Yeast Mannans Inhibit Binding and Phagocytosis of Zymosan by Mouse Peritoneal Macrophages

SUN-SANG J. SUNG, ROLF S. NELSON, and SAMUEL C. SILVERSTEIN
Department of Cellular Physiology and Immunology, The Rockefeller University, New York, New York 10021

ABSTRACT We have examined the effects of various mannans, glycoproteins, oligosaccharides, monosaccharides, and sugar phosphates on the binding and phagocytosis of yeast cell walls (zymosan) by mouse peritoneal macrophages. A phosphonomannan (PO_4:mannose ratio = 1:8.6) from *Kloeckera brevis* was the most potent inhibitor tested; it inhibited binding and phagocytosis by 50% at concentrations of ~3–5 μg/ml and 10 μg/ml, respectively. Removal of the phosphate from this mannan by mild acid and alkaline phosphatase treatment did not appreciably reduce its capacity to inhibit zymosan phagocytosis. The mannan from *Saccharomyces cerevisiae* mutant LB301 inhibits phagocytosis by 50% at 0.3 mg/ml, and a neutral exocellular glucomannan from *Pichia pinus* inhibited phagocytosis by 50% at 1 mg/ml. Cell wall mannans from wild type *S. cerevisiae* X2180, its *mnn2* mutant which contains mannan with predominantly 1 → 6-linked mannose residues, yeast exocellular mannans and *O*-phosphonomannans were less efficient inhibitors requiring concentrations of 1–5 mg/ml to achieve 50% reduction in phagocytosis. Horseradish peroxidase, which contains high-mannose type oligosaccharides, was also inhibitory.

Mannan is a specific inhibitor of zymosan binding and phagocytosis. The binding and ingestion of zymosan but not of IgG- or complement-coated erythrocytes can be obliterated by plating macrophages on substrates coated with poly-L-lysine (PLL)-mannan. Zymosan uptake was completely abolished by trypsin treatment of the macrophages and reduced by 50–60% in the presence of 10 mM EGTA. Pretreatment of the macrophages with chloroquine inhibited zymosan binding and ingestion. These results support the proposal that the macrophage mannose/*N*-acetylglucosamine receptor (P. Stahl, J. S. Rodman, M. J. Miller, and P. H. Schlesinger, 1978, *Proc. Natl. Acad. Sci. U. S. A.* 75:1399–1403, mediates the phagocytosis of zymosan particles.

The phagocytosis of zymosan (yeast cell walls) has been classified, together with the ingestion of particles such as latex, starch, and particles with denatured surfaces, as "nonspecific" phagocytosis, to distinguish it from receptor-mediated phagocytosis of particles coated with known ligands such as immunoglobulin G (IgG) or complement (22). Yeast cell walls are composed predominantly of glucose- or mannose-containing polysaccharides. The discovery of cell surface receptors that mediate the pinocytosis of mannose- or mannose phosphate-containing oligosaccharides (9, 13, 15, 25) raised the possibility that zymosan uptake might be mediated by one or more of these receptors. Exploring this question, Warr (32) showed that the binding of intact yeast particles to rat alveolar macrophages, cells that are especially rich in mannose receptors, could be blocked by mannose and by proteins containing "high mannose" type oligosaccharides. The experiments described in the present report extend and amplify Warr's findings in several ways. We show that mannan from *Kl. brevis* is an especially potent and specific inhibitor of the binding and ingestion of *Saccharomyces cerevisiae* and *Kloeckera brevis* zymosans, and that this inhibitory activity is unaffected by mild acid hydrolysis and phosphatase treatment of *Kl. brevis* mannan. Moreover, we show that macrophages plated on substrates coated with mannose-containing oligosaccharides or incubated in medium containing chloroquine are specifically depleted in their capacity to bind and ingest zymosan. In sum, our results indicate that zymosan ingestion is receptor-mediated; they suggest that this uptake is mediated by the Man/GlcNAc receptor described by Stahl et al. (25).

MATERIALS AND METHODS

NCS mice and sheep erythrocytes were obtained from the Rockefeller University animal facility. Brewer thioglycollate medium and proteose peptone were from Difco Laboratories, (Detroit, MI); anti-sheep erythrocyte IgG was from Cordis

THE JOURNAL OF CELL BIOLOGY · VOLUME 96 JANUARY 1983 160-166

Laboratories Inc., (Miami, FL); mannan from *S. cerevisiae* X2180 was from either Sigma Chemical Co. (St. Louis, MO) or Dr. Clinton Ballou of the University of California, Berkeley. Mannans from mutants of *S. cerevisiae* X2180, *mnn2* (21) and LB-301 (2), as well as phosphomannan 8.6 from *Kl brevis* (28) were generous gifts of Dr. Clinton Ballou. Mannans were dialysed overnight against 1 N acetic acid and lyophilized before use. The structures of some of these mannans are shown in Fig. 1. *Kl. brevis* strain 55–45 was a generous gift of Dr. Herman Phaff of the University of California, Davis; *Kl. brevis* zymosan particles were prepared by autoclaving a 25% (vol/vol) log phase *Kl. brevis* suspension in Dulbecco's phosphate-buffered saline without Ca^{++} and Mg^{++} (PD), followed by reduction with mercaptoethanol and alkylation with iodoacetamide as described (17). *S. cerevisiae* zymosan was purchased from Sigma Chemical Co. and washed as described (18). Yeast exocellular mannans (23, 24) were generous gifts of Dr. M. Slodki of the Northern Research Laboratory; poly-L-lysine (PLL) (mol wt 90,000) was obtained from Miles Laboratories Inc. (Elkhart, IN); Gold Seal glass coverslips from Becton, Dickinson & Co. (Oxnard, CA); *Escherichia coli* alkaline phosphatase and 3X crystallized trypsin from Worthington Biochemical Corp. (Freehold, NJ); horseradish peroxidase type II, mannose-6-phosphate, α-methyl-D-mannopyranoside grade III, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride and bovine serum albumin (BSA) from Sigma Chemical Co.; D-mannose from Pfanstiehl Laboratories, Inc. (Waukegan, IL). [^{125}I]-NaI (carrier free) was purchased from Amersham Corp. (Arlington Heights, IL) and iodogen from Pierce Chemical Co. (Rockford, IL). All other chemicals were obtained from commercial sources and were of the purest grade available. Fetal bovine serum (FBS) (Flow Laboratories, Inc., Rockville, MD) was heated at 56°C for 30 min before use.

Mannose oligosaccharides were obtained from the acetolysis of *S. cerevisiae* mannan (Sigma Chemical Co.) by the method of Kocourek and Ballou (16) followed by chromatography on a column (2.5 × 100 cm) of P-2 (−400 mesh, Bio-Rad Laboratories, Richmond, CA) eluted with water at 48°C. The carbohydrate peaks were pooled, lyophilized, and analyzed by thin-layer chromatography on silica gel G plates developed with butanol/acetic acid/water (100:50:50) using stachyose, raffinose, melibiose, and mannose as standards (14). The individual oligosaccharides visualized by α-naphthol-sulphuric acid (14) were essentially homogeneous and migrated with R_f values similar to the corresponding standards with the same degree of polymerization. The oligosaccharides did not contain any phosphate as determined by the method of Bartlett (3).

Cell Cultures: Mouse peritoneal macrophages were harvested by the method of Cohn and Benson (5) and plated on coverslips (13 mm diameter) in 35-mm dishes essentially as described (18). Resident and proteose peptone-elicited (7) macrophages were plated at a density of 2×10^5 peritoneal cells per coverslip. Thioglycollate-elicited macrophages were plated at a density of 1×10^6 cells per 35-mm dish (18). Macrophage cultures were incubated at 37°C for 16–24 h; before use, the coverslip cultures were washed as described below. For experiments employing PLL-, PLL-mannan-, and PLL-bovine serum albumin (PLL-BSA)-coated substrates, cells were plated on appropriately coated 13-mm coverslips (see below) in 16-mm Costar wells at 3×10^5 cells per well (19) in Eagle's minimal essential medium (MEM) with 10% FBS, incubated at 37°C for 4 h, and washed to remove nonadherent cells. Despite vigorous washing, many lymphocytes remained adherent to these PLL-, PLL-mannan- or PLL-BSA-coated coverslips. For this reason these cultures were further incubated at 37°C in MEM-10% FBS overnight. After this period the lymphocytes detached, leaving a population of viable, well-spread cells >95% of which are macrophages. For experiments using macrophages on untreated coverslips, cells were plated at the densities described above in MEM-10% FBS for 4 h at 37°C, washed twice with MEM, and incubated in MEM-10% FBS overnight.

Binding and Phagocytosis Assays: Prior to use, coverslip cultures were washed by dipping in cold MEM and placed in 16-mm Costar wells with 0.5 ml of medium containing MEM and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (pH 7.4) with or without inhibitors as appropriate and preincubated for 15 min at 4°C for binding assays or at 37°C for phagocytosis assays. For binding, 20 μl of a 5% (vol/vol) suspension of particles was added per well and attachment was allowed to proceed for 60–90 min at 4°C. For phagocytosis, 20 μl of a 2% (vol/vol) suspension of particles was added to each well for 30 min at 37°C. Phagocytosis of IgG-coated erythrocytes was measured as described (18). At the end of the experiment the cultures were washed with PD and fixed in 2.5% glutaraldehyde in PD. Ingestion and binding were scored with a phase-contrast microscope by counting the number of particles bound or ingested by 100 macrophages. Under phase-contrast optics, ingested zymosan particles can be readily distinguished from bound particles by the location of the whole particle within the periphery of the macrophage, by the loss of the characteristic grayish color of uningested particles, and by the presence of a vacuole around the particle. The binding of complement-coated erythrocytes, prepared as described (4), was performed by adding 20 μl of 5% (vol/vol) complement-coated erythrocytes to resident macrophages on coverslips in 500 μl of MEM-25 mM HEPES, pH 7.4-2% FBS. The cultures were incubated at 37°C for 30 min, washed in PD, and fixed in 2.5% glutaraldehyde in PD. The phagocytic or binding index is defined as the number of particles ingested or bound by 100 macrophages.

Preparation of Coverslips Coated with Poly-L-lysine, Poly-L-lysine-bovine Serum Albumin and Poly-L-lysine-mannan: Poly-L-lysine-coated coverslips were prepared by the method of Michl et al. (19). Mannan from *S. cerevisiae* was clarified by centrifugation of a 100 mg per ml aqueous solution at 23,000 *g* for 20 min; the mannan in the supernatant was reprecipitated with Fehling's solution, washed in methanol/acetic acid (5/1), dialyzed against 1 N acetic acid, and lyophilized (16). 100 μl of a solution containing 50 mg/ml BSA or mannan and 10 mg/ml of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride were layered over PLL-coated coverslips and incubated at 4°C overnight. The amount of mannan or BSA coupled was quantitated by adding 1×10^7 cpm of iodinated mannan (2.3×10^5 cpm/μg) or BSA (4.4×10^5 cpm/μg) to each ml of the coupling mixture and counting the coverslips for radioactivity. ~4.3 ± 0.8 μg mannan or 9.0 ± 3.0 μg BSA were coupled per coverslip.

Mild Acid Hydrolysis and Alkaline Phosphatase Treatment of Mannan: *Kl. brevis* mannan (20 mg in 1 ml of 0.01 M HCl) was hydrolysed for 30 min at 100°C, dialyzed exhaustively against water, and lyophilized. 8 mg of this acid-hydrolysed mannan was dissolved in 0.8 ml of 0.75 M Tris HCl, pH 8.0 and incubated with 49 U of *E. coli* alkaline phosphatase (sp act 31 U/mg) at 37° for 24 h. The reaction was terminated by incubating the mixture for 3 min in a boiling water bath. The mannan was dialyzed exhaustively against 1 M acetic acid and lyophilized. The carbohydrate and phosphorus content of each preparation was quantitated. The mannose/phosphate ratio was 9.1 for the initial mannan, 8.2 for the acid-treated mannan, and 23.5 for the mild acid- and alkaline phosphatase-treated mannan. The final product contained about 35% of the phosphate of the acid-treated mannan.

General Methods: Iodination of mannan and BSA was performed as described (11). Phosphorus content of mannan was measured by the method of Bartlett (3). Carbohydrates were quantitated by the phenol-sulphuric acid method (6). Alkaline phosphatase activity was measured as described in the Worthington Enzyme manual.

Cells for trypsinization were washed three times with MEM, followed by treatment with 1 mg/ml trypsin in MEM-25 mM HEPES, pH 7.4 at 37°C for 30 min. The trypsin-containing medium was aspirated and excess trypsin neutralized with MEM-10% FBS. Cells were washed twice again with MEM before use.

For chloroquine inhibition assays, 8×10^4 resident macrophages were plated on 13-mm coverslips in MEM-10% FBS, incubated overnight, washed two times with MEM, and incubated in 16-mm Costar wells for 1 h at 37°C in 500 μl of MEM containing 25 mM HEPES (pH 7.4), 2% FBS to which chloroquine and 1 or 10 mg/ml *S. cerevisiae* mannan were added as indicated. The cells were then washed three times with cold MEM and further incubated at 37°C in control or chloroquine-containing medium to which *S. cerevisiae* or *Kl. brevis* zymosan was added to measure phagocytosis and binding, respectively.

RESULTS AND DISCUSSION

To identify oligosaccharides that might be useful as probes of the macrophage zymosan uptake system we screened a number of mannans for their capacity to inhibit phagocytosis of *S. cerevisiae* zymosan. Significant inhibition of zymosan phagocytosis was observed with mannans from *Kl. brevis*, wild type, and sugar transferase mutants of *S. cerevisiae*, and *Pichia pinus* (Table I). Other exocellular mannans and phosphomannan monoesters obtained from *Hansenula capsulata*, *Torulopsis pinus*, *Pichia sp.*, *Hansenula minuta*, and *Pichia mucosa* were weak inhibitors (data not shown).

To explore further the relationship between mannan structure and inhibitory potency we studied the dose dependence of inhibition of zymosan uptake by mannans from wild type and mutant *S. cerevisiae*. Mannan from wild type *S. cerevisiae* at a concentration of 1 mg/ml inhibited zymosan ingestion by ~50% (Fig. 2). Mannan from the *S. cerevisiae mnn2* mutant, which has a 1 → 6-linked linear array of mannosyl residues in its outer chain but lacks the 1 → 2- and 1 → 3-linked mannosyl oligosaccharide branches found in wild type mannan (Fig. 1), was about twofold less effective than wild type mannan (Fig. 2). Mannan from another *S. cerevisiae* mutant LB-301, derived from *mnn2*, has a truncated outer chain of 1 → 6-linked mannosyl residues. LB-301 mannan was approximately 10-fold

TABLE I

*Inhibition of Resident Macrophage Phagocytosis of S. cerevisiae Zymosan by Yeast Mannans**

Source of mannan	NRRL no. of yeast strain	Type of mannan	Phosphate content	Phagocytosis (% control) at mannan concentration‡ 5	3	1
			μmol/mg mannan		*mg/ml*	
Cell wall mannans						
Kl. brevis 55-45	—	O-phosphonomannan	0.59	2	3	5
S. cerevisiae X2180	—	O-phosphonomannan	0.13	18	30	43
Exocellular mannans						
P. pinus	Y-2579	Neutral glucomannan	0.01	6	15	20
		O-phosphonoglucomannan	0.42	6	18	42
H. holstii	Y-2448	Neutral mannan	0.04	46	73	ND§
		O-phosphonomannan	0.68	27	53	ND
		O-phosphonomannan monoester	0.45	37	72	ND
Sp. sp.‖	Y-6493	O-phosphonoglucogalactan	0.71	12	31	71

* Overnight cultures of macrophages at 8×10^4 macrophage per 13-mm coverslip in MEM-10% FBS were washed with MEM, pretreated with the mannans in 0.5 ml of MEM-2% FBS-25 mM HEPES, pH 7.4 for 15 min at 37°C before the addition of 20 μl of 2% *S. cerevisiae* zymosan. The macrophages were allowed to phagocytose for 30 min at 37°C and then washed in PD and fixed in 2.5% glutaraldehyde in PD. The number of zymosan particles ingested was scored by counting the number of particles ingested by 100 macrophages with phase-contrast microscopy.

‡ The results represent the mean of three experiments.

§ ND, not determined.

‖ This is not a mannan, but a galactan containing exclusively glucose, galactose, and phosphate.

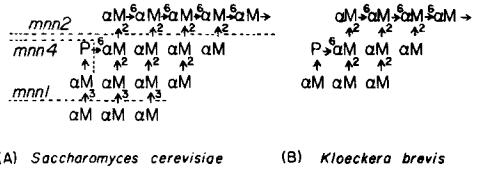

FIGURE 1 Structure of the outer chains of (*A*) *S. cerevisiae* X2180 mannan and mannans produced by its mutants (1); and (*B*) *Kl. brevis* mannan (28).

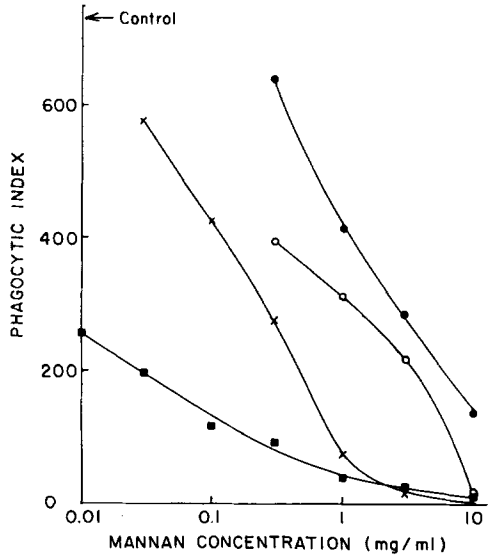

FIGURE 2 Inhibition of resident macrophage phagocytosis of *S. cerevisiae* zymosan by different mannans. Resident macrophages on 13-mm diameter glass coverslips were preincubated in 0.5 ml of MEM containing 2% FBS and 25 mM HEPES, pH 7.4 in Costar wells with or without inhibitor mannan for 15 min at 37°C. 20 μl of 2% *S. cerevisiae* zymosan in PD were then added and the cultures were incubated for an additional 30 min at 37°C. The cultures were then washed, fixed, and assayed microscopically for zymosan ingestion. ○, *S. cerevisiae* X2180 mannan; ●, mannan from the *mnn2* mutant of X2180; ×, mannan from the LB-301 mutant of X2180; ■, *Kl. brevis* mannan with a mannose/phosphate ratio of 8.6:1.

more potent than wild type or *mnn2* mannan as a phagocytosis inhibitor. The most effective phagocytosis inhibitor was a mannan fraction with a mannose:phosphate ratio of 8.6:1 purified from *Kl. brevis* (28). It is at least two orders of magnitude better, on a weight basis, than wild type *S. cerevisiae* mannan in inhibiting zymosan phagocytosis (Fig. 2).

Inflammatory macrophages elicited with thioglycollate medium or proteose peptone broth are larger and more phagocytic (4) than resident macrophages. To determine whether mannan affects zymosan uptake by these cells we incubated them with *S. cerevisiae* zymosan in the presence of varying amounts of *Kl. brevis* or *S. cerevisiae* mannans. Both types of mannan inhibited zymosan ingestion by thioglycollate-elicited and proteose peptone-elicited macrophages (Fig. 3). However, the two types of inflammatory macrophages differed in their susceptibility to mannan inhibition of zymosan uptake. Thioglycollate-elicited macrophages were inhibited by lower concentrations of mannan than were resident macrophages, whereas the reverse was observed with proteose peptone-elicited macrophages (Fig. 3). It is relevant, and should be noted here, that Ezekowitz et al. (8) found marked variation in the activity of Man/GlcNAc receptors on mouse macrophages depending upon

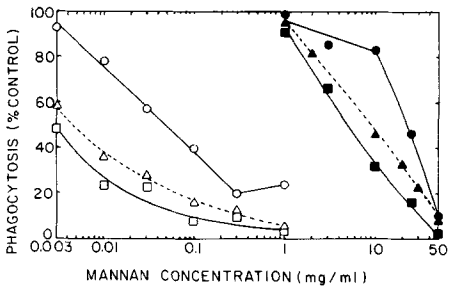

FIGURE 3 Phagocytosis inhibition of different cell types by yeast mannans. Inhibition of *S. cerevisiae* zymosan ingestion was performed as in Fig. 2. ○, ● proteose peptone-elicited macrophages; △, ▲, resident macrophages; □, ■ thioglycollate-elicited macrophages. Open symbols, ○, △, □, inhibition by *Kl. brevis* mannan; closed symbols ●, ▲, ■, inhibition by *S. cerevisiae* X2180 mannan.

their source and the agents used to elicit them. Similarly, the data in Fig. 3 suggest that the number and/or affinity of macrophage receptors for zymosan varies, depending upon the agent used to elicit the macrophages.

Mannans Selectively Inhibit Phagocytosis of Zymosan

To determine whether mannans are general inhibitors of macrophage phagocytosis, or whether they act selectively on a subset of macrophage membrane receptors, we incubated resident macrophages with IgG-coated sheep erythrocytes in the presence of varying concentrations of *Kl. brevis* mannan. This mannan inhibited phagocytosis of *S. cerevisiae* zymosan, but had no inhibitory effect on Fc receptor-mediated ingestion of IgG-coated erythrocytes (Fig. 4). This experiment indicates that mannans do not inhibit phagocytosis per se; it suggests that the mannans affect a specific subset of receptors on the macrophage surface.

Mannans Inhibit Binding of Zymosan to the Macrophage

S. cerevisiae zymosan does not bind efficiently to macrophages at 4°C (<250 particles bound per 100 resident or proteose peptone-elicited macrophages). Because *Kl. brevis* mannan is a much more potent inhibitor of zymosan uptake than *S. cerevisiae* mannan, we reasoned that cell walls prepared from *Kl. brevis* might have a higher affinity for the macrophages than *S. cerevisiae* zymosan. Indeed, when *Kl. brevis* zymosan was incubated with macrophages at 4°C, the *Kl. brevis* zymosan was bound efficiently to the macrophages. Resident and proteose peptone-elicited macrophages bound 390 ± 130 and 420 ± 160 *Kl. brevis* zymosan particles per 100 macrophages, respectively, whereas thioglycollate-elicited macrophages bound 200 ± 150. Thus, consistent with the results described in Fig. 3, thioglycollate-elicited macrophages bind fewer zymosan particles than resident or proteose peptone-elicited macrophages.

Kl. brevis mannan is a much more potent inhibitor than *S. cerevisiae* mannan of binding of *Kl. brevis* zymosan to macrophages. The concentration causing 50% inhibition of *Kl. brevis* zymosan binding to proteose peptone-elicited macrophages was ~100 μg/ml for *S. cerevisiae* mannan and ~3–5 μg/ml for *Kl. brevis* mannan. *Kl. brevis* mannan completely inhibited the binding of *Kl. brevis* zymosan to resident, thioglycollate-elicited, and proteose peptone-elicited macrophages at concentrations above 20 μg/ml (data not shown).

Modulation of Mannan Receptors by Substrate Adherent Ligands

The results presented to this point confirm that specific mannan-inhibitable receptors mediate the binding of zymosan particles to the macrophage surface; they suggest that these receptors mediate the internalization of zymosan particles as well. To examine these issues further we have examined the effects of ligand-coated surfaces on zymosan uptake, a technique that has proved useful in analyzing the physiology of macrophage Fc and complement receptors (19) and of the chicken hepatocyte galactose-binding receptor (33). Macrophages were plated on coverslips coated with PLL to which mannan was cross-linked; the macrophages were then incubated at 37°C with zymosan particles. Few of the macrophages

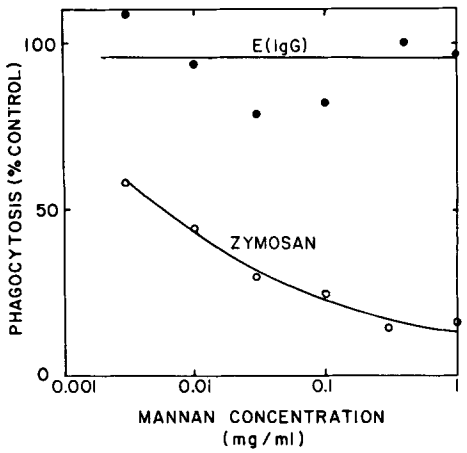

FIGURE 4 Effects of *Kl. brevis* mannan on ingestion of *S. cerevisiae* zymosan or IgG-coated erythrocytes by resident macrophages. Experimental protocol is the same as in Fig. 2. The amount of IgG-coated erythrocytes added was 20 μl of 2% particles. Uningested IgG-coated erythrocytes bound on the macrophages were lysed by hypotonic lysis in water. The total number of particles ingested by macrophages in control medium was approximately 400 and 600 per 100 macrophages for zymosan and IgG-coated erythrocytes, respectively. ○, zymosan ingestion; ●, IgG-coated erythrocyte ingestion.

plated on these mannan-coated coverslips for 2 h before the addition of zymosan ingested any of these particles (Table II). Macrophages incubated on PLL-mannan for 24 h before the addition of zymosan gave qualitatively similar results (Table II). In contrast, the capacity of macrophages plated on PLL-mannan to bind complement-coated erythrocytes and to ingest IgG-coated erythrocytes was unimpaired (Table II). These results confirm that the receptors that mediate zymosan binding and ingestion can be modulated by substrate adherent ligands without altering the activities of other classes of macrophage membrane receptors (i.e. Fc and complement receptors); moreover, in conjunction with the data in Fig. 4 they show that these receptor systems operate independently of one another.

Chloroquine Inhibits Zymosan Phagocytosis

Chloroquine elevates macrophage intralysosomal pH (20) and promotes "down regulation" of macrophage Man/GlcNAc receptors (29), presumably by trapping the receptors within intracellular compartments (12, 29). As shown in Fig. 5*a*, chloroquine inhibits zymosan phagocytosis in a dose-dependent manner, reaching 75% inhibition at 250 μM. Preincubation of macrophages in chloroquine enhances its inhibitory effect. The inhibitory effect of chloroquine was further increased by including mannan in the preincubation medium. Under these last conditions, 10 μM chloroquine was sufficient to produce 50% inhibition of zymosan phagocytosis.

Macrophages incubated at 4°C with 250 μM chloroquine bind as many *Kl. brevis* zymosan as control macrophages, indicating that the drug does not interfere directly with the interaction of zymosan with macrophage receptors. As expected, macrophages preincubated at 37°C for 1 h with 100 μM chloroquine exhibited a 95% reduction in binding of *Kl. brevis* zymosan (data not shown).

In all cases, the inhibitory effects of chloroquine, or of chloroquine plus mannan, on zymosan binding (data not shown) and phagocytosis (Fig. 5*b*) were reversible. Chloroquine had no effect on the activity of other macrophage surface receptors examined. For instance, treatment of resident mac-

TABLE II

Phagocytosis of Resident Macrophages Plated on PLL-Mannan

	macrophages phagocytosing	Average no. of particles ingested per macrophage	Phagocytic index	Control phagocytic index
	%			%
Zymosan ingestion by macrophages plated for 2 h* on:				
PLL	93	3.5	320	100
PLL-BSA	84	3.0	250	78
PLL-mannan	13	1.8	23	7
Zymosan ingestion by macrophages plated for 24 h‡ on:				
PLL	85	6.1	522	100
PLL-BSA	86	4.4	382	73
PLL-mannan	41	2.5	102	19
IgG-Coated erythrocyte ingestion by macrophages plated for 24 h§ on:				
PLL	99	12.2	1,210	100
PLL-BSA	93	9.4	874	72
PLL-mannan	96	11.8	1,130	93

	macrophage binding	Average no. of particles bound per macrophage	Binding index	Control binding index
	%			%
Complement-coated erythrocyte binding¶ by macrophages plated for 24 h on:				
PLL	100	18.3	1,830	100
PLL-mannan	100	15.7	1,570	86

* 8×10^4 resident macrophages were incubated on 13-mm coverslips coated with the indicated ligands at 37° in MEM-10% FBS for 2 h. After washing three times with MEM, these coverslips were placed in 0.5 ml of MEM-2% FBS-25 mM HEPES, 7.4 in 16-mm costar wells. 20 μl of 2% *S. cerevisiae* zymosan in PD were added and phagocytosis was allowed to proceed for 30 min at 37°. Only cells with well-spread membrane and macrophage morphology were counted.

‡ Macrophages were plated on PLL, PLL-BSA and PLL-mannan coverslips as described above. After incubation at 37° for 24 h, phagocytosis was performed as indicated. The results represent the average of four experiments.

§ Macrophages on the indicated coverslips were prepared the same way as those used in ‡ above. For each coverslip in a 16-mm costar well containing 0.5 ml of MEM-2% FBS-25 mM HEPES, pH 7.4, 20 μl of 2% IgG-coated erythrocytes were added. After phagocytosis for 30 min, coverslips were dipped in water, washed with PD, fixed in glutaraldehyde, and counted as described in Fig. 4.

¶ Macrophages prepared as in ‡ above were incubated with 20 μl of 2% complement-coated erythrocytes for 30 min at 37°C, washed with PD, fixed in glutaraldehyde, and counted as described in Fig. 4.

rophages with chloroquine or with chloroquine plus mannan did not alter binding or ingestion of IgG-coated erythrocytes (data not shown), confirming again (cf. Fig. 4) that the receptor that mediates zymosan uptake functions independently of other macrophage receptor systems.

Zymosan Uptake is Abolished by Trypsin and Reduced by Calcium Chelation

The capacity of rat alveolar macrophages to bind mannose-BSA and *Candida krusei* is inhibited by trypsin treatment of the macrophages or by chelation of Ca^{++} in the medium (26, 32). Incubation of resident macrophages with trypsin reduced the capacity of these cells to bind and ingest *S. cerevisiae* zymosan by 93% and 72% (mean of three experiments), respectively (data not shown). Incubation of macrophages in calcium-free medium had no inhibitory effect on zymosan binding or phagocytosis. In contrast, addition of EGTA to the medium caused a 52–61% decrease in zymosan binding and a 43–45% decrease in zymosan ingestion by macrophages. The inhibitory effects of EGTA were overcome when excess Ca^{++} was added to the medium.

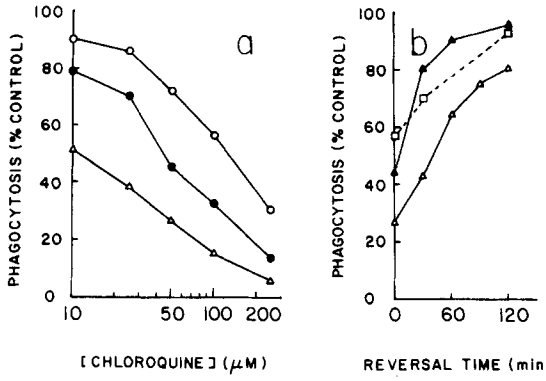

FIGURE 5 (*a*) Dose response of chloroquine inhibition of phagocytosis of *S. cerevisiae* zymosan by mouse peritoneal macrophages. Washed 24-h cultures of macrophages on 13-mm coverslips (2×10^5 peritoneal cells/coverslip) in 16-mm Costar wells were preincubated at 37°C for 1 h with 500 μl of medium containing various concentrations of chloroquine in control medium (MEM-25 mM HEPES, pH 7.4-2% FBS) with (△) or without (●) 10 mg/ml mannan. The coverslips were then washed three times with cold MEM, and the medium was replaced with 500 μl of control medium containing chloroquine without mannan and 0.08% (vol/vol) *S. cerevisiae* zymosan. Phagocytosis was performed for 30 min at 37°C. Macrophages in ○ were not preincubated with chloroquine or mannan, and phagocytosis was performed in chloroquine-containing medium. Phagocytosis is expressed as % of the control phagocytic index, which is 390 for these experiments. The results are the average of four experiments. (*b*) Phagocytosis reversal of chloroquine inhibition. Macrophages preincubated for 1 h in 50 μM chloroquine (▲), 10 mg/ml mannan (□), or 50 μM chloroquine plus 10 mg/ml mannan (△) were washed three times with cold MEM and placed in warm control medium. Zymosan particles were added at 0, 30, 60, 90, and 120 min after preincubation and phagocytosis was carried out for 30 min. The reversal time denotes the total amount of time after preincubation, and the phagocytic index for control is 420. The results are the average of two experiments.

The Man/GlcNAc receptor described by Townsend and Stahl (30) requires Ca^{++} for ligand binding. Thus the failure of EGTA to completely block zymosan binding and ingestion might result from participation of another receptor system in this process. The mannose phosphate receptor described by Kaplan et al. (15) has no Ca^{++} requirement for ligand binding. To determine whether this receptor has any role in zymosan uptake, we incubated resident macrophages with EGTA and 1 mg/ml phosphomannan monoester from *Hansenula holstii* or 25 mM mannose-6-phosphate. Addition of these phosphorylated saccharides did not enhance the inhibitory effect of EGTA (data not shown).

Macrophage Receptors that Mediate Zymosan Uptake Recognize Neutral and Not Phosphorylated Mannans

To search further for a possible role for a mannose phosphate receptor in zymosan uptake we compared the effects of mannose-6-phosphate and mannose phosphate-containing oligosaccharides with mannose, mannose-containing oligosaccha-

rides, dephosphorylated mannan, and a mannose-containing glycoprotein on zymosan binding and phagocytosis. 50 mM mannose-6-phosphate was slightly less inhibitory than equal concentrations of mannose or mannobiose (Table III). None of these saccharides was an effective inhibitor of zymosan binding or ingestion. Mannotriose and mannotetraose, derived from the acetolysis of *S. cerevisiae* mannan, were good inhibitors at 50 mM (Table III) but were roughly comparable to mannose in inhibitory potency when their concentrations were corrected for their mannose content (e.g. 10 mM mannotetraose equals 40 mM mannose). D-Glucose, L-fucose and D-galactose at 50 mM had no effect on either the binding or phagocytosis of zymosan by macrophages (data not shown).

To examine the effect of mannan phosphorylation on zymosan uptake we used mild acid hydrolysis and alkaline phosphatase to degrade *Kl. brevis* mannan (mannose:phosphate ratio = 8.6:1) in a stepwise fashion. Mild acid treatment resulted in only a small reduction in the inhibitory potency of *Kl. brevis* mannan (Fig. 6). Alkaline phosphatase treatment of this acid-hydrolyzed mannan removed 65% of the phosphate groups but caused no further change in the inhibitory potency of the mannan (Fig. 6).

In a related series of experiments, exocellular mannans with high phosphate content that had been hydrolysed with mild acid to expose their mannose-6-phosphate groups were tested for their ability to inhibit the phagocytosis of zymosan particles by macrophages. They were no more inhibitory than the corresponding neutral mannans (Table I). *O*-Phosphonomannan monoesters, such as one from *Hansenula holstii* that potently inhibits β-glucuronidase uptake by the fibroblast mannose-6-phosphate receptor (50% inhibition = 0.2 μg/ml [reference 10]), were also ineffective. On a weight basis, the best inhibitors of phagocytosis among several exocellular mannans tested were the *O*-phosphonoglucomannan and the neutral glucomannan from *Pichia pinus*. *Pichia pinus* mannan contains almost exclusively glucose as the nonreducing end group (24). A glucogalactan from *Sporobolomyces sp.* (23) that has predominantly glucose at the nonreducing terminal and contains no mannose also inhibited phagocytosis to an appreciable extent (Table I). Thus glucose appears to compete for the binding site for zymosan particles.

TABLE III

Effects of Glycoproteins and Sugars on the Phagocytosis And Binding of Zymosan by Macrophages *

Inhibitor	Concentration	Phagocytosis (% control)	Binding (% control)
Horseradish peroxidase	30 mg/ml	13 ± 8	1 ± 0.7
	10 mg/ml	28 ± 2	7 ± 6
D-Mannose	50 mM	57 ± 24	65 ± 29
Mannobiose‡	50 mM	ND§	50¶
Mannotriose	50 mM	ND	11¶
	25 mM	ND	53 ± 16
	10 mM	ND	121¶
Mannotetraose	50 mM	ND	7¶
	25 mM	ND	22 ± 6
	10 mM	ND	69 ± 8
α-Methyl-D-mannopyranoside	50 mM	101 ± 8	89 ± 25
Mannose-6-phosphate	50 mM	68 ± 16	80 ± 23

* Macrophages (8×10^4 macrophages per 13-mm coverslip) in MEM-10% FBS were incubated overnight at 37°C, washed, pretreated with the listed compounds in 0.5 ml of MEM-2% FBS-25 mM HEPES, pH 7.4 for 15 min at 37°C for phagocytosis and 4°C for binding before the addition of particles. For binding, 20 μl of 5% *Kl. brevis* zymosan was added to proteose peptone-elicited macrophages and the cultures were allowed to stand at 4°C for 90 min. For phagocytosis, 20 μl of 2% *S. cerevisiae* was added to resident macrophages and the cultures were incubated at 37°C for 30 min. The number of particles ingested or bound was scored by phase-contrast microscopy. 50-mM sugar solutions were adjusted to a final osmolarity of 300 mosmole by dissolving the sugars in a solution of 3 parts MEM-2% FBS-25 mM HEPES pH 7.4 and 1 part distilled water. The results represent the mean of two to three experiments and are expressed as mean ± standard deviation.

‡ Acetolysis products of *S. cerevisiae* mannan were preincubated with resident macrophages (24 h in culture) for 15 min. at 4°C before addition of *Kl. brevis* zymosan.

§ Not done.

¶ Experiments were performed once.

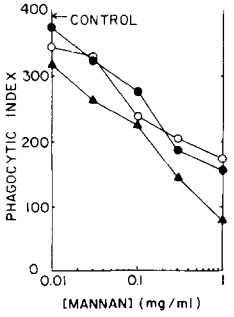

FIGURE 6 Phagocytosis inhibition by *Kl. brevis* mannan treated with mild acid and alkaline phosphatase. 20 mg of *Kl. brevis* mannan were treated with 0.01 M HCl at 100° for 30 min. After dialysis, an aliquot of the acid-treated mannan in 0.8 ml of 0.75 M Tris HCl pH 8.0 was treated with 49 U of alkaline phosphatase for 24 h. Phagocytosis inhibition was performed as in Fig. 2. ▲ *Kl. brevis* mannan; ○ acid hydrolysed; ●, acid hydrolysed and alkaline phosphatase-treated *Kl. brevis* mannan. The mannan used was from a slightly less inhibitory batch.

Horseradish peroxidase is reported to contain no phosphorylated oligosaccharides (34), and we have confirmed that the peroxidase used in our experiments has no detectable phosphate. It is a very effective inhibitor of zymosan binding and phagocytosis (Table III). Moreover, in experiments to be reported elsewhere, we have found that pinocytosis of HRP by mouse macrophages can be inhibited to a significant degree by mannan. Thus mannan and HRP bind to the receptors exhibiting similar ligand specificity.

In summary, the results reported here show that zymosan binding and phagocytosis can be inhibited by micromolar concentrations of *Kl. brevis* mannan and by somewhat higher concentrations of other mannose-containing oligosaccharides and glycoproteins; that phosphorylated mannose residues do not contribute to the inhibitory effects of the saccharides or glycoproteins used in these experiments; that the macrophage surface structures that mediate zymosan uptake can be modulated by substrates coated with mannan; that they can be "down regulated" by mannan in the presence of chloroquine, destroyed by trypsin, and inhibited by Ca^{++} chelation. These treatments have no effect on the binding or phagocytosis of IgG-coated erythrocytes by macrophage Fc receptors. These findings indicate that zymosan binding and phagocytosis are mediated by a specific membrane receptor and that the receptor is similar in its ligand-binding requirements, trypsin sensitivity, and requirement for Ca^{++} to the Man/GlcNAc receptor described by Stahl and his colleagues (25, 26). We conclude that zymosan uptake is mediated by this Man/GlcNAc receptor. Thus the mannose receptor, like the Fc and complement receptors (27, 31), mediates both adsorptive pinocytosis of soluble molecules and phagocytosis of particulate materials.

We would like to thank Drs. Clinton E. Ballou and Morey E. Slodki for so generously providing us with the yeast mannans and Dr. Herman Phaff for sending us his *Kl. brevis* strain. We would also like to thank Dr. Zanvil A. Cohn for his support of this work, Miss Betty Broyles for preparing the manuscript, and Ms. Judy Adams for making the prints.

This work was supported in part by the United States Public Health Service grant AI 08697.

Received for publication 8 July 1982, and in revised form 16 August 1982.

REFERENCES

1. Ballou, C. E. 1980. Genetics of yeast mannoprotein biosynthesis. *In* Fungal Polysaccharides. P. A. Sanford and K. Matsuda, editors. American Chemical Society, Washington, D.C. 1–14.
2. Ballou, L., R. E. Cohen, and C. E. Ballou. 1980. *Saccharomyces cerevisiae* mutants that make mannoprotein with a truncated carbohydrate outer chain. *J. Biol. Chem.* 255:5986–5991.
3. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466–468.
4. Bianco, C., F. M. Griffin, Jr., and S. C. Silverstein. 1975. Studies of the macrophage complement receptor. Alteration of receptor function upon macrophage activation. *J. Exp. Med.* 141:1278–1290.
5. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes. *J. Exp. Med.* 121:153–170.
6. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350–356.
7. Edelson, P. J., R. Zweibel, and Z. A. Cohn. 1975. The pinocytic rate of activated macrophages. *J. Exp. Med.* 142:1150–1164.
8. Ezekowitz, P. A. B., J. Austyn, P. D. Stahl, and S. Gordon. 1981. Surface properties of Bacillus Calmette-Guérin activated mouse macrophages. Reduced expression of mannose-specific endocytosis, Fc receptors and antigen F4/80 accompanies induction of Ia. *J. Exp. Med.* 154:60–76.
9. Fisher, H. D., A. Gonzalez-Noriega, W. S. Sly, and D. J. Morré. 1980. Phosphomannosyl-enzyme receptors in rat liver. Subcellular distribution and role in intracellular transport of lysosomal enzymes. *J. Biol. Chem.* 255:9608–9615.
10. Fisher, H. D., M. Natowicz, W. S. Sly, and R. K. Bretthauer. 1980. Fibroblast receptor for lysosomal enzymes mediates pinocytosis of multivalent phosphomannan fragment. *J. Cell Biol.* 84:77–86.
11. Fraker, P. J., and J. C. Speck, Jr. 1978. Protein and cell membrane iodinations with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril. *Biochem. Biophys. Res. Commun.* 80:849–857.
12. Gonzalez-Noriega, A., J. H. Grubb, V. Talkad, and W. S. Sly. 1980. Chloroquine inhibits lysosomal enzyme pinocytosis and enhances lysosomal enzyme secretion by impairing receptor recycling. *J. Cell Biol.* 85:839–852.
13. Hubbard, A. L., G. Wilson, G. Ashwell, and H. Stukenbrok. 1979. An electron microscope autoradiographic study of the carbohydrate recognition systems in rat liver. I. Distribution of ^{125}I-ligand among the liver cell types. *J. Cell Biol.* 83:47–64.
14. Jacin, H., and A. R. Mishkin. 1965. Separation of carbohydrate on borate impregnated silica gel G plates. *J. Chromatogr.* 18:170–173.
15. Kaplan, A., D. T. Achord, and W. S. Sly. 1977. Phosphohexosyl components of a lysosomal enzyme are recognized by pinocytosis receptors on human fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 74:2026–2030.
16. Kocourek, J., and C. E. Ballou. 1969. Method of fingerprinting yeast cell wall mannans. *J. Bacteriol.* 100:1175–1181.
17. Lachmann, P. J., and M. J. Hobart. 1978. Complement technology. *In* Handbook of Experimental Immunology, 3rd Edition. D. M. Weir, editor. Blackwell Scientific Publications. Oxford. 5A.9–5A.10.
18. Michl, J., D. J. Ohlbaum, and S. C. Silverstein. 1976. 2-Deoxyglucose selectively inhibits Fc and complement receptor-mediated phagocytosis in mouse peritoneal macrophages. I. Description of the inhibitory effect. *J. Exp. Med.* 144:1465–1483.
19. Michl, J., M. M. Pieczonka, J. C. Unkeless, and S. C. Silverstein. 1979. Effects of immobilized immune complexes on Fc and complement receptor function in resident and thioglycollate-elicited mouse peritoneal macrophages. *J. Exp. Med.* 150:607–621.
20. Okuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. U. S. A.* 75:3327–3331.
21. Raschke, W. C., K. A. Kern, C. Antalis, and C. E. Ballou. 1973. Genetic control of yeast mannan structure. Isolation and characterization of mannan mutants. *J. Biol. Chem.* 248:4660–4666.
22. Silverstein, S. C., R. M. Steinman, and Z. A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* 46:669–722.
23. Slodki, M. E. 1980. Structural aspects of exocellular yeast polysaccharides. *In* Fungal polysaccharides. P. A. Sanford and K. Matsuda, editors. American Chemical Society, Washington, D.C. 183–196.
24. Slodki, M. E., R. M. Ward, J. A. Boundy, and M. C. Cadmus. 1972. Extracellular mannans and phosphomannans: structural and biosynthetic relationships. *In* Proc. Int. Ferment. Symp. 4th: Fermentation Technology Today. G. Terui, editor. Soc. Fermentation Technology, Osaka. 597–601.
25. Stahl, P. D., J. S. Rodman, M. J. Miller, and P. H. Schlesinger. 1978. Evidence for receptor-mediated binding of glycoproteins, glycoconjugates and lysosomal glycosidases by alveolar macrophages. *Proc. Natl. Acad. Sci. U. S. A.* 75:1399–1403.
26. Stahl, P., P. H. Schlesinger, E. Sigardson, J. S. Rodman, and Y. C. Lee. 1980. Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages: characterization and evidence for receptor recycling. Cell. 19:207–215.
27. Steinman, R. M., and Z. A. Cohn. 1972. The interaction of particulate horseradish peroxidase (HRP)-anti HRP immune complexes with mouse peritoneal macrophages *in vitro*. *J. Cell Biol.* 55:616–634.
28. Thieme, T. R., and C. E. Ballou. 1972. Subunit structure of the phosphomannan from *Kloeckera brevis* yeast cell wall. *Biochemistry.* 11:1115–1120.
29. Tietze, C., P. Schlesinger, and P. Stahl. 1980. Chloroquine and ammonium ion inhibit receptor-mediated endocytosis of mannose-glycoconjugates by macrophages: apparent inhibition of receptor recycling. *Biochem. Biophys. Res. Commun.* 93:1–8.
30. Townsend, R., and P. Stahl. 1981. Isolation and characterization of a mannose/*N*-acetylglucosamine/fucose binding protein from rat liver. *Biochem. J.* 194:209–214.
31. Van Snick, J. L., and P. L. Masson. 1978. The effect of complement on the ingestion of soluble Ag-Ab complexes and IgM aggregates by mouse peritoneal macrophages. *J. Exp. Med.* 148:903–914.
32. Warr, G. A. 1980. A macrophage receptor for (mannose/glucosamine)-glycoproteins of potential importance in phagocytic activity. *Biochem. Biophys. Res. Commun.* 93:737–745.
33. Weigel, P. H. 1980. Rat hepatocytes bind to synthetic galactoside surfaces via a patch of asialoglycoprotein receptors. *J. Cell Biol.* 87:855–861.
34. Welinder, K. G. 1979. Amino acid sequence studies of horseradish peroxidase amino and carboxyl termini. Cyanogen bromide and tryptic fragments, the complete sequence and some structural characteristics of horseradish peroxidase C. *Eur. J. Biochem.* 96:483–502.