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Growth Characteristics of Recent Sputum Isolates of *Mycobacterium tuberculosis* in Guinea Pigs Infected by the Respiratory Route

V. BALASUBRAMANIAN,† ERNST H. WIEGESHAUS, AND DONALD W. SMITH*

Department of Medical Microbiology and Immunology, University of Wisconsin—Madison, 1300 University Avenue, Madison, Wisconsin 53706

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The consideration of virulence must distinguish between infectivity and the ability to cause progressive disease once the infection is established. Several investigators have reported the presence of naturally occurring isolates which differ in virulence for guinea pigs. Isolates from south India which differed with respect to gross disease and number of bacilli recovered from spleen after an intramuscular infection also differed in their efficiencies to initiate an infection, once inhaled and retained. Also, this difference was correlated with differences in the rate of multiplication at the site of implantation and rate of multiplication at sites of hematogenous seeding, as well as the extent of hematogenous seeding. The number of metastatic foci was identified as a quantitative measure of hematogenous seeding, which was not confounded by the rate of multiplication of bacilli. Even allowing for the fourfold-reduced efficiency of low-virulence tubercle bacilli to produce a lesion, this measure clearly revealed a significantly reduced ability of the low-virulence tubercle bacilli to disseminate via the bloodstream.

Virulence encompasses two features of a pathogenic organism: its infectivity and the severity of the disease it produces. In considering virulence we must distinguish between infectivity and the ability to cause progressive disease once the infection is established. Prior to 1948, isolates of *Mycobacterium tuberculosis* from patients were found to have a uniformly high virulence for susceptible animals (7, 8, 19). Dhayagude and Shah (4) first reported that strains from Indian patients often exhibited reduced virulence for guinea pigs. Several investigators have observed the presence of naturally occurring isolates differing in virulence for guinea pigs (2, 4, 13, 15, 16). Mitchison and coworkers observed that 60 to 70% of isolates of tubercle bacilli from the sputum of patients in south India were of significantly reduced virulence for guinea pigs (10). There is evidence to show that infection in guinea pigs with tubercle bacilli of low virulence either by a parenteral injection of a massive dose (10) or by the respiratory route with a low dose (13) is less progressive than that produced by the high-virulence strains. However, evidence that low-virulence tubercle bacilli are any less infective than the high-virulence strains is conflicting (6, 9).

Wiegeshaus et al. (21) observed that the number of tubercle bacilli recovered from the spleens of guinea pigs 6 weeks after infection is a good predictor of survival time of the guinea pigs. Prabhakar et al. (14) observed a strong relationship between the root index of virulence (11) and the $\log_{10}$ number of bacilli recovered from the spleen (BE-spleen). They suggested that BE-spleen was a measure of the virulence of an isolate. On the basis of this observation, Smith et al. (20) interpreted the virulence of an isolate as the capacity of that isolate to disseminate via the bloodstream.

The objective of our study was to study naturally occurring recent sputum isolates of tubercle bacilli from south India in order to increase understanding of the mechanisms by which tubercle bacilli cause disease. Specifically, we have examined quantitatively in a guinea pig model of respiratory infection the replication of tubercle bacilli at the site of primary implantation in the lung, hematogenous dissemination, replication at sites of hematogenous seeding, and granulomatous changes at sites of seeding.

MATERIALS AND METHODS

Selection of isolates. From our culture collection of isolates received from south India, 10 isolates representative of high virulence and 10 of low virulence as classified in the high-challenge dose intramuscular assay developed by Mitchison (11) were selected for the present study.

Cloning of isolates and preparation for storage at −70°C. Challu et al. (3) observed that a proportion of isolates from south India were mixtures of two or more strains of tubercle bacilli in terms of colony morphology, phage type, and virulence. As this was a possible reason for the large variance observed within a group of animals infected with the same isolate (unpublished data), all isolates were carried through a cloning process described earlier (14). Briefly, single-cell suspensions of the isolates were plated at a limiting dilution, and an isolated colony (representative of the predominant colony type) was transferred to 7H-9 broth. After incubation for 8 to 10 days at 37°C in 5% CO_2, the broth was centrifuged twice for 30 min at 2,500 × *g* and the sediment was resuspended in fresh 7H-9 broth. The resuspended broth was homogenized and filtered through a 5.0-μm-pore-size membrane filter. Aliquots (0.6 ml) were dispensed into vials for storage at −70°C. After an initial period of 7 days, on two occasions 3 weeks apart a vial of each suspension was rapidly thawed at 37°C, mixed, diluted, and plated to give two estimates of the CFU per milliliter of frozen stock. There was no difference between the two estimates.

Respiratory infection virulence assays. Specific-pathogen-

* Corresponding author.
† Present address: Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461.

free female guinea pigs, weighing 250 to 300 g, were obtained from the Charles River Breeding Laboratories, Wilmington, Mass. These were randomly allocated to cages, identified by a number tattooed in the ear, and allowed a 2-week adjustment period prior to infection. Groups of guinea pigs were infected via the respiratory route by procedures described earlier (21), with one of several low- or high-virulence cloned isolates. On the basis of the number of CFU in the frozen suspension and a priori information for the aerosol chamber used in this study regarding the relationship between viable counts in the nebulizer fluid and the number of primary lesions induced for H37Rv (21), the nebulizer fluid was adjusted to a concentration expected to result in the inhalation and retention of 3 to 5 CFU, each unit capable of multiplying and initiating a primary lesion in the lungs. All animals were skin tested with 5 tuberculin units (TU) of purified protein derivative 1 day prior to necropsy. Five or 6 weeks after challenge, groups of animals were killed by exposure to histamine aerosol and then by exposure to CO_2. Lung lobes from each animal were transferred to sterile petri dishes, and X rays of the inflated lung lobes were prepared according to procedures described earlier (17). Primary lesions were enumerated on the X ray and on the lungs. Two of the largest primary lesions and the spleen from each animal were excised for quantitative recovery of tubercle bacilli.

Deep culture technique for recovery of low numbers of mycobacteria from tissues. About 18 h after challenge, whole lung lobes were excised for quantitative recovery of tubercle bacilli by a modification of a technique described earlier (18), which permitted the recovery of as few as 2 or 3 tubercle bacilli. Briefly, individual lung lobes were homogenized in 5.0 ml of 2% albumin and transferred to a 150-cm^2 sterile tissue culture flask (Corning) containing 50.0 ml of oleic acid-albumin agar (no longer commercially available but can be replaced with 7H10 agar) supplemented with 0.2% penicillin and 1% cycloheximide maintained at 50°C. The contents were mixed, and the flasks were incubated on their larger surface, thus forming a shallow layer of medium. This enables the development of deep and surface colonies of mycobacteria.

Calculation of growth constant for each isolate. The growth constant (k) represents the rate at which the natural logarithm of a cell population increases with time (t) during the exponential phase. The number of tubercle bacilli (X) increases with time according to the following relationship: $X_n = e^{kt} X_{n-1}$, where n-1 and n are succeeding time intervals and $k = (\ln X_n - \ln X_{n-1})/t$.

Definitions. Virulence was defined as the $\log_{10}$ number of tubercle bacilli recovered from spleens of animals at 6 weeks after challenge.

The infectivity index was defined as the ratio of the number of CFU per milliliter of nebulizer fluid to the number of primary lesions observed in the lungs of guinea pigs at 6 weeks after challenge.

Lesion-inducing efficiency (LIE) was defined as the ratio of the mean number of CFU recovered from whole lungs 18 h after challenge to the mean number of primary lesions observed in the lungs of guinea pigs at 6 weeks after challenge.

Statistical analysis. The experiments were carried out according to the principles of blinded experimentation and randomization. The number of tubercle bacilli recovered from various tissues was transformed into $\log_{10}(X + 1)$ values and subjected to correlation analysis by using standard statistical software (5).

RESULTS

In the first experiment, groups of four guinea pigs were infected via the respiratory route with 1 of 20 cloned isolates obtained from patients in south India and representative of high and low virulence as classified by the intramuscular assay developed by Mitchison (11), such that 3 to 5 CFU were expected to be inhaled and retained in the lungs of each animal. This was expected to yield 3 to 5 primary lesions per animal. Five weeks after challenge, gross and X-ray observations of the lungs as well as culture of whole lung homogenates revealed that 35% of the animals infected with one or another low-virulence isolate had missed infection. In contrast, only 5% of the animals infected with one or another high-virulence isolate missed infection. The infectivity index based on the ratio between the number of tubercle bacilli in the infecting inoculum and the number of primary lesions per animal is plotted against virulence in Fig. 1. The correlation (r) between the infectivity index and BE-spleen (measure of virulence) was −0.85. The ratio of the infectivity index of low-virulence isolates to that of high-virulence isolates was 5.57. These findings were reproduced in a subsequent experiment. Further, it was also observed that the infectivity index was independent of the dose of infection (data not shown).

Because the infectivity index was calculated from the concentration of tubercle bacilli in the nebulizer fluid in the experiments described above, the observed differences in the infectivity index of isolates differing in virulence could have been due to the differences in the survival of tubercle bacilli during the aerosolization procedure. Thus, in the next experiment groups of six guinea pigs were infected via the respiratory route with one of eight isolates used in the first experiment, such that 30 to 50 CFU was expected to be inhaled and retained in the lungs of each animal. Another measure, LIE, was utilized to determine the differences, if any, in the infectivity among strains differing in virulence. Table 1 shows that whereas the LIE for the high-virulence isolate was 1, the LIE for low-virulence isolates ranged from 1.5 to 4. The correlation between LIE and the infectivity index for various isolates was 0.80. The correlation between LIE and BE-spleen was −0.848, and the correlation between infectivity index and BE-spleen was −0.967. On the basis of the number of tubercle bacilli recovered from the spleen 6 weeks after challenge, the isolates were confirmed as being of low or high virulence. The relationship between infectivity and virulence was reproduced in four different experiments.

Figure 2 indicates that on the basis of the analysis of variance, the mean number of tubercle bacilli recovered from the spleen as well as primary lung lesions of animals infected with the two high-virulence isolates (HV1 and HV9) was significantly greater than the mean number recovered from the spleens of animals infected with low-virulence isolates. The next experiment focused on the progression of infection in animals infected with high- or low-virulence isolates. Groups of animals were infected according to procedures described above and killed at various intervals between 8 and 70 days following infection. On the basis of the number of tubercle bacilli recovered from whole lung homogenates at 8, 10, and 12 days after challenge, the growth constants for each of four isolates were calculated. A steady growth rate was observed for all four isolates (Fig. 3). However, the rate of low-virulence isolates was slower than that of the high-virulence isolates. The mean k values calculated from 8 to 10 and 10 to 12 days reveal a two- to

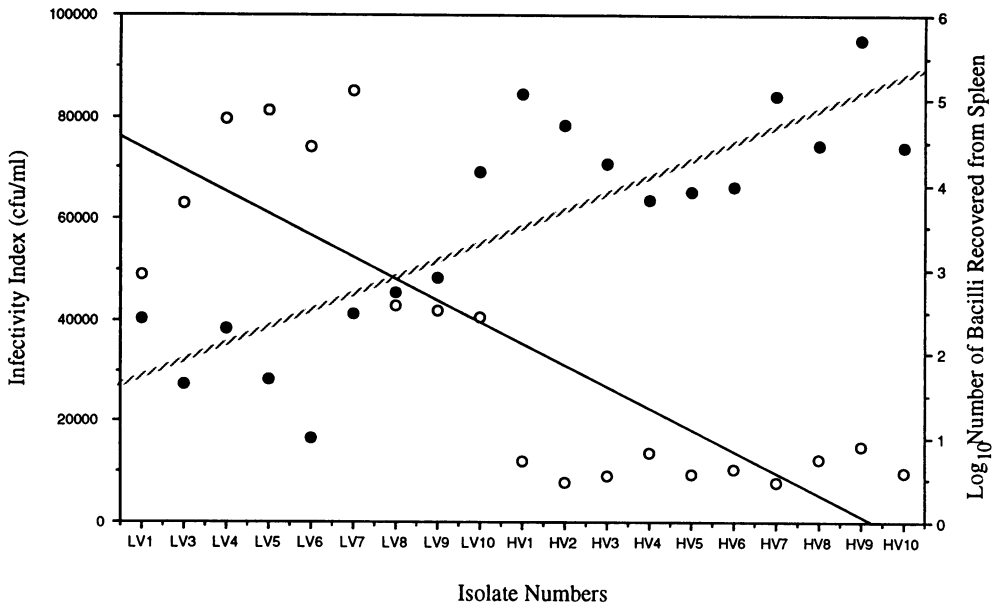

FIG. 1. Inverse relationship between infectivity and virulence. ○, infectivity index; ●, BE-spleen.

threefold difference ($P < 0.001$) between the growth constants of high- and low-virulence isolates.

The change with time in the number of bacilli recovered from the site of initial implantation (primary lesions) during the first 70 days of infection is shown in Fig. 4. At 12 days the number of tubercle bacilli recovered from primary lesions in animals infected with high-virulence isolates was 100-fold higher than that recovered from animals infected with low-virulence isolates. However, the peak bacterial numbers in the primary lesions for the two groups of isolates were not significantly different. The time of onset of bacteriostasis, i.e., 22 days of infection, coincided with the skin test conversion to a dose of 5 TU of purified protein derivative and was not significantly different for the two groups of animals. At this time, the primary lesions from both the groups were similar in terms of the number of bacteria and gross disease. However, the fate beyond this time was very different for the two groups. The number of bacilli in the primary lesions in the case of the high-virulence isolates remained at a steady level, while that in the low-virulence group rapidly declined.

The change with time in the recovery of tubercle bacilli from the spleen during the 70 days following infection is shown in Fig. 5. The onset of hematogenous seeding, as evidenced by the first recovery of bacilli from spleen, occurred between 17 and 22 days for all the four isolates. Six weeks after infection, the number of tubercle bacilli recovered from the spleen of animals infected with the two high-virulence isolates was 1,000-fold greater than those recovered from animals infected with low-virulence isolates. The extent of dissemination as revealed by the number of bacilli recovered from the spleen includes the number of bacilli transported via the bloodstream. The number of bacilli transported via the bloodstream is best revealed from the number of metastatic foci observed on the lungs. Figure 6 shows that the degree of opacity on the X rays of lungs from animals infected with high-virulence isolates is significantly different from that observed in the case of low-

TABLE 1. Comparison of LIE, infectivity index, and virulence of isolates

Isolate	Nebulizer fluid bacterial concn (10^5 CFU/ml)	Mean CFU/whole lung[a]	No. of lesions/lung[b]	LIE[c]	Infectivity index (10^4)[d]	BE-spleen ($\log_{10}$ values)[e]
LV3	4.60	26.0 + 4.2	18.33 + 6.50	1.42	2.51	4.23 + 0.93
LV5	4.70	46.2 + 18.2	11.00 + 4.33	4.20	4.27	3.11 + 1.02
LV6	4.00	25.3 + 7.6	10.00 + 4.24	2.53	3.98	3.73 + 0.98
LV8	4.10	33.0 + 7.0	12.50 + 5.73	2.64	3.31	4.29 + 0.63
LV9	4.30	35.2 + 17.1	14.67 + 2.33	2.40	2.95	3.81 + 0.72
LV11	4.10	51.0 + 21.2	14.00 + 6.60	3.64	2.95	3.88 + 0.45
HV1	2.10	30.4 + 11.7	30.50 + 5.71	1.00	0.69	5.92 + 0.28
HV9	3.00	NA[f]	34.00 + 8.04	NA	0.89	5.93 + 0.28

[a] Estimated mean CFU per whole lung = mean CFU per half lung × 2.
[b] Actual number of primary lesions observed in the lungs of animals killed 6 weeks postinfection.
[c] LIE, mean CFU recovered from whole lung at 18 h/mean number of primary lesions observed at 6 weeks.
[d] Infectivity index, mean nebulizer fluid concentration (CFU/ml)/mean number of primary lesions observed at 6 weeks.
[e] BE-spleen, bacterial enumeration of the spleen, 6 weeks postinfection.
[f] NA, not available.

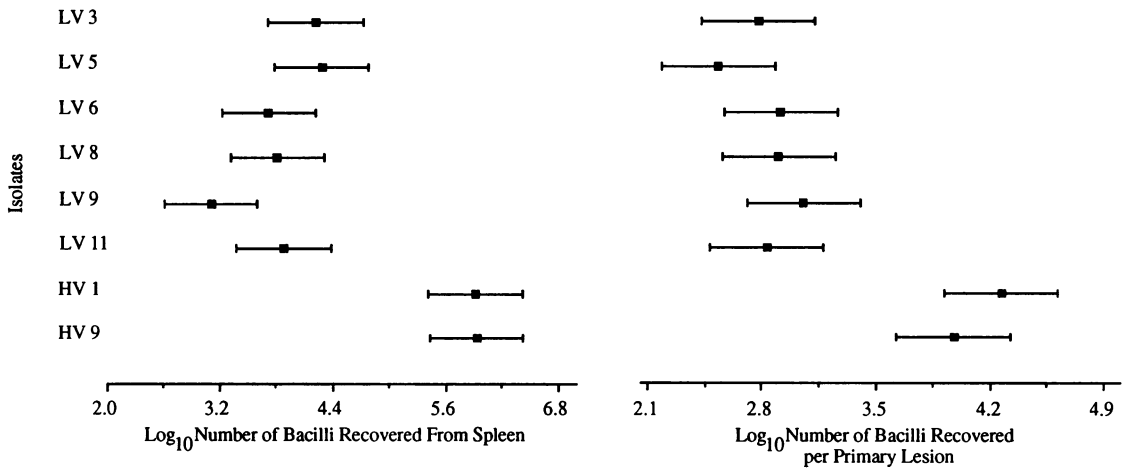

FIG. 2. Log_{10} number of tubercle bacilli recovered from spleen and primary lesions of animals infected with one of eight cloned south India isolates. Means and 95% confidence intervals are based on pooled standard deviations.

virulence isolates. The extensive opacity is due to the large number of metastatic foci.

DISCUSSION

Knowledge of the fate in vivo of naturally occurring isolates differing in virulence increases understanding of the fundamental mechanisms by which tubercle bacilli cause disease. The present study specifically focused on recent sputum isolates from south India which are known to differ in terms of gross disease produced in guinea pigs (12), as well as the number of tubercle bacilli recovered from the spleens of guinea pigs 6 weeks after intramuscular or respiratory infection (1, 14). This study utilized an experimental model involving infection of animals by the respiratory route with small numbers of mycobacteria. This experimental model had the advantage of simulating the conditions of infection in humans.

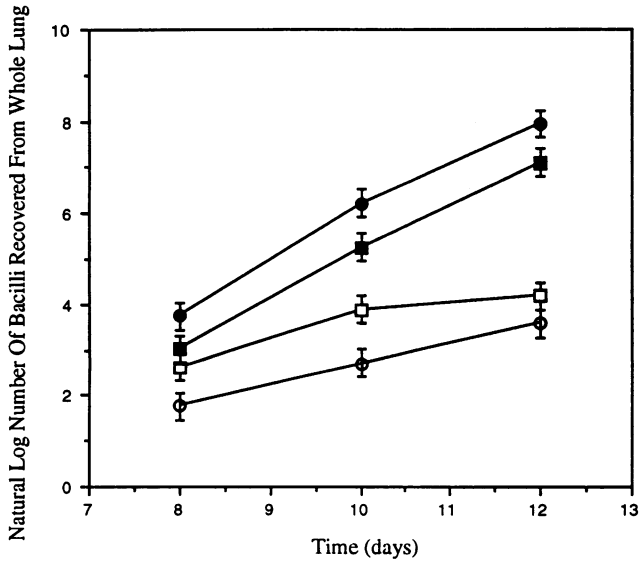

FIG. 3. Growth rate of various isolates of tubercle bacilli in the lungs of guinea pigs. ○, LV5; □, LV9; ●, HV1; ■, HV9.

In this study, infectivity was defined as the capacity of the tubercle bacillus to produce a primary lesion in the lungs of guinea pigs infected by the respiratory route. In order to assess the infectivity of different strains, two measurements, namely, infectivity index and LIE, were made. By either assessment it was observed that the low-virulence isolates were also less infectious. Gangadharam et al. (6) reported that the infectivity of drug-sensitive south Indian strains correlated with the pathogenicity of these strains. In their study, infectivity was defined as the number of tubercle bacilli recovered from the lungs of guinea pigs 4 weeks after respiratory infection and virulence was defined as the number of tubercle bacilli recovered from the spleen 6 weeks after challenge. However, Mitchison (9) pointed out that the appropriate measure of infectivity in the study by Gangadharam et al. should have been the number of surface tubercles (primary lesions) on the lungs. According to the measure suggested by Mitchison, the data obtained by Gangadharam et al. led to the conclusion that there was no correlation between infectivity and virulence (9).

Our findings, which utilized the enumeration of primary lesions for the measure of infectivity, disagree with the conclusions drawn by Mitchison (9) from the study by Gangadharam et al. (6). This could be due to our use of cloned isolates which minimized the variance and thus allowed a difference as low as fourfold to be observed consistently. This difference may have been inapparent in the study by Gangadharam et al. because primary cultures were used. Primary cultures from south India often contain mixed populations in terms of virulence for guinea pigs and other biochemical properties (3). We have observed that infection with primary cultures often results in a large variance (unpublished data). Such variance may have hidden the differences in the infectivity among strains in the study by Gangadharam et al. (6).

One of the principal questions asked in our study was whether the differences in the extent of disease observed were due to the differences in the in vivo generation time of the various isolates. The growth constant (k) was calculated, and the intervals chosen were restricted to the exponential phase of the growth, a phase during which it was assumed that there would be unrestricted doubling of tubercle bacilli at the sites of implantation. Low-virulence isolates repli-

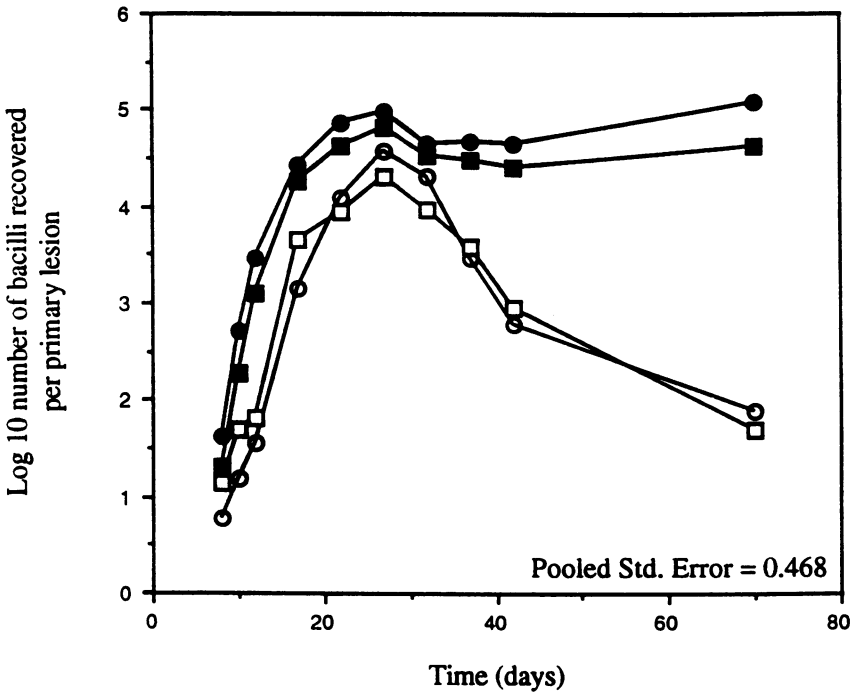

FIG. 4. Change with time in the growth of tubercle bacilli in primary lung lesions. See legend to Fig. 3 for symbols.

cated more slowly in the animals. Despite the difference in the initial rates of multiplication, the peak bacterial number and the extent of tissue damage observed in the primary lung lesions at the onset of bacteriostasis were not different for the four isolates. It is possible that the onset of bacteriostasis in the primary lesions could be a result of the maximum number of bacteria that could be accommodated in a lesion. The onset of bacteriostasis coincided with skin test conversion in the animals. Therefore, the onset of bacteriostasis may be due to the activation of a specific cell-mediated immune response, which is possibly controlled quantitatively and not qualitatively by the infecting strain. However, the survival of the tubercle bacilli in the face of activated cell-mediated immune response is clearly dependent on the virulence of the isolate. In this respect, the differences in virulence could be related to the antigenic makeup that is not presented differently by the macrophage but that evokes the effector cell functions differently.

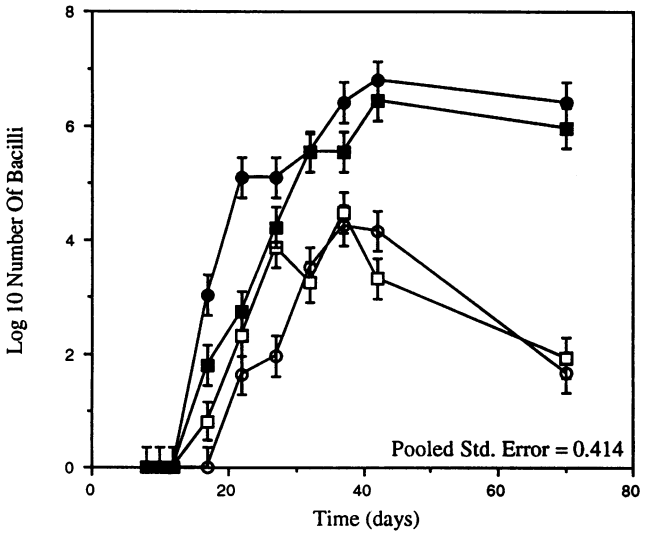

FIG. 5. Change with time in the number of tubercle bacilli recovered from the spleen. See legend to Fig. 3 for symbols.

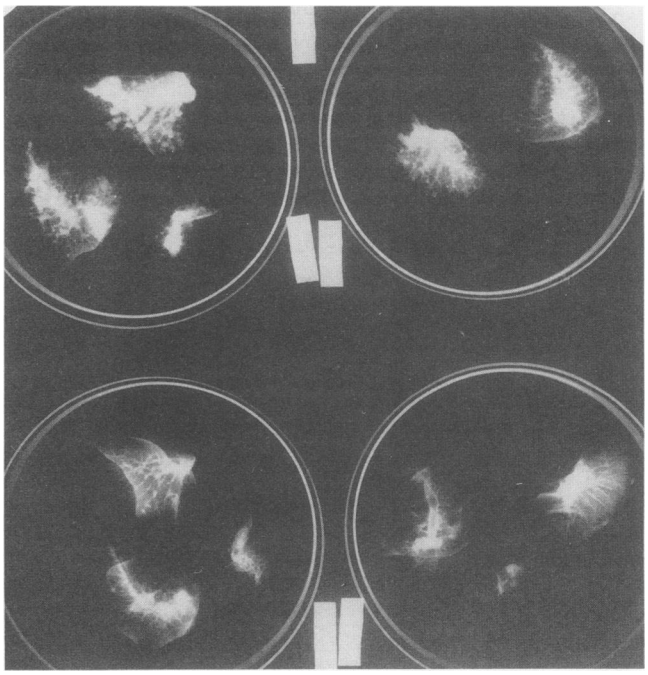

FIG. 6. Extent of gross disease due to metastatic foci in the lungs of guinea pigs infected with high (top)- or low (bottom)-virulence isolates.

The continued decline in the number of low-virulence tubercle bacilli in the primary lesions set the stage for either a low-level chronic infection or sterilization. On the other hand, in the case of high-virulence isolates, the continued presence of a high number of tubercle bacilli in the primary lung lesions provided a continuous source for dissemination, as reflected in the steady increase in the number of organisms recovered from the spleen. The number of tubercle bacilli recovered from the spleen indicate that low virulence for guinea pigs may be related to a low number of tubercle bacilli transported via the bloodstream. The number transported via the bloodstream is more appropriately inferred from the number of metastatic foci in the lungs. It was reasoned that the biologically relevant bacilli transported to the lung are only those bacilli capable of producing metastatic lesions. Thus, the low-virulence phenotype was related to a reduced number of bacilli transported via the bloodstream.

Any study focusing on the identification of virulence genes in order to understand the basis for the survival of tubercle bacilli in vivo and the resulting disease should take into account that virulence is indeed a multiphenotype. Depending on the variable chosen to quantify virulence and depending on the stage of infection, different sets of genes may be identified.

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