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Effect of Ethambutol on the Viable Cell Count in *Mycobacterium smegmatis*

JAMES O. KILBURN* AND JOSEPH GREENBERG

Center for Disease Control, Atlanta, Georgia 30333

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Soon after a strain of *Mycobacterium smegmatis* was exposed to ethambutol (EMB), the number of viable cells increased dramatically above the number in a drug-free control. This rapid rise did not occur when the culture was maintained at 4°C instead of 37°C, when an EMB-resistant mutant was used, when auxotrophs were exposed in medium lacking nutrients essential for growth, nor when the levo form of EMB was used. EMB caused no increase in deoxyribonucleic acid synthesis, nor in septum formation of dividing cells. Treated cells changed morphologically, resulting in a lower surface area-to-volume ratio. Whereas EMB did not eliminate cell clusters, the cluster size decreased markedly as detected by filtration and Coulter counter measurements. We concluded that EMB causes a reduced surface-to-volume ratio, leading to reduced cell cohesion and a consequent reduction in cluster size, reflected in an increase in colony-forming units.

Ethambutol (EMB) is an effective antituberculosis drug and, in combination with isoniazid, is commonly used in many chemotherapeutic regimens. EMB is effective only against mycobacteria (3). Only the D isomer shows activity (5). Forbes et al. (1) noted an increase in viable cell counts, above those in drug-free control, in a culture of *Mycobacterium smegmatis* exposed to EMB. This unexplained rapid rise and its relationship to cell viability were examined.

MATERIALS AND METHODS

Bacteria and growth conditions. Both EMB-susceptible and the EMB-resistant mutants (resistant to 3 μg of EMB/ml) were grown in liquid 7H-9 medium (Difco). The albumin-dextrose enrichment was omitted, and 0.05% Tween 80 (Atlas Powder Co., Wilmington, Del.) and 0.2% glycerol were substituted. Cultures were grown at 37°C in a shaking water bath to an optical density (OD) of 0.5. Before use, they were diluted with fresh 7H-9 medium to an OD = 0.1 or 0.025, corresponding to 1.5×10^7 and 2.5×10^6 colony-forming units (CFU) per ml, respectively. The numbers of CFU were determined by serial dilution plate counts on Dubos- Middlebrook agar (Difco). The plates were incubated at 37°C for 2 days before the colonies were counted. In filtration experiments, 5-ml culture samples were aseptically filtered through 5.0-μm Metricel membrane filters (Gelman Instrument Co., Ann Arbor, Mich.).

Electronic cell counting. Cell size distribution in EMB-treated and control cultures was determined in a Coulter counter, model TAII, equipped with an X-Y recorder (Coulter Electronics, Inc., Hialeah, Fla.). The aperture setting was 30 μm, and Isoton was used as the diluent.

Electron microscopy. Samples of control and EMB-treated *M. smegmatis* were centrifuged at 9,000 × g for 30 min, and the pellets were resuspended in 2 ml of distilled water. A drop of each suspension was added to Formvar-coated copper grids and negatively stained with 2% aqueous uranyl acetate (pH 4.0) for 15 s. The grids were blotted dry, examined, and photographed with a Philips model 200 transmission electron microscope operating at an accelerating voltage of 60 kV.

Measurement of DNA synthesis. A modified membrane-filter technique of Roodyn and Mandel (4) was used. A 60-ml culture (OD = 0.1) was exposed for 60 min to 0.6 m Ci of [*methyl*-^{3}H]thymidine (2.0 Ci/mmol, New England Nuclear, Boston, Mass.). The cells were washed two times by centrifuging in the cold at 18,000 × *g* for 15 min and resuspending the cells in equal volumes of cold medium. After the final wash, the culture was divided into two equal parts, and 0.25 μg of EMB/ml was added to half. Both were incubated at 37°C. Incorporation of label into deoxyribonucleic acid (DNA) was determined by pipetting 1.0-ml samples into an equal volume of 10% trichloroacetic acid and maintaining the mixture for 15 min in an ice bath. The samples were then collected on membrane filters (0.45-μm, Millipore Corp., Bedford, Mass.) and washed with 15 ml of cold 1.0% trichloroacetic acid. Total cell counts were determined by collecting 1.0-ml samples on membrane filters and washing the samples with 15 ml of cold 1.0% trichloroacetic acid. After air-drying, the filters were added to vials containing 10 ml of toluene-based Spectrofluor (Amersham/Searle, Des Plaines, Ill.), and the radioactivity was determined in an Isocap/300 liquid scintillation

counter (Searle Analytic, Des Plaines, Ill.). Nonincorporated counts were determined by subtracting incorporated counts (trichloroacetic acid-insoluble) from the total counts.

RESULTS

Effect of EMB on cell viability. *M. smegmatis* at a concentration of approximately 2×10^6 organisms/ml was treated in growth medium with EMB concentrations from 0.1 to 3.0 μg/ml (Fig. 1). There was no discernible difference between cultures treated with 0.1 μg/ml and the untreated controls. At concentrations of 0.25 μg/ml, there was a rapid rise in CFU discernible in 2 h and reaching a peak at about 4 h. Thereafter, there was a decline in CFU until after 24 h, at which time cultures treated with 0.25 and 0.5 μg of EMB/ml increased in CFU. Treatment with 1.5 μg and 3.0 μg of EMB/ml resulted in death; there were fewer CFU after 48 h than there were at 0 h. The following should also be noted in Fig. 1: (i) from 0 to 12 h the kinetics of CFU was identical for concentrations of EMB from 0.25 to 3.0 μg/ml; (ii) the untreated culture did not reach the same density of CFU as cultures treated with ≥ 0.25 μg of EMB/ml until after 12 h, whereas it took untreated cells 8 h longer to reach the CFU density achieved by the treated cultures in 4 h.

Figure 2 shows CFU when the original cell density was about 2×10^7 /ml, which is 10 times that used in the experiments shown in Fig. 1; the EMB concentration was 3 μg/ml. Though the early kinetics—the rapid, almost 10-fold rise in CFU, followed by a slight decline between 2 and 4 h—was the same as with less-dense cell populations, there was recovery, and CFU increased in numbers after 6 h at the higher cell density. At lower cell densities, the CFU declined progressively after 4 h with no evidence of recovery (Fig. 1). The data in Fig. 1 and 2 clearly show that although the rapid rise always preceeded cell death in populations treated with EMB, some recovery was possible after the rapid rise. The amount of recovery was a function of both the concentration of EMB and of the density of the cell population.

Conditions affecting the rapid rise. The rapid rise was not detected when cells were treated with 3 μg of the levo form of EMB/ml, which is not effective in killing mycobacteria (data not shown).

A mutant of *M. smegmatis*, selected for re-

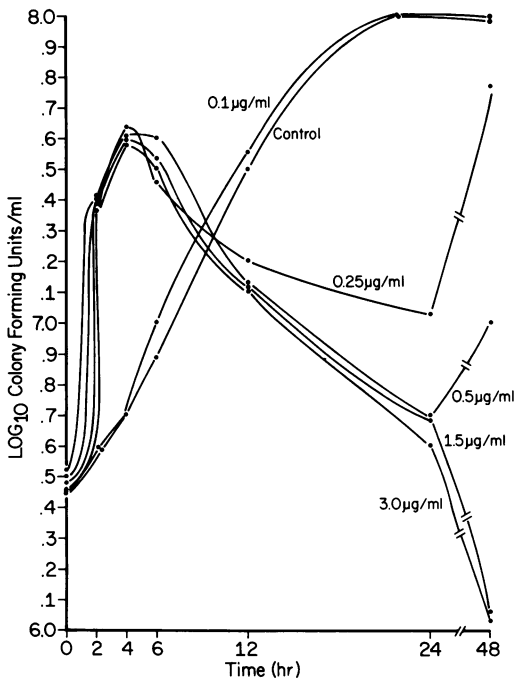

FIG. 1. *Dose response of a low-density cell population. An initial cell density of 2.5 × 10⁶ CFU/ml was exposed to concentrations of EMB ranging from 0.1 to 3.0 μg/ml; samples were removed, diluted, and plated at the indicated times.*

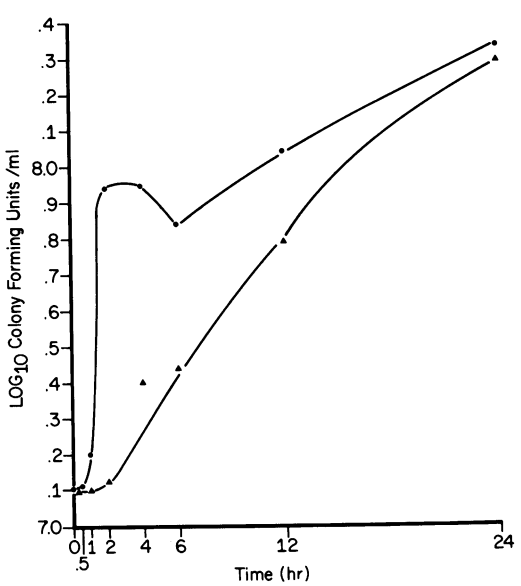

FIG. 2. *Effect of 3.0 μg of EMB/ml on a high-density cell population. An initial cell density of 1.5 × 10⁷ CFU/ml was exposed to 3.0 μg of EMB/ml (closed circles) and treated as in Fig. 1. Samplings at 0.5 and 1.0 h are included. The control is represented by closed triangles.*

sistance to 3 μg of EMB/ml, exhibited no rapid rise until exposed to 12 and 24 μg of EMB/ml (Fig. 3).

The rapid rise requires metabolizing cells, as is evidenced by its absence in cultures treated with 3 μg of EMB/ml and maintained at 4°C (data not shown). Furthermore, an auxotrophic mutant, P22, treated with 3 μg of EMB/ml of medium lacking the essential amino acids, leucine, arginine, and histidine, did not exhibit the rapid rise (Fig. 4).

Static cultures exhibited the same rapid rise as shaken cultures, indicating that the physical effects of shaking did not cause the rapid rise (data not shown).

From these data, it may be concluded that the rapid rise observed to begin between 30 to 60 min after exposure of cells to EMB is not a physical effect and depends on a stereospecific form of the drug reacting with actively metabolizing cells.

Effect of EMB on DNA synthesis. Since the rapid rise is elicited only in metabolizing cells, it is possible that EMB initiates rapid cell division. The data for untreated controls in Fig. 1 indicate that, under the conditions of the experiment, the generation time for *M. smegmatis* is about 4 h. To accomodate the hypothesis that EMB increases the rapidity of cell division, the generation time would have to be 2 h and would be reflected in an increased rate of DNA synthesis. Therefore, cells preloaded with [^{3}H]thymidine and exposed to EMB should incorporate this precursor from the pool to the acid-insoluble fraction more rapidly than the untreated cells. As seen in Fig. 5, this did not occur. Therefore, the rapid rise is not attributible to an increased rate of cell division.

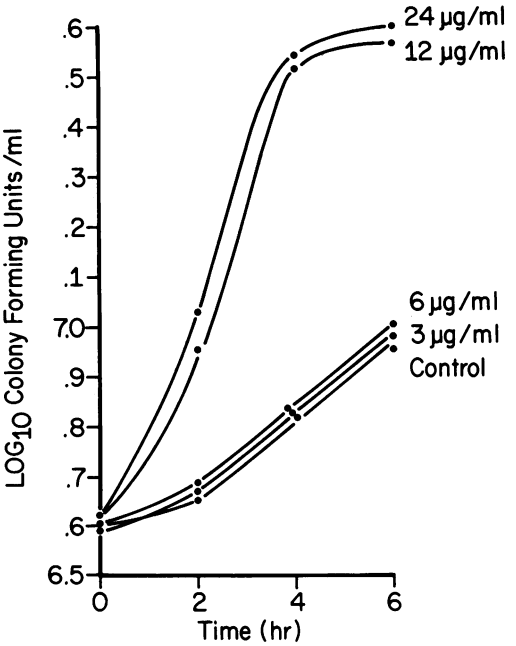

FIG. 3. *Effect of EMB concentration on the rapid rise in EMB-resistant mutant cells. The EMB-resistant mutant was selected by plating a susceptible population on Dubos-Middlebrook agar containing 3 μg of EMB/ml. Liquid cultures of the EMB-resistant mutant were exposed to the indicated EMB concentrations, and platings were made at the indicated times.*

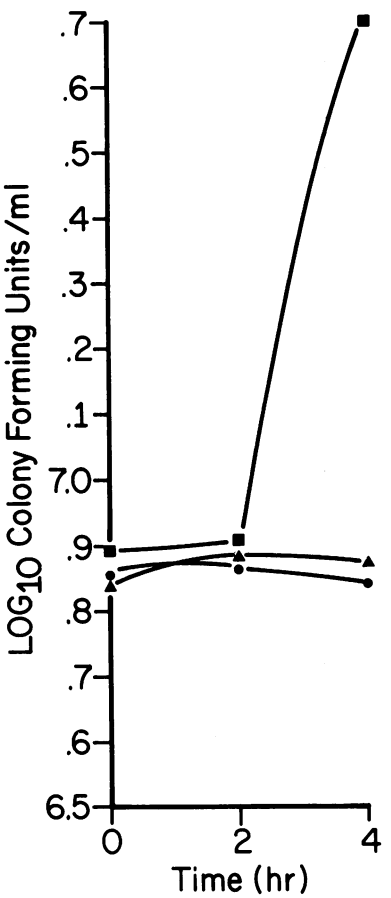

FIG. 4. *Effect of EMB on metabolizing and nonmetabolizing cells. M. smegmatis (Arg$^-$,His$^-$,Leu$^-$) was grown in medium supplemented with the essential amino acids. The culture was centrifuged and resuspended in the same medium without the essential amino acids and grown for 3 h to deplete the pool. The culture was then divided into three portions and treated as follows: (■) supplemented with the amino acids and plus 3 μg of EMB/ml, (▲) nonsupplemented and without EMB, and (●) nonsupplemented plus 3 μg of EMB/ml.*

Effect of EMB on cell morphology. Another possibility explaining the rapid rise is that indi-

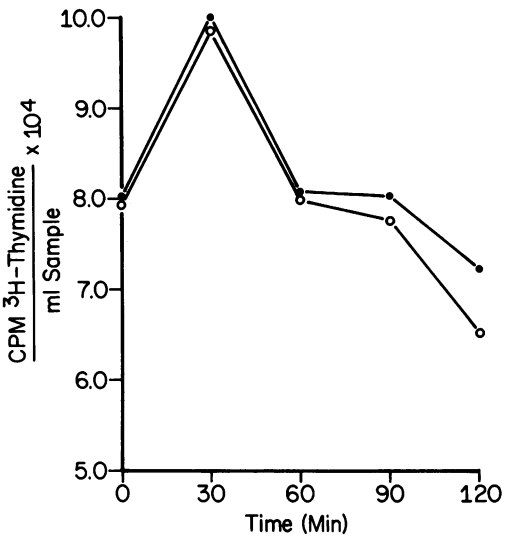

FIG. 5. *Effect of EMB on [³H]thymidine incorporation. An initial cell concentration of 1.5 × 10⁷ CFU/ml was preloaded with [³H]thymidine as described in Materials and Methods. Half was exposed to 0.25 μg of EMB/ml (open circles); the other half served as control (closed circles). One-milliliter samples were removed at the indicated times and assayed for label incorporation. The "pool" content at time 0 was 5.0 × 10⁴ CPM/ml.*

vidual cells of *M. smegmatis* are actually multinucleate filaments, requiring only the formation of septa to mature and to separate as individual CFU. EMB could accelerate the process of septation and dechaining. This hypothesis would require that each cell "unit" is potentially ≥10 CFU. To examine this possibility, cells treated with 3 μg of EMB/ml for 4 h were compared with untreated cells in an electron microscope. A sufficient number of cells could be examined at a magnification of ×9,000 to detect septation or a large number of small cells. Representive fields of treated and untreated cells are shown in Fig. 6. Cell clusters occurred in both preparations. No evidence of very small cells could be found in either the treated or untreated preparation, and no convincing evidence of incipient septation was observed in the control preparation. What was observed was that the treated cells were shorter and wider than the control. The average dimension of 10 untreated cells was 3.5 × 0.48 μm, of the treated cells, 2.2 × 0.65 μm. Treating both as ideal cylinders, the volumes of the treated and control cells are almost identical. However, the surface area of the treated cells is ≤80% that of the controls.

Effect of EMB on cluster size. The possibility that the rapid rise associated with EMB was attributible to the breaking up of clusters of cells was examined. Despite the fact that the variant of *M. smegmatis* used in these experiments was "smooth" and that Tween 80 was used in the liquid culture medium, the cells, treated with EMB or not, tended to cluster. This can be seen in the electron microscope pictures (Fig. 6) and could be seen through a light microscope in liquid drops of culture in which there was no distortion from drying. Nevertheless, that EMB treatment does reduce the size of clusters can be seen from the next two experiments.

In the first experiment, a culture treated with 3 μg of EMB/ml was compared with an untreated culture by passing a sample, at times given in Table 1, through a 5-μm membrane filter. The filtrates were appropriately diluted and plated for viable cell counts. About 10% of the cells in the untreated culture could pass through the filter, and this percentage remained the same through 6 h of growth and a tripling of the viable unit count. In the treated culture, the proportion of filterable cells doubled (22%) relative to the control within 2 h, increased slightly more (25%) at 4 h, but dropped to less than the control at 6 h (5%). These results are like the kinetics of the whole population shown in Fig. 1 and Table 1, in which there was a rapid rise of viable cells up to 4 h followed by a drop. Clearly, the increase in cells filterable through a 5-μm filter does not account for the 12- to 15-fold increase in total viable cells in the treated population. This leads to the conclusion that the increase in viable units in the treated culture is not attributable to an increase in single cells, all of which would have passed through the filter. It suggests rather that larger units are being deaggregated to smaller units, but still only a small portion of these are small enough to pass through the filter.

When units were selected by filtration through a 5-μm filter before their treatment with 0.25 μg of EMB/ml, it was observed (Fig. 7) that no significant rapid rise occurred and, unlike the situation with the general population, no recovery occurred after 24 h. This suggests that there is little deaggregation of small units and that some protection from the lethal effects of EMB is afforded cells in larger clusters.

The idea that EMB reduces the size of clusters of cells is confirmed by examining size distributions in a Coulter counter in cultures treated with 3 μg of EMB/ml for 4 h as compared with the untreated culture. As the data in Table 2 show, the volume sizes of units in the untreated culture varied over a 256-fold range;

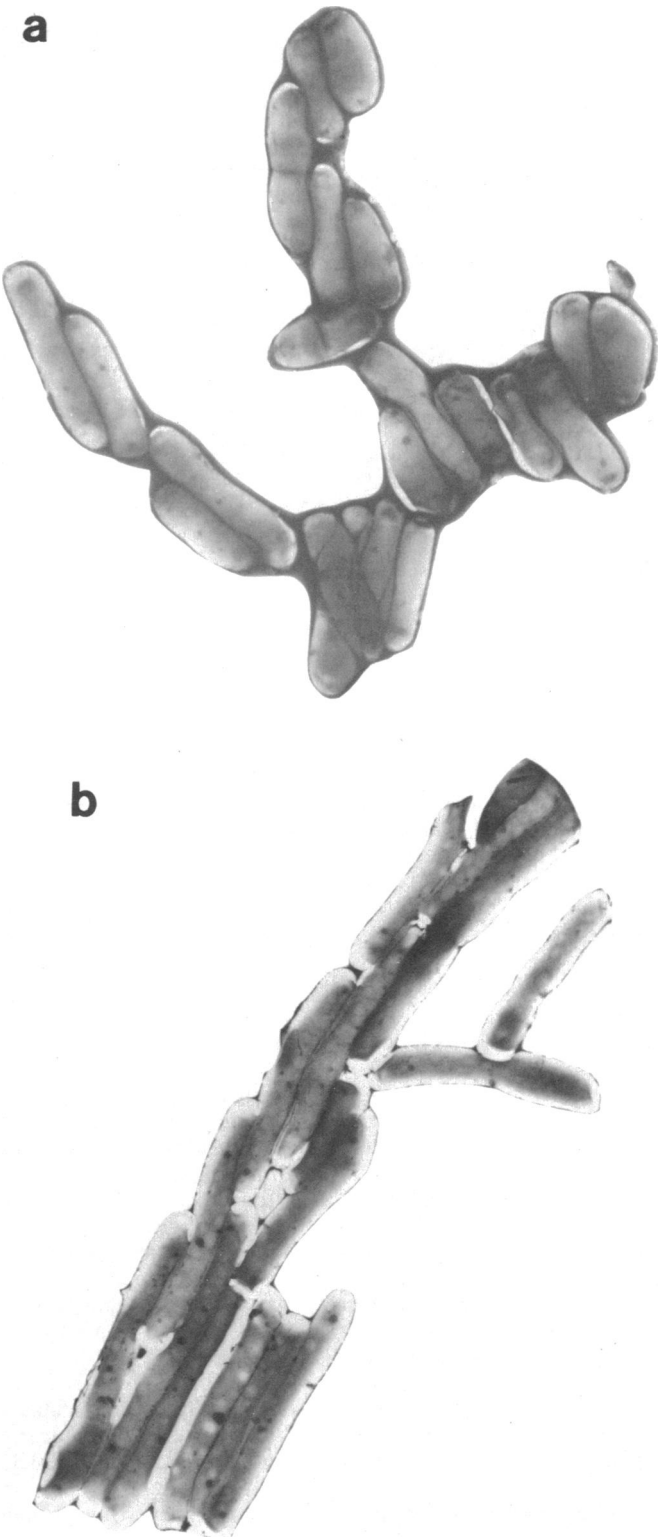
a
b

TABLE 1. *Comparison of untreated total and untreated filterable populations to EMB-treated total and filterable populations*

Time (h)	Populations			
	Untreated total	Untreated filterable	Treated total	Treated filterable
0	5.7×10^{6}[a]	6.1×10^{5}	5.7×10^{6}	6.1×10^{5}
2	5.4×10^{6}	5.0×10^{5}	6.7×10^{7}	1.5×10^{7}
4	9.5×10^{6}	9.2×10^{5}	6.8×10^{7}	1.7×10^{7}
6	1.6×10^{7}	1.6×10^{6}	4.8×10^{7}	2.3×10^{6}

[a] Expressed as CFU/ml of culture. The treated culture was exposed to 3 μg of EMB/ml. Five-milliliter samples were filtered through 5-μm Gelman membrane filters at the given times. The filtrates, along with total population samples, were diluted and plated as previously described.

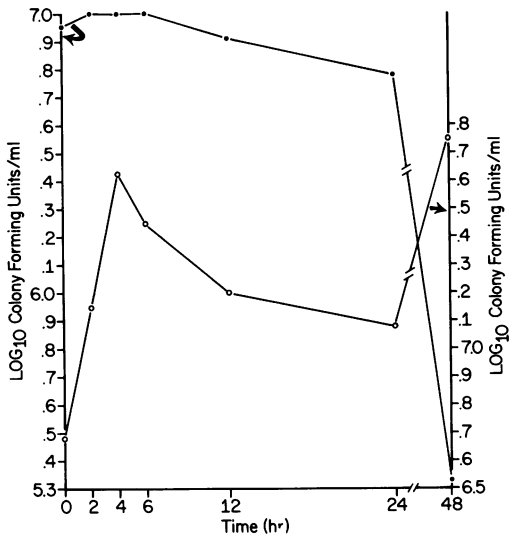

FIG. 7. *Comparison of preselected cells to a nonselected population after exposure to EMB. Cells were selected for size by passing 100 ml of an overnight culture through a 5-μm membrane filter. The filtrate was concentrated by centrifuging and resuspending in 20 ml of fresh medium. The nonselected population (open circle) was an overnight culture diluted with fresh medium to a cell density of approximately 1.5×10^{7} CFU/ml. Both cultures were exposed to 0.25 μg of EMB/ml, sampled, and plated at the indicated times. The arrows indicate the correct axis.*

more than half the population was in cluster units larger than 16 times the smallest measurable units. In the treated culture, the largest clusters were only 32 times the smallest units,

TABLE 2. *Comparison of particle-volume distribution (expressed as percent) in EMB-treated and untreated populations*

Volume (μm³)	Cultures[a]	
	Untreated (%)	Treated (3 μg of EMB/ml) (%)
0.52	7.0	13.0
1.047	10.0	33.0
2.094	16.0	30.0
4.189	20.0	17.0
8.378	17.0	5.0
16.760	12.0	2.0
33.510	10.0	0
67.020	4.0	0
134.00	4.0	0

[a] Both treated and untreated cultures were diluted to an initial cell density of 1.5×10^{7} CFU/ml. A 0.5-ml sample was removed from each culture after 4 h of incubation, diluted with 20 ml of Isoton, and run through a Coulter counter with an aperture setting of 30 μm.

and almost half the population (46%) was in units smaller than twice the minimum. Assuming that each cluster produces a colony and assuming no difference in the total number of viable cells in the two populations, it is clear that the reduction in cluster size can account for the rapid rise in viable colonies in the treated population. The fact that even the treated cells tend to cluster obscures the kinetics of killing by EMB, since even one viable cell in a cluster will probably yield a viable colony.

DISCUSSION

The picture that emerged from these experiments is the following: within 2 h after exposure to EMB the shape of the treated cells changed; they became shorter and wider. Although their volume did not change, their surface area lessened. It is reasonable to consider clumping a function of adhesion which depends on surface area. A reduction of surface area would contribute to the breaking up of clumps of cells to smaller units. This was reflected in a rapid rise in the number of units which, when plated, form a colony.

The change in shape of EMB-treated cells was so readily apparent even in the light microscope, that no experimental attempt was made to explain the failure of Gale and McLain (2) to observe this in their electron microscope preparations. It is noted, however, that these investigators used a much higher concentration of EMB (1,000 μg/ml) for a much longer time (10 h) than we did. Furthermore, their electron

FIG. 6. *Comparison of EMB-treated (a) and untreated (b) M. smegmatis. Culture was exposed to 3 μg of EMB/ml for 4 h. Note the change in the EMB treated to a more spherical shape. The magnification is ×9,000.*

microscope preparations were fixed and sectioned as opposed to negative staining used in the present study.

Alternative explanations that have been considered for the rapid rise in CFU are not supported by the data. There is no evidence of a rapid increase in DNA synthesis, which might indicate that EMB stimulates cell division. Nor was any evidence found that EMB causes the fragmentation into single CFU of multinucleated cells. In fact, no evidence was found for the extremely large or extremely small cells in an untreated population that would be necessary to support the fragmentation hypothesis. The idea that EMB causes a reduction in the size of clumps is suggested by the data from the Coulter counter, demonstrating that large, multicell clumps in untreated cultures are dispersed into smaller clusters. The large clusters become sufficiently dispersed to account for the increase in numbers of colonies formed during EMB treatment.

No explanation is yet offered for the change in shape of treated cells. This is being further investigated, particularly the possibility that EMB causes some change in a structural component of the cell wall which results in a relaxation of its rigidity. The results reported in this paper offer some guidelines to further studies. Clearly, the effects of EMB are not simply physical and dispersal of clumps is not the result of shaking. The L-form of EMB does not cause dispersal or cell death, so the effect is sterospecific. Dispersal was not observed at 4°C, nor in an auxotrophic mutant deprived of its essential amino acids, nor in a mutant resistant to EMB. With regard to the last observation, when the concentration of EMB was increased sufficiently to approach that needed to kill cells, dispersal occurred. Cell death occurs in susceptible and resistant cells only at doses slightly larger than the dose required to cause dispersal of clumps. Much of the kinetics of the survival of mycobacteria treated with EMB is obscured by complications arising from the fact that the cells are clumped and, even when dispersed, do not present a uniform population of single cells. The picture is further obscured by the yet unexplained observation that survival is a function of the density of the population.

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