Metronidazole Is Bactericidal to Dormant Cells of Mycobacterium tuberculosis

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Very abrupt exposure to anaerobic conditions has a lethal effect on actively growing cultures of Mycobacterium tuberculosis. However, incubation under conditions in which oxygen is depleted gradually causes M. tuberculosis to shift down from active replication to dormancy. The dormant bacilli are resistant to the bactericidal effects of oxygen and also exhibit partial or complete resistance to the bactericidal effects of isoniazid and rifampin. On the other hand, metronidazole, a drug specific for anaerobes, kills dormant tubercle bacilli under anaerobic conditions, but it has no effect on actively growing aerobic cultures. The lethal effect of metronidazole under anaerobic conditions is enhanced by rifampin. The possible implications of these findings on the phenomenon of latency in tuberculosis are discussed.

The introduction of specific chemotherapy for tuberculosis approximately 50 years ago accelerated the decline in the incidence of that disease in industrialized countries. In the past few years that decline has been arrested, and in some populations there has been an alarming increase in tuberculosis (3, 19). This increase has been further complicated by the occurrence of outbreaks of infections with multiple-drug-resistant tubercle bacilli (5, 6, 18). In some of these cases the infections are manifested only by a conversion of the tuberculin test reaction of exposed subjects. These individuals are at risk of developing active drug-resistant disease at a later date; prevention of this serious consequence by chemoprophylaxis is compromised by the multiple-drug-resistant status of the infecting agent. It was recently reported that a "majority of the cases (of tuberculosis) reported annually in the United States arises from the pool of persons who have been infected in the remote past. In the United States, the number of such persons with latent infection is estimated to be between 10 and 15 million" (1). The latent disease associated with the presence of dormant Mycobacterium tuberculosis in the body has long been a source of concern. Animal models have been used to demonstrate both immunologically induced latency (16, 21) and postchemotherapeutic latency (8, 9, 12-14), with the associated late reactivation of disease. Grosse (7) has concluded that "in the mouse, a very effective chemotherapy is more likely to achieve a latent state of infection than to achieve a real sterilization." Conventional antimycobacterial agents are highly effective against tubercle bacilli only when the bacilli are actually growing. In vitro models of antibiotic treatment of nonreplicating tubercle bacilli which rely on nonphysiologic temperatures or on pH manipulation to achieve "dormancy" have been described; the physiologic parameters of these models have not been presented (2, 10).

Although actively replicating tubercle bacilli die rapidly when they are abruptly deprived of oxygen (24, 25), they have been shown to shift into a state of dormancy when they are allowed to adapt to a gradually decreasing supply of oxygen (22). Tubercle bacilli that have settled through a self-generated oxygen depletion gradient in unagitated culture tubes undergo an orderly metabolic shift-down, and as they accumulate in the bottom of the tubes they enter a homogeneous physiologic state of dormancy. The homogeneity of this state was proved by the demonstration that the bacilli undergo synchronized division about 12 h after they are resuspended in well-aerated medium, having first undergone a period of RNA synthesis (23). A first cycle of DNA synthesis occurs only after completion of the first division of cells. The dormant cells produce an antigen that is absent from actively growing aerated cultures (27) and exhibit a 10-fold increase in glycine dehydrogenase, which appears to function in the glyoxylic acid cycle of these organisms (25). The latter response is compatible with the established shift away from oxygen-dependent pathways to anaerobic or facultative anaerobic pathways in host-derived M. tuberculosis compared with that in cultured bacilli (20). This in vitro model is probably representative of the adaptation of tubercle bacilli to growth-suppressive conditions in inflammatory and necrotic tissues. Viable tubercle bacilli have been recovered from approximately 20% of blocked, oxygen-deprived tuberculous lesions in surgical specimens from human subjects in whom sputum the bacilli could no longer be detected (26).

Some drugs that do not exhibit significant inhibitory activity against M. tuberculosis under conventional conditions of aerobic cultivation may be capable of either repressing the shift-down of the bacilli into dormancy during oxygen depletion or killing the dormant bacilli under anaerobic or microaerophilic conditions. These drugs could be of value, if they are used in combination with conventional anti-tuberculosis drugs, in preventing the persistence of dormant bacilli and the later emergence of active disease. This report describes the encouraging results obtained when our in vitro model of dormancy of M. tuberculosis was used to explore the effect of metronidazole, a member of the nitroimidazole class of agents, the antibacterial activity of which is selectively directed against anaerobes (4).

MATERIALS AND METHODS

Strain of M. tuberculosis. All experiments were conducted with M. tuberculosis H₃₇Rᵥ from the culture collection main-
tained in the Long Beach VA tuberculosis research laboratory. Small aliquots of seed stocks were maintained at 7°C and were subcultured once in liquid medium with agitation until they reached an optical $A_{580}$ of 0.4 to 0.6, corresponding to a concentration of about $3 \times 10^8$ to $5 \times 10^8$ CFU/ml, before inoculation to an experimental culture. The usual test inoculum represented a 1:100 dilution of this first-passage culture into fresh medium.

**Culture medium.** All liquid culture experiments were conducted in Dubos Tween-albumin broth (DTA) prepared from Dubos broth base (Difco, Detroit, Mich.) and Dubos medium albumin (Difco). The complete medium contained the following (per liter): asparagine, 2 g; Casitone (Difco), 0.5 g; Na$_2$HPO$_4$, 2.5 g; KH$_2$PO$_4$, 1 g; ferric ammonium citrate, 50 mg; MgSO$_4$, 10 mg; CaCl$_2$, 0.5 mg; ZnSO$_4$, 0.1 mg; CuSO$_4$, 0.1 mg; Tween 80, 0.2 g; bovine albumin fraction V, 5 g; and glucose, 7.5 g (final pH, 6.6 ± 0.2). The medium was aseptically dispensed in 10-ml aliquots to sterile 20-by-125-mm screw-cap culture tubes unless specified otherwise. In preparation for the counting of colonies on agar, dilutions of liquid culture were made in basal medium from which the albumin and glucose were omitted.

For colony counting, culture dilutions were plated onto Dubos oleic-albumin agar, which was prepared from Dubos oleic agar base (Difco) supplemented with Dubos oleic albumin complex to a final concentration of 50 μg of oleate and 5 mg of albumin per ml. The medium was dispensed in 3-ml amounts to each of the 12 wells of sterile tissue culture plates (Falcon 3043). The agar surfaces were inoculated with 20 μl of selected dilutions of test culture and were examined twice weekly as described below.

**Conditions of incubation.** For continuous aeration, the caps of the culture tubes were loosened slightly and were held in place with a small strip of tape. The cultures were agitated in a model G24 rotary shaker-incubator (New Brunswick Scientific Co., Inc., Edison, N.J.) at 37°C and 250 rpm. Growth was monitored by measuring the optical $A_{580}$ in a Coleman Jr. IIA spectrophotometer (Coleman Instruments, Maywood, Ill.). For some experiments in which the effects of the abrupt interruption of aeration of actively growing cultures were to be studied, agitated cultures were removed from the rotary shaker and were placed upright in a conventional incubator at 37°C, and the cells were allowed to settle and deplete their available oxygen.

In order to establish homogeneous populations of dormant cells, freshly inoculated tubes were incubated in an upright position without agitation and with care not to disturb the sediment that formed or to aerate the medium, as described previously (22). When dormant cells were needed for study, the supernatant suspension that contained slowly replicating bacilli (22) was removed from 28-day-old stationary (i.e., nonagitated) cultures and was discarded; the dormant cells that had settled under gravity into the sediment at the bottom of the tube were then resuspended in fresh medium for experimental treatment.

Anaerobic incubation of the culture suspensions was carried out in a desiccator containing copper-plated iron wool (17). Immediately after the copper sulfate treatment of the iron wool and its placement in the desiccator, the air was evacuated and was replaced with sterile filtered nitrogen. Anaerobic conditions were confirmed by observing the decolorization of a monitor suspension of *M. tuberculosis* containing methylene blue (2.5 μg/ml) as described previously (25).

**Colony counts.** Colonies on agar plates were counted by examination of the inverted plates on a dissecting microscope under ×10 magnification by using transmitted light. The colonies were counted twice a week, starting 14 days after inoculation, until the counts stabilized.

**Drugs.** For preliminary estimation of MICs under aerobic conditions, small volumes (100 μl or less) of selected twofold dilutions of filter-sterilized stock drug solutions were added to 10-ml tubes of DTA. Stock solutions of metronidazole, tinidazole, and ornidazole (12,800 μg/ml) and isoniazid (80 μg/ml) were prepared in H$_2$O; rifampin (100 μg/ml) was dissolved in ethanol. The inoculated cultures were incubated with continuous aeration, the $A_{580}$ was determined three times a week, and growth curves were plotted; on the basis of a doubling time of 18 h (22), a 120-h lag of the growth curve of a drug-containing culture behind that of the control corresponds to an apparent 100-fold difference in viable inoculum size, i.e., an apparent inhibition of 99% of the inoculum, as long as the slopes of the respective curves are parallel. The lowest concentration of a drug that caused this lag was recorded as the MIC. Under these conditions, the MICs for isoniazid and rifampin were determined to be 0.1 and 0.016 μg/ml, respectively.

**RESULTS**

An experiment was conducted to determine the effect of metronidazole against tubercle bacilli incubated under aerobic and $O_2$-limiting conditions. Tubes of DTA that contained 64, 32, and 16 μg of metronidazole per ml, respectively, as well as drug-free controls were inoculated with *M. tuberculosis* and were incubated at 37°C with continuous aeration. Completely overlapping growth curves were seen with all cultures during the period of aeration; the drug had no effect on the growth of the bacilli under these conditions (Fig. 1). In separate experiments, concentrations of metronidazole of as high as 512 μg/ml had no effect on the aerobic growth of the bacilli. After 124 h of incubation with agitation, when the $A_{580}$ approached 0.80, the aeration was interrupted, and the dissolved $O_2$ was rapidly depleted by the bacilli; the methylene blue in an indicator tube of the culture was completely decolorized within about 3 h. After 112 h of stationary incubation under $O_2$-depleted conditions, the partially settled cultures were resuspended, diluted 1:100 in fresh prewarmed drug-free DTA, and incubated under continuous aeration. The subcultures exhibited drug dose-dependent displacement of a parallel family of curves (Fig. 1).
suggesting either that different proportions of the bacilli in the tubes were killed by the metronidazole when O2 was depleted or that all of the bacilli in a tube were damaged to a dose-dependent extent and required different amounts of time to repair the damage before resuming growth. Similar experiments were conducted with two other nitroimidazole drugs, tinidazole and ornidazole. These yielded essentially the same types of responses seen with metronidazole, confirming that the phenomenon is characteristic of this class of drugs.

In order to determine which of the alternative explanations for the dose-dependent delay in the resumption of growth proposed above was correct, we performed another experiment in which the actual survival of bacilli was established by dilution and plating of the cultures after exposure of the settling cultures to metronidazole (32 \( \mu g/ml \)). In addition, the known tuberculocidal drugs isoniazid (0.4 \( \mu g/ml \)) and rifampin (0.1 \( \mu g/ml \)) were tested alone or in combination under the same conditions of O2 depletion. In that experiment the drugs were added just before the interruption of aub of the mid-log-phase culture to minimize possible drug decay. The bacilli were then permitted to settle without any agitation at 37°C for 72 h, after which time the tubes were stirred and sampled for dilution and plating. Additional tubes of culture mixed with either isoniazid or rifampin alone were also incubated with continuous shaking for 72 h to establish the bactericidal effects of the drugs on these dense populations of actively replicating cultures. A sample of the actively growing culture was diluted and plated immediately before the addition of drug to establish the original bacillary count.

The microaerophilic conditions established by settling of the original dense, actively growing drug-free culture, which contained 7.3 \( \times 10^6 \) CFU/ml, resulted in a 59% decline in the number of CFU/ml after 72 h. The 72-h exposure to isoniazid or rifampin alone during continued agitation of dense cultures led to approximately 90% declines in the number of CFU per milliliter (Fig. 2A).

The effects of the antimicrobial agents on the settled cultures were calculated as the proportional decline compared with that in the culture that was permitted to settle without the addition of drugs (Fig. 2B). Isoniazid and rifampin alone had small effects on the viabilities of the settled cultures, with declines in CFU per milliliter in the range of 42 to 58%; metronidazole alone yielded a decline of 69%. However, when metronidazole was added in combination with either isoniazid or rifampin, a decline in the CFU per milliliter of over 95% was seen. In contrast, a combination of isoniazid and rifampin was no more bactericidal than either of these two drugs used alone. These results suggest that metronidazole was bactericidal to a segment of the mixed population of cells that had shifted down to the dormant state and that the bacilli that had not yet shifted down were affected indiscriminately and to a comparable extent by the bactericidal effect of either isoniazid or rifampin.

In order to confirm the bactericidal selectivity of metronidazole for dormant tubercle bacilli, a homogeneous population was prepared by pooling a harvest of resting bacilli from the sediment of 28-day-old unagitated cultures prepared as described in Materials and Methods. Two-milliliter aliquots of the pool were rapidly distributed to 2-ml vials containing sufficient volumes of drug stock solutions to yield final concentrations of 8 \( \mu g \) of metronidazole per ml, 0.4 \( \mu g \) of isoniazid per ml, or 0.1 \( \mu g \) of rifampin per ml alone or in combination; drug-free controls were also prepared. Each combination was prepared in triplicate, and all vials were incubated anaerobically; individual vials of each combination were removed for dilution and plating after 2, 4, and 8 days of incubation. The brief exposure of the bacilli to air during handling was not sufficient to cause them to shift out of the dormant state (25). As seen in Fig. 3, only 0.20% of the dormant bacilli survived 8 days of anaerobic exposure to 8 \( \mu g \) of metronidazole per ml alone; i.e., the drug caused a 2.7-log-unit decline in the number of CFU. Under these conditions, 0.4 \( \mu g \) of isoniazid per ml alone had no appreciable bactericidal effect on the bacilli, nor did it exhibit an additive effect when it was combined with metronidazole. On the other hand, 0.1 \( \mu g \) of rifampin per ml alone caused an approximately threefold (i.e., a 0.44-log-unit) decrease in 8-day anaerobic survival of the dormant bacilli compared with that of the drug-free control, but in combination it caused a 10-fold enhancement of the bactericidal effect of metronidazole, i.e., for a total of a 3.68-log-unit decline in the CFU per milliliter.

**DISCUSSION**

After decades of complacency during which tuberculosis was considered to be under control and well on the path toward eradication, there has been an alarming increase in the incidence of the disease and a disturbing trend toward the emergence of multidrug resistance. The disease in most individuals who are infected with *M. tuberculosis* does not progress to the active state. However, the bacillus may remain in the host for decades in a quiescent state with the potential for revival and initiation of clinical disease. Only when a means is found to prevent the dormancy of the tubercle bacillus or to kill the organism when it is in the dormant state will the goal of eradication of tuberculosis become a realistic prospect. The data generated in the present study suggest that drugs that can
contribute to the attack on dormant tubercle bacilli and latent tuberculosis can be found.

Metronidazole was chosen for the present study because it is known to be selectively active against anaerobes (4), and the shift-down to dormancy of M. tuberculosis represents a metabolic adaptation to survival under conditions of severe O\(_2\) depletion, presumably with a low order of anaerobic maintenance metabolism. Furthermore, metronidazole is a drug of known clinical efficacy and safety and ready availability. The response to this agent appears to be common to the general class of nitroimidazole drugs, since comparable results were seen in screening experiments with tinidazole and ornidazole.

The data in Fig. 2 illustrate the bactericidal actions seen when rifampin, isoniazid, and metronidazole were added alone or in combinations to a dense culture of actively growing M. tuberculosis and then the agitation was abruptly terminated, with a consequent rapid depletion of O\(_2\). Presumably, only part of the population was able to complete the shift-down to anaerobic dormancy under these conditions. Both isoniazid and rifampin, which have different mechanisms of action, appeared to attack the same subpopulation of bacilli in this heterogeneous culture, i.e., the subpopulation that had not shifted down; the effect of a combination of these two drugs is no greater than that of either drug alone. Metronidazole attacked a different subpopulation, the members of which had shifted down from active aerobic growth to dormancy, so when metronidazole was used in combination with either isoniazid or rifampin, each attacked the subpopulation that was refractive to the effect of the other.

When a homogeneous population of dormant tubercle bacilli was exposed to these drugs alone or in combination, metronidazole exhibited marked bactericidal effects which were not enhanced by the addition of isoniazid (Fig. 3). On the other hand, rifampin, which had a small effect on dormant bacilli when it was used alone, significantly potentiated the bactericidal action of metronidazole in combination. Metronidazole exerts its action by virtue of the severe damage to the bacillary DNA resulting from the exposure to unstable products of the reduction of the nitro group in this drug (4). The necessary reduction of the drug has been attributed to the ferredoxins produced by anaerobic bacteria such as clostridia (4). The induction of a product with a comparable reducing potential (−430 to −460 mV) in tubercle bacilli that have adapted to anaerobic survival remains to be demonstrated. Metronidazole has also been reported to interfere with the enzymes involved in DNA repair (4). Rifampin, which binds to and inhibits an RNA polymerase (11), may also interfere with the repair of the DNA damaged by the reduced metronidazole product(s).

The results obtained in the study reported here suggest a potential for a two-pronged attack on tubercle bacilli, i.e., the use of metronidazole in combination with other, conventional antituberculosis drugs to exert a lethal action against bacilli in both the active and the dormant stages. We found no antagonism between metronidazole and either isoniazid or rifampin. In the case of a mixed population exhibiting different degrees of adaptation to anaerobic survival, such as would be expected to occur in different lesions or even different regions of the same lesion, in an individual suffering from active tuberculosis, the conventional antituberculosis drugs would attack the actively replicating organisms, and the metronidazole should attack those bacilli that have shifted down to dormancy in areas of O\(_2\) depletion. In the case of latent disease, i.e., when clinical disease is not evident, for example, following conversion of the tuberculin test, a small population of occult organisms may be lying dormant in microfoci of oxygen limitation, but with the potential to shift up, start replicating, and initiate the disease process. Under these circumstances, metronidazole could exert a sterilizing effect, either alone or with an additive or synergistic effect of rifampin, as seen in the experiments whose results are illustrated in Fig. 3.

Metronidazole is readily absorbed after oral administration, with doses of 250 to 500 mg yielding peak levels in serum of between 6 and 11 μg/ml (15, 28). Intravenous administration of 7.5 mg/kg of body weight leads to peak levels in serum of 20 to 25 μg/ml, with a half-life of 8 h (28). Thus, the peak ranges attainable with this drug are within the concentrations found to be bactericidal for dormant M. tuberculosis in the present study. It remains to be determined if extended oral administration of metronidazole alone or in combination with other drugs, with the resultant series of pulsed peak exposures, leads to bactericidal effects comparable to those seen with constant in vitro exposure. Since the related drugs tinidazole and ornidazole were found to exert effects comparable to those of metronidazole, it may be useful to screen other members of the nitroimidazole class of compounds against dormant tubercle bacilli in a search for products that may yield even more sustained bactericidal levels of drug.

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REFERENCES

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