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Host-Parasite Relationships in Experimental Airborne Tuberculosis

I. Preliminary Studies in BCG-Vaccinated and Nonvaccinated Animals

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ABSTRACT

SMITH, D. W. (University of Wisconsin, Madison), E. WIEGESHAUS, R. NAVALKAR, AND A. A. GROVER. Host-parasite relationships in experimental airborne tuberculosis. I. Preliminary studies in BCG-vaccinated and nonvaccinated animals. J. Bacteriol. 91:718–724. 1966.—Previous studies from this laboratory on immunogenicity and allergenicity of defatted mycobacterial vaccines involved subcutaneous challenge of guinea pigs and killing of the animals 6 weeks later to evaluate the amount of disease. This type of experiment has been discontinued in this laboratory in favor of an airborne challenge type of experiment, with the advantages that animals can be challenged with small numbers of bacilli by a natural route, and the number of primary lesions, the rate of spread from those lesions, and the rate of bacillary multiplication can be used to evaluate protection. Experiments to determine uniformity of infection showed that a fair degree of uniformity resulted when seven guinea pigs were exposed simultaneously, and were studied 3 weeks later to determine numbers of primary lesions and bacilli in the tissues. A less satisfactory degree of uniformity was obtained when more animals were exposed at one time. BCG-vaccinated and nonvaccinated animals were studied to determine the earliest time and the optimal time for killing the animals to detect the effects of vaccination. In guinea pigs, the degree of protection assessed by lesion counts is time-dependent, but the degree of protection assessed by viable counts of bacilli in the tissues was relatively constant 3 to 12 weeks after infection. Mice vaccinated subcutaneously with BCG were not protected against infection at any interval between 2 and 19 weeks. Guinea pigs vaccinated subcutaneously with the same lot of vaccine were protected as judged by counts of viable bacilli in the tissues 3 weeks after infection.

Progress in the elucidation of the factors responsible for acquired resistance in tuberculosis has not been commensurate with efforts made. A survey of the literature leads to the conclusion that every product of the tubercle bacillus (cell wall, protoplasm, lipids, polysaccharides, and tuberculin) has been shown to be protective in the hands of at least one investigator. The reviews of Weiss (14) and Crowle (1) serve as sources to the literature. It is possible that protective substances are found in all components of the tubercle bacillus, but experience with other infectious agents suggests that this is very unlikely. If only certain fractions are protective, then these reviews fail to point the direction for future work. Some reasons for this failure include the following.

(i) Few laboratories use the same criteria or definition of protection; in fact, the number of evaluation methods almost equals the number of laboratories studying the problem. (ii) Investigators often do not include control vaccines of known potency. (iii) Fractionation techniques often are inadequate to give clean separation of immunogenic materials. (iv) Insufficient attention is given to statistical treatment of the data.

It is conceivable that little more progress will be made in this field unless more attention is given to these problems, especially the first. Much thought must be given to the question: What is protection against tuberculosis and how can it be measured? This is not to say that each investigator should adopt a single standard method for evaluating protection, but careful consideration should be given to whether a

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method is intended to be relevant to tuberculosis. Protection against tuberculosis could mean any of the following: prevention of infection, increase in survival time, increase in the ID_{50} dose, reduced rate of bacillary multiplication, prevention of spread from primary lesions, or failure to develop clinically apparent infection. (In this work, "primary lesions" refers to the lesions which result from the multiplication of inhaled viable tubercle bacilli.)

Previous studies from this laboratory (3, 12, 13) on immunogenicity and allergenicity of defatted mycobacterial vaccines involved subcutaneous challenge of guinea pigs followed by killing of the animals after 6 weeks to evaluate the extent of development of the disease. Although it is possible in such experiments to use relatively low levels of challenge, the route of administration is unnatural, the evaluation method usually employed (estimation of extent of gross and microscopic disease) is subjective, and normal statistical methods are not applicable to the data. For these reasons, subcutaneous challenge experiments have been discontinued in this laboratory in favor of an airborne challenge type of experiment. The advantages of this method are that animals can be challenged with small numbers of bacilli by a natural route and the data can be evaluated by normal statistical methods. Determination of the number of primary lesions and viable counts of tissues provide answers to questions of whether infection has been prevented, the rate of bacillary multiplication, and whether there has been spread from the primary lesion.

The pioneering investigations in experimental airbone infection have been those of Lurie (7) and Wells (15). O'Grady and Riley (10) reviewed the work in this field.

The purpose of this report is to present results of preliminary experiments on airborne infection of BCG-immunized and normal animals.

MATERIALS AND METHODS

Animals. Male and female guinea pigs, primarily albino, weighing 500 to 800 g, obtained from a local supplier, were fed standard guinea pig chow and water supplemented daily with 160 μg/ml of vitamin C. They were randomly allocated to experimental groups, and they were housed either two or three per cage. CF-1 female mice, 18 to 25 g, obtained from Carworth Farms (New City, N.Y.), were used in one experiment. They were caged in groups of five and were fed Purina chow and water.

Vaccine. Lyophillized BCG vaccine (supplied by the Tice Laboratory of the Institution for Tuberculosis Research, affiliated with the University of Illinois and Research Foundation, Chicago, Ill., now designated as the source of Reference Standard Vaccine by the American Trudeau Society Research Division Study Group on Evaluation of Methods for Demonstrating Protective Activity of Mycobacterial Antigens) was resuspended in diluent to a final concentration of 1 mg/ml. Each vaccinated animal received 0.1 ml of the vaccine by either the subcutaneous or the intradermal route.

Tuberculin tests. Tuberculin tests after vaccination were made with either 0.1 or 5 μg of purified protein derivative (PPD) injected intradermally in 0.1 ml of diluent. Observations of the test sites were made usually after 24 hr.

Preparation of challenge suspension. In some of the early experiments, inocula were taken from cultures maintained on Oleic Acid Albumin Agar (Difco) or on American Trudeau Society (ATS) medium. Maintenance of challenge cultures on these media was discontinued because of the possible progressive attenuation of the cultures and because components of ATS medium interfered with nephelometric measurements. *Mycobacterium tuberculosis* strain H37Rv (obtained from W. Steenken, Trudeau Laboratory), strain Erdman (obtained from A. Crowle, Webb-Waring Institute, Denver, Colo.), and strain 199-RB (obtained from the National Institutes of Health) were maintained by semimonthly transfers of a thin surface pellicle on Sauton medium. To prepare a challenge suspension, a loopful of the young pellicle was transferred to 5 ml of Dubos broth (Difco) and was homogenized with a Teflon-glass homogenizer; 0.2 ml was inoculated into 10 ml of Dubos broth in 3-oz (ca. 90 ml) prescription bottles. After 7 days of incubation at 37 C, the contents of several bottles were homogenized and filtered through a membrane of 5-μ pore size to remove clumps of bacilli. The resulting suspension was adjusted to 20 nephelos units (Coleman model 9 Nephocolorimeter and Coleman Nephelos Standards) by addition of Dubos broth, and was then further diluted to 10^{-3} with dilute Dubos broth (20% broth in saline). A few drops of antifoam (Dow-Corning Antifoam AF Emulsion) were added to the final suspension. The number of viable tubercle bacilli in the suspension was determined from colony counts made on Oleic Acid Albumin Agar.

Operation of infection chamber. Five to thirty animals were exposed at one time in three vertically spaced compartmented baskets in a model A3 airborne infection chamber (Tri-R Instruments Co., Jamaica, N.Y.) (8). A nebulizer-venturi unit constructed from a Vaponephrine nebulizer (Vaponephrine Co., Upper Darby, Pa.) was used to nebulize the suspension. The unit (which should be capable of nebulizing at least 1.3 g of water per 5 min under the stated conditions) was operated with 30 psi of air pressure (primary air) at a flow rate of 5 liters/min. The aerosol was drawn through the chamber at 24 liters per min (secondary airflow) by means of a vacuum pump. To improve mixing, a fan [4-inch (10 cm) blade, 3,000 rev/min] was mounted on the baffle plate at the bottom of the chamber. An electric incinerator (4) placed in the line between the chamber and pump rendered the air noninfectious before it was discharged. At the end of the nebulization period

(cloud buildup), the primary air was stopped, and airflow through the chamber was continued for 45 min.

Autopsy. At various periods after infection, groups of animals were killed by the intraperitoneal injection of 2.0 ml of pentobarbital sodium (60 mg/ml). Body weight, spleen weight, and when possible the number of primary lesions on the surface of the lungs were recorded. Specimens of lung, liver, spleen, and mediastinal lymph nodes were fixed in formalin for histopathology.

Enumeration of viable tubercle bacilli in the tissues. At autopsy, the right lower lobe of the lung and a portion of spleen were removed aseptically and cultured. Each tissue was ground for 2 to 3 min in a Teflon-glass homogenizer with 5 ml of 2% bovine albumin fraction V solution. The homogenates were diluted in gelatin-saline (0.1% gelatin), and appropriate dilutions were plated on Oleic Acid Albumin Agar by the drop technique recommended by Fenner (2), or by spreading 0.2 ml over the surface of the medium. After 12 to 14 days of incubation at 37 C, colonies were counted at appropriate dilutions, and the results were used to calculate the number of viable bacilli in the tissue.

RESULTS

The purpose of the first three experiments was to gain some insight into the degree of uniformity of infection that could be expected between animals and between experiments, and to gain experience in the quantitative enumeration of viable tubercle bacilli in the tissues.

Ten guinea pigs were infected by the respiratory route with H37Rv. In an attempt to assess the number of viable bacilli inhaled, three animals were killed 1 day after infection, and homogenates of the right lower lobe of the lung were plated on Oleic Acid Albumin Agar. No colonies developed even from the undiluted homogenates. The remaining seven animals were killed 3 weeks after infection. Primary lung lesions were counted, and the number of viable bacilli in the lung and spleen was determined. The results of this experiment (Table 1) indicated a fair degree of uniformity both in the number of lesions that developed and in the number of bacilli that were cultured.

To determine whether similar results could be obtained with another strain of mycobacteria, animals in the next experiment were exposed to an aerosol prepared from *M. tuberculosis* strain 199-RB, and were killed 3 weeks after infection. The results (Table 2) indicated that the number of primary lesions and the number of bacilli cultured from the tissues were comparable to those seen in Table 1, where H37Rv was the challenge strain, and where the concentration of organisms in the challenge suspension was similar.

Although a fairly satisfactory degree of uniformity was obtained with a limited number of animals exposed in a given basket in the chamber, questions were raised about possible differences of exposure of animals placed at different levels in the chamber. Therefore, seven guinea pigs in the top basket and seven in the bottom basket were exposed to an aerosol of H37Rv and were killed 3 weeks later. Table 3 shows that the total number of lesions was about one-third that seen in the first experiment. It should also be noted that the number of bacilli in the nebulizer suspension was about one-third less than in the first experiment. No difference was seen between the mean number of lesions on the lungs of animals in the top and bottom baskets. The coefficient of variation of the number of lesions was greater than when there were only seven animals in the chamber.

The purpose of the next two experiments was to

TABLE 1. *Number of primary lesions and viable tubercle bacilli in tissues of guinea pigs killed 3 weeks after airborne infection with H37Rv*

Guinea pig	Primary lung lesions		No. of bacilli (logarithms)	
	RLL*	Total	Lung	Spleen
A-1	5	24	5.33	4.12
A-2	5	30	5.19	4.27
A-3	6	24	5.20	3.39
B-1	5	22	4.90	4.29
B-2	5	22	5.16	3.38
C-2	6	31	5.43	3.15
C-3	6	24	5.02	2.78
Mean and SD	5.4 ± 0.5	25 ± 4	5.18 ± 0.18	3.62 ± 0.60

* Right lower lobe.

TABLE 2. *Number of primary lesions and viable tubercle bacilli in tissues of guinea pigs killed 3 weeks after airborne infection with Mycobacterium tuberculosis 199-RB*

Guinea pig	Primary lung lesions		No. of bacilli (logarithms)	
	RLL*	Total	Lung	Spleen
S-332	6	27	4.78	3.40
S-333	8	40	5.10	4.30
S-334	7	32	4.88	4.20
S-335	8	38	5.10	5.44
S-336	7	32	5.18	3.85
S-337	8	39	4.78	5.77
S-338	7	25	5.00	5.74
Mean and SD	7.3 ± 0.8	33 ± 6	4.97 ± 0.16	4.67 ± 0.96

* Right lower lobe.

TABLE 3. *Number of primary lesions and viable tubercle bacilli in tissues of guinea pigs exposed in top and bottom baskets of airborne infection chamber to aerosol of H37Rv and killed 3 weeks later*

Position	Guinea pig	Primary lung lesions		No. of bacilli (logarithms)	
		RLL*	Total	Lung	Spleen
Top	S-304	3	7	5.00	2.70
	S-305	3	10	4.65	2.48
	S-306	2	12	4.54	4.54
	S-307	2	8	—	1.70
	S-308	1	6	4.40	3.88
	S-309	3	9	3.40	2.40
	S-310	1	7	4.18	—
Mean and SD		2.1 ± 0.9	8.4 ± 2.1	4.36 ± 0.54	2.95 ± 1.05
Bottom	S-311	2	10	4.65	2.60
	S-312	0	5	4.30	4.78
	S-313	1	4	4.88	2.40
	S-314	4	9	5.13	4.54
	S-315	4	9	4.48	2.54
	S-316	3	6	2.70	—
	S-317	0	6	4.10	3.30
Mean and SD		2.0 ± 1.7	7.0 ± 2.3	4.31 ± 0.79	3.36 ± 1.05

* Right lower lobe.

FIG. 1. *Photograph of fixed specimens of lungs of guinea pigs killed 5 weeks after airborne infection (498 and 499 BCG-vaccinated, 641 and 642 nonvaccinated).*

gain experience in the study of vaccinated and nonvaccinated animals challenged by the respiratory route, specifically to determine the earliest time and the optimal time for killing the animals to detect the effects of vaccination. In the first experiment, one group of BCG vaccinated guinea pigs and a comparable nonvaccinated group were exposed to infection 8 weeks later with H37Rv. Twenty-one animals from each group were killed 3 weeks after infection to determine the uniformity of infection on a larger sample of animals. Thereafter, seven animals from each group were killed at each interval. At 3 weeks, the mean number of primary lesions in the vaccinated animals was 5 ± 3, whereas in the nonvaccinated animals it was 9 ± 4. Comparison of numbers of primary lesions in nonvaccinated animals at different intervals indicates an increase from 9 ± 3 lesions at 4 weeks to 15 ± 7 lesions at 5 weeks, with no further increase at 6 weeks. Figures 1 and 2 show the gross appearance of lungs of vaccinated and nonvaccinated animals 5 and 8 weeks after infection. The results of this experiment are presented in Table 4. Little change was seen in the numbers of bacilli cultured from the lungs and spleens of nonvaccinated animals. Bacillary populations in the lungs of vaccinated animals were relatively constant at a level 100-fold lower

at most intervals for the first 12 weeks, followed by a period of progressive decline. Viable bacilli in the spleens of vaccinated animals remained at a 1,000-fold lower level at most intervals, with a suggestion of increase in the last three periods.

In the next experiment, CF-1 mice vaccinated with BCG cells by the subcutaneous route and a nonvaccinated group were infected 5 weeks later with *M. tuberculosis* (Erdman strain) and were killed at various intervals. The results presented in Table 5 indicate little difference between vaccinated and nonvaccinated mice in the number of bacilli in lungs and spleens at most intervals.

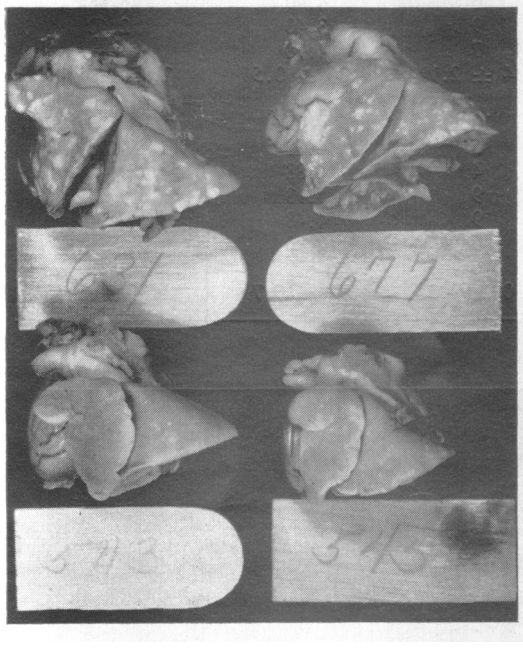

FIG. 2. *Photograph of fixed specimens of lungs of guinea pigs killed 8 weeks after airborne infection (631 and 677 nonvaccinated, 543 and 545 BCG-vaccinated).*

The last experiment to be reported compared intradermal and subcutaneous routes of BCG vaccination. Eight guinea pigs were vaccinated by each route. The animals were infected with H37Rv 7 weeks after vaccination and were killed 3 weeks later. The results presented in Table 6 indicate that the number of primary lesions among the three groups was not markedly different. The numbers of viable bacilli cultured from lung and spleen were strikingly different between vaccinated and nonvaccinated animals, but not between the groups vaccinated by different routes.

Table 7 shows the relationship between the number of primary lesions and the concentration of the suspension nebulized for the experiments with guinea pigs.

DISCUSSION

The results of preliminary experiments suggest a fair degree of uniformity of infection when seven animals are exposed and studied 3 weeks later for numbers of primary lesions and bacillary populations in the tissues. As the number of animals exposed at one time is increased, however, experience shows that the uniformity of infection is less satisfactory. To conduct long-term experiments on the natural course of the disease in vaccinated and nonvaccinated animals, large numbers of animals will have to be infected under very uniform conditions. Further work and further modifications in equipment will be required before a satisfactory degree of uniformity is achieved.

TABLE 4. *Logarithm of the number of viable tubercle bacilli in lung and spleen of vaccinated and nonvaccinated guinea pigs* at several intervals after infection with H37Rv*

Weeks post-infection	Lung†		Spleen†	
	Nonvaccinated	Vaccinated	Nonvaccinated	Vaccinated
3	5.34 ± 0.44‡	3.29 ± 0.72	3.96 ± 1.08	0.47 ± 1.03
4	5.42 ± 1.21	3.53 ± 0.61	5.34 ± 0.93	0.00
5	4.14 ± 1.93	3.42 ± 0.66	4.54 ± 1.00	0.47 ± 0.12
6	4.16 ± 0.44	3.41 ± 0.72	5.27 ± 0.67	1.70 ± 2.21
8	3.79 ± 0.47	3.26 ± 0.90	3.95 ± 0.72	1.04 ± 1.36
10	5.32 ± 0.81	3.05 ± 0.85	4.97 ± 1.43	1.37 ± 1.82
12	4.85 ± 0.52	2.96 ± 0.60	3.68 ± 1.74	0.34 ± 0.90
14	5.28 ± 0.57	2.81 ± 0.76	4.49 ± 0.46	1.40 ± 1.79
17	4.84 ± 0.68	0.88 ± 1.60	4.37 ± 1.01	1.74 ± 2.17
20	4.97 ± 0.55	0.90 ± 1.28	4.58 ± 1.03	2.48 ± 1.14

* Seven animals per group except at 3 weeks when there were 21 per group.
† Calculated as the total number of bacilli per spleen or right lower lobe of the lung.
‡ Standard deviation.

TABLE 5. *Logarithm of the number of viable tubercle bacilli in lung and spleen of vaccinated and nonvaccinated mice at several intervals after infection with Mycobacterium tuberculosis (Erdman strain)*

No. of weeks post-infection	Lung		Spleen	
	Nonvaccinated	Vaccinated	Nonvaccinated	Vaccinated
2	5.34 ± 0.27	5.25 ± 0.29	0.00	0.39 ± 1.02
3	4.84 ± 2.37	5.25 ± 0.55	3.15 ± 2.48	4.70 ± 0.68
4	6.19 ± 0.52	5.14 ± 0.41	4.00 ± 0.48	2.93 ± 0.82
6	5.74 ± 0.19	4.99 ± 0.57	4.04 ± 0.35	3.32 ± 0.87
10	5.14 ± 0.32	3.84 ± 0.66	3.53 ± 0.42	2.92 ± 1.38
19	4.16 ± 0.58	4.04 ± 0.61	2.90 ± 0.10	2.85 ± 0.79

TABLE 6. *Number of primary lesions and viable tubercle bacilli in the tissues of BCG-vaccinated and nonvaccinated guinea pigs killed 3 weeks after infection with H37Rv*

Group	Total lung lesions*	No. of bacilli (logarithms)	
		Lung	Spleen
BCG, intradermal	8	3.28	0.00
BCG, subcutaneous	5	3.24	1.54
Nonvaccinated	13	5.65	4.72

* Secondary airflow double that of previous experiments.

TABLE 7. *Relationship between the number of primary lesions and the concentration of the nebulizer suspension*

Expt in Table	Nebulizer fluid (viable units/ml × 10^3)	Total lung lesions 3 weeks after infection
1	25	25
2	35	33
3	9.5	8
4	10	8
6*	30	13

* Secondary airflow was doubled for this experiment.

Evidence was obtained in one experiment (see Table 4) that at several intervals after infection tubercle bacilli were present in the tissues of nonvaccinated guinea pigs at a level at least 100-fold higher than in vaccinated animals. This is similar to the results reported by Middlebrook (9) in airborne-infected guinea pigs examined 3 weeks after infection.

In these experiments, vaccinated guinea pigs examined 3 weeks after infection did not exhibit the 10-fold reduction in the number of primary lesions reported by Middlebrook (9). Ribi et al. (11) reported that 4 weeks after airborne infection pulmonary lesions are absent in the lungs of 80% of mice vaccinated intravenously with BCG. Larson and Wicht (6) reported that, although none of the vaccinated mice examined at 4 weeks exhibited pulmonary tubercles, all vaccinated mice studied 10 weeks after infection had lesions. Lesion counts are difficult to assess in guinea pigs because the number of primary lesions in vaccinated animals is probably maximal at 2 to 3 weeks, after which they begin to diminish in number and size with macroscopic evidence of resolution. The number of primary lesions in nonvaccinated animals is low at 3 weeks and increases up to 6 weeks. Further consideration of this question will probably require histopathological observations of sections of the lung surfaces. This information would be important because it would permit an interpretation of whether or not vaccination prevents the development of a proportion of inhaled viable particles, and hence whether vaccination could prevent infection.

The data in Table 4 suggest that populations of bacilli in the lungs of vaccinated animals began to decrease at 17 and 20 weeks, whereas in the same period populations of bacilli in the spleens of the same animals began to increase. Assessment of the significance of these observations will require experiments conducted over longer periods of time and with larger groups of animals.

Guinea pigs have continued to be the animal of choice in these experiments because acquired resistance and delayed hypersensitivity can be studied in the same animal, and because they respond to vaccines administered by routes usually employed in the immunization of human subjects. It has been shown in these experiments that mice were not protected against respiratory challenge when they were vaccinated by the subcutaneous route with the same lot of vaccine which protected guinea pigs (see Tables 5 and 6). Results reported by Larson and Wicht (6) indicate that H37Ra and BCG produced protection

against airborne infection when given by the intravenous or respiratory route, but not when given by subcutaneous, intraperitoneal, or oral route.

One disadvantage of experiments involving airborne infection is the associated health hazard for laboratory personnel. Precautions must be taken during the exposure and the subsequent handling of animals. In these experiments, the airborne infection chamber was operated in the same adequately ventilated room where the infected animals were to be housed. Precautions routinely taken in this work include: vaccination of all skin test-negative persons, use of protective clothing (gown, trousers, surgical gloves, boots), effective face mask (*see* 5), and germicidal lamps mounted so that the upper portion of each animal room is exposed to ultraviolet irradiation. The effectiveness of these procedures to date is indicated by the fact that none of the personnel involved has shown any evidence of disease on routine chest X ray taken every 4 months.

The underlying philosophy of this work is that the use of the respiratory route of challenge together with a low level of infection (5 to 10 primary lesions) in animals will lead to observations more likely to be relevant to the natural course of pulmonary tuberculosis in man. Evaluation of host response to experimental airborne tuberculosis will include a determination of the number of primary lung lesions that develop and the number of viable bacilli in the tissues at various intervals after infection. These data would permit the following assessments of protection: (i) prevention of infection, (ii) rate of spread from the primary lesion, (iii) rate of bacillary multiplication in vivo.

Future studies will be concerned with the development of techniques leading to a reproducible predictable infection in the range of 5 to 10 primary lesions in large numbers of animals. This infection system will be used to characterize the natural course of the disease in nonvaccinated animals. Detailed knowledge of the natural course of the disease is essential for recognition of the true influence of vaccination and for determining the stage of the disease likely to present the best point of departure for studies of mechanisms of acquired resistance in tuberculosis.

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