Effect of Dihydrostreptomycin on Phagocytosis of Mouse-Peritoneal Macrophages In Vitro

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The effect of the antibiotic dihydrostreptomycin on the phagocytic and bactericidal ability of peritoneal macrophages obtained from mice has been investigated. In subliminal concentrations which did not influence the bacterial growth, the drug caused macrophages to ingest and kill bacteria (Escherichia coli) at a higher rate than did macrophages without antibiotic. The differences for phagocytosis and intracellular killing of E. coli with and without a subliminal amount of dihydrostreptomycin were statistically significant. Macrophages pretreated with the antibiotic did not demonstrate any enhancement of phagocytosis.

In dealing with infections, antibiotics—known for their bactericidal or bacteriostatic abilities—are widely used. Cellular and humoral defense mechanisms on the other hand play an important role in killing bacteria. They consist of a number of partly unknown reactions which begin with the uptake of antigen by the macrophages and end with both production of specific antibodies and mediators of cellular immunity. The question of whether antibiotics influence cellular immunity in any way arises when chemotherapeutics are administered. Using antibiotics has sometimes been considered disadvantageous for the organism to produce a high level of antibodies since killing of bacteria by antibiotics would not leave sufficient antigenic material for the macrophages (9, 19, 22).

In a previous study, we investigated the influence of the antibiotics polymyxin and gentamicin on the phagocytic activity of mouse-peritoneal macrophages which were infected with Escherichia coli (1). We demonstrated that gentamicin and polymyxin added in subliminal amounts caused macrophages to ingest and kill bacteria at a higher rate than without antibiotic. In this study, we used dihydrostreptomycin, another antibiotic from the aminoglycosid group from which is known that this substance does not penetrate into macrophages (6) to exclude a stimulation of macrophage activity by the drug.

**Materials and Methods**

**Mice.** Mice of the NMRI strain from our own inbreeding, weighing 35 to 40 g, were used.

**Test organisms.** E. coli 018 (kindly supplied by F. Ørskov, Copenhagen) was kept on a solid agar maintenance medium. Prior to use, some colonies of the strain were transferred into 10 ml of Brain Heart Infusion broth (BHB, Difco) with a platinum loop and incubated at 37 C for 16 hr. Then a 1:10 dilution in the same medium was prepared, incubated for 1 hr in a water bath at 37 C, and cooled in a deep freezer for 10 min to stop any further growth. A dilution (generally 1:2) of this suspension with cooled BHB was brought to an optical density of 0.15 at 650 nm in a Coleman Junior spectrophotometer (model J 6/20). This equals a concentration of ca. 10^6 organisms/ml.

**Antibiotic.** Dihydrostreptomycin was obtained from Bayer AG, Leverkusen, West Germany (batch no. III 73 1C). Stock solutions of this substance were frozen in small bottles at -20 C until required for use.

**Sera.** Specific rabbit immune serum was prepared against E. coli 018 in a standard method. Indirect hemagglutination-titer (15) was 1:5,120. Bovine serum (Behring Werke, Marburg/Lahn, West Germany) was also used.

**Macrophages.** Mice were killed by cervical fracture. Peritoneal macrophages were obtained by a modification of the method described by Degré and Whitby and Rowley (7, 21). A 2-ml amount of Eagle's minimal essential medium (Behring Werke, Marburg/Lahn, West Germany) containing 5 IU of heparin (Liquemin from Hoffmann-La Roche, Basel, Switzerland) was injected into the peritoneal cavity. After a few seconds, 1.5 ml was withdrawn. The cell suspensions of six mice were pooled and gently shaken to prevent sedimentation of the cells. Sterile, heat-inactivated (56 C) bovine serum (20%) was added.

**Phagocytosis by macrophages in monolayers.** A 1-ml amount of the suspension containing 10^6 to 3 X 10^6 macrophages was pipetted into Leighton tubes in which a flying cover slip covered the entire bottom area. The mixtures were incubated at 37 C for 3.5 hr. The cover slips were removed aseptically, washed in...
medium, and placed into fresh sterile Leighton tubes. The macrophages were attached to the surface of the cover slips; other cells were dead or had been washed off (16, 17). A 0.1-ml amount of the bacterial suspension in Eagle's medium was given into each of the Leighton tubes which contained the antibiotic and 20% inactivated rabbit immune serum. Controls without antibiotic were set up. The total volume in each tube was 1 ml. Every test was carried out in three tubes and compared with controls which contained no macrophages. The pH of the suspension was 6.5. This pH was attained by addition of a mixture of 1% of each citric acid and lactic acid in equal volumes (4). The tubes were incubated stationary at 37 C. After a given time, they were cooled in an ice bath, and the number of viable bacteria was counted. Supernatant fluid (0.1 ml) of appropriate dilution was given on Endo-agar plates. The cover slips were removed aseptically, washed three times in saline, and placed upside down on the same medium. Then, with a platinum loop, the cover slips were rubbed vigorously against the surface of an Endo-agar plate to dislodge the macrophages.

The difference between the number of bacteria from the test and the controls containing no macrophages is due to phagocytosis. The bacteria recovered from the cover slips represent the number of viable phagocytized bacteria (see Table 1). The difference in number between total and living phagocytized bacteria gives the amount of organisms killed within the macrophages after phagocytosis. The number of macrophages was standardized in each case (ca. 1.5 \( \times 10^6 \)).

**Tests.** Viability tests with Trypan blue (EGA-Chemie KG, Kepler und Reif, Steinheim-Albuch, West Germany) were carried out. As a statistical test, the \( t \) test for paired observations (one-tailed test procedures) and the chi-square test were used (15).

**RESULTS**

In the set up we used, optimal phagocytosis could be observed after 90 min. In all further experiments, phagocytosis was allowed to occur for this length of time. The optimal pH was 6.5. During this period, no extracellular proliferation

**TABLE 1. Calculation of phagocytosis and intracellular killing of Escherichia coli 018 by peritoneal mouse macrophages with and without addition of a subliminal amount* of dihydrostreptomycin**

<table>
<thead>
<tr>
<th>Determinations</th>
<th>Total bacterial count in control tube (L)</th>
<th>Total bacterial count in test tube (R)</th>
<th>Phagocytized bacteria (L - R)</th>
<th>% Phagocytized bacteria (L - R)/L ( \times 100 )</th>
<th>Viable phagocytized bacteria (CS)</th>
<th>% Viable phagocytized bacteria (CS/L - R) ( \times 100 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without antibiotic</td>
<td>3,230</td>
<td>2,820</td>
<td>410</td>
<td>13</td>
<td>262</td>
<td>64</td>
</tr>
<tr>
<td>With antibiotic</td>
<td>3,700</td>
<td>2,440</td>
<td>1,260</td>
<td>34</td>
<td>207</td>
<td>16</td>
</tr>
</tbody>
</table>

* 0.3 \( \mu \)g/ml.

**FIG. 1. Per cent phagocytosis of E. coli 018 by peritoneal mouse macrophages after addition of a subliminal dose of dihydrostreptomycin (0.3 \( \mu \)g/ml). Minimal inhibitory concentration of dihydrostreptomycin against E. coli 018 is 20.0 \( \mu \)g/ml.
of microorganisms occurred, since this time interval corresponds with the "lag phase" of the bacterial population. Furthermore, the bacterial count in analogous prepared control tubes did not change during this incubation time. The following results could be observed. (i) Addition of a subliminal amount of dihydrostreptomycin (e.g., 0.3 μg/ml) into the medium increased the rate of phagocytosis of E. coli by macrophages (Fig. 1). The minimal inhibitory dose of di-

![Fig. 2. Influence of a subliminal amount of dihydrostreptomycin (0.3 μg/ml) on intracellular killing of E. coli 018 by peritoneal mouse macrophages.](image)

![Fig. 3. No enhancement of phagocytosis of E. coli 018 by macrophages pretreated with antibiotic.](image)
hydrostreptomycin against E. coli 018 in our set up with 3,000 to 4,000 microorganisms/ml was 2.0 μg/ml, and the minimal inhibitory concentration in an overnight culture with 10^5 microorganisms/ml was 20.0 μg/ml. In control tubes without macrophages, this dose of dihydrostreptomycin (0.3 μg/ml) did not influence the number of bacteria; the bacterial counts were identical in tubes with and without antibiotic. To verify the difference of bacterial counts between phagocytosis with and without addition of the subliminal amount of dihydrostreptomycin, the t test for paired observations (one-tailed test procedures) was used (15). The calculation resulted in a t value of 3.74. The critical t value by 14 degrees of freedom and a significance level of P = 0.025 is 2.15 (15). Hence it follows that there is a significant difference between phagocytosis with and without antibiotic (P < 0.025). (ii) Phagocytized bacteria are killed by macrophages at a certain rate (16). Bacteria which had been phagocytized under the influence of antibiotics were killed intracellularly at a higher rate compared to bacteria phagocytized without an antibiotic (Fig. 2). The difference in intracellular killing with and without addition of a subliminal antibiotic concentration is statistically significant. The calculation was carried out by the chi-square test (15). The critical chi-square value for 1 degree of freedom is 6.63. All calculated chi-square values were higher. Again the concentration of dihydrostreptomycin was below the level required for killing of the bacteria. Control tubes without macrophages but with the same amount of antibiotic did neither increase nor decrease in number of bacteria. Only a combination of cells and antibiotics had this effect (Table 1).

An activation of macrophages by the antibiotic could be excluded. Macrophages pretreated with 0.3 μg of dihydrostreptomycin per ml for 2 hr did not show any enhanced phagocytosis (Fig. 3).

**DISCUSSION**

Macrophages are part of the body's defense mechanism. They are functionally similar to tissue histiocytes such as Kupffer cells and also similar to circulating monocytes (23). It is known that they take up antigen and break it down to metabolites of lower molecular weight (8). The information is passed on and finally results in the production of specific antibodies (5, 10) and mediators of cellular immunity. In defending an organism against foreign particles, the function of macrophages is of utmost importance. Bacteria have to be opsonized to be phagocytized by macrophages. This fact explains the influence of the serum. The effect of specific immune serum is probably due to cytophilic antibodies (12), which attach themselves to the surface of the macrophages and help to engulf bacteria.

In infectious diseases, antibiotics are administered. It has been suggested that an immediate therapy with antibiotics which eliminates microorganisms might decrease the supply of antigen for macrophages, and this in turn would make a rapid functioning of the body's own defense mechanisms less effective (9, 19, 22). Since in those parts of the body which have poor blood circulation, chemotherapy sometimes results only in subinhibitory levels of antibiotics (20), it is of clinical importance to know what can happen to microorganisms in vivo which have not been reached by a full effective dosage of antibiotics. It is presumed that in those parts the microorganisms stay alive and multiply to a large extent.

Another factor in the elimination of bacteria is macrophages. This investigation and an earlier report (2) show that phagocytosis of bacteria by macrophages is increased in the presence of subliminal amounts of antibiotics (Table 1). We also observed a higher percentage of intracellular killed bacteria with subliminal amounts of antibiotic.

The result with dihydrostreptomycin is remarkable because this drug has a protein binding of 60% (20) and is additionally inactivated in an acid milieu.

Since macrophages have to ingest and kill microorganisms to "process" them into a form that is immunogenic (5, 18, 23), there is no doubt that antibiotic therapy will at least ease the body's defense to fight infection.

In addition, we could show in a previous report that heat-labile bactericidal serum constituents support the effect of antibiotics against gram-negative microorganisms (1). In certain immune deficiency states, antibiotics prove less effective in curing infections in spite of in vitro sensibility of the microorganism against the drug (3). This clinical observation demands further investigations on bactericidal potency of macrophages.

Since in our set up the antibiotics were administered before phagocytosis, these results do not contradict the findings of others reporting that phagocytized bacteria are protected from killing within the macrophages, when, in contrast to our procedure, antibiotics are only added after phagocytosis has occurred (11, 13). Bacteria are supposedly only damaged slightly by subliminal amounts of antibiotics without being killed or stopped in growth. Now however, bacteria appear to be phagocytized and killed by macrophages at a higher rate.

A stimulation of the phagocytic activity of
macrophages by dihydrostreptomycin is improbable because this substance does not penetrate into macrophages in this low concentration and short time of exposure (6). Macrophages pretreated with dihydrostreptomycin did not show any enhanced phagocytosis (Fig. 3).

The conclusion is drawn that macrophages and a subliminal amount of antibiotic have a synergistic effect in killing bacteria.

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LITERATURE CITED