Accelerated Phagocytosis of Amyloid-β by Mouse and Human Microglia Overexpressing the Macrophage Colony-stimulating Factor Receptor*

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Microglia surrounding Aβ plaques in Alzheimer’s disease and in the APPV717F transgenic mouse model of Alzheimer’s disease have enhanced immunoreactivity for the macrophage colony-stimulating factor receptor (M-CSFR), encoded by the proto-oncogene c-fms. Increased expression of M-CSFR on cultured microglia results in proliferation and release of pro-inflammatory cytokines and expression of inducible nitric-oxide synthase. We transfected mouse BV-2 and human SV-A3 microglia to overexpress M-CSFR and examined microglial phagocytosis of fluorescein-conjugated Aβ. Flow cytometry and laser confocal microscopy showed accelerated phagocytosis of Aβ in mouse and human microglia because of M-CSFR overexpression that was time- and concentration-dependent. In contrast, microglial uptake of 1-μm diameter polystyrene microspheres was not enhanced by M-CSFR overexpression. Microglial uptake of Aβ was blocked by cytochalasin D, which inhibits phagocytosis. M-CSFR overexpression increased the mRNA for macrophage scavenger receptor A, and fucoidan blocking of macrophage scavenger receptors inhibited uptake of Aβ. M-CSFR antibody blocking experiments demonstrated that increased Aβ uptake depended on the interaction of the M-CSFR with its ligand. These results suggest that overexpression of M-CSFR in APPV717F mice may prime microglia for phagocytosis of Aβ after immunization.

Alzheimer’s disease (AD) is characterized by amyloid-β peptide (Aβ) plaques surrounded by microglia. Aβ is thought to be directly neurotoxic and activated microglia are hypothesized to have negative effects on neurons through the release of effectors of inflammation (1). However, as brain macrophages microglia can clear Aβ by phagocytosis, primarily through macrophage scavenger receptors (MSR) (2–6). Immunization of transgenic mice modeling AD with Aβ results in clearance of plaques from the brain (7). Whereas some results suggest that microglial phagocytosis may be key in clearance of Aβ after immunization (8), other findings indicate that circulating antibodies may result in movement of Aβ out of the brain (9). This controversy has stimulated renewed interest in uptake of Aβ by microglia.

A distinctive phenotypic feature of microglia surrounding Aβ plaques in APPV717F transgenic mice and in AD is enhanced expression of the macrophage colony-stimulating factor receptor (M-CSFR), translation product of the c-fms proto-oncogene (10, 11). Microglial M-CSFR expression is also increased after experimental ischemic and traumatic brain injury (12, 13). M-CSFR regulates proliferation, activation, and survival of cells in the monocyte-macrophage lineage through tyrosine kinase activation of diverse signal transduction pathways including: Src kinase, Ras-ERK, phosphoinositide 3-kinase, and p38 MAP kinase (14–16). Deletion of M-CSFR expression results in decreased numbers of cells of the mononuclear phagocyte lineage (17). In AD macrophage-stimulating factor (M-CSF), the ligand for M-CSFR expressed by neurons and glia is also increased (18). Simultaneous increases in M-CSF and M-CSFR expression in the brain could result in significant changes in microglial function.

We recently demonstrated that overexpression of M-CSFR by cultured microglia increases proliferation, stimulates release of pro-inflammatory and chemotactic cytokines, and induces a paracrine inflammatory response in a microglial-organotypic co-culture system (19). In the present study we sought to determine the effects of M-CSFR overexpression on Aβ phagocytosis by cultured mouse and human microglia. We hypothesized that M-CSFR-induced activation of microglia would increase their capacity to clear Aβ from culture medium. Although Aβ immunization clinical trials have been discontinued in humans for the present, identifying factors that enhance microglial clearance of Aβ may be of benefit in devising alternative means of decreasing Aβ burden in the brain in AD.

EXPERIMENTAL PROCEDURES

Microglial Cell Lines, Plasmid Transfections, and Tissue culture—The c-fms expression plasmid pTK1 was a gift from Dr. Rao Tekmal (Emory University, Atlanta, GA), and contains the complete mouse c-fms sequence under the control of an SV40 promotor (20). Transfections were carried out using mouse BV-2 and human SV-A3 microglial cells. The BV-2 immortalized microglial cell line has been characterized previously (21–24). Because of phenotypic changes that occur at higher passages in BV-2 cells,7 BV-2 cells used in these experiments were from passage 25 or lower. The human SV-A3 cell line was a gift from Dr. Robert Nelson (Pfizer Central Research, Groton, CT). The SV-A3 line was an immortalized line from fetal brain microglia, and phenotypic features have been previously described (19,19). Transient transfections with the pTK1 plasmid were carried out

* This work was supported by National Institutes of Health Grants MH57833, MH40041, and AG17824 and the Alzheimer’s Association.

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The abbreviations used are: AD, Alzheimer’s disease; M-CSFR, macrophage colony-stimulating factor receptor; M-CSF, macrophage colony-stimulating factor; Aβ, amyloid β; Fluo-Aβ, fluorescein-conjugated amyloid β; PBS, phosphate-buffered saline; MSR-A, mouse scavenger receptor type A.
using LipofectAMINE PLUS™ reagent (Invitrogen). BV-2 or SV-A3 cells were plated at 65% confluency in 6-well tissue culture Eagle’s medium. BV-2 cells were grown in Dulbecco’s modified Eagle’s medium (Applied Scientific, South San Francisco, CA) supplemented with 5% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 4 mM glutamine (Applied Scientific, South San Francisco, CA) and 1% penicillin/streptomycin (Sigma). SV-A3 cells were cultured with Dulbecco’s modified Eagle’s/10-12 medium (Invitrogen) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. For each transfection reaction, 0.2 µg of pTK1 plasmid pre-coated with 6 µl of the PLUS reagent and 5 µl of LipofectAMINE were used per 5 × 10^5 microglial cells. Control transfections were performed in parallel with an equal volume of LipofectAMINE PLUS reagent only (transfection medium; designated by M in the figures). Transfections using the pZeosV plasmid (Invitrogen) that contains the pTK1 backbone sequence originally used in c-fms subcloning were included as a second control, although we previously showed that pZeosV does not induce a microglial proinflammatory response (19). Cells were transfected with the pZeosV plasmid using the same procedure as described for pTK1. After the addition of transfection reagents, cells were returned to the incubator for 24 h, and then used for phagocytosis studies.

Cell Surface M-CSFR—Microglia that were transfected with the pTK1 plasmid were then compared with nontransfected microglia for M-CSFR expression to determine transfection efficiency. Immunocytochemical visualization of M-CSFR was demonstrated previously (19). To quantify cells overexpressing M-CSFR, 24 h post-transfection BV-2 cells were washed with 1× PBS buffer and incubated for 1 h with 1× PBS buffer with 0.5 × 10^5 goat serum (Zymed Laboratories Inc.), and incubated with rabbit anti-mouse M-CSFR antiserum (1:500) (Upstate Biotechnology Inc., Lake Placid, NY) for 16 h at 4 °C. The M-CSFR antibody has specificity for the extracellular domain of the receptor (25). Subsequently cells were incubated for 1 h at 37 °C with 1:500 dilution of Cy3-labeled goat anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA). Cells were then washed three times with 1× PBS buffer on ice, then detached with trypsin and collected by centrifugation. Finally, cells were resuspended in 0.5 ml of 1% paraformaldehyde solution and analyzed for M-CSFR overexpression by flow cytometry as described below. A population of 10,000 cells per sample was used to acquire two-dimensional contour forward and orthogonal scatter diagrams. The nonviable subpopulation of cells was excluded by setting the gated forward scatter value to 60. Cellular autofluorescence in the Cy3 channel was 4.32 units for control and c-fms-transfected microglia.

Phagocytosis Assay for Fluorescein-conjugated Aβ—Microglial phagocytosis of Aβ was determined using Fluo-Aα, a direct fluorescein conjugate that can be visualized with a flow cytometer or with fluorescence microscopy (PerkinElmer Life Sciences). Immediately before use, Fluo-Aα 1-40 or 1-42 was resuspended in 2% DMSO solution in Earl’s buffer (140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂, 25 mM HEPES, pH 7.4) at a concentration of 1 µg/ml. The solution was incubated for 30 min at 37 °C according to the manufacturer’s instructions. This preparation was used at a concentration between 10 and 100 nM in phagocytosis assays.

Another Aβ preparation, similar to that described by Webster et al. (26), was utilized to study phagocytosis at higher Aβ concentrations. This preparation consisted of preaggregated Fluo-Aα 1-40 added to nonlabeled Aβ 1-40 (Bachem, Torrance, CA) that had been aggregated by 60 h incubation at 4 °C, followed by 8 h incubation with gentle shaking at 37 °C as previously described (24). The stock solution of this preparation consisted of 100 nM Fluo-Aα 1-40 in 10 µM aggregated unlabeled 1-40. The stock solution was diluted 10-fold in culture medium prior to the phagocytosis assay.

For the phagocytosis assay, mouse BV-2 cells were plated at 65% confluence in 6-well tissue culture dish and grown using the conditions described above. After 24 h of transfection with c-fms expression plasmid, the control plasmid pZeosV, or treatment with transfection medium alone, the medium was removed and BV-2 cells were washed four times with 1× Dulbecco’s PBS (Invitrogen). Immediately before the phagocytosis assay, cells were equilibrated for 1 h at 37 °C in 750 µl/well of Earl’s buffer that had been supplemented with 0.2% bovine serum albumin and 0.01% glutaraldehyde. Subsequently, Fluo-Aβ reagents were added and cells were incubated at 37 °C in the dark.

To monitor the time course and concentration dependence of Fluo-Aβ phagocytosis in mouse microglia, nontransfected BV-2 cells were treated with Fluo-Aβ at final concentrations of 10, 20, or 100 nM, and harvested after 30 min, 60 min, or 24 h of incubation. To examine the effects of M-CSFR overexpression, BV-2 cells were transfected with the pTK1 plasmid, exposed to 15 nM Fluo-Aβ 1-40, and harvested after 30 min, 60 min, or 24 h. In experiments with SV-A3 human microglia, transfection and phagocytosis assay conditions were the same as those for BV-2 cells, except that the final concentration of Fluo-Aβ was 10 nM and the exposure time was 60 h. To compare phagocytosis of Aβ (pTK1—fms-transfected) to that of Aβ (c-fms-transfected) BV-2 cells, 10 nM Fluo-Aβ 1-40 was applied for 180 min at final concentrations of 10 nM labeled peptide and 1 mM M-CSFR in the medium. Because rapidly proliferating c-fms-transfected BV-2 cells were not viable for more than few hours in serum-free medium, all 24-h phagocytosis experiments were performed in heat-inactivated serum-containing medium. Control samples for extracellular surface binding of Fluo-Aβ contained nontransfected BV-2 cells that were incubated in triplicate wells with 15 nM Fluo-Aβ for 24 h at 4 °C. To study Aβ uptake by MSR, BV-2 cells were pretreated with 100 µM of the MSR ligand fucoidan (Sigma) for 20 min prior to exposure to 20 nM Fluo-Aβ for 120 min. To demonstrate that uptake of Fluo-Aβ involves phagocytosis, microglia were pretreated with 5 µM cytochalasin D (27) (Sigma) using the same conditions as for fucoidan.

The phagocytosis assay was terminated by placing cells on ice and promptly removing medium containing Flu-Aβ. Cells were washed 4 times with cold 1× PBS and removed residual Fluo-Aβ and FBS from the cell suspension by gentle pipetting in 500 µl of 1× PBS buffer on ice, transferred to 5-ml polystyrene round-bottom tubes (BD Pharmingen), and immediately analyzed by flow cytometry.

Flow Cytometry Analysis—Flow cytometry was performed using a 10-color flow cytometer, a hybrid instrument consisting of a FACStar Plus bench (BD Pharmingen), MoFlo electronics (Cytometry, Fort Collins, CO) and a custom electronics and computer system (Stanford Cell Sorting Facility, Stanford, CA) (28). The emitted fluorescence intensity was measured in triplicate using 10,000 cells per sample. Data were collected on the fluorescence-activated cell sorter desk (36), calibrated so that fluorescence measurements were in the linear range above a value of 1.0. Subsequent statistical analysis of scatter plots and fluorescence histograms was performed using FlowJo 3.3 software (Tree Star Inc., San Carlos, CA). The viability of each sample was evaluated from the cell populations. Scatter plots obtained on the ungated cell population. In all cases, cell populations were found to be uniform in size and without large aggregates, and the number of nonviable cells and cellular debris was found to be less than 10% of the total. For quantitative analysis, the mean fluorescence values for each sample were collected from the ungated cell population and corrected by subtracting the corresponding cell autofluorescence value to obtain net signal. The autofluorescence values for nontransfected BV-2 cells were similar and had an average value of 4.27 units, except in Fig. 4A for which the autofluorescence was 5.6. Autofluorescence of human SV-A3 microglia was 3.31 units. Absolute mean fluorescence intensities (given in figure legends) for control microglia were assigned a value of 1.0. Normalized fold change values were then calculated according to the equation: normalized fold fluorescence change = (mean transffected sample fluorescence intensity − cellular autofluorescence)/mean control sample fluorescence intensity − cellular autofluorescence). Each experiment consisted of cell samples run in triplicate, and each experiment was performed three times. Graphs show mean normalized fold fluorescence change with standard deviations (S.D.) from triplicate experiments relative to transfection medium (M) treated control microglia.

Poly styrene Microsphere Phagocytosis—To examine microglial uptake of a substrate other than Aβ, we used polystyrene microspheres. The stock suspension of fluorescent 1-µm diameter polystyrene microspheres (blue-green Fluospheres Fluorescent Microspheres, Molecular Probes, Eugene, OR) contained 1 × 10^7 microspheres per µl of water (diluted in 0.9% NaCl). The cell suspension was grown to confluence and transfected with the pTK1 c-fms plasmid as described above. The phagocytosis assay was performed identically to that for Fluo-Aβ except that a suspension containing 0.5 µl of microsphere stock suspension per ml of medium was applied to the BV-2 cells. Fluorescence intensity measurements were taken using flow cytometry after 24 h of incubation, and calculations of fluorescence fold change were performed as described for the Fluo-Aβ assay. As a positive control, Fluo-Aβ experiments were

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performed in parallel to microsphere uptake experiments using the same batch of cells.

**Laser Confocal Microscopy**—To visualize phagocytosis of Aβ by microglia we used laser confocal microscopy. BV-2 and SV-A3 cells were cultured and transfected as described above, except that cells were grown on glass coverslips on the bottom of tissue culture dishes. Cells were treated with 25 nM Fluo-Aβ and incubated for 3 h at 37 °C. Cells were then washed 4 times with 1× PBS buffer at 4 °C and then fixed for 20 min with a 4% paraformaldehyde solution (Sigma) in 1× PBS. The coverslips were mounted on glass slides using SlowFade (Molecular Probes, Eugene, OR), and examined on a Zeiss LSM 510 laser confocal microscope (Carl Zeiss Inc., Thornwood, NY) using a ×63 oil immersion objective. Images were captured in the fluorescein isothiocyanate channel. For comparison of c-fms-transfected and control BV-2 and SV-A3 microglia, images were captured using identical acquisition settings for contrast, brightness, pixel, and pinhole dimensions. Images of microglia without Fluo-Aβ treatment acquired with identical settings were used to assess background autofluorescence.

**SYBR Green Real-Time Quantitative Reverse Transcriptase-PCR**—Mouse BV-2 microglia were grown to 65% confluency and transfected with the pTK1 plasmid or the pZeoSV control plasmid as described above. After 24 h, total RNA was extracted using the TRIzol reagent (Invitrogen). RNA samples were diluted to 1 μg/μl in RNase-free water, and reverse transcription was performed from 1 μg of RNA as previously described (19). Individual 25-μl quantitative SYBR Green real-time PCR reactions consisted of 5 μl of cDNA (50 ng/μl), 12.5 μl of 2× Universal SYBR Green PCR Master Mix (PE Applied Biosystems, Foster City, CA), and 3.75 μl of 50 nM optimized forward and reverse primers with specificity for the mouse macrophage scavenger receptor type A (MSR-A) mRNA (GenBank™ accession number AF203781). Primer sequences designed using Primer Express software (PE Applied Biosystems) were: forward813 5′-GACAAATTGGCTTCCCTGGA-3′, and reverse1044 5′-CCCGAACCCTCCGGCTT-3′, where numbers indicate the position of the 5’ nucleotide. A 64-base pair amplification was generated using these primers. Quantitative PCR was performed on an ABI 5700 Instrument (PE Applied Biosystems) using a 3-stage program provided by the manufacturer: 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Specificity of the amplification product was confirmed using dissociation reaction plots, with a distinct single peak indicating a single PCR product. Values for each sample were compared using a standard curve (37). Each sample was tested in triplicate PCR wells, and samples obtained from three independent experiments were used to calculate the mean ± S.D.

**M-CSFR Antibody Blocking**—BV-2 microglia were grown to a final density of 1 × 10⁸ cells per well in 6-well tissue culture dishes and transfected with the pTK1 c-fms expression plasmid as described above. After 24 h, transfection medium was removed, and after washing cells at 4 °C with 1× PBS buffer, blocking medium was added consisting of 750 μl of serum-free medium and 5 μl of the rabbit anti-Mouse M-CSFR antibody (Upstate Biotechnology). Transfected and control microglia were grown in the presence of the M-CSFR blocking reagent for 24 h at 37 °C to account for the greatly increased cellular surface expression of M-CSFR after transfection with pTK1 plasmid (19). As a control, microglial cells were treated with an equal dilution of normal rabbit serum (Sigma). Phagocytosis measurements were taken as described above after 120 min of incubation with 5 nM Fluo-Aβ.

**Statistical Analysis**—All experiments were performed with triplicate cell culture samples, and each experiment was replicated on at least three separate occasions. Means of triplicate experiments were compared using two-tailed unpaired t tests, or with one or two-way analyses of variance. For post-hoc comparisons among means, the rigorous Scheffe adjustment for multiple testing was used.

**RESULTS**

Fig. 1 summarizes flow cytometry analysis of M-CSFR overexpression on mouse BV-2 microglia detected using the antibody to the extracellular domain of the receptor. Fig. 1A shows that after 24 h of transient transfection with the c-fms expression plasmid pTK1, there was an increase in Cy3 fluorescence (x axis) of c-fms-transfected microglia relative to nontransfected cells, demonstrating M-CSFR overexpression. The contour diagrams are based on 10,000 cells per sample, and were gated to exclude nonviable cells with a forward scatter value of 60 and less. Examination of the ungated cell population showed that on average less than 10% of the cells were excluded by gating. The fluorescence histograms shown in Fig. 1B demonstrate
that the average Cy3 fluorescence increased from 5.67 in nontransfected cells to 51.5 units in transfected cells. After subtracting the average autofluorescence of 4.32 units, the average net Cy3 intensity increased from 1.35 to 47.18 units. We chose a liberal estimate of 20 for the maximum fluorescence intensity of nontransfected cells. Based on the number of pTK1-transfected microglia that had fluorescence intensities greater than 20, the average transfection efficiency was 89.9%.

To compare size, granularity, and overall morphology of transfected and control cells, forward/orthogonal scatter profiles of control and M-CSFR overexpressing BV-2 microglia were overlaid (Fig. 1C). After exclusion of nonviable cells there was complete overlap in the scatter plots. This demonstrates no change in the size or structure of BV-2 microglia because of M-CSFR overexpression.

BV-2 mouse and SV-A3 human microglia readily ingested Fluo-αβ at low nanomolar concentrations. This response was enhanced by overexpression of M-CSFR. Fig. 2 shows uptake of Fluo-αβ by nontransfected BV-2 cells, measured after 30 and 60 min and 24 h at concentrations of 10, 20, and 100 nM. Uptake occurred in a time- and Fluo-αβ concentration-dependent manner; a maximum mean normalized fold fluorescence change of 6.64 ± 0.04 was measured in the 100 nM Fluo-αβ-treated sample for 24 h. Two-way analysis of variance revealed significant effects because of concentration, time, and a time by concentration interaction (all p < 0.001). Post-hoc Scheffe tests showed that overall the increase after 24 h was significantly different from that after 30 and 60 min (Scheffe adjusted p < 0.05). The 30- and 60-min time points did not differ significantly. Overall, the 10, 20, and 100 nM concentrations gave significantly different results (adjusted p < 0.05). The interaction was because of a significantly greater increase at the 100 nM concentration after 24 h than after 30 and 60 min.

Phagocytosis of αβ was sharply increased after microglia were transiently transfected with the pTK1 c-fms plasmid. Fig. 3 shows confocal imaging of Fluo-αβ in control and c-fms-transfected microglia, and demonstrates an increase in intracellular Fluo-αβ in mouse BV-2 (Fig. 3, A and B) and in human SV-A3 (Fig. 3, C and D) cells as a result of M-CSFR overexpression (Fig. 3, B and D). Uptake of Fluo-αβ by nontransfected SV-A3 cells was extremely low (Fig. 3C), making it difficult to acquire detailed images of the nontransfected state, even with confocal microscopy.

Increased phagocytosis of Fluo-αβ because of M-CSFR overexpression was quantified by flow cytometry. Fig. 4A shows a fluorescence histogram from a representative experiment utilizing 10,000 BV-2 microglia that were c-fms transfected (red) or nontransfected (blue), and exposed to 25 nM Fluo-αβ for 180 min. pTK1-transfected microglia showed no difference in basal autofluorescence from control cells because of their similar size and shape. A positive shift along the x axis indicates an increase in mean fluorescence intensity from 8.7 units in nontransfected to 15.5 units in transfected microglia, which after subtracting the cellular autofluorescence value of 5.6 units results in a net increase of 3.2-fold for c-fms-transfected microglia. Fig. 4B shows data from triplicate experiments in which BV-2 cells were transfected to overexpress M-CSFR, and fluorescence intensity was measured after 30 min, 60 min, and 24 h of exposure to 15 nM Aβ. Analysis of variance showed significant main effects for transfection, for time, and a transfection by time interaction (all p < 0.001). Overall, c-fms overexpression significantly increased ingestion of Aβ. However, the difference between transfected and nontransfected cells was greater at 24 h than at 60 and 30 min. Flow cytometry measurements taken after 5, 10, and 15 h of incubation showed a progressive increase in Fluo-αβ ingestion by c-fms-transfected cells, although uptake was not as great as at 24 h (data not shown). Increased phagocytosis of Fluo-αβ because of M-CSFR overexpression was also observed with flow cytometry using SV-A3 human microglia. As shown in Fig. 4C, an average 13.75 ± 3.57-fold increase in mean normalized fluorescence was observed in SV-A3 after c-fms transfection (p < 0.005).

To verify that fluorescence was because of internalized rather than surface-bound Fluo-αβ, BV-2 microglia were incubated for 24 h at 4 °C before flow cytometry. Fig. 4B shows that...
surface bound Fluo-Aβ showed a normalized fold fluorescence change of 0.45 ± 0.07. Because microglia incubated with Fluo-Aβ for the same amount of time at 37 °C had a 2.73 normalized fold fluorescence change value, cell surface Aβ accounted for only 16.6% of signal at the point of maximal Fluo-Aβ internalization. Furthermore, Fluo-Aβ uptake upon c-fms

**FIG. 4.** Flow cytometry analysis showing increased Aβ phagocytosis in microglia overexpressing M-CSFR. A, fluorescence histograms of Fluo-Aβ phagocytosis in transfection medium-treated (blue) and c-fms-transfected (red) BV-2 microglia demonstrate positive shift upon overexpression of M-CSFR. Histograms are from a representative experiment in which cells were treated for 180 min with 25 nM Fluo-Aβ. The x-axis represents on a logarithmic scale fluorescence intensity with respect to relative cell number (y-axis). B, uptake of 15 nM Fluo-Aβ measured after 30 min, 60 min, and 24 h from triplicate samples of transected and nontransfected cells. The normalized value of 1 was equal to 4.92 fluorescence units. The right-hand bar shows uptake by BV-2 cells treated with 10 nM Fluo-Aβ alone. Nontransfected BV-2 cells ingested the 1 μM Aβ preparation more avidly than 10 nM Fluo-Aβ alone. Transfection resulted in a further increase in phagocytosis of both preparations. The normalized fluorescence value of 1 equals 5.34 units. B, uptake of 1-μm diameter fluorescent polystyrene microspheres by BV-2 cells maintained at 4 °C for 24 h of incubation. There was no difference in microsphere uptake between cells treated with transfection medium and c-fms-transfected cells. C, comparison of Fluo-Aβ 1-40 and 1-42 uptake. The increase in uptake after c-fms transfection was similar for both peptides. BV-2 cells transfected with the control plasmid pZeoSV were similar to medium-treated control cells. Normalized fold fluorescence of 1 equals 5.17 units.

**FIG. 5.** Increased phagocytosis of micromolar concentrations Fluo-Aβ. Polystyrene microsphere uptake. Comparison of Aβ 1-40 and Aβ 1-42 uptake. A, increase in mean fluorescence in control and c-fms-transfected BV-2 microglia after 180 min exposure to 10 nM Fluo-Aβ in 1 μM aggregated Aβ 1-40. Results are presented in parallel to cells treated with 10 nM Fluo-Aβ alone. Nontransfected BV-2 cells ingested the 1 μM Aβ preparation more avidly than 10 nM Fluo-Aβ alone. Transfection resulted in a further increase in phagocytosis of both preparations. The normalized fluorescence value of 1 equals 5.34 units. B, uptake of 1-μm diameter fluorescent polystyrene microspheres by BV-2 cells maintained at 4 °C for 24 h of incubation. There was no difference in microsphere uptake between cells treated with transfection medium and c-fms-transfected cells. C, comparison of Fluo-Aβ 1-40 and 1-42 uptake. The increase in uptake after c-fms transfection was similar for both peptides. BV-2 cells transfected with the control plasmid pZeoSV were similar to medium-treated control cells. Normalized fold fluorescence of 1 equals 5.17 units.
transfection was dramatically reduced by microglial pretreatment with the phagocytosis inhibitor cytochalasin D as shown in Fig. 4D (p < 0.0001).

Nontransfected and c-fms-transfected microglia avidly ingested a 1 μM concentration of unlabeled aggregated Aβ 1-40 containing a final 10 nM concentration of Fluo-Aβ (Fig. 5A). The uptake of Fluo-Aβ by nontransfected microglia was significantly greater when it was presented in combination with the 1 μM aggregated Aβ than when it was presented alone at a 10 nM concentration (p < 0.002). When cells were transfected to overexpress M-CSFR, there was a further significant increase in uptake of the 1 μM aggregates (p < 0.02). BV-2 cells also readily ingested 1-μm diameter fluorescent polystyrene microspheres. However, there was no significant difference between control cells and cells overexpressing M-CSFR in uptake of the microspheres after 24 h (Fig. 5B). Similar results were obtained after 90-min, 10-h, and 15-h incubations (data not shown). Parallel experiments performed with the same batch of cells showed a large increase in uptake of Fluo-Aβ after c-fms transfection.

M-CSFR overexpression resulted in increased phagocytosis of Fluo-Aβ 1-42 as well as Fluo-Aβ 1-40. Fig. 5C shows results from BV-2 cells treated with transfection medium, c-fms transfected, and transfected with the control vector pZeoSV. Fluo-Aβ 1-42 and Fluo Aβ 1-40 were internalized by microglia with similar efficiency, and c-fms transfection increased ingestion of both peptides (Scheffe adjusted p < 0.05). Transfection with the control vector pZeoSV that contains the complete DNA backbone of the pTK1 plasmid but lacks the c-fms cDNA did not significantly increase phagocytosis of either peptide over values from transfection medium-treated control cells.

To determine whether the M-CSFR-induced increase in Fluo-Aβ phagocytosis was dependent on MSR, BV-2 microglia were treated with the MSR ligand fucoidan prior to the addition of Fluo-Aβ. As shown in Fig. 6A, fucoidan resulted in a significant decrease in mean phagocytosis in c-fms-transfected (p < 0.05) and nontransfected (p < 0.05) cells, although uptake was not abolished. Fig. 6B shows that overexpression of M-CSFR on BV-2 microglia also resulted in a significant increase in MSR-A mRNA as determined with real-time reverse transcriptase-PCR (p < 0.03).

To demonstrate that increased Fluo-Aβ phagocytosis in c-fms-transfected microglia was dependent on interactions of the M-CSFR with its ligand, M-CSFR antibody blocking experiments were performed. As shown in Fig. 7, BV-2 microglia exposed to the M-CSFR blocking antibody showed a decrease in Aβ phagocytosis relative to cells without blocking (Scheffe adjusted p < 0.05), and relative to cells treated with normal rabbit serum (adjusted p < 0.05). There was no significant difference between untreated and serum-treated cells in Fluo-Aβ uptake.

**DISCUSSION**

These results demonstrate that overexpression of the M-CSFR on cultured mouse and human microglia results in increased phagocytosis of Aβ. Recently, there has been great interest in microglial phagocytosis of Aβ because microglia may clear Aβ from the brain in APPV717F mice after immunization (7, 8). Microglia in APPV717F mice show increased M-CSFR expression (10). Increased microglial M-CSFR expression may be a key factor in the ability of APPV717F microglia to efficiently clear Aβ from the brain after immunization.

We used a direct fluorescein conjugate of Aβ to detect microglial phagocytosis by flow cytometry and confocal microscopy. The kinetics of BV-2 mouse microglial phagocytosis of Fluo-Aβ were similar to those reported for primary microglia derived from adult human brain (3). We also observed that BV-2 mouse and SV-A3 human microglia rapidly ingest Aβ at concentrations in the range of 10 to 100 nM. These concentrations are similar to those found in APPV717F brain homogenates from
animals younger than 8 months of age (29). Experiments using nanomolar Aβ concentrations may model conditions encountered by microglia in APPV717F mice immunized beginning early in life, a treatment that largely prevents Aβ deposition (7). Immunization is also effective in decreasing, although not eliminating, Aβ deposits when initiated in older APPV717F animals. We showed that increased expression of M-CSFR is also effective in enhancing microglial phagocytosis of Aβ at the micromolar concentrations likely present in older transgenic mice. Furthermore, M-CSFR overexpression enhances microglial ingestion of the Aβ 1-40 and 1-42 species, both of which are present in AD and transgenic mice modeling AD.

MSR-A are important in microglial internalization of Aβ (4, 26). Pretreatment of BV-2 microglia with the MSR ligand fucoidean resulted in a decrease in the uptake of Fluo-Aβ by nontransfected cells, and a proportionately similar decrease in uptake by cells overexpressing M-CSFR. This indicates that the increase in phagocytosis of Aβ because of M-CSFR occurs in part through MSR. The accelerated uptake of Aβ after c-fms transfection may have been due in part to the increased MSR-A expression we detected in transfected cells. Others have also reported increased MSR-A expression after activation of the M-CSFR (30). Uptake of Aβ not blocked by fucoidean may have been because of interactions between Aβ and other microglial receptors. Although we used serum-free medium or media containing heat-inactivated serum, microglia express Clq and its receptor (31), which could also have been involved in uptake of Aβ (26). These results suggest that increased M-CSFR expression activates multiple pathways involved in the internalization of Aβ.

Cytochalasin D inhibits actin polymerization and hence interferes with phagocytosis (27). We found that cytochalasin D treatment resulted in an ~70% reduction in Aβ ingestion by BV-2 cells overexpressing M-CSFR. This level of Aβ phagocytosis inhibition is similar to that observed with cytochalasin D for primary rodent microglia (26). These results, along with those showing low cell surface binding and confocal images showing Fluo-Aβ internalization, demonstrate that M-CSFR-induced internalization is phagocytic.

BV-2 cells have been shown to avidly ingest 1-μm fluorescent microspheres by phagocytosis (22, 32). Surprisingly, uptake of microspheres was not enhanced by M-CSFR overexpression. This could mean that overexpression of M-CSFR results in preferential uptake of Aβ aggregates. However, additional phagocytic substrates such as Candida albicans, Escherichia coli, myelin, and zymosan will need to be tested to determine whether M-CSFR overexpression results in Aβ-specific uptake.

Increased Aβ phagocytosis in microglia overexpressing M-CSFR is dependent on interaction with its ligand, M-CSF, as shown by antibody blocking of the extracellular domain of the M-CSFR on BV-2 cells. This is of significance in that M-CSF levels are up-regulated in AD brain (18), and hence could augment increased phagocytosis induced by increased M-CSFR expression. Furthermore, Aβ increases expression of M-CSF by microglia (33) as does M-CSFR overexpression (19). Antibody blocking of the M-CSFR did not completely abolish the increase in phagocytosis induced by c-fms transfection. The very strong increase in M-CSFR induced by c-fms transfection in BV-2 cells (19) most likely resulted in continual recruitment of new receptors to the cell surface, making complete blocking difficult. Furthermore, there is evidence for intracytoplasmic autocrine interactions between M-CSF and the M-CSFR (34). These autocrine interactions, which could also occur in microglia overexpressing c-fms in AD, would not be affected by extracellular antibody blocking experiments.

Microglia have been implicated in clearance of Aβ in transgenic mice after immunization (7, 8). However, a competing hypothesis holds that circulating Aβ antibodies do not enter the brain but rather form a “sink” that results in net transport of Aβ from the central nervous system to the circulation (9). If microglia do play a significant role in Aβ clearance after immunization, anti-Aβ antibody levels in the brain are likely to be very low. Our data suggest that microglia in AD and in APPV717F mice that overexpress M-CSFR are likely to be “primed” to rapidly ingest Aβ. This may explain why amounts of antibody entering the brain after vaccination can have a major effect on Aβ clearance.

We did not determine the fate of Aβ taken up by microglial cells overexpressing M-CSFR. Aβ taken up by cultured microglia could be degraded, remain intact intracellularly (35), or be processed and released in a form with a modified propensity for aggregation. In APPV717F mice, whatever the fate of Aβ internalized by microglia, the area occupied by extracellular aggregated Aβ decreases after Aβ immunization (7). Aβ immunization clinical trials in humans were recently halted because of a “central nervous system inflammatory state.” Although the nature of this reaction is unclear, it is conceivable that in AD patients increased activation of microglia near Aβ deposits after immunization could contribute to an inflammatory meningitis or encephalitis. We are currently examining the effects of Aβ on cultured microglia overexpressing M-CSFR, because these may differ from the effects previously reported for nontransfected microglia (24).

In summary, overexpression of the M-CSFR by microglia resulted in enhanced phagocytosis of Aβ, but not of polystyrene microspheres. M-CSFR-induced phagocytosis of Aβ occurred at a wide range of peptide concentrations, and both Aβ 1-40 and 1-42 were ingested with equal avidity. These effects were dependent on interactions between the M-CSFR and its ligand, and in part on MSR. The role of microglia in clearance of Aβ after immunization of APPV717F mice has been questioned because of the low Aβ antibody titers likely to exist in the brain (9). However, our results suggest that microglia in APPV717F mice that overexpress M-CSFR should show aggressive uptake of Aβ even at low intracerebral antibody titers. Experiments are in progress to determine the effects of M-CSFR overexpression on microglial uptake of opsonized Aβ. If opsonization results in a further enhancement of Aβ uptake by cultured microglia overexpressing M-CSFR, then it is unlikely that high antibody concentrations are required for clearance of Aβ by microglia after immunization of APPV717F mice.

Acknowledgments—We thank Dr. Rajeshwar Rao Tekmal, Emory University School of Medicine, for providing pTK1 c-fms expression plasmid; Dr. Robert Nelson, Pfizer Central Research, Groton, CT, for providing human SV-A3 microglia, and Grace Perez, Clara Poon, and Feifei Zhao for technical assistance. We also thank Mark Gilbert, Dr. David Parks, and the staff of the Stanford Shared Cell Sorting Facility for assistance with flow cytometry and data analysis, and Dr. Robert Malenka and the Nancy Pritzker laboratory for assistance with confocal microscopy.

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Accelerated Phagocytosis of Amyloid-β by Mouse and Human Microglia Overexpressing the Macrophage Colony-stimulating Factor Receptor
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doi: 10.1074/jbc.M200868200 originally published online May 24, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M200868200

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