AUTORADIOGRAPHIC DETECTION OF INTRACELLULAR AND EXTRACELLULAR BACTERICIDAL ACTION OF NEUTROPHILS

A. A. Pal'tsyn, A. K. Badikova,
N. V. Chervonskaya, T. S. Ustinova,
and É. K. Uchaneishvili

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A technique of electron-autoradiographic demonstration of the bactericidal ability of neutrophils was described previously [2]; the basis of the method is that a short time after a suspension of neutrophils is added to a suspension of bacteria under conditions favoring phagocytosis, the RNA precursor ^{3}H-uridine is added to the incubation mixture for a short time. After preparation of autoradiographs the level of RNA synthesis is determined in phagocytosed and free bacteria, and this provides an indicator of their viability during interaction with neutrophils.

This paper suggests a method which substantially increases the accuracy of determination of bactericidal activity by the use of light microscopy and autoradiography on semithin sections. Changes in the method compared with that described previously are of a minor nature: the preparations are exposed in darkness for 4 days rather than 5 h, and they are developed by paraphenylenediamine developer, suggested previously for developing electron-microscopic autoradiographs [1]. This technique is of fundamental importance for the autoradiographic determination of intracellular and extracellular bactericidal activity. The level of bactericidal activity is expressed as the ratio of living to dead bacteria, i.e., the ratio of the number of labeled and nonlabeled bacterial cells in a particular zone (in phagosomes adsorbed on the surface of the phagocyte or in the intercellular space). If the autoradiographs are developed by ordinary developers, such as the widely used D-19, very short exposure times of the preparations in darkness must be used, so that there should be one or, at most, two grains of silver above a labeled bacterium. With a larger number of grains of silver, because of their considerable size and the small size of the bacterium located beneath them (in the case of cocci), the bacterium becomes indistinguishable, being almost or completely covered by grains, as in Fig. 1 (because of the small size of the objects under discussion, namely grains of silver and bacteria — the article is illustrated by electron-microscopic autoradiographs).

The use of electron-microscopic autoradiographs developed with paraphenylenediamine (producing smaller grains) and exposed for a long period, so as to facilitate the appearance of a larger number of grains above a living bacterium, in order to study bactericidal activity showed that the viability of bacteria was reduced gradually under the influence of the bactericidal agents, depending on the duration of their action, their concentration, and the state of the bacterium itself (Fig. 2). This conclusion is proved by the fact that not only unlabeled and intensely labeled bacteria are present in an electron-microscopic autoradiograph prepared under these conditions, but also microorganisms with intermediate densities of labeling. This diversity of the degree of incorporation of the label into individual microorganisms can be regarded as an advantage of the method of autoradiography as an indicator of microbial viability compared with bacteriological methods, which divide them into living and dead only, and thus distorting and schematizing what in fact is taking place in the microorganisms in reality. Let us return to the problem of development. If the presence of viability is judged from the appearance of one or two grains of silver above a bacterial cell, preparations must be developed when this kind of label is found only above the most viable microorganisms. All microbial cells with somewhat reduced viability will be classed as dead (unlabeled). This will lead to an erroneous exaggeration of the true degree of bactericidal

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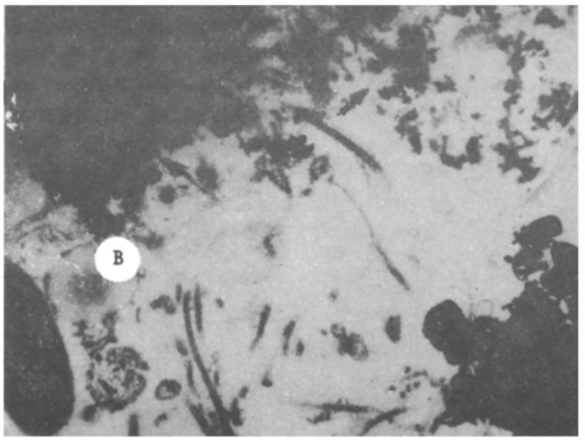

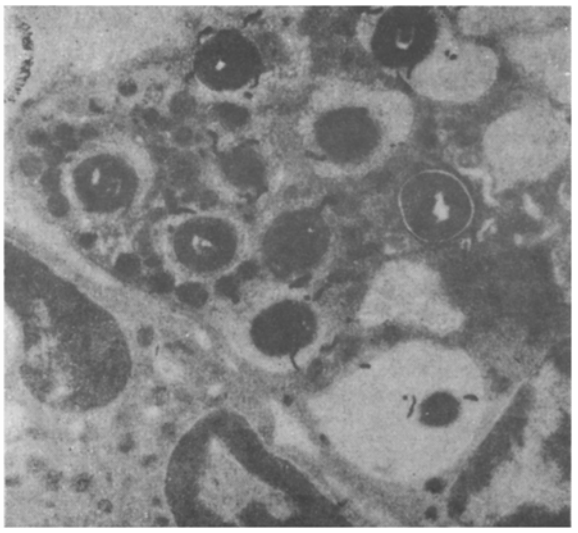

Fig. 1 Fig. 2

Fig. 1. Electron-microscopic autoradiograph reflecting RNA synthesis in bacteria. D-19 developer. Unlabeled bacterium (B) can be clearly seen. Labeled bacteria covered by grains of silver, either almost completely (short arrow), or completely (long arrow). The number of grains cannot be determined. Magnification: 15,000.

Fig. 2. Electron-microscopic autoradiograph reflecting RNA synthesis (viability) of staphylococci ingested by a neutrophil (N). Paraphenylenediamine developer. Differences in density of grains of silver above individual cocci are evidence of a difference in their state. The number of grains can be counted above each bacterial cell. Magnification: 12,000.

activity of the agent concerned. Such a mistake will be clearly seen if electron-microscopic and light-microscopic autoradiographs prepared from the same material are compared: the number of living bacteria in the former will be much greater than in the latter.

Another mistake in the determination of viability of bacteria, also arising with the use of coarse-grain developers, but due to the random character of radioactive fission, is more dangerous. It may appear that a bacterium above which one grain of silver is found contained a smaller total number of radioactive atoms than an unlabeled bacterium located next to it, but in the first bacterium one atom was able to disintegrate in a short exposure time and emitted a β-particle in the direction of the photographic emulsion, whereas in the second bacterium, either fission did not take place or the β-particle was directed away from the photographic emulsion. Besides the situation described above, coarse-grain development deprives the autoradiographic method of one of its advantages, namely that quantitative parameters of viability of individual bacterial cells are measured.

Fine-grain development with paraphenylenediamine was not used to prepare light-microscopic autoradiographs because the grains formed by this substance are invisible if the preparations are examined by the usual method. If, however, ordinary illumination of the preparations from below through a condenser is combined with a flow of light directed from above through the objective, the fine grains of silver, reflecting light falling on them, become fully distinguishable because of the bright halo surrounding them. With this type of illumination they appear like luminescent points against the background of the dark blue bacterium, whereas large grains in preparations illuminated only through the condenser have the appearance of black dots, which are less distinguishable against the same background.

The small size of the grains formed by paraphenylenediamine makes it possible to see the grains themselves and also bacteria located beneath them clearly, even when not one or two, but several grains are located above a bacterium.

Labeling of viable bacteria in light-microscopic autoradiography by many (up to 10-20) grains of silver sharply reduces the contribution of the two errors examined above and allows inequality of action of bactericidal factors to be studied depending on time. Such a preparation, of course, cannot replace electron-microscopic autoradiography, which demonstrates

the detailed structure of bacteria and of the phagocyte and, because of this, enables conclusions to be drawn regarding the mechanisms of bactericidal activity (for example, the presence or absence of marked degranulation). However, in cases where only the degree of intra- and extracellular bactericidal activity needs to be determined, without going into more details, the method of light-microscopic autoradiography with a fine-grain developer actually has a definite advantage over electron-microscopic autoradiography, for, with much less work, it enables an investigation to be conducted on a large number of objects (an essential condition for obtaining reliable data).

The suggested method and the results obtained by it show that, in a certain sense, electron-microscopic autoradiography and even the intermediate variant described here (light-microscopic autoradiography with electron-microscopic development) may actually prove to be more sensitive than the traditional light-microscopic autoradiography. Of course, in its standard meaning, sensitivity (minimal detectable concentration of radioactivity) of light-microscopic autoradiography is higher than that of electron-microscopic autoradiography. However, the possibility of finding minimal differences between concentrations of radioactivity in objects such as bacteria, which evidently must also be called sensitivity, is significantly higher in electron-microscopic autoradiography or in our suggested method of light-microscopic autoradiography with the use of a fine-grain developer.

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POSSIBLE EFFECT OF A MECHANICAL FACTOR ON COMPLETENESS OF DORSAL SKIN RESTORATION IN MICE

E. A. Efimov, T. V. Bukina, and V. E. Kobzar'

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Completeness of skin restoration during healing of full-thickness wounds may vary from a scar to perfect regeneration. This will depend on a number of experimental conditions: the species of animal, the location of the wound defect, and the intensity of contraction of the wound [2]. We know that healing of full-thickness wounds of the dorsal skin in mice and rats usually ends with the formation of a small connective-tissue scar [1, 2]. However, it has been shown experimentally that completeness of skin restoration in mammals can be deliberately influenced. For instance, by restraining wound contraction or by stimulating wound healing with small doses of the antioxidant dibunol, the outcome of skin regeneration can be changed and instead of an epithelized connective-tissue scar, it is possible to obtain regeneration of the correct skin type with the formation of skin derivatives: hairs and sebaceous glands [3, 4]. Such changes in the repair process in the skin have been shown to be possible if action is confined to young regenerating tissues, before granulation tissue has been converted into fibrous tissue [2].

We also know that severe trauma to the tissues of the regenerating amphibian limb by means of needles can stimulate regeneration and affect its completeness [5]. However, the influence of such a mechanical factor on the course and outcome of skin regeneration has not been studied in mammals. In our view, thanks to such procedures, the regenerating tissues

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