Immunology 1986 **58** 117–124

Activation of human polymorphonuclear leucocytes by particulate zymosan is related to both its major carbohydrate components: glucan and mannan

J. D. WILLIAMS, N. TOPLEY, H. M. ALOBAIDI & M. J. HARBER *Department of Renal Medicine, KRUF Institute, Royal Infirmary, Cardiff*

Accepted for publication 2 January 1986

SUMMARY

Unopsonized particulate zymosan and its major carbohydrate component glucan were phagocytosed under serum-free conditions by adherent polymorphonuclear leucocytes (PMN) in a dose- and time-dependent manner. Preincubation of PMN monolayers with mannan did not cause a reduction in the phagocytosis of either particle. The phagocytic response was inhibited by preincubation of the cells with trypsin at a concentration that did not inhibit the phagocytosis of sheep erythrocytes coated with IgG or of latex particles. Homology of the recognition mechanisms for glucan and zymosan was confirmed when cells cultured on fixed glucan or on fixed zymosan failed to ingest either particle to more than 40% of control phagocytosis. Similarly, zymosan and glucan activated PMN in suspension, in a dose- and time-dependent manner, to generate reactive oxygen species which were measured as luminol-dependent chemiluminescence (CL). There was, however, a four-fold greater CL response to zymosan. Preincubation of PMN with mannan resulted in a significantly decreased CL response to zymosan, while the response to glucan was unaffected. The CL response was also sensitive to a range of concentrations of trypsin. In contrast, two other complex polysaccharide particles (barley-derived β-glucan and algae-derived laminarin) were not phagocytosed by PMN, nor did they cause the generation of CL, despite the fact that they possessed the capacity, in common with zymosan and glucan, to activate the alternative pathway of complement. The identification of a trypsin-sensitive recognition mechanism on the surface of human PMN for unopsonized zymosan and glucan represents a response not hitherto characterized. Furthermore, our data indicate that the phagocytosis of unopsonized zymosan by human PMN is dependent primarily on its glucan content, but that its capacity to activate the respiratory burst may involve mannan and the recruitment of a second cell surface recognition mechanism.

INTRODUCTION

Particulate ligands and phagocytic cells interact through a variety of mechanisms that depend both on the recognition units on the cell surface and on the nature of the interacting particle. Human polymorphonuclear leucocytes (PMN) and mononuclear phagocytes possess on their surface distinct phagocytic receptors for the Fc portion of IgG (Mantovani, 1975; Huber & Fundenberg, 1968) as well as receptors for surface-bound cleavage products of the third component of complement. These include the CR1 and CR3 receptors, recognizing particle-bound C3b and iC3b, respectively (Ross & Lambris, 1982). Such complement receptors are generally thought to act as opsonic receptors (Ehlenberger & Nussenzweig, 1977), but under certain conditions may function directly as phagocytic receptors (Newman, Becker & Halme, 1985). Circulating human monocytes also possess a trypsin-sensitive phagocytic receptor which recognizes target activators of the alternative pathway of complement, including zymosan, rabbit erythrocytes and desialated sheep erythrocytes (Czop, Fearon & Austen, 1978; Czop & Austen, 1980), and which, when stimulated, initiates the generation and release of inflammatory mediators (Williams, Czop & Austen, 1984; Czop & Austen, 1985a). In contrast, the interaction between human PMN and unopsonized zymosan particles has received less attention. In one study zymosan particles interacted only with PMN after centrifugation at 400 *g*, and the mechanism described was not trypsin sensitive (Roos *et al.*, 1981). Phagocytosis does, however, occur when zymosan and PMN are brought together, either at much lower speeds or in suspension; an interaction that results in the release of

Abbreviations: CL, chemiluminescence; c.p.s., counts per second; *E. coli*, *Escherichia coli*; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis-amino-tetraacetate; E^s, sheep erythrocytes; GVB, veronal-buffered saline with 11 mM glucose; KRPG, Krebs–Ringer phosphate buffer with 11 mM glucose; PBS, phosphate-buffered saline; PMN, polymorphonuclear leucocytes.

Correspondence: Dr John Williams, Dept. of Renal Medicine, Royal Infirmary, Newport Road, Cardiff CF2 1SZ, U.K.

leukotrienes (Williams *et al.*, 1985) and in the generation of reactive oxygen species (Ross, Cain & Lachmann, 1985; Harber & Topley, 1986).

The phagocytosis of particulate zymosan by murine peritoneal macrophages (Rabinovitch & DeStefano, 1973) is thought to take place through a mechanism that involves the surface mannosyl/fucosyl receptor (MFR), based on the capacity of yeast mannan to inhibit the ingestion (Sung, Nelson & Silverstein, 1983), and on the modulation of MFR activity in macrophages cultured on fixed zymosan (Berton & Gordon, 1983). Other studies have indicated that the phagocytosis of zymosan by human monocytes is dependent on locally secreted complement components, and that the mechanism of ingestion is related to both the CR3 and the MFR receptors (Ezekowtiz *et al.*, 1983).

The exact nature of the cell surface recognition mechanism on human PMN is unclear, but strong evidence has recently been presented to implicate the CR3 receptor (Ross *et al.*, 1985). In addition, the characteristics of the activating ligand have yet to be fully defined, although the recognition unit on human monocytes has been shown to be inhibitable by a β-glucan (Czop & Austen, 1985b). In the present study, we have investigated the capacity of particulate, unopsozined zymosan and its major polysaccharide components glucan and mannan, to stimulate human PMN in serum-free conditions by quantifying phagocytosis and by measuring luminol-dependent chemiluminescence (CL), a technique which provides an estimate of the generation of reactive oxygen species by inflammatory cells (Allen, 1982).

MATERIALS AND METHODS

Preparation of PMN

Normal human PMN were isolated from citrated peripheral blood by dextran sedimentation of erythrocytes, rendered plasma-free and platelet-poor by washing with phosphate-buffered saline, pH 7·3, lacking calcium and magnesium (PBS), and purified by density gradient centrifugation at 400 *g* for 35 min at 23° on Ficoll–Hypaque. The PMN pellet was rendered erythrocyte-free by hypotonic lysis, counted in a modified Neubauer counting chamber and resuspended in PBS at 1×10^7 cells/ml. Using cytocentrifuged preparations (Cytospin II, Shandon Southern Products Ltd, Runcorn, Cheshire) more than 95% of these cells were identified as PMN by their morphology.

The PMN used for phagocytosis studies were washed and resuspended in RPMI–1640 medium containing 0·2% bovine serum albumin (BSA crystalised; Miles Laboratories Inc., Slough, Berks) (RPMI–BSA) at a concentration of 2×10^6 cells/ml. Three millilitres of this suspension were layered onto 50 mm Petriperm tissue culture dishes (W.C. Heraeus, GmbH) and were incubated at 37° in a humidified chamber of 5% CO_2 for 30 min. The dishes were washed three times with 2 ml of RPMI–BSA to remove non-adherent cells before the addition of target particles. About 75% of the layered cells ($4{\cdot}5 \times 10^6$) remained attached to the petri-dishes, as determined by quantitation of non-adherent cells. More than 97% of the adherent cells were identified as PMN by their morphology.

The PMN used to assess luminol-dependent CL were stored prior to assay at 5×10^6 cells/ml in PBS at 4° after hypotonic lysis. Cells stored under these conditions showed consistent CL responses for up to 8 hr after preparation (Harber & Topley, 1986).

Preparation of target particles

Zymosan A particles (Sigma Chemical Co., Poole, Dorset) were boiled, washed and stored in 0·15 M NaCl and counted in a modified counting chamber under phase contrast microscopy. Glucan particles (Sigma), which are an homopolysaccharide extract of the yeast *Saccharomyces cereviciae*, were suspended in 0·15 M NaCl, subjected a 1-min period of sonication in an MSE 150 watt ultrasonic disintegrator (MSE, Crawley, Sussex) at 16 μ peak to peak activity to disrupt clumps, and counted. β-Glucan (Sigma), an extract of barley, was suspended in buffer, subjected to sonication and counted. Glucose analysis (Dubois *et al.*, 1956) of the hydrolysed pellet and supernatant revealed that glucan derived from *Saccharomyces cereviciae* was totally insoluble, while β-glucan derived from barley was 50% soluble in buffer or distilled water. Both soluble and insoluble fractions from the β-glucan were analysed separately for their capacity to activate PMN. Laminarin (P.-L. Biochemicals Inc., Milton Keynes, Bucks.), a particulate homopolysaccharide extract of algae, was suspended in buffer, washed and the particles counted. Mannan (Sigma), a soluble, branched homopolysaccharide of mannose subunits, was dissolved in buffer at the appropriate concentration.

Sheep erythrocytes (E^s) (Tissue Culture Services, Slough, Berks.) were washed and stored in Veronal-buffered saline, pH 7·5, containing 0·1% (w/v) gelatin (GVB) and 0·04 M ethylenediaminetetraacetic acid (EDTA), (GVB–EDTA). E^s-bearing IgG (E^s-IgG) were prepared by incubating 5×10^8 E^s/ml in GVB-EDTA with equal volumes of rabbit 7S anti-sheep haemolysin (Sera Lab Ltd, Crawley Down, W. Sussex) diluted 1/60 in GVB–EDTA for 30 min at 37°, then for 30 min on ice. Cells were then washed three times and stored in GVB–EDTA at 4°. Latex beads (1·09 μm diameter; Sigma) were diluted to a concentration of 1×10^9/ml in buffer immediately before use.

A uropathogenic strain of *Escherichia coli* (SC) isolated in our laboratory was grown for 18 hr in pooled normal human urine to suppress fimbrial synthesis. Under these culture conditions, this strain was not an activator of the alternative complement pathway, was not phagocytosed by human PMN, and did not induce generation of CL. Opsonization was performed in 75% pooled human serum: 25% Krebs–Ringer phosphate buffer, pH 7·3, with 11 mM glucose (KRPG), for 30 min at 37°, and the bacteria washed three times in buffer before use. This resulted in a marked increase in the susceptibility of *E. coli* SC to phagocytosis and in its ability to stimulate CL generation.

Assay of phagocytosis

The PMN monolayers were incubated with 1·5 ml of various concentrations of target particles in RPMI–1640 supplemented with 5 mM $MgCl_2$ (RPMI-Mg) for the designated times. The monolayers were then washed three times with PBS, dried, fixed in 95% methanol, stained with Wright's stain and the number of particles ingested by at least 200 PMN/plate were determined by visual enumeration at a magnification of 400× with a light microscope. Results were expressed as the percentages of PMN ingesting two or more particles/cell. Phagocytosis was confirmed in two ways: (a) incubation of cells for 15 min after ingestion with 2·5% w/v trypsin failed to decrease the percentage

of PMN ingesting two or more particles/cell; (b) monolayers stained with trypan blue and examined by phase-contrast microscopy allowed the distinction of non-phagocytosed particles from those ingested by the cells with an obvious uptake of dye by the attached or free particles. Again, the percentage of cells ingesting two or more particles/cell remained the same.

PMN monolayers were incubated with increasing concentrations of mannan over a dose range of 1–10 mg/ml in RPMI-Mg for 20 min. Zymosan particles and glucan particles were then added and phagocytosis was assessed after an appropriate time interval.

The monolayers of PMN were treated with 1·5 ml trypsin (specific activity $3{\cdot}38 \times 10^6$ mol/min/mg, using benzoyl-(D)-L-arginine-*p*-nitroanilide HCl as a substrate, pH 7·5, 40°) (Sigma) in the concentration range 100 ng/ml–500 μg/ml in RPMI-Mg for 20 min at 37°, washed three times with RPMI-1640, and then used in the phagocytic assay.

In order to demonstrate the affinity of zymosan and glucan for the same cell surface recognition mechanism, PMN were cultured on either zymosan or glucan immobilized on tissue culture plates by the modified procedure of Berton & Gordon (1983) adapted from Michl *et al.* (1979). Briefly, 60-mm tissue culture dishes (Corning Glass Works, Corning, NY) were treated with poly-L-lysine (Sigma) and then with gluteraldehyde. After washing, the plates were incubated with 2 ml of either yeast glucan or zymosan at a concentration of 5 mg/ml overnight at 37°. The excess particles were then removed by washing, leaving a firmly bound layer of glucan or zymosan. Free aldehyde groups were quenched as described, the dishes sterilized with 70% alcohol and then stored. Plates were thoroughly washed with buffer before use. Control plates coated with gluteraldehyde but without particles were also prepared. Following the incubation of cells with particles, plates were washed to remove excess particles and incubated in PBS containing 0·25% trypsin–1·5 mM EDTA for 15 min to detach cells. Cytocentrifuge preparations of washed cells were stained with Wright's stain and examined for phagocytosis.

CL assay

CL was measured using a Lumac Biocounter M2010 (Lumac/3M, NL) at 37°, with readings in relative light units (RLU) being obtained using the rate mode.

1 RLU = 10 photon counts per second (c.p.s).

Samples for CL assays contained 200 μl KRPG, 100 μl PMN (5×10^6/ml PBS) and 100 μl of 10 μM 5-amino-2,3-dihydro-1,4 phthalazinedione (luminol, Sigma) in KRPG. All samples were preincubated for 6 min in a waterbath at 37° before the addition of the stimulant in order to achieve temperature equilibration and to record the background CL. The target particles or ligands, diluted to an appropriate concentration in KRPG, were added to the cuvettes at 15 second intervals, and each reaction mixture was assessed for CL emission at precise 2 min intervals. Under these conditions, eight samples could be assayed simultaneously. In the absence of added ligands, there was no spontaneous generation of CL by PMN incubated in buffer alone for up to 30 min. The respiratory burst of human neutrophils in response to these phagocytic stimuli as measured by CL was completely inhibited by superoxide dismutase, thus indicating that the superoxide radical is an important intermediate in the oxidation of luminol (Harber & Topley, 1986).

In separate experiments, PMN at 5×10^6/ml were preincubated with mannan in a concentration range from 1 to 10 mg/ml for 30 min at 37°. The cells were then washed five times and resuspended at 5×10^6 cells/ml in PBS prior to the CL assay.

Suspensions of PMN at 1×10^7/ml were treated with trypsin at concentrations ranging from 100 ng/ml to 10 μg/ml in PBS for 30 min at 37°, washed twice in PBS, recounted in a Neubauer counting chamber and resuspended to a concentration of 5×10^6 cells/ml in PBS prior to the CL assay.

Assessment of complement activation

The capacity of the different polysaccharides, both soluble and insoluble, to activate the alternative pathway of complement was assessed according to the method of Riches & Stanworth (1980). Briefly, the total available alternative pathway haemolytic complement was estimated using rabbit erythrocytes and a range of dilutions of human serum chelated with 10·0 mM ethylene glycol-bis-amino tetraacetate (EGTA) and 7 mM magnesium chloride. Having established that dilution of serum which lyses 50% of a known concentration of these erythrocytes (CH50), the capacity of the test ligands to deplete this diluted serum of its complement activating capacity was established over a range of ligand concentrations.

RESULTS

Phagocytosis of particles by PMN

Zymosan particles and glucan particles were phagocytosed by human PMN in a dose-dependent manner, and in the complete absence of exogenously added serum opsonins (Fig. 1a). Identical preparations of cells did not phagocytose particulate β-glucan or particulate laminarin over a similar dose range (5×10^7/ml to 1×10^9/ml). At the higher particle concentrations (5×10^8/ml and above), there was disruption of the monolayer with fewer cells remaining adherent. At concentrations of $2{\cdot}5 \times 10^8$ particles/ml a mean ± SEM ($n=4$) or 54 ± 5% and 57 ± 4·3% of adherent neutrophils had ingested two or more glucan and zymosan particles, respectively. At this particle concentration, the ingestion of both zymosan and glucan was time-dependent, and the time-courses of ingestion were comparable (Fig. 1b), reaching a plateau of ingestion by 15 min for zymosan and by 20 min for glucan.

Effect of mannan on the phagocytosis of zymosan and glucan by PMN

Preincubation of the PMN monolayers from three separate donors for 20 min with concentrations of yeast mannan ranging from 1 to 10 mg/ml did not inhibit phagocytosis of zymosan or glucan at a particle concentration of $2{\cdot}5 \times 10^8$ particles/ml.

Effect of trypsin on the phagocytosis of zymosan and glucan by PMN

Adherent PMN were treated with trypsin, washed, and incubated at 37° for 20 min with $1{\cdot}25 \times 10^8$ zymosan particles/ml, $1{\cdot}25 \times 10^8$ glucan particles/ml, 1×10^8 latex particles/ml and 5×10^8 E^s-IgG/ml. These concentrations of particles gave equal amounts of ingestion in the absence of trypsin. The phagocytosis by PMN pretreated with trypsin was compared with that seen in parallel petri-dishes pretreated with buffer for the same

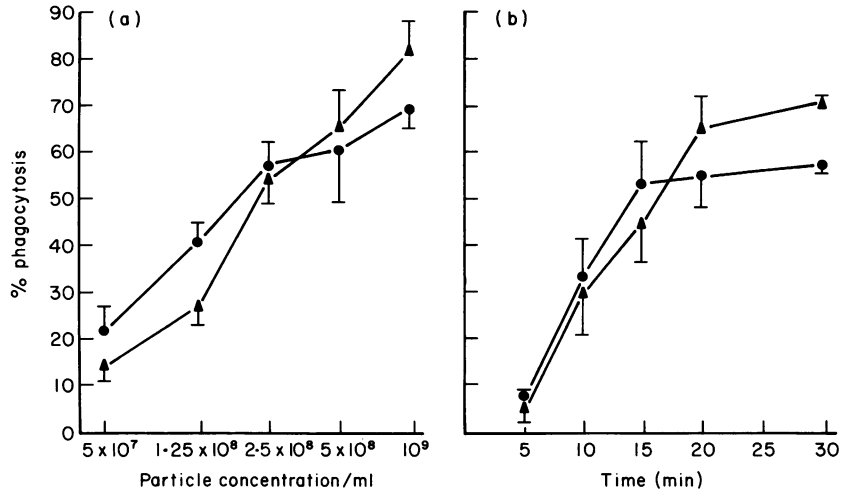

Figure 1. (a) Dose effect of unopsonized zymosan particles (●) and unopsonized yeast glucan particles (▲) on the capacity of monolayers of PMN to phagocytose two or more particles/cell after 20 min incubation. (b) Time-course of the proportion of PMN ingesting two or more unopsonized zymosan particles (●) and unopsonized yeast glucan particles (▲) per cell at a concentration of $2{\cdot}5 \times 10^8$ particles/ml. The results are expressed as the mean ± SEM of separate experiments with cells from four different donors.

period of time, three experiments being performed with each type of particle using cells from three different donors. The phagocytosis of latex and E^s-IgG was unaffected by trypsin pretreatment over the dose range tested. In contrast, 20 μg/ml trypsin decreased the ingestion of zymosan and glucan: mean reductions of 56·4% and 44% from the respective control values (Fig. 2). In three separate experiments, increasing the trypsin concentration incrementally to 500 μg/ml caused further dose-dependent mean reductions in the phagocytosis of zymosan and glucan to 76% and 87% respectively, of the control values (results not shown).

Phagocytic response by PMN cultured on fixed glucan or fixed zymosan

Control plates and particle-coated plates were incubated with 2 ml of RPMI-BSA containing PMN at a concentration of 3×10^6/ml. The period of incubation was extended to 4 h to allow capping of receptors whilst preserving maximal PMN adherence. Incubation of cells beyond this time resulted in detachment of PMN and disruption of the monolayer. PMN cultured on control plates coated with poly-L-lysine/gluteraldehyde alone phagocytosed glucan and zymosan to a degree comparable with PMN cultured on uncoated plates. In two separate experiments, at a particle concentration of $2{\cdot}5 \times 10^8$/ml, a mean of 75% and 83% of the control PMN ingested two or more zymosan or glucan particles per cell, respectively. In contrast, the phagocytosis of free zymosan by PMN cultured on fixed zymosan was reduced to 26·5% of cells ingesting two or more particles per cell. Similarly, the ingestion of free glucan by cells cultured on fixed glucan was reduced to 29% of cells. If PMN were cultured on zymosan and exposed to free glucan, only 25·8% of cells phagocytosed two or more particles, while only 15% of cells cultured on fixed glucan ingested free zymosan.

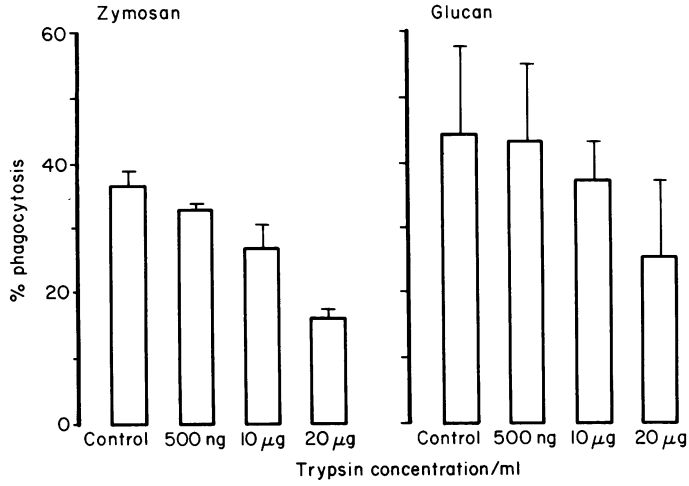

Figure 2. Dose effect of trypsin on the capacity of monolayers of PMN to phagocytose two or more zymosan particles (●) or yeast glucan particles (▲) per cell. The results expressed are the mean ± SEM of separate experiments with cells from three different donors.

The phagocytosis of both serum-opsonised zymosan and latex was unaffected by the culture of PMN on fixed particles. All the cells cultured on fixed particles exhibited vacuolation.

Generation of CL by PMN in response to zymosan and glucan

Human PMN generated CL in response to particulate zymosan and yeast glucan in the total absence of exogenous serum opsonins and in a dose-dependent manner (Figure. 3a). β-glucan and laminarin, both in a particulate form, and mannan in a soluble form failed to generate any CL response from PMN over the same dose range. The CL response of PMN to glucan reached a peak of 33,600 ± 2970 c.p.s. (mean ± SEM, $n=4$) at a concentration of 5×10^8 particles/ml, a particle:cell ratio of 500:1. In contrast, the CL response to zymosan increased in a linear manner, reaching a maximum CL of 98,160 ± 30,380 c.p.s. (mean ± SEM, $n=4$) at a concentration of 1×10^9 particles/ml, the highest concentration of zymosan tested. The generation of CL by PMN stimulated with either zymosan or glucan particles was also time-dependent (Fig. 3b). Peak CL occurred at 4 min following stimulation of PMN by glucan at a concentration of 5×10^8 particles/ml. The CL response of PMN to particulate zymosan at a concentration of 1×10^9 particles/ml did not peak until 8 min, but was four-fold greater than that of glucan.

Effect of mannan on the CL response of PMN to zymosan and glucan

Preincubation of PMN with mannan inhibited their response to zymosan with a mean 34% inhibition of CL at a mannan concentration of 10 mg/ml. In sharp contrast, mannan preincubation did not affect the response of PMN to glucan (Fig. 4).

The effect of trypsin on the CL response of PMN to zymosan and glucan

The trypsin sensitivity of the CL response of human PMN was examined using zymosan, glucan and latex particles, together with opsonized *E. coli* SC. Pretreatment of PMN with trypsin in the concentration range 100 ng/ml–10 μg/ml did not interfere with the generation of CL by the PMN in response to latex particles or opsonised SC. There was, however, a dose-dependent decrease in CL generated from trypsin pretreated PMN in response to zymosan and glucan at a particle concentration of $1{\cdot}25 \times 10^8$ (Fig. 5), such that at a concentration of 10 μg/ml trypsin there was significant mean decreases of 44·6% and 48·5% in glucan- and zymosan-generated CL, respectively. Further incremental increases in the concentration of trypsin up to 500 μg/ml resulted in dose-dependent decreases in generated CL of 78% and 80%, respectively, of the control values for glucan and zymosan.

Complement activation

Ligands were incubated over a range of concentrations (10–2000 μg/ml) with chelated normal human serum, and demonstrated varying degrees of dose-dependent activation of the alternative complement pathway (Fig. 6). Zymosan, the most potent of the activators, achieved 50% consumption at a concentration of 165 μg/ml. The same degree of activation of complement was achieved by barley-derived glucan and yeast glucan at concentrations of 235 and 285 μg/ml, respectively. Laminarin, the least potent of these activators, required a concentration of 1850 μg/ml to reach 50% consumption. Over the same dose range, mannan failed to consume >12% of complement function, as did E^s and latex particles.

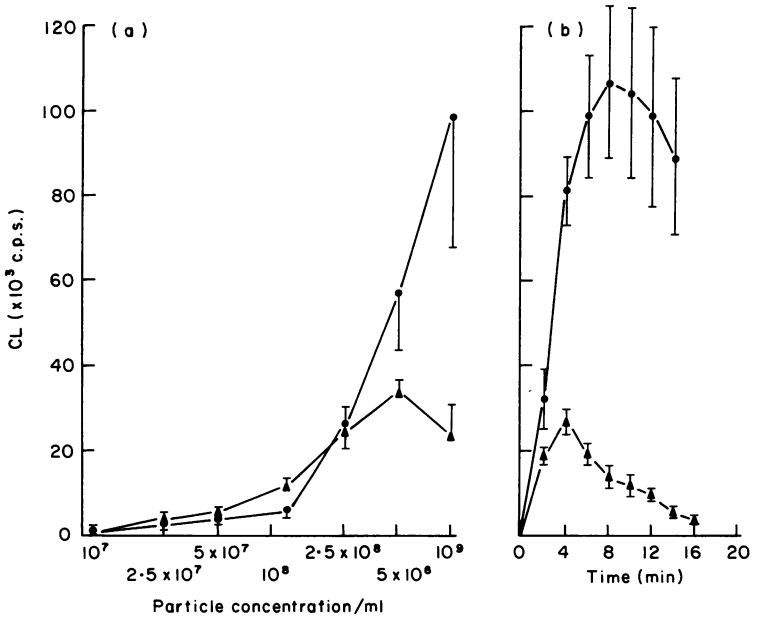

Figure 3. (a) Dose effect of unopsonized zymosan particles (●) and unopsonized yeast glucan particles (▲) on the capacity of PMN at a concentration of 1×10^6 cells/ml to generate luminol-dependent CL. (b) Time-course of generation of luminol-dependent CL by PMN stimulated with unopsonized zymosan particles (●) and unopsonized yeast glucan particles (▲) at concentrations of 1×10^9 and 5×10^8 particles/ml, respectively. Results of (a) and (b) are expressed as the mean ± SEM of five and seven separate experiments, respectively, with cells from separate donors.

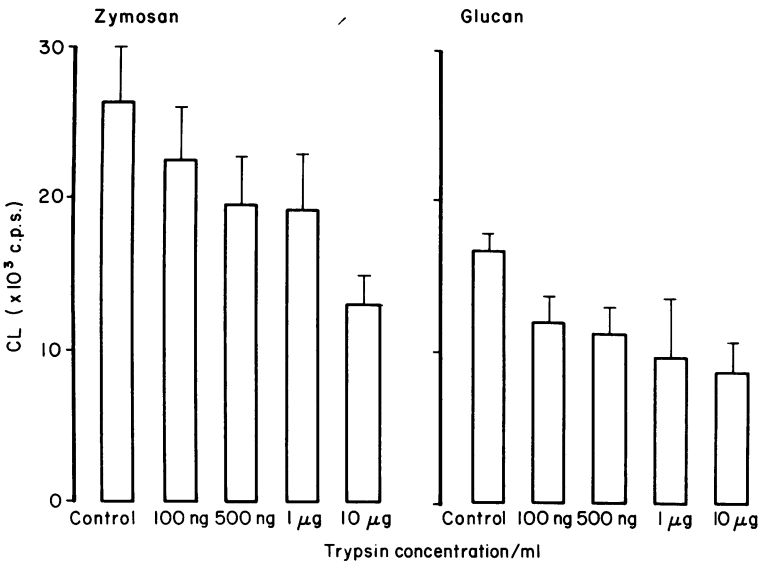

Figure 4. Dose effect of trypsin preincubation on the capacity of PMN to generate luminol-dependent CL in response to unopsonized zymosan particles (●) and to unopsonized yeast glucan particles (▲). The results are expressed as the mean ± SEM of separate experiments with cells from six separate donors.

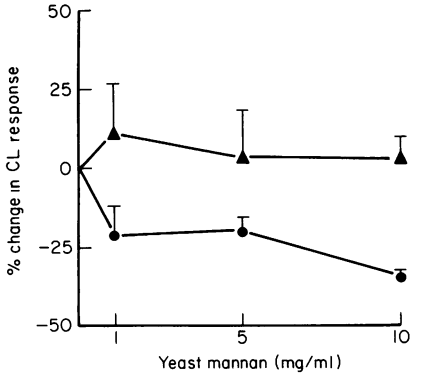

Figure 5. Dose effect of mannan preincubation on the capacity of PMN to generate CL in response to zymosan (●) and to yeast glucan (▲). The results are expressed as the mean ± SEM percentage change of the CL response in four separate experiments with cells from different donors.

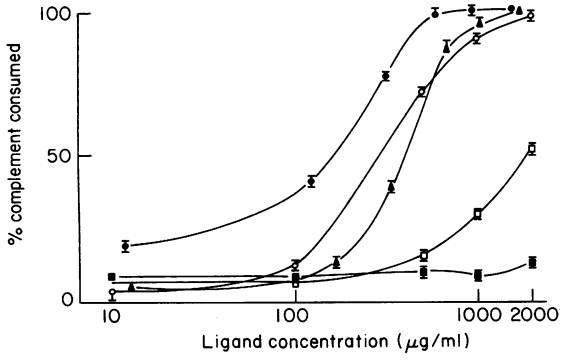

Figure 6. Dose-dependent activation of the human alternative complement pathway by (●) zymosan, (○) barley-derived glucan, (▲) yeast glucan, (□) laminarin and (■) mannan. Results are expressed as the percentage of consumed complement in EGTA-chelated human serum.

DISCUSSION

The demonstration of a trypsin-sensitive recognition mechanism on human PMN for particulate zymosan and yeast glucan identifies a response not previously characterized. The ingestion of both particles was time- and dose-dependent with comparable kinetics and dose effect. In contrast, the other insoluble β-linked glucose homopolysaccharides examined (barley-derived glucan and laminarin) were not ingested by PMN, suggesting that the recognition mechanism on these cells for the phagocytosis of such particles requires a relatively high degree of structural specificity. It is indicated that the recognition mechanisms for the ingestion of zymosan and yeast glucan are associated by the significant reduction in the phagocytosis of free glucan or free zymosan by PMN cultured on fixed zymosan and by a decrease in the ability of cells cultured on fixed glucan to ingest either free glucan or free zymosan. In the same experiments, the phagocytosis of latex particles and serum-opsonized zymosan was unaffected by the culture of PMN on either fixed glucan or zymosan, indicating that the mechanism of ingestion was not affected and that the inhibition of phagocytosis was selective. Since the zymosan receptor of human monocytes has been characterized in terms of its trypsin sensitivity (Czop *et al.*, 1978; Williams *et al.*, 1984), the effect of trypsin on the capacity of PMN to ingest zymosan and glucan was examined and compared with its effect on the phagocytosis of E^s-IgG and latex particles. The ingestion of yeast glucan and zymosan were equally sensitive to low concentrations of trypsin with a similar inhibition of phagocytosis, whilst the phagocytosis of latex particles (hydrophobic interaction) and E^s-IgG (Fc receptor) were unimpaired. Higher concentrations of trypsin resulted in a further dose-dependent inhibition of the phagocytosis of both zymosan and glucan, indicating that the major recognition mechanism of such particles on the surface of these cell is a membrane protein.

The activation of human PMN by zymosan and yeast glucan

also resulted in the generation of luminol-dependent CL, which again occurred in a dose- and time-dependent manner. Barley-derived glucan, laminarin and yeast mannan all failed to stimulate CL generation. In contrast to the phagocytic response, however, there were significant differences in the respiratory burst produced by PMN in response to stimulation by zymosan and glucan with a three- to four-fold greater response by PMN to zymosan. On the other hand, the trypsin sensitivity of the CL response of PMN to these stimuli was similar and parallelled the trypsin sensitivity of the phagocytic response. Thus, it seems clear from the above results that the ingestion of zymosan and yeast glucan is through a mechanism that involves either the same cell surface receptor or very closely associated recognition mechanisms. The differences observed in the respiratory burst generated by PMN in response to the two stimuli, however, require further explanation.

Zymosan, a 3–5 μm cell wall fragment of *Saccharomyces cereviceae*, has been analysed and found to contain a mixture of polysaccharides, protein and lipid (DiCarlo & Fiore, 1957). Glucan, a β-linked branched chain polysaccharide of glucose subunits, which constitutes the inner portion of the cell wall, forms up to 60% of the dry weight of zymosan. A second polysaccharide, mannan, composed of α-linked mannose subunits, constitutes another 20%. In their investigations of the capacity of components of yeast cell walls to activate the reticuloendothelial system of rodents, Riggi & DiLuzio (1961) demonstrated that the major activating component of zymosan was glucan, and that neither mannan nor lipid contributed significantly to this activation process. The role of mannan as a component of zymosan in the activation of inflammatory cells is complex. The presence of receptors for mannose-terminated glycoconjugates on rodent mononuclear phagocytes is well documented (Stahl & Gordon, 1982), although their identification on rodent PMN has been questioned, and where they are probably present at a much lower level (Berton & Gordon, 1983). Such receptors appear only on isolated human monocytes after several days in culture (Shepherd *et al.*, 1982; Ezekowitz *et al.*, 1983) and their presence on human PMN has yet to be established. It has been demonstrated that yeast mannan of various sources will inhibit the phagocytosis of zymosan by mouse peritoneal macrophages (Sung *et al.*, 1983), whilst others, although failing to demonstrate inhibition of phagocytosis, did establish an association between the MFR and the mechanism of activation of macrophages by zymosan (Berton & Gordon, 1983). A recent study by Ross *et al.* (1985), whilst establishing an association between the polymorph CR3 receptor and the phagocytosis of zymosan, failed to demonstrate inhibition of receptor function with relatively high concentrations of mannan. In the present study, the preincubation of PMN with concentrations of soluble yeast mannan up to 10 mg/ml failed to inhibit the phagocytosis of either yeast glucan or zymosan. In contrast, the preincubation of PMN with high concentrations of mannan significantly inhibited the generation of luminol-dependent CL in response to zymosan, whereas the response to yeast glucan was unaffected. These results indicate that, although the major recognition mechanism on PMN for the phagocytosis of zymosan is for glucan, the CL response is at least partly dependent on the mannan component of the zymosan particle. It has been suggested that the capacity of mannan to inhibit zymosan and glucan phagocytosis by monocytes is related to the contamination of mannan by glucan (Czop *et al.*, 1985a). The opposite effect of mannan on the CL responses of PMN to both particles, however, makes this an unlikely explanation in the present study and supports a role for mannan in the activation of these cells.

The glucan component of *Saccharomyces cerevicea* cell wall is heterogenous. Eighty-five percent is a branched β-(1–3)-glucan containing 3% of β-(1–6)-glucosidic linkages (Manners *et al.*, 1973a). The minor component consists of a branched β-(1–6)-glucan with about 19% β-(1–3)-glucosidic linkages (Manners *et al.*, 1973b). Although both particulate zymosan and yeast glucan are of the same parent cell source, their preparation results in significant modification to their structure, including a loss of the minor (1–6) component during the preparation of particulate yeast glucan (Bacon *et al.*, 1979), and a marked variation in the degree of branching between different glucan preparations (Manners *et al.*, 1973a). It therefore seems likely that a second reason for the difference in the CL response of PMN to zymosan and yeast glucan may, in part, be related to the structure of each glucan and its interaction with the cell surface receptor. The requirement for a specific structural arrangement is further emphasized by the inability of barley-derived glucan (70% β-(1–4) and 30% β-(1–3)-glucosidic linkages) and laminarin (β-(1–3)-glucosidic linkages) to promote phagocytosis or to initiate CL. It is also of interest to note that these two latter compounds, whilst unable to activate PMN, are potent activators of the alternative pathway of complement (Fig. 6). This suggests that, although there appears to be a functional association between particulate activators of the alternative complement pathway and their capacity to interact with phagocytic cells (Czop *et al.*, 1978), this relationship is by no means absolute, and that recognition by the cells requires a greater degree of structural conformity of the particle than that which is required to activate the alternative pathway of complement. It has also been suggested that human monocytes require local opsonization of complement components by zymosan to facilitate both particle adherance and internalization (Ezekowitz *et al.*, 1983). The capacity of PMN to synthesize all the required alternative pathway proteins has yet to be established; furthermore, the recent studies of Ross *et al.* (1985) demonstrate that the interaction of zymosan and neutrophils is independent of C3 opsonization.

The present study clearly demonstrates that the human PMN has on its surface a trypsin-sensitive recognition mechanism for a specific β-linked glucan. The capacity of fixed glucan and zymosan to inhibit the phagocytosis of either ligand, and the similarity of the phagocytic and CL responses in respect to their trypsin sensitivity, suggests that the major component of zymosan responsible for cell activation is glucan. The differential effect of mannan preincubation on the PMN CL response, however, suggests that the mannan component of zymosan also plays an important role in the trans-membrane activation of the respiratory burst in these cells. This latter finding demonstrates that, although stimulus response coupling with respect to phagocytosis probably only requires a single cell surface recognition mechanism, the respiratory burst can be enhanced by the recruitment of a second receptor.

REFERENCES

ALLEN R.C. (1982) Biochemiexcitation: chemiluminescence and the study of biological oxygenation reactions. In: *Chemical and Biological*

Generation of Excited States (eds W. Adam and G. Cilento). p. 309. Academic Press, New York.

BACON J.S.D., FARMER V.C., JONES D. & TAYLOR I.F. (1969) The glucan components of the cell wall of bakers yeast (*Saccharomyces cerevisiae*) considered in relation to its ultrastructure. *Biochem. J.* **114,** 557.

BERTON G. & GORDON S. (1983) Modulation of macrophage mannosyl-specific receptors by cultivation on immobilised zymosan. Effects on superoxide-anion release and phagocytosis. *Immunology*, **49,** 705.

CZOP J.K., FEARON D.T. & AUSTEN K.F. (1978) Opsonin-independent phagocytosis of activators of the alternative complement pathway by human monocytes. *J. Immunol.* **120,** 1132.

CZOP J.K. & AUSTEN K.F. (1980) Function discrimination by human monocytes between their C2b receptors and their recognition units for particulate activators of the alternative complement pathway. *J. Immunol.* **125,** 124.

CZOP J.K. & AUSTEN K.F. (1985a) Generation of leukotrienes by human monocytes upon stimulation of their β-glucan receptor during phagocytosis. *Proc. natl. Acad. Sci. U.S.A.* **82,** 2751.

CZOP J.K. & AUSTEN K.F. (1985b) A β-glucan inhibitable receptor on human monocytes: its identity with the phagocytic receptor for particulate activators of the alternative complement pathway. *J. Immunol.* **134,** 2588.

DICARLO F.J. & FIORE J.V. (1957) On the composition of zymosan. *Science*, **127,** 756.

DUBOIS M., GILLES K.A., HAMILTON J.K., REHEOS P.A. & SMITH F. (1956) Colorimetric method for determination of sugars and related substances. *Ayt. C.* **28,** 350.

EHLENBERGER A.G. & NUSSENZWEIG V. (1977) The role of membrane receptors for C3b and C3d in phagocytosis. *J. exp. Med.* **145,** 357.

EZEKOWITZ R.A.B., SIM R.B., HILL M. & GORDON S. (1983) Local opsonization by secreted macrophage complement components: role of receptors for complement in uptake of zymosan. *J. exp. Med.* **159,** 244.

HARBER M.J. & TOPLEY N. (1986) Factors affecting the measurement of chemiluminescence in stimulated human polymorphonuclear leukocytes. *J. Bioluminescence Chemiluminescence*, **1** (in press).

HUBER H. & FUNDENBERG H.H. (1968) Receptor sites on human monocytes for IgG. *Int. Arch. Allergy appl. Immunol.* **34,** 18.

MANNERS D.J., MASSON A.J. & PATTERSON J.C. (1973a) The structure of a β-(1–3)-D-glucan from yeast cell walls. *Biochem. J.* **139,** 19.

MANNERS D.J., MASSON A.J., PATTERSON J.C., BJORNDAL H. & LINDBERG G. (1973b) The structure of β-(1–6)-D-glucan from yeast cell walls. *Biochem. J.* **135,** 31.

MANTOVANI B. (1975) Different roles of IgG and complement receptors in phagocytosis by polymorphonuclear leukocytes. *J. Immunol.* **115,** 15.

MICHL J., PIECZONKA M.M., UNKLESS J.C. & SILVERSTEIN S.C. (1979) Effects of immobilized immune complexes on Fc and complement-receptor functions in resident and thioglycolate-elicited mouse peritoneal macrophages. *J. exp. Med.* **150,** 607.

NEWMAN S.L., BECKER S. & HALME J. (1985) Phagocytosis by receptors for C3b (CR1), iC3b (CR3) and IgG (Fc) on human peritoneal macrophages. *J. Leuk. Biol.* **38,** 267.

RABINOVITCH M. & DESTEFANO M.J. (1973) Particle recognition by cultured macrophages *J. Immunol.* **110,** 695.

RICHES D.W.H. & STANWORTH D.R. (1980) A simple new method of measuring the capacity to activate the alternative complement pathway. *Immunol. Lett.* **1,** 363.

RIGGI S.J. & DILUZIO N.R. (1961) Identification of a reticuloendothelial stimulating agent in zymosan. *Am. J. Physiol.* **200,** 297.

ROOS D., BOT A.A.M., VAN SHAIK M.L.J., DE BOER M. & DAHA M.R. (1981) Interaction between human neutrophils and zymosan particles: the role of opsonins and divalent cations. *J. Immunol.* **126,** 433.

ROSS D.G. & LAMBRIS J.D. (1982) Identification of a C3bi-specific membrane complement receptor that is expressed on lymphocytes, monocytes, neutrophils & erythrocytes. *J. exp. Med.* **155,** 96.

ROSS D.G., CAIN J.A. & LACHMANN P.J. (1985) Membrane complement receptor type three (CR3) has lectin like properties analogous to bovine conglutinin and functions as a receptor for zymosan and rabbit erythrocytes as well as a receptor for ic3b. *J. Immunol* **134,** 3307.

SHEPHERD V.L., CAMPBELL E.J., SENIOR R.M. & STAHL P.D. (1982) Characterization of the mannose/fucose receptor on human mononuclear phagocytes. *J. Reticuloendothel. Soc.* **32,** 423.

STAHL P. & GORDON S. (1982) Expression of a mannosyl-fucosyl receptor for endocytosis on cultured primary macrophages and their hybrids. *J. Cell Biol.* **83,** 49.

SUNG S.J., NELSON R.S. & SILVERSTEIN S.C. (1983) Yeast mannans inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. *J. Cell Biol.* **96,** 160.

WILLIAMS J.D., CZOP J.K. & AUSTEN K.F. (1984) Release of leukotrienes by human monocytes on stimulation of their phagocytic receptor for particulate activators. *J. Immunol.* **132,** 3034.

WILLIAMS J.D., LEE T.H., LEWIS R.A. & AUSTEN K.F. (1985) Intracellular retention of the 5-lipoxygenase pathway product, leukotriene B4, by human neutrophils activated with unopsonized zymosan. *J. Immunol.* **134,** 2624.