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Binding of *Mycobacterium avium-Mycobacterium intracellulare* to Human Leukocytes

ANTONINO CATANZARO[1]* AND SAMUEL D. WRIGHT[2]

Department of Medicine, Division of Pulmonary and Critical Care Medicine, University of California, San Diego, San Diego, California 92103,[1] and Laboratory of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021[2]

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We examined nonopsonic binding of *Mycobacterium avium-Mycobacterium intracellulare* (MAI) by human leukocytes. Macrophages (Mϕ) avidly bound fluorescently labeled MAI in the absence of serum proteins. Binding appeared to be mediated by a lineage-specific, proteinaceous receptor on Mϕ, since (i) binding of labeled bacteria could be competitively inhibited by unlabeled MAI, (ii) treatment of Mϕ with trypsin ablated the ability of Mϕ to bind MAI, and (iii) the capacity to bind MAI was observed on monocytes, Mϕ, and stimulated polymorphonuclear cells but not on lymphocytes or unstimulated polymorphonuclear cells. The receptor for MAI appeared mobile in the plane of the membrane, since spreading of Mϕ on a carpet of immobilized, unlabeled MAI down modulated binding of labeled MAI added in suspension. The receptor required neither calcium nor magnesium for activity and appeared different from other known receptors for intracellular pathogens.

The soil and water in large regions of the United States are contaminated with *Mycobacterium avium-Mycobacterium intracellulare* (MAI) (1). Epidemiologic studies in these regions by using delayed-type hypersensitivity to antigens derived from MAI suggest that infection with MAI is very common (15). Despite repeated contact with this organism, clinical infection among the general population is very infrequent (15). However, when host defenses are altered as in patients with chronic lung diseases (10) or under conditions that depress cell-mediated immune responses, the balance shifts and chronic or disseminated (16) disease develops. This is particularly the case for acquired immunodeficiency syndrome (AIDS), where overwhelming infection with MAI is found in more than half of the victims (7, 25).

MAI is an intracellular pathogen that resides principally in the macrophage (Mϕ), as is apparent from the examination of infected tissues of both AIDS and non-AIDS patients (24). In vitro studies demonstrate that Mϕ provide an excellent environment for replication of MAI (3, 5, 9). An intracellular location may help MAI evade host defense mechanisms. Here we examine the earliest interaction between MAI and Mϕ, the binding of unopsonized bacteria. We show that MAI interact with a protease-sensitive receptor restricted to phagocytes. The receptor is mobile in the plane of the membrane, does not require divalent cations, and mediates very rapid attachment.

MATERIALS AND METHODS

Reagents. Reagents were obtained from the following sources: phorbol myristate acetate (PMA), fluorescein isothiocyanate, Tris, and poly-L-lysine, Sigma Chemical Co. (St. Louis, Mo.); trypsin, Millipore Corp. (Freehold, N.J.); Dubos base and Middlebrook 7H10, Difco Laboratories (Detroit, Mich.); Tween 20, Bio-Rad Laboratories (Richmond, Calif.); and neuraminidase (*Vibrio cholerae*), Boehringer Diagnostics (La Jolla, Calif.). Terasaki plates were purchased from Miles Scientific, Div. Miles Laboratories Inc. (Naperville, Ill.). Sheep erythrocytes were from blood collected from sheep kept at the Laboratory Animal Research Center of the Rockefeller University. HAP buffer consists of Dulbecco phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 8 mM phosphate, pH 7.4) containing 3 mM glucose, 0.5 mg of human serum albumin (Armor Pharmaceuticals, Kankakee, Ill.) per ml, and 0.3 U of aprotinin (Sigma) per ml. PD refers to PBS without calcium or magnesium.

Growth and labeling of mycobacteria. MAI 13528-1079 var. 4 was provided by Anna Tsang, National Jewish Hospital, Denver, Colo. Isolates were cultured in Dubos broth containing 1% Tween at 37°C in 5% carbon dioxide for 7 to 14 days. Once each day, cultures were vigorously agitated by Vortex mixing. MAI were harvested by centrifugation at 2,000 rpm for 10 min. Cultures that were to be used without label were suspended in PBS. Cultures that were to be labeled with fluorescein were suspended in 1 mg of fluorescein isothiocyanate per ml–50 mM sodium carbonate–100 mM sodium chloride, pH 9.2, incubated at room temperature for 30 min, and then washed three times in PBS. The final suspension was sonicated for 4 s, and the optical density at 600 nm was determined. Colony counts were performed by plating 15 μl of serial 1:10 dilutions on Middlebrook 7H10 and culturing as described above. A suspension of mycobacteria with an optical density at 600 nm of 0.2 yielded a colony count of 5×10^9. Viability was not altered by the fluoresceination procedure.

Phagocytes. Human buffy coats were obtained from the New York or San Diego Blood Bank. Monocytes were isolated by sequential Ficoll-Hypaque gradient and Percoll gradient (23). For the experiments shown in Fig. 1, 2, 3, and 5, contaminating T lymphocytes were further depleted by rosetting, using neuraminidase-treated sheep erythrocytes (SRBC). SRBC previously incubated at 37°C in neuraminidase were incubated with monocytes, and the resulting

* Corresponding author.

rosetted T lymphocytes were removed by centrifugation on a cushion of Ficoll-Hypaque. Monocytes were suspended in 12.5% human serum in RPMI 1640 and cultured for 4 to 10 days in Teflon beakers. These cells are hereafter referred to as Mϕ. Polymorphonuclear (PMN) cells were prepared by the method of English and Anderson (4) and used the same day.

Assay for attachment of MAI to phagocytes. Mϕ harvested from Teflon beakers after 4 to 7 days were washed and suspended at 0.5×10^6 to 1.0×10^6/ml in HAP. A 5-μl sample of Mϕ suspension was added to each well of a Terasaki plate, and cells were allowed to spread for 45 min at 37°C. In other experiments, monocytes or PMN were collected from fresh blood as described above and plated in a similar manner. Monocytes were used at 10^6/ml, and PMN were used at 2×10^6/ml. Monolayers were washed with PBS, and MAI were added at a ratio of approximately 1,000 per Mϕ. These conditions allow a synchronous, heavy challenge to the Mϕ, and binding under these conditions reflects the ability of Mϕ to bind bacteria, not the availability of bacteria. Plates were then incubated for 5 to 90 min at 37 or 4°C. Wells were washed vigorously three times with copious amounts of PBS and fixed with 2.5% glutaraldehyde. The attachment of MAI was scored by fluorescence microscopy. Phagocytes in a field were identified and counted, the light was reduced, and UV light turned on. The number of phagocytes with fluorescent MAI was determined. The percent phagocytes with at least one MAI attached was enumerated. In addition, the number of MAI that were cell associated was counted. Attachment index (AI) is reported as the number of MAI that were bound per 100 phagocytes. Since each Mϕ bound more than 30 bacteria, enumeration was difficult. In all experiments shown, enumeration was facilitated by adding a mixture of labeled and unlabeled bacteria in a ratio of 1:7. The AI determined for fluorescently labeled MAI was then multiplied by the ratio of labeled to unlabeled bacteria to yield the true AI. In some experiments, monolayers of Mϕ were formed on a carpet of immobilized MAI. Plastic wells were first treated with 1 mg of poly-L-lysine per ml for 30 min and washed. Various concentrations of MAI were added and allowed to adhere for 30 min. Microscopic examination of the washed wells revealed an even coating of bacteria at various densities. Mϕ were added, and monolayers were established on these surfaces as described above.

An alternative assay for attachment of MAI to Mϕ used cells in suspension. A 100-μl sample of Mϕ (10^6/ml) was mixed with 100 μl of labeled MAI an optical density of 0.2 in a plastic tube (12 by 75 mm) and incubated for 20 min at 4°C on a rotating platform. Cells were washed twice (1,000 rpm, 5 min) to remove the bulk of unattached MAI, and Mϕ were analyzed in a FACScan flow cytometer (Becton Dickinson and Co., Oxnard, Calif.).

RESULTS

Unopsonized MAI bind to Mϕ. MAI were labeled, added to monolayers of Mϕ, and incubated at 37°C in the absence of serum. Microscopic observation showed that Mϕ bound more than 30 MAI during a 45-min incubation. This observation was confirmed by using a suspension assay of binding (data not shown). Essentially all Mϕ bound MAI, suggesting that the capacity to recognize these bacteria was shared by all members of the Mϕ population. Some images showed MAI bound to the cell periphery, but others showed the MAI gathered in a perinuclear region, suggesting that phagocytosis had occurred. Since the intracellular or extracellular location of MAI could not be determined with certainty, we counted all cell-associated organisms and report the data as AI.

To reduce the number of labeled MAI per Mϕ to a level that could be easily enumerated, a lower number of labeled MAI were mixed with a compensating number of unlabeled MAI before presentation to the Mϕ (Fig. 1). This approach

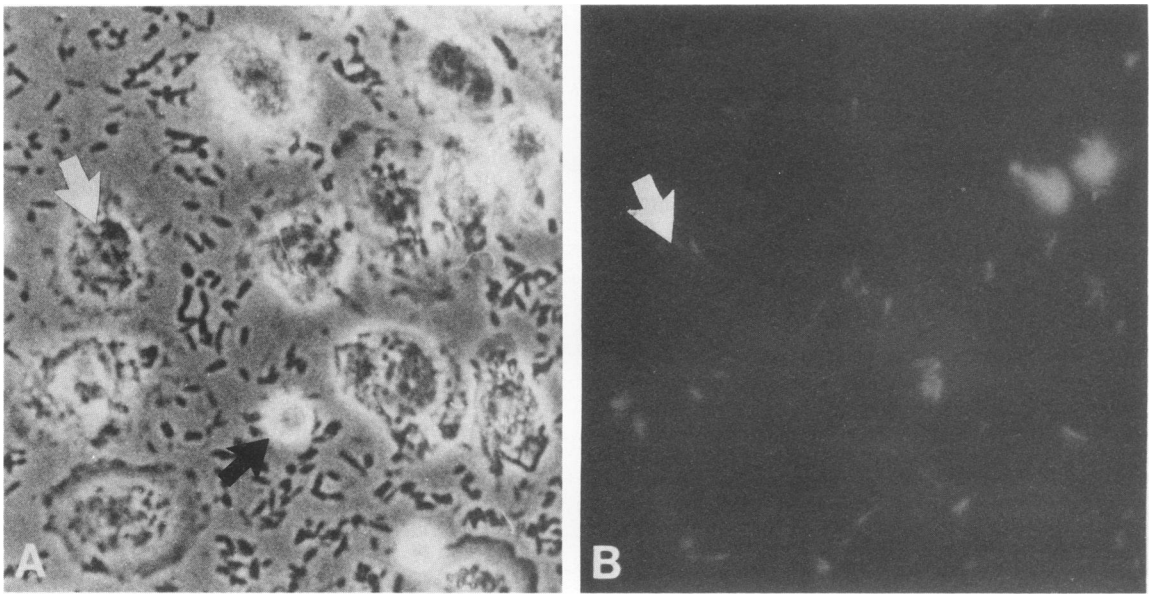

FIG. 1. Photomicrograph of an Mϕ monolayer incubated with MAI. A monolayer of Mϕ was established, and a mixture of labeled and nonlabeled MAI was added for 30 min at 37°C. Each panel shows the same field under phase-contrast illumination (A) or epillumination for fluorescence (B). The white arrow points to three labeled MAI that appear to have been ingested. Lymphocytes in the field did not bind MAI (black arrow).

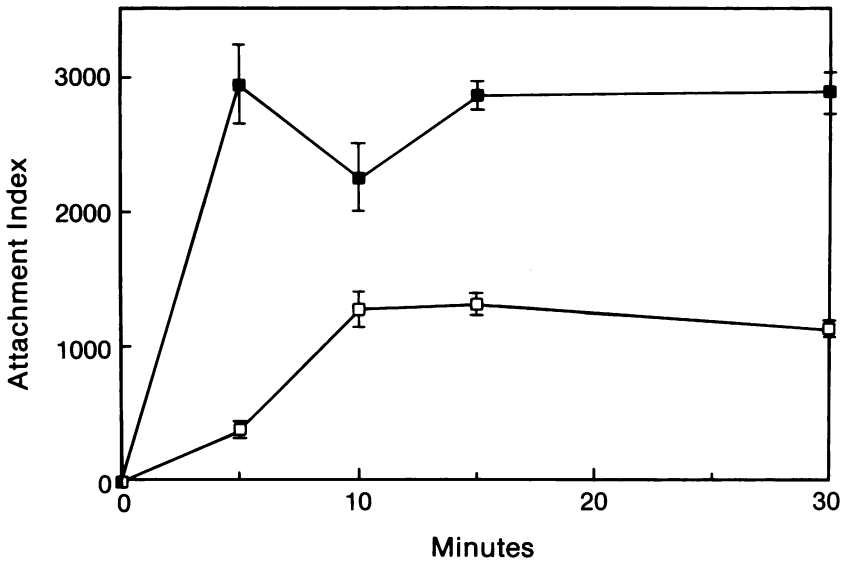

FIG. 2. Time course of binding MAI to Mϕ. Monolayers of Mϕ were established, and labeled MAI were added. After incubation of the plates at 4°C (□) or 37°C (■) for the times indicated, AI was measured. Each point is the mean ± standard deviation of four experiments. Cells held at 4°C showed no indication of phagocytosis, but those held at 37°C appeared to ingest many MAI (see text).

lowered the number of labeled bacteria within Mϕ without altering the frequency of contact between phagocyte and bacteria. Labeled and unlabeled bacteria appeared to interact with Mϕ by the same mechanism, since the binding of a fixed concentration of labeled bacteria was competitively inhibited by the addition of unlabeled bacteria (data not shown).

Binding of MAI to Mϕ occurred very rapidly at 37°C, reaching completion by 5 min (Fig. 2). Reduction of the temperature to 4°C slowed both the rate and the extent of binding about threefold. The decrease in extent of binding caused by lowered temperature may have been due to the inhibition of phagocytosis, since microscopic examination of the cultures held at 4°C showed no bacteria in a perinuclear location (data not shown). Binding of MAI at low temperature, however, was avid, and the attachment mechanism must be considered relatively temperature independent.

Unopsonized MAI are recognized by a proteinaceous receptor specific to phagocytes. Treatment of Mϕ with trypsin reduced the binding of MAI in a dose-dependent fashion (Fig. 3). This result suggests that a protein on the surface of Mϕ serves as a receptor for MAI or that the receptor is associated with a protein. To determine the cellular distribution of this receptor, we measured the binding of MAI to monolayers of monocytes, macrophages, lymphocytes, and PMN. Both monocytes and Mϕ bound MAI avidly, but neither lymphocytes nor PMN bound appreciable numbers of MAI (Fig. 4). The expression of receptors on the surface of PMN can be dramatically altered by exposure of the cells to PMA (21). We observed that PMA treatment of PMN enabled the cells to bind MAI. These data suggest that the receptor for MAI is restricted to phagocytes and is inducible on PMN.

Receptors for MAI are mobile in the plane of the membrane. When Mϕ are spread on surfaces coated with immunoglobulin G or complement, the respective receptors for these two ligands diffuse to the adherent surface of the Mϕ and are trapped by interaction with ligand. This leaves the apical surface of the phagocyte specifically depleted of Fc or complement receptors (19, 22). To determine whether MAI are recognized by a mobile receptor, Mϕ were allowed to spread on surfaces coated with MAI, and the attachment of labeled MAI to the apical surface was measured. Surface-bound MAI caused strong, dose-dependent down modulation of the binding of bacteria to the apical portion of the Mϕ (Fig. 5). These observations confirm that MAI are recognized by a specific receptor and indicate that the receptor is mobile in the plane of the membrane.

Binding of MAI does not require divalent cations. Mϕ bind unopsonized *Escherichia coli* (20), *Histoplasma capsulatum*

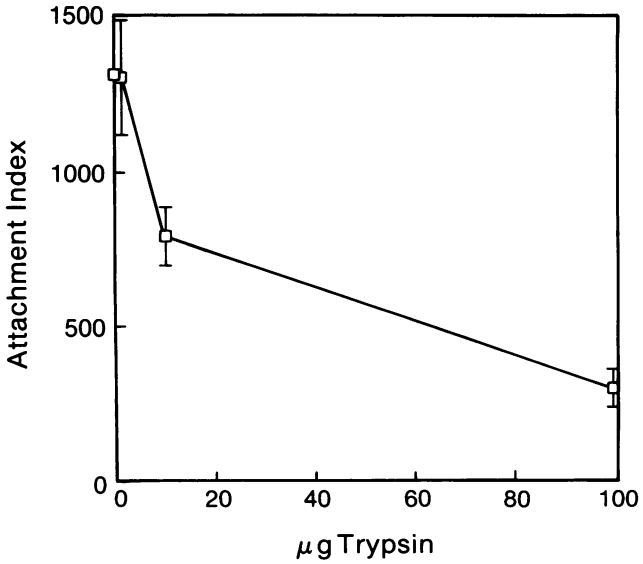

FIG. 3. Demonstration that treatment of Mϕ with trypsin inhibits binding of MAI. An established monolayer of Mϕ was treated with 0, 1, 10, or 100 μg of trypsin per ml of PBS for 15 min at 37°C. Trypsin was removed by washing with HAP, labeled MAI were added and incubated at 4°C for 20 min, and AI was determined. Trypsin inactivated by incubation with soybean trypsin inhibitor did not affect MAI binding (data not shown).

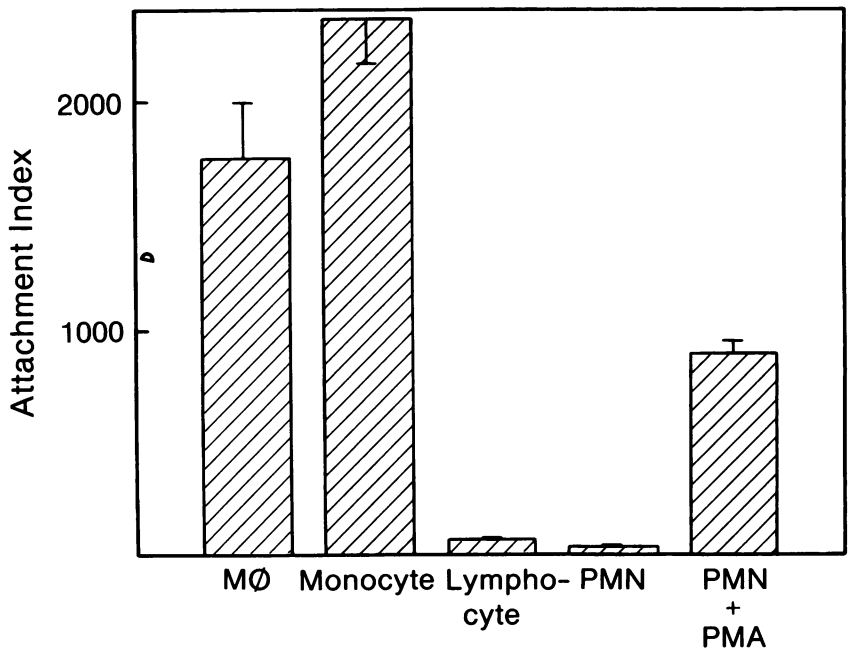

FIG. 4. Binding of MAI to leukocytes. Monolayers of monocytes, Mϕ, lymphocytes, or PMN were established as described in Materials and Methods, MAI were added and incubated at 4°C for 20 min, and AI was determined. Each bar is the mean ± standard deviation of four experiments. Monocytes and PMN were from freshly drawn blood; Mϕ were from day 5 cultures. The lymphocytes observed were contaminants in the Mϕ culture. Where indicated, PMN were stimulated with PMA (30 ng/ml) for 5 min at 37°C before assay.

(2), *Staphylococcus aureus* (6), and *Leishmania* species (11, 17) via the CD18 complex of leukocyte antigens. All known binding functions of CD18 molecules are inhibited in the absence of divalent cations. To determine whether this family of receptors plays a role in recognition of MAI, we measured binding of bacteria in the presence and absence of divalent cations.

Monolayers of Mϕ were established and washed with 1 mM EDTA in PD. Monolayers were replenished with PD or with PD and 0.5 mM $CaCl_2$ or 0.5 mM $MgCl_2$ or both. Labeled MAI were also washed in 1 mM EDTA in PD and then washed in PD before being added to Mϕ. AI was measured after incubation at 4°C for 20 min. AI in each buffer was as follows: PD with Ca^{2+} and Mg^{2+}, 3,850; PD with Ca^{2+}, 4,142; PD with Mg^{2+}, 4,378; PD alone, 3,259. AIs were comparable in each population and comparable to those obtained with cells maintained in PBS throughout the experiment (data not shown). The ability of Mϕ to bind EC3bi was used as a control to demonstrate that the conditions of the experiment functionally removed Ca^{2+} and

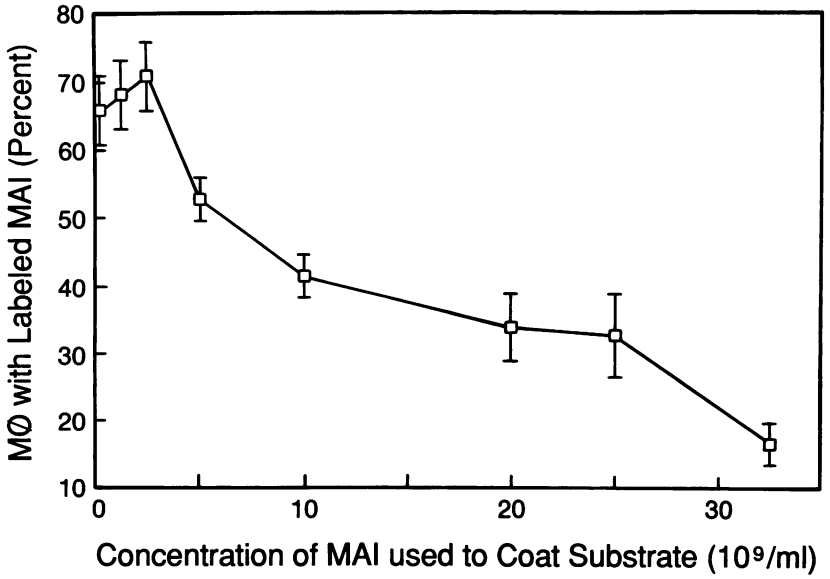

FIG. 5. Mobility of binding sites for MAI in the plane of the membrane. Increasing concentrations of MAI were attached to the culture surface, using poly-L-lysine as described in Materials and Methods, and a monolayer of Mϕ was established on the carpet of MAI. Binding of labeled MAI to the Mϕ was then measured after incubation at 4°C for 20 min.

Mg^{2+}. In these controls, Mϕ binding of EC3bi was inhibited (data not shown).

Binding of MAI was unaffected by the removal of Ca^{2+}, Mg^{2+}, or Ca^{2+} and Mg^{2+} from the incubation medium. The CD18 complex is thus unlikely to play a critical role in the recognition of unopsonized MAI by Mϕ.

DISCUSSION

MAI are intracellular parasites of macrophages, and here we show that unopsonized MAI show strong affinity for monocytes and macrophages in vitro. This property may allow rapid colonization of appropriate host cells during infection. The opsonins immunoglobulin G and complement may also serve to target MAI to Mϕ (14), but our studies suggest that opsonins are not necessary for the observed tropism of MAI.

Several observations suggest that unopsonized MAI are recognized by a specific receptor protein on Mϕ. The bacteria are not bound by lymphocytes or inactivated PMN, ruling out a nonspecific interaction with cell membranes. Binding is also abolished by trypsinization of the Mϕ, suggesting that a protein is involved in recognition. Finally, the capacity of Mϕ to bind MAI is down modulated upon spreading of cells onto immobilized carpets of MAI. By analogy with results for Fc receptor, CR1, and CR3, this finding suggests that the binding sites for MAI can diffuse in the membrane and be depleted from the apical surface by redistribution to the basal surface of the phagocyte. This observation indicates that the receptors for MAI are mobile and further confirms that bulk components of the membrane, such as phospholipids, do not serve as binding sites for MAI.

Members of the CD18 family of antigens on Mϕ serve as receptors for several intracellular parasites. However, CD18 molecules are unlikely to play a role in the binding of MAI. CD18 function is inhibited in the absence of divalent cations and 4°C (18, 20), but these conditions do not block binding of MAI. Further, depletion of CD18 molecules from the apical surface of Mϕ by spreading on anti-CD18 monoclonal antibodies does not inhibit binding of MAI (unpublished observations). The mannosyl-fucosyl receptor is also unlikely to serve as the receptor for MAI, since this receptor is not expressed on monocytes (13), a cell type that avidly binds MAI. The nature of the receptor is now under study.

Phagocytosis is controlled and modulated by receptors on the phagocyte. We have demonstrated that MAI are bound avidly to Mϕ by a receptor. The avidity of binding of MAI by Mϕ may account for the fact that histologic examination of tissue from infected individuals showed the majority of organisms to be intracellular (24). Although our studies show strong binding of MAI to Mϕ, they do not rule out the expression of binding sites on other cell types. Indeed, Mapother and Songer (8) showed that human intestinal epithelial cell monolayers bind MAI. This finding may explain the common intestinal infestation of AIDS patients with MAI (12, 16).

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