Synchronized Replication of *Mycobacterium tuberculosis*

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When *Mycobacterium tuberculosis* was grown in Tween-albumin broth without any agitation, the bacilli replicated in the upper, oxygen-rich portion of the medium at a rate that was just balanced by the rate at which the bacilli settled toward the bottom of the tube. When the organisms that accumulated in the sediment were suspended and diluted into fresh medium, they exhibited synchronous replication. The bacilli initiated ribonucleic acid synthesis immediately upon suspension, but marked deoxyribonucleic acid synthesis was not apparent until after the first cellular division was completed, about 14 h after suspension.

It has long been recognized that *Mycobacterium tuberculosis* dispersed in detergent-containing liquid medium exhibits net arithmetic growth when incubated without continuous agitation (3). Aeration of the culture by shaking or stirring results in logarithmic growth (7). It was shown recently that tubercle bacilli undergo early logarithmic replication in dispersed cultures, even without active aeration, until the population density reaches approximately 4 × 10^7 viable units per ml (10). The generation time during that period is 17 to 18 h. Thereafter, the replication of bacilli slows down with the limitation of O_2 until the generation time reaches about 33 h. The population in the supernatant culture medium then stabilizes as bacilli settle to the bottom of the tube at a rate just balancing the replication in the upper layer. Thus, the bacilli that are dispersed in the medium continue to replicate in the geometric mode, but the viable bacilli that have settled into the sediment have stopped replicating; the net result is a linear, rather than geometric, increase in total number of bacilli in the tube. The fact that the bacilli that have settled slowly through an O_2 gradient stop replicating but retain their viability suggested to us that the organisms in the sediments might enter a uniform physiological state and might thus be capable of initiating synchronous growth on suspension and dilution into fresh medium. This is a report of experiments which demonstrate that such is indeed the case.

**MATERIALS AND METHODS**

All studies were conducted with the H_37Rv strain of *M. tuberculosis*. Dubos Tween albumin (DTA) broth was dispensed in 10-ml amounts to screw-capped tubes (20 by 125 mm). Inocula were prepared by subculturing from a stock Lowenstein-Jensen culture through two passages in DTA. The second passage culture was divided into small portions and frozen at -70°C. Sufficient thawed suspension was added to culture tubes to yield a calculated initial absorbance at 580 nm of 0.002 unless otherwise specified.

The microaerophilic sediments used in the synchrony experiments were obtained by incubating the cultures at 37°C for 14 to 21 days without agitation or disturbing them in any way. On the day of the experiment the supernatant fluid was aspirated and discarded, leaving the sediment and only a trace of fluid undisturbed. The sediment was then suspended in fresh prewarmed (37°C) DTA broth, and the absorbance at 580 nm was determined. Suitable corrections were made for linearity (10), and dilutions were made in DTA appropriate for plating or isotope uptake experiments.

Counts of viable units were determined by preparing dilutions in DTA and plating onto Dubos Oleic albumin agar. Cultures were kept for up to 6 weeks or until colony counts had stabilized.

Nucleic acid synthesis was monitored by adding 1 μCi of sterile [6-3H]uracil (23 Ci/mmol) or [methyl-3H]thymidine (22 Ci/mmol) (Amersham/Searle, Arlington Heights, Ill.) per ml of culture medium. Cell-bound substrates were collected by filtering 2- or 5-ml samples through 0.45-μm B-Bac-T-Flex membrane filters (Schleicher and Schuell, Inc., Keene, N. H.) and washing the filters thoroughly with distilled water. The filters were dissolved in 1 ml of 2-ethoxyethanol for at least 2 h and then mixed with Aquasol-2 scintillation cocktail (New England Nuclear, Boston, Mass.) for counting. When [6-3H]uracil was used, one sample was filtered directly and a duplicate sample was incubated for 20 to 24 h at 37°C in 0.3 N KOH before filtering to permit distinction between deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (8).

All results were corrected for zero-time absorption of label by the filter.

**RESULTS**

Synchronized replication. When tubed cultures of *M. tuberculosis* were incubated
without any mixing for 14 to 21 days, most of the bacillary mass rested at the bottom of the tube, and only the supernatant fluid. The bacilli in the microaerophilic sediments were suspended and diluted rapidly in warm DTA to yield a calculated initial cell density of 100 to 200 viable units per ml. Portions were plated at zero time, and the dilute suspensions were reincubated and sampled at regular intervals. Figure 1 is a composite illustration of data from two such experiments, with the points normalized. There was no evidence of replication of bacilli for the first 8 h after reconstitution and dilution of the resting sediments. Thereafter, a series of twofold-stepped increases was seen with a periodicity of approximately 20 h.

Nucleic acid synthesis. Experiments to demonstrate the temporal relationships between nucleic synthesis and cell division were complicated by the fact that uracil is not only incorporated into RNA but is methylated and incorporated into mycobacterial DNA as well (4). Furthermore, only negligible amounts of exogenous thymidine are incorporated into mycobacterial DNA. The interference with use of exogenous thymidine in bacterial DNA synthesis is not uncommon and has been associated with induction of thymidine phosphorylase, which hydrolyzes the substrate (1). Several attempts were made to incorporate [3H]thymidine into both synchronized and nonsynchronized actively replicating cultures of M. tuberculosis, but the rate of degradation of substrate was too rapid to permit recovery of appreciable amounts of cell-bound label with bacillary suspensions that were dilute enough to permit establishment of synchronous replication.

To estimate nucleic acid synthesis, it was necessary to rely on [3H]uracil incorporation and to distinguish between labeled RNA and DNA by extracting the RNA from the cells with 0.3 N KOH. Microaerophilic bacillary sediments were reconstituted in fresh warm DTA and diluted in the same medium supplemented with 1 μCi of [6-3H]uracil per ml to a cell concentration of 10^7 viable units per ml. The DNA synthesis was expressed as disintegrations per minute per milliliter recovered on the filters after incubation of the suspension with 0.3 N KOH. The RNA was expressed as the difference between total label recovered on the filter without KOH treatment and that of the KOH-treated preparation. The results are shown in Fig. 2 as a composite of data from three experiments normalized to the mean count after 48 h of incubation. The synthesis of RNA started immediately after reconstitution of the resting bacilli. The DNA synthesis, on the other hand, did not begin until a time corresponding to the completion of the first cell division illustrated in Fig. 1. Thereafter, DNA synthesis continued in a linear fashion, with the rate increasing abruptly at points corresponding to the completion of each twofold step.

The failure of the RNA curve to show such clear-cut increases in rate is probably due to turnover of previously synthesized RNA concomitant with synthesis of new RNA.

DISCUSSION

The question of "cure" of tuberculosis is clouded by the known ability of M. tuberculosis to persist for long periods of time within closed lesions after progression of disease has stopped (9, 12). This has led to speculations on the existence of dormant forms of tubercle bacilli that can survive under conditions of O2 deprivation and are not eliminated by antimicrobial agents (2, 5, 6). Abrupt depletion of the O2 available to tubercle bacilli that are growing vigorously in vitro results in their rapid death and autolysis (11). On the other hand, when they settle slowly through an O2 gradient, replication stops, but viability does not appear to be impaired for at least several weeks (10). The data derived from the present study demonstrate that these bacilli are not arrested in a random state, but rather appear to have put their affairs in order during the settling period and to have arrived in the microaerophilic sediment in a uniform stage of their replication cycle; i.e., they are synchronized.

The bacilli appear to have synthesized a full complement of DNA during the settling process, since no appreciable incorporation of the [3H]uracil-derived label into DNA was demonstrated until the time when a cell division, as reflected by increasing viable unit counts, had occurred. This is in contrast to the incorpora-
tion of $^3$H]uracil into RNA, which began immediately upon suspension of the dormant organisms. This suggests that a period of induction of enzyme synthesis is necessary before cell division can occur.

It is not yet clear whether the nonreplicating bacilli in the microaerophilic sediments are analogous to the persistent bacilli in quiescent human disease. If so, they could provide a useful model for study of in vivo antibiotic action and of host-parasite interactions at the cellular level. The possible differences in enzyme, and thus protein composition, between dormant and actively multiplying tubercle bacilli might permit the isolation of antigens which reflect the state of activity of these organisms in the human host.

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**LITERATURE CITED**


