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Astrocytes Regulate Microglial Phagocytosis of Senile Plaque Cores of Alzheimer's Disease

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We have developed an *in vitro* model in which isolated senile plaque (SP) cores are presented to rat microglial cells in culture. Microglia rapidly phagocytosed, broke apart, and cleared SP cores. However, when cocultured with astrocytes, microglial phagocytosis was markedly suppressed, allowing the SPs to persist. Suppression of phagocytosis by astrocytes appears to be a general phenomena since microglia in the presence of astrocytes showed reduced capacity to phagocytose latex beads as well. The astrocyte effect on microglia is related in part to a diffusible factor(s) since astrocyte- but not fibroblast-conditioned media also reduced phagocytosis. These results suggest that while microglia have the capacity to phagocytose and remove SPs, astrocytes which lie in close association to microglia may help prevent the efficient clearance of SP material allowing them to persist in Alzheimer's disease. © 1998 Academic Press

Key Words: **beta amyloid; macrophages; neurodegenerative diseases; CNS-injury; debris removal; reactive gliosis.**

INTRODUCTION

Amyloid β (Aβ) accumulation in senile plaques (SP) is considered a key step in the pathogenesis of Alzheimer's disease (AD) (55). This contention is supported by numerous molecular and genetic studies linking mutations in the β-protein precursor (βPP) to increased production of Aβ (8, 19, 46, 55, 64). While most studies have focused on Aβ formation, maintenance of the SP depends not only on factors which promote Aβ production, but also on factors which hinder efficient clearance (31). Therefore, SP maintenance is likely to be a complex interaction of Aβ with the surrounding dystrophic neurites (12), processes of reactive astrocytes (9, 42, 52), and activated microglia (12, 28, 34, 43, 49). As the resident macrophages of the brain (21, 62, 68), microglia are likely candidates in Aβ catabolism. However, even though activated microglia are a common component of SPs, there is little evidence for efficient Aβ removal by them in AD (16, 27).

The efficiency of microglial phagocytosis and debris removal seems to be related to their state of activation. Early in development or following certain types of injury in the adult when debris removal is rapid, microglia have a round or ameboid morphology and express high levels of various macrophage markers (4, 6, 13, 15, 20, 22, 24, 30, 32, 33, 35, 38, 45, 60, 63, 68). In contrast, so-called "resting" microglia in the adult CNS have a ramified or process bearing morphology with a small cell body, low levels of macrophage markers (13, 45, 62, 72), and poor phagocytic ability (7, 13, 23, 50, 51, 69). Following axotomy via a nonpenetrating injury, e.g., dorsal rhizotomy or infection, resting microglia become activated (5, 13, 20, 68). Although such activated cells express high levels of macrophage markers, they remain process bearing and continue to show limited phagocytosis (13, 20, 37). The cell processes often found on microglia associated with SP are similar to those showing limited phagocytic activity (28, 34). Yet microglia in the adult CNS are able to phagocytose debris following certain severe insults in the adult, e.g., a penetrating injury or stroke, (20, 22, 24). Indeed, when infarcts occur in AD brain, SP actually can be rapidly removed by microglia that transform into macrophages (70; P. Gambetti, personal communication). Understanding the mechanism(s) that modulate microglial activation and clearance of Aβ is critical to understanding SP homoeostasis.

Since astrocytes can modify microglial/monocyte behavior (1, 26, 39, 44, 58, 65, 67), we examined the role of astrocytes as potential modulators of microglial interactions with SPs *in vitro.* We found that purified rat microglia with a round, macrophage-like morphology rapidly phagocytose and clear SP cores. In contrast, when cocultured with rat cortical astrocytes microglia had a process bearing morphology and phagocytosis was dramatically suppressed. Therefore, SP persistence *in vivo* may be the result of a complex interplay that occurs between glial cells associated with the SP.

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METHODS

Isolation of Senile Plaque Cores

SP cores were isolated using a modification of the procedure of Selkoe *et al.* (56). Portions of frontal and temporal lobe from three patients with confirmed, severe Alzheimer's disease were obtained at autopsy and frozen. The meninges and large blood vessels were removed along with large portions of white matter. Grey matter was finely minced and stirred for 2 h in 2% SDS, 1 mM Tris (pH 7.6) to a final volume of 5×. Tissue was homogenized by 20 passages in a Dounce homogenizer (A pestle). The homogenate was brought to 97°C for 10 min, sieved through 110-μm nylon mesh, and centrifuged at 300*g* for 30 min to pellet the SP core-containing fraction. The samples were then washed in (0.1% SDS, 150 mM NaCl, 0.02% NaN_3) and centrifuged again at 300*g* for 10 min. The samples were sieved through a 35-μm nylon mesh and then applied to a noncontinuous sucrose gradient (1.2 M, 1.4 M, 1.6 M, 1.8 M sucrose) and centrifuged again at 72,000*g* for 60 min. All interfaces were collected, pelleted, and washed 3× with 1 mM Tris (pH 7.6), 0.1% SDS to remove sucrose. Most of the SP cores as well as some lipofuscin were at the 1.4 M–1.6 M interface as demonstrated by Congo red birefringence. To enhance the purity of the SP cores as well as to remove small or broken cores and clumps, a Bectin–Dickinson FACStar Plus was used. SP cores were sorted by fluorescence and size (56), with a flow rate of 1000 particles per second. SP cores were stored at 4°C until use.

Immunocytochemistry

Cells and SP attached to coverslips were fixed in 2% paraformaldehyde in 1 M PBS, pH 7.2, for 1 h and rinsed with 1 M PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 30 min. The coverslips were further blocked with 10% normal goat serum for 30 min prior to addition of primary antibodies or lectin. The lectin *G. simplicifolia* (Isolectin B_4, biotin conjugated, Sigma) was specific for microglia and did not bind to astrocyte cultures (61). Additionally, ED1 and OX-42 (Chemicon) were used as markers of microglia (45). Cellular purity was assessed by using phase microscopy and antisera to GFAP (Accurate), an astrocyte marker, and O1, an oligodendrocyte marker (gift of Robert Miller). SP cores were identified with the monoclonal 4G8 (36) or antisera to Aβ. Primary antibodies and lectin (20 μg/ml) were incubated overnight at 4°C. The peroxidase anti-peroxidase method (59) with diaminobenzidine as a cosubstrate was used to visualize the immunoreaction. Biotinylated lectin and ED1 were visualized with Vectastain ABC streptavidin-alkaline phosphatase (Vector Labs) with Fast Blue as a chromagen.

Microglia Cultures

Microglia were obtained using a modification of the procedure of Giulian and Baker (23). Mixed cultures were prepared from the cortex of PO Sprague–Dawley rat pups, and grown in poly-L-lysine-coated flasks with DMEM (Gibco) containing 20% fetal calf serum. After 6–7 days, the cultures were placed in an orbital shaker at 37°C for 1 h. Microglia were separated by collecting the media, since astrocytes and other cells remain attached to the substrate. Microglia were seeded onto poly-lysine-coated coverslips and after 1 h, when a majority of microglia had adhered, the media was changed to further eliminate contaminating, nonadherent cells. Microglia obtained in this manner were consistently >95% pure. Unless indicated, approximately 2–3 × 10^4 cells per well were grown in 24-well Falcon plates.

Astrocyte Cultures and Conditioning

Astrocytes were obtained from neonatal rat pups. Mixed glial cultures were prepared as indicated above except they were grown in DMEM with 10% FCS for 2 weeks to allow for growth of astrocytes. Microglia and other cells were removed through shaking. About 5 × 10^4 astrocytes per well were grown with SP cores for 1 week prior to the addition of microglia to determine the effects of astrocyte conditioning/coculture. Astrocyte-conditioned media was collected after 4 days in culture and applied to microglia cultures 1–2 h after seeding to determine the effects of soluble, astrocytic factors.

Fibroblast Cultures

Fibroblasts were obtained from the meninges which were stripped from the neonatal rat brain, diced with a razor blade, and added to poly-lysine-coated culture flasks with DMEM and 10% FCS. Fibroblasts adhered to the flask and the media was changed after 1 day to remove large aggregates of cells. Fibroblast-conditioned media was also obtained after 4 days.

Quantification of Senile Plaque Cores

The fate of SP cores was assayed by binding 1–2 μl of SP core stock solution (Approximately 500 ± 30 SD) to 15-mm glass coverslips previously coated with poly-L-lysine. These were incubated in the presence of 2–3 × 10^4 microglial cells per well in a 24-well plate. At various time points, the coverslips were fixed in 2% paraformaldehyde and processed for immunocytochemistry. At the time of fixation, and during all media changes, the media was collected and frozen for later analysis. Four coverslips were used for each time point. This paradigm was repeated for two complete sets yielding a total of eight coverslips per time point.

Following immunocytochemistry and Congo red stain-

ing for Aβ, the SP cores remaining were counted on a Zeiss Axiophot microscope using the 20× objective and the 2× optivar. Lectin/alkaline phosphatase staining was used to determine if microglia surrounded SPs (intracellular) or were either not associated with microglia or simply in close juxtaposition (extracellular). Aβ immunoreactive material within microglia which appeared as condensed, small vesicles within the cell (>5 μm total diameter) was scored as 1 SP. Multiple SP cores were scored as such only when each of the Aβ aggregates was ~10 μm diameter. In parallel cultures, SP cores were incubated in media alone or astrocyte cultures in the absence of microglia. Spent media was saved and analyzed for intact SP cores by centrifugation at 5000*g* for 10 min. The pelleted material was smeared and stained with Congo red solution and viewed under cross-polarized light.

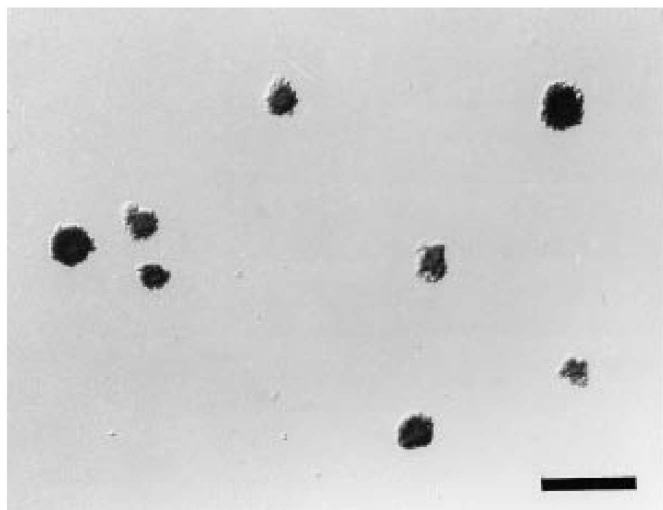


FIG. 1. Isolated senile plaque cores following FACS sorting and immunocytochemistry for Aβ. Scale bar, 50 μm.

Phagocytosis of Latex Beads

Latex beads (Sigma) of the approximate diameter as SP cores (11 μm diameter) were used to compare phagocytosis of SP cores versus that of dissimilar material as well as to determine the percentage of phagocytic cells in conditioned media. Prior to use, 500 μL of a 2.5% suspension of beads was activated with borate buffer (pH 8.0) and incubated with 300 μg/mL bovine serum albumin for 2 h at 20°C. The beads were rinsed three times with Tris–HCl, pH 7.2, and stored in Tris buffer, at 4°C until use.

Microglia were seeded at approximately 3×10^4 cells per well in 1 ml of DMEM with 10% FCS. After 2 h, the media was replaced with fresh media or that conditioned for 4 days by either astrocytes or fibroblasts (which had been passed through a 0.2-μm filter). After 24 h, 1-μl (approximately 10^5) beads were added to each well. On day 2, the percentage of cells phagocytosing beads was determined. The first 100 randomly selected cells were counted by a "blind" observer and scored as having 0, 1, or >1 bead per cell.

Latex particles of 0.5 μm diameter (Polysciences) were used to quantitate the extent and rate of phagocytosis. Since these smaller beads were supplied in excess and microglia could phagocytose >10 beads (rather than 1–2 seen with 11-μm beads), they serve as a better index for the extent of phagocytosis and kinetic analysis than the larger beads. After a 24-h incubation of the microglial cells with astrocytes or astrocyte-conditioned media, latex particles were added. The cells were fixed after 6 or 24 h and analyzed. The total number of beads within randomly selected microglia was determined.

Electron Microscopy

Cultures were fixed in 4% paraformaldehyde with 3% glutaraldehyde, rinsed, and postfixed with 1% osmium tetroxide in 0.1% sodium cacodylate buffer. The coverslips were then dehydrated in ethanol followed by infiltration in Spurr medium. Thin sections were made, stained with lead citrate and uranyl acetate, and observed on a JEOL 100 electron microscope.

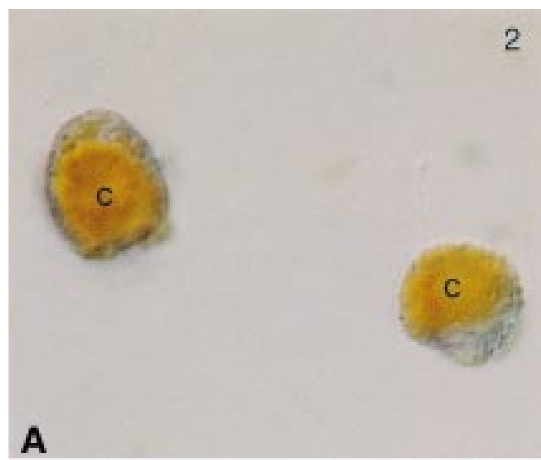
RESULTS

Isolation of SP Cores for Use in an in Vitro Model

SP cores from AD brain were isolated by the method of Selkoe *et al.* (56), which yields a population with a fairly uniform diameter (from 5 to 20 μm). They were Congo red positive, displaying the characteristic apple-green birefringence in a Maltese cross-pattern under polarized light and were also Aβ immunoreactive (Fig. 1). This morphology is virtually identical to SP cores in AD brain. When placed in DMEM/F12 media alone, over 95% of SP cores remain bound to coverslips and intact for at least 30 days.

FIG. 2. *In vitro,* SP cores (c, brown) are phagocytosed by rat cortical microglia (blue, GS1B4 isolectin) (A) and subsequently appear as numerous small vesicles containing Aβ (arrow) (B). Scale bar, 10 μm.

FIG. 6. *In vivo*, astrocytes show increased GFAP immunoreactivity (brown) in processes surrounding SP cores (c, core) (A). *In vitro*, rat astrocytes (GFAP, brown) also surround SP cores (blue) (B). Microglia (HLADR, purple) associated with SP cores (brown) *in vivo* are often ramified (C). Similarly, microglia (ED1, blue) cultured on monolayers of astrocytes (unstained, arrowheads) have a ramified morphology even near SP cores (brown) (D). Note: in neither case is there evidence of phagocytosis of SP cores. Scale bars, 30 μm.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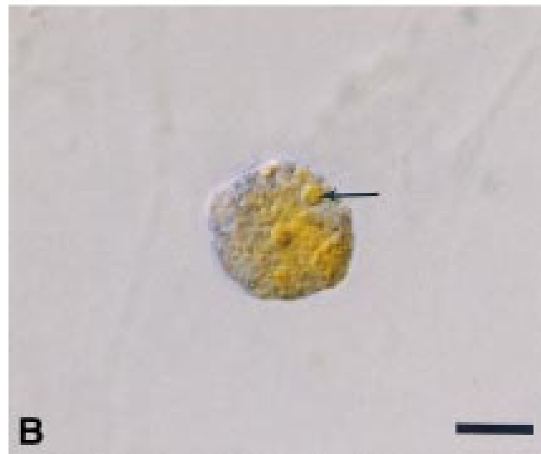
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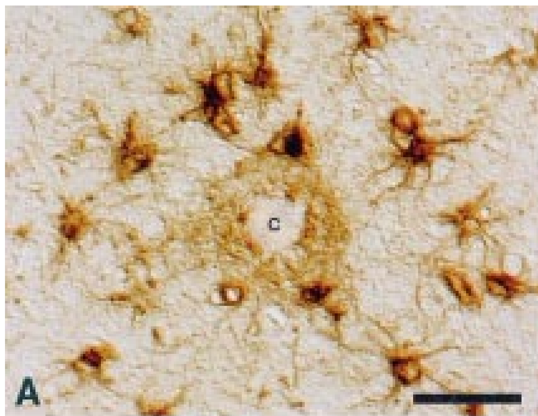
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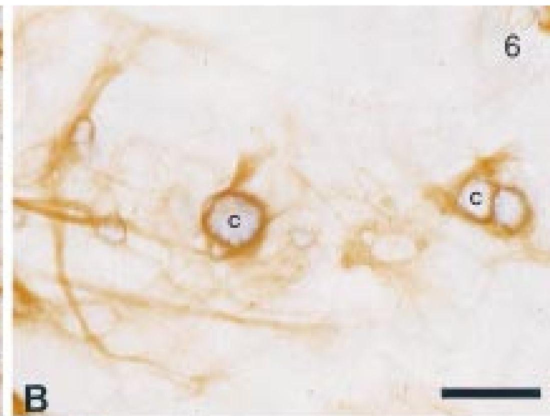
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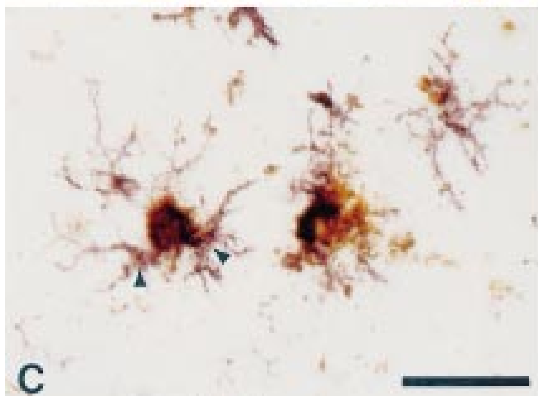
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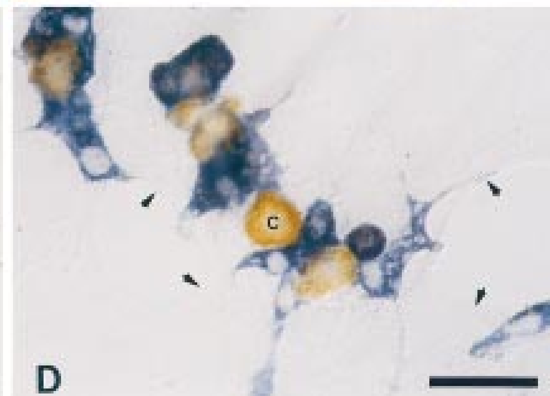
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Microglia Phagocytose and Degrade SP Cores

Rat microglial cells have the ability to phagocytose SP cores and began to internalize them within 2 h after plating. Although most microglia contained 1 core per cell (Fig. 2A), a few cells phagocytosed multiple cores (not shown). With extended time, the SP cores were broken apart and incorporated into smaller intracellular vesicles (Fig. 2B) a phenomenon that was also observed ultrastructurally (see below). Most cells contained a single Aβ containing vesicle (roughly 6–12 μm). Others had numerous small (1–2 μm) Aβ containing vesicles. The number of cells with these "broken" cores was low, suggesting that SP cores are rapidly cleared once they have been partitioned into smaller vesicles.

Electron microscopy verified that the SP cores were intracellular (Fig. 3A). At early time points, intact SP

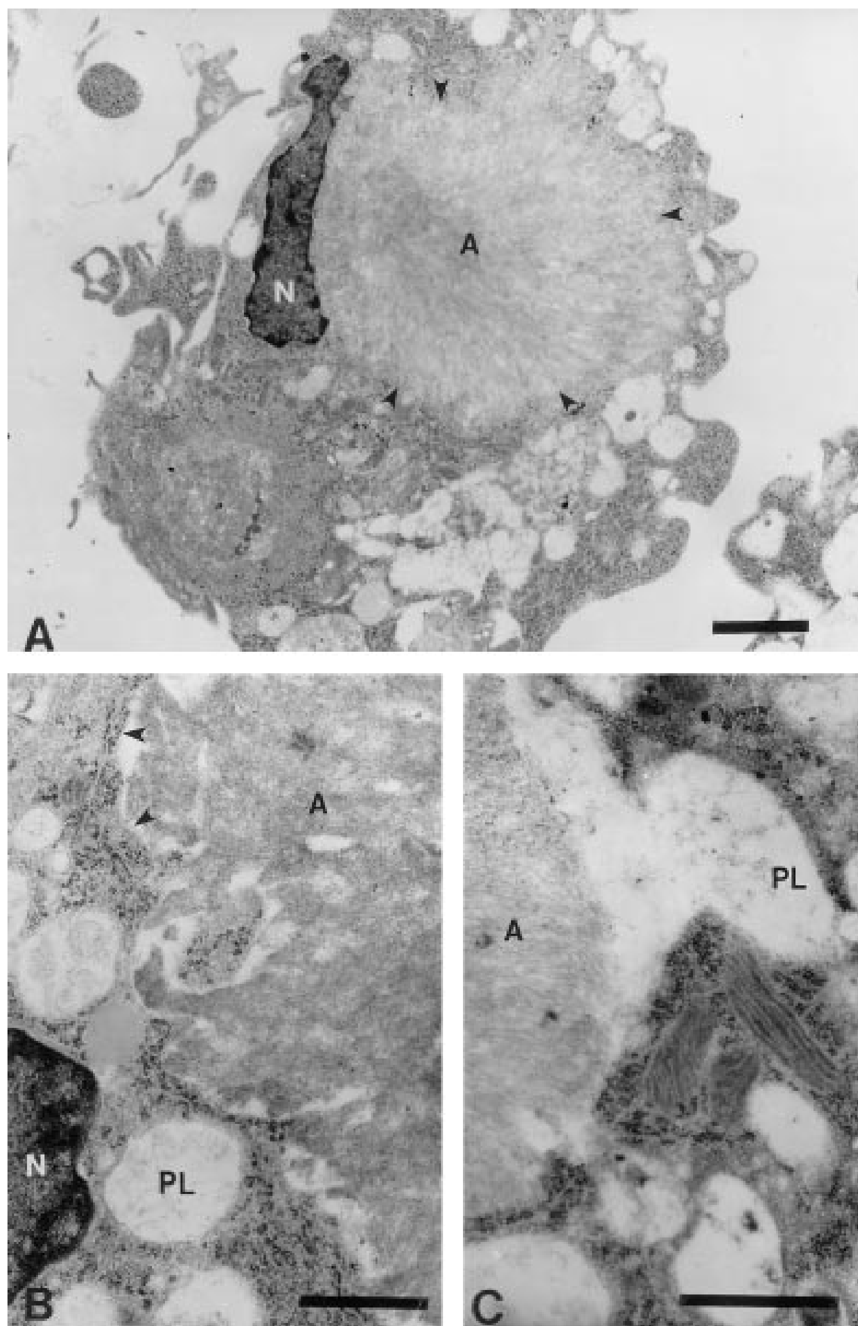

FIG. 3. Ultrastructural examination of internalized SP cores shows Aβ fibrils (A, arrowheads) (A) within a membrane bounded vesicle (arrowheads) (B). Phagolysosomes (PL) could be seen fusing with the SP containing vesicle (C). N, nucleus. (A) Scale bar, 2 μm. (B, C) Scale bar, 1 μm.

cores could be observed in apparent membrane bounded structures residing entirely within the cell (Fig. 3B). The SP cores occupied most of the cytoplasmic space, displacing the nucleus to one side. Phagolysosomes, characteristic of microglia, could be seen fusing with the SP core containing vesicle (Fig. 3C), suggesting that lysosomal enzymes may be involved in SP breakdown.

Temporal Distribution of SP Core Phagocytosis and Breakdown

To determine the kinetics of SP core clearance from microglial cultures, we examined their temporal distribution. Extracellular SP cores decreased continuously and exponentially during the culture period, consistent with rapid phagocytosis by microglia (Fig. 4A). In conjunction, the number of intracellular SP cores increased steadily within the first 24 h, but then stabilized for the next 7 days in culture (Fig. 4B), and then decreased.

After 3 days in culture, the total number of SP cores (extracellular and intracellular combined) decreased exponentially; less than 10% remained by day 30 (Fig. 4C). This distribution of SP cores is consistent with phagocytosis, internal processing leading to breakdown, and subsequent turnover or removal of SP from the cell.

Phagocytosis May Be the Rate Limiting Step

In order to determine if the rate-limiting step in SP core clearance is the rate of phagocytosis or subsequent breakdown, the rate of SP core clearance was followed for a range of microglial cell densities. At higher cell densities, nearly 100% of the SP cores were phagocytosed within 24 h while with low cell densities it was <60% over the same time period. The initial rate of SP core dissolution was dependent on the number of cells per well (Fig. 5A), suggesting that phagocytosis is a rate-limiting step in SP core clearance with a minimum SP core half-life of 2.4 days (Fig. 5B). However, even with maximum cell density (100,000 cells per well) and virtually all of the SP cores internalized within hours, there was a small fraction of cores which persisted intracellularly for at least 14 days. It is unclear whether this is a subset of resistant SP cores or a subgroup of microglia with less efficient digestive capacity.

Response of Glial Cells to SP Cores in Vitro

In order to mimic the cellular complexity of the SP we presented isolated SP cores to microglia in mixed glial cultures. Rat cortical astrocytes were grown on SP cores which had been bound to coverslips. Astrocytes wrapped processes around the SP cores but did not phagocytose them, a situation that is reminiscent of astrocytes associated with SPs in AD brain (compare Figs. 6A and 6B). These cells appeared to adopt a

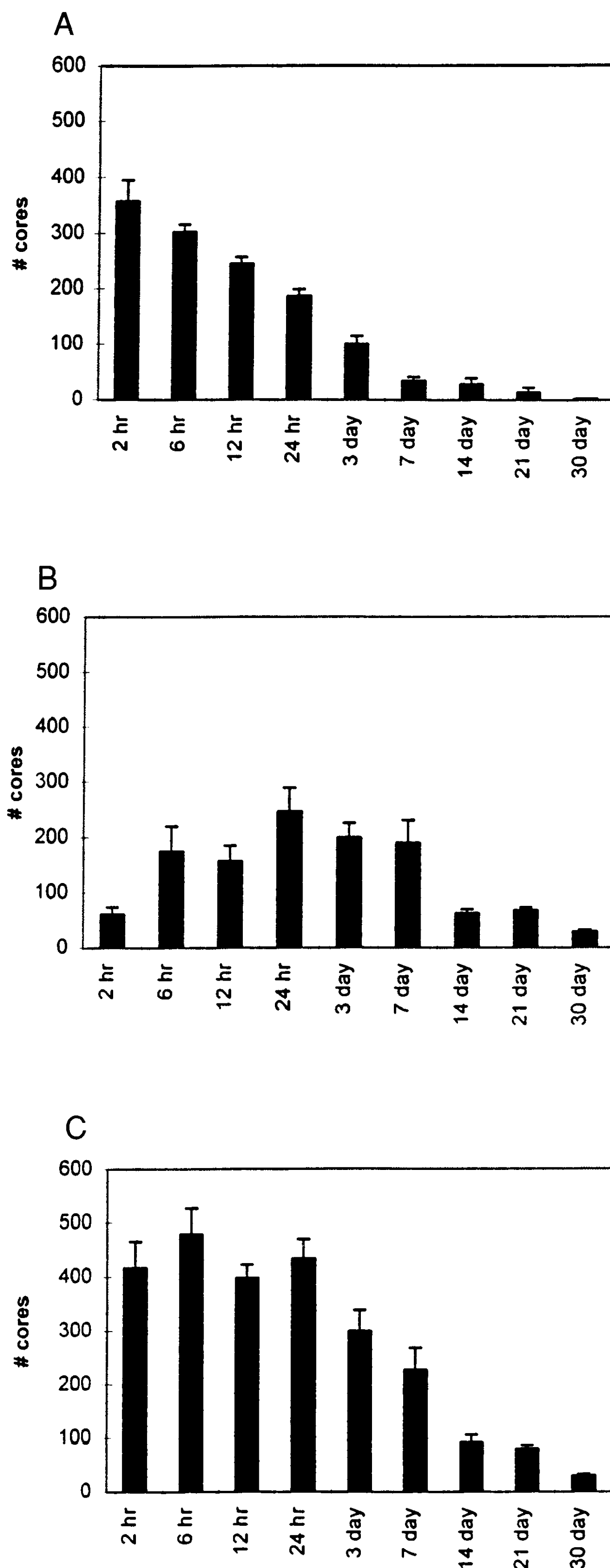

FIG. 4. Time course of microglial SP phagocytosis expressed as extracellular (A), intracellular (B), and total SP cores (C). Each time point is an average from eight coverslips.

reactive phenotype as indicated by their morphology and increased GFAP immunoreactivity (Fig. 6B) in the immediate vicinity of the SP core. SP cores were not toxic to astrocytes since these cells survived with SP cores through 30 days *in vitro.* Further, DIC microscopy and immunocytochemistry indicated that the astrocytes did not completely cover the SP cores but left them partially exposed providing a surface for microglial contact.

Microglia seeded onto an established monolayer of astrocytes with SP cores adopted a process bearing morphology even when in close contact with SP cores (Fig. 6D). The microglial processes were long and branched, consistent with previous studies which have shown that astrocytes induce microglial ramification (26, 39, 58, 65). During the 14-day period of the experiment, few microglia phagocytosed SP cores in the presence of astrocytes. Surprisingly, microglia often clustered tightly around the SP cores but, nonetheless, they did not phagocytose them (Fig. 6D). Both micro-

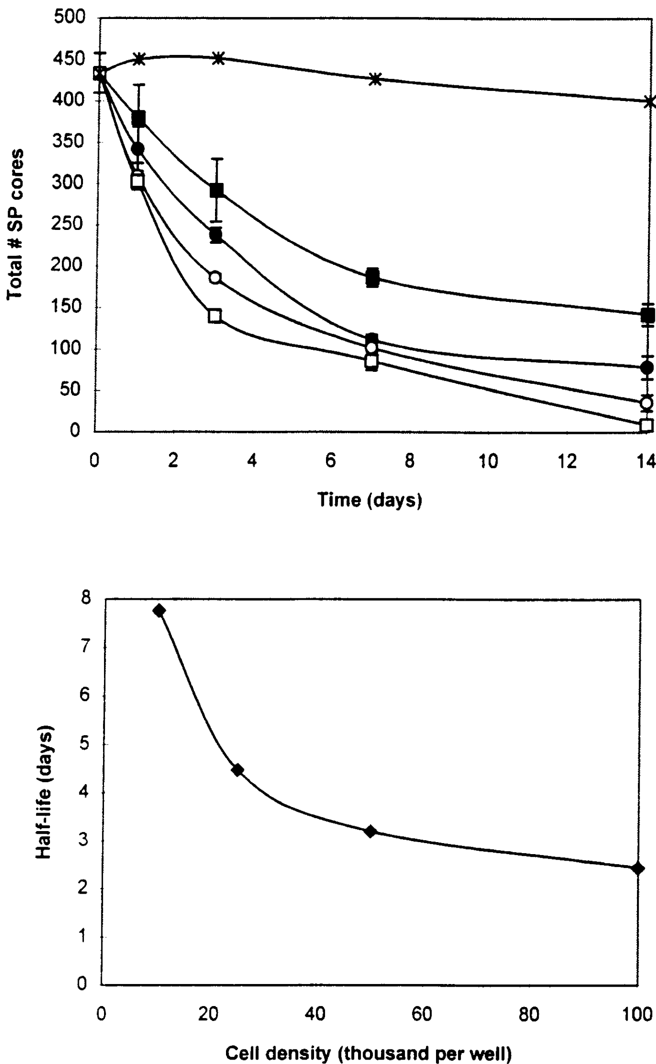

FIG. 5. SP core clearance was dependent on microglial density (A) as reflected in a decreasing half-life (B). In A, each time point represents an average from six coverslips from two experiments. (*no microglia; ■, 1×10^3 cells/well; ●, 2.5×10^3 cells/well; ○, 5×10^4 cells/well; □, 1×10^5 cells/well.)

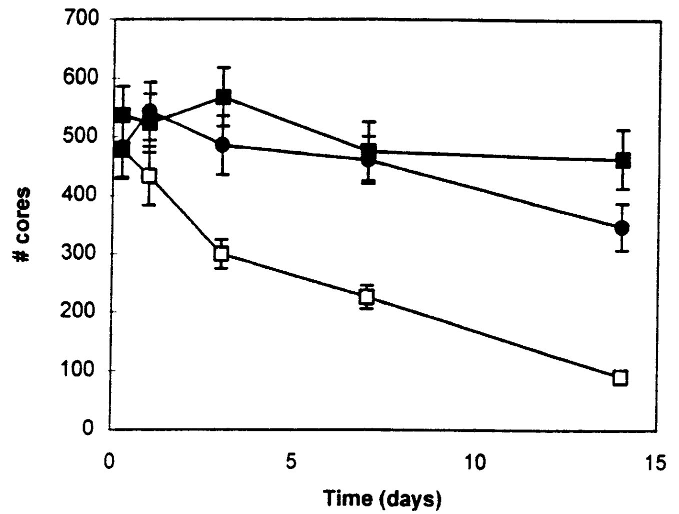

FIG. 7. In contrast with the clearance of SP cores by microglia, if SP cores were cultured with astrocytes for 7 days prior to addition of microglia virtually no SP cores were cleared. (□, microglia only; ●, microglia + astrocytes; ■, no microglia.)

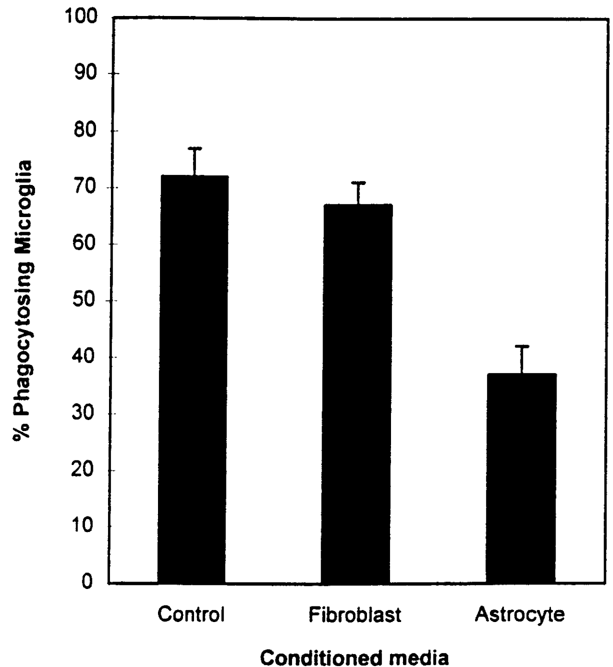

FIG. 8. Microglia were grown in astrocyte conditioned, fibroblast conditioned, or control media for 24 h prior to addition of polystyrene beads. Astrocyte-conditioned media reduces the percentage of microglia phagocytosing 11-μm polystyrene beads compared to nonconditioned media or fibroblast conditioned media. Note: microglia which had phagocytosed a bead had a round morphology. (Representative data, $N = 6$, performed in triplicate.)

glial clustering and their process bearing morphology are characteristics of microglia in AD brain (Fig. 6C). With extended time in coculture, some microglia did eventually phagocytose SP cores; however, the decreased phagocytosis of SP cores in the presence of astrocytes was reflected in their persistence. Time course experiments indicated the SP cores were not readily broken down or cleared from microglia cocultured with astrocytes (Fig. 7).

Effect of Astrocytes/Astrocytic Factors on Microglial Phagocytosis

To determine whether astrocyte factors decrease microglial phagocytosis generally, we used 11-μm latex beads as a model of SP cores but having the advantage that they cannot be broken down. While microglia readily phagocytosed these beads, neither astrocytes nor fibroblasts did. In cocultures, microglia growing on fibroblast monolayers quickly phagocytose the beads; however, microglia growing on astrocyte monolayers showed virtually no internalization of the beads. The ability of astrocytes to suppress phagocytosis is, in part, diffusible since the percentage of phagocytic microglia was significantly lower in astrocyte-conditioned media compared with fibroblast conditioned media (Fig. 8). In all cases, suppression of phagocytosis was temporally correlated with microglial transformation from a round to a ramified morphology. These results were consistent with other findings that ramified microglia have impaired phagocytic ability (23, 69) since microglia grown on astrocytes were ramified while those on fibroblasts were round.

We further analyzed the effects of astrocytes on the extent and rate of microglial phagocytosis using 0.5-μm latex particles. The smaller size allows the microglia to phagocytose numerous beads which provides a more precise evaluation of the kinetics of phagocytosis. Microglia were incubated for 24 h in the presence of astrocytes, astrocyte-conditioned media, or control media prior to addition of the latex particles. Astrocyte cocultures suppressed phagocytosis at least three-fold compared to control media. A similar, although less dramatic effect was also seen in astrocyte-conditioned media (Fig. 9A) after 6 h. In both astrocyte cocultures and conditioned media, phagocytosis was still significantly suppressed after 24 h; however, by this time, the differences between astrocyte coculture and astrocyte-conditioned media were less obvious (Fig. 9B). While diffusible astrocytic factors appear to suppress microglial phagocytosis in general, direct astrocytic contact was clearly more effective in suppression of phagocytosis as well as induction of a ramified morphology (Fig. 10).

DISCUSSION

We have shown that microglial cells from rat cortex can rapidly phagocytose whole SP cores. Once internalized, the SP cores were partitioned into smaller vesicles and cleared from the cell. However, when in the presence of astrocyte-conditioned media or especially when direct contact occurred between astrocytes and microglia, microglial phagocytosis was strongly suppressed with a coincident increase in their ramification. This suggests that both diffusible and astrocyte contact-

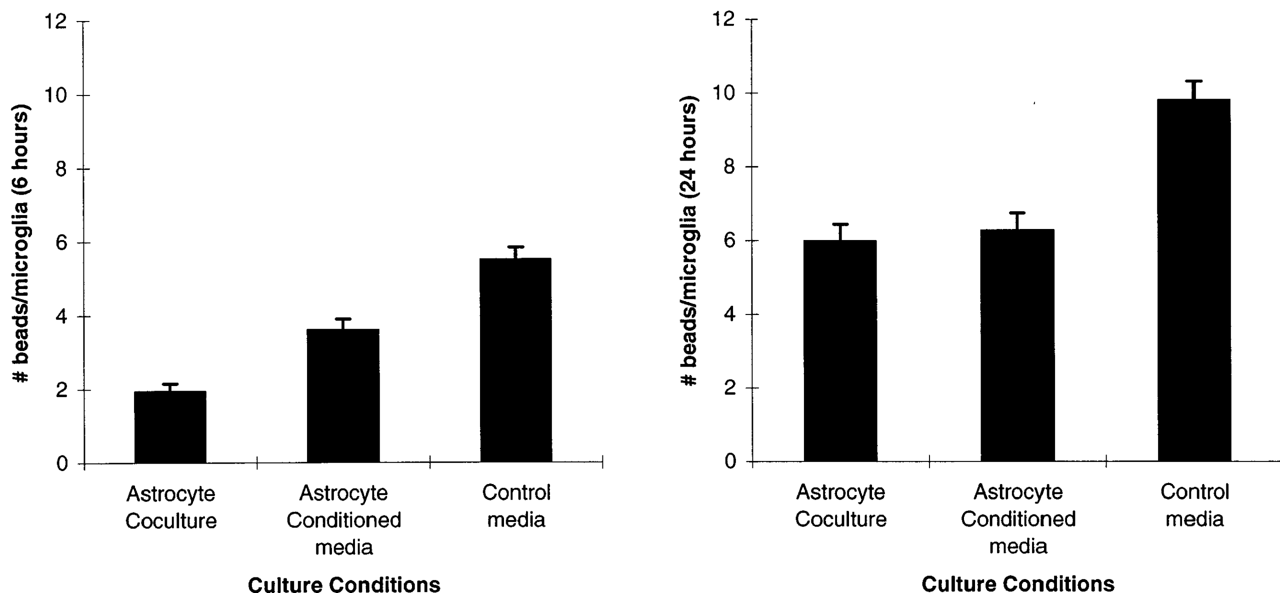

FIG. 9. Phagocytosis of 0.5-μm latex particles conditioned for 24 h with astrocytes, astrocyte-conditioned media, or control media. After 6 h, astrocyte-conditioned media suppresses microglial phagocytosis; however, not to the same extent as astrocyte cocultures until 24 h (B), while both are significantly less than control. (Representative data, performed in triplicate.)

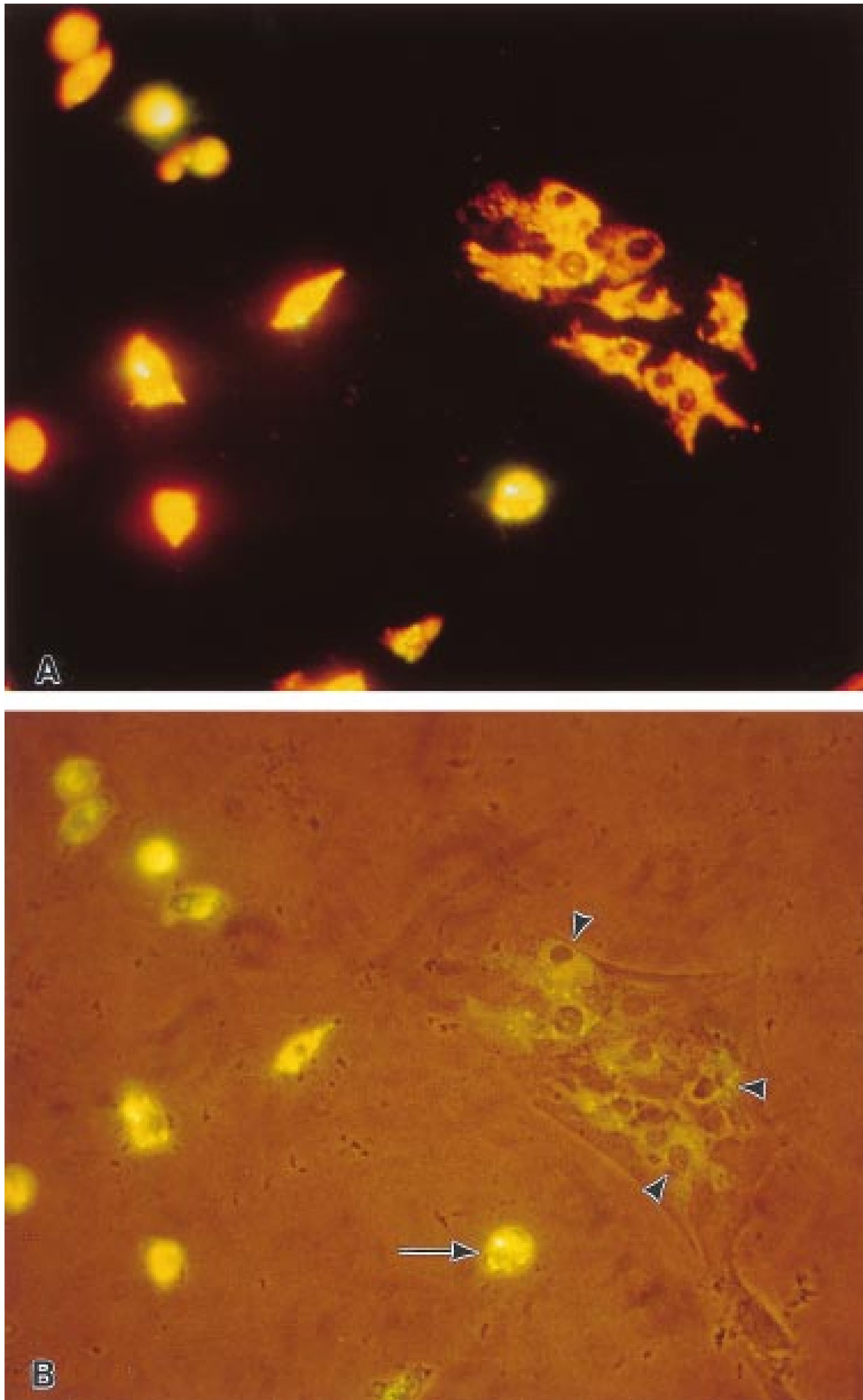

FIG. 10. As early as 1 day in coculture with astrocytes, microglia (red, ED-1) adopt a ramified morphology when in direct contact with astrocytes (A) while other microglia in the same culture not in contact with astrocytes remain round (B, arrow). Note the large number of latex beads (green) phagocytosed by microglia not in contact with astrocytes.

mediated factors play significant roles in regulating microglial phagocytosis (26, 67) *in vitro,* a phenomena that may be relevant to the turnover of SPs within the brain.

Several roles for microglia in the pathogenesis of AD have been proposed, including production of Aβ fibrils (16, 71), proteolytic processing of βPP (12), synaptic stripping (47), destruction of neurons through complement activation (29, 43, 53), and the production of cytokines and neurotoxins (2, 25). Relatively little attention has been paid to phagocytic activities of microglia possibly because the ramified microglia associated with SPs, although activated (34, 41), are in a largely nonphagocytic state. Observations of minimal Aβ phagocytosis *in vivo* (27) contrast with the robust debris removal capacity that microglia actually possess following traumatic injury such as a stroke or penetrating injury. Indeed, following a stroke, microglia rapidly phagocytose SP in a rapid time frame consistent with our *in vitro* model (70). Significantly, both penetrating injury and stroke can lead to astrocyte death or migration from the immediate site of injury (40), which could, in turn, eliminate their regulatory role and allow for a more rapid rate of debris removal.

Our results are consistent with previous studies where dog microglia in culture (16) and rat microglia *in situ* phagocytosed SP cores (18). However, in both studies, the authors stressed the persistence of Aβ rather than its removal and suggested that Aβ was resistant to microglial proteases even though in the latter study Aβ was present in small, intracellular vesicles. While we also found that some SP cores could persist for as long as 30 days *in vitro,* this represented only a small subpopulation of SP cores. Further, since the SP cores were initially of a uniform size, but once eaten, became incorporated into progressively smaller vesicles, we suggest that these observations are consistent with the idea that SP cores can be readily broken down by their host phagocyte (3, 57). The similar regulatory capacity of astrocytes on microglia in the presence of both SP cores and latex beads suggest astrocyte inhibition of microglial phagocytosis is not Aβ-specific. Astrocytes could regulate microglial phagocytosis through a number of possible mechanisms. Astrocytes could down-regulate microglial scavenger receptors or (5, 11, 48) alter levels of phagocytosis-related trophic factors (14). Alternatively, astrocytes may induce ramification which blocks the formation of pseudopods thought to be required for phagocytosis (59).

Although the specific astrocytic factor(s) that induce microglial ramification are unknown, a number of candidate molecules exist including mCSF1 (39), fibronectin (10), and TGF-β (66). In addition, a recent paper clearly implicated astrocytic extracellular matrix proteins (67) since fixed astrocyte monolayers as well as astrocyte matrix alone induced ramification. Our results are consistent with these findings, since astrocyte monolayers were much more effective than astrocyte-conditioned media in suppressing phagocytosis. Although it is well established that astrocytes can induce ramification of microglia (26, 39, 58, 64), ours is the first to show that astrocytes concomitantly suppress microglial phagocytosis.

The implications for astrocyte regulation of microglial phagocytosis and debris removal within the CNS goes well beyond its potential role in AD. Following an injury to the PNS, macrophages rapidly adopt a round morphology and remove debris and necrotic tissue quickly (7, 20, 51, 72). In contrast, a lesion to the CNS such as severing (7, 50) or crushing (17) the optic nerve results in a much slower rate of resolution distally. Our data implicating astrocytes in the general suppression of microglial phagocytosis suggests that astrocytes may be indirectly responsible for the persistence of SP as well as other forms of debris in the brain.

ACKNOWLEDGMENTS

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