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CD14 Receptor-Mediated Uptake of Nonopsonized *Mycobacterium tuberculosis* by Human Microglia

PHILLIP K. PETERSON,[1,2]* GENYA GEKKER,[1] SHUXIAN HU,[1] WEN S. SHENG,[1] W. ROBERT ANDERSON,[3] RICHARD J. ULEVITCH,[4] PETER S. TOBIAS,[4] KRISTEN V. GUSTAFSON,[5] THOMAS W. MOLITOR,[5] AND CHUN C. CHAO[1,2]

Departments of Medicine[1] and Pathology,[3] Hennepin County Medical Center, Minneapolis, Minnesota 55415; University of Minnesota Medical School, Minneapolis, Minnesota 55455[2]; Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037[4]; and College of Veterinary Medicine, University of Minnesota, St. Paul, Minnesota 55108[5]

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This study was carried out to determine the role of CD14 receptors in the uptake of nonopsonized *Mycobacterium tuberculosis* by human microglia. Treatment of microglial cell cultures with antibodies to CD14 or with soluble CD14 significantly blocked infection by *M. tuberculosis* H37Rv, suggesting that CD14 receptors could facilitate entry of nonopsonized tubercle bacilli into macrophages within the brain.

Infection of the central nervous system is one of the most lethal forms of tuberculosis, especially in children (9). In tuberculous meningitis, bacilli have been shown to gain access to the subarachnoid space via rupture of adjacent tuberculomata within the parenchyma of the brain (9, 13) rather than by hematogenous spread, which occurs in other forms of bacterial meningitis. However, the type of cells involved in infection of the brain by *Mycobacterium tuberculosis* is unknown. Infection of mononuclear phagocytes is generally regarded as a critical step in the pathogenesis of *M. tuberculosis* (5, 14). Several groups of investigators recently have characterized the receptors on blood monocytes and in certain populations of tissue macrophages which are involved in complement-mediated or opsonin-independent uptake of *M. tuberculosis* (7, 16–18). In tissues in which complement levels are likely to be low, such as brain tissue, nonopsonic recognition of mycobacteria by macrophages may be particularly important. Little is known, however, about the mechanism of recognition of *M. tuberculosis* by microglial cells, the resident macrophages of the brain. On the basis of studies showing that human microglial cells release tumor necrosis factor alpha in response to lipopolysaccharide (LPS) (11) and the demonstration that lipoarabinomannan, a phosphatidylinositol-anchored lipoglycan of the *M. tuberculosis* cell wall, stimulates tumor necrosis factor alpha production by mononuclear phagocytes through an interaction with CD14 membrane receptors (23), we tested the hypothesis that CD14 receptors on human microglial cells facilitate the uptake of nonopsonized *M. tuberculosis.*

Microglial cell uptake of *M. tuberculosis.* An initial series of experiments was carried out to characterize the uptake of nonopsonized *M. tuberculosis* by human fetal microglial cells. The virulent strain of *M. tuberculosis* H37Rv (ATCC 25618) was cultured and prepared for experiments by previously described methods (10) and by adding Tween 80 to prevent bacterial clumping (22). Under protocols approved by the Human Subjects Research Committee at our institution, human fetal microglial cells were obtained from the brain tissue of 18- to 22-week abortuses as described previously (11). Briefly, microglia were first cultured for 14 days in the presence of other brain cell types and then isolated and plated overnight in culture medium (10% heat-inactivated fetal bovine serum [FBS; HyClone Laboratories, Logan, Utah] in Dulbecco's modified Eagle medium [DMEM; GIBCO Laboratories, Grand Island, N.Y.]). The cell monolayers to which tubercle bacilli were added consisted of a population in which >95% of the cells (i) were viable (assessed by their ability to exclude trypan blue dye), (ii) stained positively with CD68 antibodies (Dako, Carpinteria, Calif.), a human macrophage marker, and (iii) stained negatively for nonspecific esterase, a monocyte marker. Tubercle bacilli were then added to microglial cell cultures at a ratio of 10 bacteria to 1 microglial cell and incubated (37°C, 5% CO_2) for various times. Microglial cells were then washed three times with DMEM, fixed with 4% paraformaldehyde, and stained for *M. tuberculosis* with auramine-rhodamine (Becton Dickinson, Cockeysville, Md.). The percentage of microglial cells infected by *M. tuberculosis* was determined by counting a minimum of 100 consecutive microglial per well by fluorescence microscopy, and the total number of bacilli per 100 infected cells was enumerated by counting a minimum of 100 consecutive infected microglia. The mean ± standard error for duplicate samples was determined in each experiment.

Bacterial uptake first was assessed after 2, 6, and 18 h of incubation by using microglial cells obtained from three different fetal brain specimens. Bacterial uptake was found to be gradual over the first 6 h (about 10% of the cells were infected); however, by 18 h of incubation, approximately 50% of the microglial cells were infected and each of the infected cells contained 1 or 2 bacilli. When FBS was omitted from the culture medium, no discernible effect on the rate of uptake or the number of bacilli per 100 infected cells was observed (data not shown). The uptake of nonopsonized *M. tuberculosis* was investigated further with microglial cells obtained from 13 additional brain specimens. After 18 h of incubation in culture medium containing 10% heat-inactivated FBS, 53% ± 3% (range, 39% to 69%) of microglia were infected. To determine whether the auramine-rhodamine fluorescence microscopy technique for assessing microglial cell uptake of these bacilli measured adherent or internalized bacteria, studies using

* Corresponding author. Mailing address: Department of Medicine, Hennepin County Medical Center, 701 Park Ave., Minneapolis, MN 55415. Phone: (612) 347-2877. Fax: (612) 347-2020.

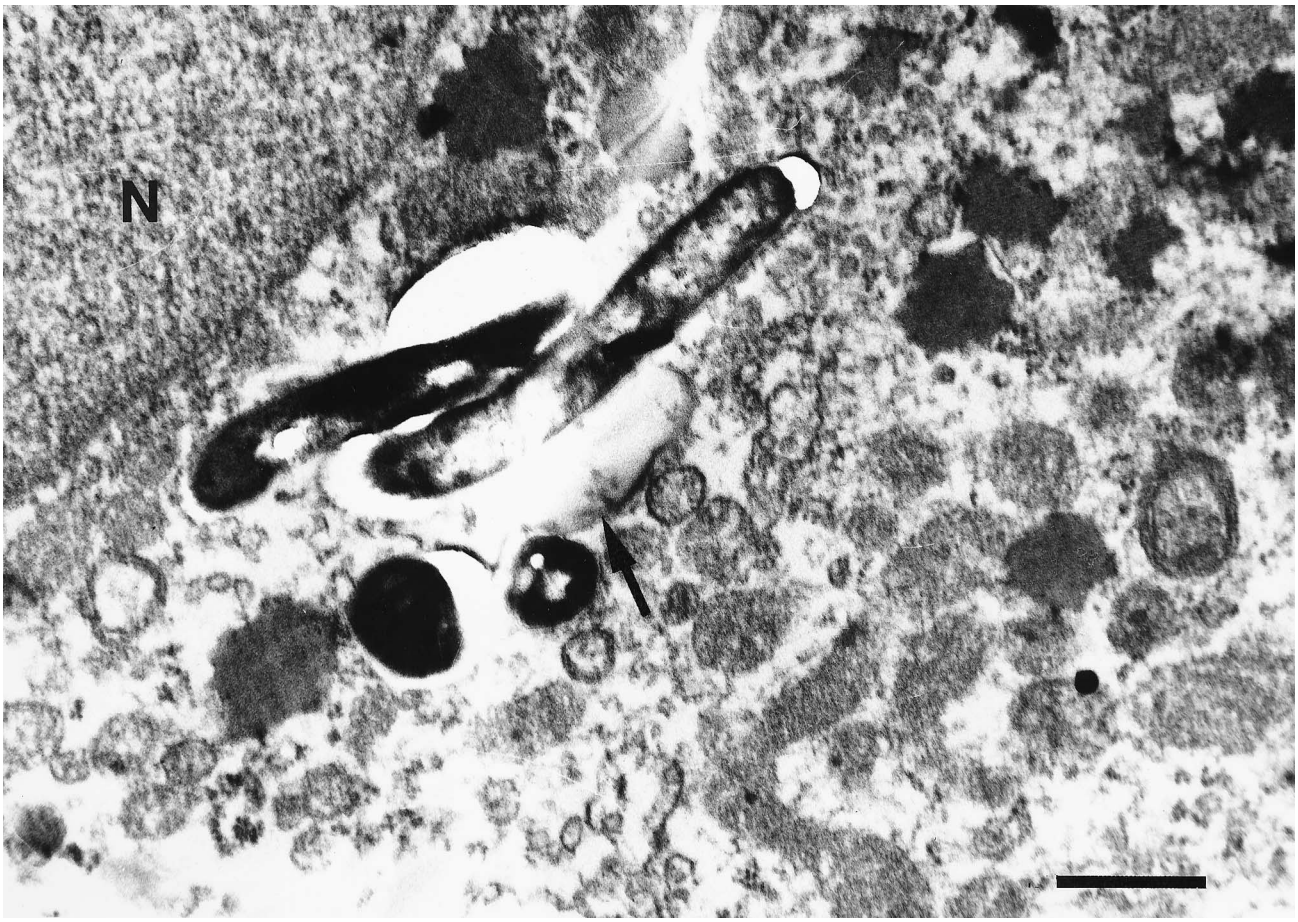

FIG. 1. Electron micrograph of a fetal microglial cell demonstrating tubercle bacilli within a cytoplasmic vacuole (arrow). Bacteria were added to microglial cell cultures containing 10% heat-inactivated FBS, and the disposition of bacilli was assessed by transmission electron microscopy after 18 h of incubation. N, cell nucleus. Bar, 0.56 μm.

transmission electron microscopy were performed by standardized techniques. After 18 h of incubation of bacilli with fetal microglial cells, transmission electron microscopy studies revealed that all bacilli were located intracellularly within vacuoles (Fig. 1). Thus, at 18 h of incubation of *M. tuberculosis* in microglial cell cultures containing 10% heat-inactivated FBS, fluorescence microscopy appeared to identify bacilli that had been internalized.

Involvement of CD14 receptors. To test the hypothesis that CD14 receptors on microglial cells are involved in the uptake of nonopsonized *M. tuberculosis*, the inhibitory effect of monoclonal antibodies specific for CD14 receptors was evaluated. Anti-CD14 antibodies (MY4, mouse immunoglobulin G2b [IgG2b]; Coulter Cytometry, Hialeah, Fla.) were added to microglial cell cultures containing 10% heat-inactivated FBS, and uptake was determined after 18 h of incubation. For comparison, monoclonal antibodies to the complement receptors CR3 (CD11b [Leu-15], mouse IgG2a; Becton Dickinson, Mountain View, Calif.) and CR4 (CD11c [Leu-M5], mouse IgG2b; Becton Dickinson) also were studied. Anti-CD14 antibodies (5 μg/ml) inhibited the uptake of *M. tuberculosis* H37Rv by 64% ± 7%, whereas neither anti-CR3 nor anti-CR4 antibodies had any effect on microglial cell uptake of nonopsonized bacilli (Fig. 2A). Treatment of microglial cells with each of these antibody preparations for 18 h had no effect on cell viability (assessed by trypan blue dye exclusion). No further inhibition of bacterial uptake was observed when a higher concentration of anti-CD14 antibodies (20 μg/ml) was used, and the inhibitory activity of anti-CD14 antibodies was lost at antibody concentrations of <1.0 μg/ml (data not shown). Also, the uptake of sheep erythrocytes with rabbit IgG antibodies was found to be unaffected by anti-CD14 antibodies (50.5% ± 1% of microglial cells incubated in culture medium containing MY4 antibodies [5 μg/ml] or in culture medium alone [control] contained sheep erythrocytes with rabbit IgG antibodies after 1 h of incubation at a sheep-erythrocyte-to-microglial-cell ratio of 100:1), indicating that the inhibitory effect of anti-CD14 antibodies on the uptake of nonopsonized *M. tuberculosis* was not nonspecific. Taken together, these findings suggest that CD14 receptors play a major role in microglial cell uptake of nonopsonized *M. tuberculosis* H37Rv.

To determine the percentage of human fetal microglial cells which express CD14, CR3, and CR4 receptors, microglial cells were treated with the respective monoclonal antibodies and analyzed by fluorescent-activated cell sorting (FACS). For this experiment, microglial cells were aliquoted at 3×10^5 cells per Teflon-coated tube in 0.5 ml of RPMI medium. Specific monoclonal antibodies and their isotype controls (5 μl of antibody [1 μg/μl] in 200 μl of buffer) were added to cells at 4°C for 35 min, washed twice, fixed with 1% paraformaldehyde, and studied with a Becton Dickinson FacSTAR Plus. While 92.6% ± 1.8% and 81.6% ± 1% of the cells stained positively with

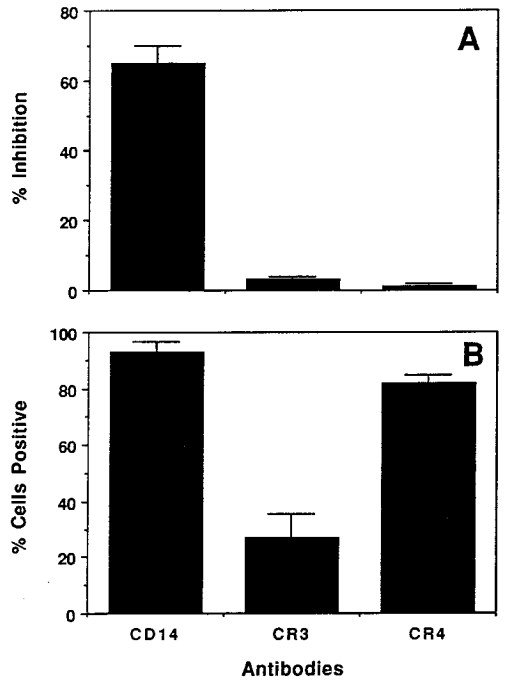

FIG. 2. Interaction of monoclonal antibodies with specific cell membrane receptors on microglial cells. (A) Effect on uptake of *M. tuberculosis*; (B) effect on binding to microglial cells. (A) Human fetal microglial cells were incubated in culture medium containing 10% heat-inactivated FBS in the absence (control) or presence of anti-CD14 (5 μg/ml), anti-CR3 (10 μg/ml), or anti-CR4 (10 μg/ml) monoclonal antibodies. After 18 h of incubation, bacterial uptake (percentage of microglia infected) was determined. Data are expressed as percent inhibition of uptake by antibodies relative to the corresponding control value and are means ± standard errors for three to five separate experiments. (B) Microglial cells were treated with the indicated monoclonal antibodies, and the percentage of cells that stained positively was determined by FACS. Less than 1% of cells stained positively with isotype control antibodies. Data are means ± standard errors for three separate experiments with microglia from different brain specimens.

monoclonal antibodies to CD14 and CR4 receptors, respectively, only 27.1% ± 8.5% of microglial cells stained positively with anti-CR3 antibodies (Fig. 2B). Thus, although a large majority of fetal microglial cells express both CD14 and CR4 receptors, only CD14 receptors appear to participate in the uptake of nonopsonized *M. tuberculosis* H37Rv.

The finding that uptake of nonopsonized *M. tuberculosis* was blocked exclusively by antibodies specific to CD14 receptors does not exclude the participation of CR4 or CR3 receptors in the uptake of bacilli opsonized with complement, nor does this finding rule out the possibility that complement facilitates the uptake of bacilli via CD14 receptors. Indeed, the relatively low rate of uptake of nonopsonized *M. tuberculosis* observed in the present study, in which complement was omitted from the culture medium, contrasts with the rapid rate of phagocytosis that has been reported for other types of macrophages (7, 17), suggesting either that there is a lack of such a contribution of complement in our studies or that the phagocytic capacity of microglial cells is intrinsically different from that of macrophages recovered from other body sites. Since we previously have shown that nonopsonized *Toxoplasma gondii* is taken up relatively rapidly by human fetal microglial cells, i.e., about 40% of the cells harbor tachyzoites after only 1 h of incubation (3), it appears that the rate of phagocytosis is also influenced by the type of target presented to these cells.

To evaluate whether microglial cells constitutively express

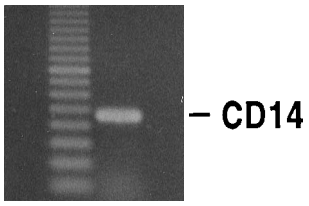

FIG. 3. Constitutive expression of CD14 receptor mRNA by microglia. Total RNA was harvested from microglial cell cultures, and reverse transcriptase PCR analysis was performed. Left lane, 100-bp ladder marker; right lane, RNA derived from microglial cell cultures. Results are representative of three experiments.

CD14 receptor mRNA, reverse transcriptase PCR was performed with primer sets derived from the human CD14 receptor gene (sense set, 5′-TTCCAGTGTGTGTCTGCAGTAGAGG-3′; antisense set, 5′-TCCAGGATTGTCAGACAGGTCTAGG-3′). Total RNA was isolated, as previously described (2). Reverse transcription of 1 μg of RNA was performed with oligo(dT)$_{12–18}$ primer (Clontech, Palo Alto, Calif.). The reaction mixture was incubated at 42°C for 60 min, and then the reaction was terminated at 95°C for 5 min in a programmable Tempcycler (Coy Corp., Ann Arbor, Mich.). The cDNA was subjected to 35 amplification cycles, with each cycle consisting of 94°C for 45 s, 65°C for 45 s, and 72°C for 90 s. A 10-μl aliquot of PCR product was loaded in 1.5% agarose gel for electrophoresis, and the amplified DNA fragments were visualized with ethidium bromide stain. The size of the DNA fragment for CD14 receptor is 485 bp. Reverse transcriptase PCR analysis demonstrated a single transcript of 474 bp, which corresponds to the predicted size (Fig. 3).

To investigate further the involvement of CD14 receptors in the opsonin-independent uptake of *M. tuberculosis*, soluble CD14 was added to microglial cell cultures containing serum-free DMEM. Soluble CD14 was obtained from cell-free supernatants of CHO-K1 cells stably transfected with DNA encoding human CD14 (8) and was purified as described previously (12). At 18 h of incubation, soluble CD14 (20 μg/ml) significantly blocked infection of microglial cells (62% ± 6% inhibition, $P < 0.01$ [results from three separate experiments]). Concentrations of soluble CD14 greater than 20 μg/ml were no more inhibitory, and the inhibitory effect of soluble CD14 was lost at concentrations below 1.0 μg/ml (Fig. 4A). Next, we investigated whether LPS-binding protein (LBP), a glycopeptide which forms complexes with gram-negative bacterial LPS and markedly facilitates its interaction with CD14 (21), would potentiate the uptake of *M. tuberculosis*. LBP was purified from acute-phase rabbit serum as described previously (20). For this experiment, bacilli were added to microglial cell cultures that contained DMEM supplemented with 1% human albumin to keep the LBP dispersed in the culture medium, and uptake was measured at 6 h. At a concentration of 5 μg/ml, LBP was found to potentiate the uptake of bacilli (23% ± 0.7% of microglia infected versus 12% ± 0.7% of control cells, $P < 0.01$ [results from two separate experiments]) (Fig. 4B). Thus, LBP appears to enhance *M. tuberculosis* recognition by microglial cells; however, this serum protein is not required as an opsonin since initial experiments demonstrated that FBS was not necessary in the culture medium for bacterial uptake.

Human microglial cells have been shown previously to express CR3 and CR4 receptors (2); however, previous immunohistological analysis of adult human brain tissue obtained at autopsy failed to identify CD14 on microglial cells (6). The reason for the discrepancy between the findings in this earlier

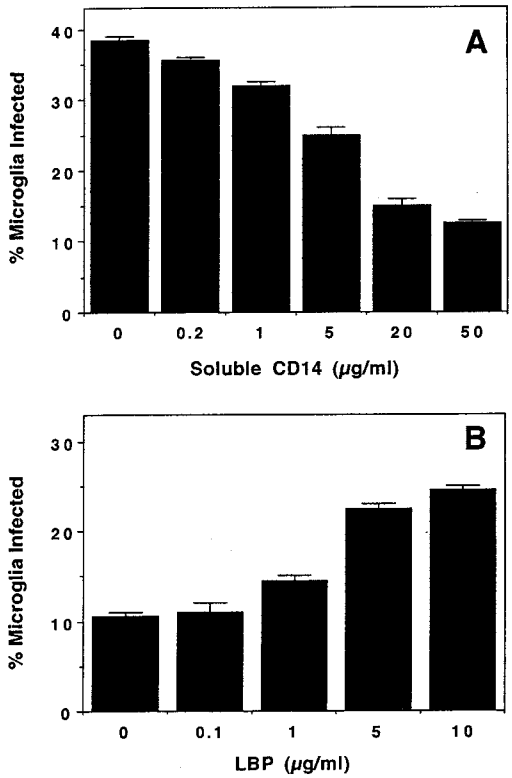

FIG. 4. Effect of soluble CD14 (A) and LBP (B) on microglial cell uptake of *M. tuberculosis*. (A) Soluble CD14 was added, at the indicated concentrations, to microglial cell cultures containing DMEM, and the percentage of microglial cells infected was measured after 18 h of incubation. Data are means ± standard errors of duplicate values and are representative of three separate experiments. (B) LBP was added, at the indicated concentrations, to microglial cell cultures containing DMEM supplemented with 1% human albumin, and uptake was measured after 6 h of incubation. Data are means ± standard errors of duplicate values and are representative of two separate experiments.

study and the findings in the present study is unknown. The use of anti-CD14 monoclonal antibodies which recognize different epitopes (Mo-2 antibody used previously [6] versus the broadly reactive MY4 monoclonal antibody in the present study) could be one explanation. In studying CD14 expression in cells of the monocytic lineage, it has been recommended that antibodies with broad reactivities for multiple epitopes, such as MY4 monoclonal antibodies, be used (24). Since our studies were carried out with microglial cells obtained from fetal brain specimens, it is also possible that CD14 receptors are expressed only in the early stages of brain development and that microglial cells from adult brain tissue lack this receptor. To test this possibility, the effect of anti-CD14 monoclonal antibodies on uptake of *M. tuberculosis* H37Rv was studied with microglial cells that had been isolated from a postmortem brain specimen of a 79-year-old adult by using techniques previously described (11). After 18 h of incubation in 10% heat-inactivated FBS, 85% ± 7% of the control microglial cells were infected by *M. tuberculosis*, compared with only 34% ± 2.8% of the microglial cells cultured in medium containing anti-CD14 antibodies (5 μg/ml) (i.e., there was 60% inhibition of uptake by treatment with anti-CD14 antibodies). Also, anti-CD14 antibodies reduced the total number of bacilli associated with 100 cells (447 ± 19 versus 126 ± 9 bacilli per 100 infected microglial cells in control and anti-CD14 antibody-treated cell cultures, respectively). Finally, immunocytochemical studies with this adult brain specimen revealed that >80% of microglial cells stained positively with MY4 anti-CD14 monoclonal antibodies, whereas only <2% of cells stained positively with isotypic control antibodies.

While the immunopathogenesis of tuberculosis is complex and involves many types of immune cells and mediators, infection of mononuclear phagocytes appears to be critical, since it is only in these cells that *M. tuberculosis* appears to grow in vivo (15). The findings in the present study suggest that microglial cells may play a pivotal role in the pathogenesis of *M. tuberculosis* infection in the central nervous system and that CD14 receptor-mediated uptake may be an important mechanism of infection of these cells by nonopsonized tubercle bacilli. Since monoclonal antibodies to this receptor and soluble CD14 inhibited microglial cell uptake by only about 60%, it appears that microglia also internalize nonopsonized bacilli via other receptors, such as mannose receptors (16) or CR3 receptors of the type described by Stokes et al. (19), which have been identified on other macrophage populations. Although the rationale underlying the hypothesis that CD14 receptors are involved in the uptake of *M. tuberculosis* by human microglial cells was based upon the demonstration by other investigators (23) that the *M. tuberculosis* cell wall lipoglycan lipoarabinomannan stimulates the release of tumor necrosis factor alpha from mononuclear phagocytes, it is unknown whether lipoarabinomannan serves as the bacterial ligand for uptake via CD14 receptors on microglial cells. Mannose-capped lipoarabinomannan recently has been shown to function as the ligand which mediates uptake via mannose receptors on human blood monocyte-derived macrophages (18). Additional studies are necessary to determine whether nonopsonized *M. tuberculosis* is also internalized by receptors other than CD14 and to ascertain the fate of tubercle bacilli that enter microglial cells via the CD14 receptor route. Also, although the normal brain parenchyma is likely to be devoid of opsonins, the effect of complement on phagocytosis of tubercle bacilli by microglial cells should be investigated, since activated microglia are capable of synthesizing complement components (1) and since complement-mediated opsonic activity for other bacterial species has been demonstrated in cerebrospinal fluid from patients with acute bacterial meningitis (25).

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