PHAGOCYTOSIS OF *LEGIONELLA PNEUMOPHILA* IS MEDIATED BY HUMAN MONOCYTE COMPLEMENT RECEPTORS

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Intracellular bacterial pathogens, such as the agents of Legionnaires' disease, tuberculosis, leprosy, and psittacosis, multiply within and destroy host mononuclear phagocytes. The receptors on the mononuclear phagocytes and the ligands on the bacteria mediating uptake of these human pathogens have not been described.

In this study, we have examined receptors involved in the phagocytosis of *Legionella pneumophila*, an intracellular bacterial pathogen that multiplies in human monocytes and alveolar macrophages (1, 2) and causes Legionnaires' disease. Monocytes phagocytize *L. pneumophila* by an unusual process termed "coiling phagocytosis" in which pseudopods coil around the organism as it is internalized (3). After entry into monocytes, *L. pneumophila* is enclosed within a specialized phagosome, formation of which involves the sequential interaction of the phagosome with monocyte smooth vesicles, mitochondria, and ribosomes (4). *L. pneumophila* multiplies in these phagosomes, which do not fuse with lysosomes (5) nor undergo normal acidification (6). *L. pneumophila* multiplies intraphagosomally until the monocyte ruptures.

We report in this paper that monocyte complement receptors (CR),[1] CR1 and CR3, mediate phagocytosis of *L. pneumophila*; mAbs against these receptors inhibit adherence of *L. pneumophila* to monocytes and, subsequently, the ability to enter monocytes and multiply intracellularly.

Materials and Methods

Media. Charcoal yeast extract agar, egg yolk buffer with or without 1% BSA, and RPMI 1640 were made or purchased as described (1). No antibiotics were used in the cell cultures.

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[1] *Abbreviations used in this paper:* E-C3b, C3b-coated sheep erythrocytes; E-C3bi: C3bi-coated sheep erythrocytes; E-IgG: IgG-coated sheep erythrocytes; HSA, human serum albumin; CR3, complement receptor type 3; CR1, complement receptor type 1.

Reagents. Mannan (No. 3640, Sigma Chemical Co., St. Louis, MO) was suspended at 40 mg/ml and used at a final concentration of 4–6 mg/ml in adherence assays. Human fibronectin (M_r 440,000, No. 341635; Calbiochem-Behring Corp., La Jolla, CA) was added to monocyte monolayers in selected experiments at a final concentration of 200 μg/ml.

Sera. Sera from immune and nonimmune adult volunteers were separated, filtered, stored, and handled in such a manner as to preserve complement activity (4). When required, complement was inactivated by incubating at 56°C for 30 min. Nonimmune sera had an indirect fluorescent antibody titer of ≤1:64 (4); immune sera had a titer of 1:512.

Monocytes. Mononuclear cells were isolated from heparinized blood on Ficoll–sodium diatrizoate gradients, and the monocyte fraction was cultured in 16-mm diameter flat-bottomed wells (Linbro; Flow Laboratories, Inc., McLean, VA) or on No. 2 glass cover slips in RPMI 1640 with 20% autologous or AB, nonimmune serum (1, 7). Monolayers contained ~10^5 monocytes per coverslip or culture dish well. In experiments designed to modulate Fc receptors, mononuclear cell suspensions were prepared as above, diluted to a concentration of 3×10^6 cells/mm^2, cultured for 24 h in Teflon wells (Savillex Corp. Minnetonka, MN), and plated on antigen-antibody-coated or control cover slips.

Bacteria. L. pneumophila, Philadelphia 1 strain, was grown in embryonated hens' eggs, passed once only on charcoal yeast extract agar, and suspended at the working concentration in egg yolk buffer (1).

Antibodies. mAbs anti-CRI, anti-CRII, anti–transferrin receptor (Becton Dickinson Immunocytometry Systems, Mountain View, CA), and OKM-1 (Ortho Diagnostic Systems Inc., Westwood, MA), were purchased. mAb OKM-10 was generously provided by Dr. Gideon Goldstein, Ortho Pharmaceuticals, Raritan, NJ. mAb D-12 (equivalent to Becton Dickinson's Leu-15) in ascitic fluid was generously donated by Dr. Loran Clement, Department of Pediatrics, UCLA School of Medicine. mAbs OKM-1 (IgG2b), OKM-10 (IgG2), and D-12 (IgG2a) are all directed against the CR3 receptor for C3 fragments (8–10). Anti-CR1 (IgG1) is directed against the C3b or CRI receptor (11), anti-CRII (IgG2a) is directed against the C3d or CR2 receptor (12), anti–transferrin receptor (IgG2a) is directed against the human transferrin receptor (13), and anti-DR (IgG2a) is directed against the DR antigen (13). All commercially obtained mAbs were dialyzed twice against ≥100 volumes of PBS to remove azide.

FITC-labeled anti-C3 and anti-IgG were purchased from Atlantic Antibodies, Scarborough, ME. Rabbit anti–sheep RBC IgG was the generous gift of Dr. Samuel Wright, The Rockefeller University, New York. FITC anti-*L. pneumophila* rabbit Ig was obtained from the Centers for Disease Control, Atlanta, GA (lot 82-0073). Nonspecific mouse IgG was obtained from Cappel Laboratories, Malvern, PA.

Assay for Inhibition of L. pneumophila Adherence to Monocytes by mAbs. Monocyte monolayers were cultured on plain glass coverslips in RPMI medium containing either 20% heat-inactivated, or 20% fresh serum at 37°C in 5% CO_2–95% air for 24 h and then incubated at 37°C for 30 min with various mAbs in concentrations varying from 0.5 to >50 μg/ml. Then, 10^8 CFU of *L. pneumophila* were added to the monolayer (bacteria/monocyte ratio, 200:1) and the mixture was incubated at 100 rpm on a rotating platform at 37°C for 60 min in 5% CO_2–95% air. The monolayers were then washed vigorously and fixed in 10% formalin. *L. pneumophila* were stained with fluorescein-labeled anti-*L. pneumophila* antibody and the percentage of monocytes with ≥1 adherent bacterium and the average number of bacteria per monocyte on each cover slip were determined by counting a minimum of 200 monocytes per cover slip under fluorescence microscopy (Fluophot; Nikon, Tokyo, Japan).

In selected experiments, monocytes were incubated with *L. pneumophila* in the absence of serum, in which case 10–20 mM Hepes was added to the medium. In experiments requiring preopsonization of bacteria, *L. pneumophila* were opsonized in 20% fresh, nonimmune serum, washed twice in RPMI, and resuspended to the original concentration before addition to the monocyte monolayer. In some of these experiments, preopsonized bacteria were heat-treated for 30 min at 56°C before addition to monocytes.

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Assay for Modulation of Monocyte CR1, CR3, and Fc Receptors. Cover slips coated with *L. pneumophila* or *Escherichia coli* membranes were prepared by adhering the membranes, isolated as described (14), to glass cover slips by the method of Michl et al. (15) using poly-L-lysine (70,000 M_r; Sigma Chemical Co.) and 2.5% glutaraldehyde. Control cover slips were prepared the same way but without bacterial membranes. IgG-coated sheep erythrocytes (E-IgG) were prepared by incubating a 5% suspension of washed erythrocytes at 37°C for 30 min with an equal volume of rabbit anti-sheep IgG diluted 1:199 in PBS without calcium and magnesium. Erythrocytes coated with C3b (E-C3b) or C3bi (E-C3bi) were prepared as described (16).

Monocytes were plated on coverslips coated with bacterial membranes or on control cover slips and incubated with E-IgG, E-C3b, or E-C3bi at an erythrocytes/monocyte ratio of 80:1 at 37°C for 90 min without agitation in the presence of heat-inactivated serum. The monocytes were washed and the number of E-C3b or E-C3bi bound to monocytes or the number of E-IgG bound or ingested by monocytes was enumerated. Ingested E-IgG were enumerated after hypotonic lysis of extracellular E-IgG. At least 200 monocytes on each of duplicate or triplicate cover slips were evaluated.

In separate experiments designed to assess the importance of the Fc receptor to phagocytosis of *L. pneumophila*, monocytes were plated on coverslips coated with antigen-antibody complexes. Human serum albumin (HSA; Calbiochem-Behring Corp.) at 1 mg/ml was attached to No. 2 glass coverslips using the method of Michl et al. (15), followed by blocking of unreacted sites with 0.2 M glycine in 0.01 M sodium-phosphate buffer (pH 7.2), washing with PBS, and finally the addition of rabbit anti-HSA at 1 mg/ml for 30 min. The coverslips were then washed with RPMI and monocytes were allowed to attach for 60 min at 37°C. Control coverslips were coated with HSA alone.

Assay for Intracellular Multiplication of L. pneumophila. Monocyte monolayers were cultured for 24 or 48 h in 20% heat-inactivated, AB, nonimmune serum and then incubated at 37°C for 30 min in 5% CO_2–95% air with or without mAbs (11 μg/ml) or mouse IgG (168 μg/ml). The cultures were infected with *L. pneumophila* (10^4 CFU) and assayed for CFU daily for 3 d. Monolayers were also inspected daily for evidence of cell damage.

In selected experiments designed to evaluate the influence of mAbs on multiplication of intracellular *L. pneumophila*, anti-CR3 was added after the monocytes were infected with *L. pneumophila* rather than before. Monocytes were infected, washed to remove extracellular bacteria, and treated with anti-CR3 (11 μg/ml).

Electron Microscopy. Fresh monocytes treated with anti-CR3, anti-CR1, or anti-HLA-DR at a concentration of 20 μg/ml for 30 min at 37°C, were washed and then combined with *L. pneumophila* at a bacteria/monocyte ratio of 1,000:1 in the presence of fresh nonimmune serum. The samples and a control specimen with monocytes not exposed to mAbs were then incubated and prepared for electron microscopy as described (3). At least 100 monocytes from each sample were examined.

Statistical Analysis. Mean, standard deviation, standard error, and t-statistics were calculated using standard formulas.

Results

Monocyte CR1 and CR3 Receptors Mediate Adherence of L. pneumophila. To determine if any of several known monocyte receptors, and in particular complement receptors, mediate adherence of *L. pneumophila*, we examined the capacity of mAbs against several monocyte receptor antigens to inhibit the adherence of live *L. pneumophila* to monocytes in monolayer culture. A representative experiment is shown in Table I and cumulative data using eight mAbs is shown in Table II. mAbs against the CR3 and CR1 receptors, which recognize C3bi and C3b, respectively, strongly and consistently inhibited *L. pneumophila* adherence to monocytes. Anti-CR3 antibodies D-12 (Leu-15), OKM-1, and OKM-10 inhibited *L. pneumophila* adherence by 64–74% and anti-CR1 antibod-

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TABLE I

mAbs Against CR1 and CR3 Receptors Inhibit L. pneumophila Adherence to Monocytes

Monoclonal antibody	Target antigen	Monocytes with ≥1 adherent *L. pneumophila* ± SD	Mean number *L. pneumophila*/monocyte ± SD	Inhibition of adherence (decrease from control)
		%		%
None (Control)		81.0 ± 0.1	3.29 ± 0.42	0
D-12 (Leu 15)	CR3	32.1 ± 6.6	0.60 ± 0.10	82
Anti-CR1	CR1	42.0 ± 2.3	0.97 ± 0.02	71
Anti-CR1/D-12	CR1 & CR3	22.7 ± 4.1	0.37 ± 0.15	89
Anti-transferrin receptor	Human transferrin receptor	77.0 ± 5.8	2.75 ± 0.84	16
Anti-CRII	CR2	78.1 ± 0.2	3.02 ± 0.20	8

Monocytes in monolayer culture on coverslips were incubated with *L. pneumophila* in the presence of the mAbs indicated at a concentration of 11 μg/ml or without mAb (control). The percentage of monocytes with adherent bacteria and the average number of bacteria per monocyte were determined by fluorescence microscopy. Data are the mean of duplicate or triplicate coverslips.

TABLE II

Inhibition of Legionella pneumophila adherence to Monocytes by mAbs

Monoclonal antibody	Target antigen	Ig Subtype	Inhibition of *L. pneumophila* adherence (Mean ± SE)*
			%
D-12 (Leu-15)	CR3	IgG2a	74 ± 7
OKM-10	CR3	IgG2	67 ± 12
OKM-1	CR3	IgG2b	64 ± 8
Anti-CR1	CR1	IgG1	68 ± 1
Anti-CR1/D-12	CR1/CR3	IgG1/IgG2a	73 ± 9
Anti-transferrin receptor	Human transferrin receptor	IgG2a	29 ± 7
Anti-CRII	CR2	IgG2a	16 ± 6
Anti-HLA-DR	HLA-DR	IgG2a	12 ± 9
Mouse IgG	Nonspecific	—	16 ± 7

L. pneumophila adherence to monocytes in the presence of the various mAbs indicated was assayed as in Table I. The mAbs were used at a concentration of 11 μg/ml, and the mouse IgG was used at a concentration of 168 μg/ml. Data for each antibody are the mean inhibition of *L. pneumophila* adherence ± SE obtained in two to nine independent experiments, each of which was done in duplicate or triplicate.

* Percent decrease in mean number of *L. pneumophila* per monocyte from control monocytes incubated without antibody.

ies inhibited by 68% (Table II). In most experiments, a combination of anti-CR3 and anti-CR1 antibodies resulted in little or no greater inhibition (mean 73%) than anti-CR3 or anti-CR1 antibody alone. mAbs against the DR antigen, the CR2 receptor (17, 18) and the transferrin receptor, and nonspecific mouse IgG had only modest effects on *L. pneumophila* adherence (average inhibition 12–29%, Table II).

The IgG subtype was not relevant to the capacity of mAbs to inhibit adherence. The three anti-CR3 antibodies, all of which strongly inhibited adherence, were of two different subtypes (2a and 2b). mAbs against the HLA-DR antigen,

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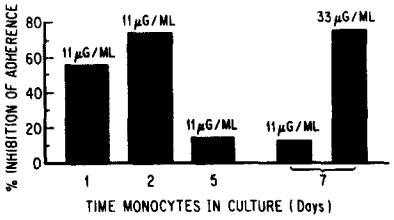

FIGURE 1. Capacity of anti-CR3 mAb (D-12) to inhibit the adherence of *L. pneumophila* to monocytes kept in culture for various lengths of time. Monocytes were cultured for 1, 2, 5, or 7 d and incubated with anti-CR3 antibody at the concentration indicated above the bars for 30 minutes at 37°C, and infected with *L. pneumophila* as noted in Tables I and II. 11 μg/ml of anti-CR3 strongly inhibited *L. pneumophila* adherence to monocytes cultured for 1 or 2 d but not 5 or 7 d. A higher dose, 33 μg/ml, was required to inhibit strongly adherence to monocytes cultured for 7 d. Results are representative of two separate assays each done in duplicate or triplicate.

transferrin receptor, and CR2 receptor that had minimal effects on adherence, were of the same subtype (IgG2a) as anti-CR3 antibody D-12, which had the strongest inhibitory effect on adherence.

Anti-CR3 antibodies inhibited *L. pneumophila* in a dose-dependent fashion and at relatively low concentrations. As little as 1 μg/ml of D-12 and 2 μg/ml of OKM-10 inhibited adherence by 67 and 88%, respectively. The inhibitory effects plateaued at 5–10 μg/ml with all three anti-CR3 antibodies, and did not increase above 90% inhibition even with concentrations of D-12 or OKM-10 of ≥80 μg/ml.

The anti-CR3 receptor antibodies were not lethal to monocytes. After incubation with as high a concentration as 44 μg/ml of D-12, 98% of monocytes excluded trypan blue. Moreover, anti-CR3 receptor antibodies did not inhibit monocyte ingestion of *L. pneumophila* when the bacteria were coated with antibody and complement (see below).

Specific antibody in the presence of complement greatly enhances phagocytosis of *L. pneumophila* by monocytes (19). Interestingly, mAb against the monocyte CR3 receptor inhibited binding of such antibody-coated bacteria to monocytes by only 12 ± 8% (mean ± SE) in the presence of fresh serum. One explanation for this may be that monocyte receptors for the Fc portion of Ig are able to mediate adherence of such antibody-coated bacteria. Alternatively, the amount of C3 fixed to the bacteria in the presence of specific antibody may be great enough to overcome the CR3 receptor inhibition induced by the mAbs.

Monocyte monolayers cultured for 7 d required more anti-CR3 mAb than did monolayers cultured for 1 d to achieve the same degree of inhibition of *L. pneumophila* adherence (Fig. 1). Whereas 11 μg/ml of anti-CR3 antibody inhibited *L. pneumophila* adherence to monocytes cultured for 1 d by 56% and for 2 d by 74%, this amount of anti-CR3 antibody inhibited *L. pneumophila* adherence to monocytes cultured for 7 d by only 13% in the same experiment. Increasing the amount of anti-CR3 antibody to 33 μg/ml resulted in 74% inhibition of *L. pneumophila* adherence to 7-d monocytes, similar to that seen with 1-d monocytes (Fig. 1). The amount of anti-CR1 antibody required to inhibit *L. pneumophila* adherence was not different in monocytes cultured for 1 or 7 d.

We used electron microscopy to determine if anti-CR3 altered coiling phagocytosis or resulted in an increased number of attached but uningested bacteria. The decreased number of bacteria ingested by monocytes in the presence of anti-CR3 were still ingested by coiling phagocytosis, which did not differ qualitatively from that seen in control specimens or from that reported previously

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TABLE III

Modulation of Fc Receptors by Antigen-Antibody Complexes Does Not Significantly Reduce Legionella pneumophila Attachment

Substrate	*L. pneumophila* per monocyte		IgG-coated sheep RBC per monocyte		
	Bacteria preopsonized in nonimmune fresh serum	Bacteria preopsonized in immune heated serum*	Ingested	Rosetted	Total
HSA‡	0.22	0.88	1.53	2.65	4.18
HSA/Anti-HSA	0.15	0.21	0.04	0.75	0.79
Percent Reduction	32%	76%	97%	72%	81%

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, IgG-coated *L. pneumophila* (bacteria preopsonized in high-titer immune serum) or *L. pneumophila* not coated with detectable IgG (bacteria preopsonized in nonimmune serum lacking detectable antibody), and assayed for associated RBC or *L. pneumophila* as described in Materials and Methods. Data represent the mean of duplicate measurements. Monocytes plated on antigen-antibody complexes displayed a marked reduction in capacity to bind or ingest IgG-coated RBC (columns 4–6) or IgG-coated *L. pneumophila* (column 3), but only a slight reduction in capacity to bind *L. pneumophila* not coated with IgG (column 2). This experiment is representative of three independent experiments.

* IFA titer = 1:20,000. Serum was heated at 56°C for 60 min before use.

‡ Human serum albumin.

(4). In the presence of anti-CR3 there was no increase in the proportion of bacteria that were attached but not ingested, suggesting that anti-CR3 inhibits phagocytosis primarily by decreasing adherence to the monocytes.

The role of Fc receptors was thought to be small in these experiments in which bacteria were opsonized in nonimmune sera. To confirm this, we investigated the influence of modulating monocyte FcR on *L. pneumophila* adherence to monocytes. We modulated FcR by plating monocytes on antigen-antibody complexes consisting of HSA and anti-HSA. Whereas monocytes plated on antigen-antibody complexes bound or ingested 87 ± 8.1% fewer E-IgG, such monocytes bound only 28 ± 5.5% fewer *L. pneumophila* than did control monolayers plated on HSA alone (Table III). The small decrease in *L. pneumophila* adherence after FcR modulation confirmed that FcR play little role in the phagocytosis of *L. pneumophila* after opsonization with nonimmune serum.

Mannan at 4–6 mg/ml final concentration did not significantly inhibit *L. pneumophila* adherence to monocytes (28 ± 16% inhibition). Addition of fibronectin at a final concentration of 200 μg/ml did not increase unopsonized *L. pneumophila* adherence.

L. pneumophila Membrane Substrates Modulate Monocyte CR1 and CR3 Receptors and Monocyte Phagocytosis of L. pneumophila. To further examine the interaction between the *L. pneumophila* surface and monocyte CR1 and CR3 receptors, we plated monocytes on *L. pneumophila* membrane substrates and examined the capacity of these monocytes to bind complement-coated erythrocytes. We reasoned that if *L. pneumophila* binds to monocyte CR1 and CR3 receptors, the CR1 and CR3 receptors of monocytes plated on *L. pneumophila* membrane substrates would migrate to the undersurface of the monocyte and be unavailable for binding C3b and C3bi-coated erythrocytes. Indeed, monocytes plated on *L. pneumophila* membrane substrates bound 70 ± 15% fewer C3b-coated erythro-

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TABLE IV

L. pneumophila Membrane Substrates Modulate Monocyte CR1 and CR3 Receptors

Exp.	Monocytes	Monocyte binding of E-C3b, E-C3bi, and E-IgG and ingestion of E-IgG*			
		Bound			Ingested
		E-C3b	E-C3bi	E-IgG	E-IgG
1	Control monocytes	1.58 ± 0.11	0.18 ± 0.08	1.19 ± 0.19	1.97 ± 0.60
	Monocytes plated on *L. pneumophila* membranes	0.35 ± 0.01	0.07 ± 0.05	0.76 ± 0.23	1.60 ± 0.47
2	Control monocytes	0.53 ± 0.30	0.10 ± 0.02	1.96 ± 0.01	2.90 ± 0.33
	Monocytes plated on *L. pneumophila* membranes	0.05 ± 0.08	0.03 ± 0.01	1.78 ± 0.84	2.04 ± 0.09
3	Control monocytes	1.42 ± 0.65	0.60 ± 0.12	0.43 ± 0.18	3.20 ± 0.05
	Monocytes plated on *L. pneumophila* membranes	0.83 ± 0.22	0.44 ± 0.06	0.50 ± 0.13	2.99 ± 0.15
	Mean percent inhibition from control ± SD	70 ± 15	53 ± 23	13 ± 22	19 ± 12

Monocytes were cultured on coverslips coated with *L. pneumophila* membrane substrates or on control coverslips and then incubated with E-C3b, E-C3bi, or E-IgG. The monocytes were washed to remove nonadherent erythrocytes, and the number of erythrocytes of each type bound per monocyte and the number of E-IgG ingested per monocyte were enumerated. Data are the mean for duplicate or triplicate coverslips; 200–600 monocytes per coverslip were enumerated.

* Mean number of erythrocytes per monocyte ± SD.

cytes and 53 ± 23% fewer C3bi-coated erythrocytes than control monocytes (Table IV). In contrast, monocytes bound to *L. pneumophila* substrates and control monocytes bound and ingested comparable numbers of IgG-coated erythrocytes, indicating that the *L. pneumophila* membranes do not induce a generalized inhibition of adherence or phagocytosis.

L. pneumophila membrane substrates also modulate the ability of monocytes to phagocytize live *L. pneumophila*. Monocytes plated on these substrates bound 87–92% fewer live *L. pneumophila* than control monolayers. For example, in a representative experiment, control monocytes bound 0.43 bacteria per monocyte, monocytes plated on *E. coli* (K-12) membranes bound 0.39 bacteria per monocyte, whereas monocytes plated on *L. pneumophila* membranes bound only 0.04 bacteria per monocyte, a reduction of >1 $\log_{10}$.

L. pneumophila Adherence Correlates with Fixation of Complement Component C3 to the Bacterial Surface. The finding that complement receptors CR1 and CR3 mediate *L. pneumophila* adherence implied a role for C3 or a C3-like molecule on the *L. pneumophila* surface. To investigate this, we first measured adherence of *L. pneumophila* in the presence of fresh serum, heat-inactivated serum, or no serum (Table V). Adherence of *L. pneumophila* was markedly reduced in heat-inactivated serum (84% reduction) or in the absence of serum (97% reduction; Table V).

To confirm that this reduction in adherence was due to the absence of an opsonin on the bacterial surface, we examined the adherence of *L. pneumophila*

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TABLE V

Complement Fixation Correlates with Adherence of L. pneumophila to Monocytes: Adherence of L. pneumophila to Monocytes in Fresh, Heat-inactivated, and No Serum

Serum	Bacteria per monocyte (mean ± SD)	Inhibition of adherence*
		%
Fresh serum	4.23 ± 0.21	
Heat-inactivated serum	0.64 ± 0.18	85
No serum	0.11 ± 0.03	97

Monocytes in monolayer culture were incubated with *L. pneumophila* in the presence of fresh, heat-inactivated, or no serum and the mean number of adherent bacteria per monocyte was enumerated. Data are the mean ± SD for triplicate coverslips; at least 200 monocytes per coverslip were enumerated.

* Percent decrease in mean number of bacteria per monocyte for *L. pneumophila* incubated in heat-inactivated or no serum in comparison to *L. pneumophila* incubated in fresh serum.

TABLE VI

Complement Fixation Correlates with Adherence of L. pneumophila to Monocytes: Adherence of Preopsonized and Unopsonized L. pneumophila to Monocytes in the Presence of Heat-inactivated Serum

L. pneumophila	Bacteria per monocyte (mean ± SD)	Inhibition of adherence*
		%
Preopsonized	3.45 ± 0.12	—
Unopsonized	0.19 ± 0.03	95

Monocytes were incubated with preopsonized or unopsonized *L. pneumophila* in the presence of heat-inactivated serum and the number of adherent bacteria per monocyte was enumerated as above. Preopsonized *L. pneumophila* were obtained by incubating the bacteria in fresh nonimmune serum and washing them. Data are the mean ± SD for triplicate coverslips; at least 200 monocytes per coverslip were enumerated.

* Percent decrease in mean number of bacteria per monocyte for unopsonized *L. pneumophila* in comparison to preopsonized *L. pneumophila*.

that were preopsonized. *L. pneumophila* were incubated in fresh nonimmune serum, vigorously washed, and then added to the monocytes in the presence of heat-inactivated serum. Such preopsonized *L. pneumophila* were readily bound by monocytes (Table VI). In contrast, unopsonized *L. pneumophila* that were incubated with monocytes under the same conditions were poorly bound; adherence of unopsonized *L. pneumophila* was 18-fold less than that of preopsonized bacteria. Thus monocytes appeared to phagocytize *L. pneumophila* very efficiently in heat-inactivated serum, provided the bacteria were preopsonized.

These studies suggested that adherence of *L. pneumophila* to monocytes was dependent on the deposition of a serum opsonin that was either heat-labile itself or was deposited by a heat-labile system of proteins such as complement. To further define this, we studied the adherence of *L. pneumophila* that had been heated to 56°C for 30 min after opsonization in fresh, nonimmune serum.

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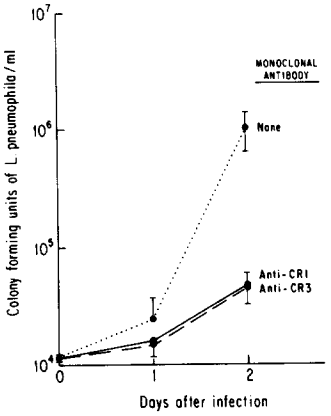

FIGURE 2. Inhibition of intracellular multiplication of *L. pneumophila* by mAbs directed against monocyte membrane CR1 or CR3 receptors which recognize the third component of complement. Monocyte monolayers were incubated with mAbs for 30 min at 37°C before infection with *L. pneumophila*. CFU of *L. pneumophila* in the cultures were assayed immediately after infection and daily thereafter for 2 d. Data are the mean ± SE for triplicate cultures and representative of two separate experiments.

Monocytes bound heat-treated, preopsonized *L. pneumophila* (0.34 ± 0.12 bacteria per monocyte) as avidly as untreated preopsonized *L. pneumophila* (0.34 ± 0.23 bacteria per monocyte). Therefore, heat inactivation of serum decreases the deposition of serum opsonins but does not alter them after they have bound to the bacterial surface.

These experiments were consistent with the hypothesis that fragments of complement component C3 are the primary ligands recognized by CR1 and CR3 and that these ligands mediate adherence of *L. pneumophila* to monocytes. Recent studies from this laboratory (20) have demonstrated by ELISA and by Western blot analysis that fragments of complement component C3 fix to the *L. pneumophila* surface when the bacteria are incubated with fresh nonimmune serum; relatively little C3 fixes to the bacterial surface in the presence of heat-inactivated serum.

mAbs Against Monocyte CR1 and CR3 Receptors Inhibit L. pneumophila Intracellular Multiplication. Since *L. pneumophila* multiplication is exclusively intracellular under tissue culture conditions, mAbs that inhibit *L. pneumophila* phagocytosis should also inhibit multiplication in monocytes by decreasing bacterial access to the intracellular milieu of monocytes. We tested this hypothesis by adding mAbs against CR1 and CR3 receptors to monocyte cultures before infecting the monocytes with *L. pneumophila* (Fig. 2). Indeed, in the presence of mAb against the CR3 receptor (D12 or OKM1) or the CR1 receptor, *L. pneumophila* multiplication in monocyte monolayers was strongly inhibited. The presence of large amounts (168 μg/ml) of nonspecific mouse IgG had no inhibitory effect on *L. pneumophila* multiplication. These results provided an independent confirmation of the adherence assay used above.

To determine if mAbs against CR1 or CR3 receptors inhibit intracellular multiplication of *L. pneumophila* that have already been ingested, we added the mAbs to monocyte monolayers after the monocytes were infected. Addition of mAbs under these conditions resulted in no decrease in intracellular multiplication of ingested bacteria, indicating that mAbs have no effect on bacterial multiplication after the bacteria are internalized.

Paralleling these results, monocyte monolayers infected with *L. pneumophila* in the presence of anti-CR1 and anti-CR3 mAbs were protected from destruction.

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3 d after infection, control monocyte monolayers were completely destroyed whereas monocyte monolayers treated with the anti-CR1 and with anti-CR3 receptor antibodies remained intact.

Discussion

This study demonstrates that monocyte CR1 and CR3 receptors mediate phagocytosis of *L. pneumophila.* The extent to which these receptors mediate phagocytosis of other bacterial intracellular pathogens is unknown. However, preliminary studies indicate that complement receptors and complement mediate phagocytosis of *Mycobacterium tuberculosis* (21). CR3 as well as LFA-1 and p150,95 antigens have been shown to bind *E. coli* (22) and *Histoplasma capsulatum* (23). Furthermore, monocyte CR3 and mannosyl-fucosyl receptors have been shown to mediate phagocytosis of promastigotes of the intracellular parasite *Leishmania donovani* (24). Thus, complement receptors may play a general role in mediating phagocytosis of intracellular pathogens.

Whether monocyte complement receptors play a role in inducing the coiling phenomenon that characterizes phagocytosis of *L. pneumophila* is unclear. Interestingly, ingestion of *L. donovani* promastigotes, which as noted above involves complement receptors, may take place by coiling phagocytosis. The whorls of macrophage membranes seen with phagocytosis of *L. donovani* and originally attributed to flagellar motion may instead reflect coiling phagocytosis (25). Equally intriguing, ingestion of *Candida albicans* may take place by coiling phagocytosis, at least under certain circumstances (26), and involve complement receptors. Recently, this organism has been found to have a CR3-like molecule on its surface (16). Thus, complement receptors may be involved in inducing the coiling phenomenon.

mAbs against either CR1 or CR3 receptors strongly inhibited *L. pneumophila* adherence and the simultaneous addition of mAbs against both of these receptors resulted in little or no additional inhibition (Table II).

mAbs against CR3 and CR1 receptors are not known to crossreact. Possibly, the CR1 and CR3 receptors are functionally linked in ways not yet understood or both receptors may be required for phagocytosis of *L. pneumophila.* Along these lines, Blackwell et al. (24) reported that two receptors are involved in the phagocytosis of *L. donovani.* Inhibition of either the CR3 or mannosyl-fucosyl receptors strongly inhibited *L. donovani* adherence and the two antibodies together were not more inhibitory than either antibody alone (24).

In this study, three mAbs directed against the CR3 receptor inhibited *L. pneumophila* adherence. In previous studies, two of these antibodies, OKM-10 and Leu-15, have been shown to inhibit rosetting of complement-coated erythrocytes, but the third antibody, OKM-1, has had variable inhibitory effects on erythrocyte rosetting. Interestingly, OKM-1 had the least inhibitory effect on *L. pneumophila* adherence of the three antibodies (64 ± 8%), but the degree of inhibition was highly significant in comparison to control antibodies (Table II, $p < 0.01$, unpaired t test).

Monocytes and nonactivated macrophages bind but do not ingest complement-coated erythrocytes. However, the situation with erythrocytes may not be generally applicable to microbes. Monocytes readily ingest complement-coated en-

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capsulated *E. coli* in the absence of IgG; ingestion of this encapsulated *E. coli* is strictly dependent on serum ligands (27). Moreover, in the case of *L. pneumophila*, it is possible that receptors in addition to complement receptors participate in phagocytosis.

C3 from two sources, heat-inactivated serum and monocytes, may have been involved in modulating CR1 and CR3 receptors in the presence of *L. pneumophila* membranes. Heat-inactivated serum retains a low but significant capacity to fix C3 to *L. pneumophila* (20). Monocytes secrete complement components of the alternative pathway and C3 from this source may fix to an appropriate target (10).

The complement receptor pathway may be a preferred route of entry for intracellular parasites. Under conditions in which complement receptors for C3b and C3bi mediate phagocytosis, ligation of these receptors does not consistently result in release of superoxide, hydrogen peroxide, or mediators of inflammation such as arachidonic acid (28–30). Thus, entry via complement receptors may allow intracellular parasites to avoid the adverse consequences of the metabolic oxidative burst. Along these lines, *Toxoplasma gondii* has been shown to enter phagocytes without eliciting an oxidative burst (31), although it is not known whether CR1 or CR3 receptors play a role in phagocytosis of this organism. *L. pneumophila*, *T. gondii*, and *C. psittaci* may share a common pathway in mononuclear phagocytes (32, 33). All three organisms reside in phagosomes lined by mitochondria at one stage, all three inhibit phagosome-lysosome fusion, and at least two (*L. pneumophila* and *T. gondii*) inhibit phagosome acidification. It therefore seems possible that these or other intracellular parasites also share common entry mechanisms.

Summary

We have examined receptors mediating phagocytosis of the intracellular bacterial pathogen, *Legionella pneumophila*. Three mAbs against the type 3 complement receptor (CR3), which recognizes C3bi, inhibit adherence of *L. pneumophila* to monocytes by 64 ± 8% to 74 ± 11%. An mAb against the type 1 complement receptor (CR1), which recognizes C3b, inhibits adherence by 68 ± 1%. mAbs against other monocyte surface antigens do not significantly influence adherence. Monocytes plated on substrates of *L. pneumophila* membranes modulate their CR1 and CR3 receptors but not Fc receptors; such monocytes bind 70% fewer C3b-coated erythrocytes and 53% fewer C3bi-coated erythrocytes than control monocytes. Adherence of *L. pneumophila* to monocytes in nonimmune sera is dependent on heat-labile serum opsonins; adherence is markedly reduced in heat-inactivated serum (84% reduction) or buffer alone (97% reduction) compared with fresh serum. mAbs against CR1 and CR3 receptors also inhibit *L. pneumophia* intracellular multiplication and protect monocyte monolayers from destruction by this bacterium. This study demonstrates that human monocyte complement receptors, CR1 and CR3, mediate phagocytosis of *L. pneumophila*. These receptors may play a general role in mediating phagocytosis of intracellular pathogens.

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