Autoradiographic Evidence for the Impermeability of Mouse Peritoneal Macrophages to Tritiated Streptomycin

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ABSTRACT

Cultured mouse peritoneal macrophages were found to be relatively impermeable to streptomycin. Based on radioactivity measurements and radioautographic evidence, macrophages were impermeable to tritiated dihydrostreptomycin for periods up to 20 hr of incubation. Little or no intracellular streptomycin could be detected even when incubation was carried out in the presence of therapeutic blood levels of carrier dihydrostreptomycin. When the cultured mouse macrophages were allowed to phagocytize staphylococci, yeast cells, or polystyrene latex particles in the presence of tritiated streptomycin, the impermeability of the cells to the antibiotic was not affected. These observations suggested that the process of phagocytosis does not facilitate the intracellular accumulation of streptomycin, as seems to be the case for the fixed phagocytic cells of the liver.

The efficacy of streptomycin in the treatment of intracellular infections such as tuberculosis and tularemia is an undisputed fact verified by extensive laboratory experiments and clinical trials. Although streptomycin may not currently be the antibiotic of choice for various reasons (drug-resistant strains, adverse side effects, etc.), this antibiotic does exert a bactericidal effect on several pathogenic species for which the most part are located within phagocytic cells during active infection. The difficulty arises when one tries to understand the antibacterial action of streptomycin on intracellular organisms vis-a-vis the commonly held view that mammalian cells are relatively impermeable to streptomycin and its derivatives. Presumably, this impermeability also accounts for the low toxicity of the antibiotic for animal cells.

The penetration of streptomycin into mammalian cells has been tested experimentally, but the results have been inconsistent. Conclusions reached by various investigators have run the gamut of possibilities: (i) that streptomycin is concentrated within phagocytic cells in quantities sufficient to be bactericidal (10, 16); (ii) that streptomycin can penetrate the cell membrane but is not bactericidal for the intracellular organisms which are contained within phagocytic vacuoles (11); (iii) that streptomycin does not penetrate and has no effect whatever on intracellular microorganisms (4, 14). These investigators used a variety of experimental models involving several phagocytic cell types and bacterial species, which presumably can account at least in part for the divergent results.

In a previous investigation in which we measured the kinetics of phagocytosis and intracellular digestion of viable bacteria by the isolated perfused rat liver, attempts were made to inhibit extracellular multiplication of microorganisms by the addition of bactericidal concentrations of streptomycin to the perfusing fluids. This has become a routinely used procedure to eliminate the complicating factor of extracellular multiplication in studies concerned with the process of bacterial digestion within phagocytic cells. Mackaness (15) reviewed the pros and cons of this experimental procedure, and his considered opinion is that data obtained in this fashion must be interpreted with extreme caution, since in many cases it is impossible to determine the relative contribution of the antibiotic and the phagocytic cells to bacterial destruction. In our experi-

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ments, it was observed that streptomycin in the perfusing serum exerted a significant bactericidal effect on the microorganisms phagocytized by the Kupfer cells of the liver. Further experiments with the isolated perfused liver provided evidence that streptomycin was not by itself concentrated within the Kupfer cells in quantities sufficient to affect phagocytized organisms. The impermeable nature of the phagocytic cells of the liver to streptomycin, however, could be circumvented by the presence of nonviable bacteria in the perfusate; this suggests that the antibiotic penetrated the liver cells and was concentrated during the phagocytic process (17). A mechanism which might account for the penetration of the streptomycin into the liver cells is the “piggy-back” phagocytosis phenomenon described by Sbarra et al. (19). Their results showed that compounds which, under ordinary circumstances, cannot enter phagocytic cells could be concentrated intracellularly during the invagination of the cell membrane and the subsequent phagocytosis of latex particles.

In view of the fact that most investigators have employed either blood leukocytes or peritoneal macrophages rather than fixed phagocytic cells of the reticuloendothelial system for the study of intracellular disposition of phagocytized bacteria, it was considered of interest to determine whether macrophages behaved in a similar fashion as did the Kupfer cells of the perfused liver. In the present investigation, rather than rely on the bactericidal action of streptomycin as an index of intracellular concentration, tritiated streptomycin was employed so that radioactivity could be measured or visualized by radioautographic procedures.

**Materials and Methods**

*Animals.* Swiss-albino mice (20 to 25 g) used as the source of peritoneal macrophages were obtained from Hamilton Laboratories, Hamilton, Ohio.

*Collection of macrophages.* Mice were injected intraperitoneally with 1.0 ml of nutrient broth (Difco). At 5 hr after the injection of the irritant, the animals were sacrificed and the peritoneal macrophages were immediately harvested. The cells were collected from the abdominal cavity in Eagle’s Minimum Essential Medium (MEM) (7) containing 0.5 units of heparin per ml. The cells were pooled and maintained at 4 C until cultured.

*Cell cultures.* The cell suspension was distributed aseptically in 4.0 ml quantities in 60-mm petri dishes containing cover slips (three per dish). All glassware was presterilized. The cells were allowed to settle onto the cover slips for approximately 1 hr at 37 C under 5% CO₂ tension. The tissue culture fluid was then drained and replaced with MEM plus 40% horse serum. The high concentration of horse serum favors the outgrowth of macrophages rather than fibroblasts (1). The cells were cultured for 2 to 3 days at 37 C with the tissue culture fluid being replenished daily.

*Phagocytosis and incubation with tritiated streptomycin.* A variety of experimental conditions were devised to establish whether streptomycin penetrated the phagocytic cells. The macrophage cultures were incubated at 37 C with tritiated (H) streptomycin in the tissue culture fluid (MEM) at a concentration of 5 μc/ml. The tagged antibiotic was the dihydro derivative of streptomycin and was uniformly labeled (specific activity, 290 mc/m mole; Nuclear Research Chemicals, Orlando, Fla.). To determine whether the process of phagocytosis influenced the penetration of streptomycin, the macrophage cultures were also incubated with the tritiated streptomycin during phagocytosis of either *Staphylococcus aureus*, yeast cells, or polystyrene latex particles (PLP; Difco). The staphylococci were grown at 37 C in Trypticase Soy Broth (BBL) for 16 hr and were washed twice with buffered saline and resuspended in the MEM-H₃-streptomycin mixture to be used in the experiment. The yeast cells were nonviable organisms derived from a Zymosan preparation (Fleischmann Laboratories, New York N.Y.). The yeast cells and PLP were also suspended in the tissue culture medium. In both cases, a standard turbidity of 25 units was used (measured at 660 nm in a Klett-Summerson colorimeter). The various combinations of macrophage cultures, particles, and tritiated streptomycin, and the incubation periods involved are described in the text.

*Radioautography.* After incubation, the cover slip cultures were rinsed in MEM and fixed in a saturated aqueous solution of picric acid for several minutes. Picric acid was chosen as the fixative since it reacts with derivatives of streptomycin in a stoichiometric manner to give water-insoluble precipitates (Johnson, personal communication). This is an important consideration, since water-soluble compounds would have a tendency to leach out of the cells during the radioautographic processing and staining. After fixation, the cover slips were rinsed in distilled water, dried, and mounted on glass slides with Permoun, with the cell monolayers facing upwards.

The macrophage cultures were prepared for autoradiography in a dark room equipped with Wratten series no. 2 safelights. Nuclear emulsion (NTB-3, Eastman-Kodak Co., Rochester, N.Y.) was liquefied in a water bath at 42 C and was stirred gently (to release trapped air bubbles) for 1 hr before use. Slides were prewarmed to 42 C and dipped in emulsion for 2 sec. Excess emulsion was drained and wiped off the back of the slides with a soft cloth. Coated slides were then allowed to dry in a horizontal position in an enclosed chamber through which air circulated. Humidity in the chamber was kept relatively high to prevent cracking of the emulsion during the drying procedure (12). Slides were placed in light-tight slide boxes containing a desiccant and were stored at 4 C. After the exposure periods of 4 weeks, the slides were developed in D-19 developer (Eastman-Kodak Co.) equilibrated to 20 C. The autoradiograms were stained by the Giemsa method after photographic development.
Protein determinations and radioactivity measurements. The protein content of the macrophage cultures was determined by the method of Lowry et al. (13), modified for tissue cultures by Oyama and Eagle (18). The cells were digested in 1 N NaOH and prepared for liquid scintillation counting in the dioxane-toluene-naphthalene-thixotropic gel mixture of Tye and Engel (20). Radioactivity was determined in a model 574 Tri-Carb spectrometer (Packard Instrument Co., Downers Grove, Ill.) and is expressed as counts per minute per milligram of protein.

Chromatographic assay of tritiated streptomycin. The commercial preparation of labeled antibiotic was subjected to chromatographic analysis to insure that the radioactivity was associated with hydrogen atoms of tritiated dihydrostreptomycin and not with any degradation products. A sample of the H\textsuperscript{3}-dihydrostreptomycin was added to carrier dihydrostreptomycin (Nutritional Biochemicals Corp., Cleveland, Ohio) and spotted on Whatman no. 1 filter paper strips in 10-\textmu g quantities. After equilibration overnight, the mixture was chromatographed in a solvent mixture composed of n-butanol (100 ml), water (14 ml), and p-toluenesulfonic acid (2.3 g) for 48 hr. After drying, the paper strips were cut into 10-cm lengths and placed on the agar surface of large petri plates containing Streptomyacin Assay Agar (Difco) which had been seeded with a lawn of Bacillus subtilis (ATCC 6633). After incubation for 3 hr at 37 C, the paper strips were removed and the plates were incubated for another 24 hr to allow the outgrowth of the lawn. The plates were examined for zones of inhibition of bacterial growth, which were then correlated with the distance from the origin of the chromatographic paper strips. The strips were cut at intervals of 2.5 cm from the origin and eluted in 2.0 N NaOH held at 60 C overnight. The residue of paper was removed by centrifugation, and 0.2-ml samples were prepared for liquid scintillation counting in the DTN mixture of Tye and Engel (20) to which no thixotropic gel was added. Radioactivity of the samples was measured as described. In all cases, the peak of radioactivity corresponded exactly with the zone of bacterial inhibition (Fig. 1). This control experiment provided unequivocal evidence that the radioactivity was associated only with the active form of the antibiotic.

RESULTS

Incubation of the mouse peritoneal macrophages for relatively short periods of time did not result in the intracellular accumulation of tritiated streptomycin either in the absence or in the presence of particulate materials. Macrophages incubated with H\textsuperscript{3}-streptomycin alone (5 \textmu g/ml) for 1 hr demonstrated no intracellular radioactivity as visualized by radioautography. Cells incubated with Staphylococcus aureus, yeast cells, or polystyrene latex particles and the labeled antibiotic for 45 min also failed to accumulate any radioactive material, in spite of the fact that phagocytosis had taken place in a normal fashion. Microscopic examination of the autoradiograms showed that, although most cells were engorged with the particles, no radioactive streptomycin had penetrated the cell membrane. In view of the fact that previous experiments with the isolated rat liver suggested a facilitated uptake of streptomycin by Kupfer cells during phagocytosis of bacteria (17), the results obtained with the peritoneal macrophages were surprising. Several possibilities which might account for the results were tested experimentally. First, it was considered that a concentration gradient might be necessary in order for the antibiotic to be accumulated intracellularly; i.e., there might be a necessity for a relatively high concentration of streptomycin in the extracellular menstruum. Second, in view of the short incubation period which had been employed, it was considered that a longer period of contact between the phagocytic cells and the H\textsuperscript{3}-streptomycin might be required. To test these possibilities, experiments were performed in which the incubation period with H\textsuperscript{3}-streptomycin was extended to 20 hr and 30 \textmu g/ml of carrier dihydrostreptomycin was added to the tissue culture fluids. This concentration of antibiotic was chosen since it represents the upper level attained in the blood during therapeutic situations (8). In spite of these experimental manipulations, no significant quantities of streptomycin were accumulated intracellularly. Table 1 shows the radioactivity of the peritoneal macrophages after incubation with the H\textsuperscript{3}-streptomycin alone, or with the tagged antibiotic and either S. aureus or yeast cells for 20 hr. It can be seen

![Graph showing correlation of radioactivity and bactericidal activity of dihydrostreptomycin eluted from chromatographic paper strips.](http://jb.asm.org/)

**Fig. 1. Correlation of radioactivity and bactericidal activity of dihydrostreptomycin eluted from chromatographic paper strips.**
TABLE 1. Impermeability of cultured peritoneal macrophages to H3-streptomycin in the absence or presence of particulate materials in the extracellular menstruum

<table>
<thead>
<tr>
<th>Incubation mixture</th>
<th>Radioactivity (counts per min per mg of protein)</th>
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<tbody>
<tr>
<td></td>
<td>Menstruum</td>
</tr>
<tr>
<td>H3-streptomycin</td>
<td>&gt;2 × 10⁴</td>
</tr>
<tr>
<td>H3-streptomycin + Staphylococcus aureus</td>
<td>&gt;2 × 10⁴</td>
</tr>
<tr>
<td>H3-streptomycin + yeast cells</td>
<td>&gt;2 × 10⁴</td>
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* Cell cultures incubated at 37°C for 20 hr. In all cases, the MEM tissue culture fluid contained H3-streptomycin (5 μc/ml) and carrier streptomycin (30 μg/ml).

Evidence has been presented which suggests that cultured mouse peritoneal macrophages are impermeable to tritiated dihydrostreptomycin and antibiotic represented approximately 0.5% of the total added.

The radioautographic data also show that negligible quantities of streptomycin entered the macrophages. Figure 2 shows a normal culture of peritoneal macrophages before exposure to either the tagged antibiotic or particulate materials. Figure 3 is an autoradiogram prepared from a cell culture incubated with H3-streptomycin alone, and Fig. 4 and 5 are autoradiograms of the macrophage cultures incubated with H3-streptomycin and S. aureus and yeast cells, respectively. The same results were obtained with polystyrene latex particles. It is clear that the peritoneal macrophages were impermeable to the antibiotic either in the absence of particulate materials, or in the presence of particles which were subsequently phagocytized. These results indicate that the process of phagocytosis did not facilitate the uptake of streptomycin by the mouse peritoneal macrophages. In addition, the experimental results show that the cultured macrophages remained essentially impermeable to the antibiotic for a period of 20 hr.

DISCUSSION

that the radioactivity for all three groups was essentially the same, i.e., ≈ 10,000 counts per min per mg of protein. This level of radioactivity is negligible when one calculates the ratio of intracellular to extracellular activity. The extracellular menstruum after the addition of 5 μc/ml of H3-streptomycin had a radioactivity of > 2 × 10⁴ counts per min per ml. Therefore, the intracellular radioactivity for all three groups was essentially the same, i.e., ≈ 10,000 counts per min per mg of protein. This level of radioactivity is negligible when one calculates the ratio of intracellular to extracellular activity. The extracellular menstruum after the addition of 5 μc/ml of H3-streptomycin had a radioactivity of > 2 × 10⁴ counts per min per ml. Therefore, the intracellular menstruum represented approximately 0.5% of the total added.

FIG. 2. Cultured mouse peritoneal macrophages; 3-day normal cell culture. Giemsa; × 1,280.

FIG. 3. Autoradiogram of peritoneal macrophage culture incubated with tritiated streptomycin (5 μc/ml) for 20 hr. Giemsa; × 1,280. Black particles seen outside of the cells are exposed silver grains denoting loci of radioactivity. Note the absence of exposed grains within the macrophages.
do not show any evidence of intracellular accumulation after lengthy periods of contact with significant quantities of the antibiotic in the extracellular menstruum. It should be noted that this conclusion is based either on the absence of radioactivity or on the morphological evidence provided by radioautography and not, as is usually the case, on bactericidal effects of the antibiotic on intracellular microorganisms. A recent publication by Holmes et al. (9) provides good evidence that peripheral white blood cells remain impermeable to streptomycin and penicillin for as long as 24 hr. This conclusion was reached without the complication of intracellular digestion of the bacteria, since the cells employed were obtained from patients with a fatal granulomatous disease (3) which is characterized by an inability of the blood leukocytes to digest phagocytized microorganisms. Our results therefore would appear to be in agreement with theirs, and suggest further that all mobile phagocytic elements are relatively impermeable to streptomycin.

The findings that phagocytosis of microorganisms or other particulate material did not facilitate the uptake of streptomycin suggests that the "piggy-back" phagocytosis phenomenon does not occur with mouse peritoneal macrophages. This is not consistent with the results of previous experiments with the isolated perfused rat liver, in which evidence was obtained indicating that bactericidal quantities of streptomycin accumulated within Kupffer cells during the phagocytosis of particulate matter. It is difficult to account for this apparent discrepancy of results obtained with the mobilized peritoneal macrophages on the one hand and the fixed phagocytic cells of the liver on the other. Several possibilities however, can be advanced. It may be that the fixed phagocytic elements have different permeability properties than do the mobile phagocytic cells, owing to variations in the configuration of the cell membranes. Another possibility is that the physicochemical properties of dihydrostreptomycin (used in the present experiments) and streptomycin sulfate (used in the liver perfusion experiments) are sufficiently different to explain the apparent differential permeability of the two types of phagocytic cells. If this were the case, it would be the chemical configuration of the two streptomycin derivatives and not the permeability.
properties of the cells which would account for the results.

It is not likely that different degrees of pinocytosis can account for the differential response of fixed and mobile phagocytic elements, although this remains a possibility. Cohn and Benson showed that pinocytosis of serum proteins and lysozyme by cultured mouse peritoneal macrophages is a reversible process related directly with the concentration of serum in the tissue culture menstrum (5, 6). If this concentration effect of serum also holds for pinocytosis of low molecular weight compounds such as streptomycin, then the cultured macrophages in our experiments should have accumulated streptomycin to a greater degree than the Kupfer cells of the perfused liver. This supposition is based on the fact that the serum concentration employed was higher with the peritoneal macrophages (40%) than with the perfused liver (25%). As has been described, however, streptomycin penetrated the Kupfer cells but not the macrophages. The intracellular accumulation of streptomycin by the liver cells, moreover, occurred only during phagocytosis of particulate materials. Therefore, the only other explanation which would invoke the pinocytic process is the assumption that the two types of phagocytic cells differ with respect to the kinds of compounds which can be concentrated by this mechanism.

On the basis of these results and those of others (4, 10, 15), it may be concluded that the use of streptomycin to inhibit extracellular multiplication of streptomycin-sensitive microorganisms is a valid technique for studying intracellular bacterial digestion when either blood leukocytes or peritoneal macrophages are used as the phagocytic cells. On the other hand, when fixed phagocytic elements of the liver, spleen, or lymph nodes are employed for the same types of investigation, it may be judicious to avoid the use of extracellular streptomycin, since there is tentative evidence that these cell types may accumulate the antibiotic intracellularly during phagocytosis.

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LITERATURE CITED