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Superoxide Production in Pulmonary Alveolar Macrophages and Killing of BCG by the Superoxide-Generating System With or Without Catalase

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The superoxide production of BCG-infected and noninfected alveolar macrophages was measured by superoxide dismutase-inhibitable nitro blue tetrazolium reduction. The cells were incubated with or without cell-free bronchial lavage fluid (pulmonary washings). When control alveolar macrophages were infected by BCG, superoxide production was decreased markedly, probably due to bacterial cytotoxic factors. In contrast, the production of superoxide in alveolar macrophages exposed to pulmonary washings was increased and not appreciably influenced by BCG infection. Superoxide production by alveolar macrophages was dependent on time and on the protein concentration in the pulmonary washings. In controls, it was inversely proportional to the infecting dose of BCG. We observed previously that alveolar macrophages activated by pulmonary washings inhibited intracellular growth of BCG. We now present evidence that enhanced production of superoxide contributes to such inhibition, especially in the presence of catalase at acid pH. These findings are pertinent to the defense of inflamed lungs, where serum and serum immunoglobulin G transuded from blood into alveolar spaces probably induce such activation on alveolar macrophages.

Alveolar macrophages (AM) exposed to pulmonary washings (PW) from normal rabbit lungs show a higher capacity than that of unexposed AM to inhibit the growth of intracellular BCG. The capacity of the inhibition is correlated with the intensity of nitro blue tetrazolium (NBT) reduction by the AM (2). Baehner et al. reported that most of the NBT reduction by polymorphonuclear leukocytes is due to superoxide (5). Recent observations concerning superoxide production by polymorphonuclear leukocytes and mononuclear phagocytes have raised the possibility that superoxide or its by-products play a role in bacterial killing (7, 8, 13, 14). If so, in AM exposed to PW, the higher capacity to inhibit the growth of intracellular BCG may be due to superoxide or its by-products.

The present study evaluates whether AM exposed to PW produce superoxide and whether superoxide or its by-products participate in the killing of BCG. The superoxide generated by AM was assayed by observing the inhibition of NBT reduction by superoxide dismutase (SOD) (5), an enzyme catalyzing the conversion of superoxide to hydrogen peroxide and oxygen (22). The killing of BCG by superoxide or its by-products was assayed by the xanthine oxidase–purine system (3), supplemented with or without catalase.

The experimental data reported in this paper show that the AM exposed to PW produce superoxide and that superoxide or its by-products participate in the killing of BCG, especially in the presence of catalase. The antimycobacterial mechanisms in the activated AM are discussed with respect to the superoxide-generating catalase system.

MATERIALS AND METHODS

Animals. Female New Zealand white rabbits, weighing 2 to 3 kg, were used in all experiments.

Bacteria. Eighty milligrams of lyophilized BCG strain (Japan BCG Laboratory, Tokyo, Japan) was dissolved in cold saline. The BCG suspension was allowed to stand at 4°C for 20 min and was then centrifuged at 35 × *g* for 5 min to remove the larger bacillary clumps. After the opacity of the supernatant was read in a spectrophotometer at 570 nm, it was adjusted to appropriate optical densities by the addition of saline. An optical density of 1.52 represented 2.3×10^7 viable bacilli. Heat-killed BCG were prepared by heating in boiling water for 10 min.

Culture media. RPMI 1640 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 1% L-glutamine and 10% fetal calf serum, heat-inactivated for 30 min at 56°C, was used for all experiments. Penicillin (100 U/ml) was added.

Harvest of AM. AM were harvested by the method of Myrvik et al. (25), using saline and gently flushing the lungs via the trachea. The cells were washed three

times with Hanks balanced salt solution and suspended in culture medium. Differential counts of AM averaged 95% macrophages, 4% lymphocytes, and 1% polymorphonuclear leukocytes.

Preparation of PW. PW were prepared as previously described (2). In brief, normal rabbit lungs were washed five times by bronchial lavage, each with 25 ml of saline. The pooled lavages without blood contamination were centrifuged at 400 × *g* for 10 min and passed through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) to remove mucus, dust, and bacteria. The filtrate was concentrated by ultrafiltration on a Diaflo membrane (MX50; molecular weight > 50,000; Amicon Corp., Lexington, Mass.). After another passage through a 0.45-μm membrane filter, the solutions were designated PW and frozen at −20°C until use. Protein was determined by the procedure of Lowry et al. (21), with bovine serum albumin as the standard.

Preparation of the BCG-infected AM. BCG suspension was added to a suspension of AM and incubated in Falcon plastic tubes (no. 2001; Falcon Plastics, Oxnard, Calif.) at 37°C for 1 h. The ratios of bacteria to AM ranged from 5:1 to 50:1, but the ratio of 15:1 was used throughout unless otherwise indicated. After incubation, the suspensions were centrifuged at 125 × *g* for 5 min. The supernatant was discarded, and the pellets were washed three times with Hanks balanced salt solution to remove nonphagocytized bacilli. Two milliliters of the BCG-infected AM (0.75×10^6 cells per ml) in culture medium was introduced into a plastic Petri dish (no. 3001, 35 by 10 mm, Falcon Plastics). Without washing the dishes with Hanks balanced salt solution, the monolayers of BCG-infected AM were cultivated either with PW or with saline. The monolayers of the AM which phagocytized heat-killed BCG and the monolayers of noninfected AM were prepared by the same procedure. The BCG-infected AM and the AM which phagocytized heat-killed BCG were smeared and stained with carbolfuchsin for the identification of acid-fast bacilli under the microscope.

Quantitative and qualitative NBT reduction by AM. Quantitative NBT (Sigma Chemical Co., St. Louis, Mo.) reduction by AM was performed by the method of Baehner and Nathan (6). Two milliliters of 0.3% NBT solution was added to each Petri dish. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 1 ml of 1 N HCl. All AM and the extracellularly reduced NBT formazan were removed from the Petri dishes with a rubber policeman and collected into the tubes containing 2 ml of 1 N HCl. The precipitated formazan was pelleted by centrifugation at 1,000 × *g* for 10 min and suspended in 2.5 ml of pyridine and read at 515 nm (2). Qualitative NBT reduction by AM was observed by a modification of the supravital method of Park et al. (27), as previously reported (1). An AM with clumped NBT formazan was designated as a positive cell.

Measurement of superoxide production by AM. Superoxide generated by AM was assayed by observing the extent of inhibition of the reduction of NBT by SOD (Sigma) (5). SOD was added to the one of the duplicate AM monolayers at a concentration of 100 μg/ml and incubated at 37°C for 20 min, followed by the quantitative NBT reduction test described above. As a control, the same volume of saline (0.1 ml) was added to another chamber. The difference of absorbance at 515 nm in the absence and presence of SOD was taken as a measurement of superoxide.

Assay for killing of BCG by superoxide generating in the xanthine oxidase-purine system. The xanthine oxidase-purine system was carried out by the method of Babior et al. (3) supplemented with or without catalase. Xanthine oxidase, purine, SOD, crystalline catalase, and mannitol were purchased from Sigma Chemical Co. Assay mixtures contained 50 μl of xanthine oxidase, 50 μl of 1.0 mM purine solution, 50 μl of 67 mM sodium acetate buffer (pH 4.0 to 6.5), 100 μl of 0.75 mM potassium phosphate buffer (pH 7.8), and 100 μl of BCG solution (optical density, 0.010 at 570 nm). One hundred micrograms of SOD, 1,650 U of catalase, or 15 mM mannitol was added to the assay mixtures as a scavenger of superoxide, hydrogen peroxide, or hydroxyl radical, respectively. The final volume of each assay mixture was adjusted to 500 μl by addition of saline. Before use, all materials except for xanthine oxidase were sterilized. SOD, catalase, mannitol, and buffers were sterilized by filtration with a 0.45-μm membrane filter (Millipore). Other materials were sterilized by autoclaving.

To assay killing of BCG, the mixtures were incubated at 37°C for 30 min, and the appropriate dilutions were incubated on Ogawa medium at 37°C for 4 weeks.

NBT reduction by each of the assay mixtures was also measured in the absence and presence of SOD, catalase, or mannitol. After 1 ml of NBT solution was added to each assay mixture at 0°C, it was incubated at 37°C for 30 min. Reduced NBT formazan was measured quantitatively, as described above.

Statistical method. The standard error of the mean and *P* values were calculated by using Student's *t* test (1).

RESULTS

Effects of PW and BCG infection on superoxide production by AM. PW were added to the monolayers of BCG-infected AM and noninfected AM in a protein concentration of 300 μg/ml of culture medium, and the NBT reduction by the cells was measured in the absence and presence of SOD 24 h after incubation. Monolayers to each of which was added the same volume (usually 0.1 ml) of saline instead of PW were used as controls.

Superoxide measured by the difference of absorbance at 515 nm in the absence and presence of SOD is shown in Table 1. Superoxide production by the noninfected AM in the control monolayers was relatively low. However, when the noninfected AM were incubated with PW, superoxide production by the cells was enhanced markedly ($P < 0.001$). PW alone neither reduced NBT nor inhibited the activity of SOD. Therefore, it was concluded that in the present experimental system superoxide was generated by the AM exposed to PW. The effect of PW was also observed on the superoxide production by the BCG-infected AM. Superoxide production by

TABLE 1. *Superoxide production by BCG-infected AM or noninfected AM incubated with and without PW*[a]

AM	NBT reduction [optical density at 515 nm/(1.5 × 10^6 cells)]						P values
	Control			+ PW			
	−SOD	+SOD	Difference (superoxide production)	− SOD	+ SOD	Difference (superoxide production)	
I. Noninfected AM	0.068 ± 0.002	0.038 ± 0.003	0.030 ± 0.004	0.229 ± 0.032	0.129 ± 0.022	0.100 ± 0.012	<0.001
II. BCG-infected AM	0.035 ± 0.003	0.022 ± 0.001	0.013 ± 0.003	0.177 ± 0.017	0.079 ± 0.016	0.098 ± 0.013	<0.001
III. AM-phagocytized heat-killed BCG	0.043 ± 0.005	0.031 ± 0.005	0.012 ± 0.003	0.155 ± 0.013	0.093 ± 0.003	0.062 ± 0.009	<0.001
P values							
I vs II	$P < 0.001$		$P < 0.007$	$P > 0.12$		$P > 0.48$	
I vs III	$P < 0.001$		$P < 0.006$	$P < 0.04$		$P < 0.03$	
II vs III	$P > 0.14$		$P > 0.41$	$P > 0.18$		$P < 0.04$	

[a] Superoxide generated by AM was assayed by measuring the inhibition of the reduction of NBT by SOD. The NBT reduction test was carried out 24 h after incubation with or without 300 μg of PW per ml. SOD (200 μg) was added to the AM monolayers 20 min before beginning the NBT reduction test. Incubation for the NBT test was for 30 min at 37°C. The ratio of bacteria to AM was 15:1. Most of BCG were internalized by AM, and the percentages of phagocytizing AM and the numbers of bacteria per phagocytizing AM at 0 h were 14.5% and 3.7 ± 0.3 (II) and 16.4% and 4.5 ± 0.6 (III), respectively ($P > 0.15$). Each value represents the mean of four experiments with its standard error.

the BCG-infected AM in the control monolayers decreased by more than 60% when compared with those of noninfected AM in the controls ($P < 0.007$). On the other hand, the superoxide production by the BCG-infected AM exposed to PW was seven times the superoxide production by BCG-infected AM in the controls ($P < 0.001$), and the values were not different from those of noninfected AM exposed to PW, in which enhanced production of superoxide was observed. The intensity of NBT reduction by the BCG-infected AM exposed to PW was time dependent and dependent on the concentration of PW. It was inversely proportional to the infecting dose of BCG (Fig. 1). Therefore, superoxide production by AM may be also dependent on these factors. As shown in Table 1, superoxide production by AM which phagocytized heat-killed BCG was decreased more than that by AM which phagocytized live BCG ($P < 0.04$). This suggests that the additional inhibitory substances were produced by heating BCG.

These results indicate that when normal AM are infected by BCG, the superoxide-generating system in the AM is inhibited markedly. On the other hand, the enhanced superoxide-generating system in AM activated by PW was not appreciably inhibited by BCG infection. The present data and our previous observation that AM exposed to PW showed enhanced inhibition of intracellular growth of BCG (2) suggest that an enhanced production of superoxide is one of the antimycobacterial mechanisms of AM. To evaluate this possibility, the following experiments were carried out.

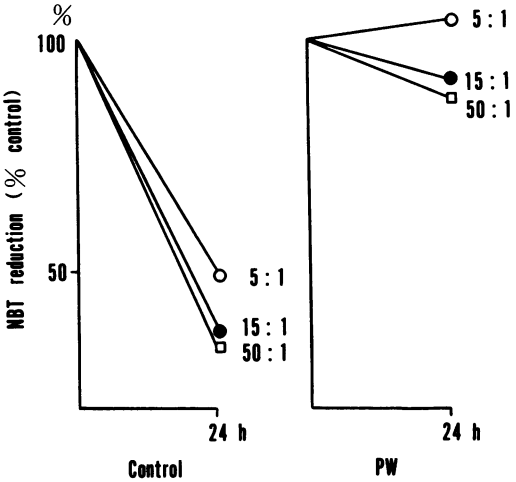

FIG. 1. *Influence of the infecting dose of BCG on the NBT reduction of the AM incubated with or without 300 μg of PW per ml. The ratios of BCG to AM are given in the figure. The NBT reduction test was carried out quantitatively, and the result is shown as the percentage of control. Each value represents the mean of duplicates of one experiment. The duplicates were within 3% of each other.*

Superoxide and its by-products as bactericidal agents for BCG. NBT reduction by the xanthine oxidase–purine system was performed in the absence and presence of SOD. NBT was reduced markedly by the assay mixtures of xanthine oxidase, but the NBT reduction was inhibited by SOD (Table 2). Catalase, a scavenger of hydrogen peroxide, and mannitol,

TABLE 2. *NBT reduction and killing of BCG in the xanthine oxidase-purine system with or without catalase*[a]

System	NBT reduction (optical density at 515 nm)	Colonies ($\times 10^3$)
Xanthine oxidase-purine		
Complete system[b]	0.156 ± 0.007	11 ± 2
− Xanthine oxidase	0.007 ± 0.001	16 ± 1
− Purine	0.022 ± 0.001	8 ± 0.8
Boiled xanthine oxidase[c]	0.020 ± 0.005	16 ± 1
+ SOD (100 μg)	0.033 ± 0.005	15 ± 1
+ SOD (50 μg)	0.083 ± 0.008	ND[d]
+ Catalase (1,650 U)	0.156 ± 0.007	2 ± 0.8
+ Mannitol (15 mM)	0.135 ± 0.008	15 ± 1
+ Inactivated SOD[e] (100 μg)	0.052 ± 0.003	ND
+ Inactivated SOD (50 μg)	0.155 ± 0.015	ND
Control (buffer)	0.005 ± 0.001	16 ± 1
Catalase + buffer	0.005 ± 0.001	14 ± 1
SOD + buffer	0.005 ± 0.001	13 ± 1
Mannitol + buffer	0.005 ± 0.001	15 ± 1
Xanthine oxidase-purine with catalase		
Complete system[f]	0.140	7 ± 1
Catalase (1:10 dilution)	ND	9 ± 2
Catalase (1:100 dilution)	ND	15 ± 1
Boiled xanthine oxidase[c]	0.010	18 ± 1
Boiled catalase[c]	0.130	14 ± 1
+ SOD (100 μg)	0.030	15 ± 1
+ Mannitol (15 mM)	0.115	15 ± 1
Control (buffer)	0.005	19 ± 2

[a] Each value represents the mean ± standard error of two experiments, each in duplicate, except for the NBT reduction values for the xanthine oxidase-purine system with catalase, where each value represents the mean of duplicates in one experiment.

[b] Assay mixtures of the complete system contained 50 μl of xanthine oxidase (13 U/ml), 50 μl of purine, 50 μl of sodium acetate buffer (pH 5.3), and 100 μl of potassium phosphate buffer (pH 7.8). After BCG was added, the assay mixtures were incubated at 37°C for 30 min. To assay NBT reduction, 1 ml of 0.3% NBT solution was added to each of the assay mixtures without BCG and incubated at 37°C for 30 min.

[c] Inactivated in boiling water for 1 min.

[d] ND, Not done.

[e] SOD was inactivated by autoclaving at 120°C for 20 min.

[f] Catalase (50 μl of 22,000 U/ml) was added to the xanthine oxidase-purine system.

a scavenger of hydroxyl radical, did not inhibit the NBT reduction. These results suggest that the substance which reduced NBT in the present system was mostly superoxide. Heat-inactivated SOD also inhibited NBT reduction partly, but not completely. It might be explained that the native SOD inhibit the NBT reduction through their enzymic activities, whereas the boiled enzymes exert their nonspecific inhibitory effects, since the activity of SOD is destroyed by boiling the enzyme (3, 22).

The assay for the killing of BCG by superoxide or its by-products was performed with the same procedure. The results are shown in Table 2. When BCG were incubated in the xanthine oxidase-purine system, 30% of the BCG were killed, and the killing was inhibited by SOD or mannitol. Unexpectedly, however, additional killing was observed in the assay mixtures of the xanthine oxidase-purine system supplemented with catalase at acid pH, and the killing was dependent on the concentration of catalase. The bactericidal activity was depressed by heat inactivation of either xanthine oxidase or catalase or by the addition of SOD or mannitol. These results indicate that both superoxide (or its by-products) and catalase are required. Neither SOD, catalase, nor mannitol killed BCG by itself at acid pH. Killing was observed, however, when purine was omitted from the assay mixtures. Therefore, the assay mixture probably contains other compounds which may accumulate in the vicinity of the bacteria (as a consequence of their metabolic process) and serve as substrates for xanthine oxidase, an enzyme of very broad specificity (3).

Requirement of both superoxide (or its by-products) and catalase was also confirmed when the killing was assayed with various pH values. Greater killing was observed at pH 4.0, 4.5, and 5.0, which are optimal for catalase (18), although relatively weak killing was also found at pH 6.0 and 7.0 (Fig. 2). In contrast, the amount of reduced NBT in these assay mixtures was inversely proportional to acid pH (Fig. 3). Therefore, the maintenance of a very low steady-state concentration of superoxide (or its by-products) by the superoxide-generating system seems to be important in the present experimental system, similar to the findings of Klebanoff (18) in his hydrogen peroxide-generating catalase system.

From these results, we conclude that superoxide or its by-products kill BCG extracellulary, especially in the presence of catalase at acid pH.

DISCUSSION

This study indicates that in vitro infection with BCG decreases the production of superoxide by AM but has little effect on the enhanced production of superoxide by AM activated by bronchial lavage fluid (PW). This result and our previous observations that the antimycobacterial activity of AM activated by PW is correlated

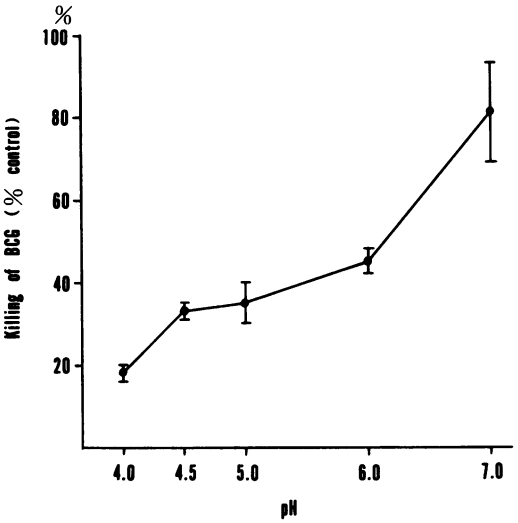

FIG. 2. *Effect of pH on the killing of BCG in the xanthine oxidase-purine system supplemented with catalase. The assay mixtures contained both sodium acetate buffer (pH 4.0 to 6.5) and potassium phosphate buffer (pH 7.8). Xanthine oxidase (0.65 U), purine (50 μl of 1.0 mM), and catalase (1,100 U) were also added. BCG in the amount of 1.5 × 10^4 viable organisms were present. Incubation was at 37°C for 30 min. The appropriate blank controls of pH had no effects on the killing of BCG. Each value represents the mean ± standard error of two experiments run in duplicate.*

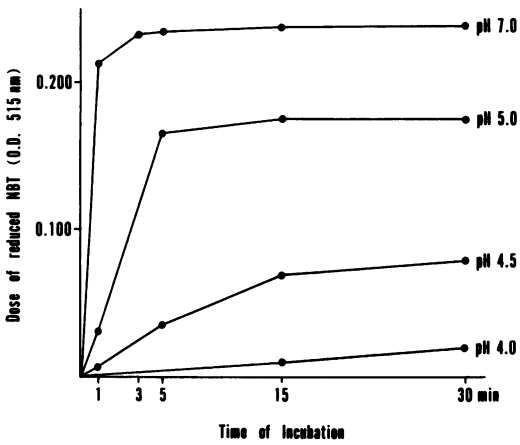

FIG. 3. *Effect of pH on the time course of NBT reduction in the xanthine oxidase-purine system supplemented with catalase. Each value represents the mean of one experiment run in duplicate.*

with enhanced NBT reduction (2) suggest that the superoxide or its by-products generated by the AM may participate in the killing of BCG. Superoxide (or its by-products) was shown to kill extracellular BCG in vitro, especially in the presence of catalase.

Superoxide was measured by SOD-inhibitable NBT reduction rather than by cytochrome *c* reduction, since the culture medium used in the present study, as well as PW, influenced the absorbance at 550 nm used for the detection of reduced cytochrome *c*. Superoxide is a potent reducing agent of NBT (9, 23, 28). NBT reduction by xanthine oxidase was inhibited markedly by SOD, but NBT reduction by AM was only partly inhibited by SOD. This difference may be explained by the experiments of Baehner et al. (5): in phagocytizing polymorphonuclear leukocytes, superoxide accounts for only 60% of NBT reduction, and the rest is due to the presence of an as-yet-uncharacterized reductase.

Human monocytes release less superoxide during phagocytosis than do polymorphonuclear leukocytes (15, 29). Mouse AM release large amounts of superoxide during phagocytosis of zymosan particles, but guinea pig AM do not (8). Thus, there are obviously considerable species differences. In our experimental system with rabbit AM, superoxide production by normal AM decreased significantly when AM phagocytized BCG. The inhibition might be due to the cytotoxic effects of certain BCG components: (i) heat-killed BCG were more inhibitory than were viable BCG; (ii) NBT reduction by AM is inversely proportional to the infecting dose of BCG; and (iii) cord factor (one of cytotoxic factors of BCG [26]) inhibits the oxidative metabolism of cells (17). On the other hand, when AM were exposed to PW, the production of superoxide was markedly increased and not appreciably influenced by BCG infection. This increase may be due to the stimulation of plasma membrane receptors for the Fc portion of immunoglobulins, because the active substance which enhances NBT reduction by AM exposed to PW is the immunoglobulin G in the PW (2). Production of superoxide by mononuclear phagocytes, like that by polymorphonuclear leukocytes, is influenced not only by phagocytosis but also by cell surface stimulation by chemical agents (13, 30) or immunoglobulin (15).

An enhanced transudation of serum into the alveolar spaces and bronchial trees occurs during the early stages of most inflammatory diseases of the lungs (16, 24). The immunoglobulin G in such serum could enhance the activation of AM (2) and increase their ability to kill bacteria via the superoxide-generating system with or without catalase (this report).

Our previous observations indicate (i) that peritoneal exudate cells exposed to PW (2) or lymphokines from phytohemagglutinin-activated lymphocytes (1) show enhanced NBT reduction only 18 to 24 h after incubation with the stimulants, (ii) that NBT reduction by the per-

itoneal exudate cells is less than NBT reduction by AM, and (iii) that AM exposed to lymphokines show decreased NBT reduction. These results suggest that the production of superoxide by mononuclear phagocytes is due to changes in the plasma membrane or metabolic pathways of the cells. Superoxide production by mononuclear phagocytes is relatively well correlated with the intensities of NBT reduction by the cells.

Superoxide or its by-products are known to kill certain bacteria, such as *Escherichia coli., Staphylococcus aureus,* and *Streptococcus viridans* (3, 14). Our results show that BCG was killed extracellularly by substances produced in the xanthine oxidase–purine system, especially at acid pH when catalase was added, and that the bactericidal activity was markedly depressed by heating either xanthine oxidase or catalase or by adding SOD or mannitol. These findings suggest that superoxide (or its by-products) is one of the killing agents and that its activity can be enhanced or supplemented by other agents. Klebanoff reported that catalase has bactericidal activity when combined with a hydrogen peroxide-generating system (18). Catalase reacts with H_2O_2 to form an enzyme–substrate complex which reacts with a second molecule of H_2O_2 to form oxygen and water. When the H_2O_2 is maintained at a very low steady-state concentration by an H_2O_2-generating system, a number of substances can successfully compete with the second molecule of H_2O_2 for oxidation by the catalase–H_2O_2 enzyme–substrate complex. Under the latter conditions a microbicidal effect is observed (20). Our results are consistent with the experiments of Klebanoff: (i) hydrogen peroxide was formed in the xanthine oxidase–purine system (3), (ii) a very low steady-state production of superoxide (or its by-products) was maintained at acid pH, and (iii) enhanced killing was observed.

Johnston et al. (15) reported that phagocytizing polymorphonuclear leukocytes remove oxygen from the surrounding medium and convert it, probably, first to superoxide (4, 14) and then to hydrogen peroxide (19). Hydrogen peroxide and superoxide may interact to form the hydroxyl radical, a potent oxidizing agent (14). The spontaneous dismutation of superoxide is believed to result in singlet oxygen formation (15). If so in AM, the superoxide-generating catalase system (or the H_2O_2-generating catalase system) would be one of the main antimycobacterial mechanisms of these phagocytes. Rabbit AM are rich in catalase (11), which is transferred in part into the phagocytic vacuole after phagocytosis, where the intravacuolar pH is in the vicinity of the pH optimum of catalase (20).

The superoxide-generating system is associated with the cell surface (12), and aerobic bacteria contain SOD (10). For intracellular killing of BCG by mononuclear phagocytes, the levels of superoxide produced by the membranes of phagocytic vacuoles must exceed the amount of superoxide destroyed by the SOD of bacilli.

To our knowledge, there has been no report that the superoxide-generating system with or without catalase kills BCG, but our experimental results are consistent with this conclusion.

ACKNOWLEDGMENT

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