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Research Article

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Evidence that the Superoxide-Generating System of Human Leukocytes Is Associated with the Cell Surface

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ABSTRACT Superoxide anion ($O_2^{\cdot-}$) generation by human peripheral blood polymorphonuclear leukocytes is enhanced when these cells encounter appropriate soluble or particulate stimuli. $O_2^{\cdot-}$ generation requires intact, viable cells and proceeds independently of phagocytosis. To investigate the possibility that the $O_2^{\cdot-}$ -generating system is associated with the outer surface of the polymorphonuclear leukocyte plasma membrane, we have examined the effects upon $O_2^{\cdot-}$ production of p -diazobenzenesulfonic acid, a reagent which can react predominantly with proteins of the external cell membrane. When normal human polymorphonuclear leukocytes were preincubated with cytochalasin B (to minimize endocytosis) and then exposed to the surface-active lectin, concanavalin A, the cells were stimulated to generate $O_2^{\cdot-}$ in a concentration- and time-dependent fashion and selectively to discharge the granule-associated enzyme, lysozyme, into the surrounding medium. These responses, as well as cellular binding of [3H]concanavalin A, could be blocked by α -methyl-D-mannoside. Brief treatment (<5 min at 4°C) of the cells with p -diazobenzenesulfonic acid (1.0–5.0 mM) significantly interfered with concanavalin A-mediated $O_2^{\cdot-}$ generation but had no influence upon lysozyme release or upon binding of [3H]concanavalin A. The diazonium salt did not alter cell viability or the specific activity of the cytoplasmic enzyme, lactate dehydrogenase (inhibitable under conditions which allowed entry of this reagent into the cytosol). p -Diazobenzenesulfonic acid, therefore, very likely exerted its effects at the cell surface of the intact polymorphonuclear leukocyte, selectively inhibiting $O_2^{\cdot-}$ production (either directly or indirectly) without influencing another response to lectin-cell

contact: release of lysozyme. These results support the possibility that a polymorphonuclear leukocyte ectoenzyme is responsible for $O_2^{\cdot-}$ production.

INTRODUCTION

There is now considerable evidence that the burst of oxidative metabolism which accompanies phagocytosis by polymorphonuclear leukocytes (PMN)¹ is probably initiated at the cell surface. A variety of particulate as well as surface-reactive soluble stimuli are capable of provoking increased consumption of oxygen, generation of H_2O_2 , and increased utilization of glucose via the hexose monophosphate shunt pathway in the absence of phagocytosis (1–8). More direct evidence that the PMN cell surface participates in these reactions was provided recently by the demonstration by Briggs et al. (9) of an apparent ecto-enzyme which generates H_2O_2 via oxidation of NADH. A likely intermediate in this reaction is the superoxide anion ($O_2^{\cdot-}$) which can undergo spontaneous dismutation to yield H_2O_2 , or which can be converted to this product by the action of superoxide dismutase (10). $O_2^{\cdot-}$ generation by human PMN is also enhanced when these cells encounter appropriate soluble or particulate stimuli (8, 11–15). $O_2^{\cdot-}$ generation requires intact, viable cells and proceeds independently of phagocytosis. In fact, $O_2^{\cdot-}$ recovery in the medium surrounding PMN is enhanced when formation of phagocytic vacuoles is inhibited, as after treatment of cells with cytochalasin B (13). These observations, as well as the demonstration that $O_2^{\cdot-}$ does not appear to be released from cells as a consequence of degranulation or cell disruption, support the possibility that $O_2^{\cdot-}$ production takes place on the outer surface of the PMN cell membrane as well as in phagocytic vacuoles which are formed by invaginations of

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¹ Abbreviations used in this paper: Con A, concanavalin A; DBSA, p -diazobenzenesulfonic acid; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PMN, polymorphonuclear leukocyte (s).

this membrane (13, 16). We have investigated this possibility by examining the effects of *p*-diazobenzene-sulfonic acid (DBSA) upon generation of $O_2^{\cdot -}$ by PMN. This reagent reacts predominantly with proteins of the external cell membrane (17, 18). As a stimulus for $O_2^{\cdot -}$ generation, we have selected concanavalin A (Con A), a lectin known to enhance the oxidative metabolism of PMN (19). We have found that DBSA treatment significantly inhibited Con A-mediated $O_2^{\cdot -}$ generation without influencing another PMN response to surface stimulation by this ligand: secretion of the granule-associated enzyme, lysozyme (20).

METHODS

Preparation of leukocyte suspensions. 32 ml of venous blood from healthy adult donors was allowed to sediment at room temperature after mixing with 8 ml acid citrate dextrose (National Institutes of Health formula A) and 20 ml 6% dextran (average mol wt, 234,000, Sigma Chemical Co., St. Louis, Mo.) in 140 mM NaCl. 35 ml of 0.87% NH_4Cl was added to 15-ml aliquots of the leukocyte-rich supernates and the mixtures were centrifuged at 155 g for 10 min. Pellets were washed once with 140 mM NaCl and finally suspended in phosphate-buffered saline (Dulbecco's PBS, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 0.6 mM $CaCl_2$ and 1.0 mM $MgCl_2$, and adjusted to pH 7.4. This buffer was used throughout. In some experiments purified preparations of PMN were obtained by means of Hypaque/Ficoll gradients (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) (21), allowing studies of cell suspensions containing $98 \pm 1\%$ PMN without contaminating platelets or erythrocytes.

Preparation of DBSA. DBSA was prepared by the indirect method (17) as described previously by DePierre and Karnovsky (18). Actual yields of DBSA obtained by this method were determined by titration with histidine hydrochloride (Sigma Chemical Co.) (22). Yields of DBSA determined by this method were found generally to be within 75–80% of predicted values and correlated quite well with yields determined by employing radiolabeled sulfanilic acid (^{35}S -sulfanilic acid, Amersham/Searle Corp., Arlington Heights, Ill.) (18).

Treatment of leukocytes with DBSA. Aliquots of the leukocyte suspensions containing approximately 4×10^6 PMN were dispensed into 10×75 -mm polypropylene tubes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) and brought to desired temperature before the addition of DBSA. The pH of the DBSA solution was adjusted so that the final pH of the reaction mixtures was approximately 7.4. After appropriate periods of incubation of cells with DBSA, 2 vol of 10% fetal calf serum (Grand Island Biological Co.) in PBS buffer was added and the reaction mixtures were centrifuged at 4°C for 10 min at 155 g. The cell pellets were washed once with 10% fetal calf serum in PBS and once with buffer alone before being suspended in appropriate volumes of buffer. Control cell suspensions, not exposed to DBSA, were treated similarly.

Determination of leukocyte $O_2^{\cdot -}$ generation. Duplicate reaction mixtures containing leukocytes, with or without pretreatment with DBSA, were incubated with 5.0 $\mu g/ml$ cytochalasin B (ICI Research Laboratories, Alderley Park, Cheshire, England) in 0.1% dimethyl sulfoxide (Matheson, Coleman & Bell, East Rutherford, N. J.) at 37°C for 10 min before addition of 75 μM horse heart ferri-cytochrome *c* (type III, Sigma Chemical Co.) and (σ) Con A (Pharmacia Fine

Chemicals, Inc.). This concentration of dimethyl sulfoxide did not influence cytochrome *c* reduction, enzyme release, or enzyme assays (see below). α -methyl-D-Mannoside (Sigma Chemical Co.) was also added to some reaction mixtures. Incubations were terminated by placing the tubes in ice, then they were centrifuged at 4°C for 10 min at 755 g. Cell-free supernates were assayed for cytochrome *c* reduction as previously described (13). Specificity of cytochrome *c* reduction was monitored by assays of supernates from reaction mixtures to which had been added 10 $\mu g/ml$ superoxide dismutase (Truett Laboratories, Dallas, Tex.). $O_2^{\cdot -}$ generation has been expressed as nanomoles cytochrome *c* reduced per 10^6 PMN.

Measurement of leukocyte enzyme release. The extracellular release of the PMN granule-associated enzyme, lysozyme (EC 3.2.1.17) was measured as previously described (13) in duplicate reaction mixtures identical to those employed for the determination of $O_2^{\cdot -}$ generation, but in the absence of cytochrome *c*. Release of the cytoplasmic enzyme, lactate dehydrogenase (LDH, EC 1.1.1.27) was also measured (23) and used as an indicator of cell viability (24). Cell viability was also assessed by trypan blue exclusion. Enzyme release has been expressed as the percent of total activity released by the detergent, 0.2% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) or by sonication in simultaneously run duplicate reaction mixtures. Protein in cell-free supernates and in lysed cell pellets was measured (25) and employed to calculate enzyme-specific activities.

Binding of Con A to leukocytes. To suspensions of either normal or DBSA-treated PMN which had been preincubated with cytochalasin B were added 1.0 μCi [3H]Con A (New England Nuclear, Boston, Mass.) plus 30 μg of unlabeled lectin. After 15 min of incubation at 37°C, the leukocytes were sedimented (155g for 10 min), washed three times with buffer, and allowed to dry at room temperature. The dried pellets were digested with 0.5 ml of 0.2 N NaOH for 2 h at 56°C, followed by the addition of 0.2 ml of 3.0% acetic acid and 0.5 ml of distilled water. 1.0-ml aliquots were added to 20 ml Bray's solution (26) and counted in a Beckman LS-100 liquid scintillation counter (Beckman Instruments, Inc., Spincor Div., Palo Alto, Calif.). Results have been expressed as counts per minute per 10^6 PMN.

Binding and uptake of DBSA. For these experiments, DBSA was prepared with 14.7 mCi/mmol ^{35}S -sulfanilic acid (Amersham/Searle, lot 0976). Suspensions of purified PMN (from Hypaque/Ficoll gradients) were treated with radiolabeled DBSA ($\approx 1,500$ cpm/nmol) and washed as described above. Total cellular binding of DBSA was measured in NaOH digests of washed cell pellets (as above). In addition, some suspensions were subjected to four cycles of freezing (in dry ice/acetone) and thawing before division into 100,000-g supernates and pellets (27). This treatment was sufficient to release total assayable LDH. Supernates and digested pellets were counted before and after addition of trichloroacetic acid (TCA, 20% wt/vol). Results have been expressed as TCA-precipitable counts per minute per 5×10^6 PMN.

RESULTS

DBSA treatment of PMN. To determine the optimal conditions for treating PMN with DBSA, various combinations of time, temperature, and concentration were tested. Incubation at 37°C for greater than 10 min with 1.0 mM DBSA led to slight but reproducible reductions in the total cellular activity and specific activity of PMN cytoplasmic LDH (Table I), indicating that

TABLE I
Uptake of DBSA by PMN and Effects upon Enzyme Activities

Treatment of PMN*	Enzyme-specific activities†		Uptake of ³⁵ S-DBSA, cpm §	
	LDH	Lysozyme	Pellet	Supernate
None	1.21±0.21	6.52±0.31	—	—
DBSA (1.0 mM, 15 min, 37°C)	0.99±0.18	6.10±0.40	11,014±346	1,190±96
DBSA (5.0 mM, 2 min, 4°C)	1.23±0.23	6.50±0.29	1,053±36	-4±7

* PMN were treated as described with either routinely prepared DBSA or DBSA prepared with ³⁵S-sulfanilic acid. Cells were disrupted by sonication or by freezing and thawing. Protein and enzyme activities were assayed in supernates after centrifugation at 2,000 g for 10 min.

† LDH, absorbance units/microgram protein. Lysozyme, micrograms crystalline hen egg-white lysozyme equivalents/100 µg protein. Mean±SEM, n = 3.

§ TCA-precipitable counts in 100,000 g pellets and supernates of treated cells after freezing and thawing (20% TCA, wt/vol).

under these conditions, DBSA had penetrated into the cells. As in previous studies (18), if PMN were disrupted either by freezing and thawing or with Triton X-100, the activity of LDH could be promptly inhibited by DBSA (completely with 1.0 mM DBSA and significantly with 0.01 mM DBSA). Entry of DBSA into cells was confirmed by experiments employing a radio-labeled reagent. When PMN were incubated for 15 min at 37°C with 1.0 mM ³⁵S-DBSA, approximately 10% of cell-associated, TCA-precipitable counts were detected in 100,000-g supernates of cells after freezing and thawing. Similar time- and temperature-dependent entry of this reagent into cells have been reported for guinea pig peritoneal PMN (18) and for human erythrocytes (17). Consequently, to reduce the possibility for permeation across the plasma membrane, we elected to expose cells to DBSA for very brief durations and at reduced temperatures. DBSA has been demonstrated to be a nonpenetrating reagent under these conditions (17, 18, 28). At 4°C, for example, and for durations of less than 5 min, PMN could be exposed to 5.0 mM DBSA without any alterations in cellular integrity (measured by trypan blue exclusion and by leakage of cytoplasmic LDH) or changes in the specific activity and content of cellular enzymes (lysozyme and LDH). Furthermore, no TCA-precipitable counts were detected in 100,000-g supernates after freezing and thawing PMN which had been exposed to 5.0 mM ³⁵S-DBSA for 2 min at 4°C. Consequently, these conditions were employed in the experiments described below.

Effect of DBSA treatment upon Con-A-mediated PMN O₂⁻ generation and lysozyme release. Con A stimulated PMN to generate enhanced amounts of O₂⁻ (measured as superoxide dismutase-inhibitable cytochrome c reduction) and to release selectively the granule-associated enzyme, lysozyme, in a time- and concentration-related fashion (Figs. 1 and 2). These

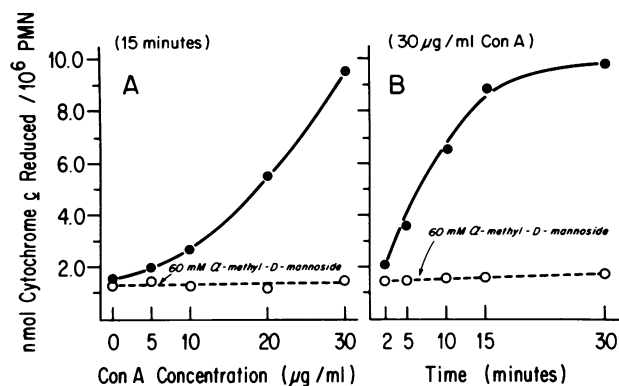


FIGURE 1 Superoxide generation by cytochalasin B-treated PMN exposed to Con A in the presence (○) or absence (●) of 60 mM α-methyl-D-mannoside: (A) versus concentration of Con A (15 min incubation), (B) versus duration of incubation (30 µg/ml Con A). Results are expressed as nmoles cytochrome c reduced per 10⁶ PMN.

responses to Con A were abolished if 60 mM α-methyl-D-mannoside was also present in the reaction mixtures. In the absence of cells, Con A or α-methyl-D-mannoside neither influenced cytochrome c reduction nor lysozyme activity. When DBSA-treated cells were exposed to Con A, O₂⁻ generation was reduced, whereas lysozyme release was unaffected (Table II). Inhibition of O₂⁻ generation varied (but not significantly) with the concentration of DBSA which was employed to pretreat the PMN, but was not associated with cytotoxicity (measured as "leakage" of cytoplasmic LDH). Results identical to those described above were obtained in experiments employing cell suspensions which contained 98±1% PMN (from Hypaque/Ficoll gradients). When DBSA was em-

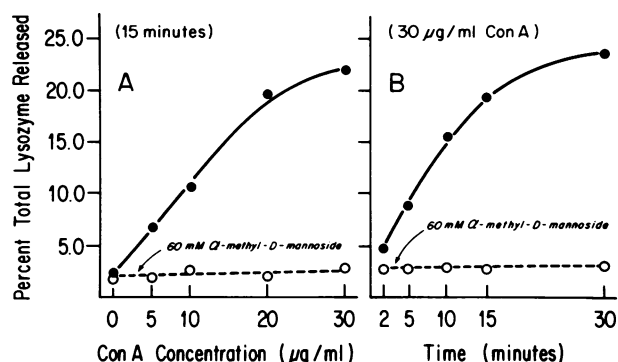


FIGURE 2 Lysozyme release from cytochalasin B-treated PMN exposed to Con A in the presence (○) or absence (●) of 60 mM α-methyl-D-mannoside: (A) versus concentration of Con A (15 min incubation), (B) versus duration of incubation (30 µg/ml Con A). Lysozyme activity in cell-free supernates is expressed as the percent of total activity released by 0.2% Triton X-100 in simultaneously run duplicate reaction mixtures (Table II).

TABLE II
Influence of DBSA Treatment upon Con A-Mediated PMN Enzyme Release and Superoxide Generation

Treatment of PMN*	Superoxide generation, nmol cytochrome <i>c</i> reduced/ 10 ⁶ PMN/15 min	Total enzyme activity released,† %	
		Lysozyme	LDH
None (control)	1.2±0.1	1.3±0.6	1.0±0.1
Con A (30 µg/ml)	8.3±0.7	22.8±1.8	1.1±0.2
DBSA (5.0 mM) + Con A	4.4±0.6§	24.2±1.7	0.9±0.1
(2.5 mM) + Con A	5.0±0.7	22.4±2.3	1.0±0.1
(1.0 mM) + Con A	5.9±0.7¶	23.9±3.3	0.9±0.2

* PMN were treated as indicated with DBSA for 2 min at 4°C, preincubated with 5.0 µg/ml cytochalasin B for 10 min at 37°C, and exposed to Con A for 15 min at 37°C.

† Expressed as percent of total (100%) activity released by Triton X-100: 100% lysozyme = 7.4 µg±1.4 crystalline hen egg-white lysozyme equivalents/2 × 10⁶ leukocytes. 100% LDH (lactate dehydrogenase) = 874±89 AU/2 × 10⁶ leukocytes. Mean±SEM, *n* (number of experiments) = 10.

§ *P* vs. Con A alone < 0.001 (Student's *t* test).

^{||} *P* vs. Con A alone < 0.005; vs. DBSA (5.0 mM) + Con A > 0.25 (Student's *t* test).

¶ *P* vs. Con A alone < 0.01; vs. DBSA (5.0 mM) + Con A > 0.05 (Student's *t* test).

ployed at concentrations greater than 5.0 mM, superoxide generation was reduced even further (results not shown). This, however, was associated with indications of cytotoxicity (enhanced release of LDH and decreased trypan blue exclusion). Consequently, this effect of DBSA was not pursued further. Whereas DBSA per se was capable of oxidizing cytochrome *c*, appropriate experiments excluded this phenomenon as accounting for the observed results. Concentrations of DBSA below 0.05 mM had no effect upon cytochrome *c* (either reduction or oxidation) nor did DBSA-treated PMN when these were mixed with normal cells exposed to Con A. Predicted amounts of cytochrome *c* reduction were observed in the latter experiments (a representative experiment is shown in Table III). These results indicated not only that our method of washing DBSA-treated cells was sufficient to remove any unbound diazonium salt which could potentially interfere with cytochrome *c* reduction, but also that bound DBSA had no apparent effect on the assay system (i.e., did not influence cytochrome *c* reduction by normal cells). Furthermore, additional control experiments excluded the possibilities that DBSA treatment influenced the enzyme assays or that there was selective adsorption of enzymes to cells after their release into the suspending buffer.

Binding of radiolabeled Con A to PMN did not appear to be influenced by prior treatment of the cells with DBSA (Fig. 3). Furthermore, binding of Con A to normal or to DBSA-treated cells was appropriately inhibited by α -methyl-D-manniside. Virtually complete inhibition of binding was observed in the presence of concentrations of this sugar exceeding 60 mM.

DISCUSSION

Con A has previously been shown capable of stimulating oxygen consumption and hexose monophosphate shunt activity in PMN (19), as well as provoking discharge from these cells, by exocytosis, of the granule-associated enzyme, lysozyme (20). Our results indicate that the lectin is also a potent stimulus for O₂^{•-} production by PMN, even under conditions (cytochalasin B treatment) which retard its internalization (20). Stimulation of O₂^{•-} production was appropriately inhibited by α -methyl-D-mannoside, a sugar which effectively competes with cell "receptors" for Con A (29). Our results provide, moreover, another example of enhanced O₂^{•-} generation after PMN cell-surface stimulation as well as further evidence that the PMN O₂^{•-}-generating system is associated with the cell surface.

The hypothesis that the PMN O₂^{•-}-generating system is localized to the external surface of the plasma membrane is attractive. Surface generation of O₂^{•-} (and of its product, H₂O₂) would allow for its concentration within phagocytic vacuoles and provides a convenient explanation for the extracellular recovery of this highly reactive free radical. There is, in fact, no readily apparent alternative hypothesis which would account for the ability of O₂^{•-} to diffuse from cells (which contain superoxide dismutase in their cytoplasm) without producing lethal membrane damage. Indeed, Salin and McCord (30) have provided evidence of O₂^{•-}-mediated lethal injury to PMN. Direct evidence for such a PMN ectoenzyme is lacking, but there is strong supportive evidence. For example, the recently described surface NADH oxidase of the human PMN, which produces H₂O₂, could very well be an O₂^{•-}-generating system (9). It is likely that O₂^{•-} is an important intermediate in H₂O₂ generation (8, 9, 13, 31).

Inhibition of intact cell enzyme activity by "non-penetrating" reagents represents one experimental

TABLE III
*DBSA-Treated Leukocytes: Influence on the Reduction of Cytochrome *c* by Normal Leukocytes which Have Been Stimulated with Con A*

Reaction mixtures*	nmol Cytochrome <i>c</i> reduced/10 ⁶ PMN/15 min
A = Normal PMN (4 × 10 ⁶) + Con A (30 µg/ml)	9.6
B = DBSA (5.0 mM)-treated PMN (4 × 10 ⁶) + Con A (30 µg/ml)	4.0
A + B (8 × 10 ⁶ PMN)	6.9†

* PMN were exposed to DBSA for 2 min at 4°C and washed. Cells were preincubated with cytochalasin B (5.0 µg/ml) for 10 min before exposure to Con A for 15 min at 37°C.

† Expected value = 6.8 nmol/10⁶ PMN.

method that can be employed to test whether a cellular enzyme has active sites directly exposed to the external milieu (18). DBSA, under appropriate conditions, is such a nonpenetrating reagent. It is capable of forming covalent bonds with sulfhydryl, amino, and phenolic groups in proteins and its polar nature would hinder translocation across the hydrophobic bilayers of intact cells (32, 33). DBSA has previously been employed to label surface proteins of the human erythrocyte (17) and to demonstrate that adenosine monophosphate was an ectoenzyme of guinea pig PMN (18).

We have found that brief (<5 min) treatment at 4°C of human PMN with DBSA significantly interfered with $O_2^{\cdot -}$ generation when these cells were subsequently exposed to Con A. Under conditions sufficient to inhibit Con A-mediated $O_2^{\cdot -}$ production significantly, DBSA neither altered cell viability nor the specific activity of the PMN cytoplasmic enzyme, LDH. This enzyme was inhibitable by DBSA under conditions (prolonged incubation at 37°C or after treatment with Triton X-100) which allowed access of this reagent to the cytosol (Table I). Although it appeared that DBSA exerted its effects at the cell surface of the intact PMN to selectively inhibit $O_2^{\cdot -}$ production, other possibilities had to be considered. In contrast to adenosine monophosphate activity of the guinea pig PMN (18), $O_2^{\cdot -}$ -generating activity of human PMN depends upon exogenous cell surface stimulation (13). Consequently, DBSA may have acted primarily upon surface proteins which are "linked" to, or activate, the enzyme system rather than upon the enzyme per se. Thus, some independent measurements of stimulus-membrane interactions were required. This formed the basis for the strategy of employing Con A as a stimulus. First, Con A binds predominantly to sugar moieties which were unlikely to be affected by DBSA (29). Second, binding of Con A could be quantitatively measured. Third, phagocytosis or internalization of Con A are not prerequisites for its stimulatory effects. To further minimize phagocytosis or internalization as variables, cytochalasin B was employed (13, 20).² Finally, another response of PMN to cell surface stimulation by Con A could be measured, i.e., the extracellular release of lysozyme. Our experiments revealed that DBSA neither influenced binding of Con A to PMN nor the release of lysozyme that occurs as a consequence of such binding. These measurements of

² In studies not described here, DBSA was found to markedly interfere with phagocytosis by PMN of serum-treated zymosan particles and opsonized paraffin oil droplets, thus excluding these as possible stimuli. The effects of Con A and of DBSA upon PMN in the absence of cytochalasin B were qualitatively and quantitatively similar to those observed in the presence of this compound. However, since measurements of internalization of Con A in these experiments were not performed, these data are not presented.

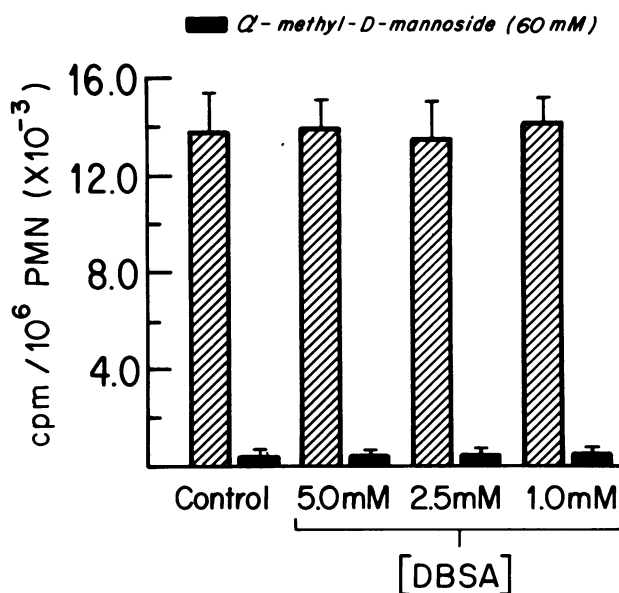


FIGURE 3 $[^3H]$ Con A binding to normal (control) and DBSA-treated PMN in the presence (solid bars) and absence (hatched bars) of 60 mM α -methyl-D-mannoside. PMN were exposed to DBSA for 2 min at 4°C. Cells were preincubated with cytochalasin B (5.0 μ g/ml) for 10 min before exposure to $[^3H]$ Con A (1.0 μ Ci) for 15 min at 37°C.

stimulus-membrane interactions support the possibility that DBSA acted primarily at the cell surface of intact PMN to selectively inhibit $O_2^{\cdot -}$ production without influencing other responses to cell surface stimulation. No conclusion, however, can be drawn from these experiments regarding possible effects of DBSA upon surface proteins which "activate" the $O_2^{\cdot -}$ -generating system. Furthermore, since greater than 50% inhibition of $O_2^{\cdot -}$ generation by DBSA was associated with cytotoxicity, these experiments do not exclude the possibility that some $O_2^{\cdot -}$ is produced at a site which was not accessible to the "nonpenetrating" reagent. Nevertheless, our results do support the hypothesis that the PMN $O_2^{\cdot -}$ -generating system is an ectoenzyme and provide additional evidence that the burst of oxidative metabolism which normally accompanies particle contact and phagocytosis by PMN is initiated at the cell surface.

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