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Bactericidal Activity of Human Macrophages: Analysis of Factors Influencing the Killing of *Listeria monocytogenes*

MARTIN J. CLINE

Cancer Research Institute and Department of Medicine, University of California School of Medicine, San Francisco, California 94122

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A technique is described for the measurement of listericidal activity of human macrophages grown from blood monocytes. Phagocytosis of *Listeria monocytogenes* was inhibited by a glycolytic poison (NaF) but was unaffected by anaerobic conditions, cyanide, or 2,4-dinitrophenol (DNP). Killing by macrophages was slower than that by neutrophils, and *Listeria* phagocytized by macrophages began to synthesize deoxyribonucleic acid within 3 hr of the time of ingestion. Differentiated macrophages ingested and killed more organisms per cell than newly isolated monocytes. Maximal killing of *Listeria* required oxygen but was unaffected by cyanide or DNP. Macrophages isolated from patients with chronic intracellular infection (leprosy, tuberculosis, fungal diseases) and from patients with active Hodgkin's disease were more bactericidal than macrophages from normal subjects.

The mechanisms by which neutrophils are known to kill microorganisms involve the generation of H_2O_2 induced by phagocytosis (11), the interaction of H_2O_2 and myeloperoxidase (8, 9), and, in some species, a family of cationic proteins (14). In contrast, virtually no details are known of the microbicidal mechanisms of mammalian macrophages. Certain features of the interaction of macrophages and bacteria have been characterized. For example, it is known that mononuclear phagocytes from sublethally infected animals are more effective killers of bacteria than cells from uninfected animals (2, 10). The enhanced killing, which is directed against a variety of facultative intracellular parasites in addition to the specific infecting organism, can be induced only by living parasites and not by dead vaccines and can be transferred by lymphoid cells but not by serum. Although enzymatic differences between macrophages from bacteria-resistant and bacteria-susceptible animals have been identified (1), there is no evidence that these differences are the bases of the altered bactericidal activity.

The present series of studies was undertaken to characterize the interaction between the human macrophage and a facultative intracellular parasite. The following questions were asked in regard to killing of bacteria by macrophages. (i) Is killing influenced by the degree of cellular maturation? (ii) Does killing depend on oxidative mechanisms? (iii) Is myeloperoxidase involved in the bactericidal process? (iv) Is killing altered by intracellular infection or other diseases?

MATERIALS AND METHODS

Tissue-culture media were obtained from Grand Island Biological Co. (Berkeley, Calif.), and bacteriological media were obtained from Difco. Carrier-free ^{32}P was obtained from Squibb. Orthoanisidine for the myeloperoxidase assay (9) was obtained from Eastman Organic Chemicals (Rochester, N.Y.).

Isolation and cultivation of leukocytes. Monocytes were isolated from the peripheral blood of normal subjects, patients with active leprosy or tuberculosis (usually untreated), patients with active Hodgkin's disease (usually receiving chemotherapy), and from one patient with monocytic leukemia. Isolation techniques have been described previously (4). Cells in a concentration of 3×10^6/ml were cultivated in 1-ml Leighton tubes in McCoy's medium containing 30% pooled normal AB serum (ABM), penicillin (50 units/ml), and streptomycin (50 μg/ml). The cells were used between the 3rd and 18th day of culture (usually between the 4th and 10th), by which time they had begun to differentiate into larger macrophages with numerous cytoplasmic extensions and lysosomal granules (M. J. Cline, *In* Red Cross Sci. Symp., 3rd, Formation and destruction of blood cells, J. B. Lippincott, Philadelphia, *in press*). Neutrophils were isolated from peripheral blood as previously described (3).

Growth of bacteria. Two strains of *Listeria* were used: one was from the reference stock of the Uni-

versity 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 grown overnight in Trypticase soy broth in the presence or absence of ^{32}P (100 μCi/ml). Before use, the bacteria were thoroughly washed in ZøBell's solution (15), and the concentration of organisms was determined from the optical density at 620 nm and from colony counts. After five washes by centrifugation (9,000 $\times$ *g* for 7 min), the level of radioactivity in the supernatant fluid did not change, and 99.6% of the radioactivity was cell-associated. Incubation with carrier-free ^{32}P in concentrations up to 300 μCi/ml did not impair bacterial viability or replication.

Bacterial killing assay. To determine the number of organisms ingested by an unknown number of macrophages adherent to cover slips of the cultivation tubes in any given time, ^{32}P-labeled bacteria were incubated with the cells. Bacteria not associated with cells were then washed off, and the amount of radioactivity associated with macrophages was determined. A standard curve was developed to determine the number of colony-forming units (CFU) equivalent to a given amount of radioactivity. The number of CFU phagocytized (ingested by or bound to) by macrophages was then determined by a simple calculation: CFU (phagocytized) = [CFU (standard)/radioactivity (standard)] $\times$ radioactivity (phagocytized). The macrophages were disrupted, and the number of viable cell-associated CFU were determined by making pour plates. The percentage of cell-associated *Listeria* killed was determined by the calculation: per cent *Listeria* killed = [CFU (phagocytized) $-$ CFU (observed)] $\times$ 100/CFU (phagocytized).

Human macrophages in Leighton tubes were washed by decantation in warm Hanks solution and resuspended in 1 ml of fresh ABM containing no antibiotics. After several hours at 37 C, 0.1 ml of ^{32}P-labeled *Listeria* (5×10^7 CFU) was added to each of the tubes, and the incubation was continued with intermittent shaking for 90 min. Since the lag phase of *Listeria* in ABM 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 (an insignificant number of glass-adherent macrophages was lost by this procedure) and 6 ml of ZøBell's solution was added. The macrophages were then sonically disrupted at an energy level and for a time period which did not injure *Listeria*. The sonically treated solutions containing viable radioactive bacteria were appropriately diluted in ZøBell's solution, and pour plates were made in 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. All bactericidal assays were performed in triplicate, with three to six pour plates made from each Leighton tube. The extreme values (calculated per cent killing) for a population of macrophages obtained from a single subject varied no more than $\pm$10% from the mean in any given experiment. To eliminate the possibility that prior exposure of macrophages to streptomycin influenced their ability to kill *Listeria*, a limited number of experiments were performed in which the macrophages were grown in medium containing only penicillin, washed, and resuspended in antibiotic-free medium. The results were no different from those obtained under the standard conditions of assay.

Appropriate controls established that, in the absence of macrophages, less than 0.5% of the radioactivity was bound to glass and there was no detectable loss of viable bacteria.

RESULTS

The time course of phagocytosis and killing of ^{32}P-labeled *Listeria* over a period of 120 min is shown in Fig. 1. Under the conditions employed, 90 to 100% of the macrophages were phagocytic by 90 min and multiple organisms were ingested by each cell. Phagocytosis of living organisms was unaffected by hypoxic conditions (PO_2 < 16 mm of Hg) but was depressed by glycolytic inhibitors such as 20 mM NaF (91% inhibition).

To determine the time course of killing of intracellular *Listeria* by macrophages over several hours, phagocytosis was allowed to proceed for 90 min, at which time the cells were washed. More than 99% of the nonphagocytized organisms were removed by washing. The incubation was then resumed in medium containing penicillin (50 units/ml) to suppress the replication of the few remaining extracellular organisms. At various intervals, the percentage of viable organisms was determined (Fig. 2). By 4 hr, between 20 and 30% of the phagocytized organisms were still viable. By 24 hr, the number of viable CFU was still approximately 10% of that initially ingested by the macrophages. At this latter time period, the number of viable *Listeria* probably represented both the organisms initially phagocytized and those arising from intracellular replication. Killing by neutrophils was more rapid and was essentially complete (96 $\pm$ 4%) within the 90-min period of phagocytosis.

The following evidence was obtained for bacterial replication within macrophages. Because macrophages have only a very limited capacity to incorporate thymidine, the onset of deoxyribonucleic acid synthesis by intracellular microbes could be determined by the addition of ^3H-thymidine at various intervals after phagocytosis was completed. Labeled organisms could then be identified intracellularly by radioautography. By this technique (Fig. 3), labeled intracellular *Listeria* cells were detected as early as 90 min after

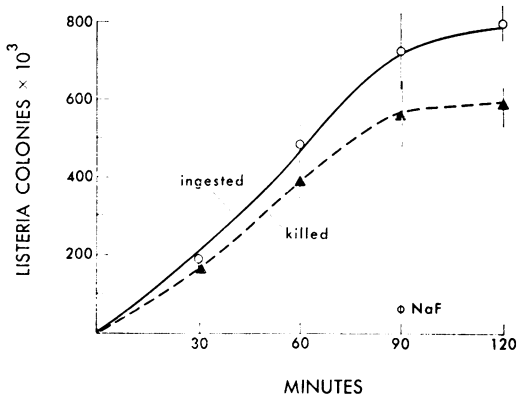

FIG. 1. *Phagocytosis and killing of Listeria monocytogenes by human macrophages during 120 min of incubation. The effect of 20 mM NaF on phagocytosis is shown at 90 min.*

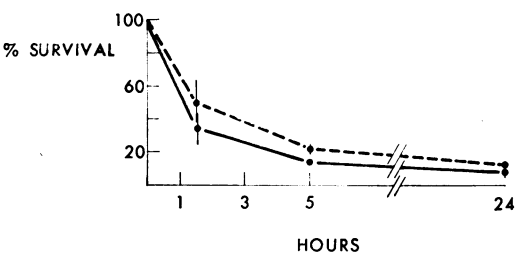

FIG. 2. *Killing of Listeria monocytogenes by two populations of human macrophages during a 24-hr period. The upper curve is derived from studies of a population of macrophages 5 days of age; the lower curve is derived from studies of macrophages 9 days of age.*

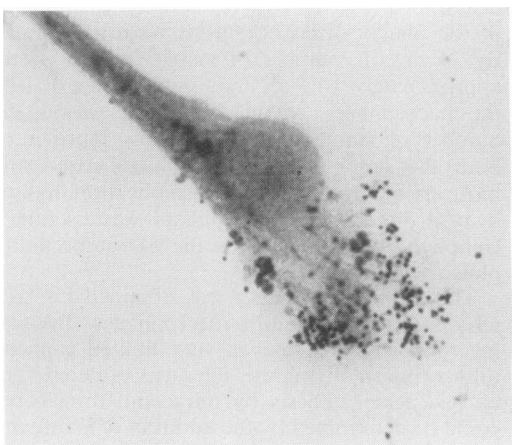

FIG. 3. *Radioautograph of macrophage after phagocytosis of viable Listeria and dead Staphylococcus aureus; cell exposed to ^{3}H-thymidine for 3 hr. Grains are localized over rodlike Listeria. S. aureus cells are unlabeled. Original magnification, × 1,250.*

phagocytosis. After 24 hr, few intracellular labeled organisms were observed, suggesting that, although a fraction of intracellular *Listeria* remained viable at this time period (as determined by colony count), replication was impaired. Occasional grains were observed in the cytoplasm of the macrophages overlying small structures which were interpreted as fragments of bacteria. This observation suggested that bacteria which initially replicated intracellularly were subsequently killed and degraded.

Influence of monocyte-macrophage differentiation on killing. Monocytes of various ages in in vitro culture were compared in their ability to kill *L. monocytogenes*. A standard 90-min period for phagocytosis and killing was used in all studies. The age of the cells in culture between 3 and 18 days had little influence on the *fraction* of ingested organisms killed (Fig. 4). It was clear, however, from morphological observations that differentiated macrophages ingested many more organisms than did newly isolated monocytes (Fig. 5); for example, in one series of experiments, 94 ± 2% of macrophages and only 64 ± 3% of monocytes had ingested *Listeria*. Of the phagocytic macrophages, 67% had phagocytized more than six bacteria, whereas only 9% of monocytes showed comparable phagocytosis. It was concluded that older macrophages were capable of killing a greater absolute number of *Listeria* per cell than were less differentiated cells, but that the fraction of ingested *Listeria* killed was relatively independent of macrophage age.

Another difference between newly isolated monocytes and mature macrophages was noted. It was evident from morphological observation that a significant fraction of infected monocytes, but not differentiated macrophages, left the glass surface after phagocytosis of viable organisms. These monocytes were presumed to have lysed

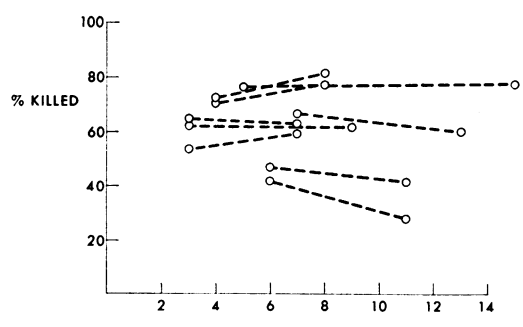

FIG. 4. *Influence of macrophage age (in vitro) on the killing of Listeria. The dotted lines connect cells of different ages obtained from the same donor.*

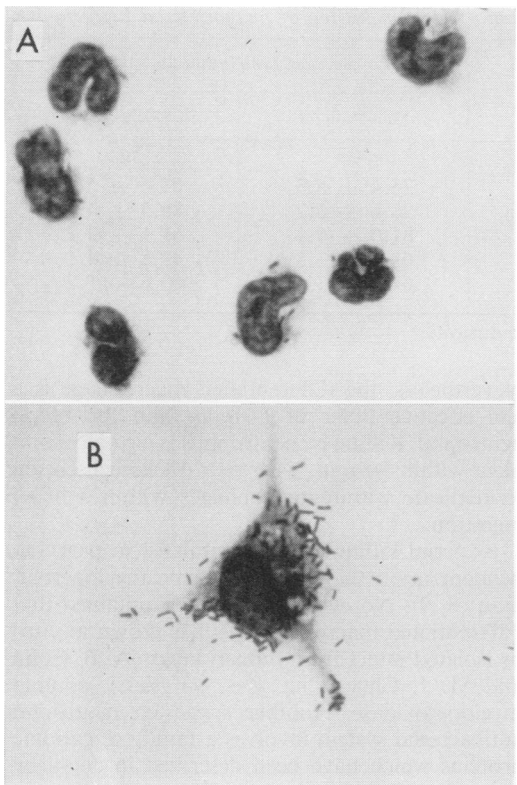

FIG. 5. *Listeria phagocytized in 90 min by monocytes (A) and macrophages (B). Giemsa stain. Original magnification, × 1,250.*

TABLE 1. *Effect of phagocytosis of Listeria on glass adherence of ^{51}Cr-labeled mononuclear phagocytes*[a]

Cells	Age in vitro (days)	Distribution of radioactivity	
		Glass	Supernatant
		%	%
Monocytes.....	2	13	87
Macrophages....	9	92	8

[a] Adherence was measured 7 hr after addition of bacteria to cells.

since they could not be recovered from the supernatant even after concentration of the fluid. This phenomenon was quantitated by prelabeling the cells with ^{51}Cr before the addition of bacteria and determining the distribution of radioactivity (glass-associated versus supernatant) after phagocytosis. The results shown in Table 1 confirmed the morphological observations.

Oxygen requirement. Hypoxic conditions were achieved by incubating macrophage cultures with 5% CO_2 in nitrogen for 60 min before and 90 min after the addition of *Listeria*. Control cultures were incubated with 5% CO_2 in air. The partial pressure of O_2 was determined on similarly handled cultures containing no bacteria. Hypoxic conditions (PO_2 < 16 mm of Hg) had no effect on phagocytosis but clearly depressed the killing of ingested *Listeria* (Table 2).

KCN and 2,4-dinitrophenol (DNP) were added to human macrophage cultures in concentrations of 1 and 0.2 mM, respectively; these concentrations have been used in the study of animal leukocyte systems (7). As shown in Table 3, these compounds were without effect on the phagocytosis or killing of *Listeria* by macrophages.

Effects of disease. Monocytes were isolated from normal subjects, patients with Hodgkin's disease, and patients actively infected by intracellular parasites. The cells were cultivated in vitro from 4 to 9 days and then tested for their ability to kill *L. monocytogenes* (Fig. 6). The difference between normal subjects and patients with Hodgkin's disease and the difference between normal subjects and patients with intracellular infection were both statistically significant (P < 0.01) when the *Listeria* strain UCLM-1 was used. Similar results were obtained with 4524B, but insufficient data were acquired for statistical analysis.

Macrophage myeloperoxidase. Although histochemical and specific immunochemical techniques reveal that monocytes contain small amounts of myeloperoxidase (9, 12), macrophages grown in culture cannot be demonstrated to contain the enzyme. That the absence of enzyme is not an artifact of in vitro cultivation is demonstrated by two observations. Newly isolated human alveolar macrophages have no histochemically detectable myeloperoxidase. Macrophages grown in vitro show an increase in activity of other lysosomal enzymes concomitantly with a loss of detectable myeloperoxidase (M. J. Cline, *In* Red Cross Sci. Symp., 3rd, Formation and destruction of blood cells, J. B. Lippincott, Philadelphia, *in press*).

DISCUSSION

The monocyte-macrophage may be regarded as a continuum of cells at various stages of maturation (5). This cell species originates in the bone marrow (13), has a brief existence in the circulation (monocyte), and spends the bulk of its life span in the tissues, where it undergoes differentiation into a macrophage (6). Mammalian blood monocytes cultured in vitro undergo a similar sequence of maturation characterized by enlargement, more active phagocytosis, and accumulation of lysosomes and associated acid hydrolases (5).

TABLE 2. *Effect of hypoxic conditions (PO_2 < 16 mm of Hg) on killing of phagocytized Listeria*

Source of macrophages	*Listeria* strain	Phagocytized *Listeria* killed in 90 min[a]	
		5% CO_2 in air	5% CO_2 in nitrogen
		%	%
Normal	UCLM-1	75.4 ± 5.3	63.6 ± 5.2
Chronic fungal disease-1	UCLM-1	96.6 ± 6.2	86.3 ± 4.2
Chronic fungal disease-2	UCLM-1	81.0 ± 6.1	61.1 ± 3.2
Monocytic leukemia	UCLM-1	61.1 ± 3.3	43.8 ± 4.1
Monocytic leukemia	4524B	64.1 ± 3.5	60.6 ± 1.2

[a] Results expressed as the mean ± two standard deviations.

TABLE 3. *Effect of KCN and 2,4-dinitrophenol on phagocytosis and killing of Listeria monocytogenes by macrophages*

Addition to incubation medium	*Listeria* phagocytized in 90 min[a]	*Listeria* killed in 90 min[a]
	CFU	%
Saline	$3.15 \times 10^5 \pm 0.45$	55 ± 8
KCN (1 mM)	$3.69 \times 10^5 \pm 0.70$	60 ± 6
2,4-Dinitrophenol (0.2 mM)	$2.99 \times 10^5 \pm 0.64$	60 ± 3

[a] Results expressed as the mean ± two standard deviations.

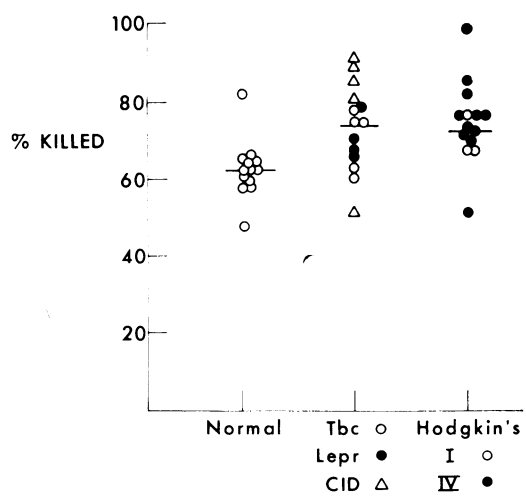

FIG. 6. *Listeria killed in 90 min by macrophages from various sources. Abbreviations: Tbc, tuberculosis; Lepr, leprosy; CID, chronic inflammatory disease; I, stage I Hodgkin's disease; IV, stage IV Hodgkin's disease.*

From the observations reported here, it is clear that maturation is accompanied by functional changes. The mature macrophage can ingest more organisms than the newly isolated monocyte and retains its structural integrity better after phagocytosis of large numbers of viable organisms. Nevertheless, the differentiated macrophage is a less effective killer of *Listeria* than the blood neutrophil. Killing by neutrophils is virtually complete within 90 min, whereas *Listeria* cells begin to replicate within macrophages within 3 hr of ingestion.

Bacterial killing by neutrophils is, in part, dependent upon the cellular enzyme myeloperoxidase (8, 9). No evidence has been obtained that differentiated macrophages, either grown in vitro or isolated intact from human lungs (A. B. Cohn and M. J. Cline, Clin. Res., *in press*), contain myeloperoxidase. Another suggested neutrophil antibacterial system involves a family of cationic proteins which have been described in detail by Zeya and Spitznagel (14). These investigators found no evidence for a similar class of proteins in macrophages.

From evidence reported here, it would appear that killing of a facultative intracellular parasite by human macrophages is, in part, dependent upon the availability of oxygen, although an inhibitor of the cytochrome system (KCN) and an uncoupler of oxidative phosphorylation (DNP) did not affect killing. A similar phenomenon has been observed in systems involving neutrophils and certain species of bacteria. The oxygen requirement of the neutrophil is thought to be part of a cellular H_2O_2-generating system. Generation of H_2O_2 by macrophages has not been clearly demonstrated as yet, although there is preliminary evidence that certain redox agents, which apparently enhance intracellular H_2O_2 generation, also enhance macrophage listericidal activity (M. J. Cline, Clin. Res., *in press*).

The observation that macrophages from patients with Hodgkin's disease killed *Listeria* better than normal cells was unexpected and has no readily available explanation. The observation that macrophages from patients with intracellular infections are superior in their killing ability to macrophages from normal subjects agrees with results obtained in animal systems (2, 10). However, it is possible that the results obtained with human cells are an artifact of in

vitro culture conditions. For example, tissue culture may select the hardiest or most differentiated cells. On the other hand, if macrophages from infected patients are functionally superior, it suggests that the commitment of an infected host to produce more effective macrophages is made at an early stage, at the level of the circulating monocyte or earlier. Once the commitment is made, persistent exposure to a parasite may not be necessary for maturation into a macrophage with enhanced bactericidal capabilities.

ACKNOWLEDGMENTS

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