AN EXPERIMENTAL MODEL OF CHEMOTHERAPY ON DORMANT TUBERCULOUS INFECTION, WITH PARTICULAR REFERENCE TO RIFAMPICIN

Eiko KONDO and Koomi KANAI

Department of Cellular Immunology, National Institute of Health, Kamiosaki, Shinagawa-ku, Tokyo 141 and National Institute of Health, Nonthaburi, Bangkok, Thailand

(Received May 13, 1988. Accepted August 1, 1988)

SUMMARY: Mice were infected intravenously with a streptomycin (SM)-dependent strain of tubercle bacilli which had been starved of the antibiotic. The inoculum persisted in the spleen for a fairly long period, especially keeping almost the initial level of viable counts in the first few weeks. Isoniazid (INH) administration exerted little effect on such fate of the infection at a bactericidal dose to the same strain multiplying under the supply of SM. Rifampicin (RFP) was, however, highly effective in either case. Similar results were obtained in the corresponding in vitro experiments. The data suggest that this animal model is a convenient system for screening drugs effective on the dormant tuberculous infection.

INTRODUCTION

The chemotherapy of tuberculous infection is expected to be always effective. In practice, however, the effects of drug-administration are sometimes less satisfactory than they should be. Several reasons for that are considered, e. g., such factors that make drug concentrations below the effective level at the bacillary location, the emergence of drug-resistant tubercle bacilli, and a phenomenon called “microbial persistence”. In the last case, the bacteria in closed
lesions can survive in a reduced state of metabolism (dormant), thereby they are much less sensitive to substantially effective drug concentrations (1,2).

The phenomenon of microbial persistence is compatible with the well-known facts that eradication of tubercle bacilli is difficult even in clinically cured cases, and that tuberculosis is now the disease of the older generation due to bacteriological relapse of once arrested infection.

Antituberculous drugs have been developed mainly on the basis of their effectiveness on the acute phase of experimental mouse tuberculosis, not on the dormant stage of infection. In this regard, using an SM-dependent strain of SM-starvation as inoculum, we examined a mouse model for such dormant infection. In this model, INH did not express its essential effectiveness (3). More recently, we observed that RFP was effective on such a model of dormant infection. This result led us to an expectation that the experimental model as above might be a good system to screen for such drugs that are active on persistent tuberculous infection.

**MATERIALS AND METHODS**

*Microorganisms*: The SM-dependent strain (18b) of tubercle bacilli and the kanamycin (KM)-resistant strain (Ravenel R-KM) of *M. bovis* employed here were those described in our previous report (3).

*Culture medium*: Glycerol-egg medium (Ogawa-type) was used for stock-culture and also for enumeration of viable units. Sauton synthetic liquid medium was used mainly to maintain working strains by subculture. Tween-albumin medium was used for particular experimental purposes as described later. SM was incorporated into these media in appropriate concentrations to support the growth of 18b strain.

*Measurement of bacterial growth and suspension density*: Bacterial growth in Tween albumin liquid medium was measured by reading its turbidity with a Coleman spectrophotometer set at 420 nm. The density of bacterial suspension for inoculation was adjusted according to the known standardized turbidity of bacterial suspension at 1 mg per ml.

*Preparation of SM-starved cell suspension of 18b strain*: The basic principle of preparation of SM-starved 18b cells is to allow the strain a residual growth at 37°C in the absence of added SM for some period. In case of Sauton synthetic liquid medium on whose surface 18b strain was making pellicle growth, the medium was replaced with fresh one without SM, and the strain was allowed to continue the growth for a further week or two. The homogenous bacterial suspension was
prepared from the washed harvest of this culture to desired concentrations. In case of Tween-albumin medium, a 1-mg or 0.1-mg per ml density of growth was left standing in SM-free environment at 37 C for 3 days. In some other case, a more thick suspension such as 10 mg per ml was prepared from SM-starved culture on Sauton medium in Tween-albumin medium without SM as suspending medium.

Experimental animals: Mice were of the commercially available dd strain, male, weighing 20 g on the average. They were housed, 10 per box, on wood shavings in metal boxes with wire-grid tops, and fed with pellet diet and water.

Enumeration of viable units in infected mouse organs: Mice were sacrificed by cervical dislocation to remove aseptically the spleen and the lungs. The organs were weighed, washed with distilled water, and placed into a mortar or a glass-homogenizer. Each original tissue homogenate was prepared as to make a 1:10 dilution in distilled water, and it was passed through a funnel with a sheet of gauze. When required, two of three serial 10-fold dilutions were prepared from this filtrate, and 0.1-ml aliquots of each dilution were inoculated onto three slants of glycerol-egg medium. The number of colonies developed after incubation at 37 C for 5 weeks allowed calculation of the viable units present in the tissue. Strain 18b and Ravenel R-KM strain were selectively isolated on the medium containing SM or KM in 100 μg per ml, respectively.

Antituberculous drugs: SM sulfate, KM sulfate, INH, ethambutol (EB), pyrazinamide (PZA), and RFP were used as aqueous solutions.

For in vitro experiments with RFP, it was dissolved in 0.5% Na-carboxymethyl-cellulose, and its 0.2-ml aliquot was added to 10 ml of test culture medium to get desired concentration.

In the case of animal injection, INH was dissolved in distilled water so a 0.1 ml contain the dose for ip administration. RFP and PZA were each dissolved in propylenglycol (150 mg/50 ml) under warming and further diluted with 1.5 liters of distilled water to make a 0.1 mg per ml solution. This was given to mice as drinking water for therapy. On an average, each mouse daily consumed 5 ml of it. Therefore, we estimated the daily uptake-dose of RFP and PZA at 0.5 mg. For SM injection to support the growth of 18b in mice, 0.2 ml of an aqueous solution of 50 mg per ml was administered intraperitoneally.

EXPERIMENTAL

SM Requirement of the Strain 18b for in vitro Growth

The SM-dependent strain 18b requires SM in a concentration of at least 40 μg per ml for the satisfactory growth on glycerol-egg medium (4). Therefore, it has been maintained by subculture on glycerol-egg medium or Sauton synthetic liquid medium containing SM in 100 μg per ml. In the present study, observation on such
dependency was extended to the case of Tween-albumin medium. The medium was dispensed in 6-ml amounts into test tubes of middle size and they were added with SM to make serial concentrations of 0, 5, 10, 20, 40, 80 and 160 µg per ml. These tubes were inoculated with 0.1 mg of SM-starved 18b cells.

The bacterial growth in each tube was then followed daily for 30 days of incubation at 37 C. The results are shown in Fig. 1. As shown here, vigorous growth of the 18b strain was obtained in SM concentrations higher than 20 µg per ml. Though limited growth was observed in 10 µg SM per ml after a prolonged period of time, no macroscopical growth was obtained in 5 µg per ml or without SM for 30 days.

Fig. 1. The growth of an SM-dependent strain (18b) of tubercle bacilli in Tween-albumin medium containing various concentrations of SM.
Survival Nature of 18b in SM-starvation

A vigorous growth of 18b in 40 μg SM per ml was once washed with fresh SM-free Tween-albumin medium and resuspended in a medium of the same kind to 1 mg per ml density. The tube was left standing in an incubator at 37°C. Thereafter, turbidity and viable cell counts of this suspension were determined on days 1, 3, 6 and 10. The results are shown in Fig. 2. No substantial change in either viable counts or turbidity was observed indicating that this SM-dependent strain can survive in the absence of enough SM, if not multiply. In other words, SM-starvation has only a bacteriostatic effect on this particular strain under the given experimental condition.

Comparison of in vitro Bactericidal Effects of Antituberculous Drugs on 18b in the Presence or Absence of SM

A 1 mg/ml mycobacterial suspension in SM-containing (100 μg/ml) Tween-albumin medium was prepared from a subculture of 18b on SM-containing (100 μg/ml) Sauton liquid medium. The suspension was incubated at 37°C. One milliliter of this culture was added to 100 ml of SM-containing Tween-albumin

Fig. 2. Survival of 18b strain in the absence of SM.
medium. After being shaked well, it was dispensed into 10 test tubes in the amounts of 10 ml. These tubes were incubated for 3 days. Turbidity of the starting culture was 0.038 at 420 nm. One of them served as drug-free control, and another one was added with 0.2 ml of 0.5% carboxymethyl cellulose as the RFP-free control. The remaining eight tubes were added with 0.2 ml of test drug solutions in the concentrations of 2 and 4 µg per ml for KM, 1, 2 and 4 µg per ml, respectively for INH or RFP.

Experiments of the same type as above were conducted simultaneously with SM-starved cells. Turbidity of the starting culture was 0.180 at 420 nm.

These inoculated tubes were incubated at 37 C in a slant position with occasional shaking. Enumeration of viable units in each tube was carried out with time intervals by sampling 0.2-ml aliquots each time. The results are shown in Fig. 3.

Fig. 3. Bactericidal effects of KM, RFP and INH on 18b in the presence or absence of SM.
Here, it was very clear that each drug had a marked bactericidal effect on the SM-supported growing culture in the concentrations of 1 to 2 µg per ml, reducing viable counts down by 2 to 3 log levels. Against the SM-starved resting cultures, however, INH and KM were much less effective reducing their viability even in the higher concentrations of 8 to 16 µg per ml. Only RFP was again active in this respect reducing viable counts down by a 2 log level in 2 to 8 µg per ml.

Chemotherapeutic Effect of RFP and INH on the Experimental 18b Infection of Mice with or without Simultaneous Administration of SM

Seventy-eight mice were divided into two groups (A and B) of equal 39. The animals of one group (A) were infected intravenously with 0.1 mg of 18b grown for 2 weeks on Sauton synthetic liquid medium supplemented with SM in 100 µg per ml. The mice of the other group (B) were infected in the same way, but with an inoculum of 18b which had been made SM-starved by residual growth for one week on the SM-lacking medium.

On the first day of infection, three mice were sampled from each group to be subjected to the enumeration of viable units just settled in the spleen. Then, each group was further divided into three subgroups of equal 12 animals for treatment with INH, or RFP, still or to be left untreated. In group A, SM was administered daily in the dose of 10 mg per mouse. Chemotherapy was started 3 days after infection in the dose of 0.2 mg of INH or 0.5 mg of RFP. In group B, treatment was conducted in the same way as above, but without simultaneous SM administration.

Then, with one week intervals, viable units in the spleen were enumerated on three mice from each subgroup to follow the fate of 18b. The results are shown in Fig. 4. As shown here, the event we had experienced in vitro was observed likewise in this mouse infection model. When SM was administered to mice to support the growth of inoculum, both INH and RFP exerted such a marked therapeutic effect as to decrease the spleen viable counts down to 3 to 4 log lower levels of that of the untreated group at 3 weeks. When SM was not given to mice, the effect of INH was very slight, but RFP was still as effective as in the former case.

This type of experiment was conducted again with some modification. Eighty-five mice were infected intravenously with a mixed inoculum consisting of 0.1 mg of SM-starved 18b and 0.005 mg of Ravenel R-KM strain of *M. bovis*. Five days later, the mice were divided into four groups of equal 20. One group was untreated to serve as control. The mice of the remaining three groups were subjected to the treatment by administration of INH (0.25 mg daily), RFP (0.5 mg daily) or the combination of INH (0.25 mg daily) and PZA (0.5 mg daily). On the
day of infection and then with 10-day intervals, three or four mice were sampled for viable counts in the spleen. Each time, both KM (100 µg/ml)-containing and SM (100 µg/ml)-containing media were employed for differential isolation of the two kinds of inoculum bacteria. The results are shown in Fig. 5. In this experiment, one strain of mycobacteria multiplied up to the two log higher level of viable counts in 10 days and another strain persisted after showing one log decline of the initial viable counts in the first 30 days. INH and INH plus PZA were not effective on the latter strain, but RFP was again highly effective in reducing viable counts in the tissue. On the other hand, all these three regimens were so effective to the multiplying inoculum. Though the data are not shown, the same tendency of chemotherapeutic effect was obtained concerning the fate of inoculum in the lung.
DISCUSSION

In the present study, an attempt was made to employ an SM-dependent strain of tubercle bacilli, after being made SM-starved, as an inoculum to produce a mouse model of "dormant infection". Our concept of dormant infection here is the continuous survival of pathogenic microorganisms in the host tissue without any active multiplication, probably keeping a low level of metabolic activity.

The reason why we are interested in such an animal model is concerned with the well-known phenomenon first demonstrated and named "microbial persistence" by McCune and Tompsett, and McDermott (2). These authors found in
their mouse experiments that some population of infecting tubercle bacilli can persist even under intensive and prolonged chemotherapy despite of their essential drug sensitivities. In other words, a difficulty of bacterial eradication from the infected tissue sites was thus demonstrated experimentally, well reflecting the situation of clinical tuberculosis and epidemiological features.

This microbial persistence is generally explained that most of chemotherapeutics are active only against microorganisms under multiplication. This has been shown repeatedly by in vitro experiments (5-9). We have introduced an animal model which represents this situation by employing an SM-dependent strain (18b) of tubercle bacilli as infection inoculum (10). Such our animal model demonstrated that INH was not effective on the “resting” tubercle bacilli in the host even in the bactericidal dose against the same strain under multiplication. In the present study, we definitely observed that 18b can retain its viability for a prolonged period of SM-starvation in vitro, even though it can not multiply. Therefore, we have had a reliable model system in hand for infecting mice with an inoculum preconfirmed of its resting state. Using this experimental animal system, we demonstrated a unique effectiveness of RFP against such SM-starved resting bacilli both in vitro and in vivo.

There is a good reason to believe that tubercle bacilli can survive keeping resting condition for long periods of time in closed caseous lesions of low oxygen tension (11-14). Interesting enough in this connection, there have been some reports (15-19) suggesting that SM-starvation of SM-dependent microorganisms induces the changes of respiratory metabolism which include a lack of carbon monoxide-sensitivity of respiration, an enhancement of glycolytic activity, a deficiency in the electron-transport system, etc. In order words, in the environment of SM-limitation, SM-dependent microorganisms appear to show a metabolic pattern similar to that in low oxygen tension.

This information may give a good support for our animal model with 18b as dormant tuberculous infection. RFP was still active on the cells of this particular state of metabolism. RFP inhibits bacterial DNA-dependent RNA polymerase activity (20), especially the chain initiation of RNA synthesis (21). In this context, RFP-sensitive RNA synthesis may be operating in the SM-starved resting cells.

Another interesting fact is that RFP is also active on leprosy in which causative organisms are very slow in multiplication (22). It is understandable that leprosy bacilli do not need the active generation of energy through the highly efficient respiratory route for their multiplication, and that they are, therefore, sensitive to RFP, if not to INH.
Such mechanism may it be, the present experiments encourage our idea that the combination of INH and RFP should be a recommendable regimen for tuberculosis chemotherapy, since it is effective on both multiplying and resting stages of infecting tubercle bacilli. It has been generally accepted that PZA has the mycobactericidal activity similar to RFP acting on resting cells as well (23). Though it is not proved in the present system, the dose of PZA may have not been enough to get the therapeutic effect.

REFERENCES