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Enhanced In Vitro Phagocytosis of *Listeria monocytogenes* by Human Monocytes in the Presence of Ampicillin, Tetracycline, and Chloramphenicol

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An in vitro system was developed to test the phagocytic activity of human macrophages grown from blood monocytes in the presence of the antibiotics ampicillin, tetracycline, and chloramphenicol. ^{3}H-labeled *Listeria monocytogenes* served as test organism. Subinhibitory amounts of the antibiotics enhanced the phagocytic activity significantly ($P < 0.025$). Macrophages pretreated with the drugs in identical concentrations showed the same phagocytic activity as control cells in the absence of the drugs. Because the drug concentrations used were similar to those that may be attained in man at certain places of inflammation, enhanced phagocytosis in the presence of antibiotics may have clinical significance.

In previous investigations we demonstrated the influence of gentamicin and polymyxin (3) and of dihydrostreptomycin (1) on the phagocytic activity of mouse peritoneal macrophages infected with *Escherichia coli*. Enhanced phagocytic activity and intracellular destruction of the test organism was demonstrated. In the present study the effect of ampicillin, tetracycline, and chloramphenicol, in subeffective doses, on phagocytic activity of human mononuclear phagocytes was determined with *Listeria monocytogenes* as test organism.

MATERIALS AND METHODS

Antibiotics. Ampicillin (batch no. 1515) and tetracycline (batch no. 3965) were obtained from Bayer AG, Leverkusen, West Germany. Chloramphenicol (batch no. 141268) was obtained from Boehringer Mannheim, Mannheim, West Germany. Stock solutions of these drugs were frozen in small bottles at −20 C until required for use. For each experiment the stock solution was diluted in tissue culture (TC) medium 199 (Difco) to a final concentration which influenced neither growth nor number of viable bacteria during the time period of phagocytosis.

Media. TC medium 199 and Hanks solution were obtained from Difco, fetal calf serum (FCS) was from GIBCO, and 35% bovine albumin solution was from Pentex, Kankakee, Ill. ^{3}H-labeled uracil was obtained from Amersham Buchler.

Human monocyte isolation and cultivation. Monocytes were isolated from the peripheral venous blood of healthy adults and one neonate by a slight modification of the method of Bennet and Cohn (5). Homogeneous populations of monocytes were obtained by albumin gradient centrifugation. A 50- to 80-ml (25 ml from the umbilical vein for the neonate) amount of blood obtained by puncture of the vena cubiti were collected in a sterile, siliconized glass cylinder containing 500 IU of heparin (Liquemin, Hoffmann-La Roche, Basel, Switzerland) and a one-fifth volume of 3% dextran in saline. After spontaneous sedimentation of the erythrocytes for 60 to 80 min at 37 C, the leukocyte-rich plasma was removed and centrifuged in 15-ml portions at 100 × *g* for 15 min at 12 C. The packed cells were carefully washed in an equal volume of TC medium 199 containing 10% FCS and 20 U of Liquemin per ml at 150 × *g* for 15 min at 12 C. Washed cells were allowed to dry in an inverted position, resuspended in 35% bovine albumin solution without any clumping, and diluted with TC medium 199 to a final albumin concentration of 28%. The final leukocyte concentration was about 5×10^7 white blood cells per ml. The less dense monocytes were concentrated in a surface pellicle by centrifugation at 2,400 × *g* for 35 min at 12 C, subsequently removed by means of a curved siliconized Pasteur pipette, and pooled in 10 ml of TC medium 199 plus 10% FCS at 12 C. Cells were collected by centrifugation at 150 × *g* for 15 min at 12 C and resuspended to a concentration of 1.8×10^6 cells/ml. Portions of 1 ml were placed into Leighton tubes containing a flying cover slip, gassed by 5% CO_2 in air, and allowed to adhere to the glass surface for 2 h at 37 C. Then they were washed free of serum proteins as well as contaminating lymphocytes with 1.5-ml volumes of warm Hanks solution by continous gentle shaking. New complete medium was replaced, and tubes were gassed and closed by silicone rubbers. Cell monolayers were kept in culture up to 48 to 72 h at 37 C for morphological and functional differentiation.

All manipulations were done under aseptic conditions, especially because of the lack of any antibiotic in the medium. All glassware except the Leighton

tubes were siliconized, Leighton tubes and silicone rubbers were carefully cleaned of any detergent, and cover slips were washed in 90% ethanol before sterilization.

Growth of test organism. *L. monocytogenes* (strain no. 1/2a 2459, a generous gift from D. Braveny [Institute for Hygiene, Klinikum Rechts der Isar, Technical University of Munich, Munich, West Germany]) was kept on blood agar (Difco). Prior to use some colonies of the strain were transferred into 2 ml of brain heart infusion broth (BHI, Difco), diluted to one-sixth of its original concentration in the presence of 80 μCi of ^{3}H-uracil, and incubated for 18 h at 37 C. Under these conditions growth and radioactive labeling of the bacteria were suitable for our tests. Before use, the bacteria were thoroughly washed by centrifugation at 6,000 $\times$ *g* for 10 min in cold BHI until the level of radioactivity in the supernatant did not undergo change. The suspension was brought to an optical density of 0.1 at 620 nm in a Coleman Junior spectrophotometer (model J 6/20). This equals a concentration of 10^8 organisms per ml. A standard curve was developed to determine the number of colony-forming units equivalent to a given amount of radioactivity. During the time period of phagocytosis, no growth of organisms occurred.

Light microscopy. To investigate different problems (e.g., purity of monolayers, differentiation in macrophages, and phagocytosis of test organisms), cell monolayers on flying cover slips were washed in Hanks solution, dried in air, fixed in cold absolute methanol, and stained by the Giemsa method.

Phagocytosis. The macrophages for each test series with the three antibiotics and the controls without antibiotics were from the same donor. All tests were performed in triplicate. Viability tests with trypan blue showed the percentage of living cells. Preparations for light microscopy were made after phagocytosis. The number of cell-associated and -ingested bacteria was determined as follows: cover slips with 48-h-old macrophage monolayers, containing 10^5 to 3 $\times$ 10^5 cells, were removed aseptically and placed into fresh tubes containing 1 ml of either TC medium 199, 40% FCS, 10^7 ^{3}H-labeled *L. monocytogenes* and antibiotics or the same solution without antibiotics. Controls without macrophages were set up. Subsequently cultures were incubated for a period of 90 min at 37 C with intermittent shaking. During this time no growth of bacteria occurred, because the lag phase of *L. monocytogenes* in the test medium at 37 C is at least 110 min. After a period of 90 min, the tubes were placed in an ice bath, and the cover slips were removed, washed five times in saline, and placed in Packard vials containing a dioxane-based liquid scintillator (Aquasol Universal LSC cocktail, New England Nuclear Corp., Boston, Mass.). By this procedure free bacteria were washed off, and 95 to 98% of the radioactivity was bound to macrophages. The amount of radioactivity determined allowed the following calculation: cell-associated bacteria = no. of colony counts (standard)/radioactivity (standard) $\times$ radioactivity (phagocytized). The extreme values (calculated per number of bacteria phagocytized) for a population of macrophages obtained from a single subject varied no more than $\pm$15% from the mean in any given experiment. To eliminate the possibility that prior exposure of macrophages to the drugs tested influenced their ability to phagocytize bacteria, cell cultures were kept free of any antibiotic.

RESULTS

Influence of ampicillin, tetracycline, and chloramphenicol on the phagocytosis of L. monocytogenes by human mononuclear phagocytes. For these studies, cells were obtained from healthy subjects (five female and four male adults as well as one neonate) and used in the tests on the 3rd day of culture. At this time about 90% of the macrophages of the dense monolayer on the cover slip of each tube were viable. Most of these cells were active as phagocytes and showed a high rate of associated bacteria, the mean being 8 to 12 per cell. The effects of antibiotics on phagocytosis were studied with the drugs in concentrations which did not affect the number of microorganisms in an 18-h culture of 10^5 bacteria/ml.

The results of a representative experiment on the effects of ampicillin and tetracycline in subinhibitory concentrations on the phagocytosis by human mononuclear phagocytes of ^{3}H-labeled *L. monocytogenes* are summarized in Table 1. The mean of the absolute number of phagocytosed bacteria in the absence of antibiotics was used as the base level and defined as 100%. It may be seen that ampicillin and tetracycline enhanced phagocytosis by 41 and 75%, respectively.

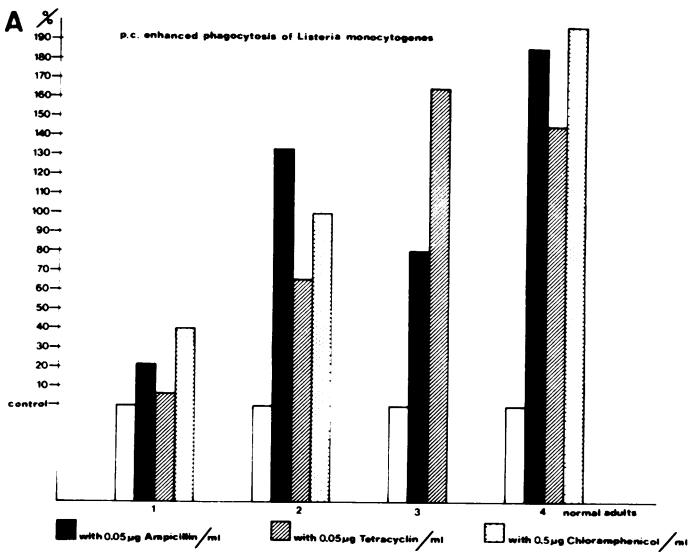
The results of all experiments are summarized in Fig. 1. In the upper portion of the figure (A) are shown the results with cells from adult males, and in the lower portion (B) are shown the results with cells from adult females and a neonate. Shown is the percentage of increased phagocytosis in the presence of the three antibiotics (shaded columns) and of the controls without antibiotics (white column). It is evident that in all instances phagocytosis was enhanced, in the presence of ampicillin, tetracycline, and chloramphenicol, to a lesser or greater degree and that the magnitude of enhancement differed between cells of various individuals. The difference in number of phagocytosed bacteria in the presence and absence of antibiotics was statistically significant ($P < 0.025$). Mention should be made of the fact that macrophages, pretreated with antibiotics in identical concentrations and washed free of drugs prior to incubation with the test organism, showed the same phagocytic activity as did control cells not treated with the antibiotics.

TABLE 1. *Calculation of phagocytosis of ^3H-labeled Listeria monocytogenes by human mononuclear phagocytes with and without addition of a subinhibitory amount of ampicillin and tetracycline*[a]

Determination	Counts/min				Absolute no. of phagocytized bacteria	Phagocytosis (%)
	Tube 1	Tube 2	Tube 3	Mean		
Without antibiotic	2,706	2,700	2,795	2,734	108,160	100
With 0.05 μg of ampicillin/ml	3,243	3,324	4,241	3,603	151,645	141
With 0.05 μg of tetracycline/ml	3,646	4,001	5,392	4,346	188,799	175
Control without macrophages[b]	124	867	960	650		

[a] Human monocytes were grown from a single person. Tests were performed in triplicate. CFU, Colony-forming units; RA, radioactivity.

[b] CFU (standard): $1,3 \times 10^7$; radioactivity (standard): $2,6 \times 10^5$ counts/min. CFU (standard)/RA (standard) counts/min × RA (phagocytized) counts/min = CFU (phagocytized).



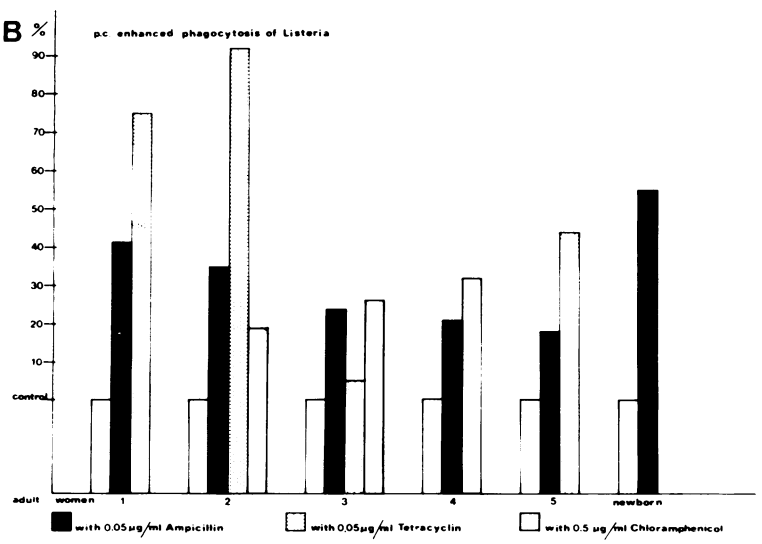


FIG. 1. *Percentage of enhanced phagocytosis of ^3H-labeled Listeria monocytogenes by human monocytes after addition of subinhibitory doses of ampicillin (0.05 μg/ml), tetracycline (0.05 μg/ml), and chloramphenicol (0.5 μg/ml). The minimal inhibitory concentrations against this strain of L. monocytogenes are: ampicillin, 1.0 μg/ml; tetracycline, 1.0 μg/ml; and chloramphenicol, 3.0 μg/ml.*

DISCUSSION

It is firmly established that the phagocytic ability of macrophages plays a key role in resistance to certain bacterial infections. Cellular immunity to some of these diseases is elicited by viable microorganisms, and not as well by dead vaccines (6, 11, 12). The in vitro cultivation of peripheral blood monocytes results in morphological and functional activities similar to those resulting from the transformation of monocytes into tissue macrophages at extravascular sites in vivo (5, 7, 17, 20). These changes include an increase in cell size (18). Studies of certain immunodeficient states revealed that the therapeutic usefulness of antibiotics depends on an established host-defense mechanism (2).

Phagocytosis may be enhanced during bacterial infection by the following. (i) Among the humoral factors are cytophilic antibodies (10) as well as opsonizing immunoglobulins and complement (4, 8, 15, 16). (ii) A persistent pool of antigen (13, 14) and especially partially damaged, but still viable microorganisms (6), may activate the production and maturation of monocytes. (iii) As shown in the present investigation, antibiotics in subinhibitory concentrations may enhance the phagocytic activity of human macrophages. Indeed, these drug concentrations may be obtainable in certain tissues of patients.

The mode of action of antibiotics in subinhibitory concentrations on phagocytosis remains to be elucidated. It has been shown, however, that the effect is not due to action on the macrophages themselves (1). It is important to note that the antibiotics enhanced phagocytosis of unrelated microorganisms, *L. monocytogenes*, as shown here, and *Escherichia coli*, as shown previously (1). Antibiotics of different modes of action yielded similar results.

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