Scanning Electron Microscopy of the H37Ra Strain of *Mycobacterium tuberculosis* Exposed to Isoniazid

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The morphology of cells of the H37Ra strain of *Mycobacterium tuberculosis* exposed to 0.5 μg of isonicotinic acid hydrazide (isoniazid) per ml was examined by scanning electron microscopy (SEM). Cells that were exposed to isoniazid for 3 h showed no detectable change, whereas cells exposed to the drug for 24 h exhibited diverse morphological features. From our examination of these SEM pictures, we have reconstructed the probable sequence of morphological changes to be as follows: (i) the wrinkling of the cell surface was ascribed as the earliest observable change, (ii) the cell surface then became very rough and ragged, (iii) eventually the cytoplasmic material was extruded from the cell, (iv) this event produced a collapsed cell, (v) the cells began to fragment, (vi) the fragmented cells then coalesced to form an amorphous mass of cell debris.

Evidence obtained to date suggests that the tuberculocidal action of isoniazid (isonicotinic acid hydrazide; INH) on tubercle bacilli is due to inhibition in the synthesis of the mycolic acids (13–15). The mycolic acids are the major lipid components of the lipoid-rich mycobacterial murein layer, wax D, and trehalose mycolate (cord factor) (1, 4, 8, 11). These cellular fractions are part of the general structure of the mycobacterial cell envelope (7, 10, 11). The INH inhibition of growth of tubercle bacilli involves a lag period of 2 to 36 h (2, 12). Therefore, cells exposed to INH would grow or develop for a single generation time in the absence of mycolic acid synthesis. This should result in a gross imbalance in the composition of the cell envelope which in turn could become evident by morphological changes.

Scanning electron microscopy (SEM) has been used effectively in studying the morphological changes during the growth of other microorganisms (3, 6, 9). In the present study, we have applied this technique to examine the effects of INH on the *Mycobacterium tuberculosis* H37Ra.

**MATERIALS AND METHODS**

**Growth and sampling of bacterial culture.** *M. tuberculosis* H37Ra was grown in Middlebrook 7H9 medium enriched with Tween 80 (polyethylene Sorbitan monoooleate; BBL) and ADC (Difco) on a bench-top environmental shaker, model G-25 (New Bruns-

![Fig. 1. Inhibition of growth of *M. tuberculosis* by isoniazid. A 200-ml culture of H37Ra strain was divided into two 100-ml portions on the fourth day of growth. One set was the control and to the other set was added isoniazid to give a final concentration of 0.5 μg/ml. Growth was followed by measuring absorbancy at 650 nm, and sampling for scanning electron microscopy analysis commenced on the fourth day of growth.](#)
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wick Scientific Co., New Brunswick, N.J.) at 37 C and 150 rpm. Frequent transfers were made to assure well-dispersed cultures. Growth of the bacteria was followed in Nephlo-flasks by measuring absorbancy at 650 nm with a Coleman model 44 spectrophotometer. A 1.0-ml inoculum of a culture of H37Ra having an absorbancy value of 0.33 was used to initiate growth in 200 ml of the 7H9 supplemented medium. When the absorbancy of the new culture reached a value of 0.045 (about 4 days), it was divided into two 100-ml portions in sterile 300-ml Nephlo-flasks. To one set, sterile INH was added to a final concentration of 0.5 
μg/ml, and both control and INH-treated H37Ra cells were incubated on the shaker. The measurement of growth and sampling for SEM were done at 24-h intervals for 7 days. Sampling involved aseptically taking 5.0 ml of the culture and adding it to 0.50 ml of formaldehyde. The formalized samples were then stored at 5 C until sampling was completed.

SEM. To prepare the specimens of H37Ra for SEM, a sample (0.25 ml) of the formalized broth cultures was washed two times with 15 ml of distilled

Fig. 2. Scanning electron micrographs of control cells of M. tuberculosis at two different magnifications. Bar = 1.0 μm.

Fig. 3. Scanning electron micrographs of cells of M. tuberculosis exposed to isoniazid for 24 h. Bar = 1.0 μm.
water by centrifuging. Each sample was then suspended in 1.0 ml of water, left overnight at 4 C, and then centrifuged 30 min at 3,000 rpm onto a piece of microscopic slide (5 by 5 mm) in a 0.25-dram vial (9 by 30 mm). The supernatant fluid was siphoned off, the sample was dehydrated with alcohol and cleared in xylene, and the xylene was replaced with Freon 11 in a critical-point bomb (5; R. S. Merkal et al., Amer. Rev. Resp. Dis., in press). After venting the Freon gas above the critical point, the slide was glued to the mount and coated with gold. All specimens were treated simultaneously so that observed differences between specimens were not due to differences in preparation. These specimens were examined with a Cambridge Stereoscan electron microscope operating at 20 kV. Kodak Ektopore film was used for photomicrographs.

Chemicals. INH was purchased from Calbiochem, Los Angeles, Calif. All other chemicals were reagent grade.

RESULTS AND DISCUSSION

Growth of M. tuberculosis and its exposure to INH. The INH concentration used in this study was 0.5 µg/ml. At this concentration, the mycolic acid synthesis has been shown to be completely inhibited in M. tuberculosis H37Ra in about 60 min (13). The viability of the cells was reduced by 70% after 3 h of exposure to the drug (13). The effect of INH at this concentration on the growth of M. tuberculosis is shown in Fig. 1. Cell growth slowed down after 24 h and was completely halted after about 48 h. When INH was added, the cell population was 3.5 x 10⁸ cells/ml. This value increased to 5.1 x 10⁹ cells/ml (0.73 generation time) after 24 h and to 6.4 x 10⁹ cells/ml (0.92 generation time) after 3 days. We have confined our examination to relatively young cultures of M. tuberculosis (early log growth). This measure was necessary in order to prevent the formation of the extracellular polysaccharides which tended to coat the surface of older cells of M. tuberculosis, thus making SEM examination difficult.

SEM examination of INH-treated cells of M. tuberculosis. Preliminary SEM examination of INH-treated cells indicated that definite morphological changes occurred which might be attributed to the action of the drug. It also indicated to us that very early changes would be difficult to detect (drug exposure to 3 h). Upon viewing the field at low magnification, the morphology of the cells varied from one extreme of healthy cells to the other of cell debris, even in control cultures. This lack of uniform population of cells caused us to examine the cultures under carefully controlled conditions. Therefore, both control and INH-treated cells were derived from the same culture of M. tuberculosis. The growth curve of such an experiment is shown in Fig. 1.

The SEM examination revealed that in general there were some degraded cells in all control specimens and some healthy cells in all INH-treated specimens. However, on the whole, about 95% of the cells in the control specimens

![Fig. 4. Scanning electron micrographs of cells of M. tuberculosis exposed to isoniazid for 96 h. Bar = 1.0 µm.](http://aac.asm.org/)
were healthy, whereas about 70 to 80% of the material in the INH-treated specimens eventually was in the form of unrecognizable cell debris. This difference could be seen as early as 24 h after exposure to INH. Photographs were taken mainly of what could be recognized as cells or cell parts. The ratio of debris to healthy cells increased with time of INH exposure but stayed relatively constant in the control specimens.

The 24-h INH-treated cells showed the most diverse and abundant morphological change. With increase in the exposure time to INH, this change decreased to show mostly fragmented cells or cell debris. Therefore, we have closely examined the cells that were exposed to INH for 24 h to show the various morphological features which may tell us how the degradation of the cells proceeds in the presence of INH.

Figure 2 illustrates the appearance of healthy cells of *M. tuberculosis*. The main features of these cells are the smooth surface and the normal rodlike shape. Some of the diverse structural features of cells exposed to the drug for 24 h are shown in Fig. 3. Figure 3A shows cells that have surface wrinkles, whereas Fig. 3B shows a cell with rough surface features. Figure 3C shows the extrusion of the cytoplasmic material from the rupture point of the cell. This event leads to a collapse of the cells (Fig. 3D and E). It should be mentioned that the extrusion shown in this illustration (Fig. 3C) probably was caused by centrifugation during the preparation of the specimen. Also evident in the INH-treated cells was the presence of cells that were bulging and the ends (Fig. 3D). Occasionally, highly twisted morphology was observed only in INH-treated cells (Fig. 3F). Figure 3G shows a cell that is beginning to fragment. These pictures illustrate the general observation that, within the first 24 h of exposure, the INH-sensitive cells undergo rather dramatic changes in their surface appearance, after which time fragmentation occurs (see Fig. 4A) and they aggregate into large, amorphous cell debris (Fig. 4B). We can conclude that SEM is unable to differentiate the subtle difference between a healthy cell and a cell that is growing in the absence of mycolate synthesis (one exposed to INH for about 3 h [13]). The earliest morphological change that the SEM can detect appears to be the surface wrinkles of cells. The results of this study did not allow us to determine whether or not the morphological changes observed are caused by the INH inhibition of mycolic acid synthesis.

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