-stimulated PBM is presented in Fig. 2.

Since neural killing continued over a "chronic" (30-day) time scale, and proceeded despite replacement of conditioned media with fresh media every 3 days, it was reasoned that the applied PBM must have penetrated into interstitial spaces of the brain sections. Using confocal immunofluorescence microscopy with an antibody that specifically recognizes only human monocytes and monocyte-derived cells, we determined that Aβ-stimulated PBM did, indeed, infiltrate into the recesses of the brain tissue. This is indicated in Fig. 3, which shows an optical section through a brain slice that was stained with anti-Mo-2 antibody. Brightly stained monocytes are seen against the dark background of the brain parenchyma. No staining was observed in rat brain slices that were not cocultured with PBM (data not shown).

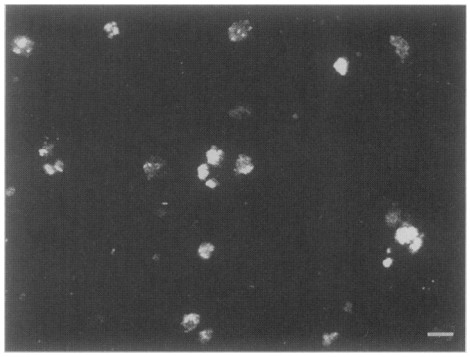

FIG. 3. Monocyte migration into rat brain sections. PBM previously stimulated with 20 μM Aβ were cocultured with rat brain sections for 10 days. After this time, the sections were rinsed with PBS, fixed, permeabilized, and then stained with a fluorescein-conjugated antibody that specifically recognizes human Mo-2 (CD14), a monocyte marker. Rat monocytes, brain perivascular macrophages, and microglia are not stained by this antibody—only the applied human PBM are. The infiltrating stained PBM were visualized by confocal fluorescence microscopy as described in *Materials and Methods.* (Bar = 5 μm.)

The neurotoxic effects of the Aβ-stimulated PBM suggested that exposure to Aβ might cause PBM to become "activated." To explore this possibility, we evaluated whether Aβ-stimulated PBM exhibited homotypic aggregation and/or membrane perturbation, two qualitative indicators of monocyte activation (5). Fig. 4 *B* and *D* clearly shows that PBM exposed to 20 μM Aβ fuse into clumps and exhibit a ruffled texture, reminiscent of a state of immune activation. In marked contrast, PBM exposed to BSA remain as single cells with a relatively smooth surface (Fig. 4 *A* and *C*). Thus, Aβ may function as an immune stimulus that activates PBM to a neurotoxic state.

DISCUSSION

Results presented here indicate that exposure of m0 to Aβ can lead to heightened neurotoxicity when such stimulated cells

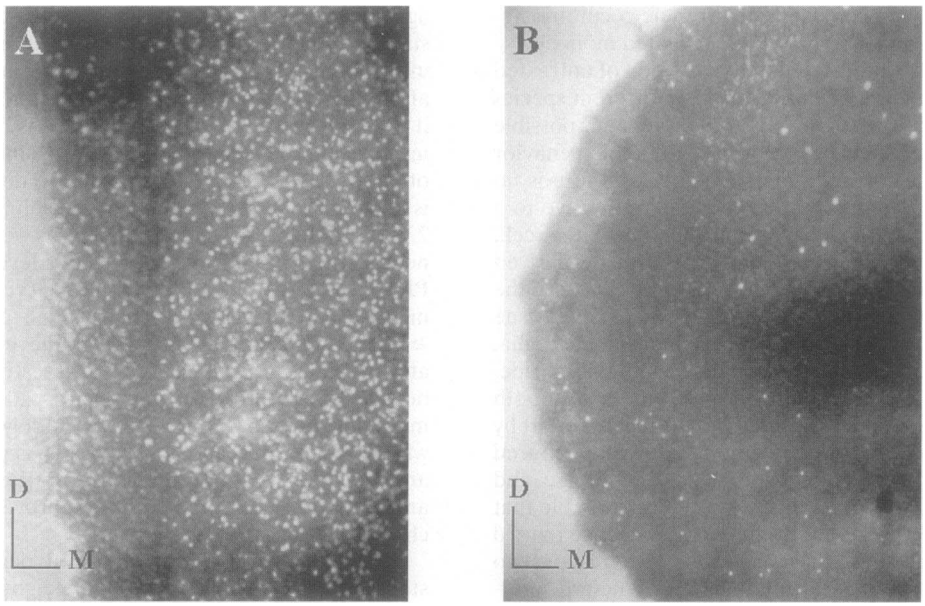

FIG. 2. Neurocytopathology of Aβ-stimulated PBM. PBM stimulated for 3 days with 20 μM Aβ (*A*), or an equivalent amount of reverse peptide (*B*), were cocultured with rat brain sections for 30 days as described in Fig. 1 and in *Materials and Methods.* At this time, sections were stained with EthD-1/calcein solution. The EthD-1 concentrates in the nuclei of dead or dying cells and fluoresces bright red. Using a rhodamine filter, only EthD-1 fluorescence (dead/dying cells) is shown here. Clearly, the density of brightly fluorescent nuclei is dramatically heightened in *A* versus *B*, reflecting the higher degree of cell death resulting from co-culturing rat brain sections with Aβ-stimulated PBM. Intersecting bars in the bottom left corner each represent 10 μm; D and M indicate the dorsal and medial orientations, respectively, of the tissue.

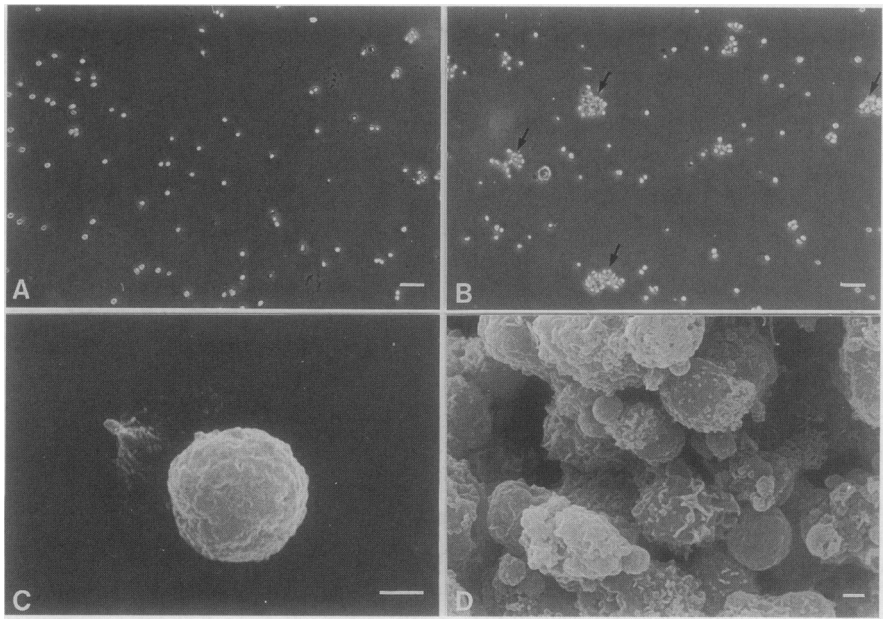

FIG. 4. Aβ-induced aggregation of PBM. Phase–contrast light microscopy (*A* and *B*) and scanning electron microscopy (*C* and *D*) indicate the formation of large clusters of PBM in response to Aβ. (*A* and *C*) Control PBM exposed to BSA (20 μM) and (*B* and *D*) PBM exposed to Aβ (20 μM) for 3 days. Arrows denote large clusters in *B* that are only observed in Aβ-treated PBM. Treatment of PBM with reverse peptide (20 μM) failed to induce similar aggregates (data not shown). (Bars in *A* and *B* = 10 μm; bars in *C* and *D* = 1 μm.)

are cocultured with brain tissue. These findings have important implications regarding the cellular and molecular pathogenesis of AD.

The enhanced neural cell killing observed in cocultures of rat brain sections and Aβ-stimulated human PBM appears to be largely, if not solely, the result of a specific PBM reaction to Aβ and not due to any constitutive PBM activity or problems with histocompatibility related to the xenogeneic nature of the coculture system. This argument is bolstered by the fact that the effects of Aβ-stimulated PBM were routinely matched against those elicited by control cells, which included both untreated PBM and PBM exposed to equimolar concentrations of BSA or Aβ reverse peptide. Furthermore, recent investigations employing analogous xenogeneic coculture systems to assess the neurotoxic activity of activated monocytes/macrophages have also indicated that the addition of untreated phagocytes to neural cultures derived from a different species failed to induce any cell death (6, 18). While it remains possible that Aβ-stimulated PBM engage in neurocytopathic behavior only toward CNS tissue from a different species, there is no evidence to indicate that m0 that are equally responsive to a given stimulus are species-selective regarding their target cell. On the contrary, it has been observed that both rat-derived microglia and peritoneal macrophages, when activated by the immunostimulant Zymosan A, are equally toxic to CNS tissue from rat and chicken (6). Furthermore, murine microglia, when stimulated with interferon γ and lipopolysaccharide, have been reported to exert similar cytopathic effects on both murine and human neural cell cultures (18). A recent report by Shaffer *et al.* (15) has additionally demonstrated that cultured rat microglia and human monocytic cells phagocytose and process Aβ to similar extents. Thus, we feel it improbable that human PBM exposed to Aβ are preferentially reactive toward rat-derived CNS tissue, and consider the observed response to be generally indicative of Aβ-stimulated PBM–neural interactions. This is not to say, however, that the neurocytopathic response of PBM, or m0 in general, to Aβ is unique in any way. Indeed, Aβ may be just one of many physiologic stimuli that can activate m0 to a neurocytopathic state. Lastly, though, the absolute carryover of free Aβ was not quantitated here, we consider it unlikely that contaminating free peptide was a major contributing factor to the observed neural cell death. Supporting this contention, heightened neural cell death was observed only when neural tissue was exposed to monocytes that had previously been stimulated with 2.0 μM Aβ (Fig. 1), but not when 2.0 μM of free peptide was added directly to cultures (data not shown).

That a level of 2.0 μM Aβ or higher was required to achieve the observed neurotoxic effects may be due, in part, to a need for Aβ to adopt a defined structure in order to promote a specific monocyte response. Underscoring this possibility, Giordano *et al.* (19) recently reported that while both Aβ and reverse Aβ aggregate and can form β pleated sheets, only Aβ can form the fibrillar structures found in AD. If, indeed, aggregated Aβ is acting as a phagocytic-dependent activation stimulus, then such a structural requirement would not be uncommon, as particle size and shape are known to greatly affect the extent of m0 phagocytosis (20). Furthermore, while it is appreciated that the concentrations of Aβ employed here may exceed the level of soluble Aβ found in biological fluids of patients with AD, it is important to note that neurotoxicity is related to the aggregate—not the soluble—state of Aβ (21, 22). It should be reemphasized here, as well, that at no time was neural tissue directly exposed to this level of Aβ, but only to PBM previously stimulated with this level of peptide. Thus, it may be that only at particularly high concentrations—e.g., ≥2.0 μM—does Aβ adopt a unique conformation and/or attain the appropriate aggregate size to activate PBM to a neurocytotoxic state. In this regard, 2.0 μM Aβ (or higher) may more closely mimic the amyloidogenic state *in vivo* to which brain m0 are actually exposed. As yet, however, no data are available regarding the concentration of insoluble Aβ in amyloid fibrils deposited in the cerebrovasculature and parenchyma of AD brains.

The observation that neural cell death induced by Aβ-stimulated PBM continued at a heightened pace over the course of a 30-day period is evidence that Aβ can produce neurotoxic effects *in vitro* on a relatively chronic time scale. In fact, by this time damage had become so extensive that large areas of tissue actually dissociated from the brain section, a condition not seen with control PBM. This finding lends credence to the argument that Aβ is the etiologic agent causing

the progressive neuropathology of AD. Hence, while the incipient event in AD may be the extracellular deposition of Aβ into insoluble amyloid, this activity may set into motion a cascade of immune-mediated neurocytopathic events that continue over an extended period of time. Such delayed, downstream effects do not preclude Aβ from also exerting more immediate, direct toxic actions on different neural cell types (23–26). Thus, both acute toxic reactions and chronic inflammatory responses to the presence of Aβ in the brain may collectively contribute to forming the biological substrate of AD. A recent report stating that inflammatory processes can induce alterations in amyloid precursor protein expression in brain (27) further suggests that inflammatory reactions and amyloidogenesis might be linked in a viscious cycle that causes progressive neurodegeneration. In support of this hypothesis, a recent cotwin control study has shown an inverse association of anti-inflammatory treatments and AD (28), and a clinical trial of indomethacin, a nonseroidal anti-inflammatory drug, has indicated this agent protects mild to moderately impaired AD patients from the degree of cognitive decline found in a placebo-treated control group (29).

By what mechanism does Aβ-stimulated PBM engage in neurotoxic activity when cocultured with brain slices? While this question remains to be resolved, several reports have revealed that activated and/or infected m0 can release soluble substances that are toxic to neural cells in culture (5, 30–35). Thus, in the simplest scenario, Aβ-stimulation/activation of PBM may signal these cells to release neurotoxins. Substances with demonstrated neurotoxic activity that are released from stimulated m0 include tumor necrosis factor-α, interleukin 1, reactive oxygen and nitrogen intermediates, glutamate, and quinolinic acid (2, 33, 35, 36). Alternatively, Aβ-stimulated PBM might act in a paracrine manner, releasing substances that in turn cause the release of toxic compounds from nearby parenchymal neural cells—e.g., astrocytes. The significant degree of cytokine crosstalk between astrocytes and microglia (37), as well as the capacity of astrocytes to synthesize and release a battery of neurotoxic substances similar to that liberated by activated m0 (38), is consistent with such a notion. A physical interaction between Aβ-stimulated PBM and specific neural cell types may further be required for release of neurotoxic factors. In this regard, contact-dependent neural cell killing by human immunodeficiency virus-infected monocytes (33, 34) has been reported. Additionally, this laboratory has recently described the binding of activated monocytic cells to astroglia (39)—an event that might trigger the release of either monocyte- or astrocyte-derived toxins. The ability of the PBM to infiltrate the intercellular spaces of the brain sections in our culture system allows for such adhesive events to take place. However, it remains to be determined whether neural cell death occurs only in immediate proximity to the invading PBM or is more diffuse.

Though PBM were used as the singular source of m0 in this report, it is recognized that both microglia and perivascular macrophages also engage in varied examples of CNS destruction *in vivo*. Such degradative activity includes the synaptic remodeling that occurs during ontogenesis and after CNS injury, as well as the degeneration that follows cerebral ischemia (40). Thus, all classes of m0 that enter the CNS seemingly have an intrinsic capacity to cause neural cell damage or death. Whether or not these cells ultimately engage in neurocytopathic behavior, however, appears to be dependent upon their receiving the appropriate signals. Results from this study suggest that accumulation of Aβ peptide into insoluble amyloid deposits might function as just such a signal and be able to stimulate m0-mediated neural cell death *in vivo*. Other investigations describing both the appearance of activation antigens on brain macrophages and microglia surrounding amyloid deposits *in vivo* (41–43), as well as Aβ-induced stimulation of peritoneal macrophages and microglia *in vitro* (9–11), are consistent with this hypothesis, and highlight the prospect that m0 may be important effector cells in the pathogenesis of AD. Accordingly, therapeutic intervention aimed at regulation of m0 activity may hold considerable promise.

We wish to gratefully acknowledge the expert technical assistance of Ms. Gretchen Burger and Ms. Leslie Niego. An additional debt of gratitude is owed to Mr. Joseph Biegel (Polaroid Medical Imaging, Newton, MA) for his help in generating high resolution images of EthD-1-stained brain slices. This work was supported by grants from the Sandoz Foundation and the Travelers Center on Aging to J.A.L. and from the American Cancer Society (CB 12) and the Proctor and Gamble Company to J.S.P.

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