Phagocytosis of Leprosy Bacilli Is Mediated by Complement Receptors CR1 and CR3 on Human Monocytes and Complement Component C3 in Serum

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Abstract

Mycobacterium leprae, **an obligate intracellular pathogen, invades and multiplies within host mononuclear phagocytes. To understand *M. leprae* invasion better, we have investigated the role of phagocyte receptors and bacterium-bound ligands in phagocytosis of *M. leprae* by human monocytes. Complement receptors CR1 and CR3 mediate adherence and phagocytosis of *M. leprae* in nonimmune serum. Two MAbs used in combination against CR3 inhibit adherence by up to 90±3%. Two MAbs used in combination against CR1 and CR3 inhibit adherence by up to 70±1%. Single MAbs against CR1 or CR3 consistently inhibit adherence by 38–55%. In contrast, MAbs against other monocyte surface molecules, alone or in combination, do not significantly influence adherence. As studied by electron microscopy, 100% of monocyte-associated *M. leprae* are ingested in the presence of nonimmune serum and MAbs against CR3 markedly inhibit ingestion. Complement receptors CR1 and CR3 also mediate the low level of adherence observed in the absence of serum.**

Serum complement component C3 serves as a ligand on the bacterial surface in monocyte phagocytosis of *M. leprae.* Adherence of *M. leprae* to monocytes is enhanced by preopsonization (3.1±1.1-fold increase) and is markedly reduced in < 0.5% fresh serum (66±7% reduction) or heat-inactivated serum (68±3% reduction). Adherence is also markedly reduced in C3- or factor B-depleted serum; repletion with purified C3 or factor B increases adherence 4.3±0.8- and 2.6±0.2-fold, respectively. C3 is fixed to *M. leprae* by the alternative pathway of complement activation, as determined by a whole bacterial cell ELISA.

By electron microscopy, monocytes ingest *M. leprae* by conventional phagocytosis.

This study demonstrates that (*a*) human monocyte complement receptors CR1 and CR3 mediate phagocytosis of *M. leprae;* (*b*) complement component C3 on the bacterial surface serves as a ligand for complement receptors; (*c*) complement component C3 binds to *M. leprae* by the alternative pathway of complement activation; and (*d*) monocytes phagocytize *M. leprae* by conventional phagocytosis. (*J. Clin. Invest.* 1990. 85:1304–1314.) *Mycobacterium leprae* • phagocytosis • complement receptors • complement component C3 • monocytes • leprosy

Introduction

Leprosy continues to afflict millions of persons worldwide and exerts a major socioeconomic impact on developing countries. The causative agent, *Mycobacterium leprae,* is a slowly growing obligate intracellular bacterium that resides principally within host mononuclear phagocytes. The bacterium is found in enormous numbers within these phagocytes in lepromatous leprosy, the most severe form of the disease. The initial step in invasion of mononuclear phagocytes is phagocytosis, a process mediated by specific receptor–ligand interactions. Receptor–ligand interactions important to phagocytosis of *M. leprae* are not understood.

Several receptors on the surface of monocytes have been found to be important in mediating phagocytosis of intracellular parasites. Complement receptors CR1 and CR3 have been found to mediate ingestion of *Legionella pneumophila* and *Mycobacterium tuberculosis* and complement receptor CR3 has been found to mediate ingestion of *Leishmania donovani, Leishmania major,* and *Histoplasma capsulatum* (1–6). These complement receptors demonstrate multiple and overlapping ligand specificities for fragments of complement components C3 and C4. CR1, a glycoprotein with four allotypes, binds primarily C3b, but also C4b and C3bi (7). CR3 consists of two noncovalently linked glycoprotein chains (α and β) which demonstrate specificity primarily for C3bi, but also C3dg and C3d (7, 8). Other monocyte receptors involved in uptake of intracellular pathogens include the lymphocyte function associated antigen-1 (LFA-1)[1] and p150,95 antigen, which have been shown to mediate adherence of *Histoplasma capsulatum* (3).

Several molecules on the surface of intracellular pathogens have been implicated as ligands for mononuclear phagocyte receptors potentially involved in phagocytosis. These include serum-derived molecules such as complement component C3 (1, 2, 6, 9–12) and fibronectin (13, 14), and glycosylated parasite surface molecules (15–18).

Phagocytes ingest intracellular pathogens by one of two processes, as viewed by electron microscopy. Phagocyte pseudopodia coil around the organism as it is internalized (19, 20), a process called coiling phagocytosis, or phagocyte pseudopodia move circumferentially and more or less symmetrically about the organism and fuse at the distal side (21–23), a pro-

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1. *Abbreviations used in this paper:* E, erythrocyte(s); LFA-1, lymphocyte function-associated antigen 1; PD, PBS without Ca^{++} and Mg^{++} ions.

cess called conventional phagocytosis. The method by which *M. leprae* enter mononuclear phagocytes has not been studied previously.

In the present series of experiments, we have examined the receptors and ligands mediating phagocytosis of *M. leprae* by human monocytes and the method of phagocytosis. We shall demonstrate that: (*a*) complement receptors CR1 and CR3 mediate phagocytosis of *M. leprae* in the presence and absence of serum; (*b*) complement component C3 on the surface of *M. leprae* acts as a ligand for these receptors; (*c*) C3 is fixed to the surface of *M. leprae* by the alternative pathway of complement activation; and (*d*) *M. leprae* is ingested by a conventional form of phagocytosis.

Methods

Media. Dulbecco's phosphate-buffered saline with Ca^{++} and Mg^{++} ions (PBS), PBS without Ca^{++} and Mg^{++} (PD), and RPMI 1640 medium with L-glutamine (RPMI) were obtained or prepared as described previously (24, 25). RPMI medium was used alone or with 10 or 20 mM Hepes buffer (Sigma Chemical Co., St. Louis, MO), pH 7.2. No antibiotics were added to any medium in any of the experiments.

Reagents. Zymosan (Sigma Chemical Co.); laminarin (Calbiochem-Behring Corp., San Diego, CA); EDTA (Sigma Chemical Co.); EGTA (Sigma Chemical Co.). Human complement component C3 and human factor B were purchased from Cordis Laboratories, Inc., Miami, FL. Formaldehyde solution, 37% wt/wt was obtained from Fisher Scientific Co., Fair Lawn, NJ. Auramine-rhodamine stain was generously donated by George Berlin, Department of Clinical Microbiology, UCLA School of Medicine. Potassium permanganate (Mallinckrodt Inc., St. Louis, MO) solution was prepared and stored in the dark. Trypan blue (Sigma Chemical Co.) was used as a 0.4% solution.

Sera. Serum from three healthy adult volunteers who were purified protein derivative-negative and had no known exposure to leprosy patients was used in all experiments except those using C3-depleted sera. The serum was separated, filtered, stored, and handled so as to preserve complement activity (24). Heat-inactivated serum was prepared by heating serum at 56°C for 30 min and factor B- depleted serum was prepared by heating serum at 50°C for 20 min. (26). C3-depleted serum (C3 removed by affinity chromatography) was purchased from Organon Teknika Corp., West Chester, PA.

Monocytes. Mononuclear cells were isolated from heparinized blood on Ficoll-sodium diatrizoate gradients as previously described (25). The monocyte fraction was obtained by adherence and cultured in RPMI containing 10% autologous nonimmune serum for 24 h on No. 2 glass coverslips in 16-mm diam flat-bottomed wells (Linbro, Flow Laboratories, McLean, VA) as described (25, 27). Approximately 5.0×10^5 monocytes were added to each well.

In certain experiments, mononuclear cells were cultured in teflon wells (Savillex Corp., Minnetonka, MN) for 24 h as described (1). The teflon wells were then placed on ice for 30 min, and mononuclear cells were removed with a pasteur pipette and washed twice with RPMI to remove serum. The monocytes in the mononuclear cell preparation were then adhered to No. 2 glass coverslips as above.

Bacteria. Armadillo-derived *M. leprae* was kindly provided by Dr. Patrick Brennan (Colorado State University, Fort Collins, CO) through the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Contract No. AI-52582. The *M. leprae* were purified from fresh nonirradiated armadillo spleens and livers (28) and stored in buffered water with 0.1% Tween 80 at 0°C. The suspension was then diluted to the appropriate concentration for use in experiments.

Antibodies. MAbs anti-CR1, anti-CR2, Leu-15, anti-Leu-M5, anti-transferrin receptor, and anti-HLA-DR (IgG2a) were purchased from Becton Dickenson Immunocytometry Systems, Mountain View, CA. Anti-HLA-DR (IgG2b) and anti-lymphocyte function-associated antigen-1 (anti-LFA-1) were purchased from AMAC, Inc., Westbrook, ME. OKM1 was purchased from Ortho Diagnostic Systems Inc., Raritan, NJ. MAb OKM10 (29) was generously provided by Dr. Seth Rudnick, Ortho Pharmaceuticals, Raritan, NJ. MAb IB4 (30) was kindly provided by Dr. Samuel D. Wright, The Rockefeller University, New York. OKM1, OKM10, and Leu-15 recognize different epitopes on the α-chain of CR3 (8). IB4 recognizes the β-chain of the CR3/LFA-1/p150,95 family of surface glycoproteins (8). Anti-LFA-1 recognizes the α-chain of LFA-1 (31). Anti-Leu-M5 recognizes the α-chain of p150,95 (32). Anti-CR1 recognizes CR1 (33). Anti-CR2 recognizes complement receptor 2 (CR2) (34, 35), anti-transferrin receptor antibody recognizes the human transferrin receptor (36), and anti-HLA-DR antibody recognizes the DR antigen (36, 37). All MAbs containing sodium azide were dialyzed four times against $\geq$ 100 vol of PBS before use. MAbs purchased from AMAC, Inc. contained bovine serum albumin and no sodium azide; when these MAbs were used, an equivalent final concentration (0.005–0.010%) of bovine serum albumin (Sigma Chemical) was added to control wells. Rabbit anti-sheep RBC IgG was the generous gift of Dr. Samuel D. Wright.

Assay of M. leprae adherence to monocytes. Monocytes in monolayer culture were incubated on plain glass coverslips in RPMI-10 mM Hepes medium containing 10% fresh serum at 37°C in 5% CO_2-95% air for 24 h. The monocytes were then washed vigorously with RPMI and incubated at 37°C under various conditions with *M. leprae* at 40 rpm on a rotating platform for 30 min and then under stationary conditions for 90 min. 5.0×10^6 *M. leprae* were used in experiments in which serum was present and 5.0×10^7 *M. leprae* were used in experiments in which serum was absent. After incubation, the monocytes were washed vigorously to remove nonadherent bacteria and fixed in 10% formalin. Coverslips were allowed to dry and *M. leprae* were stained with auramine-rhodamine by flooding coverslips with the stain for 20 min and rinsing with distilled water. Coverslips were then flooded with fluorochrome acid alcohol for 3 min, rinsed, flooded with potassium permanganate for 2 min, rinsed, and dried (38). The percentage of monocytes with one or more than one adherent bacterium and the average number of bacteria per monocyte on each coverslip were determined by counting a minimum of 200 monocytes per coverslip under fluorescence microscopy (Fluophot, Nikon, Tokyo, Japan). The mean±SD was determined for duplicate coverslips. In certain control experiments, 7×10^6 zymosan particles, 1.4×10^7 polybead-Polystyrene Microspheres No. 17135 (Polysciences, Inc., Warrington, PA) or 1.5×10^7 IgG-coated sheep erythrocytes (E-IgG), were added to each well instead of *M. leprae.* Zymosan particles were dissolved in PBS (1 mg/ml), boiled for 30 min, and washed twice in PBS prior to use. E-IgG were prepared as previously described (1). Polystyrene microspheres were washed twice in RPMI prior to use.

In studies on the influence of MAbs on *M. leprae* adherence to monocytes, the monocytes were preincubated for 20 min at 37°C in RPMI-20 mM Hepes containing either 2.5% fresh autologous serum or no serum, and various MAbs, alone or in combination, and then *M. leprae* were added to the culture. All MAbs except IB4 were used at a final concentration of 11 μg/ml. IB4 was used at a final concentration of 5 μg/ml. This concentration was chosen so as to maximally inhibit adherence without disrupting the integrity of the monocyte monolayer, a known effect of this MAb.

In studies on the potential role of the β-glucan inhibitable receptor for zymosan (39–41), monocytes were incubated with either *M. leprae* or unopsonized zymosan in the absence of serum and a high concentration of laminarin (200 μg/ml).

In studies on the effect of divalent cations (Ca^{++} and Mg^{++}) in adherence of *M. leprae,* RPMI-20 mM Hepes containing 1 mM EDTA was added to monolayers before the addition of either preopsonized *M. leprae* or E-IgG. Monocytes were harvested from teflon wells for these experiments and the duration of the adherence assay was 1 h.

In studies on the influence of serum concentration on *M. leprae* adherence, monocytes were incubated with *M. leprae* in the presence of fresh autologous serum in concentrations ranging 0–20%.

In studies on the role of the complement system in *M. leprae*

adherence, monocytes were incubated with *M. leprae* in the presence of 2.5% heat-inactivated serum, 2.5% factor B-depleted serum, 2.5% factor B-depleted serum plus factor B (10-fold excess of factor B H_{50} units), 2.5% C3-depleted serum, or 2.5% C3-depleted serum plus C3 (twofold excess of C3 CH_{50} units).

In early experiments requiring preopsonization of bacteria, *M. leprae* were first incubated in 50% fresh nonimmune serum at 37°C for 1 h and then incubated at 56°C for 30 min to inactivate the complement cascade. The preopsonized bacteria were then added to monocytes in monolayer culture in the presence of heat-inactivated serum. Control unopsonized bacteria for these experiments were prepared by first incubating *M. leprae* in 50% heat-inactivated nonimmune serum at 37°C for 1 h and then incubating them at 56°C for 30 min. In later experiments requiring preopsonization of bacteria, *M. leprae* were first incubated in fresh serum at 37°C as above. The bacteria were then harvested by centrifugation at 12,000 *g* for 10 min at 4°C, washed twice with 1 ml PD, and resuspended in PD at a final concentration of 10^8 bacteria/ml.

Assay for influence of Fc receptor modulation on M. leprae adherence. Monocytes harvested from teflon wells were adhered to Chromerge (Fisher Scientific Co.)-cleaned glass coverslips coated with antigen–antibody complexes comprised of human serum albumin (HSA) (Calbiochem-Behring Corp.) and rabbit anti-HSA (Organon Teknika Corp) as described (1, 42). Control monocytes were plated on coverslips coated with HSA alone. The monocytes were then incubated with either preopsonized *M. leprae* (5.0×10^6) or E-IgG (1.5×10^7), as a control, in the presence of heat-inactivated serum. Adherent *M. leprae* were enumerated as in the previous assay. The total number of adherent E-IgG was enumerated by phase-contrast microscopy. Ingested E-IgG were enumerated after hypotonic lysis of extracellular E-IgG. At least 200 monocytes on each of duplicate coverslips were evaluated for adherent bacteria or E-IgG.

ELISA to detect C3 fixation to M. leprae. M. leprae (10^8 bacteria/ml) were incubated for 60 min at 37°C in 50% fresh nonimmune serum, in 50% fresh nonimmune serum containing either 10 mM EDTA or 10 mM EGTA with 7 mM $MgCl_2$, in 50% heat-inactivated serum, or in PD. The bacteria were harvested by centrifugation at 12,000 g for 10 min. at 4°C, washed twice with 1 ml PD, and resuspended in PD at a final concentration of 10^8 bacteria/ml. 0.1-ml aliquots (10^7 bacteria) were dispensed into wells of a 96-well tissue culture plate and allowed to evaporate to dryness under a laminar flow hood (24–48 h). Each well was incubated with 3% ovalbumin in PBS for 12–18 h at 4°C to block nonspecific protein-binding sites, washed three times with PBS, and then incubated with goat anti-human C3 IgG (Atlantic Antibodies, Inc., Scarborough, ME) diluted 1:800 in PBS for 1 h. The wells were washed, incubated for 1 h with alkaline phosphatase-conjugated rabbit anti-goat IgG (Organon Teknika Corp.) diluted 1:3,000 in PBS, washed again, and incubated with phosphatase substrate (Bio-Rad Laboratories, Richmond, CA) for 30 min. The reaction was terminated by the addition of NaOH (final concentration 0.2 N) and the absorbance at 405 nm was measured using a Titertek Multiscan MCC ELISA reader (Flow Laboratories, Inc., McLean, VA). The mean±SD of the absorbance for triplicate wells of each type was calculated.

Electron microscopy. In studies to determine if *M. leprae* that adhere to monocytes in the presence or absence of MAbs are ingested, monocytes were plated on 14-mm diam toluene-resistant plastic coverslips (Wako Chemical Co., Dallas, TX) as described (43). The monocytes were incubated with bacteria in 12.5% serum in the presence or absence of MAbs as in the assay for *M. leprae* adherence to monocytes described above, except that the bacteria/monocyte ratio was increased 10-fold. The monocytes were then washed, incubated for an additional 30 min, and fixed and prepared for electron microscopy as described (43). The location (on the surface of or inside the monocyte) of ≥ 300 consecutive monocyte-associated bacteria from each coverslip preparation was enumerated.

The method by which monocytes phagocytize *M. leprae* was studied as previously described (19). Briefly, freshly isolated mononuclear cells (6×10^6 mononuclear cells including ~ 2×10^6 monocytes) in RPMI containing 10% fresh serum were mixed with 2×10^8 bacteria in a total volume of 1 ml in a conical centrifuge tube at 4°C, a temperature at which phagocytosis does not proceed. The monocytes and bacteria were pelleted together by centrifugation at 4°C, rapidly warmed (0.5 min) to 37°C, a temperature at which phagocytosis proceeds, incubated for 3, 6, or 10 min, and fixed and prepared for electron microscopy.

Results

Monocyte complement receptors CR1 and CR3 mediate adherence and phagocytosis of M. leprae. To explore the role of monocyte receptors in mediating adherence of *M. leprae* to monocytes, we examined the capacity of MAbs directed against several monocyte surface molecules to inhibit bacterial adherence. Three different MAbs against the alpha chain of CR3 (OKM10, OKM1, and Leu-15) and a MAb against CR1 consistently and significantly inhibited *M. leprae* adherence, although the degree of inhibition was moderate (Table I). Mean inhibition (±SE) for MAbs against the α chain of CR3 was 55±1% for OKM10, 47±4 for OKM1, and 38±2% for

Table I. Inhibition of M. leprae Adherence to Monocytes by MAbs against CR1 and CR3

MAbs	Target antigen	Ig subtype	Inhibition of adherence*
			%
Leu-15/OKM1	CR3/CR3	IgG2a/IgG2b	90±3
Leu-15/OKM10	CR3/CR3	IgG2a/IgG2	87±3
OKM1/OKM10	CR3/CR3	IgG2b/IgG2	65±1
CR1/Leu-15	CR1/CR3	IgG1/IgG2a	70±1
CR1/OKM10	CR1/CR3	IgG1/IgG2	67±2
CR1/OKM1	CR1/CR3	IgG1/IgG2b	60±9
OKM10	CR3	IgG2	55±1
IB4	CR3/LFA-1/p150,95	IgG2a	48±6
OKM1	CR3	IgG2b	47±4
Leu-15	CR3	IgG2a	38±2
Anti-CR1	CR1	IgG1	38±4
Anti-TrR‡	TrR	IgG2a	19±13
Anti-Leu-M5	p150,95	IgG2b	15±2
Anti-LFA-1	LFA-1	IgG1	13±16
Anti-HLA-DR	HLA-DR	IgG2a	1±4
Anti-LFA-1/ Anti-HLA-DR	LFA-1/HLA-DR	IgG1/IgG2b	17±16
Anti-LFA-1/ Anti-CR2	LFA-1/CR2	IgG1/IgG2a	5±7
Anti-CR2/ Anti-HLA-DR	CR2/HLA-DR	IgG2a/IgG2b	−19±1§

Monocytes in monolayer culture on coverslips were incubated with *M. leprae* in the presence of the various MAbs indicated and adherence assayed as described in the text. Each MAb was used at a concentration of 11 μg/ml except for IB4 which was used at a concentration of 5 μg/ml. Data are the mean inhibition of *M. leprae* adherence±SE obtained in two to five independent experiments, each of which was done in duplicate.

* Percent decrease in mean number of *M. leprae* per monocyte from level in control monocytes incubated without antibody.

‡ TrR, transferrin receptor.

§ Adherence was increased rather than inhibited.

Leu-15 and mean inhibition (±SE) for the MAb against CR1 was 38±4% A MAb, IB4, against the common β-chain of CR3, LFA-1, and p150,95, inhibited to a degree similar to that of single MAbs against the α-chain of CR3 (48±6%) (Table I). MAbs against the α-chain of LFA-1, the α-chain of p150,95, the transferrin receptor, and HLA-DR did not significantly inhibit *M. leprae* adherence (mean inhibition ranged from 1 to 19%) (Table I). The antibody subtype was not an important factor as MAbs of three different subtypes (IgG1, IgG2a, and IgG2b) against CR1 and CR3 inhibited adherence whereas control MAbs of the same subtypes did not (Table I).

A combination of MAbs against CR1 and CR3 or against two epitopes of CR3 yielded markedly enhanced inhibition of *M. leprae* adherence. Representative duplicate experiments are shown in Table II and cumulative data are shown in Table I. The combination of a MAb against CR1 and a MAb against the α-chain of CR3 inhibited *M. leprae* adherence by 70±1% (mean±SE) for anti-CR1 plus Leu-15, 67±2% for anti-CR1 plus OKM10, and 60±9% for anti-CR1 plus OKM1 (Table I). The most striking inhibition of adherence occurred with a combination of two different MAbs against the α-chain of CR3. The rationale for using two different MAbs against CR3, each recognizing a different epitope (30, 39), was that the two MAbs may inhibit the binding of *M. leprae* to CR3 more effectively than one MAb. The most potent combination of MAbs, Leu-15 and OKM1, reduced *M. leprae* adherence by a mean of 90±3% (Table I). In contrast, combinations of various control MAbs did not significantly reduce *M. leprae* adherence (mean inhibition ranged from −19% to 17%).

Monocyte viability as assessed by trypan blue exclusion in the presence of fresh serum and all combinations of MAbs was found not to be impaired. Monocyte phagocytic capacity, as determined by assaying the uptake of polystyrene microspheres in the presence of heat-inactivated serum, was not impaired by any MAb or any combination of MAbs (data not shown). Monocyte Fc receptor function, as determined by assaying the binding of E-IgG in the presence of heat-inactivated serum, was not impaired by MAbs. No inhibition of E-IgG binding occurred with MAbs used alone or in combination with the one exception of OKM10. This MAb inhibited by a small but consistent degree (30±3% SE) in three independent experiments. Under the same conditions, Leu-15 and OKM1, the two other MAbs directed against CR3 that were studied, resulted in no inhibition of E-IgG binding (data not shown).

To determine if *M. leprae* that adhere to monocytes are ingested, we infected monocytes in monolayer culture with *M. leprae* as in the adherence assay, except that the bacteria/monocyte ratio was increased 10-fold and monocytes were incubated an additional 30 min after washing away nonadherent bacteria to allow internalization of bacteria that may have adhered at the end of the 2 h infection period. We then examined monocyte-associated bacteria by electron microscopy (Fig. 1). 100% of ≥ 300 consecutive bacteria were intracellular, i.e., none were on the surface of the monocytes, whether the monocytes were incubated with or without MAbs. When monocytes were incubated with *M. leprae* in the absence of MAbs, 77% of cross sections of monocytes contained intracellular bacteria, and there were on average 10–15 bacteria per monocyte cross section. When monocytes were incubated with *M. leprae* in the presence of two MAbs against CR3, Leu-15 and OKM10, only 8% of cross sections of monocytes contained intracellular bacteria and there were relatively few bacteria (average of two) per monocyte cross section. When monocytes were incubated with *M. leprae* in the presence of control MAbs (anti-CR2 and anti-HLA-DR), 67% of cross sections contained intracellular bacteria and there were many bacteria per monocyte cross-section, comparable to that seen in control monocytes incubated without MAbs.

As will be shown below, optimal adherence of *M. leprae* to monocytes requires low levels of fresh serum and adherence is markedly reduced under conditions in which serum is removed. To determine whether the low level of adherence seen under serumless conditions was being mediated by complement receptors CR1 and CR3, we examined the capacity of

Table II. MAbs against CR1 and CR3 Inhibit M. Leprae Adherence to Monocytes

Experiment	MAbs	Target antigen	Monocytes with one or more than one adherent bacterium	Mean number of bacteria per monocyte	Inhibition of adherence (decrease from control)
			%	*n*	%
A	None (Control)	—	51±13	1.65±0.65	—
	Leu-15/OKM10	CR3/CR3	6±2	0.08±0.06	95
	Leu-15/OKM1	CR3/CR3	4±1	0.08±0.04	95
	Anti-CR1/OKM10	CR1/CR3	19±1	0.51±0.11	69
	Anti-CR2/Anti-LFA-1	CR2/LFA-1	52±12	1.75±0.27	−6*
	Anti-CR2/Anti-HLA-DR	CR2/HLA-DR	54±10	1.95±0.72	−18*
B	None (Control)	—	54±1	1.98±0.10	—
	Leu-15/OKM10	CR3/CR3	8±1	0.29±0.01	85
	Leu-15/OKM1	CR3/CR3	9±4	0.39±0.16	80
	Anti-CR1/OKM10	CR1/CR3	21±1	0.77±0.08	61
	Anti-CR2/Anti-LFA-1	CR2/LFA-1	56±4	1.93±0.14	3
	Anti-CR2/Anti-HLA-DR	CR2/HLA-DR	57±1	2.35±0.24	−19*

In these two duplicate experiments, monocytes in monolayer culture on coverslips were incubated with *M. leprae* in the presence of the MAbs indicated at a concentration of 11 μg/ml (each MAb) or without MAb (control). The percentage of monocytes with one or more than one adherent bacterium and the average number of bacteria per monocyte were determined by fluorescence microscopy. Data are the mean±SD for duplicate coverslips. * Adherence was increased rather than inhibited.

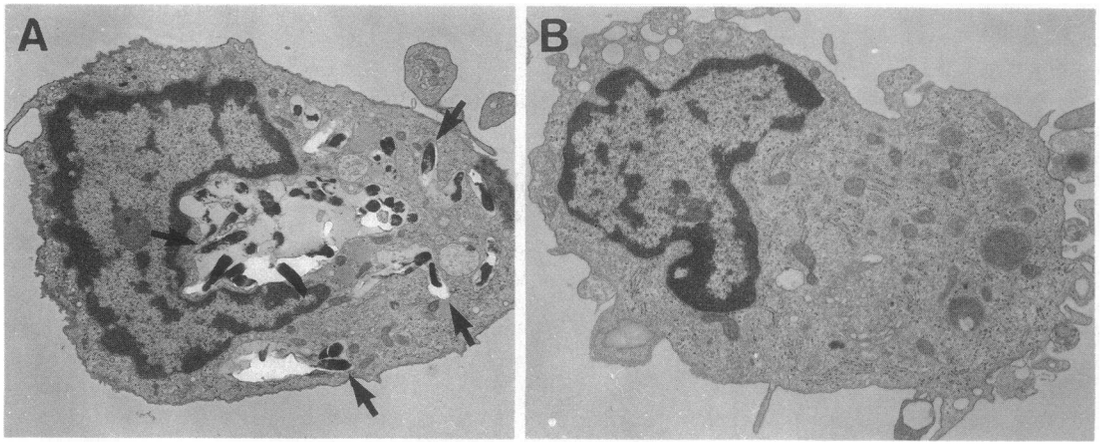

Figure 1. MAbs to CR3 inhibit ingestion of *M. leprae*. Monocytes in monolayer culture were infected with *M. leprae* in the presence or absence of MAbs to CR3 as described in the text. The monocytes were then washed, fixed, and prepared for electron microscopy. (*A*) A typical cross section of a monocyte infected with *M. leprae* in the absence of MAbs. All bacteria (*arrows*) are internalized, and the monocyte contains numerous bacteria. (*B*) A typical cross section of a monocyte infected with *M. leprae* in the presence of two MAbs against CR3 (Leu-15 and OKM10). The monocyte is devoid of attached or ingested bacteria. Cross sections of monocytes infected with *M. leprae* in the presence of two control MAbs (anti-CR2 and anti-HLA-DR) were comparable in appearance to those of monocytes infected in the absence of MAbs, i.e., comparable in appearance to micrograph *A*. (*A*) ×4,800. (*B*) ×7,200.

MAbs directed against several monocyte surface molecules to inhibit bacterial adherence in the absence of serum. We employed our usual adherence assay except that the monocytes were preincubated with MAbs in the absence of serum and *M. leprae* were added to monocytes at a 10-fold higher bacteria/monocyte ratio. This ratio was increased to compensate for the markedly reduced adherence of *M. leprae* in the absence of serum.

MAbs to CR1 and CR3 markedly inhibited *M. leprae* adherence under these conditions. One representative experiment is shown in Table III. The pattern of inhibition by MAbs against CR1 and CR3 was similar to that seen in the presence of serum except that single MAbs inhibited adherence to a greater extent. Mean inhibition (±SE) for MAbs against the alpha chain of CR3 was 71±6% for Leu-15, 70±2% for OKM10, and 62±12% for OKM1 and mean inhibition (±SE) for the MAb against CR1 was 64±11%. A combination of MAbs against CR1 and CR3 or against two epitopes of CR3 slightly enhanced the level of *M. leprae* adherence (mean inhibition ranged from 76±11% to 77±7%). Combinations of various control MAbs did not significantly reduce *M. leprae* adherence (mean inhibition 4±10%).

The function of CR3 is dependent upon the divalent cations Ca^{++} and Mg^{++} (8). Since CR3 appeared to be the predominant receptor type in our adherence assay, we examined the divalent cation-dependence for adherence of *M. leprae* by chelating the Ca^{++} and Mg^{++} in RPMI medium with 1 mM EDTA. Under these conditions, adherence of *M. leprae* was markedly reduced (86±3% SE; $n = 2$). In contrast, adherence of IgG-coated sheep erythrocytes, which are ingested by cat-

Table III. MAbs against CR1 and CR3 Inhibit M. leprae Adherence to Monocytes in the Absence of Serum

MAbs	Target antigen	Monocytes with one or more than one adherent bacterium	Mean number bacteria per monocyte	Inhibition of adherence (decrease from control)
		%	*n*	%
None (Control)	—	39±1	1.24±0.45	—
Anti-CR1	CR1	32±6	0.58±0.01	53
Leu-15	CR3	25±1	0.43±0.01	65
OKM1	CR3	30±4	0.62±0.03	50
OKM10	CR3	17±1	0.35±0.09	72
Anti-CR1/Leu-15	CR1/CR3	24±9	0.43±0.17	65
OKM1/OKM10	CR3/CR3	23±3	0.37±0.02	70
Leu-15/OKM1	CR3/CR3	13±0	0.20±0.04	84
Anti-HLA-DR/Anti-CR2	HLA-DR/CR2	41±2	1.06±0.03	15
Anti-LFA-1/Anti-CR2	LFA-1/CR2	50±10	1.44±0.36	−16*

In this representative experiment, monocytes in monolayer culture on coverslips were washed free of serum and then incubated with *M. leprae* in the presence of the MAbs indicated (11 μg/ml each MAb) or without MAbs (control). The percentage of monocytes with one or more than one adherent bacterium and the average number of bacteria per monocyte were determined by fluorescence microscopy. Data are the mean±SD for duplicate coverslips. * Adherence was increased rather than inhibited.

ion-independent Fc receptors (44), was minimally inhibited (27±2% SE; n = 2) in the presence of EDTA. These results provided further evidence for a major role for CR3 in mediating adherence of *M. leprae* to monocytes.

To determine the potential role of Fc receptors in mediating *M. leprae* adherence in the presence of nonimmune serum, we examined the influence of Fc receptor modulation on bacterial adherence. We modulated Fc receptors to the basal surface of monocytes by plating monocytes on antigen–antibody complexes consisting of HSA and anti-HSA. Whereas monocytes plated on antigen–antibody complexes bound or ingested 99±1% fewer E-IgG than control monocytes plated on antigen alone, such monocytes bound only 30±3% fewer *M. leprae* bacilli than control monocytes (Table IV). These results indicate that Fc receptors play little if any role in mediating *M. leprae* adherence under nonimmune conditions.

Finally, to examine the potential role in *M. leprae* adherence of the β-glucan-inhibitable receptor for unopsonized zymosan, which has been found on neutrophils and monocytes (39–41), we assessed *M. leprae* adherence in the presence or absence of laminarin, a soluble β-glucan from algae, which inhibits this receptor. In the presence of laminarin, zymosan adherence was reduced by 43±2% (n = 2). In contrast, *M. leprae* adherence was slightly increased (8±7% SE, n = 2). Thus, the β-glucan receptor does not appear to be involved in *M. leprae* adherence under serumless conditions.

M. leprae adherence is dependent upon fixation of complement component C3 to the bacterial surface. Having determined that complement receptors CR1 and CR3 play an important role in mediating adherence and phagocytosis of *M. leprae* by monocytes, we next explored the role of complement in this process. We first assessed the importance of serum in mediating *M. leprae* adherence by measuring bacterial uptake in the presence of various concentrations of fresh serum (Fig. 2). Adherence was highly serum-dependent. Adherence remained high at serum concentrations of ≥ 0.5%. Below this concentration, adherence was markedly reduced. In comparison to the level of adherence in 2.5% serum, adherence in 0.1% serum was reduced by 66±7% (SE; n = 2) to the level obtained in the absence of serum.

We next examined the importance of an intact complement pathway on *M. leprae* adherence by comparing adherence in the presence of fresh serum with that in the presence of heat-inactivated serum. Heat-inactivation of serum resulted in a significant reduction (68±3%) in bacterial adherence in 11 independent experiments. To determine if this reduction in adherence was due to the absence of an opsonin such as complement component C3 on the bacterial surface, we studied adherence of *M. leprae* that had been preopsonized in fresh nonimmune serum (Table V). In early experiments, we were unable to wash the preopsonized bacteria free of serum complement components by centrifugation without the bacteria clumping. We instead inactivated complement in the medium containing the preopsonized bacteria by heating it at 56°C. We reasoned that such heat treatment would not affect bacterium–C3 complexes, since these complexes, once formed, should be heat stable. We then added the bacteria in the two preparations to monocytes in monolayer culture in the presence of heat-inactivated serum. *M. leprae* preopsonized in fresh serum adhered readily to monocytes; preopsonization enhanced adherence by 3.1±1.1-fold.

In later experiments, we were able to preopsonize and then wash *M. leprae* without the bacteria clumping; this allowed us to preopsonize bacteria without heating them to 56°C. The results obtained with this preopsonization technique were similar to those obtained using the previous technique described above.

Taken together, the experiments performed to this point strongly suggested that degradation products of complement component C3 were acting as bacterium-bound ligands for CR1 and CR3 on monocytes in mediating adherence and phagocytosis of *M. leprae*. To test this hypothesis further, we assessed the role of C3 in *M. leprae* adherence. We incubated monocytes with *M. leprae* in the presence of either heterologous C3-depleted serum or heterologous C3-depleted serum to which purified human C3 was added back. In three indepen-

Table IV. Modulation of Fc Receptors by Antigen–Antibody Complexes Does Not Significantly Inhibit M. leprae Adherence

Experiment	Substrate	*M. leprae* per monocyte	IgG-coated sheep RBC per monocyte	
			Total	Ingested
A	HSA* (n)	3.60±0.37	6.07±0.06	3.22
	HSA/anti-HSA (n)	2.40±0.18	0.04±0.02	0.00
	Reduction (%)‡	33	99	100
B	HSA (n)	2.33±0.86	3.49±0.21	2.35
	HSA/anti-HSA (n)	1.70±0.42	0.07±0.03	0.04
	Reduction (%)	27	98	98
(1)	(2)	(3)	(4)	(5)

Monocytes were plated on a substrate of antigen–antibody complexes (HSA/anti-HSA) or on antigen alone (HSA) as a control, incubated with IgG-coated erythrocytes or *M. leprae* preopsonized in nonimmune serum, and assayed for associated RBC or *M. leprae* as described in Methods. Columns 3 and 4 represent the data from duplicate measurements in two independent experiments (mean±SD). Column 5 represents the mean data of 200 consecutive monocytes on a single coverslip after hypotonic lysis of extracellular E-IgG. Monocytes plated on antigen–antibody complexes displayed a marked reduction in capacity to bind or ingest IgG-coated RBC (columns 4 and 5), but only a slight reduction in capacity to bind preopsonized *M. leprae* (column 3).

* Human serum albumin.

‡ Percent reduction in mean number of particles (*M. leprae* or E-IgG) for monocytes plated on HSA/Anti-HSA in comparison to control monocytes plated on HSA only.

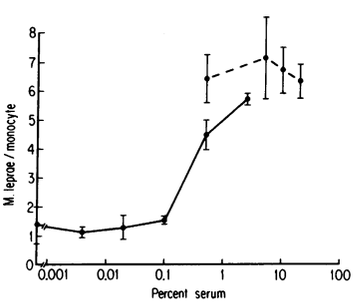

Figure 2. Adherence of *M. leprae* to monocytes is serum dependent. Monocytes in monolayer culture were incubated with *M. leprae* in the presence of fresh autologous human serum at the concentrations indicated. The solid line denotes an experiment in which the serum concentration varied from 0% to 2.5%. The dashed line denotes an experiment in which serum concentration varied from 0.5% to 20%. Adherence of *M. leprae* was assayed as described in the text. Results are mean±SD for duplicate wells.

Table V. Adherence of M. leprae to Monocytes Is Enhanced by Preopsonization in Fresh Serum

Experiment	M. leprae	Bacteria per monocyte	Fold increase in adherence*
A	Preopsonized	3.85±1.12	
	Unopsonized	0.95±0.71	4.1
B	Preopsonized	2.90±0.58	
	Unopsonized	1.46±0.23	2.0

Monocytes were incubated with preopsonized or unopsonized *M. leprae* in the presence of heat-inactivated serum and the number of adherent bacteria per monocyte was enumerated. Preopsonized *M. leprae* were obtained by incubating the bacteria in fresh nonimmune serum at 37°C for 1 h followed by incubation at 56° for 30 min to inactivate the complement cascade. Unopsonized *M. leprae* were obtained by incubating the bacteria in heat-inactivated nonimmune serum at 37°C for 1 h followed by incubation at 56°C for 30 min. Data are the mean±SD for duplicate coverslips in two independent experiments; at least 200 monocytes per coverslip were enumerated. * Fold increase in mean number of bacteria per monocyte for preopsonized *M. leprae* compared to unopsonized *M. leprae*.

dent experiments, C3 repletion consistently increased *M. leprae* adherence (Fig. 3). Overall, adherence was increased 4.3±0.8-fold (SE).

To confirm that C3 was fixed to *M. leprae,* we studied C3 fixation to the bacteria directly by means of a whole bacterial cell ELISA specific for human C3. Fig. 4 shows the results of one representative experiment. Significant amounts of C3 were fixed to *M. leprae* when the bacteria were incubated in fresh serum but C3 fixation was markedly reduced to near baseline levels when the bacteria were incubated in heat-inactivated serum.

To determine the roles of the classical and alternative pathways of complement activation in C3 fixation, we studied C3 fixation to bacteria in serum containing EDTA, which inhibits activation of both pathways, and in serum containing EGTA and $MgCl_2$, which inhibits activation of only the classical pathway. In the presence of EDTA, C3 fixation was markedly reduced to baseline levels (Fig. 4). However, in the presence of EGTA and $MgCl_2$, as much C3 was fixed to the bacteria as in serum alone. In contrast, under the same conditions (EGTA and $MgCl_2$), C3 fixation to E-IgG was reduced to the level obtained in heat-inactivated serum in this ELISA (data not shown). These results indicate that C3 is fixed to *M. leprae* by the alternative pathway of complement activation.

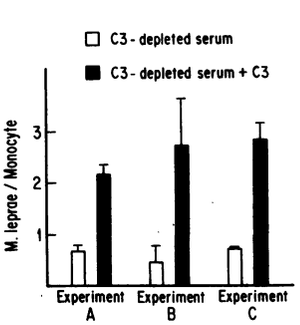

Figure 3. Adherence of *M. leprae* is dependent upon human C3. In three independent experiments (*A–C*), monocytes in monolayer culture on coverslips were incubated with *M. leprae* in the presence of either fresh heterologous serum depleted of C3 or fresh heterologous serum depleted of C3 to which purified C3 was added back. Adherence was assayed as described in the text. Data represent the mean±SD for duplicate wells.

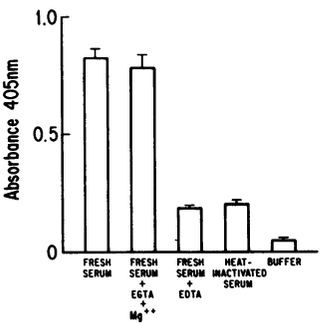

Figure 4. M. leprae fixes complement component C3, as demonstrated by ELISA. *M. leprae* were incubated for 1 h in fresh, nonimmune human serum, in fresh nonimmune serum containing 10 mM EGTA and 7 mM $MgCl_2$, in fresh nonimmune serum containing 10 mM EDTA, in heat-inactivated nonimmune serum, or in buffer (PD) only. The bacteria were vigorously washed and assayed for associated human C3 in a whole bacterial cell ELISA that utilized goat anti-human C3 as primary antibody and alkaline phosphatase-conjugated rabbit anti-goat IgG as secondary antibody. Data are from one representative experiment and show the mean±SD of the absorbance for triplicate measurements.

To confirm the importance of the alternative pathway of complement activation in *M. leprae* adherence, we studied the influence on adherence of using serum depleted of a critical component of the alternative pathway: factor B. We incubated monocytes with bacteria in the presence of fresh autologous serum, fresh autologous serum depleted of factor B, or fresh autologous serum depleted of factor B to which purified factor B was added back. In three consecutive experiments, adherence of *M. leprae* in the presence of Factor B-depleted serum was inhibited by 65±5% (SE) from the control level with whole serum (Fig. 5). When purified factor B was added back to factor B-depleted serum, adherence increased 2.6±0.2-fold (SE) and approached control levels (90±8% of control). Thus, the alternative pathway of complement activation mediates fixation of C3 to *M. leprae* and, in turn, adherence of the bacteria to monocytes.

M. leprae are phagocytized by conventional phagocytosis. To determine whether monocytes phagocytize *M. leprae* by a conventional or coiling form of phagocytosis, we studied ingestion by electron microscopy using an assay designed to synchronize phagocytosis and allow for visualization of a large number of organisms fixed during the process of ingestion. We found that *M. leprae* are phagocytized by a conventional appearing form of phagocytosis (Fig. 6). As in a previous study

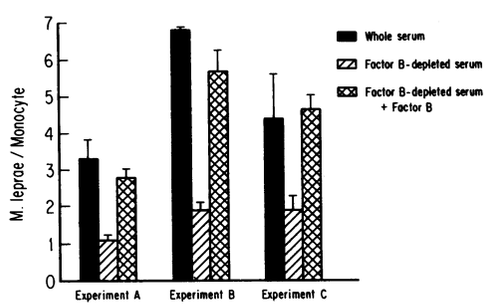

Figure 5. Adherence of *M. leprae* is dependent upon human factor B. In three independent experiments (*A–C*), monocytes in monolayer culture on coverslips were incubated with *M. leprae* in the presence of fresh autologous serum, fresh autologous serum depleted of factor B, or fresh autologous serum depleted of factor B to which purified factor B was added back. Adherence was assayed as described in the text. Data represent the mean±SD for duplicate wells.

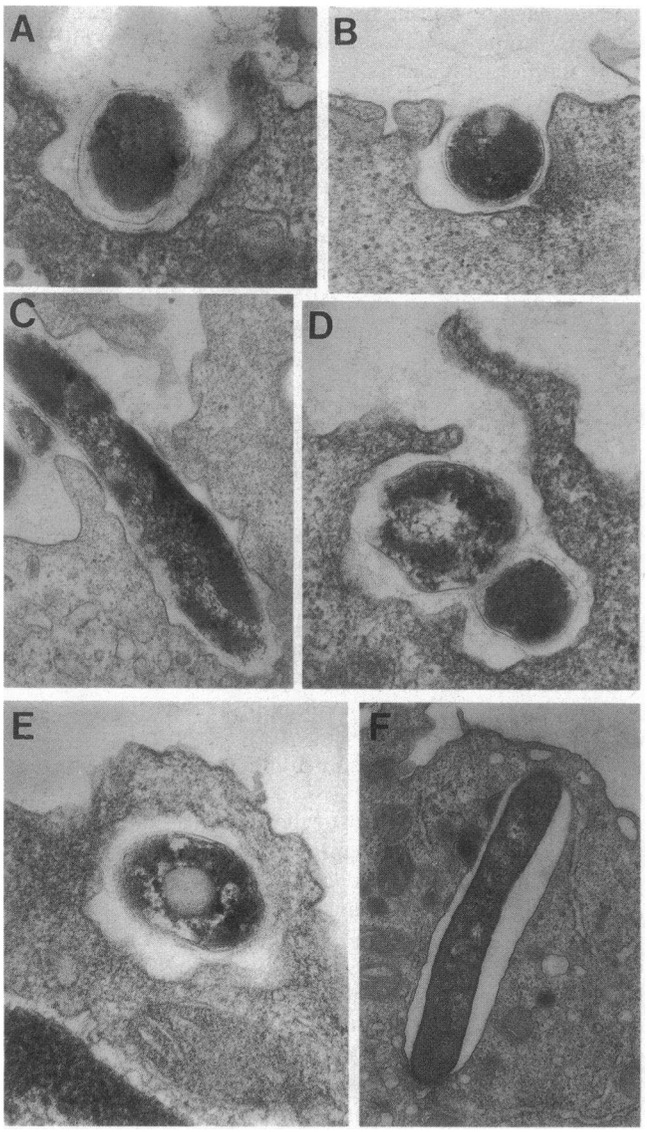

Figure 6. Human monocytes ingest *M. leprae* by conventional phagocytosis. *M. leprae* were mixed and sedimented with monocytes at 4°C, rapidly warmed to 37°C to allow phagocytosis to proceed, incubated for 3, 6, or 10 min, and fixed and processed for electron microscopy, as described in the text. (*A*) An early stage of ingestion. The bacterium is tightly adherent to the monocyte surface. (*B–D*) More advanced stages of ingestion. The bacteria are in the process of being internalized. (*E* and *F*) The final stages of ingestion. The bacteria are completely enclosed within membrane bound vacuoles or phagosomes. (*A*) ×51,600. (*B*) ×46,400. (*C*) ×28,800. (*D*) ×46,000. (*E*) ×57,600. (*F*) ×15,200.

on phagocyte ingestion of bacteria, two simultaneous processes appeared to be involved: the extension of pseudopods about the organism and the displacement of the cytoplasm at the zone of contact of the phagocyte and the organism such that the organism appears to sink into the phagocyte (19).

Discussion

The receptors and ligands involved in mediating phagocytosis of *M. leprae* by human monocytes have not been explored previously. Our findings demonstrate that monocyte surface receptors CR1 and CR3 mediate attachment and ingestion of *M. leprae* both in the presence of nonimmune serum and in the absence of serum. Single MAbs against CR1 or CR3 moderately inhibit *M. leprae* adherence, and combinations of MAbs against CR1 and CR3 very strongly inhibit adherence. In certain experiments, two MAbs against the α-chain of CR3 inhibited adherence by > 1 log. The rationale for using two different MAbs against CR3 was that one MAb against a single epitope of CR3 may not completely block binding of *M. leprae* to this receptor. Myones et al. (45) have recently reported that three MAbs used in combination against the α-chain of CR3 more effectively inhibits rosetting of C3bi-coated erythrocytes to human monocytes than any one of the MAbs. It is also possible that two MAbs in combination block more complement receptors than one MAb. However, single MAbs were used at concentrations considerably above the saturating levels (1).

In the presence of nonimmune serum not all combinations of MAbs against CR3 yield equivalent degrees of inhibition. The combination of Leu-15 with either OKM1 or OKM10 yields much greater inhibition (87–90%) than each MAb alone. However, the combination of OKM1 and OKM10 yields a degree of inhibition that is only slightly greater (65%) than each MAb alone. Perhaps, the spatial distribution of epitopes recognized by the combination of Leu-15 with either OKM1 or OKM10 is such that these combinations of MAbs more effectively block the binding site of *M. leprae* than the combination of OKM1 and OKM10.

Each of the four MAbs directed against CR3, when used alone, inhibited *M. leprae* adherence to a similar extent both in the presence and absence of serum. In contrast, in a study by Wright et al. (30), OKM10, which these investigators believe recognizes a CR3 determinant at or near the ligand-binding site for this receptor, more effectively inhibited adherence of C3bi-coated sheep erythrocytes than did OKM1 and IB4 in a serum-free system. This difference in the effects of MAbs against CR3 on *M. leprae* and erythrocyte adherence may be explained in several ways. First, the steric interaction between a test particle and a monocyte receptor is complex and may be quite different for *M. leprae* and erythrocytes. Secondly, the amount and/or distribution of C3 degradation products on the surface of a test particle may impact on ligand–receptor interaction and this may be different for *M. leprae* and erythrocytes. Third, the ligand–receptor interaction observed may vary somewhat depending on the nature of the *in vitro* assay performed and the age of the monocyte in tissue culture.

The combination of a MAb against CR1 and a MAb against CR3 also yields greater inhibition of *M. leprae* adherence than either MAb alone. This suggests that these two receptors may cooperate in *M. leprae* ingestion. This might occur in at least two ways. First, both receptors may recognize the same ligand, C3bi, fixed to the bacterial surface. C3bi is the major ligand recognized by CR3, but C3bi is also recognized by CR1, although to a lesser extent (8). Secondly, CR3 and CR1 may recognize different ligands on the bacterial surface, i.e., CR3 may recognize C3bi and CR1 may recognize C3b, its major ligand (8). The very strong inhibition of *M. leprae* adherence observed with 2 MAbs against CR3 suggests that C3bi represents the major ligand on the bacterium. The striking level of inhibition of *M. leprae* adherence in the presence of EDTA also supports a major role for CR3. *In vivo,* where serum factors H and I are present, C3b would normally be

degraded rapidly to C3bi. However, the degradation pathway of C3 fixed to the bacterium may depend to a great extent on the nature and local milieu of the complex formed between C3 and its acceptor molecule on the *M. leprae* surface.

Monocyte viability in fresh serum, assessed by trypan blue exclusion, was not impaired by the presence of MAbs used alone or in combination. Monocyte function also remained intact in the presence of MAbs, as assessed by two criteria. First, monocytes incubated with or without all combinations of MAbs avidly phagocytized polystyrene microspheres and to an equivalent degree. Secondly, monocytes incubated with single MAbs or combinations of MAbs exhibited no reduction in capacity to bind E-IgG except when treated with OKM10. This MAb resulted in a small but consistent degree of inhibition (30±3%) in three experiments. Two other MAbs against CR3, Leu-15 and OKM1, did not result in inhibition of E-IgG binding. Interestingly, anti-CR3 MAbs have previously been shown to inhibit E-IgG binding and/or ingestion by mouse peritoneal macrophages, human polymorphonuclear leukocytes, and human monocytes to a variable degree (46–48). Our finding that the capacity of anti-CR3 MAbs to inhibit monocyte phagocytosis of E-IgG is epitope-specific is consistent with that of Brown et al. (48) who found that MAbs M1/70 and Mo-1 but not OKM1 inhibited monocyte binding and ingestion of E-IgG.

Complement receptors have been found to play a role in mediating phagocytosis of other intracellular parasites both in the presence and absence of fresh serum. In the presence of fresh serum, CR3 has been found to be important in mediating uptake of *Legionella pneumophila* (1) *Mycobacterium tuberculosis* (2), *Leishmania donovani* (4), and *Leishmania* major (6), and CR1 has been found to be important in mediating uptake of *L. pneumophila* (1) and *M. tuberculosis* (2). In the absence of serum, CR3 has also been found to be important in mediating uptake of *Leishmania donovani* (4, 5), and CR3, LFA-1, and p150,95, all of which share a common β-chain, have been found to mediate attachment of *H. capsulatum* (3). In our experiments, MAbs against LFA-1 and p150,95 did not significantly inhibit *M. leprae* adherence in the presence of fresh serum. Furthermore, IB4, a MAb against the shared β-chain of CR3, LFA-1, and p150,95 did not inhibit *M. leprae* adherence to a greater extent than single MAbs against the α-chain of CR3.

M. leprae adherence to monocytes was markedly increased in the presence of fresh serum in our study. Compared with the level of adherence in 2.5% fresh serum, adherence was reduced by nearly 70% in the absence of serum. Where the comparison has been made, adherence of other intracellular parasites to mononuclear phagocytes has been greater in the presence of fresh serum than in its absence (1, 2, 4, 6). Inasmuch as serum and/or complement components are ubiquitous in vivo, it seems important to evaluate the relative contributions of host cell surface receptors to phagocytosis in the presence and in the absence of serum. Our study shows that complement receptors mediate *M. leprae* adherence even in the absence of serum. This may reflect interactions between complement receptors and native ligands on the *M. leprae* surface under these conditions. Alternatively, complement components secreted by the monocytes during the course of the assay may result in C3 fixation to the *M. leprae* surface. C3 may thus mediate bacterial adherence even in the absence of serum. Along these lines, Wozencraft et al. (9) have shown that complement components secreted by murine resident peritoneal macrophages result in C3 fixation to *Leishmania donovani* within 20 minutes of the addition of the parasites.

The relative importance of different receptors in mediating ingestion may vary with serum conditions. Blackwell et al. (4) reported that, in the absence of serum, blocking the CR3 or the mannosyl-fucosyl receptor results in equivalent inhibition (50–80%) of *L. donovani* promastigote binding and ingestion. In contrast, in the presence of fresh serum, blocking CR3 results in 70–95% inhibition whereas blocking the mannosyl-fucosyl receptor results in only 35% inhibition (4).

MAbs against complement receptors inhibit ingestion as well as attachment. When we infected monocyte monolayers with *M. leprae,* essentially as in the adherence assay, and then examined them by electron microscopy, we found all of the monocyte-associated *M. leprae* in intracellular membrane-bound phagosomes in monocytes in the presence or absence of MAbs. The number of *M. leprae* in monocytes incubated in the presence of MAbs against CR3 was markedly reduced in comparison to the number in control monocytes.

As previously suggested, complement receptors may be the preferred route of entry for intracellular pathogens by allowing them to avoid the toxic consequences of the oxidative burst or the release of mediators of inflammation that otherwise may accompany phagocytosis (1). Along these lines, Holzer et al. (49) have reported that ingestion of *M. leprae* by human neutrophils and monocytes fails to stimulate significant superoxide anion release (49).

Other receptors reported to mediate ingestion of various particles including microorganisms by monocytes appeared to play little or no role in mediating monocyte ingestion of *M. leprae* in our study. We evaluated the role of Fc receptors by selectively modulating them to the basal surface of the monocyte. Under these conditions, E-IgG ingestion was completely abolished but *M. leprae* adherence was only minimally decreased. In other experiments, we were unable to demonstrate any significant role in *M. leprae* adherence for the β-glucan-inhibitable receptor for unopsonized zymosan.

Our study provides strong evidence that complement component C3, fixed to the bacterial surface via the alternative pathway of complement activation, serves as a major ligand for complement receptors mediating ingestion of *M. leprae.* Ramanathan, et al. has previously shown that *M. leprae* can activate the alternative complement pathway in fresh serum (50). We initially determined that *M. leprae* adherence is serum dependent and that heat inactivation of serum results in a markedly reduced level of adherence. We then determined that preopsonization of *M. leprae* in fresh serum significantly enhances the level of adherence compared to unopsonized organisms. As activation products of complement component C3 are known to serve as ligands for complement receptors, we studied the role of C3 in adherence. We established that adherence of *M. leprae* is C3 dependent and is greatly enhanced when C3 is added back to C3-depleted serum. Utilizing a whole bacterial cell ELISA, we then directly demonstrated the presence of C3 on the bacteria. Utilizing our ELISA further, we demonstrated that C3 is fixed to *M. leprae* by the alternative complement pathway. Finally, we directly documented the importance of the alternative complement pathway in *M. leprae* adherence by demonstrating that adherence is markedly reduced in the presence of factor B-depleted serum and restored to control levels when purified factor B is added back.

In addition to *M. leprae,* complement component C3 has been shown to be important in mediating phagocytosis of other intracellular pathogens including *L. pneumophila* (1), *M. tuberculosis* (2), *M. avium* complex (11), and *Leishmania sp* (6, 9, 10).

M. leprae are ingested by a conventional form of phagocytosis as studied by electron microscopy. Monocyte pseudopodia appear to extend circumferentially about the organism until they meet at the distal side. Simultaneously, the cytoplasm at the zone of contact between the monocyte and the bacterium is displaced such that the bacterium appears to sink into the phagocyte. Other intracellular pathogens ingested by conventional phagocytosis include *M. tuberculosis, Trypanosoma cruzi,* and *Toxoplama gondii* (2, 21–23). In contrast, the intracellular pathogens *L. pneumophila* and *L. donovani* are ingested by coiling phagocytosis (19, 20). The significance of the method of ingestion to subsequent intracellular events is not known.

In conclusion, monocyte complement receptors CR1 and CR3 and serum complement component C3 mediate ingestion of *M. leprae.* This important intracellular pathogen thus takes advantage of a host receptor–ligand system to parasitize host mononuclear phagocytes.

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