ROLE OF THE ADHERENCE-PROMOTING RECEPTORS, CR3, LFA-1, AND p150,95, IN BINDING OF *HISTOPLASMA CAPSULATUM* BY HUMAN MACROPHAGES

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Histoplasma capsulatum is a dimorphic fungal zoopathogen of worldwide distribution that produces a broad spectrum of disease activity. In most cases the portal of entry is the lung, where inhaled spores and mycelial fragments of the fungus lodge within the terminal bronchioles and alveoli. Here, transition from the mycelial phase to the yeast phase ensues, presumably over a period of hours to days. The mycelial to yeast phase conversion is critical since it is the yeast form of *H. capsulatum* (Hc)[1] that is responsible for the pathogenesis of histoplasmosis in nearly all of its clinical manifestations (1).

The earliest pulmonary lesions induced by Hc yeasts in man and experimental animals are patches of pneumonitis consisting largely of parasitized macrophages (Mϕ) (2–4). Hc yeasts are thought to be phagocytized by resident pulmonary Mϕ within which they multiply with a generation time of ~9–11 h (5). Host Mϕ appear to be destroyed by the multiplying yeast forms that in turn, are phagocytosed by other Mϕ attracted to the infected site. The multiplication cycle is repeated within these cells and Hc yeasts are disseminated from the lung to other organs by the lympho-hematogenous route. In most cases, the primary lesions in lung and those at peripheral sites begin to resolve after development of measurable delayed-type hypersensitivity to antigens of Hc at 10–20 d after inoculation.

During an early phase of infection, Mϕ bind to and phagocytose Hc yeasts, thereby providing a highly permissive intracellular environment for multiplication of the organism. Later, however, after the activation of cell-mediated immune defenses, Hc yeasts that are bound and ingested by Mϕ appear to be inhibited or killed. To better understand the crucial process of initial recognition and attachment of Hc yeasts by phagocytic cells, we have studied the receptor-ligand interactions between unopsonized Hc yeasts and human Mϕ derived from peripheral blood monocytes (PBM) of nonimmune donors. The findings reported

This work was supported by grants AI-17339 and AI-22003 from the National Institutes of Health (Bethesda, MD) and grant JFRA-103 from the American Cancer Society. Address correspondence to Ward E. Bullock, M.D., Dept. of Internal Medicine, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0560.

[1] *Abbreviations used in this paper:* AI, attachment index; FcR, Fc receptor; Fn, fibronectin; Hc, *H. capsulatum*; HSA, human serum albumin; Mϕ, macrophages; NBT, nitroblue tetrazolium; PBM, peripheral blood monocytes; PLL, poly-L-lysine; PMN, polymorphonuclear.

here show that Mϕ bind avidly to unopsonized organisms by a mechanism that is both temperature- and cation-dependent. The attachment is mediated by the CR3/LFA-1/p150,95 family of leukocyte adherence-promoting glycoproteins. Each of these receptors independently can mediate binding of Hc yeasts. Neither low-avidity Fc receptors (FcR) nor mannosyl-fucosyl receptors mediate binding of yeast ligands to the Mϕ surface, and binding activity is not caused by opsonization of yeasts with complement components secreted locally by Mϕ.

Materials and Methods

Reagents. mAb 3D9 directed against CR1 (6) was provided by Drs. J. O'Shea and E. Brown, National Institutes of Health, Bethesda, MD; OKM1 and OKM10 (7) directed against the α chain of CR3, 3C10 (8) directed against a 55 kD protein unique to monocytes and Mϕ, and 3G8 (9) against the low-avidity FcR of polymorphonuclear (PMN) leukocytes were prepared as described previously (7, 9). TA-1 was purchased from Hybrid Tech (San Diego, CA), and TS-1/22 was a generous gift of Timothy Springer, Harvard Medical School, Boston, MA. Both TA-1 and TS-1/22 recognize the α chain at LFA-1 (10). Leu-M5 against the α chain of p150,95 (11) was a gift of Dr. Louis Lanier, Becton Dickinson & Co., Mountain View, CA. IB4 against β chain of the CR3/LFA-1/p150,95 glycoproteins was prepared as described (7), and mAb 60.3, also directed against β chain of these glycoproteins (12), was provided by Dr. John Hansen, University of Washington, Seattle, WA. Rabbit polyclonal antibody to human C3 was provided by Dr. Robert Sim, Oxford University, Oxford, United Kingdom. Sigma Chemical Co. (St. Louis, MO) supplied poly-L-lysine (PLL), EDTA, polymyxin B, mannan from *Saccharomyces cerevisiae* X2180, L-fucose, galactose, *N*-acetyl galactosamine, *N*-acetyl-D-glucosamine, D-glucosamine, D-glucuronic acid, α-methyl-D-mannoside, D-mannose, scopoletin, sodium *m*-periodate, horseradish peroxidase P8250 type II, PMA, sodium azide (NaN_3), and aprotinin. β-glucan was a gift of Dr. J. Czop, Harvard Medical School, Boston, MA. Mannosylated BSA was purchased from B-Y Laboratories, San Mateo, CA. The Greater New York Blood Center supplied fibronectin (Fn).

Yeasts. The yeast phase of Hc strain 217B was maintained as described (13). A clinical isolate of *Torulopsis glabrata* was provided by Dr. Timothy Kiehn, Sloan Kettering, New York. The yeasts were grown in brain/heart infusion broth (Difco Laboratories Inc., Detroit, MI) at 37°C with a gyrorotatory speed of 180 rpm. Cultures of Hc and *T. glabrata* were harvested after 36 and 18 h, respectively, and washed twice with PBS by centrifugation at 327 *g* for 5 min. A final centrifugation at 30 *g* for 1 min allowed for removal of larger cell aggregates. The yeasts were counted in a hemocytometer and resuspended in PBS to 2.2×10^8 organisms/ml for addition to Mϕ monolayers.

Cells. Human monocytes were purified from buffy coats on Percoll gradients and cultured in 12.5% human serum in Teflon beakers as described previously (14). Sheep E coated with C3b (EC3b), C3bi (EC3bi), or IgG (EIgG) were prepared as described (14).

Attachment of Unopsonized Yeasts and Ligand-coated Erythrocytes. Mϕ were harvested from teflon beakers after 4–7 d, washed, and suspended at $0.5–1.0 \times 10^6$/ml in PBS containing 3 mM glucose, 0.5 mg/ml human serum albumin (HSA) (Worthington Biochemical Corp., Freehold, NJ), and 0.3 U/ml aprotinin. Terasaki tissue culture plates (Miles Laboratories Inc., Naperville, IL) were coated with HSA (1 mg/ml) or mAbs (20–50 μg/ml) by a 60-min incubation at 20°C. The culture surfaces were washed, 5 μl of Mϕ suspension were added per well, and cells were allowed to spread at 37°C for 45 min. Attachment of test particles to monolayers of Mϕ was determined by adding 10^6 yeasts or 5×10^5 ligand-coated E per well and incubating for 45 min at 37°C. The attachment of particles to Mϕ was scored by phase microscopy as described (12). Results are reported as the attachment index (AI), i.e., the percent of phagocytes that attach particles multiplied by the average number of particles per phagocyte. This figure includes both the attached and ingested yeasts. All assays were performed in triplicate and at least 100 cells were counted for each data point.

Antigen/Antibody-bearing Culture Surfaces. IgG-bearing surfaces were prepared by sequentially treating tissue culture plastic with PLL (Miles Laboratories, Inc., Elkhart, IN), DNP (Eastman Kodak Co., Rochester, NY), and rabbit anti-DNP IgG as described by Michl et al. (15). IgM-coated surfaces were prepared in similar fashion by substituting monoclonal murine IgM anti-DNP (provided by Dr. V. Nussenswig, New York University, NY) for the IgG anti-DNP.

Nitroblue Tetrazolium (NBT) Reduction. Spectrophotometric measurement of reduced NBT was performed by modification of the method of Baehner et al. (16). A 0.1% solution of NBT (Sigma Chemical Co., St. Louis, MO) in PBS was prepared fresh daily. The test system consisted of 0.2 ml NBT solution, plus 0.2 ml Hc yeasts at 5×10^6 or 5×10^7/ml; PMA (0.1 μg/ml) was used as a positive control and PBS as a negative control. The reaction mixtures were preincubated for 10 min at 37°C in 12 × 75 siliconized glass tubes. Mϕ (5×10^5) were then added and tubes were incubated for 20 min at 37°C. Hc yeasts, without added Mϕ, were also used. The reaction was terminated by adding 0.25 N HCl in PBS. The tubes were centrifuged, aspirated, and the purple cell button was extracted twice with 1 ml of pyridine, reagent grade (Fisher Scientific Co., Fairlawn, NJ), in a boiling water bath for 10 min. Optical density of the combined extracts was determined in a spectrophotometer at 515 nm against a pyridine blank.

Similar reagents and controls were used to prepare Mϕ for visual scoring of NBT reduction. The cells were fixed for 12 h in 1% paraformaldehyde and 10^5 cells were applied to glass slides by a cytocentrifuge (Shandon Southern Instruments Inc., Sewickley, PA) at 400 *g* for 5 min. Slides were air dried, stained with modified Wright-Giemsa stain (Diff Quik, American Scientific Products Div., McGraw Park, IL) and mounted with Permount (Fisher Scientific Co.) for microscopic examination. At least 100 cells were counted and the percentage of NBT^+ cells was determined.

H_2O_2 Production. H_2O_2 was measured by a semiautomated scopoletin procedure (17). Briefly, 100 μl of Mϕ (0.5×10^6/ml) were added to wells of a 96-well culture plate, incubated at 37°C for 90 min, and washed in PBS. The PBS was removed and to each well was added 100 μl of PBS containing 240 nM of scopoletin, 10 nM NaN_3, 3 mM glucose, and 12 U/ml of horse radish peroxidase. As a control, scopoletin solution was added to wells containing PBS only. Where indicated, the following triggering agents were incorporated with the scopoletin solution: PMA at a final concentration of 100 ng/ml, Hc yeasts (5×10^5/ml–5×10^6/ml), IgG-coated glass beads (18), and Hc yeasts (5×10^6/ml) plus PMA (100 ng/ml). After challenge as above, the secretion of H_2O_2 by Mϕ was quantitated in a plate-scanning fluorometer by recording oxidation of fluorescent scopoletin to a nonfluorescent product during an interval of 120 min. Results are expressed as nM of H_2O_2 produced per 5×10^4 Mϕ.

Results

Temperature and Cation Dependence of Hc Binding. A study of the effect of temperature on the interaction between unopsonized Hc yeasts and culture-derived human Mϕ revealed dramatic differences in the AI at 4° and 37°C (Fig. 1). The binding of yeasts to Mϕ at 37°C was very rapid, such that after only 5 min of incubation, the percentage of yeasts bound was 43% of the maximum AI of 698 achieved at 60 min. Moreover, the binding of Mϕ to yeasts was avid since the AI was not diminished by as many as six washes of the culture wells. Conversely, at 4°C, there was virtually no binding of yeasts by Mϕ even after a 90 min period of incubation (AI, 33).

There is an absolute requirement for divalent cations to support optimal attachment of Mϕ to certain ligands, for example, C3bi coated erythrocytes, whereas cations are not required for binding to other substrates such as the Fc domain of IgG or C3b-coated erythrocytes (14). To ascertain the role played by divalent cations in Mϕ binding of Hc yeasts, 5-d cultured monocytes were

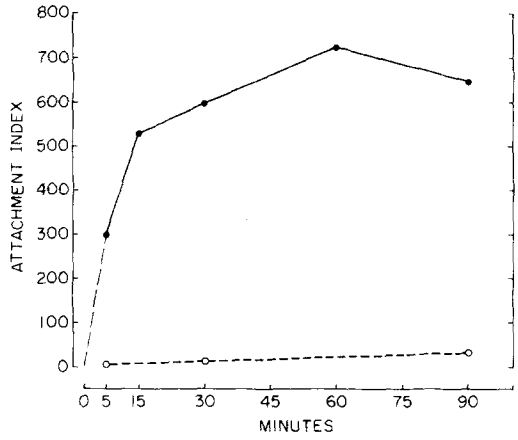

FIGURE 1. Temperature dependence of Hc yeast–binding by Mϕ. Monolayers of Mϕ (7-d cultures) were established on surfaces coated with HSA. Hc yeasts were added to cultures and the AI was determined serially over a 90-min period in cultures incubated at 0° (○) and at 37°C (●).

TABLE I
Divalent Cation Dependence of H. capsulatum Binding to Macrophages

Cations present	Attachment index	
	Exp. 1	Exp. 2
0.5 mM Ca^{2+} and 0.5 mM Mg^{2+}	490	347
0.5 mM Ca^{2+}	206	61
0.5 mM Mg^{2+}	22	110
None	66	46

Monocytes were cultured for 5 d in Teflon beakers and then were plated for 45 min in PBS containing 3 mM glucose and then washed thoroughly in phosphate buffer free of divalent cations. Hc yeast cells from broth cultures were similarly washed and suspended in buffer containing the indicated cations for addition to Mϕ and determination of the attachment index (AI, the number of Hc yeasts attached per 100 phagocytes).

coincubated with yeasts in the presence or absence of Ca and Mg ions. The results of two experiments, summarized in Table I, demonstrate that both Ca and Mg ions are required for optimal binding of Hc yeasts by Mϕ.

Effect of mAbs to Mϕ Surface Receptors on Binding of Hc. To search for receptor molecules on the surfaces of Mϕ capable of binding unopsonized yeasts of Hc, a series of mAbs directed against surface antigens of Mϕ were added individually to separate sets of wells containing adherent Mϕ immediately before addition of yeast cells. Antibody 3G8 directed against low-avidity Fc receptors was without effect on the AI (Table II). mAbs OKM1, TA-1, and Leu-M5 are directed against the CR1, LFA-1, and p150,95 glycoprotein molecules, respectively. Each caused a partial, but consistent reduction of the AI in four separate experiments. On the other hand, two mAbs (IB4 and 60.3) directed against the β chain that is a common member of the glycoprotein adherence-promoting family, greatly reduced the binding of Hc to Mϕ (Table II). These experiments suggest that the

TABLE II

The Attachment of H. capsulatum Yeasts to Macrophages is by Anti-Receptor mAbs

mAb	Antigen recognized	Attachment index
None	—	657
OKM1	α chain, CR3	488
TA-1	α chain, LFA-1	441
Leu-M5	α chain, p150,95	530
IB4	β chain, CR3/LFA-1/p150,95 family	120
60.3	β chain, CR3/LFA-1/p150,95 family	61
3G8*	Low-avidity Fc receptor	596

Monocytes were cultured for 4–7 d in Teflon beakers. The phagocytes were allowed to spread on surfaces by incubation for 45 min at 37°C as described in Materials and Methods. The adherent phagocytes were washed, placed on ice, and the indicated anti-receptor antibodies were added in a concentration of 25 μg/ml. The binding of Hc yeasts was then measured using a 45-min assay. Results are representative of four separate experiments.

* The mAb is directed against the low-avidity Fc receptor of neutrophils and reacts weakly with Mϕ (9).

CR3/LFA-1/p150,95 family of receptors participates in the recognition of Hc yeasts by Mϕ.

Inhibition of Hc Yeast Binding by Surface-bound Anti-Receptor Antibodies. Phagocytosis-promoting receptors diffuse freely in the plane of the Mϕ plasma membrane and function independently of one another in mediating particle binding (15, 19). Thus, after cells spread on a surface coated with anti-receptor mAbs, the receptors diffuse to the substrate-adherent domain of the cell and are trapped by interaction with antibody. The apical surface of the cell is thereby depleted of a single antigen (7). We used this property to remove specifically individual members of the CR3/LFA-1/p150,95 family of receptors from Mϕ.

Surfaces coated with individual mAbs to each of the three respective α chain determinants of the adherence molecules partially reduced the attachment of Hc to Mϕ (Fig. 2). However, a greater reduction in AI was achieved by coating surfaces with any two anti-α chain–directed mAbs, and the greatest reduction of AI by anti-α chain mAbs was obtained when all three were used in combination as described. In fact, the reduction in binding of Mϕ to yeasts was of similar magnitude to that produced by coupling mAb IB4 to the culture surface. The experiment depicted in Fig. 2 contains a slight anomaly in that the combination of OKM1 and Leu-M5 caused full reduction in binding of Hc. However, in three other experiments, the action of OKM1 and Leu-M5 was equivalent to that of the other pairs of antibodies. In contrast to these results, removal of Fc receptors from the apical surfaces of Mϕ had no effect whatever on the binding of organisms (Fig. 2). These findings suggest that the capacity to bind Hc yeasts is a property shared by all three members of the CR3/LFA-1/p150,95 family.

Hc and T. glabrata Are Bound by Different Surface Receptors. Mϕ isolated from several sources express a surface receptor that recognizes glycoproteins containing oligosaccharides terminating in mannose (20, 21). This mannosyl/fucosyl receptor is the principal mechanism by which human Mϕ bind to zymosan in the

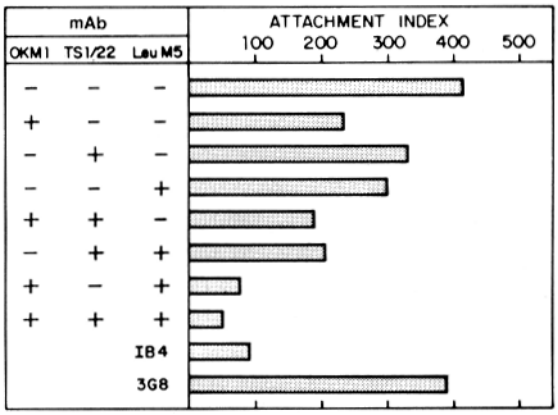

FIGURE 2. Effect on Hc yeast–binding of down–regulating Mϕ surface target antigens by mAb-coated surfaces. mAbs against α chains of the CR3/LFA-1/p150,95 family were used singly or in combination in a concentration of 50 μg/ml; IB4 and 3G8 were used singly at 50 μg/ml. The data shown are representative of four experiments.

TABLE III

H. capsulatum yeasts and T. glabrata are Bound to Macrophages by Different Surface Receptors

Reagent	Concentration	Attachment index of:			
		H. capsulatum		*T. glabrata*	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
	μg/ml				
None	—	470	522	692	420
Mannosylated BSA	50	451	510	268	ND
Mannan	1,000	473	497	133	40
mAb IB4	50	—	27	584	403

Monolayers of Mϕ (7-d cultures) were allowed to spread on surfaces for 45 min as described. Adherent phagocytes were washed, placed on ice, and to these were added mannosylated BSA (50 μg/ml), mannan (1 mg/ml), or mAb IB4 (50 μg/ml). The binding of Hc yeasts or *T. glabrata* was then measured using a 45-min assay.

absence of serum (22) and by which pulmonary Mϕ of mice bind and ingest *Candida krusei* (23). Therefore, we measured the binding of unopsonized Hc yeasts by Mϕ in the presence of soluble ligands that compete for attachment to the mannosyl/fucosyl receptor. Parallel studies were performed with the opportunistic yeast pathogen, *T. glabrata*, chosen for its size proximity to Hc yeasts and the absence of germ tube formation. Neither the neoglycoprotein, mannosyl/BSA, nor yeast-derived mannan exerted detectable blocking activity against attachment of Hc (Table III). However, mannosylated BSA considerably reduced Mϕ binding to *T. glabrata* and mannan greatly decreased the AI. Further evidence that the respective yeast forms of Hc and *T. glabrata* are bound by different receptors was provided by the finding that mAb IB4 exerted no blocking activity against the binding of *T. glabrata* as contrasted with its profound blockade of Mϕ attachment to Hc yeasts.

Freshly isolated human monocytes do not express mannosyl/fucosyl receptor

TABLE IV
Binding of H. capsulatum yeasts and T. glabrata to Monocytes and Macrophages

Cells used	Attachment index of:	
	H. capsulatum	*T. glabrata*
Monocytes	380	31
Macrophages	416	467

Monocytes freshly isolated from peripheral blood as described in Materials and Methods and 7-d cultured Mϕ were allowed to spread for 45 min. The attachment of Hc yeasts or *T. glabrata* was then measured using a 45-min assay.

TABLE V
Fc Receptor Function Is not Required for Attachment of H. capsulatum Yeasts or T. glabrata

Surface	Attachment index of:				
	H. capsulatum	*T. glabrata*	EIgG	EC3b	EC3bi
DNP	479	497	617	592	636
IgG anti-DNP	475	495	152	582	564
IgM anti-DNP	476	508	629	574	564

Mϕ cultured for 7 d were plated on DNP-anti-DNP IgG or DNP-anti-DNP IgM-coated surface and on control DNP-coated surfaces. The cells were then washed and the AI measured with Hc yeasts and *T. glabrata*. We used EIgG as a positive control of particle binding, and EC3b and EC3bi as negative binding controls. Results are representative of three separate experiments.

activity, but this receptor is expressed upon in vitro maturation of monocytes into macrophages (24, 25). Conversely, CR3 is expressed on human blood monocytes and increases further in activity during the maturation to Mϕ (14). We thus measured the binding of Hc and *T. glabrata* by freshly isolated monocytes and by Mϕ after 7 d in culture (Table IV). The binding of Hc yeasts to monocytes was extensive, whereas the AI of *Torulopsis* yeasts was very low. However, at day 7, binding of *T. glabrata* to Mϕ equalled or exceeded the binding to Hc yeasts. Taken together, these data strongly suggest that *T. glabrata* is recognized by the mannosyl/fucosyl receptor but is not recognized by CR3, LFA-1, or p150,95. Hc, on the other hand, is bound by the CR3/LFA-1/p150 family of adherence-promoting glycoproteins and is not bound by the mannosyl/fucosyl receptor.

Recognition of Hc Does Not Require FcR, β-Glucan Receptors, or Secreted Complement Proteins. To assess the role of FcR in the binding of Hc, Mϕ were allowed to spread on surfaces coated with IgG in immune complexes. The attachment of EIgG by such Mϕ was reduced by 75%, indicating that FcR had been depleted from the Mϕ (Table V). In contrast, IgM anti-DNP complexes exerted no down regulation of the binding of EIgG. Removal of FcR by fixed IgG-DNP complexes did not alter the binding of Hc yeasts to Mϕ. Thus, FcR are not required for the binding of Hc by Mϕ. These data confirm results obtained with mAb 3G8 to the low-avidity Fc receptor (Table II, Fig. 2). Data in Table V also demonstrate that the FcR does not participate in attachment of *T. glabrata*.

Czop and Austen (26) have observed that several particles that activate the

TABLE VI
Effect of Antibody to C3 on Binding by Macrophages of Yeasts, EC3b, and EIgG

Concentration of anti-C3	Attachment index of:		
	H. capsulatum	EC3b	EIgG
μg/ml			
None	492	519	480
25	494	96	441
50	508	21	471
100	454	7	440

Monocytes were cultured for 5 d in Teflon beakers and monolayer cultures were then established. The binding of Hc yeasts or the indicated ligand-coated erythrocyte was then measured in the presence of Fab anti-C3 antibody in the concentrations indicated.

alternative pathway of complement are recognized by monocytes, and that this form of recognition can be blocked by low concentrations of β-glucan. However, the AI of Mϕ to Hc yeasts was 501 in the presence of 100 μg/ml of β-glucan as compared with an AI of 491 in the control. The concentration of β-glucan used is 100-fold greater than that required to inhibit the binding of alternative pathway activators. Therefore, the β-glucan receptor does not participate in the binding of Hc yeasts to Mϕ.

Mϕ can synthesize all of the classical complement components as well as the alternative pathway constituents (27), and it has been reported (28) that complement components secreted by Mϕ in vitro can opsonize zymosan particles that activate the alternative pathway. Since Hc yeasts also activate this pathway (29), we explored the possibility that these organisms may be opsonized by secreted Mϕ products. Assays of binding were conducted in the presence of varying concentrations of Fab fragments of rabbit anti-C3 antibody. Binding of EC3b to Mϕ was virtually abrogated by 100 μg/ml of anti-C3, whereas the AI of Hc yeasts or of EIgG did not differ from control values (Table VI). It is unlikely therefore, that Mϕ attachment can be explained by local opsonization of yeasts by C3 and subsequent binding to CR3 receptors via C3bi.

Effects of Soluble Sugars on Attachment of Hc to Mϕ. The cell wall of Hc yeasts is composed of polymeric carbohydrates, primarily *N*-acetyl glucosamine and glucose in the form of chitin (30). To ask whether a sugar or amino sugar in the cell wall serves as the recognition site for receptors on Mϕ, we attempted to block the binding of Hc yeasts with high concentrations of monosaccharides. Fucose, α-methylmannoside, glucosamine, *N*-acetyl glucosamine, or glucuronic acid (all at 200 mM) produced mild depression of the AI, ranging from 3.4% with L-fucose to 24.3% with *N*-acetyl D-glucosamine (Table VII). However, sugar concentrations of 100 mM and 33 mM, respectively, induced minimal reductions of binding. Thus, the small reductions in AI produced by all sugars at 200 mM are likely to have resulted from changes in the osmolarity or ionic strength of the medium. D-mannose on the other hand, at concentrations of 100 and 200 mM, produced consistent elevations of the AI in four separate experiments of which two are shown. These experiments suggest that the receptors on Mϕ that

TABLE VII
Effect of Soluble Sugars on Binding of H. capsulatum Yeasts to Macrophages

Sugar	Concentration	Attachment index		
		Exp. 1	Exp. 2	Exp. 3
	mM			
None	—	416	526	465
N-acetyl D-glucosamine	200	315	—	
	100		469	
	33		476	
D-glucosamine	200	371	—	
	100		444	
	33		482	
D-glucuronic acid	200	322	—	
	100		419	
	33		499	
α-methyl D-mannoside	200	403	—	
	100		408	
	33		447	
L-fucose	200	402	—	
	100		420	
	33		488	
D-mannose	200	567	—	
	100		558	
	33		468	
Galactose	200			—
	100			412
	33			503
N-acetyl-galactosamine	200			—
	100			436
	33			477

Monocytes were cultured for 7 d in Teflon beakers and monolayer cultures were then established. The binding of Hc yeasts was then measured in the presence of sugars at the concentrations indicated. The pH of all sugars was adjusted to pH 7.4.

recognize Hc do not act as lectins for galactose, *N*-acetyl-galactosamine, fucose, mannose, α-methyl mannoside, *N*-acetyl glucosamine, or glucuronic acid. Additional sugar specificities are currently under study.

NBT Reduction and H_2O_2 Production by Mϕ in Response to Hc Yeasts. We asked whether Mϕ attachment and ingestion of Hc yeasts stimulated a respiratory burst with formation of toxic oxygen metabolites. The reduction of NBT to blue-purple formazan by 4-d cultured Mϕ was assessed as a measure of Mϕ superoxide activity (16). The results of qualitative and quantitative measurements of NBT

TABLE VIII

NBT Reduction by Mϕ in Response to H. capsulatum Yeasts

Stimulus	Qualitative analysis	Quantitative analysis (OD)
	Percent NBT^+ Mϕ	
None	18.8 ± 1.4	0.048 ± 0.010
Hc/Mϕ (1:1)	57.8 ± 4.7	0.153 ± 0.019
Hc/Mϕ (10:1)	86.1 ± 1.5	0.270 ± 0.024
PMA	83.9 ± 1.4	0.256 ± 0.025

Qualitative and quantitative analyses of NBT reduction by 4-d cultured Mϕ from six normal subjects were performed as described in Materials and Methods. Hc yeasts were added to Mϕ at ratios of 1:1 and 10:1. PMA was used at a final concentration of 100 ng/ml. In the absence of Mϕ, Hc yeasts alone did not raise the OD above the background levels produced by Mϕ controls. When the quantitative assay was performed with yeasts alone at 5×10^6/ml, the concentration corresponding to that added to yield one yeast per Mϕ, the OD was 0.015 ± 0.005, and at 5×10^7/ml, the OD was 0.036 ± 0.006. This result was confirmed by visual inspection, which showed negligible NBT associated with free yeasts.

reduction by Mϕ cultured from six healthy individuals are shown in Table VIII. At a 1:1 ratio of Hc yeast to Mϕ, both the percentage of Mϕ containing formazan deposits and the total production of redox compound were significantly higher ($p < 0.01$) than in unstimulated control Mϕ. The quantity of dye-reduced and percentage of formazan-positive Mϕ at a 10:1 ratio of yeasts to phagocytes were similar in amounts to those produced by incubation with PMA.

Hydrogen peroxide is one of the principal cytotoxic molecules elaborated by Mϕ. To observe whether Hc stimulate the release of peroxide, Mϕ were incubated with Hc and the time course of peroxide release was measured by the scopoletin assay (17) (Fig. 3). After a lag of 10–20 min, Hc initiated rapid release of peroxide. The time course and amount of release induced by the addition of 10 Hc per Mϕ corresponded closely with the response to IgG-coated glass beads, a potent trigger of the oxidative burst. A lower dose of Hc (1 Hc per Mϕ) caused slower release. Yeast alone evolved no hydrogen peroxide, and the yeast had no effect on the release caused by PMA.

Discussion

The early pathogenesis of disease caused by primary infection with Hc is characterized by extensive multiplication of yeast forms within host Mϕ. Thus, the primary phase of Mϕ-yeast interaction is critical to successful parasitization of Mϕs. In the present study, we have examined this interaction in vitro and have demonstrated that attachment of yeasts to Mϕ is achieved by a mechanism that is not dependent on serum opsonins. Attachment of Hc yeasts does not require FcR (Table V), mannose receptors (Tables III, VII), β-glucan receptors, or Mϕ-derived complement proteins (Table VI). The attachment of Hc does, however, require warm temperatures and the presence of divalent cations. These two properties are characteristics of the Mϕ receptors, CR3 (14, 31) and LFA-1 (32).

CR3 and LFA-1 are members of a family of cell surface proteins that function in cell adhesion. CR3 mediates attachment of C3bi-coated particles (7), LFA-1

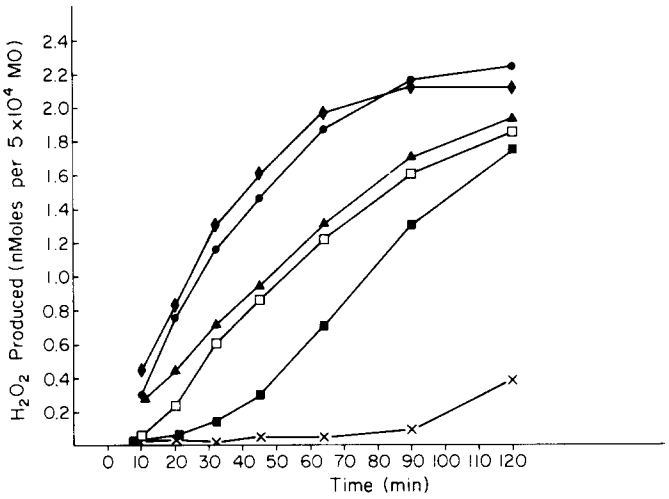

FIGURE 3. Hydrogen peroxide production by 7-d cultured Mϕ in control cultures (×), and in cultures incubated with 1 Hc yeast per Mϕ (■), or 10 yeasts per Mϕ (□). In positive controls, Mϕ cultures were stimulated with IgG-coated beads (▲) or PMA, 100 ng/ml (◆). Both act as potent triggers of H_2O_2 to production. The effect of adding 10 Hc yeasts per Mϕ to cultures stimulated with PMA was measured in an additional control (●).

mediates the attachment to tumor cells and endothelial cells (33, 34), and the function of a third protein, p150,95, has not yet been determined. Mϕ express approximately equal amounts of these three proteins on their cell surface (Wright, S. D., unpublished observations). Each molecule of the CR3/LFA-1/p150,95 family contains a unique α subunit that is associated noncovalently with a common β subunit (M_r, 95,000) in an α_1 β_1 configuration. Both the α chains and the common β chain are surface-exposed.

To test whether members of the CR3/LFA-1/p150,95 family mediate the recognition of Hc, we used soluble anti-receptor antibodies in blocking experiments. Incubation of Mϕ with specific mAb directed against the α chains of each member of the family of adherence-promoting proteins partially reduced the AI of Hc yeasts. On the other hand the mAbs IB4 and 60.3 both directed against the common β chain, greatly reduced the AI. Further studies used substrate-bound mAbs to physically remove receptors from the apical surfaces of Mϕ. Removal of CR3, LFA-1, and p150,95 by a combination of three mAbs to each of the specific α chains effected a substantial reduction in binding of yeasts (Fig. 2). A comparable blockade was achieved with the anti-β mAb IB4. mAbs directed to other surface antigens, including the low-avidity FcR, failed to reduce the AI. These findings indicate that binding of unopsonized Hc yeasts is mediated by the CR3, LFA-1, p150,95 family.

We have also observed that removal of a single member of the CR3/LFA-1/p150,95 family causes only a negligible decrease in the binding of Hc, and removal of any pair of receptors lowers but does not eliminate binding (Fig. 2). Thus, it appears that the capacity to recognize Hc is a property shared by all three members of the CR3/LFA-1/p150,95 family.

The structural basis for the observation that all three members of the

CR3/LFA-1/p150,95 family share the ability to recognize Hc is not clear. One possibility is that the recognition site for Hc is located on the shared β chain. This would explain the requirement that all three family members must be removed from Mϕ to fully inhibit binding of Hc (Fig. 2), and the capacity of soluble anti-β antibodies (IB4 and 60.3) to inhibit binding of Hc (Table II). The binding site for C3bi, on the other hand, appears to be on the α chain of CR3 since LFA-1 and p150,95 do not recognize C3bi (31), and the anti-α mAb, OKM10, directly blocks the binding of C3bi to CR3 (7). Thus, CR3 may express two distinct binding sites, one for C3bi on the α chain, and the other for Hc on the β chain. Such a view is consistent with experiments of Ross et al., who suggest that CR3 expresses a lectin-like binding site that is distinct from the C3bi-binding site (35). An alternative explanation for the data is that Hc express a variety of surface structures, some of which are recognized via the α chain of CR3, others by LFA-1, and others by p150,95. Currently, we cannot eliminate this possibility.

One of the mechanisms by which intracellular pathogens may enhance their survival within phagocytes is by a failure to trigger the secretion of toxic oxygen metabolites. We have observed, however, that Hc yeasts do induce generation of superoxide anion by Mϕ, as measured by NBT reduction (Table VIII), and also promote the release of H_2O_2 under the conditions used in the present study. In fact, the time course and extent of H_2O_2 release was comparable to that induced by IgG-coated particles (Fig. 3). Since by electron microscopic examination, many ingested Hc appear to remain viable within human Mϕ 120 min after ingestion, (Detmers, P. A., and W. E. Bullock, unpublished observations), we assume that Hc (strain 217B) is relatively resistant to destruction by toxic oxygen metabolites.

The capacity of Hc to initiate the release of peroxide was surprising in view of previous work (36) showing that ligation of CR3 by C3bi did not lead to its release. Since Hc is recognized by LFA-1 and p150,95 in addition to CR3, it is possible that these receptors differ from CR3 or can synergize with CR3 in such a fashion as to initiate release of peroxide. Alternatively, Hc may bear ligands for other types of receptors and these may initiate peroxide release after CR3, LFA-1, or p150,95 bind the yeast to the Mϕ surface. Still another explanation for these results is the hypothesis of Ross et al. (35) that CR3 contains two binding sites, one for C3bi that does not initiate an oxidative burst and a second site that does. Experiments are underway to explore these possibilities.

The biochemical nature of the structure(s) on Hc that are recognized by Mϕ remains to be determined. Since the cell wall of Hc is composed principally of carbohydrate (31), we sought evidence that receptors on Mϕ act as lectins. However, mannose, fucose, α-methyl mannoside, glucuronic acid, glucosamine, *N*-acetyl glucosamine, galactose, galactosamine, β-glucan, and yeast mannan failed to block binding (Table VII). The failure of Mϕ mannosyl/fucosyl receptors to bind Hc may derive in part from the low mannose content of Hc cell walls (1–5%) (30) as contrasted with avid binding to other yeasts with cell walls of high mannose content, for example, *Candida* species (37) and *S. cerevisiae* (38). Recent experiments (39) suggest that phosphosugars may be more likely candidates for the recognition site. CR3, LFA-1, and p150,95 all bind to bacterial LPS. The portion of LPS that is recognized is the diglucosamine bisphosphate head

structure of lipid A (31). It is thus of interest that five novel phosphoinositol-sphingolipids have been purified recently from the yeast phase of Hc (40, 41). These sphingolipids possess an identical inositol phosphoceramide core but differ by the glycosyl substitution of the polar head groups (40). Studies of the blocking activity by these compounds will be of particular value in future work.

The present studies have established that unopsonized Hc yeasts are bound to Mϕ by the CR3/LFA-1/p150,95 adherence glycoproteins. In our view, it is primarily by this mechanism that the organisms are provided a means for gaining critical entry into a host cell that is highly permissive of yeast multiplication. Thus, successful parasitization of the host is ensured before maturation of specific cell-mediated immune responses that render Mϕ capable of restricting yeast cell growth. By virtue of their location, alveolar Mϕ are presumably among the first phagocytes to come into contact with invasive yeast forms of Hc in the setting of natural infection. However, certain properties of these cells differ from monocyte-derived Mϕ or Mϕ obtained from other anatomic sites (42). Therefore, further studies are required to establish the receptor mechanism(s) and the kinetics of binding to Hc yeasts by human alveolar Mϕ.

Summary

The principal host cell of *H. capsulatum* (Hc) is the Mϕ within which the pathogenic yeast phase of the fungus multiplies during active disease. The initial interaction between Hc yeasts and Mϕ therefore is a crucial step in the pathogenesis of histoplasmosis. In the present study, we have identified the major receptor mechanism that mediates the attachment of unopsonized Hc yeasts to human monocyte–derived Mϕ from peripheral blood. Binding of Hc yeasts by Mϕ is rapid, temperature dependent, and requires both Ca and Mg ions for optimum activity. Recognition of Hc yeasts does not require Fc receptors, mannosyl/fucosyl receptors, β-glucan receptors, or secretion of C3 by Mϕ. Studies were performed on the effect of down regulating specific receptors of the CR3/LFA-1/p150,95 adherence-promoting protein family from the apical portion of Mϕ to determine the effects upon binding of Hc yeasts. Anti–β chain mAbs that recognize all three of these proteins blocked binding of yeasts. However, removal of individual receptors with antibodies against the α polypeptides caused negligible depression of binding, and removal of any pair caused only modest depression. Thus, each of the members of the CR3/LFA-1/p150,95 family is independently capable of binding Hc. The delineation of this new mechanism for nonopsonic recognition by Mϕ that is exploited by Hc yeasts will aid in future studies to identify the Hc ligand, to elucidate the stoichiometry of CR3/LFA-1/p150,95 binding, and to determine triggering mechanisms for release of toxic oxygen metabolites.

We thank Michelle T. C. Jong and Deborah E. Hendricks for excellent technical assistance and Angela Birch Smith for her help in preparation of the manuscript. We also thank Dr. Zanvil Cohn for helpful discussion in review of the manuscript.

Received for publication 10 September 1986.

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