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Effects of Isoniazid on Ultrastructure of *Mycobacterium aurum* and *Mycobacterium tuberculosis* and on Production of Secreted Proteins

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Isoniazid (INH), one of the most effective antimycobacterial drugs, specifically inhibits, at an early stage of its action, the biosynthesis of mycolic acids, specific mycobacterial lipids which play a central role in the cell envelope architecture of mycobacteria. In the present study, the consequences of the action of INH on the cell morphology of *Mycobacterium tuberculosis* and *Mycobacterium aurum* were examined. Electron microscopy was used to observe bacilli which were previously treated with either subinhibitory concentrations of INH or the MIC of the drug, leading to a decrease of 20 to 35% (by weight) of their mycolic acid contents. The earlier effect of INH on the ultrastructure of mycobacteria, as revealed by negative staining of bacilli, was the alteration of the bacterial poles; this event was observed prior to the bacteriostatic action of the drug and was accompanied by a release of material from the poles into the extracellular medium. In a later stage of the drug's action, cell deformation occurred and more extracellular material was seen. The material released following the action of the drug on susceptible mycobacterial cells was identified as being almost exclusively composed of proteins. Labeling of amino acids with ^{35}S prior to and during the action of INH on *M. aurum* and subsequent analysis of the labeled proteins led to the conclusion that they consisted of secreted proteins which were up to 20-fold oversecreted in the presence of the drug. Competitive enzyme-linked immunosorbent assay with the secreted 45/47-kDa antigen complex of *M. tuberculosis* demonstrated up to 20-fold oversecretion of these proteins. Taken together, the production of oversecreted proteins following the decrease of the cell envelope mycolate content by INH strongly suggests that mycolic acids may act as a barrier in the export of proteins secreted by mycobacteria.

Isonicotinic acid hydrazide (isoniazid; INH) has been used for the chemotherapy of tuberculosis since 1952, and it remains a key component in multiple-drug treatment regimens that, until recently, seemed close to achieving complete elimination of the disease in developed countries. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and closely related mycobacterial species are exquisitely susceptible to INH: MICs are in the range of 0.02 to 0.2 μg/ml. Other mycobacteria show a broad range of INH susceptibilities, with *Mycobacterium aurum* being one of the most susceptible nontuberculous mycobacteria, while the drug has little or no activity outside the mycobacterial genus (29, 33). The resurgence of tuberculosis and the emergence of multidrug-resistant isolates have focused attention on the need for an improved understanding of the molecular aspects of the pathogenesis of the disease and for elucidation of the factors responsible for the action of the drug and for resistance to the drug (33).

It has been firmly established that INH inhibits mycolic acid synthesis, both in whole cells (27, 30) and in cell extracts (20, 21), and that the inhibition occurs at a very early stage of the action of INH (27, 30). Furthermore, application of genetic tools has recently provided new insights into the mechanisms of action of INH (4, 7, 22, 32) and has demonstrated that one target of INH is an enzyme involved in the mycolic acid biosynthesis pathway (4, 22). These long-chain fatty acids (up to 90 carbon atoms) are the major lipid components of the mycobacterial cell wall skeleton which consists of a peptidoglycan covalently linked to a heteropolysaccharide, arabinogalactan, which in turn is esterified by mycolic acids (15, 17). Mycolic acids are postulated to play a central role in both the cell envelope architecture and permeability (17). Thus, the gross imbalance in the composition of the cell envelope of INH-treated cells may result in fundamental changes in cell physiology. Interestingly, it has been shown that some changes in the cell envelope occur before the bacteriostatic effect of INH, which appears one generation after the addition of the drug (26). These changes include (i) the increase in respiration, compatible with a change in the permeability of the cell envelope (24); (ii) the fragility of the cell wall (29); and (iii) the increased loss of cellular material into the medium and the increase of the ease with which various components could be extracted from the bacteria (31). Other changes such as the loss of the acid fastness of INH-treated cells (16) are fairly slow and may correlate with cell death (29). However, it should be noted that almost all the methods used to establish these changes indicated what happens to a population of bacteria and not to an individual bacterium. Indeed, scanning electron microscopy has shown marked changes of individual cells (28), but these results were obtained with relatively high concentrations of INH and over extended periods of time after the addition of the drug, i.e., after the drug had exerted a bacteriostatic effect.

To address the question of the action of INH on the individual cells, we investigated the morphological changes of neg-

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atively stained cells using electron microscopy throughout the culture phases and determined the molecular compositions of the extracellular and surface-exposed materials of the corresponding treated susceptible and resistant strains of *M. aurum* and the susceptible strain H37Rv of *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. aurum* A^+ (INH-susceptible strain; MIC, 1 μg/ml) and its spontaneous INH-resistant mutant (strain M_2; MIC, 1,000 μg/ml [21]) were obtained from the Institut Pasteur (Paris, France) and grown on 7H9 Middlebrook broth, enriched with 0.5% Casitone and 1% glucose, in shaking cultures at 37°C. The growth curves of the strains were determined by A_{650} measurements.

M. tuberculosis (strain H37Rv, ATCC 27294) was inoculated on Sauton's medium as surface pellicles (100 ml per flask) by the technique of Lapchine and Asselineau (9) in order to obtain comparative inocula. Briefly, cells from the mid-logarithmic growth phase were harvested by decantation, and the medium was discarded. After the addition of pentane and shaking in the presence of glass beads, the suspension was placed in ice. Equal volumes of the supernatant, free of aggregates, were used as inocula, and the cells were grown at 37°C. The growth curve was established by weighing dry bacilli after autoclaving, filtration, and drying (under vacuum in a desiccator).

Treatment of cultures with INH. INH was sterilized by passing it through a 0.22-μm-pore-size filter membrane (Millex GS; Millipore). Appropriate quantities of the drug were directly added from the stock solution to shaken 24-h cultures of *M. aurum* (during the mid-logarithmic growth phase). The MIC of INH was defined as the drug concentration that completely stopped the increase in the A_{650} after a generation time (14 h for both strains) starting from the time of addition of the drug. For biochemical analyses, cells were harvested after 9, 20, 30, and 60 h of exposure to the drug by centrifugation (8,000 × *g*, 20 min); the cell pellets and the corresponding culture filtrates were analyzed as described below. The addition of the drug was omitted in control experiments.

To obtain viable bacteria with the lowest mycolic acid content, *M. aurum* A^+ was grown in the presence of several subinhibitory concentrations of INH (0.1 to 0.9 μg/ml) that was added to the cultures at different times. From the growth curves and the percentage of inhibition of the labeling of mycolic acids, 0.2 μg of INH per ml gave the best results. Consequently, cells were grown in the presence of an initial concentration of 0.2 μg of INH per ml; this was followed by the addition of 0.1 μg of the drug per ml after each increment of the A_{650} of 1. After reaching an A_{650} of 5 (corresponding to the mid-logarithmic growth phase of this strain), 10 ml of culture was taken from the 100-ml treated culture to inoculate a fresh culture medium that contained 0.2 μg of INH per ml. This procedure was repeated three times.

To analyze the effects of INH on *M. tuberculosis*, the medium of the mid-logarithmic-phase culture was poured off and replaced with a fresh INH-containing Sauton's medium (0.1 μg/ml) while the surface pellicles remained attached to the flasks. The bacteria were then grown, with or without shaking, for 2, 18, or 48 h.

Isolation and fractionation of the extracellular and surface-exposed materials. Extracellular materials were obtained from the various culture filtrates. The filtrates of *M. aurum* were first recovered by centrifugation; the supernatant was then filtered through a 6-μm-pore-size Durieux filter no. 111 (Durieux, Paris, France). The filtrates of *M. tuberculosis* were obtained by filtration through a Durieux filter; this was followed by refiltration through a 0.2-μm-pore-size sterile filter (Nalgene, Nalge Co., Rochester, N.Y.).

Surface-exposed materials were obtained by gently shaking the drug-treated and control bacteria with 10-g glass beads (4 mm in diameter) for 1 min; the materials resulting from the mechanical treatment of the cells were suspended in distilled water and filtered through a Durieux filter and then through a 0.2-μm-pore-size sterile filter (Nalgene) as described previously (18).

Macromolecules of extracellular and surface-exposed materials were obtained by concentrating the different filtrates under vacuum to 1/10 of the original volume. A portion (1/10) of the filtrates was precipitated overnight at 4°C with 6 volumes of cold ethanol to isolate the crude polymers (11). The polymers were recovered by centrifugation (14,000 × *g*, 1 h), extensively dialyzed against 0.2 M NaCl and then distilled water, and analyzed for their carbohydrate and protein contents by colorimetric assays (8, 14). The percentage of carbohydrate and the glycosyl composition were determined by gas-liquid chromatography with erythritol as the internal standard (11).

Quantification of lipids and determination of the lipid compositions of the extracellular and surface-exposed materials were performed with the organic phase obtained by adding chloroform and methanol to the remaining portion (9/10) of filtrates to obtain a partition mixture composed of chloroform-methanol-water (3:4:3; by volume) as described previously (11).

SDS-PAGE. Samples (100 μg) of the dialyzed macromolecules were analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions with sodium dodecyl sulfate (SDS). Thus, 12 or 15% polyacrylamide gels were used for the separation of the polypeptides. The gels were stained with Coomassie blue. Molecular weight markers were obtained from GIBCO BRL (Life Technology Inc., Gaithersburg, Md.).

Western blot analysis. In the Western blot (immunoblot) experiments, the polypeptides separated by SDS-PAGE in a 10 to 20% gradient of polyacrylamide were transferred onto nitrocellulose sheets (BA85; Schleicher & Schuell) in a semidry blotting apparatus according to the instructions provided by the manufacturer (Ancos, Olstykke, Denmark), and reactive sites were blocked with 0.5% (wt/vol) Tween 20 in phosphate-buffered saline (PBS; pH 7.6). The blots were incubated for 2 h at room temperature (or overnight at 4°C) with monoclonal antibodies (MAbs) directed against specific secreted proteins: HYT 6 and HYT 27 (3), HBT 12 and HBT 10 (13), and L24b5 (2). The MAbs were diluted in PBS containing 0.37 M NaCl and 0.05% (wt/vol) Tween 20 (the culture supernatants were diluted 1:25, and the protein A culture supernatant MAbs were diluted 1:500). Bound MAbs were detected by using horseradish peroxidase-coupled rabbit anti-mouse immunoglobulins (P216; Dakopatts, Glostrup, Denmark) at a dilution of 1:1,000. A color reaction was obtained by using 3,3′,5,5′-tetramethylbenzidine.

Metabolic labeling of proteins. Protein synthesis during the treatment of *M. aurum* A^+ with INH was monitored by metabolic labeling with Pro-mix L-^{35}S in vivo cell labeling mix composed of an amino acid mixture of L-[^{35}S]methionine and L-[^{35}S]cysteine (specific radioactivity, >1,000 Ci/mmol; Amersham). Labeling was carried out in 5-ml cultures (50 μCi/ml) at time intervals of −4 to 0 h, 0 to 2 h, 4 to 6 h, 6 to 9 h, and 0 to 9 h; the initial point (0 h) was defined as the time of addition of INH. Labeled cells were recovered by centrifugation (8,000 × *g*, 15 min). For the time interval of −4 to 0 h, labeled cells were recovered by centrifugation and were resuspended in an INH-containing fresh unlabeled medium and incubated for 9 h at 37°C.

The various culture filtrates were concentrated (10 times) on a Centricon 10 membrane (Amicon Inc., Witter, Germany) and were analyzed by SDS-PAGE. The gels were autoradiographed with a FujiX Bas 1000 phosphoimaging system and TINA PCBAS software.

Labeling of mycolic acids. Mid-logarithmic-phase cultures of *M. aurum* were treated with INH for 30 min; aliquots of 10-ml cultures were then incubated with 2 μM labeled [1-^{14}C]sodium acetate (specific radioactivity, 1.9 GBq/mmol; Amersham) for 30, 60, and 90 min. The 30-min preincubation period with INH was omitted for the controls. The labeling was stopped by adding potassium hydroxide (final concentration, 1% [wt/vol]), and the bacteria were collected by centrifugation (8,000 × *g*, 20 min). The cells were then saponified to isolate the mycolic acids (see below).

Subcellular fractionation of *M. aurum*. Labeled cells of *M. aurum* A^+ were disrupted by sonication (Vibra-Cell, microtip 5, 60% duty cycle, 10 on and then 20 s off) four times at 0°C. The bacterial cells resisting this treatment, as well as the cell envelopes, were recovered by centrifugation (13,000 × *g*, 20 min). The supernatant was assumed to consist mainly of a mixture of cytoplasmic components.

Monitoring of the isocitrate dehydrogenase activity. The isocitrate dehydrogenase activity was determined by spectroscopy. To 62.5 μl of culture filtrate of *M. aurum* was added 300 μl of isocitrate (10 μM) and 25 μl of $MnCl_2$ (0.1 μM). The reaction mixture was maintained at room temperature for 5 min, 300 μl of NADP (0.9 mM) was added to the cuvette, and the A_{340} was read 3, 5, and 30 min later. In the negative and positive controls, the culture filtrate was replaced by the noninoculated Sauton's medium and cytosolic fraction, respectively.

Competitive ELISA. A competitive enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of the 45/47-kDa antigen complex of *Mycobacterium bovis* BCG and *M. tuberculosis* (10, 23) in the different extracellular materials derived from control and INH-treated cells. The method has been described previously (23). Briefly, the purified antigen complex was immobilized on a plastic surface (1 μg/ml in carbonate buffer). The optimal dilution of the rabbit serum to obtain a sensitive titration curve, i.e., the measurement of the remaining antibodies after their incubation with the preparation to be assayed, was determined to be 1/6,000. The sensitivity of the assay was in the 2-ng/ml range when phosphatase-labeled antibodies directed against rabbit immunoglobulin G (heavy and light chains) were used to determine the amounts of bound anti-45/47-kDa complex antibodies.

Hydrolysis conditions. The mycolic acid contents of control and drug-treated cells were determined on delipidated bacteria. Wet cells were extracted twice with chloroform-methanol (2:1 [vol/vol]) for 3 days, and the delipidated bacteria were saponified as described previously (6). The fatty acids were isolated and methylated, and mycolic methyl esters were recovered by precipitation of the ether solution of lipids by 10 volumes of methanol and weighed.

Samples (1 mg) of dialyzed extracellular and surface-exposed polymers were routinely hydrolyzed with 1 M trifluoroacetic acid solution at 110°C for 2 h. The hydrolysates were dried under nitrogen and subjected to trimethylsilylation (25).

Chromatography. Thin-layer chromatography was used to determine the molecular composition of the lipid extracts derived from the extracellular and surface-exposed materials (19). This analytical method was also used to analyze the mycolate profile of control and drug-treated bacteria as described previously (6).

Gas-liquid chromatography was performed on trimethylsilyl derivatives of monosaccharides with a Girdel chromatograph (model G 30) equipped with a fused-silica capillary column (25 m in length by 0.22 mm in internal diameter) containing WCOT OV-1 (0.3-μm film thickness; Spiral, Dijon, France). A temperature gradient of 100 to 280°C at 3°C/min was used.

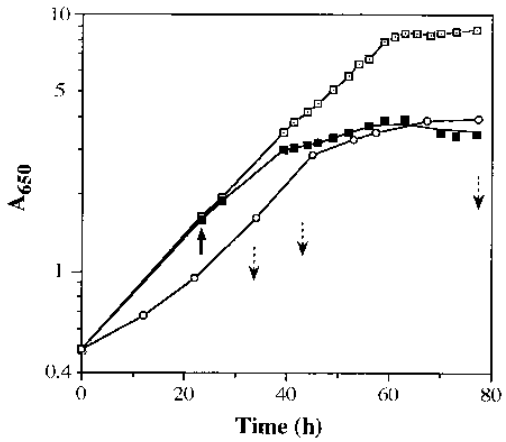

FIG. 1. Growth curves of *M. aurum*. A 200-ml culture of strain A^+ was divided into two 100-ml portions after 24 h of growth. One set was the control (□), and to the other set was added INH (solid arrow) to give a final concentration of 1 μg/ml (■). In a parallel experiment, cells were grown in the presence of 0.2 μg of INH per ml; after each increase in the A_{650} of the culture of 1, 0.1 μg of the drug per ml was added to the culture. After reaching an A_{650} of 5, 10 ml of culture was taken from the 100-ml treated culture to inoculate a fresh culture medium that contains 0.2 μg of INH per ml. This procedure was repeated three times, and the growth curve for the third culture is illustrated (○). The materials derived from the various experiments were collected at different times (dashed arrows) and analyzed by electron microscopy.

Electron microscopy. Samples of control and drug-treated cultures of *M. aurum* A^+ were examined 9, 20, and 55 h after the addition of INH; the supernatants of the control and INH-treated cultures (55 h) were also examined both before and after filtration through a 6-μm-pore-size filter. The different materials were negatively stained with phosphotungstic acid (4% [vol/vol]) and were observed with a JEOL JEM 1200 EX electron microscope operating at 80 kV. For safety reasons, cells of *M. tuberculosis* were first fixed overnight with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 4°C and were washed prior to the staining. Following this treatment, no growth was observed on the culture medium.

For thin sectioning, the cells were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) at 0°C for 2 h, postfixed for 1 h at 0°C with 1% osmium tetroxide in the same buffer, dehydrated through passages in various ethanol solutions (containing 0 to 100% ethanol), and then embedded in Epon 812. Thin sections were stained as described previously (5).

RESULTS

Among the nonpathogenic species, *M. aurum* is one of the most susceptible to the action of INH. Being a fast-growing mycobacterium, it represents a good model for studying the effects of INH on mycobacterial metabolism. In the present study, strain A^+, for which the MIC is 1 μg/ml (Fig. 1), and its spontaneous mutant M_2, which exhibits a high degree of resistance to INH (MIC, 1,000 μg/ml), were used.

In order to correlate the action of INH on whole cells of *M. aurum* and the biosynthesis of mycolic acids, [1-^{14}C]acetate was added to cultures in the presence or the absence of the MIC of INH. After 60 min of incubation with [1-^{14}C]acetate, we observed 95% inhibition of the radiolabeling of mycolic acids in the drug-treated samples of strain A^+. This result is in agreement with the data obtained when susceptible strains belonging to the *M. tuberculosis* complex were treated with INH at the MIC for the strains (0.02 to 0.2 μg/ml [29, 33]). As expected, the labeling of the mycolic acids of *M. aurum* M_2 was resistant (4% inhibition) to the action of INH at 1 μg/ml. As previously reported for *M. tuberculosis* (26), the bacteriostatic action of INH on *M. aurum* was observed only one generation time (14 h) after the addition of the drug (Fig. 1), several hours after the entire inhibition of mycolic acid synthesis. This inhibition resulted in the decrease of 20% (by weight), compared with that of the untreated control, of the covalently bound mycolic acid content of strain A^+ treated with INH for 9 h; there data are similar to those obtained for *M. tuberculosis* (26).

In order to determine the effects of lowering the mycolic acid content on the organization of the mycobacterial envelope and on the morphology of viable treated cells, experiments were undertaken to lower the global mycolate content by at least 20% (the observed diminution in bacteria treated with the MIC of INH; see above). As judged from the growth curves and the inhibition of the mycolate labeling, the addition of 0.2 μg of INH per ml to cultures and then the addition of 0.1 μg of the drug per ml at each increment of the absorbance of 1 was the assay that gave the highest percentage of inhibition of the labeling of mycolates (65 to 80%), while it allowed for the growth of cells even after three generations (Fig. 1). This resulted in a diminution of the covalently bound mycolates of 35% (by weight), compared with that of the untreated cells. Higher concentrations of the drug and/or longer treatments of the cells did not lead to a greater lowering of the mycolate content, suggesting a metabolic regulation between the biosynthesis of mycolates and cell growth.

Morphological changes induced by treatment of *M. aurum* with INH. Cells of *M. aurum* A^+ were observed by electron microscopy following their treatment at different times of growth with INH (Fig. 2) both at the MIC and at the subinhibitory concentrations of the drug. Attention was especially paid to the early effects of the drug before the bacteria stopped growing. After 9 h of treatment with 1 μg of INH per ml, i.e., when mycolic acid biosynthesis was entirely inhibited but the growth rate of the treated cells was still very close to that of the control cells (Fig. 1), the negative staining of treated cells revealed an alteration of the poles of the vast majority of treated bacteria (Fig. 2B), a phenomenon that was not observed with control cells (Fig. 2A). After a 20-h treatment of the cells with the MIC of INH, when bacterial growth inhibition had already been detected (Fig. 1), the alteration of the bacterial poles increased (Fig. 2C). In addition, cells showed a plasmolysis-like pattern characterized by the presence of highlighted regions (Fig. 2B and C). This may be due to the penetration of the negative stain inside the bacterial envelope via the cell poles and not to plasmolysis because the bacterial morphology remained intact (Fig. 2C). This phenomenon was also accompanied by the release of material from the cells, the chemical nature of which is defined below. After the full cessation of bacterial growth (55 h of treatment with INH at the MIC [Fig. 1]), the entire cell envelopes of treated cells seemed altered; more material was released from the bacteria into the culture medium (Fig. 2D). The corresponding control cells invariably appeared similar to the cells shown in Fig. 2A. No activity of isocitrate dehydrogenase, a cytosolic enzyme used as a marker for autolysis (1), was detected in the culture filtrates of control and INH-treated cells (up to 60 h after the addition of the drug). Confirmation of the absence of significant lysis was obtained by acid hydrolysis of extracellular materials. Again, no galactose or 6-*O*-methyl glucose, two sugar constituents used as markers for autolysis (11), was detected in the extracellular materials.

The different alterations observed on bacteria treated with the MIC of INH were also seen on bacteria that were treated with the subinhibitory concentrations of INH during three successive cultures (Fig. 2E and F). Because the growth of these latter cells was not stopped under these conditions (Fig. 1), it is likely that the observed alterations were more related to the lowering of the cell envelope mycolate content than to the bactericidal effects of the drug. The treatment of bacteria with the subinhibitory concentration of INH resulted in the

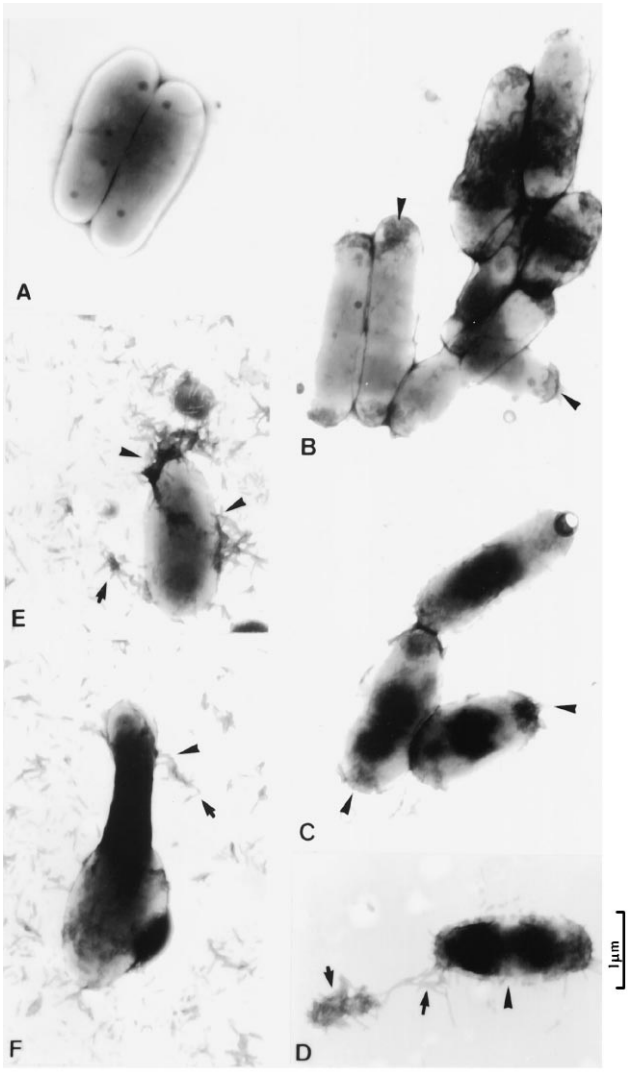

FIG. 2. Electron microscopy of negatively stained cells of *M. aurum*. Samples of control cells (A) and INH-treated cells (B to D) of strain A^+ were stained with phosphotungstic acid and observed by electron microscopy 9 h (B), 20 h (C), and 55 h (D) after the addition of INH (final concentration, 1 μg/ml). Similarly, bacteria treated with the subinhibitory concentrations of the drug (0.2 and 0.1 μg/ml) were negatively stained and observed (E and F). Control cells corresponding to the different growth phases gave pictures similar to that shown in panel A. Arrowheads indicate the alteration of the bacterial poles (B and C) and of the entire cell surface (D). Arrows indicate the material released from the bacteria into the culture medium.

presence of aberrant and deformed cells, as exemplified in Fig. 2F. The release of material and the cell damage resulting from the treatment of bacteria with the subinhibitory concentrations of INH were also observed on thin sections. The most interesting feature of the thin sections was the presence of a high proportion of deformed cells, especially star-like cells with the appearance of plasmolyzed cells (>50% of the treated cells), which certainly revealed the cell envelope weakness induced by lowering its mycolate content (Fig. 3). No obvious difference was noted, however, between the control and INH-treated cells in terms of the number and thickness of the cell envelope layers, probably because of the detection limit of the technique. It is known that, in general, the resolution and contrast available by electron microscopy of thin sections are insufficient to observe substructures in the layers.

Nature of the extracellular material of *M. aurum* released from the INH-treated cells of *M. aurum*. The electron-dense material of a fibrillar nature released from drug-treated bacteria visualized by negatively stained (Fig. 2) and thin-sectioned (Fig. 3) cells was also seen in the culture fluid filtered through a 6-μm-pore-size filter (data not shown). Upon treatment of the culture filtrate with trypsin, all semblances of fibrillar material disappeared, demonstrating the presence of protein in the released material. To further characterize the chemical composition of this material, the extracellular polymers from control cultures were compared with those derived from cells treated with drug for 9 to 60 h, because no autolysis was observed at these times. Polymers from both origins contained similar amounts of carbohydrates (roughly 6 to 8% of the dried materials) and lipids (1% of the dried materials), indicating that these two classes of polymers do not correspond to the material visualized by electron microscopy, because the latter was seen only with drug-treated samples.

The extracellular proteins from control cells and cells treated with INH (for 9 to 60 h) were compared by SDS-PAGE. When conventional amounts of materials (10 to 20 μg per well) were loaded onto the gels, only very faint polypeptide bands were revealed by staining with Coomassie blue (data not shown), suggesting that the materials were composed primarily of small peptides that derive from the Casitone in the culture medium. Chromatography of the dialyzed material derived from a fresh culture medium over Bio-Gel columns demonstrated that the peptides form large aggregates that are readily dissociable by the SDS treatment. When large amounts of materials (100 μg per well) were loaded onto the gels, marked differences in the amounts of stained proteins according to the origin of the materials were invariably observed. While faint polypeptide bands were observed in the lanes corresponding to the material from control experiments, the extracellular materials from treated cells gave distinct and important bands, demonstrating that the latter materials obviously contained much more protein than the former ones. Loading of higher amounts of extracellular material derived from control cells (300 μg per well) than from INH-treated bacteria (100 μg per well) resulted in the identification of the same major polypeptide bands stained with Coomassie blue (Fig. 4A, lanes 4 and 5). The polypeptides exhibited apparent masses of 53, 38, 27 to 33, and 22 kDa. The same difference in the amounts of extracellular proteins was observed with INH-resistant strain M_2 of *M. aurum*. A concentration of 1,000 μg of INH per ml, which corresponds to the MIC, was needed to observe an amount of extracellular proteins which is of the same order of magnitude of that of strain A^+ treated with 1 μg of INH per ml (Fig. 4A, lane 3). Concentrations of 0 and 100 μg of INH per ml gave only faint extracellular protein bands by SDS-PAGE (Fig. 4A, lanes 1 and 2, respectively). These results suggest that the increase in the amounts of extracellular proteins is correlated to the effective concentration of the drug which is necessary to inhibit the biosynthesis of mycolates. The SDS-PAGE profiles of surface-exposed proteins, which represented 56 to 60% of the dried surface-exposed materials, were similar in the control and drug treatment experiments; they differed markedly from those of the corresponding extracellular materials (Fig. 4B, lanes 1 and 2), suggesting that the increased amounts of extracellular proteins were not due to the release of surface-exposed material.

To further characterize some of the extracellular proteins. Western blot analyses were performed with MAbs directed against well-identified polypeptides of *M. tuberculosis*. The MAbs were selected on the basis of the molecular masses of the polypeptides that they recognize; accordingly, MAbs HYT

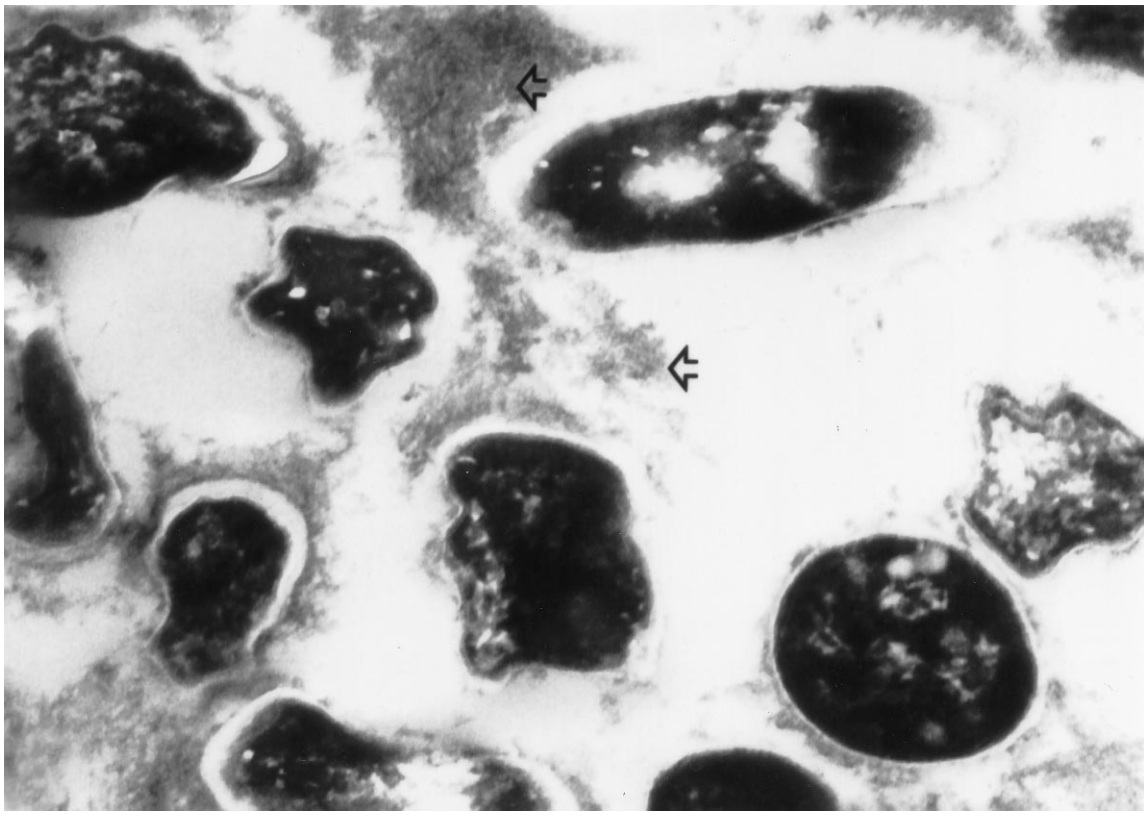

FIG. 3. Thin section of INH-treated cells of *M. aurum*. A culture of strain A^+ was treated with subinhibitory concentrations of INH (0.2 and 0.1 μg/ml), and a sample was fixed with glutaraldehyde, thin sectioned, stained with tannic acid and periodic acid-phosphotungstic acid-chromic acid (the Rambourg's method), and observed by electron microscopy (magnification, ×60,000). Arrows indicate the material released from the bacteria into the culture medium.

6, L24b5, HYT 27, HBT 12, and HBT 10, which have been raised against *M. tuberculosis* proteins of 19, 24, 30/31, 38, and 40 kDa, respectively (2, 3, 13), were used. MAb HYT 27, which recognizes the 30/31-kDa complex, so-called antigen 85, was the only MAb that reacted with the extracellular proteins of *M. aurum* (Fig. 4C, lane 2). However, MAb HYT 27 reacted with a series of polypeptides at 27, 29, 31, and 33 kDa, while it recognized only the 30/31-kDa doublet in the case of *M.*

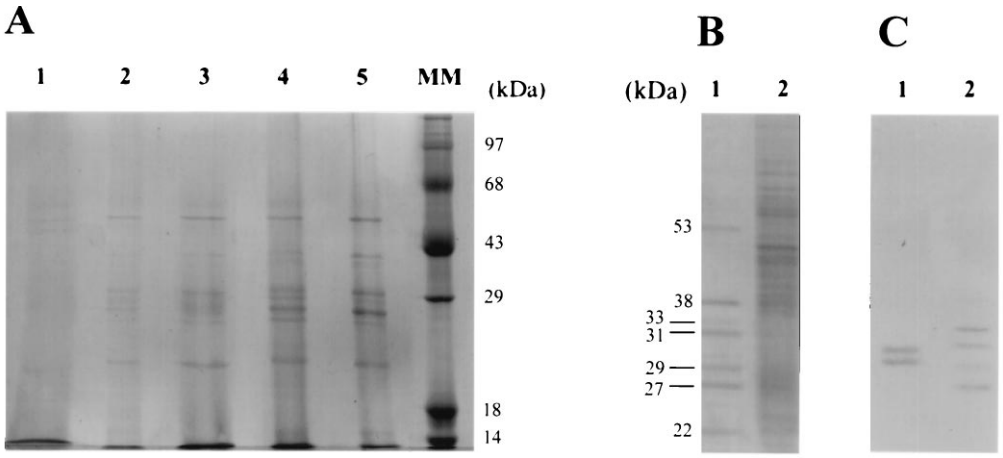

FIG. 4. SDS-PAGE of extracellular and surface-exposed proteins of *M. aurum* and immunoblot of extracellular proteins of *M. aurum* and *M. tuberculosis*. Culture filtrates and surface-exposed materials of control and INH-treated cells of *M. aurum* were concentrated, precipitated with ethanol, and dialyzed; samples of extracellular and surface-exposed materials were analyzed by SDS-PAGE, and the proteins were stained with Coomassie blue. (A) Analysis of the materials (100 μg per well) obtained from cultures of strain M_2 grown in the absence of INH (lane 1), the presence of INH at 100 μg/ml (lane 2), and the presence of INH at 1,000 μg/ml (lane 3) for 9 h and SDS-PAGE of the extracellular materials from control (300 μg; lane 4) and INH-treated (final concentration of INH, 1 μg/ml; 100 μg of material; lane 5) cells of *M. aurum* A^+ demonstrating the occurrence of the same polypeptide bands in both samples. Lane MM, molecular mass marker. (B) A total of 100 μg of extracellular (lane 1) and surface-exposed (lane 2) materials of drug-treated *M. aurum* A^+ were analyzed by SDS-PAGE, and the protein bands were stained with Coomassie blue. (C) Samples (80 μg) of extracellular materials of untreated *M. tuberculosis* H37Rv (lane 1) and INH-treated (final concentration, 1 μg/ml) *M. aurum* A^+ (lane 2) were analyzed by Western blotting on nitrocellulose with MAb HYT 27 directed against the 30/31-kDa proteins of *M. tuberculosis*.

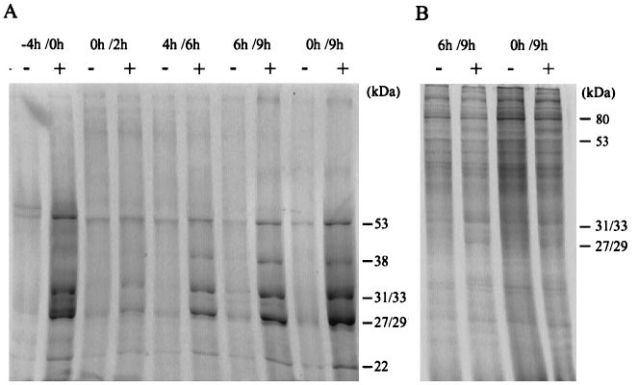

FIG. 5. Autoradiographed SDS-polyacrylamide gels of extracellular and cytosolic proteins of *M. aurum*. (A) Proteins of strain A^+ were metabolically labeled with [^{35}S]cysteine–[^{35}S]methionine for 4 h, and the cells were incubated in a fresh unlabeled medium in the presence (+) or the absence (−) of 1 μg of INH per ml for 9 h (lanes labeled −4h/0h); in parallel experiments, cells were grown in a 100-ml unlabeled culture medium for 24 h, and the cultures were divided into 5-ml portions (time zero). One series was used as a control (−), and INH (final concentration, 1 μg/ml) was added to the other series (+); labeled amino acids were added to the two series at the different time intervals, as indicated and the extracellular materials were recovered by centrifugation and analyzed (100 μg per well). (B) Cytosolic proteins from cells grown in the presence (+) or the absence (−) of 1 μg of INH per ml. INH was added at time zero, and the cells were labeled for the indicated time intervals. The cytosol materials were isolated by sonication and centrifugation and were analyzed (100 μg per well) by autoradiography.

tuberculosis (Fig. 4C, lane 1) and other mycobacterial species (3).

Metabolic labeling of proteins of *M. aurum*. To quantify the extracellular proteins of *M. aurum* A^+, INH (1 μg/ml) and [^{35}S]cysteine–[^{35}S]methionine were simultaneously added to 5-ml cultures for 9 h (the time that revealed the earliest morphological changes), and the extracellular proteins were analyzed by SDS-PAGE. The gels were stained with Coomassie blue, and the major bands in the control (INH omitted) and drug treatment experiments were quantified by autoradiography (Fig. 5A and Table 1). The level of labeling of the extracellular proteins of treated cells was, depending on the polypeptide band examined, 6 to 19 times higher than that of the corresponding proteins of the cells in the control experiment.

TABLE 1. Secretion factor of extracellular and cytosolic proteins of INH-treated *M. aurum* cells

Protein (kDa)	Secretion factor at the following time intervals[a]:					
	−4–0 h	0–2 h	4–6 h	6–9 h	0–9 h	Cytosol (0–9 h)
80						0.8
53	6.4	1.3	1.9	4.5	11	0.8
38	ND	ND	3.3	7	16	
31/32	20	1.5	5	11	19	1.2
27/28	17.5	1.5	4.5	10	13.4	1.2
22	7.3	1.4	2.2	3.3	6.1	

[a] The secretion factor is expressed as the ratio of labeling of a given protein band of extracellular material of INH-treated cells versus that of control cells. Bacteria were labeled with [^{35}S]cysteine–[^{35}S]methionine in the presence or the absence of INH (1 μg/ml) for different time intervals, as indicated, with the time of addition of INH being time zero (see also Fig. 5B). The extracellular materials and the cytosolic fractions (experiment from 0 to 9 h) were analyzed by SDS-PAGE, and the protein bands were autoradiographed. The values represent the means of two independent experiments. ND, not determined because of the failure to detect a protein band in the control sample (experiment from 0 to 2 h) or in both control and INH-treated samples (experiment from −4 to 0 h) (see also Fig. 5A).

To determine the origin of the extracellular proteins, the cells were first labeled with [^{35}S]methionine–[^{35}S]cysteine for 4 h and were then incubated in an INH-containing unlabeled medium for 9 h. This resulted in a 6- to 20-fold increase in the labeling of the major extracellular proteins, depending on the polypeptide band examined (Fig. 5A and Table 1). This result indicates that the drug induces the secretion of proteins whose synthesis was achieved before the addition of INH, as opposed to the induction of protein synthesis by the addition of the drug. This conclusion is further supported by the data obtained after a series of labeling at different time intervals following the addition of INH (Fig. 5A and Table 1). The labeling of the extracellular proteins increased with the duration of action of INH, demonstrating the progressive action of the drug. The cytosolic protein profiles of control and drug-treated cells (Fig. 5B) further confirm that the addition of the drug does not stimulate the production of secreted proteins. Compared with the nonsecreted proteins (for instance, the 80-kDa polypeptide; Fig. 5B), none of the secreted proteins, despite their oversecretion, was significantly accumulated in the cytosolic fractions of the treated cells (Table 1).

Together, these results indicate that INH induces the specific release of secreted proteins through the inhibition of the synthesis of some cell envelope constituents, mycolic acids in the present instance, that most likely represent a barrier for the secretion of mycobacterial proteins.

Morphological changes induced by the treatment of *M. tuberculosis* by INH. On synthetic Sauton's medium, the generation time of *M. tuberculosis* H37Rv was 60 h. For safety reasons, the cells were first fixed with glutaraldehyde to kill the bacilli, and they were then washed and negatively stained, a treatment that did not affect the morphologies of the control cells (Fig. 6A) but that prevented the observation of released material. Nevertheless, following the action of INH (0.1 μg/ml), the cells were obviously damaged. As observed for INH-treated cells of *M. aurum* (Fig. 2), the bacterial poles were affected when the tubercle bacilli were treated with the drug (Fig. 6B and D). The most obvious change, however, was the alteration of the cell morphology. Deformed cells were observed as early as 18 h after the addition of the drug (Fig. 6C). After 48 h of action of the drug and before the bacteriostatic effect of the drug, the morphologies of the vast majority of the cells in the bacterial population were altered. In addition, deformed and abnormal cells, probably blocked in their division, and small cells were observed (Fig. 6D and E). A failure in proper cell wall synthesis is probably at the origin of these ultrastructural changes.

It was thus concluded that profound changes in the cell morphology, similar to those observed for *M. aurum*, were induced by the treatment of *M. tuberculosis* with INH. The different alterations observed on INH-treated cells recall those visualized by scanning electron microscopy on INH-treated *M. tuberculosis* H37Ra (morphological deformations, fragmentations, and bulging poles [28]). The latter changes were observed, however, by using a high concentration of INH and after the bacteriostatic action of the drug had taken place. Thus, with the help of negative staining, it was possible to visualize the early and subtle effects of INH on cell morphology.

Effect of INH on the secreted proteins of *M. tuberculosis*. To compare the effect of INH on *M. tuberculosis* with that observed on *M. aurum*, the protein secretion in drug-treated cells of *M. tuberculosis* was investigated by measuring the concentrations of a defined secreted protein complex. The 45/47-kDa

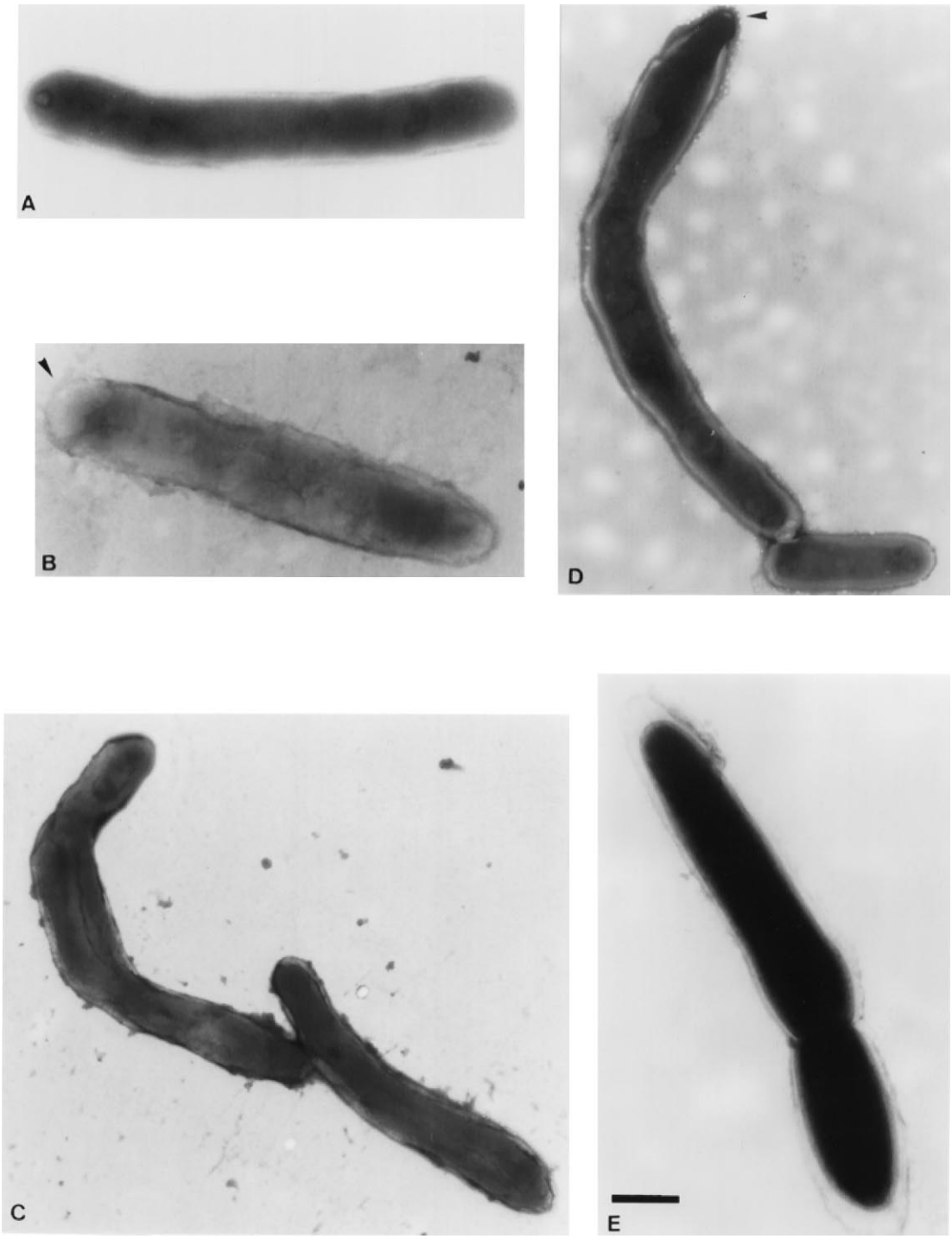

FIG. 6. Electron microscopy of negatively stained cells of *M. tuberculosis* H37Rv. Tubercle bacilli grown to the mid-logarithmic phase were incubated in fresh culture medium in the absence or the presence of 0.1 μg of INH per ml. Samples of control cells (A) and cells treated with the drug for 18 h (C) or 48 h (B and D to E) were fixed with gluteraldehyde, washed, stained with phosphotungstic acid, and observed by electron microscopy. Arrowheads indicate the alterations of the bacterial poles. Bar, 0.5 μm.

antigen complex was chosen on the basis of its presence in the early-logarithmic-phase culture filtrate (10, 23) and, more importantly, of the presence of a typical signal peptide in the structural gene coding for this complex (23). Thus, the concentrations of the 45/47-kDa antigen complex were determined in samples of control and INH-treated cells by competitive ELISA. For that purpose cultures of *M. tuberculosis* in the mid-logarithmic growth phase were harvested by pouring off the medium while the pellicles remained attached to the flasks; the medium was replaced with fresh Sauton's medium in the presence or the absence of INH (0.1 μg/ml) and bacteria were incubated, with or without shaking, for 2, 18, or 48 h. The

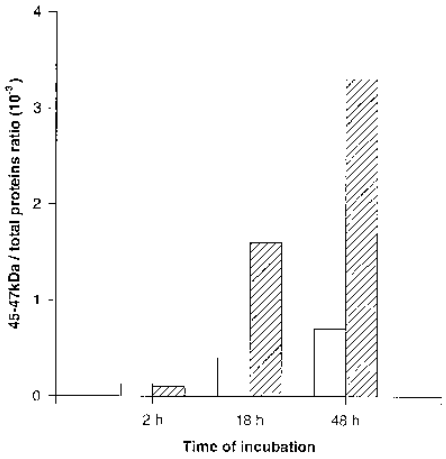

FIG. 7. Determination of the concentration of the 45/47-kDa complex in the extracellular materials of *M. tuberculosis* by a competitive ELISA. The 45/47-kDa antigen complex of *M. tuberculosis* was fixed on polystyrene wells at 1 μg/ml in a carbonate buffer. A polyclonal rabbit immune serum directed against the 45/47-kDa antigen complex was used at a constant concentration of 1/6,000 to measure the relative antigen concentrations released into the culture medium of cells that were shaken for 2, 18, or 48 h in the absence (empty symbols) or the presence (dashed symbols) of INH (final concentration, 0.1 μg/ml). Quantification is expressed as the ratio of 45/47-kDa antigens versus the total proteins.

relative concentrations of the 45/47-kDa antigen complex in the culture filtrates of control and drug-treated cells were determined.

In both experiments, only small amounts of immunoreactive molecules were present in the extracellular materials obtained from control experiments after 2, 18, or 48 h of incubation in fresh Sauton's medium (Fig. 7). In contrast, following treatment of the cells with INH for 18 and 48 h, the concentration of the secreted 45/47-kDa complex was three- to fivefold that for the corresponding control cells (Fig. 7). When bacteria were incubated as surface pellicles for 18 h, the concentration of the 45/47-kDa antigens in the INH-treated culture filtrates was found to be 16- to 20-fold that for the control cells (data not shown). The difference in the concentrations of the secreted proteins in the two experiments supports the view that many more nonsecreted surface-exposed proteins are released into the medium of shaken cultures, especially drug-treated ones, which dilute the concentration of the 45/47-kDa complex. In toto, these results demonstrate that the treatment of *M. tuberculosis* with INH induced, as in the case of *M. aurum*, the oversecretion of secreted proteins.

DISCUSSION

In order to obtain a deeper understanding of the action of INH, we investigated the effects of the drug on the mycobacterial cell envelope. This thought process was primarily motivated by the fact that a number of independent lines of investigation combined to provide evidence which suggested strongly that INH affects the cell envelope of mycobacteria (29), notably, the demonstration that INH, at an early stage of its action, inhibits the synthesis of mycolic acids (27, 30). These data provide a plausible hypothesis that accounts for most of the effects of the drug, because mycolic acids are major components of the mycobacterial cell envelope and probably play a central role in the cell wall architecture (17).

The data obtained in the present study demonstrate that the treatment of susceptible strains of *M. aurum* and *M. tuberculosis* with INH at the MIC results in different morphological changes that can be visualized by electron microscopy before the bacteriostatic effects of the drug occur. These changes start at the bacterial poles, which probably represent the weaker regions of the growing cells. The treatment of cells with INH also induces, at a later stage, alterations of the morphology of most of the cells which result in the cell deformation, probably because of defective cell wall synthesis. Examination of the nature and the origin of the material released from drug-treated cells during the early stages of INH's action demonstrated that it was composed mainly of proteins which were oversecreted following the action of the drug. Indeed, these results meet the numerous arguments in favor of a profound change in the mycobacterial cell envelope following the action of INH (29) and indicate that the drug induces a change in the permeability of the mycobacterial cell envelope. Since mycolic acids are postulated to be part of the permeability barrier of the mycobacterial cell envelope (17), it is thus conceivable that this barrier functions both for the entry and for the export of compounds.

Winder and Rooney (31) examined the effect of INH on the mycobacterial cell envelope and reported the release of alkali-extractable polysaccharides into the external medium following the action of high concentrations of INH. In the present study, no difference was found between the extracellular polysaccharide contents of control and drug-treated cells. Rather, the important decrease in the cell wall mycolic acid content selectively induces an oversecretion of secreted proteins, suggesting that mycolic acids represent a barrier to the secretion of mycobacterial proteins about which little is known.

INH does not stimulate the synthesis of secreted proteins, because prelabeled proteins, i.e., those labeled before the addition of the drug, are secreted, as are those labeled after the addition of the drug. Despite their oversecretion in the culture medium, the secreted proteins do not accumulate in the cytosol of drug-treated cells. These data suggest the existence of a pool of secreted proteins somewhere in the cells. Since any accumulation of these proteins are observed in the cytosol of untreated bacteria (Fig. 5B), the postulated pool of secreted proteins whose release is induced by INH might be located somewhere between the plasma membrane and the mycoloyl-arabinogalactan.

The selective effects of INH on the secreted proteins is somewhat unexpected in view of the current models proposed for the mycobacterial cell envelope architecture (15, 17), in which mycolic acids are believed to play an important role in the stability of the outermost layer that is believed to be composed almost exclusively of lipids. According to these models, upon the action of INH, the decrease in mycolic acid content should induce the release of the assumed lipid components of the outermost layer, a release that was not observed in the present study. Taken together, the data presented here suggest that the cell wall mycoloyl-arabinogalactan polymer does not play a major role in the architecture of the mycobacterial cell envelope outer layer, whose composition has recently been shown to consist primarily of proteins and polysaccharides (12, 18, 19). The cell wall mycoloyl-arabinogalactan contributes, however, to the permeability of the envelope and maintaining the bacterial shape. Thus, further studies are needed in order to gain a better insight into the architecture of the mycobacterial cell envelope. Ideally, the application of genetic tools should provide mutants devoid of mycolic acids. However, because the inactivation of genes coding for enzymes involved in the biosynthesis of important cell envelope components is presumed to be lethal for the cell, the desired mutants should be conditional. As an alternative, the identification of the genes coding for the enzymes involved in the biosynthesis of

these compounds may provide clues for the rational design of inhibitors of these enzymatic activities. In that connection, the characterization of InhA, an enoyl-acyl carrier protein reductase probably involved in mycolic acid synthesis (4, 22), may represent a good starting point.

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