MICROBIAL PERSISTENCE*

II. Characteristics of the Sterile State of Tubercle Bacilli

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In the preceding paper (1) evidence was presented that the phenomenon whereby large populations of tubercle bacilli can be made to "disappear" from the tissues of mice is produced by an alteration of the bacilli to a sterile[1] state. It was further shown: that the phenomenon is a specific one produced by the actions of two drugs, pyrazinamide and isoniazid; that it occurs in all or virtually all of the animals in an experimental group; and that once assumed, the sterile state endures for a period measurable in months. Whether the sterile bacilli are morphologically altered cannot be stated from the available evidence. The only indication of a host influence in maintaining the microbial sterility is the increased incidence of microbial revival when large daily doses of cortisone are given in the 3rd or 4th month after sterilization. The present paper is concerned with observations that serve to characterize more sharply this phenomenon of the sterilization of tubercle bacilli in vivo.

Materials and Methods

Basic model.—

The basic model used in these experiments has been described in the preceding report (1) and in previous publications (2, 3). In its essentials, it consists of: infecting large numbers of

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[1] In this paper and the one that precedes, the words *sterile* and *sterilization* refer only to the tubercle bacilli and are used in the biologic sense of "not capable of reproduction". Such a state may or may not be reversible. All available methods for the detection of living microbes in the tissues are based on their capacity to multiply. Once this is blocked, it obviously becomes impossible to determine directly at a particular point in time whether living microbes are present or have been exterminated. The wide interest today in microbial plasticity coupled with our quite limited technics for direct microbial observations are making it necessary to impose such restrictions on familiar words when dealing with concepts like the viability or the nonviability of microbes. The justification for using the word sterile in so restricted a fashion, therefore, is that only by so doing, can we describe with precision what has actually been observed and the limits of what is presently demonstrable.

young mice (male albino, Webster Swiss strain, Carworth Farms, New City, New York) with 1 to 3 million human tubercle bacilli (H37Rv) (0.2 ml of 10^{-1} dilution of culture in 0.1% bovine albumin) by the intravenous route and administering isoniazid, 0.0125% and pyrazinamide, 2.0% in the daily diet. Administration of the two drugs is started in less than 20 minutes after infection and is continued for 12 wk. The census of culturable tubercle bacilli in the homogenates of the lungs and spleens of periodically sacrificed sample subgroups is determined at the start of therapy or cortisone administration, during and at the completion of the period of drug administration, and at designated periods thereafter. As was previously reported (1), with a very few exceptions, no tubercle bacilli capable of multiplication in mice, guinea pigs, or cell-free or cell-containing cultures, were demonstrable at the point 12 wk after the start of drug administration or in the succeeding month. Variations of this basic model are set forth in the individual experiments below.

Drug Susceptibility Tests.—

Colonies of tubercle bacilli isolated from animal tissues onto solid oleic acid-albumin agar were subcultured in Tween®-albumin medium. After 7 days' incubation, the culture was diluted five and one half times in Tween basal medium (without albumin or glucose) at pH 5.55 for pyrazinamide and pH 7.2 for isoniazid. An inoculum of 0.1 ml of this suspension was used for each tube in the test series. (This is the equivalent of from 2×10^5 to 2×10^6 culturable units per tube.)

An autoclaved stock solution of isoniazid in distilled water, 10 mg per ml, was used. Serial twofold dilutions were made in Tween-albumin medium (pH 7.2) to give a final volume of 5 ml per tube. If the culture to be tested was believed to be in the susceptible range, the concentrations of isoniazid used ranged from 0.008 to 2.0 μg per ml, whereas if the test culture was believed to be resistant, isoniazid concentrations that ranged from 0.8 to 200 μg per ml were employed. One tube of Tween-albumin medium containing no drug was included in each test series.

The stock solution of pyrazinamide was 5 mg per ml of albumin-glucose (4.5% bovine serum albumin fraction V plus 5% glucose in physiologic saline) sterilized by Seitz filtration. This solution was diluted in albumin-glucose, and 0.5 ml of each dilution was added to 4.5 ml of Tween basal medium which had been adjusted to pH 5.55 with hydrochloric acid. The final concentrations of pyrazinamide were 500, 100, 20, and 10 μg per ml. One tube of Tween-albumin medium, adjusted to 5.55, was included in each series. A 7 day culture of *Mycobacterium tuberculosis* H37Rv was included as a control for each drug.

All cultures were examined after 7 and 14 days' incubation at 37°C. The drug susceptibility of the culture was defined as the lowest concentration of drug in which no macroscopic growth could be detected after 14 days' incubation. If this concentration was below 0.064 μg for isoniazid or 100 μg for pyrazinamide, the culture was considered to be susceptible to the corresponding drug.

Catalase production: A mixture of equal proportions of 32% hydrogen peroxide and 10% Tween 80 was used as reagent. One drop was applied to a colony growing on an oleic acid albumin agar plate. The reaction was recorded as positive if the colony and the reagent reacted by bubbling.

Niacin production: The reagents employed were: cyanogen bromide, 10% or saturated in water; para-aminosalicylic acid, 1% in H_2O; or aniline, 4% in 95% ethanol. For performance of the tests, three methods were used. In the first, 0.2 ml of a 7 day (or older) liquid culture (Tween-albumin medium or oleic acid-albumin), were placed in the well of a spot plate and 0.1 ml aniline and 0.1 cyanogen bromide were added and observed up to 10 min for yellow color. A strain giving a negative result was retested by the para-aminosalicylic acid tube method, using a 14 day Tween-albumin medium culture. In the second method, 2 ml of a 7

day or older culture in oleic acid-albumin liquid medium, 1 ml of aniline, and 1 ml of cyanogen bromide were mixed in a test tube. The third method was used for Tween-albumin medium cultures. Para-aminosalicylic acid was substituted for aniline. This method was regarded as final, if a 14 day Tween-albumin medium culture was used. A culture of *M. tuberculosis* H37Rv was the positive control for all of these methods.

OBSERVATIONS

Sequential Exposure to the Two Drugs

In the standard model employed most frequently to sterilize the populations of tubercle bacilli in the tissues, the period of two drug exposure had been fixed at 12 wk. In previously reported studies (3), it was shown that so long as the 12 wk period of total chemotherapy were kept constant, it was possible to produce the phenomenon by administration of the two drugs in sequence. The order of the sequence was critical, however, and sterilization occurred with uniformity only when it was the isoniazid that was given first. In the particular experiments cited, the period of prior isoniazid administration had been arbitrarily set at 4 wk followed by eight wk of pyrazinamide. To define the limits of the necessary period of isoniazid exposure more closely, an experiment was conducted in which isoniazid was followed by pyrazinamide for a total period of 12 wk with different groups of animals receiving the isoniazid for 4 days, 2 wk, and 4 wk respectively. Drug administration was started immediately after, i.e. within 20 min of infection. In agreement with the previous studies (3), complete sterilization of the tubercle bacilli was attained in the mice that had received the isoniazid for 4 wk. However, in the animals that had received the isoniazid for the lesser periods, culturable tubercle bacilli were present in 1 of the 7 mice in each of the two sample groups. Thus, for successful sterilization by the 12 wk regimen of chemotherapy, the necessary period of isoniazid influence is more than 2, but no more than 4, wk.

Prolongation of Two Drug Exposure to 26 Wk

In the preceding paper (1) it was noted that as late as 3 months after the cessation of drug administration, the ultimate destiny of the sterile bacilli seemed not yet decided in that, while capable of reviving, they did not always do so. This suggested that prolongation of the total period of drug exposure might exert an influence on microbial revival. Accordingly, three experiments were conducted in which administration of both drugs was continued for a total period of 26 (in one case, 25) wk.

Except for the duration of drug exposure, the procedures followed were essentially the same as in the standard model previously described (1). Drug administration was started within 20 min of infection, which was established by the intravenous route. The inoculum contained one to three million tubercle bacilli. The H37Rv (Cornell) strain of tubercle bacilli was used and the inoculum was unfiltered.

In all three experiments, there was complete sterilization of the populations of tubercle bacilli in the animal samples sacrificed at the completion of the 25 or 26 wk period of drug exposure. No microbial revival at all was observed in a 6 month follow-up period in the first experiment in which no evocation by cortisone was attempted. There was also no microbial revival at all in the second experiment in which cortisone (daily dose 1.0 mg) was administered for 4 wk to different groups of animals in the 1st and 3rd posttreatment months. In the third experiment, however, highly unusual mycobacteria, possibly altered tubercle bacilli (see below) were isolated from the spleens of 3 animals, 2 of which had received cortisone, in the 3rd posttreatment month. No culturable tubercle bacilli were found in a total of 28 mice sacrificed 14 or 18 wk after completion of therapy or from 32 other animals that had received cortisone during the 4th posttreatment month. The characteristics of these unusual mycobacteria, seen only in the posttreatment period, are presented in a subsequent section of this paper.

Microscopy at End of Drug Exposures.—At the completion of the 25 (or 26) wk of exposure, the splenic homogenates of 5 animals from two of the above experiments were examined microscopically, using either the Ziehl-Nielsen or the fluorescence procedure. Microbial forms, morphologically like tubercle bacilli, similar to those described in the preceding paper (1) were present in sufficient numbers to permit easy detection. Crude estimates of the number of microbial forms present yielded values essentially the same as after the 12 wk regimen of drug therapy, i.e. the residual population would represent less than 1% of the census of culturable tubercle bacilli in the spleen 24 hr after the initiation of infection.

Isoniazid Alone after 6 Wk.—A variation of the 26 wk regimen of chemotherapy was studied without change in either the size of the infecting inoculum or the immediate start in the administration of the two drugs. The single variation was that the pyrazinamide portion of the two drug therapy was discontinued after 6 wk and for the remainder of the 26 wk period the animals received isoniazid alone.

At the completion of the 26 wk of chemotherapy, no tubercle bacilli were cultured from the lungs or spleens of the 10 animals sacrificed. At the end of the 3rd posttreatment month, culturable tubercle bacilli were present in the spleens of 10 of 14 animals. This high incidence of microbial revival is in contrast to the absence of any microbial revival in the first 3 posttreatment months of the 3 experiments in which the 26 wk regimen included both drugs for the entire period.

In other subgroups in this experiment it was observed in agreement with previous experience (3) that sterilization of the populations cannot be induced by a 6 wk period of administration of both drugs; the spleens of 7 of 10 animals revealed culturable bacilli at that time. After the first 12 wk, however, during the latter half of which only isoniazid was administered, there were no cul-

turable bacilli in the spleens or lungs of the 10 animals sacrificed. No observations on the course of microbial revival were made on this subgroup.

Prolongation of Infection before Drug Exposure

Prolongation of the period of drug exposure to 26 wk represents a variable that could be introduced without variation in the size of the pretreatment population or its age in vivo. However, the next variable studied, prolongation of the age of the pretreatment infection, obviously carries with it other variables that are difficult to isolate. Prominent among these are a considerably higher census of tubercle bacilli at the time of first drug exposure and the presence of clearly visible inflammatory-necrotic lesions. In previous studies (3) it had been shown that the 12 wk two drug regimen produced sterilization when microscopic lesions were present as a result of allowing the infection to progress for 21 days before the drug administration was started.

Accordingly, experiments were conducted in which the infection was allowed to progress for a considerably longer period before the start of the exposure to the two drugs.

8 Wk Infection.—Forty male albino mice of the Webster Swiss strain were infected intravenously with 0.2 ml of a 10^{-1} dilution of a 7 day culture of *M. tuberculosis* H37Rv and were allowed to remain untreated for an 8 wk period. The administration of pyrazinamide and isoniazid in the standard dosage was then started and continued daily for a 12 wk period.

Nature of the Lesion.—8 wk after infection, at the time drug administration was started, the mice appeared ill as evidenced by sluggish activity, and unkempt coats. On macroscopic examination post-mortem, the spleens were increased in size and a moderate number of 1 to 2 mm lesions were scattered over all lobes of the lungs. On microscopic examination of the lung sections, the tissue appeared edematous and presented changes that were more extensive but of the same sort as were seen in the 21 day lesions (3). The cellular involvement was less diffuse in the 8 wk lesions; instead there were nodular accumulations of cells. In these nodules, many lymphocytes were mixed with histiocytes. The macrophages had foamy cytoplasm and contained many tubercle bacilli. Some of the macrophages contained what appeared to be ghosts of the previous site of cyrstals. These "clefts" had the aspect of cholesterol crystals.

Pretreatment Census.—Immediately before the start of therapy, the pulmonic populations of tubercle bacilli were approximately 15 million (1.52×10^7) culturable units per milliliter of homogenized lung. This is in contrast to the situation when therapy was started immediately after infection, at which time the number of tubercle bacilli in the entire animal body could have been little more than the one to three million organisms just introduced. The splenic populations of the 8 wk infection were at about the same level as those determined at 24 hr after infection in untreated animals.

12 Wk and 26 Wk Regimens.—The *12 wk regimen* of pyrazinamide-isoniazid resulted in complete sterilization of the populations of tubercle bacilli in the spleen and lungs of all 15 animals sacrificed at the completion of drug therapy. 3 months later, culturable tubercle bacilli were demonstrable in the tissues of 6 of the 14 animals sacrificed.

The *26 wk regimen* likewise resulted in sterilization of the tubercle bacilli

uniformly throughout the 15 mouse groups sacrificed at the 12 wk point and at the completion of therapy. In contrast to the 12 wk regimen, however, with the 26 wk regimen, microbial revival was found in only 1 of a total of 60 animals examined during a 3 month posttreatment period. This single revived strain was isolated at the end of the 2nd posttreatment month from both the lungs and spleen of an animal that had received no cortisone. The strain was resistant to both pyrazinamide and isoniazid when tested separately against each drug in vitro, and it did not produce catalase. A total of 39 of the 60 animals received large daily doses of cortisone (1.0 mg) within the 3rd posttreatment month.

15 Wk Infection

In four experiments, the infection was allowed to progress untreated for a 15 wk period before drug administration was started. At this 15 wk observation point, the animals generally appeared ill and inactive.

Nature of the Lesion.—In all experiments, the lungs of those sacrificed immediately prior to therapy, were enlarged and almost completely filled with advanced lesions. The most striking finding on postmortem examination was the extensive pulmonary involvement consisting of light grey granulomatous lesions, some of which were surrounded by areas of hemorrhage. There was hepatic and splenic enlargement but no gross lesions were present. On microscopic examination, the pulmonary disease represented an increase of the same kind of involvement as had been seen in the 8 wk lesion. In the 15 wk lesion, the tissue appeared more edematous and was moderately hemorrhagic. The inflammatory cells were seen to be predominantly perivascular and peribronchial. More plasma cells were seen than in the earlier lesions. A small amount of fibrous tissue was seen. A large number of macrophages with foamy cytoplasm contained many acid-fast bacilli. Many cholesterol "crystal ghosts" were seen. The process resembled a lipoid pneumonia, but despite the extensive involvement, there were no gross areas of necrosis.

Pretreatment Census.—At the time of first drug exposure, the pulmonic populations were 36 million, 54 million, 83 million, and 102 million culturable units of tubercle bacilli per milliliter of homogenized lung respectively; the splenic populations were 7.8 million, 3.0 million, 1.3 million, and 1.1 million per ml respectively.

12 Wk and 26 Wk Two Drug Regimen.—In the first two experiments, the *12 wk regimen* of pyrazinamide and isoniazid was employed and there was a failure to attain sterilization with uniformity. In each experiment, at the end of the 12 wk period of two drug administration, one-third of the sacrifice group (5 of 15 mice) yielded culturable tubercle bacilli. The 10 strains of persisting tubercle bacilli were present in both the lungs and the spleen. They showed no unusual growth or colonial characteristics and all were susceptible to each of the two drugs when tested in vitro. No other posttreatment observations were made.

With the 26 wk regimen there was also a failure to attain sterilization with uniformity; nevertheless the results were substantially different from those obtained with the 12 wk two drug regimen. In each experiment, 1 animal sac-

rificed at the completion of therapy revealed culturable tubercle bacilli. The "culturable bacilli" in one experiment consisted of a single atypical colony of acid-fast bacilli present only in the splenic culture. In the other experiment, the surviving strain was present in greater numbers in both spleen and lung and it grew on subculture. It was resistant to both pyrazinamide and isoniazid when tested against the two drugs separately in vitro.

In the experiment in which the sterilization failure consisted solely of one colony of a strain that was not subcultured, the posttreatment observation period was 3 months and cortisone was given to 30 animals. Culturable bacilli were found in a total of 9 of 38 animals: 5 of the 9 mice had received cortisone; the other 4 animals had not. On initial culture, these 9 isolates were present in numbers that ranged from 8 to 1824 culturable units per ml of organ homogenate. They were not tested for drug-susceptibility.

In the other experiment in which the only strain culturable at the completion of therapy was drug-resistant, 2 of the only 3 strains recovered in the posttreatment period were also drug-resistant, but only to pyrazinamide. One of the 3 strains produced catalase whereas the other 2 did not. All 3 strains were recovered from 3 of the 15 mice sacrificed at the end of the 3rd posttreatment month; there were no culturable bacilli found in the group of 10 mice sacrificed 6 months after the completion of treatment. On routine microscopic examination of the pulmonary homogenates from 2 of these animals, however, minute bacillary forms that retained the acid-fast stain could be seen. The intensified microscopic search procedures described in the preceding paper (1) were not used in this experiment.

Sterilization Failures

The term "sterilization failure" is used to identify those experiments in which any tubercle bacilli could be cultured from any of the animal groups sacrificed at the completion of the period of two drug exposure. Excluding the 2 experiments with suboptimal periods of drug exposure, there were 5 (of a total of 35) in which there was sterilization failure (Table I). In 4 of these 5 experiments, drug-resistant bacilli were isolated; the strain present in the fifth experiment was not subcultured and was not tested. In 2 of the 5 experiments, the surviving strains were clearly identifiable as tubercle bacilli; the strains in the other 3 experiments, not clearly identifiable as tubercle bacilli, were unusual mycobacteria as described immediately below. The unusual mycobacteria encountered were of two varieties.

The first variety was isolated at the completion of therapy in two consecutive experiments in 1961 in which the 12 wk two drug regimen was started immediately after infection. In each experiment, on one culture of pulmonary homogenate a large solitary colony appeared. Unlike the second variety, both of these 1961 strains were readily subcultured; indeed, they grew rapidly at room temperature on Petragnani medium. They produced catalase, but no

niacin, and both strains were resistant to isoniazid concentrations higher than 200 μg per ml and pyrazinamide concentrations of 500 μg per ml. By contrast, all bacilli that were isolated during the posttreatment period in both experiments were clearly identifiable tubercle bacilli wholly susceptible to pyrazinamide and isoniazid.

If the two unusual strains, isolated at the completion of the 12 wk regimen of therapy, represented bizarre forms of drug-resistant tubercle bacilli, it would be expected that some drug-resistant strains would have been found in the posttreatment period. From both the context in which they arose and their biologic properties, it is suspected that these 2 strains do not represent tubercle bacilli but are so called "atypical" mycobacteria.

The situation favors the opposite interpretation for *the second variety* of unusual mycobacteria encountered. This variety was found in 1 of 15 animals sacrificed at the completion of the 25 wk two drug regimen for the 15 wk infection described above. This strain, a slow

TABLE I

Sterilization Failures

Characteristics of Mycobacterial Strains Recovered at Completion of Treatment in Five Experiments

Experiment No.	Age of pretreatment infection	Treatment period	Characteristics	
			End of treatment	Later posttreatment period
		wk		
1 and 2	<20 min	12	1 colony, atypical, resistant to both drugs	typical, drug susceptible
3	<20 min	12	Typical, resistant to pyrazinamide (another isolate susceptible)	Typical, resistant to both drugs
4	15 wk	25	Slow growth no subculture	Not subcultured
5	15 wk	26	Typical, resistant to both drugs	Typical, resistant to pyrazinamide

grower, is believed to represent tubercle bacilli that had been altered by the 25 wk period of two drug exposure in vivo.

The 2 strains of drug-resistant bacilli, clearly tubercle bacilli, that were isolated at the completion of therapy have been described above in connection with the individual experiments. The 12 wk two drug regimen had been used in one experiment and the 26 wk regimen in the other. In both experiments the finding of the drug-resistant strain in 1 of the animals at the completion of therapy was associated with a high incidence of drug-resistance among the tubercle bacilli isolated in the posttreatment period. In the experiment with the 12 wk regimen there was a second animal that yielded tubercle bacilli at the completion of therapy and this strain was susceptible to pyrazinamide and to isoniazid when tested in vitro.

In sum: in 4 of the 5 experiments in which the sterilization failure requires explanation, mycobacteria resistant in vitro to one or both drugs were isolated at the completion of treatment. In the fifth experiment, the sole strain surviving at the completion of treatment could not be subcultured and its drug susceptibility was not established. Only 2 of the drug-resistant strains were clearly identifiable as tubercle bacilli. It should be noted that in one of these experiments in which a drug-resistant strain was present, a drug-susceptible strain was also isolated from another animal at the completion of the 12 wk of two drug therapy.

Unusual Mycobacteria Isolated in the Posttreatment Period

No unusual mycobacteria were found in the posttreatment period in any of the experiments with the 12 wk two drug regimen. With the 26 wk regimen, however, there were 3 isolations that merit description. These 3 strains or isolates represent "failure" of a sort in that they were the only culturable bacilli found in a posttreatment observation period of at least 4 months in any of the 3 experiments in which the 26 wk two drug regimen was started immediately after infection.

All 3 isolates appeared to be the same: they were found in 3 of the 64 animals examined in the posttreatment period of one experiment; and 2 of the 3 animals yielding the strain, had received cortisone. In each case, the strain appeared as a single large colony of small nonchromogenic bacilli that retained the acid-fast stain. All 3 isolates formed catalase but failed to produce niacin or show "cord" formation. They were resistant to high concentrations of isoniazid and pyrazinamide in vitro and when subcultured on Petragnani medium, they grew in 1 wk at room temperature. Obviously, these mycobacteria were closely similar if not identical to the 2 strains, thought to be nontuberculous mycobacteria of the first variety, that were isolated at the completion of the 12 wk regimen in the 2 successive experiments in 1961. Caution seems advisable in concluding that these 3 "posttreatment" strains or isolates were not altered tubercle bacilli, however, in view of the long period of exposure to the two drugs (26 wk) experienced by the populations of tubercle bacilli in this experiment.

Long-Term Survival of Tubercle Bacilli

Observations made on another subgroup of an experiment presented above are reported here because they illustrate the difficulty of establishing that tubercle bacilli are *not* present during the long posttreatment periods necessary to study the sterilization phenomenon. The drug regimen used was the suboptimal one in which the pyrazinamide portion of the drug pair had been discontinued after only 6 wk. This was followed, in the subgroup in question, by the administration of isoniazid for a 1 yr period. The drug exposure had been started immediately after the establishment of the infection.

Despite the suboptimal nature of the regimen in terms of sterilization, no culturable tubercle bacilli were obtained in sample animal groups sacrificed at 12 or at 26 wk in the roughly 13 month period of chemotherapy. 5 days after the completion of the 13 months of treatment there were no culturable tubercle bacilli in the spleens or lungs of a 5 animal sacrifice group.

There were also no culturable bacilli found in a 10 animal group sacrificed at the end of the 2nd posttreatment month. At this point, the standard daily dosage of 1.0 mg of cortisone was started. Eight animals died in the ensuing 3 wk and an additional 5 were sacrificed shortly thereafter. In 12 of the 13 there were no culturable bacilli; in 1 animal (death on day 22 of cortisone) a solitary colony of the second variety described previously, was present in a splenic culture and failed to grow on subculture. In an additional group of 5 animals that received no cortisone, no culturable bacilli were found when sacrificed 3 months after the completion of therapy. Thus, of a total of 21 animals examined during, or at the end of, the 3rd posttreatment month, the sole finding was a solitary "unusual" colony in 1 animal.

As it is not possible to make exact predictions of the number of animals that will be available for sampling in these year-long experiments, there are rare occasions when a few mice are still available at the planned end of the experiment. There were 3 such mice in the present experiment and it was decided to continue to observe them until death. One animal died 6 months after completion of therapy and another died 9.5 months after therapy. No culturable tubercle bacilli were found in the spleens or lungs of either animal. The 3rd mouse survived until 10 months posttreatment or for approximately 2 yr after infection and the start of the 13 months treatment. Culturable tubercle bacilli were present in a calculated census of 33,156 per ml of homogenized spleen and 13,076 per ml of homogenized lung. The strain showed no unusual colonial morphology or growth characteristics. It produced both catalase and niacin. Isolates from both lungs and spleen were susceptible in vitro to isoniazid concentrations of 0.032 μg per ml, and to pyrazinamide concentrations of 100 μg per ml.

DISCUSSION

From the observations presented above and those of the preceding papers (1–3, 5), it is possible to define a number of the characteristics of the sterilization phenomenon. In the experimental conditions of the standard 12 wk two drug regimen started immediately after infection, and considering only those populations of tubercle bacilli that are in the spleen:[2]

Isoniazid does not sterilize the populations either when administered alone, or with streptomycin and/or para-aminosalicylic acid (PAS) (1, 3).

Pyrazinamide can completely sterilize the populations but it does so only irregularly when administered alone; with less than complete uniformity when given with streptomycin or PAS (1); and it sterilizes with uniformity only when preceded or accompanied by isoniazid.

Together, the two drugs do not produce the phenomenon with uniformity when the infecting population of tubercle bacilli is predominantly resistant in vitro to either one. Nor does either drug administered alone, produce it, when the population is predominantly resistant to the other drug (3, 5).

In sequence, the two drugs produce the phenomenon only when it is the isoniazid that is administered first, and when this period of isoniazid exposure is more than 2 wk but it need not be more than 4 (3, 1). Pyrazinamide thus sterilizes isoniazid-influenced tubercle bacilli, most of which are presumably not multiplying at the time.

Administered together, pyrazinamide blocks the population-reducing effects of isoniazid for 2 to 3 wk. *This blocking is produced even when the infecting strain is resistant to pyrazinamide in vitro* (5) and can also be produced by the pyrazinamide parent drug, nicotinamide (3).

[2] The most difficult to sterilize with uniformity.

Tubercle bacilli in the sterile state are nevertheless susceptible to further drug influence; extension of the two drug exposure from 12 to 26 wk virtually abolishes microbial revival in the 4 to 6 month posttreatment period.

A relationship exists between the size (or age) of the pretreatment population and the necessary period of two drug exposure. With the standard inoculum, 6 wk of two drug exposure are insufficient but 12 wk are enough. With the much larger pretreatment populations of the 15 wk infection, 12 wk of two drug exposure are insufficient but 26 wk produce markedly different results.

The relatively few sterilization failures despite presumably adequate drug exposure, have been associated with microbial strains resistant in vitro to pyrazinamide, or to both drugs.[3] None of these relatively rare failures has been associated with resistance to isoniazid alone.

Neither the host resistance acquired as a consequence of infection (1) nor the presence of macroscopic lesions per se, appears to exert an important influence on the occurrence of the sterilization. By contrast, some host factor that can be neutralized by near lethal doses of cortisone appears to play some role in the postponement of microbial revival (1).

These observations are interpreted as showing: that it is the pyrazinamide that possesses the sterilizing type of action; that this action can be exerted on populations that are undergoing little, if any, multiplication; that the range of this action can be broadened by concurrent administration of other antituberculous drugs; but that this broadening of the action of pyrazinamide cannot be carried to the point of uniform sterilization without an action by isoniazid; and that this essential isoniazid action does not take place as readily in the presence or immediately after pyrazinamide (or nicotinamide) as when the isoniazid is allowed to act on the tubercle bacilli first.

If the uniformity of the sterilization depended principally on one drug acting on individual tubercle bacilli not affected by the other, it would be expected: that isoniazid with streptomycin and PAS would produce it; and that when produced by pyrazinamide and isoniazid, the order of their sequence should not be crucial. Neither of these assumptions proved to be the case. The order of sequence is crucial and the prior exposure to isoniazid must be longer than 2 wk. The phenomenon thus appears to represent a dependent action of the two drugs.

The capacity of tubercle bacilli to assume this chemically-induced sterile state depends primarily on factors intrinsic to the microbe. Once the state is induced, the host plays some role in inhibiting microbial revival but its role is not crucial (1). The rare sterilization failures, i.e. the failure to induce sterilization with uniformity throughout a group of animals, likewise appear to

[3] In this connection, the true identity of the atypical mycobacteria is unimportant because, except for the one strain not tested, they were all either resistant to pyrazinamide or resistant to both drugs. Thus, these unusual strains represent either incidental findings that can be wholly disregarded or altered tubercle bacilli with the same pattern of drug resistance as the identifiable tubercle bacilli of the sterilization failures.

depend on factors that are intrinsic to the microbe and largely independent of the host. This centrality of the microbe is in keeping with the concept that the sterile state is a separately definable form of that larger microbial adaptive capacity known as microbial persistence (6, 7).

The characteristics that make the phenomenon unique are not the sterilization per se, but the facts that: it occurs with such uniformity in populations of this size within the 12 wk period of drug exposure; it can be induced only in tubercle bacilli of human origin; and it can be produced when the bacilli are exposed to the two chemicals in a specific sequence.

Other isoniazid-containing chemotherapies, when administered for long periods (e.g. 9 months) will result in sterile populations in a portion of the animals (8). Pyrazinamide regimens that do not include isoniazid, will also do this, even within the 12 wk period. Isoniazid alone, will sterilize the populations in the lungs (but not the spleens) of about one-half the animals in the 12 wk period in the standard model (2). In all of these circumstances, with populations of the size of the present experiments, there is a massive killing or incapacitation of the great bulk of the population in the first 4 wk of drug exposure but a residuum of tubercle bacilli will persist in a drug-susceptible but fertile state. These residual populations will be present in all animals (except those that received any pyrazinamide) at the 12 wk point and will persist in at least a small minority throughout many months of continued administration of the chemotherapies. Large doses of isoniazid (100 mg/kg) will sterilize the populations in some mice, but not in all, in the standard 12 wk period (4). Very small populations (inoculum fewer than 100 culturable units) of either bovine or human tubercle bacilli can also be sterilized by the use of isoniazid alone (9). It is not yet known, however, whether this can be done with uniformity. Presumably, in terms of initial census, there is a dividing point, below which the residual populations would not always be present at 12 wk following treatment with the isoniazid-containing chemotherapies that do not include pyrazinamide. However, with the initial census in the standard model in the present experiments, a census that is actually lower than in most tuberculous lesions, the residual populations are invariably present.

Thus, it is clear that sterilization per se is something that can be produced in tubercle bacilli in vivo by isoniazid with or without a companion drug other than pyrazinamide. In some cases, the sterilization takes place in only a portion of a population; in others, the entire population within an animal is included; and in still others, the major portion of the animals in a group are involved. It is not known whether these tubercle bacilli, rendered sterile irregularly and in some cases only after many months of chemotherapy, represent the same microbial state as the bacilli in the populations that are sterilized rapidly, completely, and with virtual uniformity by pyrazinamide and isoniazid. From the observations in the present studies, however, there is some indication that

gradations in the state exist as shown by differences in the incidence and rapidity of microbial revival. It is also clear that in sterilization, as in other forms of drug-microbe reactions involving tubercle bacilli, the size of the population and the duration and amount of drug exposure are factors that govern the outcome. The question of gradations in the sterile state will be considered in a subsequent section.

The massive killing or incapacitation of the great bulk of the microbial population that occurs in the first 4 wk of two drug exposure does not necessarily represent a dependent action of pyrazinamide and isoniazid, because it can be produced by the administration of either drug alone. Presumably, during this phase, some bacilli could be rendered sterile rather than killed by the isoniazid, as is known to occur with the pyrazinamide. Whether the invariable occurrence of the residual populations represents a direct action of the isoniazid is not known but their sterilization with uniformity within the 12 wk period, requires an action that can be provided only by isoniazid. It is this virtually uniform sterilization of these residual populations that is unique, and that appears to require a dependent action of the two drugs.

When these observations are viewed against the prominence of pyrazinamide resistance in the few sterilization failures, it seems reasonable to infer that the key factor in the uniformity of the sterilization phenomenon is the continued maintenance of susceptibility to the pyrazinamide. For, a considerable portion of the irregularity of the sterilization produced by pyrazinamide alone, is caused by the emergence of pyrazinamide-resistant tubercle bacilli (13). For example, in the unsuccessful attempts to produce sterilization at 12 wk with streptomycin as the companion to the pyrazinamide (1), 2 of the 3 strains that survived at the completion of therapy were resistant to pyrazinamide. The third was not tested.

An attractive explanation for the sterilization phenomenon, therefore, is that tubercle bacilli that have survived 4 wk of isoniazid exposure are specifically rendered incapable of exhibiting pyrazinamide-resistance on concurrent or *subsequent* exposure to the drug. If this were the case, the sterilization failures (exclusive of suboptimal time-dose regimens) would consist principally, if not exclusively, of those instances in which it had not been possible for the isoniazid to block the pyrazinamide resistance because: the bacilli were already resistant to pyrazinamide at the time of first isoniazid exposure; they emerged resistant to pyrazinamide during the early weeks of concurrent therapy while the isoniazid influence was temporarily neutralized; or they were resistant to isoniazid at the time of first exposure to that drug. In the last named circumstances, some of the bacilli could be sterilized solely by the action of the pyrazinamide alone but those in which this failed to occur, on continued drug exposure, would be at high risk of pyrazinamide resistance or, in actuality, resistance to both drugs. For these last named reasons, sterilization failure due to resistance

to isoniazid alone, would be quite unusual. The chances for failure due to pyrazinamide resistance or two drug resistance would be somewhat increased in the 15 wk infection model with its greater opportunity for the few drug-resistant cells originally present[4] to multiply (or arise *de novo*) before the start of the 26 wk period of two drug exposure. The uniformity of the sterilization observed in the standard model (drug exposure started within minutes of infection) would thus reflect the fact that failure would require the coincidence of two events: the presence of drug-resistant cells at the time of first drug exposure; and their successful avoidance of the action of the drug to which they were susceptible. In the experimental conditions of the standard model in which drug exposure is started immediately after infection, either one of these events should be rare.

None of the eventualities set forth above is at variance with the observed data; and this hypothesis that there is a sublethal action of isoniazid that can render the cell incapable of exhibiting pyrazinamide resistance is currently being studied in appropriate long term experiments.

A major characteristic of the sterile state revealed in the present studies is that populations that cannot be shown to be capable of multiplication in vivo or in vitro are nevertheless susceptible to further drug influence. Microbial revival was strikingly suppressed when the administration of the two drugs was continued for an additional 14 wk after the 12 wk point at which sterilization was demonstrable. Indeed, when the two drug exposure was started immediately after infection, revival was suppressed completely in 2 experiments and its occurrence in the third is not absolutely certain. When all of these experiments with the 26 wk two drug regimen are grouped together without regard to the pretreatment age of the infection, bacilli of some sort were cultured during the posttreatment period from 18 of the more than 300 mice examined. All of these 18 isolates (except for one not tested for drug susceptibility) were either highly abnormal in their colonial and growth characteristics, were drug resistant, or were both. This virtually complete absence of drug susceptible identifiable tubercle bacilli in the posttreatment period when the period of drug exposure had been extended by 14 wk, is in contrast to the findings in the posttreatment period, when the drug exposure had been stopped at 12 wk. In the last named circumstances, considering only the standard model, all of the more than 300 isolates appeared as characteristic tubercle bacilli except for the 9 of the 99 strains tested thus far, that showed drug resistance (6 resistant to isoniazid; 3 to pyrazinamide).

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[4] The H37Rv strain of tubercle bacilli employed in virtually all of the experiments, shows some fluctuation over time but generally contains approximately 5 culturable units per million that are resistant in vitro to isoniazid concentrations of 0.25 μg per ml. The number of units resistant in vitro at pH 5.5 to pyrazinamide concentrations of 50 μg per ml is generally slightly lower, about 3 to 4 units per million. Tubercle bacilli resistant to both drugs have not been isolated from this standard strain.

The significance of the unusual or atypical bacilli is difficult to establish. For, there is no method by which to distinguish convincingly between a tubercle bacillus that has been sufficiently "battered" by its drug exposure to lose the characteristics used to identify the phenotype, and adventitious infection with a strain of mycobacteria other than *M. tuberculosis*. On the one hand, the possibilities of adventitious infection by nontuberculous mycobacteria during the handling of the tissues postmortem or their evocation in the living animal by cortisone, cannot be excluded. Moreover, resistance *in vitro* to isoniazid or to pyrazinamide is common among these nontuberculous mycobacteria. On the other hand, it is known that tubercle bacilli resistant to isoniazid may assume several different forms, some of which may appear highly atypical (10).

As mentioned previously, the observations suggest that there may be significant gradations within the sterile state. Another indication is afforded by the long term experiment with the suboptimal regimen of 6 wk of two drug administration followed by 6.5 wk of isoniazid alone. At the 12 wk observation point, sterilization was demonstrated in the 10 animal sample. The isoniazid alone was then continued for a second 14 wk period at which time (26 wk) the sample sacrificed also revealed uniform sterilization. In the posttreatment period, however, microbial revival was high (10 of 14 mice). This apparently sterile but readily revivable infection was subjected to a second 6 month period of isoniazid alone at which time no culturable bacilli were found even after evocation attempts with cortisone. Finally, 2 yr after infection and almost 1 year after the completion of treatment, characteristic, wholly isoniazid-susceptible tubercle bacilli were isolated from the last surviving animal.

In this experiment, the sterilization was uniform at 12 and after 26 wk of drug exposure and to that extent it yielded results identical with those obtained when both drugs had been continued throughout. But at this 26 wk point, the potential for widespread microbial revival was much greater with the "suboptimal" regimen; indeed, it was even greater than is usually observed after the standard 12 wk two drug regimen. This suggests that, regardless of whether the various sterile states were all qualitatively similar, the one produced by the predominantly isoniazid regimen was considerably less stable.

With the standard 12 wk model, a sterilization phenomenon with considerable stability in effect was made even more stable by an additional 14 wk period of two drug exposure. Obviously, the degree of sterility or "gradations" of the sterile state would merely reflect the extent of the restorative processes that must take place for the resumption of fertility. Using revival time, as a measure of the extent of microbial alteration, the alteration produced by pyrazinamide and isoniazid must be extreme compared with those produced by the other isoniazid chemotherapies. Indeed, in the case of the 26 wk two drug regimen and the 20 min infection, the alterations are so extreme that they may represent the total eradication of the populations of tubercle bacilli. Thus, the familiar question of eradication versus a more durable sterility has to be faced once

again (1). Caution seems advisable in assuming the uniform eradication of the bacilli in view of their demonstrated capacity to persist in the sterile state for long periods and then revive. As the cortisone-evoking procedure has been unsuccessful following the 26 wk two drug regimen, the tedious procedure of very long term observation for "natural revival" is being employed. The appropriate experiments have not yet been conducted to determine whether the observed striking influence of the second 14 wk period of two drug administration represents direct drug action on tubercle bacilli in the sterile state, or an indirect effect produced by making the environment hostile to bacilli in the incipient stages of revival. As reported in the previous paper (1), there is good reason to believe that not all potentially revivable sterilized tubercle bacilli do in fact revive, at least during the 1st yr after sterilization. Conceivably, therefore, tubercle bacilli that persist, or are artificially held, in the sterile state may eventually be eliminated by "natural death". In the sequential experiments of the present study, however, when the pyrazinamide exposure was started at the 4 wk point, the residual populations, while not sterile, were presumably undergoing little multiplication, yet the influence of the ensuing 8 wk of pyrazinamide was unequivocal. Thus, the possibility that the influence exerted by the two drug administration past the 12 wk point, is a direct one, is by no means far fetched.

The nature of the lesion per se does not seem to be a significant factor in preventing the sterilization of the populations of tubercle bacilli therein. The lesions of the 8 wk infection, although considerably less extensive, were qualitatively indistinguishable from those of the 15 wk infection. With the 8 wk infection, at completion of the 26 wk two drug regimen, there was complete sterilization of the populations in the sample group sacrificed, as occurs in the case in the 3 wk (3) or 20 min infections. Moreover, the microbial revival was strikingly suppressed. In the 15 wk infection, revival was similarly suppressed but complete sterilization at the completion of treatment was not attained. Yet the results in the 15 wk and 8 wk infections may be closer than appears at first glance. For, in the "8 wk" experiment and in one of the 2 "15 wk" experiments, there was a single mouse that harbored a small culturable population of tubercle bacilli resistant to both drugs. The strain in the 8 wk experiment was the only isolate in the posttreatment period and represents the only instance in the total 12 yr experience, in which a strain of indentifiable tubercle bacilli resistant to both drugs, was found at any other time than as a sterilization failure at the completion of therapy. It is possible that this strain was culturable at the completion of treatment but that by chance the mouse had not been included in the sample sacrificed then. Thus, in the infections of all ages the over-all results with the 26 wk two drug regimen were thus sufficiently close to provide no support for the possibility that the lesion itself, interfered significantly with sterilization.

Although the lesion itself seems to exert no discernible influence on the sterilization process, the size of the population in the lesion at the time the

two drug exposure is started, does appear to exert an influence. This influence appears to operate in two different ways. The first, mentioned previously, consists of the greater likelihood that drug resistant bacilli may be present in sufficient numbers in the large populations of the older lesions to escape sterilization by one drug and emerge as a cause of sterilization failure. The second has to do with the relationship that appears to exist between the length of the two drug exposure and the population size when exposure is started. The limits of this apparent relationship have not been studied systematically in the present investigation. The sterilization failures with the two drug regimen when administered for only 6 wk with the standard inoculum, or for only 12 wk with the large population of the 15 wk infection, are thought to be cases in point. In each instance the result was almost completely reversed by an approximate doubling of the period of two drug exposure. As mentioned previously, it has long been recognized that population size is important in the drug-microbe reactions of tubercle bacilli; indeed, this factor has to be carefully standardized in the performance of drug-susceptibility tests in vitro (11). In the sterilization phenomenon of the present investigation it is likely that the ratio-population size/length of exposure, is a fairly crude one once a census of a few million tubercle bacilli is attained. An analysis of 22 experiments with the standard model revealed no clear correlation between the extent of microbial revival and the unavoidable interexperiment differences in the size of the "standardized" inoculum. Whether the age of the population as well as its size is a significant factor cannot be stated.

The present experiments have cast no light on the question of the morphologic state of the tubercle bacilli while in the sterile state and the relation of the sterilized bacilli to the acid-fast microbial forms visible in the tissues. This question of the microbial states during latent and dormant infections has been discussed elsewhere (6, 7) and is considered in detail in a more recent report by Pierce-Chase, Fauve, and Dubos (12). The fact that these acid-fast forms were readily demonstrable at the end of therapy in those 26 wk drug regimen experiments in which there has been no revival at all, casts some doubt on the possibility that they represent revivable tubercle bacilli.

SUMMARY

The capability of tubercle bacilli to assume a long continued sterile state in the tissues when exposed to pyrazinamide and isoniazid is a highly specific drug-microbe phenomenon in which host participation is not critical. Although it is the pyrazinamide that possesses the sterilizing type of action, the role of the isoniazid is specific and essential. The isoniazid serves to convert a phenomenon that occurs irregularly with pyrazinamide alone into one that occurs with a high degree of uniformity. The observations suggest a competition between isoniazid and the pyrazinamide (or its parent nicotinamide) for a site or entrance in or on the tubercle bacilli and for sterilization, the isoniazid apparently

must reach the site first. The rare failures to attain complete sterilization, appear to depend on the emergence of pyrazinamide-resistance which prevents the necessary dependent action of the two drugs. Populations already in the sterile state are nevertheless subject to a continued drug influence. Whether this represents a direct action on the sterile bacilli or an indirect effect produced by making the environment hostile to microbial revival, cannot be determined from the present observations.

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