Journal of Cellular Biochemistry 56:225–235 (1994)

ARTICLES

Influence of Polysaccharides on Neutrophil Function: Specific Antagonists Suggest a Model for Cooperative Saccharide-Associated Inhibition of Immune Complex-Triggered Superoxide Production

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Abstract We have previously shown that certain monosaccharides (N-acetyl-D-glucosamine and mannose) could cooperatively inhibit the ability of neutrophils to release superoxide anions in response to immune complexes. To test the possible origins of the cooperative inhibition of superoxide release, we have examined the effect of a panel of polysaccharides on superoxide release in the presence or absence of immune complexes. Although exposure to particulate β-glucan and hyaluronan triggered superoxide release from neutrophils, other polysaccharides including chitin and mannan were without effect. Both chitin and mannan, but not other polysaccharides, inhibited the immune complex-mediated stimulation of superoxide release in a dose-dependent fashion. In sharp contrast to the cooperative inhibition mediated by monosaccharides, chitin and mannan exhibited Hill coefficients of 1. This inhibition of superoxide production was not due to simple blockage of Fc receptors since fluorescent immune complexes bound equally well to neutrophils in the presence or absence of mannan or chitin as shown by epifluorescence microscopy and quantitative fluorometry. Furthermore, this inhibition of superoxide release was not observed when neutrophils were stimulated with phorbol myristate acetate and ionophore A23187 or hyaluronan. Therefore, the specific inhibition of superoxide production by mannan and chitin could not be explained by either receptor blockage or by some nonspecific effect on cells. We suggest that these molecules interfere with a step in transmembrane signaling, presumably involving the integrin CR3. The observed Hill cofficients suggest the possibility that one polysaccharide may simultaneously bind to two monosaccharide binding sites yielding a Hill coefficient of 1, whereas individual monosaccharides separately bind yielding a Hill coefficient of 2. © 1994 Wiley-Liss, Inc.

Key words: monosaccharides, superoxide anions, polysaccharides, immune complex, β-glucan

Neutrophils participate in host defense against bacterial, fungal, viral, and possibly neoplastic disease. A key element in the neutrophil's armature is the ability to produce reactive oxygen metabolites (ROMs). Included among these ROMs are superoxide anions, hydroxyl radicals, singlet oxygen, hydrogen peroxide, and HOCl [Iyer et al., 1961; Weiss et al., 1977; Tauber and Babior, 1977; Krinsky, 1974]. These ROMs participate in both host resistance mechanisms and inflammatory disease. ROM production can be triggered by several factors including chemotactic factors, cytokines, immune complexes, zymosan, and particulate β-glucan [Nathan, 1987, 1989; Goldstein et al., 1975; Ross et al., 1987].

Received January 28, 1994; accepted January 28, 1994.

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Neutrophil surface receptors trigger many physiologically relevant forms of superoxide production. For example, plasma membrane receptors for N-formyl-methionyl-leucyl-phenylalanine, C5a, iC3b, IgG, IL-1, interferon, tumor necrosis factor, and β-glucan, a component of zymosan, can stimulate superoxide production under appropriate conditions [Nathan, 1987, 1989; Goldstein et al., 1975; Ross et al., 1987]. Both β-glucan and iC3b are recognized by the same cell surface protein, CR3. The other receptors are distinct membrane proteins, although receptor-receptor interactions may take place [Zhou et al., 1992, 1993; Sehgal et al., 1993; Petty and Todd, 1993; Xue et al., 1994]. Unfortunately, their mechanism(s) of signal transduction are not understood completely. However, these various transduction mechanisms may all terminate in a calcium- and granule-dependent step required for the deposition of superoxide

anions in targets, but not necessarily for the respiratory burst per se [Maher et al., 1993; Petty et al., 1992; Liang and Petty, 1992].

In the present study our primary interest is in Fc receptor-triggered superoxide production. Neutrophils possess two classes of plasma membrane Fc receptors which are known as FcγRII (CD32) and FcγRIIIB (CD16). FcγRII is a 40,000 M_r transmembrane protein, possessing one membrane-spanning domain [Stuart et al., 1987; Stengelin et al., 1988]. FcγRIIIB (M_r = 50,000–70,000) is a glycophospholipid-linked membrane protein on neutrophils and natural killer cells [Simmons and Seed, 1988; Selvaraj et al., 1988; Huizinga et al., 1988]. Both FcRs bind monomeric IgG with low affinity and aggregated IgG with high affinity [Jones et al., 1985; Looney et al., 1986]. Several studies have indicated that both FcγRII and FcγRIIIB are capable of affecting transmembrane signals [Kimberly et al., 1990; Anderson et al., 1990; Fanger et al., 1989; van de Winkel et al., 1990; van de Winkel and Anderson, 1991].

Since FcγRIIIB is a glycophospholipid-linked membrane protein, its mechanism of transmembrane signaling presumably involves the participation of another plasma membrane protein(s). The possible role of CR3 (CD11b/CD18), in antibody-dependent functions has received support from diverse sources [Petty and Todd, 1993]. Anti-CR3 Fab fragments inhibit IgG-dependent phagocytosis [Arnaout et al., 1983; Murata et al., 1987]. Moreover, patients deficient in CR3 exhibit depressed antibody-dependent functions [Anderson et al., 1984; Kohl et al., 1986]. Furthermore, Brown and colleagues have provided evidence based on phagocytosis studies that CR3 associates with FcγRs [Brown et al., 1988; Graham et al., 1989]. Recently, we have shown that CR3 co-caps with FcγRIIIB [Zhou et al., 1993]. Furthermore, FcR ligation triggers transmembrane proximity between CR3 and microfilaments [Zhou et al., 1992]. We have also shown that the monosaccharides N-acetyl-D-glucosamine and mannose block CR3-FcγRIIIB co-capping [Zhou et al., 1993] and depress transmembrane calcium signaling and superoxide release [Sehgal et al., 1993]. This suggests the possibility that the lectin-like site of CR3 [Ross et al., 1985, 1987] may participate in receptor-receptor interactions at the neutrophil's plasma membrane. Recently, it has been shown that fibroblasts transfected with the genes encoding CR3 and FcγRIIIB are competent to phagocytose antibody-coated targets, but not cells transfected with CR3 or FcγRIIIB alone [Krauss et al., 1993]. To better understand saccharide-dependent regulation of neutrophil function, we have tested the ability of a panel of polysaccharides to influence superoxide production. Specifically, we have tested the hypothesis that certain polysaccharides non-cooperatively inhibit IgG-dependent superoxide release.

MATERIALS AND METHODS

Materials

Phorbol myristate acetate and A23187 were obtained from Sigma Chemical Co. (St. Louis, MO). Chitin, mannan, dextran, heparin, nigeran, hyaluronan, and yeast and barley β-glucans were obtained from Sigma.

Preparation of Cells

Peripheral blood was obtained from normal healthy adults using heparinized tubes. Neutrophils were isolated by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) step-density gradient centrifugation [Petty et al., 1989]. The remaining red blood cells were removed by hypotonic lysis. The purified neutrophils were > 95% viable as judged by trypan blue exclusion. Cells were then washed using calcium-free PBS at pH 7.2.

Preparation of Immune Complexes

Insoluble immune complexes (IC) were prepared at equivalence using bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and a rabbit anti-BSA IgG fraction (Organon Teknika-Cappel, Durham, NC). After incubation for 1 h at 37°C, the ICs were washed with cold PBS by centrifugation then resuspended at 50 mg/ml in PBS. The protein content of ICs was measured using the bicinchoninic acid (BCA) (Pierce Chemical Co., Rockford, IL) or Lowry methods (Sigma Chemical Co., St. Louis, MO) using BSA as a standard. The IC stock solution was diluted to a final concentration of 230 μg/ml for cell incubations. Fluorescent immune complexes were prepared in an identical fashion except that BSA was replaced with fluorescein isothiocyanate-conjugated BSA, using previously established labeling procedures [Petty et al., 1989].

Superoxide Release Assay

Superoxide release was measured by the ferricytochrome c reduction assay [Babior et al.,

1973; Johnston, 1981]. Briefly, 1×10^6 neutrophils in HBSS without phenol red were suspended in the presence or absence of various saccharides for approximately 10 min. Particulate yeast β-glucan and chitin were prepared in solution as described [Ross et al., 1987]. This was followed by addition of IC with 75 μM ferricytochrome c (type III; Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. Parallel experiments were conducted in the absence of cells (blanks) or the presence of 40 μg/ml superoxide dismutase (SOD). Incubations were terminated by placing the tubes in an ice bath. The tubes were then centrifuged at 800*g* for 15 min in a centrifuge. The optical densities (OD) of the supernates were measured with a Perkin-Elmer (Norwalk, CT) lambda 4B spectrophotometer using a 1 nm bandwidth. The OD_{550} was converted to nmol/30 minutes/10^6 cells as described [Johnston, 1981].

Fluorescence Microscopy

Cells labeled with fluorescent immune complexes were examined using a axiovert inverted fluorescence microscope (Carl Zeiss, Inc., New York, NY) equipped with a mercury epifluorescence lamp [Zhou et al., 1991]. The fluorescence images were collected using an intensified CCD camera (Geniisys, Dage-MTI, Michigan City, IN). A discriminating interference filter set was used with excitation at 485/22 nm and emission at 590/30 nm using a 510 long-pass dichroic mirror (Omega Optical, Brattleboro, VT). Differential interference contrast (DIC) photomicrographs were collected using Zeiss polarizers and a charge-coupled device camera (Model 72, Dage-MTI). The background-subtracted digitized images were averaged then stored on hard disk or streaming tape. Processed images were photographed using a Polaroid (Boston, MA) freeze-frame video recorder.

Fluorometry

Fluorometry was performed as previously described [Francis et al., 1991]. Cells (5×10^6/ml) labeled with fluorescent IC as described above were washed thoroughly then lysed with 1% Triton X-100. The fluorescence intensity of the extracts in a final volume of 2 ml was recorded using a MPF-66 spectrofluorometer (Perkin-Elmer, Norwalk, CT) with excitation and emission wavelengths set at 520 and 560 nm, respectively.

Hill Plots

Dose-response data were analyzed according to the Hill equation [Levitzki, 1984] as written for pharmacologic analysis [Mieyal, 1981]. In this analysis $\log[\Delta/(\Delta_m - \Delta)]$ is plotted against $-\log D$ where Δ is the physiological response, Δ_m is the maximal response, and D is the dose.

Statistical Evaluation

The data were expressed as mean ± SEM where n represents the number of separate experiments performed. Data were analyzed using an Apple IIe computer and the Stats Plus software package (Human Systems Dynamics, Northridge, CA). Student's paired *t*-test was used to evaluate the cellular responses; $P < 0.05$ was considered significant.

RESULTS

Effects of Polysaccharides on Superoxide Release

We first examined the ability of a panel of polysaccharides to stimulate superoxide release by neutrophils. Polysaccharides were suspended in HBSS then added to neutrophils (10^6/ml) at various concentrations. Table I shows the superoxide released by neturophils in response to polysaccharides. As previously reported by Ross et al. [1987], particulate yeast β-glucan was found to be a potent stimulator of superoxide release (11 ± 1 nmol/30 min/10^6 cells at 0.75 mg/ml). The polysaccharide hyaluronan has also been shown to stimulate neutrophil functions, including chemiluminescence [e.g., Hakansson et al., 1980a; Hiro et al., 1986]. We therefore tested the ability of hyaluronan to stimulate the release of superoxide anions from neutrophils. As Table I shows, hyaluronan at 5 mg/ml was an effective stimulator of superoxide release. Although particulate β-glucan from yeast and hyaluronan showed physiologically relevant levels of superoxide production, other polysaccharides tested did not. Nigeran, heparin, soluble β-glucan from barley, chitin, and mannan did not induce superoxide release at any concentration tested (Table I). Since the inability of these polysaccharides to induce superoxide production might have been due to some toxic effect on the cells, we examined cell viability as judged by trypan blue exclusion in the presence of these macromolecules. No effect of nigeran, heparin, soluble β-glucan, chitin, and mannan on neutrophil viability was found (data not shown). There-

TABLE I. Effects of Polysaccharides on Superoxide Production by Neutrophils*

Treatment	Superoxide (nmol/30 min/10^6 cells)	P^a	P^b
Particulate			
β-glucan[c] (0.25 mg/ml)	5 ± 1		
(0.75 mg/ml)	11 ± 1	—	n.s.
(1.25 mg/ml)	14 ± 2		
Hyaluronan (5 mg/ml)	9 ± 1	n.s.	—
Soluble β-glucan[d]			
(0.75 mg/ml)	1.6 ± 0.6	<0.001	<0.001
Dextran (5 mg/ml)	4 ± 0.5	<0.001	<0.001
Mannan (2.5 mg/ml)	1 ± 0.2	<0.001	<0.001
Chitin (2.5 mg/ml)	1 ± 0.4	<0.001	<0.001
Nigeran (5 mg/ml)	1.5 ± 0.3	<0.001	<0.001
Heparin (5 mg/ml)	1 ± 0.2	<0.001	<0.001

*n = 3 to 7 for these experiments.
[a]In comparison to β-glucan at 0.75 mg/ml. n.s., not significant.
[b]In comparison to hyaluronan at 5 mg/ml.
[c]Particulate β-glucan from yeast was employed.
[d]Soluble β-glucan from barley was employed.

fore, chitin and mannan have no endogenous ability to stimulate superoxide release.

Effects of Chitin and Mannan on Immune Complex-Triggered Superoxide Release

Chitin and mannan are polysaccharides composed of N-acetyl-D-glucosamine and mannose, respectively. We have previously shown that N-acetyl-D-glucosamine and mannose are capable of cooperatively inhibiting immune complex-triggered superoxide release from neutrophils [Sehgal et al., 1993]. Since these polysaccharides do not have an endogenous ability to trigger superoxide production, we tested the ability of these compounds to affect immune complex triggered superoxide release.

Chitin was added to cells in HBSS at various concentrations 0.25 to 15 mg/ml followed by addition of immune complexes. Superoxide production was diminished in a dose-response fashion (Fig. 1A). The dose-response profile was hyperbolic in appearance, in contrast to our earlier results using N-acetyl-D-glucosamine, the monosaccharide subunit of chitin [Sehgal et al., 1993]. Figure 1B shows a Hill plot of the data given in panel A. A slope of 1 is obtained for chitin, whereas a slope of 2 was obtained in a previous study for N-acetyl-D-glucosamine [Sehgal et al., 1993].

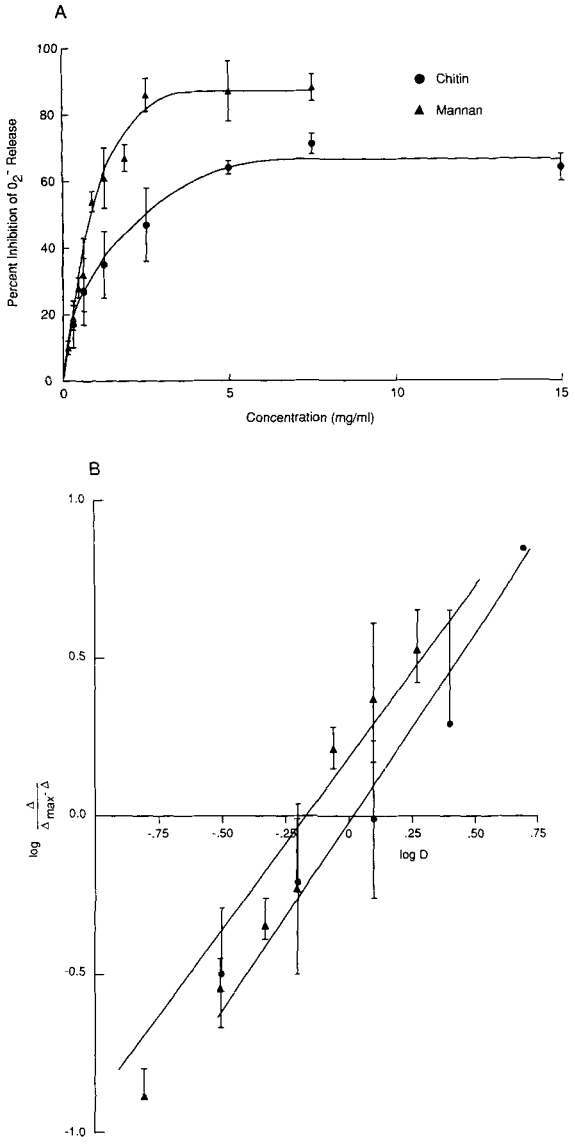

Fig. 1. Effects of chitin and mannan on IC-stimulated superoxide production. Panel A shows dose-response curves for chitin (●) and mannan (▲). The percent superoxide inhibition is plotted at the ordinate, whereas the total polysaccharide dose is given on the abscissa. Hyperbolic curves are obtained in both cases. **B** shows Hill plots of the data shown in **A**. A slope of 1.0 is obtained for both chitin- and mannan-mediated inhibition of immune complex-triggered superoxide generation. In contrast, the monosaccharides mannose and NADG exhibited sigmoidal dose-response curves and Hill coefficients of 2.0 [Sehgal et al., 1993]. n = 3 to 8.

The effect of mannan on immune complex-triggered superoxide release was also examined. Mannan has previously been shown to paralyze antibody-dependent phagocytosis [Sung et al., 1985]. Mannan was added to cells at concentrations from 0.325 to 8.0 mg/ml. Figure 1A shows a dose-response curve for immune complex-

triggered superoxide production in the presence of mannan. As these data indicate, mannan reduces superoxide release in a hyperbolic dose-dependent fashion. In comparison, mannose cooperatively inhibits superoxide release [Sehgal et al., 1993]. Figure 1B shows Hill plots of the data given in panel A. Although mannose cooperatively inhibits superoxide release, mannan exhibited a Hill coefficient of 1. This indicates that, in contrast to monosaccharides, polysaccharides inhibit superoxide release in a noncooperative fashion.

The antagonistic activity of polysaccharides on immune complex-stimulated superoxide production was found for mannan and chitin, but not many other polysaccharides (see also below). Table II shows the amounts of immune complex-stimulated superoxide production in the presence of various polysaccharides. Although chitin and mannan show significant inhibition of superoxide production, dextran, heparin, nigeran, and hyaluronan do not. This suggests that inhibitory activity is specific for these two polysaccharides.

Chitin and Mannan Do Not Affect Immune Complex Binding to Cells

Although the above data show that chitin and mannan can block immune complex-stimulated superoxide release, they have not localized the potential inhibitory step. To better define this mechanism, we have examined the ability of chitin and mannan to interfere with immune complex binding to neutrophils. Fluorescent immune complexes were prepared using fluorescein isothiocyanate-conjugated BSA as antigen, as described in Materials and Methods. Chitin and mannan were incubated with cells at 15 and 8 mg/ml, respectively, since these correspond to the highest doses used in the dose-response studies of Figure 1. As shown in Figure 2, no significant differences in immune complex binding were found for cells treated with chitin or mannan. Furthermore, there was no apparent difference in the distribution of immune complexes on untreated cells or cells treated with mannan or chitin. To provide quantitative confirmation of these qualitative fluorescence microscopic images, we performed fluorometry studies of these samples. Control samples of neutrophils not exposed to polysaccharides gave a fluorescence intensity of 89 ± 1 (arbitrary units). Cells treated with mannan (2.5 mg/ml) or chitin (2.5 mg/ml) yielded fluorescence intensities of 89 ± 2 and 88 ± 2, respectively. Therefore, these two polysaccharides can diminish the response to immune complex dependent cell activation without influencing immune complex binding. Since these large polysaccharides cannot cross the plasma membrane, they may be interfering with a post-ligand binding step at the level of the plasma membrane.

TABLE II. Effects of Polysaccharides on Immune Complex Stimulated Superoxide Production by Neutrophils*

Treatment	Superoxide release (nmol/30 min./10^6 cells)	*P*
IC	19.5 ± 1.1	—
IC + dextran	20.7 ± 1.9	n.s.
IC + heparin	19.3 ± 1.1	n.s.
IC + nigeran	19.1 ± 0.7	n.s.
IC + hyaluronan	19.3 ± 0.9	n.s.
IC + mannan	2.6 ± 1.8	<0.001
IC + chitin	7.2 ± 0.4	<0.001

*Polysaccharides were used at 5 mg/ml. n = 3 to 5 for these experiments. The *P* values are calculated in comparison to IC addition in the absence of polysaccharides. n.s., not significant.

Hyaluronan and Phorbol Myristate Acetate-Mediated Superoxide Production Are Unaffected by Chitin and Mannan

We next sought to examine the specificity of the polysaccharide-mediated inhibition of superoxide production. For example, one might imagine that the inhibition is mediated by some general effect on superoxide production or measurement. To address this possibility, we have tested the ability of chitin and mannan to block superoxide production in response to PMA and hyaluronan. PMA activates neutrophils via protein kinase C [e.g., Woronick et al., 1992] whereas hyaluronan presumably activates cells via CD44H [e.g., Underhill, 1992]; in both cases activation should be independent of plasma membrane complement and immunoglobulin receptors. Neutrophils in HBSS were treated with 1 μg/ml PMA plus 2 μM A23187 or 5 mg/ml hyaluronan. Mannan (2.5 mg/ml) and chitin (2.5 mg/ml) had no effect on the ability of PMA or hyaluronan to stimulate superoxide release (Table III). These results indicate that chitin and mannan specifically inhibit the neutrophil's ability to respond to immune complexes. Interestingly, we observed that mannan (2.5 mg/ml) could diminish particulate β-glucan (0.75 mg/ml) stimulated superoxide production. This sug-

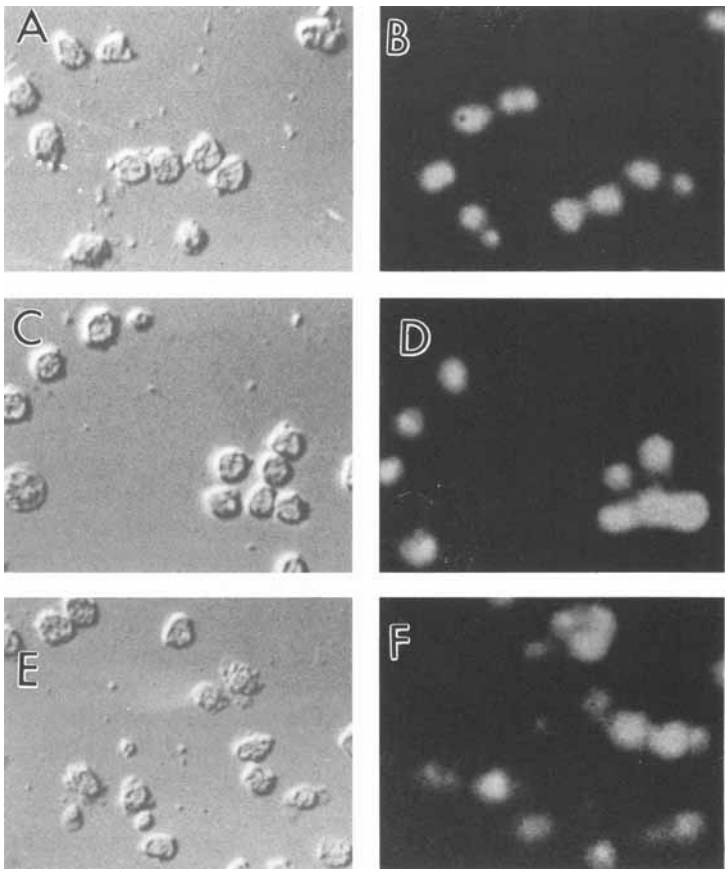

Fig. 2. Representative micrographs of fluorescent immune complex labeled neutrophils. Differential interference contrast (**A, C, E**) and fluorescence (**B, D, F**) photomicrographs are shown. A and B illustrate immune complex binding to untreated neutrophils. C and D show immune complex binding to neutrophils treated with 15 mg/ml chitin. E and F show immune complex binding to neutrophils treated with 8 mg/ml mannan. No significant differences in immune complex binding to untreated and chitin or mannan treated cells could be observed. ×1,000.

gests that mannan can also act as an inhibitor for β-glucan stimulation. Mannan could block activation sites on either particulate β-glucan or the cell membrane or it could at a second regulatory site. These results are consistent with previous studies using monosaccharides which showed that mannose blocked immune complex triggering with greater efficacy that N-acetyl-D-glucosamine.

Comparison With the Antagonistic Effects of Soluble β-Glucan and N-Acetyl-D-Glucosamine

In the preceding discussion we showed that mannan and chitin can inhibit superoxide production by neutrophils. Previous studies using polysaccharides [Ross et al., 1987; Czop and Austen, 1985] have shown that soluble barley β-glucan can inhibit superoxide and/or phagocytosis of particulate β-glucan derived from yeast. Previous studies from this laboratory have also shown that certain monosaccharides including N-acetyl-D-glucosamine (NADG) can inhibit IC-stimulated superoxide production by neutrophils [Sehgal et al., 1993]. We have therefore tested the effects of soluble barley β-glucan and NADG on the ability of IC and particulate yeast β-glucan to trigger superoxide production (Table IV). As previously reported, NADG decreases IC-stimulated superoxide production. In addition, we found that NADG also inhibits particulate β-glucan-stimulated superoxide production. We have also found that soluble β-glucan decreases both particulate β-glucan and IC-mediated superoxide production by neutrophils. However, soluble β-glucan did not inhibit the binding of fluorescent IC to neutrophils (data not shown), thus suggesting that it may operate by a similar mechanism. Therefore, soluble β-glucan is an antagonist of both particulate β-glucan and IC-stimulation of superoxide production.

TABLE III. Effects of Chitin and Mannan on Phorbol Myristate Acetate, Hyaluronan, and β-Glucan-Stimulated Superoxide Production by Neutrophils*

Treatment	Control	Superoxide release nmol/30 min/10^6 cells) +Mannan[a]	+Chitin[a]
PMA[b] (1 μg/ml)	20 ± 1	19 ± 1.5	20 ± 0.7
Hyaluronan (5 mg/ml)	9 ± 1	9 ± 0.4	9 ± 0.4
β-glucan (0.75 mg/ml)	11 ± 1	4 ± 0.3	10 ± 0.5

*n = 3 to 7 for these experiments.
[a]2.5 mg/ml.
[b]PMA was used in conjunction with 2 μM A23187.

TABLE IV. Effects of Soluble β-Glucan and N-Acetyl-D-Glucosamine on Particulate β-Glucan and Immune Complex-Stimulated Superoxide Production by Neutrophils*

Stimulus	Treatment	Superoxide release (nmol/30 min/10^6 cells)	% Inhibition
IC	None	21.5 ± 0.6	—
	Soluble β-glucan	13.1 ± 0.2	39
	NADG	10.7 ± 0.6	50
Particulate β-Glucan	None	10.6 ± 0.8	—
	Soluble β-glucan	5.2 ± 0.3	51
	NADG	5.7 ± 0.6	46

*Particulate and soluble β-glucan were used at 0.75 mg/ml. NADG was used at 150 mM. n = 3 for these experiments. Both soluble β-glucan and NADG significantly reduced superoxide production.

DISCUSSION

In the present study we have analyzed the ability of certain polysaccharides to affect the neutrophil's ability to produce superoxide anions. Two of the polysaccharides studied, particulate β-glucan and hyaluronan, can be considered as functional agonists since they can provoke superoxide production in the absence of other factors. In addition, chitin and mannan can be considered as antagonists of superoxide production since they inhibit immune complex stimulated superoxide production, but not PMA or hyaluronan-dependent superoxide release.

Particulate β-glucan and hyaluronan, but not chitin, mannan, heparin, soluble β-glucan, or nigeran, provoke the release of superoxide anions from neutrophils. β-glucan and hyaluronan stimulate superoxide production via separate receptors, CR3 (CD11b/CD18) and CD44H. β-glucan stimulates superoxide production via CR3, a member of the integrin supergene family. Ross et al. [1987] have shown that antibodies directed against CR3 block superoxide production in response to particulate β-glucan and that patients deficient in CR3 are unable to produce superoxide anions in response to β-glucan. The small level of superoxide production seen with dextran may reflect the fact that both β-glucan and dextran are polymers of glucose. As mentioned above, several lines of evidence have suggested that CR3 also participates in IgG-dependent stimulation of superoxide production [e.g., Zhou et al., 1993; Sehgal et al., 1993].

On the other hand, previous studies have indicated that hyaluronan augments neutrophil chemiluminescence [Hakansson et al., 1980a] and other functions as well [Ahlgren and Jarstrand, 1984; Hiro et al., 1986; Hakansson et al., 1980a,b; Hakansson and Venge, 1987]. Our studies indicate that hyaluronan is capable of triggering superoxide release at physiologically-relevant concentrations [Comper and Laurent, 1978], although it is more modest than that of particulate β-glucan. The less vigorous response of hyaluronan is likely accounted for by the fact that its physiological effects are mediated by a plasma membrane receptor distinct from that for β-glucan. Leukocytes recognize hyaluronan via CD44H, the hyaluronan receptor [Aruffo et al., 1989; Miyake et al., 1990; Underhill, 1992; Culty et al., 1992; Lesley et al., 1990; Webb, 1990]. CD44 is a transmembrane protein [Goldstein et al., 1989] with cytoskeletal links [Camp et al., 1991] and the ability to stimulate protein kinase activity [Turley, 1989]. Hence, CD44 apparently has all of the necessary machinery to trigger a response. This is consistent with the ability of hyaluronan to enhance complement-dependent, IgG-dependent, and yeast phagocytosis [Ahlgren and Jarstrand, 1984; Hakansson et al., 1980a] since cross-competition might be expected if β-glucan and hyaluronan bound to the same site.

We have also observed that mannan and chitin, which are polymers of mannose and N-acetyl-D-glucosamine, respectively, are able to inhibit immune complex triggered activation of neutrophil superoxide production. Previous studies have indicated that mannan and chitin could interact with leukocytes to promote cell attach-

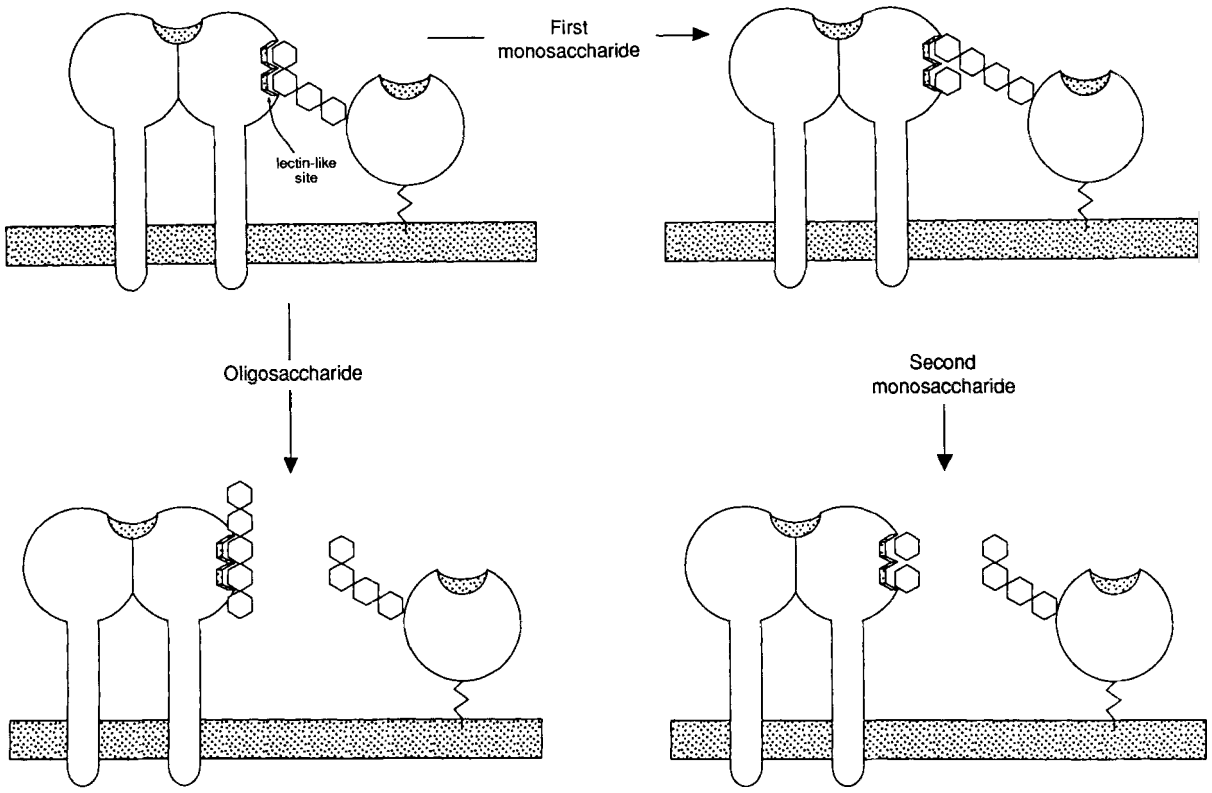

Fig. 3. A simple model of the cooperative monosaccharide mediated inhibition of superoxide release and the non-cooperative polysaccharide mediated inhibition of immune complex triggered superoxide release are shown. This model is based upon the lectin-like activity of CR3 and our hypothetical model of CR3-to-FcγRIIIB interaction (see text for discussion). We propose that two monosaccharides must bind to inhibit FcγRIIIB signaling, thus accounting for the Hill coefficient of 2.0, whereas one polysaccharide can simultaneously bind to two sites, thus suggesting a Hill coefficient of 1.0.

ment [e.g., Sung et al., 1985; Nishimura et al., 1986]. The results of the reported polysaccharide studies are consistent with our previous experiments using the monosaccharides mannose and N-acetyl-D-glucosamine [Sehgal et al., 1993]. As described above, this cannot be explained by simple receptor blockade since fluorescent immune complexes bind well in the presence or absence of these polysaccharides as determined by both fluorescence microscopy and fluorometry experiments. Nor could the inhibition by explained by some nonspecific effect since PMA- and hyaluronan-mediated stimulation of superoxide production are not affected by mannan and chitin. The half maximal inhibition of immune complex-mediated superoxide production was obtained at polysaccharide doses of 0.5 to 1 mg/ml. In contrast the half-maximal inhibition observed for monosaccharides was 5- to 10-fold higher. The increased efficacy of chitin and mannan in comparison to monosaccharides may be due to the ability of the polysaccharides to simultaneously bind to multiple sites. This is also consistent with our earlier work [Sehgal et al., 1993] which suggested the presence of two binding sites based upon the calculated Hill coefficient. We also observed that soluble β-glucan partially blocks IC-mediated superoxide production, which is consistent with its ability to inhibit particulate β-glucan stimulation of superoxide production. Whether or not soluble β-glucan also blocks receptor co-capping has not yet been established, thus β-glucan could act as either a competitive reagent or possibly as a allosteric regulator.

The dose-response curves for chitin- and mannan-mediated inhibition of immune complex-triggered superoxide production were found to be non-cooperative (Hill coefficient = 1). In contrast, our previous study using monosaccharides displayed a sigmoidal dose-response curve and a Hill coefficient of two [Seghal et al., 1993]. As illustrated in Figure 3, these two studies suggest a simple model for the previously observed cooperativity. As previously discussed [Zhou et al., 1993; Sehgal et al., 1993], we postu-

late that CR3 and FcγRIIIB interact at neutrophil surfaces via extracellular carbohydrate chains. Although the oligosaccharide chains of FcγRIIIB have apparently not yet been sequenced, it is known that FcγRIIIB binds to the lectin concanavalin A [Kimberly et al., 1989], thus suggesting the presence of mannose or similar saccharides. Using our previously proposed model of CR3-to-FcγRIIIB interactions to interpret these data, we suggest that as the monosaccharide concentration increases, one subunit of the linking oligosaccharide chain is freed from the receptor-receptor interaction. This makes it easier for a second monosaccharide to dissociate the two receptors, thereby blocking superoxide production and yielding a Hill coefficient of two. In contrast, when a polysaccharide binds to this site, both presumed monosaccharide binding sites could be simultaneously occupied, thereby blocking superoxide production and displaying a Hill coefficient of one.

These studies confirm and extend our earlier work and provide a more complete understanding of receptor-receptor interactions in cell stimulation and may contribute to rational drug development. It will be important to determine if similar interactions participate in other transmembrane signaling processes in cells.

ACKNOWLEDGMENTS

This work has been supported by NIH grant AI/CA R01-27409.

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