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Factors Which Affect Superoxide Anion Release from Rat Alveolar Macrophages

T. D. Sweeney*, V. Castranova, L. Bowman, and P. R. Miles

ABSTRACT: *In order to investigate some of the characteristics of superoxide anion release from alveolar macrophages, the effects of substances known to influence superoxide release from polymorphonuclear leukocytes (PMN) were studied in rat alveolar macrophages. There is a relatively small, but constant, amount of superoxide released from alveolar macrophages at rest. The amount released increases 5- to 6-fold and becomes maximal in about 20–30 min following exposure to unopsonized zymosan particles. The rate of superoxide release is maximal only 2 min after exposure of the cells to particles, i.e., long before particle uptake is complete. In addition to particles, release of superoxide anion can be stimulated by phorbol-12-myristate-13-acetate (PMA). Lectins and chemotactic factors, which stimulate release in PMN, have little or no effect in alveolar macrophages. Superoxide release during exposure to zymosan appears to be dependent upon extracellular Ca^{++}. Also, the release mechanism can be affected by the addition of cyclic AMP or various protein modifiers to the medium. Since many of these findings differ from those reported by others for PMN, the control of superoxide anion release from alveolar macrophages and PMN is probably different.*

INTRODUCTION

During phagocytosis, superoxide anion ($O_2^- \cdot$) is released from phagocytic cells, such as polymorphonuclear leukocytes (PMN) and alveolar macrophages [1–5]. The role of superoxide in the function of these cells has not yet been definitely established. However, there is evidence to indicate that in polymorphonuclear leukocytes it acts as a bactericidal agent [6, 7]. In addition, there is some evidence to support a relationship between superoxide release and bactericidal activity in alveolar macrophages [7]. Once superoxide anion is produced, a number of chemical reactions can occur to cause the formation of hydrogen peroxide, singlet oxygen, and hydroxyl radical, all of which may possess bactericidal activity [8–10]. Thus, superoxide may play an important role in the antibacterial activity of phagocytic cells.

The release of superoxide anion from polymorphonuclear leukocytes during phagocytosis can be affected by various substances. For example, lectins (such as concanavalin A), membrane perturbants [such as phorbol-12-myristate-13-acetate (PMA)], and chemotactic factors, [such as n-formyl-1-methionyl-1-leucyl-1-

*Present address: Department of Physiology, Harvard School of Public Health, Boston, MA.

From the Department of Physiology, West Virginia University Medical Center, Morgantown, West Virginia 26506 and Appalachian Laboratory for Occupational Safety and Health, Morgantown, West Virginia 26505.

Address correspondence to: Dr. Philip R. Miles, Department of Physiology, West Virginia University Medical Center, Morgantown, WV 26506.

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52 Vanderbilt Ave., New York NY 10017

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phenylalanine (F-met-leu-phe)], cause an increase in the release of superoxide from PMN [11–13]. Calcium also increases superoxide release, presumably because calcium influx is associated with the release mechanism [14, 15]. On the other hand, some substances cause decreases in superoxide anion release from neutrophils. For example, the sulfhydryl modifier, parachloromercuribenzene sulfonic acid (PCMBS), inhibits release by binding to membrane sulfhydryl groups [16, 17]. Addition of cyclic AMP to PMN depresses superoxide release, perhaps by blocking microtubule assembly [18, 19]. Much of what is known about the mechanisms involved in superoxide anion release from neutrophils has been derived from experiments such as those described above.

Although superoxide anion release has been studied in alveolar macrophages from several species, such as rabbits, rats, mice, guinea pigs, and humans [3–5, 20, 21], the mechanisms involved in this release remain unclear. In fact, it has generally been assumed that the mechanism of release from alveolar macrophages is similar to that in polymorphonuclear leukocytes. The objectives of this investigation were to study some of the characteristics of superoxide anion release from rat alveolar macrophages and to compare the results with those obtained in PMN by other investigators. The experimental approach used was to determine the effects of various substances known to influence superoxide release in PMN on superoxide release in rat alveolar macrophages. A preliminary report of these findings has been published previously [22].

MATERIALS AND METHODS

Male Long-Evans hooded rats (250–300 g) were anesthetized with sodium pentobarbital (0.2 mg/gm body weight) and exsanguinated by cutting the abdominal aorta. Alveolar macrophages were obtained by lavaging the lung approximately 12 times with a total of 80 ml of ice-cold Ca^{++}-free solution (145 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 9.35 mM Na_2HPO_4, and 5.5 mM glucose; pH = 7.4) [5, 23]. The cells were separated from the lavage fluid by centrifugation at 500 *g* for 5 min at 2°C and then washed twice by alternate centrifugation and resuspension in a Hepes-buffered medium [140 mM NaCl, 5 mM KCl, 5 mM Hepes (N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid), and 5.5 mM glucose; pH = 7.4]. Less than 1% of the cells obtained in this manner were PMNs; thus, a rather homogeneous population of alveolar macrophages had been obtained. Furthermore, the cells were judged to be 90–95% viable by trypan blue exclusion.

Superoxide anion release was monitored by measuring the reduction of cytochrome *c* [1, 5, 24]. Alveolar macrophages (4×10^6 cells) were added to 10 ml of Hepes-buffered medium containing 0.12 mM cytochrome *c*. This cell suspension was preincubated at 37°C for 5 min. Then substances which were used to modify superoxide release were added, when appropriate, and preincubation continued for another 10 min. After this preincubation, measurement of superoxide release was started (zero time). Then time samples were taken, centrifuged at 6000 *g* for 30 sec at 2°C, and the optical densities of the supernatants measured at 550 nm with a spectrophotometer (Model 300N; Gilford Instrument Laboratories, Inc., Oberlin, OH). The addition of 0.4 mg/ml superoxide dismutase (Sigma Chemical Co., St. Louis, MO) to the cell suspensions at zero time, i.e., after the preincubation period, completely blocked the reduction of cytochrome *c*. Therefore, the reduction of cytochrome *c* was caused by superoxide and the results are expressed as superoxide-dependent cytochrome *c* reduction in optical density units.

Superoxide anion release was measured with cells at rest (no exposure to particles) and following exposure to foreign particles. The particle used in these experiments was zymosan, an extract from the cell walls of *Saccharomyces cerevisiae* yeast (Sigma). The zymosan was prepared by boiling 50 mg in 2–3 ml of Hepes-buffered solution for 15 min. Then the zymosan was separated from the solution by centrifugation and resuspended in fresh Hepes-buffered medium. This procedure seemed to prevent the zymosan particles from clumping. In a few experiments opsonized zymosan was prepared by incubating 50 mg zymosan (prepared as described above) at 37°C for 30 min in 5 ml of rat serum. Then the zymosan was washed once in Hepes-buffered medium before use. In these experiments, zymosan (0.4 mg/ml) was added to the cell suspension at zero time, i.e., immediately after preincubation of the cells and before the zero time sample was taken. This amount of zymosan yields a particle to cell ratio of 20:1.

The following substances were obtained from Sigma Chemical Co.: N^6, $O^{2'}$-dibutyryl adenosine 3'-5'-cyclic monophosphoric acid (cAMP), theophylline, parachloromercuribenzene sulfonic acid (PCMBS), 5 5'-dithiobis-[2-nitrobenzoic acid] (DTNB), 2,4,6-trinitrobenzene sulfonic acid (TNBS), cytochalasin B, concanavalin A, phorbol myristate acetate (PMA), n-formyl-1-methionyl-1-leucyl-1-phenylalanine (F-met-leu-phe), and n-formyl-1-methionyl-1-phenylalanine (F-met-phe). SITS (4-acetamido-4'-isothiocyano-stilbene-2-2'-disulfonic acid) was obtained from ICN Pharmaceuticals Inc., Cleveland, OH. Stock solutions of PMA and cytochalasin B were made by dissolving these substances in 50 μl of dimethyl sulfoxide (DMSO) before bringing the stock solutions up to volume with water. The final concentration of DMSO added to the cell suspensions was less than 0.1%, and this had no effect on the cells or the assay. Stock solutions of all other substances were made in aqueous medium. All modifiers were tested for nonspecific effects on the assay and they had none. In addition, the effects of each of the modifiers on cell viability were determined by monitoring oxygen consumption of the cells by using a Gilson K-IC oxygraph equipped with a Clark electrode (Gilson Medical Electronics, Inc.; Middleton, WI)[5]. Incubation of either resting or zymosan-stimulated cells with modifiers does not significantly decrease oxygen consumption. Therefore, these modifiers were judged to be nontoxic to both resting and stimulated cells.

RESULTS

Time Course of Superoxide Anion Release

The release of superoxide anion from rat alveolar macrophages was measured at rest, i.e., no exposure to particles, and after exposure of the cells to unopsonized zymosan particles. The time courses for release are shown in Fig. 1. Note that there is a small amount of superoxide released from alveolar macrophages even when they are not exposed to particles, i.e., from resting cells. Upon exposure of the cells to unopsonized zymosan, there is an increase in superoxide anion release which becomes maximal in about 20 min. This time course is similar to the one obtained in PMN during exposure to opsonized zymosan particles [2, 25]. The superoxide release caused by particle exposure in alveolar macrophages is approximately 5- to 6-fold greater than the release of superoxide from resting cells. The results of these experiments indicate that the time course for superoxide release from alveolar macrophages exposed to unopsonized zymosan is similar to that for release from PMN exposed to opsonized particles. However, there is one major difference between the two cell types. DeChatelet et al. [2] have

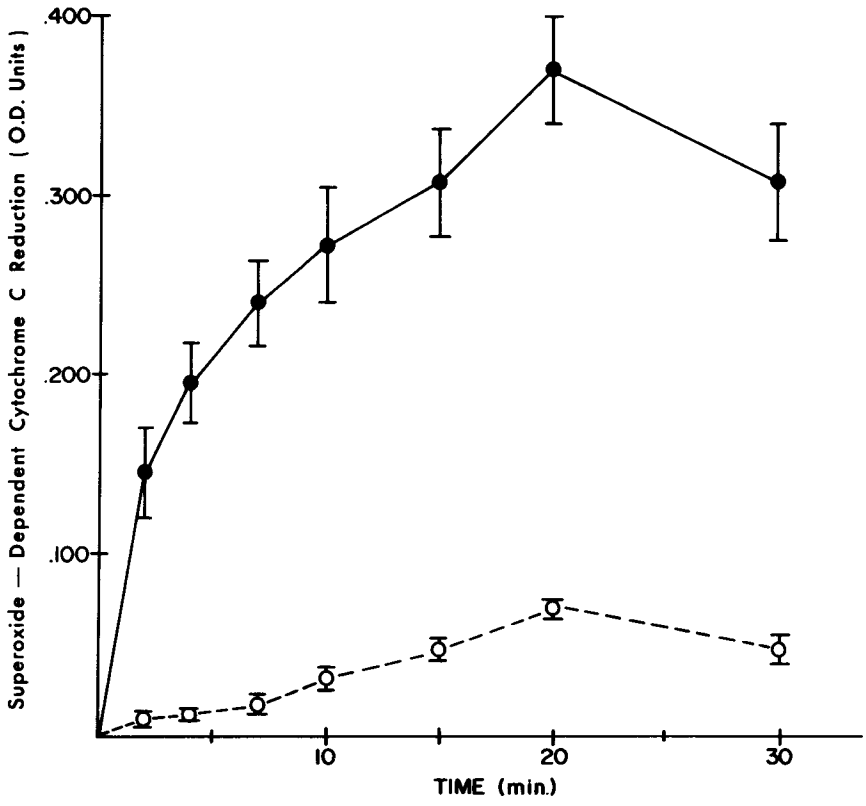

Figure 1 Superoxide anion release from resting cells (O---O) and from alveolar macrophages exposed to unopsonized zymosan (● — ●) as a function of incubation time at 37°C. In each experiment the incubation mixture consisted of 4×10^6 alveolar macrophages in a final volume of 10 ml of Hepes-buffered medium containing 0.12 mM cytochrome *c*. Unopsonized zymosan (4 mg) was added to the appropriate incubation mixtures at zero time. At each time shown, 2 ml samples of the incubation mixture were obtained, centrifuged at 6000 *g* for 30 sec, and the optical densities of the supernatants were measured at 550 nm. The points are mean values for 6 experiments and the bars represent the standard errors of the means.

reported that opsonization of particles increases superoxide release from neutrophils by as much as 25- to 30-fold while we found that opsonization results in only a 50% increase in release from alveolar macrophages (data not shown). Thus, in general, neutrophils will respond only to opsonized particles while alveolar macrophages release superoxide in response to unopsonized zymosan.

The time dependence of superoxide anion release from rat alveolar macrophages can also be presented as a rate. In Fig. 2, the rate of superoxide release is shown as a function of time. In resting alveolar macrophages superoxide is released at a low but relatively constant rate. Upon exposure to particles there is a rapid increase in the rate of superoxide release which reaches a maximum in 2 min. The rate of release then decreases with time and returns to resting level in about 20–30 min. This relationship (Fig. 2) is very similar to the one between the chemiluminescence generated by rat alveolar macrophages exposed to zymosan and time [5]. In fact, these data support our suggestion in a previous paper that chemiluminescence is the result of a reaction between superoxide anion and

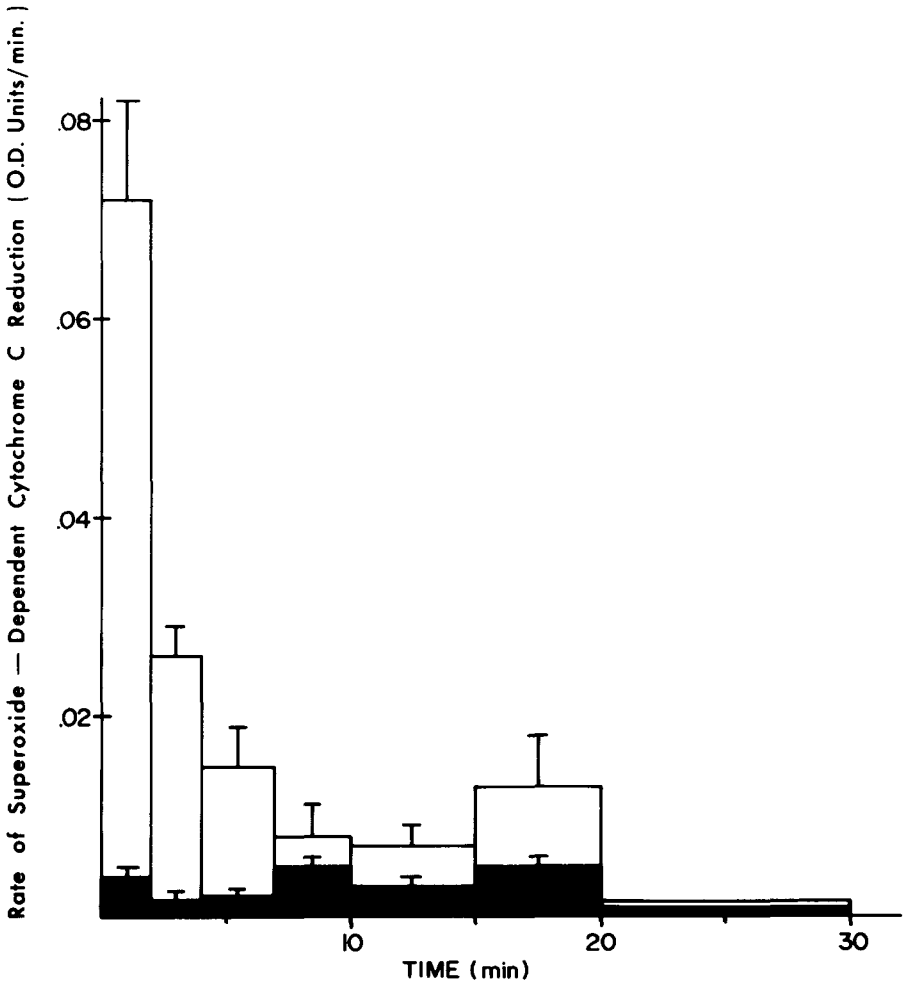

Figure 2 Rate of superoxide anion release from resting cells (solid bars) and from alveolar macrophages exposed to unopsonized zymosan (open bars) as a function of time. The rate of superoxide release was calculated from the data shown in Fig. 1 by dividing the change in optical density which occurred during a given time interval by that time. The points are mean values for 6 experiments and the bars represent the standard errors of the means.

zymosan [24]. Furthermore, the rate of superoxide release peaks long before any significant zymosan uptake occurs (i.e., uptake is complete in about 60 min) [24, 26]. Thus, in alveolar macrophages superoxide release precedes particle uptake.

Modification of Superoxide Anion Release

The effects of various substances known to influence superoxide release in PMN were studied in both resting alveolar macrophages and in cells exposed to particles. Since an incubation time of 20 min yields the maximal amount of superoxide, this incubation time was used for all measurements. Superoxide anion release from neutrophils can be stimulated by lectins, membrane perturbants, and

Table 1 Effects of Lectins, Membrane Perturbants, and Chemotactic Factors on Superoxide Release

Superoxide release was measured in resting cells and in alveolar macrophages exposed to unopsonized zymosan. The measurements were made after an incubation period of 20 min. The concentrations of substances used were those which produced maximal effects. The control values for superoxide release were 5.2 (±1.0) nmol cytochrome c reduced/10^6 cells for resting cells and 25.1 (±2.8) nmol cytochrome c reduced/10^6 cells for cells exposed to zymosan. See the Materials and Methods section for more detailed information about the methodology. The numbers shown are mean values for 6 experiments (±SEM).

	Superoxide release (% control)	
Treatment (concentration)	Rest	Exposure to zymosan
Control	100	100
Cytochalasin B(6.6 μg/ml)	40(±6)[a]	62(±9)[a]
Concanavalin A(100 μg/ml)	37(±5)[a]	28(±7)[a]
PMA (5μg/ml)	237(±45)[a]	56(±3)[a]
F-met-phe (10^{-4}M)	102(±7)	88(±6)
F-met-leu-phe (10^{-4}M)	120(±2)[a]	78(±6)[a]

[a]$P < 0.05$ vs. controls.

chemotactic factors [11, 12, 13, 17]. The effects of some of these substances on superoxide release from rat alveolar macrophages at rest and during exposure to zymosan are shown in Table 1. Only two substances, PMA and F-met-leu-phe, stimulate superoxide anion release from resting alveolar macrophages, and the stimulation by F-met-leu-phe is rather weak. Two other substances, cytochalasin B and concanavalin A, actually inhibit the release of superoxide from resting cells. All of the substances tested produce inhibition of superoxide release during exposure to particles. There are several differences between these results and those obtained in PMN. For example, concanavalin A and the chemotactic factors, F-met-phe and F-met-leu-phe, stimulate superoxide release from resting PMN but have little or no stimulatory effect in resting alveolar macrophages. In addition, cytochalasin B inhibits release during exposure to particles in alveolar macrophages but actually stimulates in neutrophils exposed to particles [12, 27]. These results indicate that there are differences in the control of superoxide release in these two cell types.

The effects of cyclic AMP, theophylline, and various group-specific protein modifiers on superoxide release were studied and the results are shown in Table 2. Treatment with cyclic AMP or theophylline, a substance which increases intracellular cyclic AMP levels by blocking phosphodiesterase [28], results in a decrease in the resting release of superoxide. However, these substances have no effect on the release of superoxide from cells exposed to particles. In contrast, addition of cyclic AMP to neutrophils exposed to particles leads to a depression of superoxide anion release [18, 19]. The sulfhydryl group modifiers, PCMBS and DTNB, produce a decrease in resting superoxide release, while the release from cells exposed to particles is inhibited by PCMBS but not affected by DTNB. The ineffectiveness of DTNB may be due to the fact that it does not penetrate cell membranes [29]. In fact, in neutrophils only permeant sulfhydryl reagents inhibit superoxide release during phagocytosis [16]. TNBS, an amino

Table 2 Effects of Cyclic AMP, Theophylline, and Group-Specific Protein Modifiers on Superoxide Anion Release
For information on superoxide release, see the caption of Table 1.

Treatment (concentration)	Superoxide release (% control)	
	Rest	Exposure to zymosan
Control	100	100
cAMP (10^{-4}M)	60(±4)[a]	104(±4)
Theophylline (10^{-3}M)	46(±9)[a]	95(±7)
PCMBS (10^{-4}M)	25(±4)[a]	11(±3)[a]
DTNB (10^{-4}M)	58(±8)[a]	91(±2)[a]
SITS (10^{-4}M)	70(±9)[a]	93(±3)
TNBS (10^{-3}M)	254(±35)[a]	117(±8)

[a] $P < 0.05$ vs. controls.

group modifier, stimulates release of superoxide from resting cells but has little effect in cells exposed to particles. The other amino modifier, SITS, has little effect and this may be due to its inability to penetrate the membrane [30]. These results indicate that the effects of cyclic AMP on superoxide release from alveolar macrophages differ from the effects in PMN. However, the effects of sulfhydryl modifiers on superoxide release appear to be similar in the two cell types. Also, many of the substances shown in Table 2, such as cyclic AMP, theophylline, DTNB, and TNBS, have some effect on resting superoxide release but do not affect release during exposure to particles.

The dependence of superoxide anion release on the divalent cations, Ca^{++} and Mg^{++}, is shown in Table 3. Superoxide release from resting alveolar macrophages is not affected by the addition of Ca^{++}, in concentrations as high as 5 mM, to the medium. However, addition of 2 mM Mg^{++} produces a 50–60% inhibition of superoxide release from resting cells. In contrast, superoxide release from cells exposed to particles is increased by addition of Ca^{++} to the medium but unaffected by Mg^{++}. The stimulatory effect of Ca^{++} during exposure to particles is similar to the effect of Ca^{++} in PMN during phagocytosis [12, 15, 31]. The results of these experiments indicate that the effects of divalent cations on superoxide release from alveolar macrophages appear to be similar to the effects in neutrophils. In addition, these results also show that the effects of divalent cations on resting superoxide release are different from their effects following particle exposure; i.e., Mg^{++} inhibits in resting cells but has no effect in cells exposed to zymosan, and Ca^{++} stimulates during particle exposure but has no effect in resting cells.

Verapamil is a substance which has been shown to block Ca^{++} uptake and superoxide release from leukocytes [15]. Therefore, we investigated the effects of verapamil in rat alveolar macrophages. Verapamil has no effect on superoxide release from resting cells. This was expected since Ca^{++} has no effect in resting cells (Table 3). In contrast, verapamil inhibits superoxide release from cells exposed to zymosan. A dose-response curve for the verapamil effects is shown in Fig. 3. The concentration of verapamil which produces about 50% of the maximal inhibition is approximately 4×10^{-4}M. These data, as well as the experiments with Ca^{++} (Table 3), suggest that Ca^{++} influx may be involved in superoxide release from cells exposed to particles but that Ca^{++} has no effect in resting cells. This result also demonstrates that verapamil has similar effects on superoxide release from both alveolar macrophages and leukocytes during phagocytosis.

Table 3 Effects of Calcium and Magnesium on Superoxide Anion Release

Superoxide release was measured in resting cells and in alveolar macrophages exposed to unopsonized zymosan. The measurements were made after an incubation period of 20 min. The control incubation medium contained no Ca^{++} or Mg^{++}. The control values for superoxide release were 6.3 (±1.2) nmol cytochrome c reduced/10^6 cells for resting cells and 17.4 (±1.6) nmol cytochrome c reduced/10^6 cells for cells exposed to zymosan. See the Materials and Methods section for more detailed information about the methodology. The numbers shown are mean values for 6 experiments (±SEM).

	Superoxide release (% control)	
Treatment (concentration)	Rest	Exposure to zymosan
Control	100	100
Ca^{++} (2mM)	87(±6)	134(±6)[a]
Ca^{++} (5mM)	108(±6)	147(±3)[a]
Mg^{++} (2mM)	44(±8)[a]	95(±3)

[a] $P < 0.05$ vs. controls.

DISCUSSION

In this investigation we have identified some of the characteristics of superoxide anion release from rat alveolar macrophages. At rest, these cells release a small but constant amount of superoxide anion. Upon exposure to unopsonized particles the amount released increases 5- to 6-fold and reaches a maximum about 20 min following the initial exposure. The rate at which superoxide is released becomes maximal only 2 min after exposure to particles and then gradually diminishes over the next 20–30 min. The relationship between rate of release and time is similar to the one between chemiluminescence generated by rat alveolar macrophages exposed to zymosan and time [5]. Thus, these data support the suggestion of Miles et al. [24] that chemiluminescence is the result of the extracellular reaction between superoxide and zymosan. These data also indicate that superoxide release becomes maximal long before the uptake of zymosan is complete; i.e., completion of uptake takes about 60 min [26]. In addition to unopsonized zymosan particles, release of superoxide anion is also caused by the membrane perturbant, PMA. However, lectins and chemotactic factors, which stimulate superoxide release in PMN, i.e., concanavalin A, cytochalasin B, F-met-phe, and F-met-leu-phe, have little or no effect in rat alveolar macrophages. Some of these results differ from those obtained by Holian and Daniele [32] which indicate that concanavalin A and F-met-phe stimulate superoxide release in guinea pig alveolar macrophages. Perhaps this discrepancy is due to a species difference or to the amounts of concanavalin A used. Finally, superoxide release can also be affected by extracellular Ca^{++} and by the addition of cyclic AMP or protein modifiers which react with sulfhydryl and amino groups to the medium.

One major finding in this work is that the control of superoxide release from alveolar macrophages probably differs in many respects from that in neutrophils. There are three lines of evidence to support this conclusion. First, the immuno-

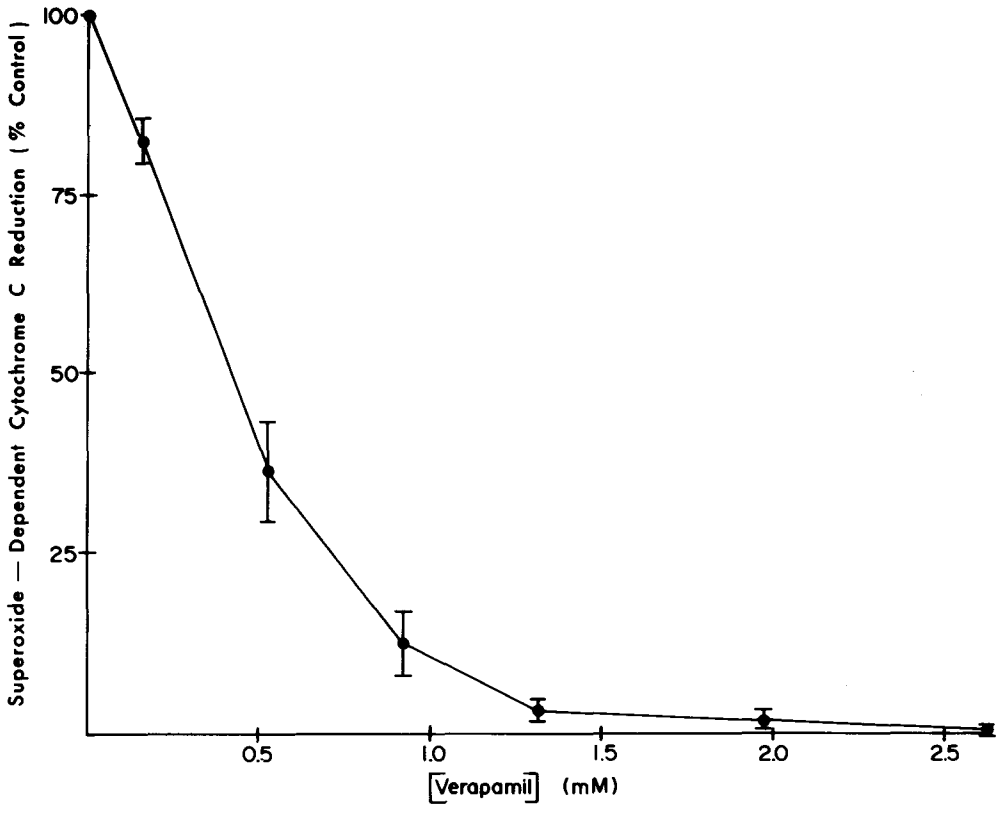

Figure 3 Dose-response curve for the effect of verapamil on superoxide anion release during exposure to unopsonized zymosan. In each experiment the incubation mixture consisted of 4×10^6 alveolar macrophages, 4 mg unopsonized zymosan, and the appropriate concentration of verapamil in a final volume of 10 ml of Hepes-buffered medium containing 0.12 mM cytochrome *c*. The control response was obtained in the absence of verapamil. After incubation of the samples for 20 min, the suspensions were centrifuged at 6,000 *g* for 30 sec, and the optical densities of the supernatants were read at 550 nm. The points are mean values for 6 experiments and the bars represent the standard errors of the means.

logical aspects of the phagocytotic responses of these two cell types are different. Leukocytes do not respond to particles which have not been opsonized [2, 27]. In fact, PMN release superoxide in response to opsonins alone; i.e., complement and immunoglobulins will stimulate release even when particles are absent [11]. Neutrophils also release superoxide in response to chemotactic factors [12, 15]. In contrast, rat alveolar macrophages do release superoxide in the presence of unopsonized particles and they do not respond to chemotactic factors. Second, the responses of the two cell types to concanavalin A, a lectin which binds to membrane glycoproteins, are different. Concanavalin A stimulates superoxide release from PMN [13] but actually inhibits release in alveolar macrophages. This result indicates that the sites involved in the particle-cell interactions are probably different in the two cell types. Third, the effects of cytochalasin B are not the same in alveolar macrophages and PMN. Cytochalasin B potentiates the effects of other stimulants for superoxide release in PMN [12, 27] but it inhibits the effect of zymosan on release from alveolar macrophages.

The results of these experiments also provide some insight as to the role of calcium in superoxide anion release from alveolar macrophages. For example, the release during exposure to particles seems to involve the movement of Ca^{++} into the cells. The evidence for this is that removal of extracellular Ca^{++} or treatment of the cells with verapamil inhibits superoxide release. In this regard, superoxide release from alveolar macrophages is like that from neutrophils; i.e., Ca^{++} uptake also appears to be required for superoxide release from PMN during phagocytosis [14, 15].

In this paper we have compared data which we obtained experimentally from alveolar macrophages with results obtained from the literature for polymorphonuclear leukocytes. In general, there are limitations to using such an approach. For example, use of different animal species, particles, particle to cell ratios, and other differences in experimental design could have an effect on the validity of these comparisons. However, in this particular case the studies on neutrophils are numerous and the results have been verified by many different investigators. The risk involved in making these comparisons are, therefore, minimal. In addition, many of the effects reported by other investigators for PMN, such as the requirement for opsonization, Ca^{++} dependence, and stimulation of superoxide release by concanavalin A, PMA, and F-met-leu-phe, have been repeated in our laboratory using methods similar to those employed in the current study [33].

It is interesting that most substances we tested affect release of superoxide from resting cells in a completely different manner than release in cells exposed to zymosan. For example, addition of Mg^{++}, cyclic AMP, theophylline, DTNB, and SITS to the incubation medium inhibits superoxide release from resting cells but has no effect on the release from alveolar macrophages exposed to zymosan. TNBS produces stimulation of release in resting cells but has little effect in cells exposed to particles. Addition of Ca^{++} or verapamil to the medium has no effect on resting release, but removal of Ca^{++} or addition of verapamil inhibits superoxide anion release from cells exposed to particles. One possible interpretation of these results is that there are two different mechanisms for superoxide anion release, one in resting cells and another in cells exposed to particles.

In summary, we have measured the time course of superoxide anion release from rat alveolar macrophages and determined some of the characteristics of this release. Our data indicate that the control of superoxide release from alveolar macrophages and polymorphonuclear leukocytes is quite different. In addition, the results suggest that there may be two mechanisms for the release of superoxide, i.e., one at rest and another in cells exposed to particles. Experiments are now being conducted in our laboratory to further study these mechanisms.

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