β-Amyloid protein (βAP) deposition is a neuropathologic hallmark of Alzheimer’s disease (AD). Yet, the source of cerebral βAP in AD is controversial. We examined the production of βAP by the BV-2 immortalized microglial cell line using a sensitive enzyme immunoassay. Constitutive production of βAP was detected in conditioned media from unstimulated BV-2 cells. Further, production of βAP was induced by treatment of cultures by lipopolysaccharide (LPS) or βAP-(25–35) and was inhibited by the calpain protease inhibitor MDL 28170. Treatment of BV-2 cells with LPS or βAP-(25–35) did not affect cell-associated β-amyloid precursor protein levels. These findings suggest that microglia may be an important source of βAP in AD, and that microglial production of βAP may be augmented by proinflammatory stimuli or by βAP itself.

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treatment condition, triplicate plates were counted, and values were averaged.

Northern Analysis—Northern analysis of RNAs from treated cells was performed as described previously (25). A βAPP hybridization probe was made by random-primed 32P labeling of a gel-isolated BamHI fragment from the human βAPP cDNA encompassing residues 1 to 500 of the extracellular domain (26). This probe detected βAPP mRNA in rat brain and without the domain coding for the Kunitz protease inhibitor. Two DNA oligonucleotide probes complementary to mRNAs coding for mouse βAPP-751 and mouse βAPP-695 previously described (27) were synthesized (Operon, Alameda, CA). 5' terminus-labeled with 32P using T4 polynucleotide kinase, and hybridized with Northern blots as described previously (28). For quantitation, autoradiograms were scanned using a Phosphorimager (Molecular Devices, Sunnyvale, CA).

Western Analysis—To examine cell-associated βAPP after 24 h of treatment with LPS, Western analyses were performed on triplicate BV-2 cultures. Cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% deoxycholate, 0.1% SDS, 1% Triton X-100), and the lysate was clarified by centrifugation at 39,000 × g for 15 min. Total protein was quantified using the Lowry method, and equal amounts of protein were loaded into each lane of an 8% polyacrylamide gel, followed by transfer to an Immobilon-P membrane (Millipore, Bedford, MA). Immunodetection was performed using the BC1 antiserum and the peroxidase-antiperoxidase method. Briefly, membranes were blocked for 1 h at 37°C in TNTB buffer (TNT buffer containing 1% BSA). Aliquots of 0.1 ml of antiserum BA1 (1:5000 dilution in TNTB buffer) were added to each well, followed by a rabbit peroxidase-antiperoxidase reaction. The specificity of the BA1 antiserum was further verified by reactivity with a synthetic truncated form of βAPP, corresponding to the peptide sequence 261-280 (14). Finally, membranes were incubated for 1 h at 37°C with 0.05% enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA) and exposed to x-ray film.

Immunoprecipitation—Metabolic labeling and immunoprecipitation of βAPP from conditioned media with the BA2 antiserum and analysis of the recovered products by 16.5% Tris/Tricine/polyacrylamide gel electrophoresis were performed as described previously (30, 31). Immunoprecipitation of βAPP from cell lysates with the BC1 antiserum raised to the cytoplasmic domain of βAPP was also performed as described previously (25). Because preliminary experiments revealed that BV-2 cells showed growth inhibition after 24 h in cysteine/methionine-free medium, cells were harvested after 6 h for all metabolic labeling and immunoprecipitation experiments. Quantitation of labeled proteins was performed using a Phosphorimager (Molecular Devices, Sunnyvale, CA).

RESULTS

βAPP Secretion—Analysis of conditioned media using the EIA demonstrated that 24-h treatment with either aggregated βAPP (25–35) or LPS alone or in combination increased βAPP production by BV-2 cells. A one-way analysis of variance showed an overall difference in mean βAPP levels in BV-2 conditioned media among the four treatment conditions (control, LPS, βAPP, and LPS plus βAPP; F = 17.67; df = 3.8; p < 0.001; Fig. 1A). βAPP levels in culture media from LPS-stimulated cells were 1.85 times greater than those in media from control cells, a statistically significant difference. βAPP levels in media from cells stimulated with βAPP or LPS plus βAPP were also significantly increased over control levels. LPS plus βAPP treatment did not significantly enhance either βAPP alone or LPS alone.

In a second set of experiments, treatment of BV-2 cells for 24 h with aggregated βAPP (25–35) resulted in mean βAPP levels 1.8 times those of control cells (n = 6 cultures; mean βAPP in control cultures = 10.7 ng/plate, S.E. = 0.46; mean βAPP with aggregated treatment = 18.7 ng/plate, S.E. = 0.89; t = 6.02, df = 7, p < 0.001). Treatment of sister cultures with unaggregated βAPP (25–35) reproducibly increased βAPP levels in conditioned media 1.5 times over control values (n = 6 cultures; mean βAPP with unaggregated treatment = 15.8 ng/plate, S.E. = 0.60; t = 5.54, df = 7, p < 0.001). However, the βAPP in conditioned media after treatment with aggregated βAPP (25–35) was significantly greater than that seen after treatment with unaggregated βAPP (25–35) (t = 5.29, df = 10, p < 0.05).

The LPS-induced increase in βAPP in conditioned media was significantly attenuated by administration of the calpain protease inhibitor MDL 28170 (31). Treatment of BV-2 cells with MDL 28170 at a concentration of 35 μM gave no indication of toxicity, either by morphological inspection or with the trypan blue viability assay. In addition, yields of total RNA were comparable between culture conditions, and Northern analysis indicated no changes in G3PDH.
Data are expressed as nanograms of both). The MDL 28170 and LPS plus MDL 28170 treatment means were less than the control mean and the mean for LPS alone (data not shown). There were no significant differences among the means for LPS, \( \beta A P-(25–35) \), and LPS plus \( \beta A P-(25–35) \) (Fig. 2). In medium harvested after 6 h, LPS treatment resulted in an approximately 2-fold increase in \( \beta A P \) signal, providing visual verification of the results obtained using a different \( \beta A P \) antiserum (BA1) and a different method of analysis (EIA).

**Morphologic Effects**—Treatment of BV-2 cells with aggregated \( \beta A P-(25–35) \) resulted in visible precipitates under phase contrast microscopy in the cultures, indicating that aggregation of the peptide had occurred. Following incubation with the aggregated peptide, the BV-2 cells began to clump within 24 h (Fig. 3C). LPS alone did not cause clumping (Fig. 3B), but appeared to enhance the \( \beta A P \) effect (Fig. 3D). MDL 28170 at a concentration of 35 \( \mu M \) had no morphological effect (data not shown).

**Cell Counts**—The effects of LPS treatment were assessed with respect to cell proliferation. This was done so as to exclude increased cell numbers as being responsible for increased \( \beta A P \) in BV-2 conditioned media. No difference was found. After 6 h of treatment with LPS, the mean cell count from three plates was \( 5.2 \times 10^5 \) cells/ml (S.E. = 0.7), whereas the mean count in control cultures was \( 4.8 \times 10^5 \) cells/ml (S.E. = 0.3). After 24 h of LPS treatment, the mean cell count from three plates was \( 6.7 \times 10^5 \) cells/ml (S.E. = 0.1), whereas the mean count from control plates was \( 8.4 \times 10^5 \) cells/ml (S.E. = 0.2). Control cultures grown in cysteine/methionine-free medium for 6 h showed a mean cell count of \( 5.0 \times 10^5 \) cells/ml (S.E. = 0.8).

**\( \beta A P \) mRNA**—LPS treatment for 6 h did not result in a significant increase in total \( \beta A P \) mRNA in BV-2 compared to control values (\( n = 3; t = 1.5, df = 4, p > 0.05 \)). However, after 24 h of LPS treatment, BV-2 total \( \beta A P \) mRNA was increased to 1.9 times that found in control cells (\( n = 3 \) replicates; \( t = 3.1, df = 4, p < 0.05 \); Fig. 4). Both \( \beta A P-751 \) (the major variant) and \( \beta A P-695 \) mRNA species were increased by LPS treatment to approximately the same degree (data not shown).

**\( \beta A P \) Immunoprecipitation**—Immunoprecipitation of \( \beta A P \) in cultures treated with MDL 28170, a further indication that no toxic effect had occurred (data not shown).

Control assays were performed on blank media which had not been exposed to cultures, or blank media with LPS or MDL 28170. These samples showed no evidence of \( \beta A P \) immunoreactivity. When conditioned media were collected from control cells after 24 h and aggregated \( \beta A P-(25–35) \) was added, there was no difference from other control cultures in \( \beta A P \) detected by the EIA. LPS, MDL 28170, and \( \beta A P-(25–35) \) added to conditioned media did not affect the performance of the EIA (data not shown).

**\( \beta A P \) Immunoprecipitation**—Immunoprecipitation of condition medium from BV-2 cells with the BA2 antiserum to \( \beta A P \) followed by Tris-Tricine gel electrophoresis revealed the presence of the characteristic \( \beta A P \) signal at approximately 4 kDa (Fig. 2). In medium harvested after 6 h, LPS treatment resulted in an approximately 2-fold increase in \( \beta A P \) signal, providing visual verification of the results obtained using a different \( \beta A P \) antiserum (BA1) and a different method of analysis (EIA).
forms of βAPP due to the relatively short 6-h interval and the nutritionally deficient medium. In contrast, βAPP species detected by Western analyses in total cellular protein harvested after 24 h were largely 105–115-kDa partially glycosylated forms, as well as mature 130-kDa βAPP (Fig. 6). This pattern is similar to that observed by Monning et al. (33) who immunoprecipitated unlabeled βAPP from BV-2 cells.

Immunoprecipitation of βAPP from cell lysates after metabolic labeling showed no difference in βAPP levels between cells treated with LPS for 6 h and control cells (Fig. 5). Likewise, triplicate Western analysis of cells treated with LPS for 24 h showed no difference in βAPP levels in comparison with controls (Fig. 6). Therefore, increased βAP in conditioned medium was not simply a result of higher levels of cellular βAPP.

**DISCUSSION**

These results demonstrate that treatment of BV-2 microglial cells with aggregated βAP-(25–35) results in a significant increase in full-length βAP in conditioned media. An increase in βAP in conditioned media was observed after treatment with unaggregated βAP-(25–35), but this increase was smaller than that observed after treatment with the aggregated peptide. The βAP-(25–35) fragment has been shown to have toxic effects on neurons (34). Our results indicate that it has important effects on microglial secretory activity in the absence of cellular toxicity.

Treatment of BV-2 cells with LPS also increased βAP in conditioned media. LPS treatment did not result in an increase in cell numbers, so the increased βAP in conditioned media must be attributed to increased production of βAP per cell. The increased βAP induced by LPS was observed after both 6 and 24 h of treatment. Combined treatment with βAP and LPS also increased βAP in conditioned media compared with controls, although the increase was not significantly greater than that observed with βAP or LPS alone.

Although there was an increase in βAPP mRNA after 24 h of LPS treatment, no significant change in βAPP mRNA was observed after 6 h of treatment, despite the fact that βAP was increased at the 6-h time point. Further, immunoprecipitation and Western analyses for βAPP showed no increase in βAPP after either 6 or 24 h of LPS treatment. Thus, the LPS-induced increase in βAP production does not appear to be driven by increased βAPP expression. A previous study suggested that when BV-2 cells are cultured on dishes coated with laminin or fibronectin, LPS treatment results in an increase in βAPP expression (33). However, in agreement with our results, no
significant change in βAPP expression was observed in that study after LPS treatment of BV-2 cells cultured in uncoated plastic dishes.

We used a number of different methods to confirm the modulation of βAP production in BV-2 cells. A βAP-(1–40)-selective EIA was employed most frequently in this study. In addition, using immunoprecipitation, we detected a protein that migrated at the βAP position. The authenticity of the βAP was further demonstrated by treatment of BV-2 cells with the calpain protease inhibitor MDL 28170. This agent, which has previously been shown to inhibit the formation of βAP (31), resulted in a significant decrease in immunoreactivity detected in BV-2 conditioned medium.

On a morphological level, treatment with βAP-(25–35) caused clumping of cultured BV-2 cells. Simultaneous treatment with βAP-(25–35) and LPS resulted in greater cell clumping than was observed with βAP-(25–35) alone, indicating an additive effect of the two agents. Clumping has previously been reported in peritoneal macrophages in response to βAP-(25–35) and was interpreted as a sign of cell activation (35). We demonstrate that βAP-induced activation of microglia results in cell clumping, too. βAP-(25–35) has shown to be chemoactive for macrophages and microglia (36), so movement of BV-2 cells toward large βAP aggregates may have also contributed to clumping. βAP-(25–35) has been shown to have many of the properties of full-length βAP, including neurotoxicity (34). However, in AD brain, full-length βAP predominates, and it is conceivable that it could have a different effect on microglia than does βAP-(25–35).

These findings support the hypothesis that inflammatory activation of microglia in AD leads to increased microglial production of βAP which is deposited in plaques. Although previous studies have indicated that microglia express βAP (18, 20, 33), heretofore it has not been known if immune activation of microglia cells results in increased βAP production. Furthermore, we present evidence that exogenous βAP may stimulate microglial βAP production. This suggests that in AD increased extracellular βAP may induce microglia to produce additional βAP, which in turn could lead to further βAP production by microglia or other cells via an autocrine or paracrine positive feedback mechanism. Because βAP has been shown to increase microglial inflammatory cytokine secretion and production of reactive nitrogen intermediates (21, 22), βAP produced by microglia may also augment the local inflammatory response surrounding the neuritic plaque, which may lead to neuronal dysfunction or injury.

It is significant that immunologic stimulation of BV-2 cells with LPS resulted in a large increase in βAP in conditioned media. BV-2 cells have many of the features of reactive, rather than resting microglia cells (24, 38). Reactive macrophages and microglia are known to contain and to secrete very high levels of proteolytic enzymes (39). The secretases which cleave βAPP to amyloidogenic fragments may be among the enzymes which are present at high levels in reactive microglia, and their activity may be enhanced after immunologic stimulation. The initiating event for βAP deposition in AD remains obscure as does the stimulus resulting in signs of immunologic activation in neuritic plaques. However, the significant increase in βAP produced by BV-2 cells when exposed to an immunogenic stimulus such as LPS suggests that activated microglia may be a major source once the pathological immune process is initiated in AD.

In conclusion, these findings indicate that microglial cells activated by inflammatory stimuli may play an important role in β-amyloidosis in AD. Anti-inflammatory agents which delay the onset of Alzheimer’s pathology (40, 41) may work by inhibiting microglial βAP production and the secretion of microglial cytokines and other proinflammatory products. In addition we demonstrate that βAP itself may act as an exogenous stimulus to its own production by cultured microglial cells. If this occurs in AD brain, secreted βAP may have important autocrine or paracrine effects which augment its own production. Further understanding of the role of microglia and cerebral inflammatory processes in AD may lead to a clearer understanding of the pathogenesis of this devastating disorder. In addition, development of drugs targeted to microglial proteases may provide a new line of treatment.

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