[Frontiers in Bioscience 3, c27-33, May 1, 1998]

LURIE S TUBERCLE-COUNT METHOD TO TEST TB VACCINE EFFICACY IN RABBITS

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Received 3/9/98 Accepted 3/14/98

TABLE OF CONTENTS

1. ABSTRACT

In human beings, infection with the tubercle bacillus usually results in inapparent disease, recognized only by a positive tuberculin reaction. However, about 10% of tuberculin-positive people develop clinically active tuberculosis (TB). The Lurie tubercle count method is probably the most accurate way to measure a vaccine's ability to prevent such clinical disease. Yet, it is rarely used. Briefly, vaccinated and control rabbits are infected by aerosol with a known quantity of virulent human-type tubercle bacilli (strain H37Rv). [Human-type bacilli are not fully virulent for rabbits.] Five weeks later, the rabbits are sacrificed, and counts are made of the number of grossly visible primary tubercles in their lungs. The best vaccines cause the greatest reduction in the number of such visible tubercles. This report describes the method, and the immunologic mechanisms involved. It also suggests how the method can be used to test TB vaccines in both mice and guinea pigs, as well as in rabbits.

2. INTRODUCTION

Vaccines have no effect on whether or not an inhaled tubercle bacillus establishes a microscopic lesion in the host. Vaccines can only prevent the development of microscopic tuberculous lesions once they have been established. These statements are conclusions derived from the following principles.

(a) Pulmonary alveolar macrophages (AM), the first cells of the host to ingest inhaled tubercle bacilli, possess no immunologic specificity. Therefore, vaccines cannot increase the power of AM to destroy tubercle bacilli, after the nonspecific effects of the vaccine have returned to normal levels.

(b) The bacillary unit that reaches the pulmonary alveoli to start a tuberculous lesion contains no more than 1 to 3 bacilli (1). Such a small number of bacilli do not contain high enough levels of antigens to call forth the immune response --- even an immune response enhanced by a vaccine. Therefore, the inhaled bacilli must multiply, thereby increasing the amount of their antigens, before memory lymphocytes, which do possess immunologic specificity, are able to affect the progress of the disease. Such multiplication is within the accumulating macrophages, and a microscopic lesion is thereby established (described more fully below). [Bacillary units containing 4 or more bacilli do not stay in the airstream, but impinge upon the (rather resistant) bronchial walls and do not reach the alveolar spaces.]

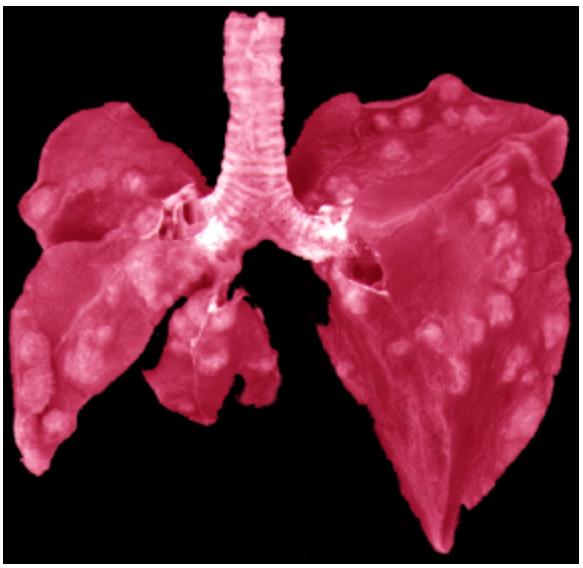

Figure 1. Formalin-fixed lungs of a rabbit that inhaled about 33,000 virulent human-type tubercle bacilli (strain H37Rv) 5 weeks previously. Upon dissection, these lungs contained 131 grossly visible primary tubercles, with no apparent secondary tubercles. The ratio of the number of bacilli inhaled to the number of primary tubercles produced was about 250. Effective BCG (and other vaccines for tuberculosis) should increase this ratio at least 5-fold (3). Small areas of caseous necrosis are visible in many of the tubercles. This photograph shows the ventral surfaces of the right upper, middle and azygous lobes on the left and of the entire left lung (upper and lower lobes) on the right. The right lower lobe (RLL) had been removed for culture. This RLL contained 23 grossly visible tubercles and 1.35 X 10^5 culturable tubercle bacilli. X 1.0

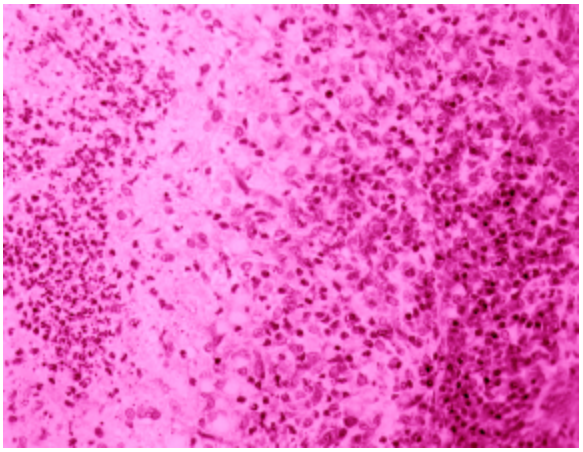

Figure 2. A tissue section of a primary lesion similar to those shown in figure 1. From left to right are (a) one of the small sites of necrosis, (b) a (surrounding) area of large epithelioid macrophages, and (x) an outside area that is densely infiltrated by smaller macrophages, lymphocytes, and plasma cells. Azure-eosin stain. X250.

TB vaccines prevent clinical tuberculosis by stopping the progression of these early pulmonary lesions while they are still small enough to remain inapparent to the host. Once such lesions are grossly visible, they are well established and often progress into clinical disease. A quantitative measure of a vaccine's ability to prevent clinical disease is therefore its ability to reduce the number of established grossly visible tubercles in the lungs.

3. DESCRIPTION OF THE TUBERCLE-COUNT METHOD

This method of assessing TB vaccine efficacy in rabbits was developed by Lurie in 1952 (1-3). He also used this method for determining the innate resistance of inbred rabbit families (4), as well as the virulence of the infecting strain of tubercle bacillus (5). Specifically, he allowed rabbits to inhale an aerosol of a given number of human-type tubercle bacilli, sacrificed the animals 5 weeks later, and counted the number of grossly visible tubercles in their lungs (figures 1 and 2). [The actual inhaled dose was then calculated from the weight of the rabbit and the number of bacilli cultured from the aerosol (6, also see 7)].

The vaccine's efficacy was then given a quantitative value. Specifically, the number of inhaled units of 1 to 3 bacilli divided by the number of grossly visible primary tubercles present at necropsy 5 weeks later provided the "ratio." This ratio is the number of inhaled bacillary units required to generate one such visible tubercle. When compared to non-vaccinated controls, rabbits that are effectively immunized need to inhale more tubercle bacilli to produce one visible tubercle, i.e., the vaccine prevented many microscopic lesions from reaching grossly visible size. The higher the ratio, the more effective was the vaccine in preventing grossly visible disease.

Bovine-type bacilli, strain Ravenel S, that are fully virulent for the rabbit produce one grossly visible primary lesion for every three bacillary units inhaled (1,6), regardless of the factors just listed. For this reason, Lurie used human-type tubercle bacilli (strain H37Rv) in which 50 to 600 bacillary units must be inhaled to produce one grossly visible primary lesion, depending on the native resistance of the rabbit (1-4,8). After BCG vaccination, Lurie's resistant strain of rabbits needed to inhale 3000 (rather than 600) bacillary units to produce one grossly visible primary pulmonary lesion; i.e. the ratio was increased 5-fold (3). Commercially available unvaccinated New Zealand White rabbits must inhale 200 to 500 units of 1 to 3 virulent human-type tubercle bacilli (strain H37Rv) to produce one primary pulmonary tubercle that is grossly visible at 5 weeks (unpublished observations with David N. McMurray).

The beauty of this tubercle count method is that it provides a direct measure of how many tubercle bacilli must be inhaled to produce a grossly evident form of the disease.

It measures the vaccine's ability to prevent short-term and long-term pulmonary tuberculosis, because 5-week tubercles caused by human-type bacilli take months to heal in the rabbit (1). In humans, such tubercles would also take many months to heal or stabilize, and some of these lesions would progress to clinically active disease. Thus, this tubercle count method seems to be a precise way to compare the efficacy of available vaccines before they are used in clinical trials.

4. RESISTANCE TO THE ESTABLISHMENT OF THE INFECTION

Clinical tuberculosis is prevented by two distinct host defense mechanisms: (a) resistance to the establishment of the infection and (b) resistance to its progress (1,9-11). The pulmonary alveolar macrophages (AM) prevent inhaled bacilli from establishing lesions. AM are highly activated cells, activated by the continuous ingestion and digestion of inhaled organic particles including microorganisms (12). In humans and rabbits inhaling virulent human-type tubercle bacilli, the AM usually kill most of the inhaled bacilli before they have had a chance to multiply appreciably. An early microscopic pulmonary lesion is established only when a strong bacillus is ingested by and multiplies within a weak alveolar macrophage (13,14). Once such a lesion is established, the host can use its broadly specific innate defenses (15,16) and its acquired immunologically-specific defenses to stop the lesion's progress and prevent grossly evident disease (see next section).

5. IMMUNE MECHANISMS CONTROLLING THE PROGRESS OF THE EARLY MICROSCOPIC PULMONARY LESION (13,14,17,18)

A small microscopic tubercle is produced wherever the bacillus multiplies appreciably in an alveolar macrophage (AM). The alveolar macrophage eventually dies, and blood-borne monocyte/macrophages emigrate from the bloodstream into the site and ingest the bacilli released from the AM. These new macrophages are not activated, so the bacillus multiplies intracellularly in a logarithmic fashion. In rabbits that have inhaled human-type bacilli, most of these early lesions never reach grossly visible size, because acquired immunity soon develops to antigens secreted by the bacillus: a) The host's tissue-damaging delayed-type hypersensitivity (DTH) reaction kills the nonactivated macrophages in which the bacillus is multiplying, thereby forming the lesion's solid caseous center(s) in which the bacillus cannot multiply; and b) cell-mediated immunity (CMI) activates the macrophages surrounding the caseous center(s) and some of these activated macrophages ingest the bacilli that escape from the caseous center(s). Since such activated macrophages can inhibit and destroy tubercle bacilli, the early microscopic lesion is often arrested at this point of its development. The acquired immunity just described converts the tuberculin reaction, but the host will not show any other evidence of clinical disease unless the early lesion continues to progress.

6. HOW VACCINES PREVENT GROSSLY VISIBLE TB LESIONS

No tuberculosis vaccine will appreciably increase the power of the alveolar macrophages (AM) to destroy tubercle bacilli in an immunologically-specific manner. AM are not immunocytes and therefore do not recognize specific antigens. However, AM can recognize certain bacterial components in a broadly specific manner, because all macrophages have some innate resistance to microorganisms (15,16). In contrast, various clones of lymphocytes have specific receptors for various antigens of the tubercle bacillus, and these lymphocyte clones expand when presented with antigens in the vaccine. In the vaccinated host, the increased numbers of antigen-specific lymphocytes cause a more rapid development of both tissue-damaging DTH (producing caseous necrosis) and CMI (producing macrophage activation). Therefore, bacillary multiplication is inhibited sooner, and fewer lesions progress to grossly visible size.

7. EFFECTS OF VACCINES ON THE 5 STAGES OF TUBERCULOSIS (13,14,17,18)

7.1 Stage 1. Destruction of the bacillus by alveolar macrophages

Vaccines have no effect on the power of alveolar macrophages to destroy tubercle bacilli, after the nonspecific adjuvant effects of the vaccine have subsided.

7.2 Stage 2. Symbiosis

Vaccines markedly shorten the stage of symbiosis, in which the bacillus is growing logarithmically in the non-activated macrophages infiltrating from the bloodstream, because acquired immunity occurs more rapidly in immunized host.

7.3 Stage 3. Initial caseous necrosis

Vaccines enable bacilli-laden macrophages to be killed sooner, causing caseous necrosis to occur sooner and stopping the logarithmic growth of the bacillus sooner. The bacillus cannot multiply in solid caseous material.

7.4 Stage 4. Progression or regression of the established primary tubercle

Vaccines hasten the activation of blood-derived infiltrating macrophages. The now-activated macrophages ingest and inhibit bacilli escaping from the solid caseous center, thereby preventing renewed intracellular bacillary growth. In other words, vaccines decrease the progression of established tubercles and hasten their regression.

7.5 Stage 5. Liquefaction and cavity formation

Vaccines, given before the onset of the disease, may or may not hasten liquefaction and cavity formation. Vaccines may or may not affect the extracellular growth of tubercle bacilli in liquefied caseum and cavities. Also, such vaccines may or may not enhance host immunity at this stage

of tuberculosis, because by this time the disease itself has caused the host to develop considerable immunity. Since susceptibility to the tuberculin-like products of the bacillus is apparently a major cause of liquefaction and cavity formation (19-21), vaccines that produce little sensitivity to tuberculin should be favored over those that produce more, if they are equally effective in preventing clinical disease (see 22).

7.5 Relevance

The tubercle-count method measures the vaccine's ability to prevent microscopic tubercles from reaching grossly visible size by affecting Stages 2, 3 and 4. In other words, it measures the vaccine's ability to shorten the symbiotic stage, hasten the onset of caseation, and activate blood-derived macrophages more rapidly.

8. DOSE EFFECTS

Tuberculosis is a local disease (23-25), controlled by the lymphocytes and macrophages participating at sites containing the bacillus. Primary pulmonary lesions are usually separated from each other by numerous alveolar spaces. Therefore, we have always thought that their development was independent of each other. Recently, however, evidence is accumulating that each lesion has systemic effects that influence the development of other primary lesions that are developing simultaneously. Specifically, when numerous primary lesions are developing in the lung, the host's ability to prevent the development of microscopic tubercles into grossly visible size is impaired (26); and/or when very few primary lesions are developing in the lung, the host's ability to prevent the development of microscopic tubercles is enhanced (26).

These effects are apparently related to the prevalence of Th1 or Th2 immune-specific lymphocytes: Low infecting doses of intracellular microorganisms favor the beneficial Th1 immune response, and high-infecting doses favor the host-detrimental Th2 immune response (27). This systemic effect in acquired resistance to pulmonary tuberculosis is more fully discussed in reference (26). [An update on Th1 and Th2 cells is presented in references 28 and 29.]

A careful immunohistochemical study will be required to investigate whether the microscopic tubercles that do not reach grossly visible size are those that locally contain more Th1 cells (which produce both tissue-damaging DTH and macrophage-activating CMI); and/or whether the tubercles that do reach visible size are those that locally contain more Th2 cells (which can suppress the Th1 response).

9. SINGLE PRIMARY TB LESIONS

Early clinical tuberculosis is usually occurs as a single Xray-visible lesion, because a period of weeks or months usually elapses before the next inhaled bacillus could establish another lesion. A few weeks after the single primary lesion begins, the host's immune forces have been enhanced so much that lesions created by additional inhaled bacilli are usually aborted at a microscopic stage, leaving the initial lesion as the only one visible in the lungs. Single primary lesions were also produced in rabbits (1,9,30) and guinea pigs (31) when these animals repeatedly inhaled an occasional virulent tubercle bacillus over periods of many weeks.

In humans, a single grossly visible primary pulmonary lesion often progresses. Therefore, the calculation of ratios, i.e., the number of inhaled bacilli required to produce such a single visible lesion, is a good quantitative measure of a vaccine's ability to prevent clinical tuberculosis.

10. TUBERCULOSIS IN DIFFERENT SPECIES

Rabbits and human beings are both rather resistant to tuberculosis. Guinea pigs are much more susceptible; and mice, although rather resistant, develop a somewhat different form of the disease. These species differences are compared in references 32 through 35.

Rabbit TB and human TB have these two characteristics in common: (a) Only a small percentage of inhaled virulent human-type tubercle bacilli is able to multiply and create at least a microscopic lesion, thereby converting the tuberculin skin test. (b) In both species, pulmonary cavities with bronchial spread readily occur, especially if the more virulent bovine-type of tubercle bacillus is used to infect the rabbits.

Guinea pigs usually develop a primary lesion for every unit of 1 to 3 bacilli that reaches the alveolar spaces (see 36-39), as do rabbits that inhale fully virulent bovine-type bacilli (1,6,8, see 5). Guinea pigs will develop cavities when infected with a low dose of bacilli (40), but bronchial spread of the disease rarely occurs in this species, because the disease spreads mainly by the hematogenous route.

Mice develop a slowly progressing form of tuberculosis (32,41) with less (caseous) necrosis and no cavity formation. We do not know how many tubercle bacilli must be inhaled by mice to produce one primary tubercle. However, unpublished preliminary experiments of Donald W. Smith (using methods described in reference 42) indicate that mice are just as susceptible as guinea pigs to H37Rv, i.e., about one unit of 1 to 3 tubercle bacilli in the pulmonary alveolar spaces will produce one primary lesion. Mice are much less sensitive to tuberculin than are humans, guinea pigs and rabbits (in that order), which is one of the reasons why their tubercles show less caseous necrosis than those of the other three species (32-34). Francis (33, see 34) states that pulmonary tubercles of mice contain more bacilli than do tubercles of rabbits and guinea pigs. If confirmed, this finding would be consistent with the less extensive caseous necrosis

in mice, because the killing of macrophages in which the bacillus is proliferating stops such intracellular bacillary multiplication (13,14,17,18).

In human beings, the number of inhaled bacillary units required to generate one grossly visible primary pulmonary tubercle is unknown. Estimates vary from 20 to 200 units, depending on the native (genetic) resistance of the host and the virulence of the infecting bacillus. Most TB researchers agree that humans are much more resistant than guinea pigs to both human- and bovine-type virulent tubercle bacilli, and that humans are somewhat more susceptible than rabbits to the human-type. [Rabbits uniformly recover from infection with human-type tubercle bacilli, although many months are required to do so (1).]

Virulent bovine-type tubercle bacilli are apparently more infectious for rabbits than for humans: One inhaled unit of 1 to 3 fully virulent bovine-type bacilli in the alveolar spaces is sufficient to establish the disease in rabbits (1,6). Such bacilli in rabbits produce a cavitary disease with spread via the bronchial tree, which is quite similar to that found in immunocompetent human beings.

Humans, guinea pigs and mice (in contrast to rabbits) show no major differences in their susceptibility to human- and bovine-type tubercle bacilli (33, see 34). In humans, the main difference in the disease produced by these two bacillary types was due to the route of infection: The human type was usually inhaled, whereas the bovine type was usually ingested in milk from tuberculous animals (43).

11. APPLICATION OF THE TUBERCLE COUNT METHOD TO GUINEA PIGS AND MICE

In guinea pigs, the Smith (44,45) and the Horwitz (46,47) groups counted the primary lesions produced by the inhalation of virulent tubercle bacilli. The Smith group showed that when guinea pigs inhaled very low doses of virulent tubercle bacilli (strain H37Rv), the number of visible primary pulmonary tubercles was reduced about 50% by prior BCG vaccination (44,45). However, the tubercle count method could be more effectively used in guinea pigs if they were made to inhale a semi-virulent strain of human- or bovine-type tubercle bacillus, preferably a strain in which at least 200 inhaled bacillary units (rather than 3 such units) would be required to produce one visible primary pulmonary tubercle in unvaccinated animals. Smith's group clearly showed that more inhaled human-type tubercle bacilli were required to generate one primary pulmonary tubercle in guinea pigs if the bacilli were of a reduced virulence (48).

Except for the preliminary experiments of D.W. Smith mentioned above in "Tuberculosis in different species," no research group, to our knowledge, has counted primary pulmonary tubercles in mice. We did, however, make rather accurate counts of primary microscopic pulmonary lesions in mice that inhaled Pseudomonas pseudomallei (49-51). Therefore, the tubercle count method could also be developed for mice that inhaled tubercle bacilli, especially if these bacilli were of somewhat reduced virulence.

12. SUMMARY

We urge TB investigators to use Lurie's tubercle count method to assess the efficacy of TB vaccines in preventing progressive tuberculosis in laboratory animals. The method provides information directly applicable to human pulmonary tuberculosis, but it has been largely neglected since 1952. It was originally published for rabbits, and still remains to be used as a standard method for evaluating TB vaccines in guinea pigs and in mice.

13. ACKNOWLEDGMENTS

Dr. Donald W. Smith made many suggestions that improved the accuracy of this report. Rena Ashworth and Ilse M. Harrop helped prepare the manuscript. The work was supported by grants AI-27165 and AI-35195 from the National Institute of Allergy and Infectious Diseases, Bethesda, MD, and in part by grant ES-03819 for the Johns Hopkins Environmental Health Sciences Center from the Institute of Environmental Health Sciences, Research Triangle Park, NC, and grant HL-10342 from the National Heart, Lung, and Blood Institute, Bethesda, MD. The lung specimen for figure 1 was provided by Dr. David N. McMurray and Susan W. Phalen, Department of Medical Microbiology and Immunology, Texas A & M University, College Station, TX.

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Key words: Tuberculosis, T.B., Vaccine, Tubercle, Rabbit, Guinea pig, Mouse

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