ELECTRON-AUTORADIOGRAPHIC DEMONSTRATION OF THE INTRA- AND EXTRACELLULAR BACTERICIDAL ACTIVITY OF NEUTROPHILS

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The intracellular bactericidal activity of polymorphonuclear leukocytes (neutrophils) is usually determined by counting colonies after lysis of phagocytes, which have ingested bacteria, in distilled water, and by inoculating agar with the lysis products [5-7]. This method has disadvantages, which may introduce considerable errors into the measurements. During separation of the neutrophils from the uningested bacteria, the neutrophil fraction contains not only phagocytosed bacteria, but also those adsorbed on the surface, and the ratio between the numbers of ingested and adsorbed bacteria is unknown. Many bacteria, especially staphylococci, tend to form clumps. When seeded on agar a clump, like a single bacterial cell, forms one colony, and this significantly distorts the value of the ratio between living and dead bacterial cells.

We have attempted to overcome these disadvantages by using autoradiography to estimate the state of the bacteria.

EXPERIMENTAL METHOD

The suggested method consists essentially of adding ^{3}H-uridine, an RNA precursor, for a short time to the incubation mixture of leukocytes and bacteria. These bacteria which have been ingested by neutrophils, and which still remain alive at the time of addition of the ^{3}H-uridine and continued to grow [4] will take up the radioactive precursor and become

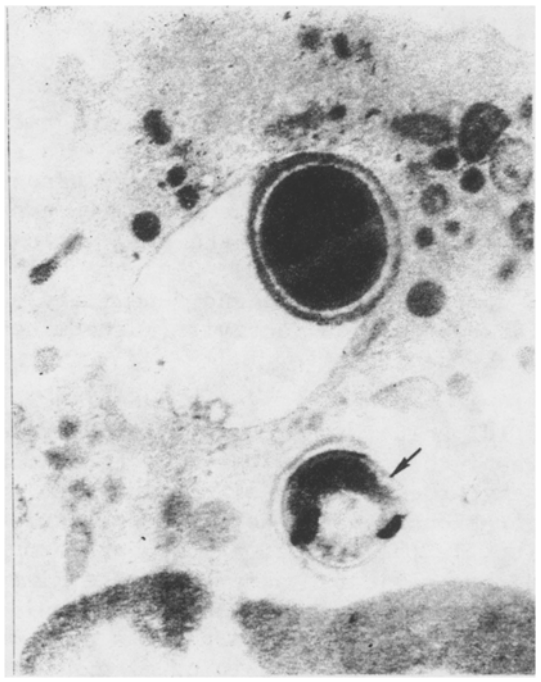

Fig. 1. Neutrophils with phagocytosed staphylococci. One staphylococcus is alive and labeled with three grains of silver (arrow); another, contained in an extensive phagosome, is unlabeled. Here and in Figs. 2 and 3: magnification 30,000.

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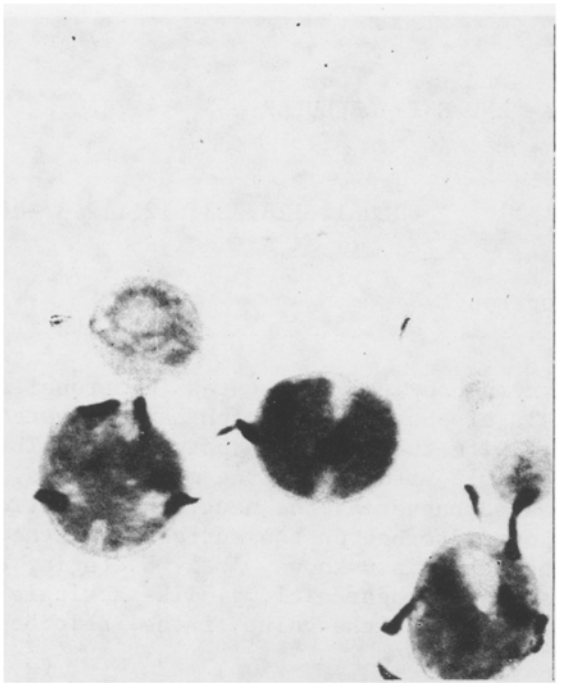

Fig. 2

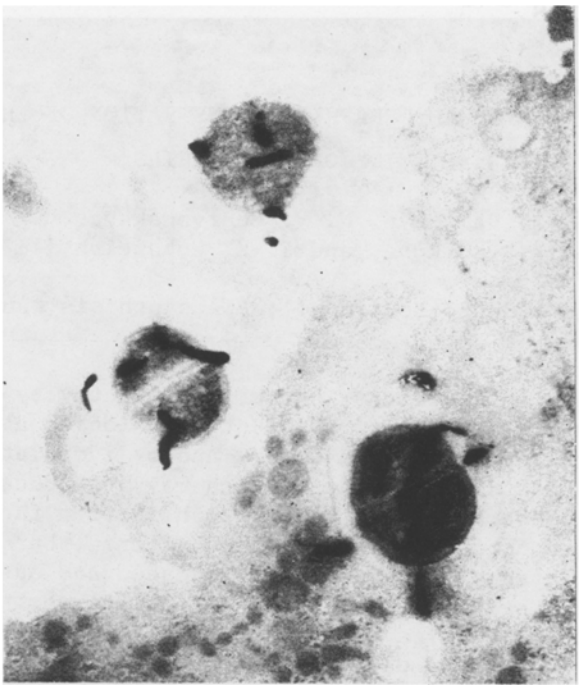

Fig. 2

Fig. 2. Labeled and unlabeled staphylococci in the intercellular space.

Fig. 3. Neutrophil with staphylococci ingested and adsorbed on the cytoplasmic surface.

labeled in the autoradiographs. Dead bacteria, or at least those which are not growing, will not take up the uridine and will remain unlabeled. By counting the numbers of labeled and unlabeled bacteria in phagosomes, without introducing the error mentioned above, it is possible to estimate the intracellular bactericidal activity of neutrophils.

By making preparations by the method described below, the presence not only of phagocytosed, but also of uningested microbial cells in them can be identified. Our first attempts to develop the method did in fact show that these prebacteria also are subjected to the action of neutrophils, and the intensity of that action can be characterized by the ratio between the numbers of labeled and unlabeled bacteria in the intracellular space. Thus, by analyzing a single preparation, it is possible to measure three very important characteristics of the antimicrobial action of neutrophils (Figs. 1 and 2): 1) the ingestive capacity, determined as the number of leukocytes which have ingested bacteria — the phagocytic index (PI), and the number of bacteria ingested — the phagocytic number (PN); 2) intracellular bactericidal activity, i.e., the ratio between the numbers of living and dead (labeled and unlabeled) bacteria in neutrophils; 3) extracellular bactericidal activity, determined as the ratio of labeled to unlabeled bacteria between the neutrophils.

Blood was collected in test tubes containing 1 ml of a neutralized 3% solution of EDTA to 10 ml of blood as the anticoagulant and 1 ml of 10% gelatin solution, to accelerate erythrocyte sedimentation. The tube containing blood was incubated for 30 min, after which the layer of plasma with leukocytes, formed above the erythrocytes, was withdrawn. The leukocytes were washed twice with medium 199, the cell concentration adjusted to $5 \cdot 10^{-6}$/ml, and a suspension of bacteria in the same medium was added to it. In the present experiments *Staphylococcus aureus* and *Bacillus pyocyaneus* cells, isolated from patients, were used. The mixture of leukocytes and bacteria in the ratio of 1:10 was incubated in the presence of opsonins (serum) or in their absence, at 37°C for 30 min, after which ^{3}H-uridine was added in a dose of 5 μCi/ml for *S. aureus* and 20 μCi/ml for *B. pyocyaneus*. The mixture containing *S. aureus* was incubated with uridine for 5 min, that containing *B. pyocyaneus* for 10 min. The incubated mixtures were then quickly cooled by immersing the tubes in running water.

The cooled incubation mixture was centrifuged at 3000 rpm for 20 min. Under these conditions uningested bacteria were sedimented along with leukocytes. The residue was fixed in 1% glutaraldehyde solution in cacodylate buffer, then washed twice with Ringer's solution, using centrifugation under the same conditions. The residue was embedded initially in gelatin and later in epoxide resins by the method described previously [1].

Autoradiographs of semithin and ultrathin sections were prepared [2, 3]. Semithin sections were exposed in darkness for 5 h in the experiments with *S. aureus* and 48 h in the experiments with *B. pyocyaneus*. The ultrathin sections were exposed for 10 days and 1.5 months, respectively.

The ingestive capacity of neutrophils and their intra- and extracellular bactericidal activity can also be analyzed in autoradiographs of semithin sections. However, this requires a microscope which will allow the preparations to be illuminated simultaneously through the condenser and through the objective. If the incident light through the objective is bright enough the grains of silver will reflect it intensively and will appear as luminous points against the background of the cells and bacteria. Under these conditions it is easy to distinguish between labeled and unlabeled bacteria, even if located close together. If only transmitted light is used, these bacteria which take up the stain will darken to some extent the grains of silver (which are black with this method of illumination) and differentiation of labeled and unlabeled bacteria will be less reliable. The accuracy of the analysis is greatly increased by the use of electron-microscopic autoradiographs (Figs. 1 and 2; Fig. 3), but this kind of work, of course, is much more laborious. It will be clear from Figs. 1-3 that in the suggested method the possibility of error due to aggregation of the bacteria is eliminated, even of those which, like staphylococci, are extremely inclined to aggregate. Identification of bacteria ingested by phagocytes, as formed on their surface, and present in the intercellular space, likewise presents no difficulty (Fig. 3).

The suggested method reflects not only the bactericidal, but also the bacteriostatic action of neutrophils, and for that reason the use of this method provides additional information about the function of these cells which cannot be obtained by other methods of investigation.

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