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Drug Potentiation of Macrophage Function

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The mechanism by which macrophages kill facultative intracellular parasites is not known. A test system is described utilizing human macrophages derived from blood monocytes and the opportunistic pathogen *Listeria monocytogenes*. In this system, bacterial killing was impaired under hypoxic conditions. Under aerobic conditions, addition of Clofazimine (B663), a phenazine derivative effective in the treatment of human leprosy, potentiated the killing of *Listeria* by macrophages. Potentiation occurred at drug concentrations usually attainable in man and which are not directly injurious to the bacteria. The enhanced bactericidal activity occurred only in the presence of oxygen; the drug induced increased leukocyte oxygen consumption which was insensitive to cyanide.

In man, as well as other mammals and higher vertebrates, the macrophage may be regarded as a spectrum of cells of various degrees of differentiation, from the blood monocyte to the giant, multinucleate tissue histiocyte (4). This cell line serves a number of functions, among them the localization and destruction of certain microorganisms. Although the neutrophil is generally regarded as the first line of defense against the common bacterial pathogens, the macrophage is thought to be the major cellular defense against the intracellular parasites, organisms such as *Mycobacterium tuberculosis*, *M. leprae*, *Listeria monocytogenes*, and *Brucella* species. The mechanism by which macrophages kill such parasites, which are sometimes capable of surviving and multiplying within the host, is largely unknown. In the course of the investigation of these mechanisms, a compound was found which enhanced the killing capacity of human macrophages. Elucidation of the metabolic changes induced by this drug has shed light on the mechanisms by which macrophages kill parasites.

MATERIALS AND METHODS

Clofazimine (B663; reference 11), an experimental compound used in the treatment of leprosy, was the generous gift of Louis Levy, U.S. Public Health Service Hospital, San Francisco, Calif. B663, which is insoluble in water, was dissolved in a small amount of absolute ethanol and then gradually diluted in serum to make a stock solution of 0.1 mM concentration. The final concentration of ethanol was less than 0.1%, at which level it had no detectable effect on macrophage function or on bacterial viability. In this form, some of the B663 remained as microparticles in suspension. In the final concentrations used (10^{-6} to 2×10^{-5} M), no particles were visible by ordinary light or phase microscopy.

Tissue culture media were obtained from the Grand Island Biological Co. (Berkeley, Calif.), and bacteriological media were obtained from Difco. Carrier-free ^{32}P was obtained from Squibb. Techniques for incubation of *Listeria* and for the bactericidal assay are described elsewhere (2) and are summarized below.

Isolation and cultivation of leukocytes. Leukocyte populations rich in neutrophils were isolated from the peripheral blood of normal subjects by sedimentation with a 0.5 volume of 3% dextran in saline and centrifugation of the white cell-rich supernatant at $150 \times g$ for 8 min. The leukocytes were suspended in McCoy's medium containing 30% pooled AB serum (ABM). Monocytes were isolated from the peripheral blood of normal subjects, from patients with active leprosy, tuberculosis (usually untreated), or disseminated fungal infection, and from one patient with monocytic leukemia. Isolation techniques have been described previously (3). Cells (3×10^6/ml) were cultivated in 1-ml Leighton tubes in ABM containing penicillin (50 units/ml) and streptomycin (50 μg/ml). The cells were used between the 5th and 10th day of culture, by which time they had begun to differentiate into larger macrophages. At this time, many of the cells had become detached from glass and there were generally between 0.3×10^5 and 3×10^5 macrophages per tube (Hanifin, Dirksen, and Cline, *unpublished data*).

Growth of L. monocytogenes. Two strains of *Listeria* were used: one from the reference stock of the University of California, San Francisco (UCLM-1); the other, 4524B, a strain virulent for mice, was the generous gift of Sidney Silverman of Fort Detrick, Md. A virulent subline of UCLM-1 was obtained by passage of bacteria through human macrophages. Both strains gave similar results.

Listeria were labeled with ^{32}P (100 μCi/ml) and washed five times by centrifugation (9,000 $\times$ *g* for 7 min) in ZøBell's solution (14); the concentration of organisms was determined from the optical density at 620 nm and from colony counts. The radioactivity was 99.6% cell-associated; the bacteria were viable and capable of replication. A standard curve was established for the number of colony-forming units (CFU) equivalent to a given amount of radioactivity.

Bacterial killing assay (2). Macrophages in Leighton tubes were washed by decantation in warm Hanks solution and resuspended for several hours at 37 C in 1 ml of fresh ABM containing no antibiotics. Labeled *Listeria* (0.1 ml or 5 $\times$ 10^7 CFU) was added to the tubes, and incubation was continued with intermittent shaking for 90 min. Since the lag phase of *Listeria* in ABM (as determined by colony counts) is at least 120 min, no growth of bacteria occurred. The tubes containing macrophages and *Listeria* and control tubes without macrophages were then washed by decantation six times with 1.5-ml volumes of warm Hanks solution, and the radioactivity was determined. The number of CFU associated with macrophages was established by the calculation: CFU (phagocytized) = [CFU (standard)/ radioactivity (standard)] $\times$ radioactivity (phagocytized). Between 1 and 10% of the added organisms were phagocytized.

After addition of 6 ml of ZøBell's solution, the macrophages were sonically disrupted without injury to the labeled *Listeria*. The sonically treated material was diluted in ZøBell's solution, and pour plates were made with Trypticase Soy Agar. One-milliliter samples of the sonically treated material were taken for liquid scintillation counting in a dioxane-based phosphor. The number of colonies on a pour plate (usually between 50 and 250) was counted after 48 hr. The percentage of cell-associated *Listeria* killed was determined by the calculation: per cent *Listeria* killed = [CFU (phagocytized) $-$ CFU (observed)/CFU (phagocytized)] $\times$ 100. All assays were performed in triplicate, with three to six pour plates made from each Leighton tube. The extreme values (calculated per cent killing) for any given macrophage population varied no more than $\pm$10% from the mean and usually less than $\pm$7% from the mean.

Hypoxic conditions. Leighton tubes containing macrophages were fitted with rubber stoppers containing inflow and outflow needles. Macrophages were incubated with humidified 5% CO_2 in air or with 5% CO_2 in nitrogen for 60 min. After the introduction of ^{32}P-labeled *Listeria* through a rubber sleeve fitted on the tube, the incubation in gas was continued for the period necessary for phagocytosis of the bacteria. Listericidal assay was then performed as described above. PO_2, PCO_2, and *p*H were determined on tubes handled similarly but containing no bacteria.

Metabolic studies with B663. The method for demonstration of cellular O_2 consumption and generation of H_2O_2 was essentially that described by Zatti, Rossi, and Patriarca (12). Consumption of O_2 by newly isolated neutrophils or monocytes (2 $\times$ 10^7/ml in ABM containing 2 or 3 mM KCN) was determined with a Clark electrode and a Gilson model KM oxygraph. After equilibration, heat-killed *Candida albicans* as a phagocytizable particle or nonparticulate B663 (final concentration, 0.5 $\times$ 10^{-5} to 2 $\times$ 10^{-5} M) in a small volume was introduced into the chamber. Recording was continued for an additional 4 or 5 min, at which time an excess of catalase was added to liberate O_2 from the accumulated H_2O_2.

RESULTS

Killing of Listeria by human macrophages. During the standard 90-min period for phagocytosis and killing, macrophages from 13 normal subjects killed 62.5 $\pm$ 7.6% (SD) of phagocytized *Listeria*. Macrophages from 14 patients with active tuberculosis, leprosy, or disseminated fungal diseases killed slightly more (73.9 $\pm$ 11.5%). In three studies, 20 to 24 hr was required to reduce the number of CFU to 10% of those originally phagocytized by normal macrophages during the 90-min period. Killing was independent of the absolute numbers of organisms ingested in the range of 0.5 $\times$ 10^6 to 5 $\times$ 10^6 (1 to 10% of the organisms added to each culture tube). It has been shown previously that the percentage of phagocytized organisms killed was independent of the age of macrophages in the range of 4 to 13 days but was, in part, dependent upon an available partial pressure of oxygen of greater than 35 mm of Hg (2).

In all but one study, 99 to 100% of the macrophages were phagocytic, usually with multiple organisms in each cell (Fig. 1). The exception occurred with macrophages from a patient with

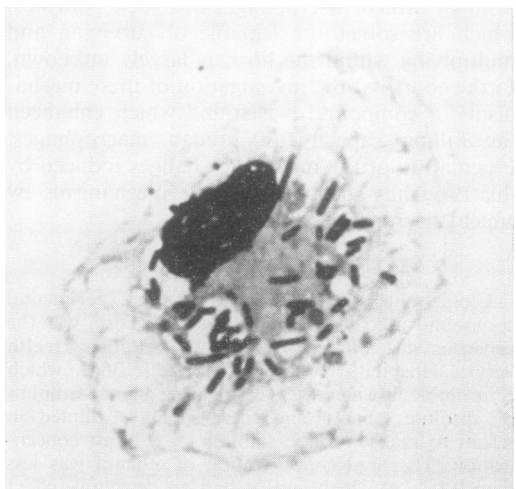

FIG. 1. *Listeria monocytogenes phagocytized by a human macrophage. Original magnification* $\times$ *1,250.*

acute monocytic leukemia, only 74% of which were phagocytic. Phagocytosis, as judged either by cell-associated radioactivity or by morphological examination of Giemsa-stained smears, was not affected by hypoxic conditions.

Effect of B663 on killing of Listeria. B663, an experimental drug used in the treatment of leprosy, is a redox agent. The effect of B663 on the killing of *Listeria* by macrophages was studied under a variety of conditions. In paired studies of macrophages from 11 subjects, addition, simultaneously with *Listeria*, of B663 in concentrations between 10^{-6} and 2×10^{-5} M greatly increased the fraction of ingested organisms killed within a 90-min period. The results for a concentration of 5×10^{-6} M are shown in Fig. 2. When analyzed by the paired *t* test, the effects of B663 were

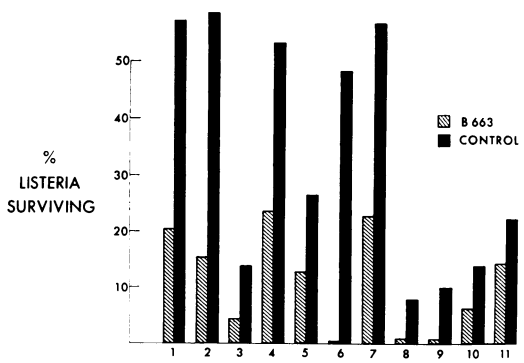

FIG. 2. *Effect of B663 (5×10^{-6} M) on the survival of Listeria monocytogenes phagocytized by macrophages from 11 normal subjects. Phagocytosis and survival were measured after a 90-min incubation period.*

TABLE 1. *Effect of various concentrations of B663 on the killing of phagocytized Listeria monocytogenes*

Monocyte source	Concn of B663	*Listeria* killed in 90 min
	M	%
Normal-1	0	52
	5×10^{-6}	56
	1×10^{-5}	87
	2×10^{-5}	100
Normal-2	0	47
	5×10^{-6}	76
	1×10^{-5}	77
Monocytic leukemia	0	78
	1×10^{-6}	77
	5×10^{-6}	85
	1×10^{-5}	87

TABLE 2. *Effect of O_2 tension and B663 on listericidal activity of macrophages*

PO_2	B663 concn	*Listeria* killed
mm of Hg	M	%
>100	0	61 ± 2
>100	5×10^{-6}	81 ± 3
<15	0	44 ± 5
<15	5×10^{-6}	42 ± 6

significant ($P < 0.01$). The enhanced killing of phagocytized *Listeria* was dependent upon the concentration of B663 (Table 1). However, only at the highest concentration of drug (2×10^{-5} M) was there an effect on the number of organisms phagocytized. At this highest concentration, phagocytosis was slightly depressed. B663 in concentrations as high as 2×10^{-5} M, in the absence of macrophages, had no effect on the viability of *Listeria* in incubations lasting 4 hr, although considerably higher concentrations of drug for longer periods of time will inhibit growth of the organism (10).

The effect of B663 on the listericidal activity of macrophages was analyzed in three ways: (i) *Listeria* were pretreated with B663 before being fed to macrophages; (ii) macrophage killing in the presence of B663 was analyzed under normal and hypoxic conditions; and (iii) macrophages were exposed to B663 and then washed free from drug and incubated with *Listeria*.

The enhanced destruction of *Listeria* by macrophages in the presence of B663 could reflect the facilitated killing of partially damaged but still viable organisms rather than a primary effect of B663 on macrophage function. To test this possibility, radioactive *Listeria* was preincubated with 5×10^{-6} M B663 for 60 min, washed by centrifugation, and resuspended in drug-free ZøBell's solution before addition to macrophage cultures. There was no significant difference observed in the killing of control and drug-treated *Listeria*, suggesting that the primary effect of drug observed during short-term incubations was on the macrophage rather than on the organism.

To determine whether the potentiation of killing activity by B663 was dependent upon available O_2, the compound was added to macrophages from a normal subject in a listericidal assay under hypoxic conditions and at ambient O_2 concentrations (Table 2). It was clear that the potentiation of killing by drug required O_2. Similar results were observed in two other experiments.

To determine whether macrophages required continuous exposure to drug to demonstrate increased killing ability, cell cultures were (i)

preincubated for 60 min with 5×10^{-6} M B663, washed free from extracellular drug and resuspended in drug-free medium for 1 hr, or (ii) preincubated for 48 hr with B663 and then reincubated in drug-free medium for 24 hr. The ability of the treated macrophages to kill phagocytized *Listeria* was variable. In half of each set of the experiments, they demonstrated more listericidal activity than untreated control cells from the same subject. In half of the experiments, they were no different from controls.

Effect of phagocytosis and of B663 on O_2 consumption. The observation that killing, but not phagocytosis, of *Listeria* by macrophages was depressed at low partial pressures of O_2 suggested a listericidal mechanism that required available O_2.

In neutrophils, particle ingestion produces a respiratory burst. The microbicidal activity of this cell is thought to be dependent upon this respiratory burst and to require an H_2O_2-generating system based on the oxidation of intracellular substrates by molecular O_2 (9). It appeared logical to question whether particle ingestion by monocytes and macrophages was also associated with changes in cellular O_2 utilization. Since phenazine derivatives can function as intracellular redox agents (6), the effect of B663 on O_2 utilization was also examined.

Particle ingestion (*Candida*) by both neutrophils and monocytes produced enhanced cellular O_2 consumption which was not inhibited by KCN in concentrations of 2 or 3 mM (Fig. 3a and c). Freshly prepared B663 (nonparticulate by light and phase microscopy) also produced a KCN-insensitive respiratory burst in both cell types. At concentrations of 0.01 mM, this burst was of lesser magnitude than that associated with phagocytosis (Fig. 3b and d).

In neutrophils, at least part of the phagocytosis-dependent O_2 consumption is used for generation of intracellular H_2O_2 (9, 12). This phenomenon can be demonstrated by partially inhibiting the H_2O_2-destroying systems of the neutrophil (such as catalase and peroxidase) with KCN and adding exogenous catalase which releases O_2 from the H_2O_2 accumulating in the medium, thus producing a decrease in the rate of oxygen consumption. A typical experiment is shown in Fig. 3a. Although B663 consistently induced enhanced neutrophil oxygen consumption, H_2O_2 generation could not be demonstrated at drug concentrations as high as 0.01 mM.

The generation of H_2O_2 by phagocytizing monocytes is less easy to demonstrate, presumably because of a slower rate of production or more efficient intracellular destroying mechanisms (Fig. 3c). Similarly, it could not be clearly demonstrated that the respiratory burst induced by B663 was associated with H_2O_2 generation.

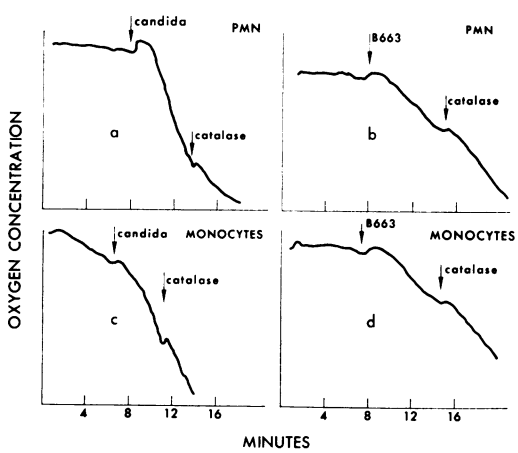

FIG. 3. *Effect of phagocytosis of Candida albicans or of exposure to B663 (0.01 mM) on O_2 consumption by human neutrophils and monocytes suspended in ABM containing 2 mM KCN. Relative oxygen concentration is shown on the ordinate. A stable base line oxygen consumption was established for each cell population before the test period. Multiple cell populations used in each type of experiment produced qualitatively similar results.*

DISCUSSION

Studies of genetic defects of neutrophil function suggest that the microbicidal activity of this cell type involves H_2O_2 generation and is partially dependent on the granule enzyme myeloperoxidase (1, 5, 7, 8). In some mammalian species (although not definitely in man), neutrophil cationic proteins may also provide a bactericidal system (13). In contrast, human macrophages lack myeloperoxidase, and cationic proteins have not been identified in mammalian macrophages (13). How, then, do these cells deal with phagocytized microorganisms? The reduced killing by macrophages under hypoxic conditions suggests an O_2-dependent bactericidal system.

B663 has been used successfully in experimental trials in this country and abroad to treat leprosy, a disease caused by an obligate intracellular parasite. It is also effective in the treatment of murine mycobacterial infection (11). Patients taking the drug for any length of time accumulate concentrations of drug in their blood which are equal to or greater than those used in the in vitro test system described here (L. Levy, *personal communication*). The observation reported here, that B663 (a phenazine drug) enhances macrophage bactericidal activity at concentrations

which are in themselves not injurious to the organism, opens up an avenue of exploration of the mechanism of macrophage bactericidal activity. B663 increased consumption of O_2 by both neutrophils and monocytes. Like the respiratory burst associated with phagocytosis, the enhanced cellular O_2 utilization induced by B663 was not inhibited by KCN, suggesting a mechanism independent of the mitochondrial system. We must still exclude the possibility that B663 produces a respiratory burst by functioning as a phagocytizable particle whose size is below the resolving power of ordinary light and phase microscopy. This possibility seems unlikely since the ability of a variety of redox agents, including phenazine derivatives, to enhance leukocyte oxygen utilization is well known (6).

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