TUBERCULOSIS INDUCED BY DROPLET NUCLEI INFECTION

Initial Homogeneous Response of Small Mammals (Rats, Mice, Guinea Pigs, and Hamsters) to Human and to Bovine Bacilli, and the Rate and Pattern of Tubercle Development*

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Plates 1 to 3

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When rats, mice, hamsters, and guinea pigs were caused to inhale either human or bovine tubercle bacilli as separate cells in fine droplet nuclei, the number of initial tubercles equalled approximately the number of bacilli calculated to have been inhaled. Moreover, the initial tubercles in the eight host-parasite combinations developed to macroscopic size within 4 weeks, apparently at a highly uniform rate, but thereafter the rate and pattern of tubercle formation varied widely (1). It seems probable, therefore, that these species do not differ in their "native" or inherent resistance to the bacilli of tuberculosis. Instead, they differ in their capacity to acquire resistance to these organisms.

Additional evidence to support this hypothesis is provided by the present study, which traces the sequence of tissue changes through the homogeneous stage of the response to infection in the eight host-parasite combinations to its transformation into the later, heterogeneous phases of the disease. This study demonstrates that the rate and pattern of tubercle formation in all eight host-parasite combinations are, indeed, highly uniform for a time, and correspond closely to the initial stages of tubercle formation in rabbits infected by bovine tubercle bacilli (2, 3).

Material and Methods

The animals used in these experiments, and their weights, were: albino mice, 15 to 20 gm.; Syrian hamsters, 80 to 100 gm.; Long-Evans rats, 150 to 200 gm.; and short haired guinea pigs, 500 to 800 gm. Commercial diets were fed.

Infections were induced by means of the Wells' apparatus and techniques, which fulfill the postulates that govern quantitative control of droplet nuclei infection (4–7). Under these conditions the number of initial tubercles has approximated liters of aerosol inhaled times

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number of bacilli per liter of aerosol; also, the average ventilation rates in liters per hour per animal have about the following values: mice, 4; hamsters, 10; rats, 12; and guinea pigs, 40. Bacilli per liter of aerosol were about equal to bacilli per milliliter of aqueous suspensions (Breed counts) multiplied by 1.6×10^{-5} (1).

The Ravenel strain of *Mycobacterium tuberculosis* (*bovis*) and the H37Rv strain of *M. tuberculosis* (*hominis*) were used to infect the animals. These organisms were grown in a liquid medium in revolving flasks; they were subcultured semimonthly (8, 9). The aqueous suspensions from which aerosols were generated were prepared as follows: a liquid culture, not more than 18 days of age, was filtered through No. 4 Whatman paper, the bacilli per milliliter of filtrate determined by the Breed method, and the suspension diluted to an appropriate level in demineralized water.[1]

The animals were infected in groups, and 3 or more of each group (human and bovine) were killed at stated intervals after infection. The lungs were deflated, then expanded by filling with Bouin's fluid, and stored in Bouin's fluid for at least 4 days. Tissues were embedded in paraffin, sectioned at 5 μ, and stained to demonstrate the bacilli and the histological changes associated with the infection. All sections were cut and stained by one technician.

The stages of tubercle development during the first 3 weeks were studied in animals that had inhaled relatively large numbers of bacilli. Mice were estimated to have inhaled about 500, rats and hamsters about 2000, and guinea pigs about 5000 viable organisms. A series of from 10 to 20 consecutive sections were cut from each of at least 4 blocks of tissue from each of these animals. Tubercle development after 21 days was studied in animals that had inhaled less than 50 organisms. These animals also were infected in groups.

Initial Response of the Lungs to Air-Borne Tubercle Bacilli

6-Day Stages:—From the 6th to the 12th day after the bacilli were inhaled they were found only in alveolar macrophages. At the 6 day stage the 1 or 2 parasitized cells found in any one focus in the lungs were estimated to contain not more than 12 organisms. Usually the parasitized cells were isolated but occasionally they were part of a small group of alveolar macrophages. Alveolar walls about these foci were unchanged (Figs. 1 and 2).

9-Day Stages:—3 days later each focus of infection was much more conspicuous. This was especially true in the lungs of rats and guinea pigs that had inhaled the bovine organism. In these two host-parasite combinations blood monocytes had thickened the alveolar walls about the foci of infection and had joined the mass of alveolar macrophages that now clustered about the several parasitized cells in each focus. In the other six host-parasite combinations alveolar walls about the infected foci were unchanged, and only alveolar macrophages had collected about the 4 or 5 parasitized cells that now formed the approximated center of each focus. The number of bacilli visible in any focus was at least twice as great as in the 6-day stage, and about equal in all host-parasite combinations (Figs. 2, 3).

12-Day Stages:—At this time each focus in the lungs occupied 1 or 2 alveolae and now, in all host-parasite combinations, the alveolar macrophages were

[1] Demineralized water is a substitute for distilled water. The apparatus used to produce it is manufactured by the Barnstead Still and Sterilizer Co.

mixed with blood monocytes and polymorphonuclear leucocytes to form rather compact masses about the parasitized cells (Figs. 4–6). The more intense reaction of guinea pigs and rats to the bovine organism also continued through this stage (compare Figs. 4 and 6). Otherwise the characteristics of the response to the infection and the number of bacilli in the lesions were much the same in all animals.

15-Day Stages:—3 days later the acute inflammatory reaction that was beginning at the 12-day stage had reached its greatest intensity. Polymorphonuclear leucocytes were the predominant cells in all lesions. The leucocytes, with smaller numbers of blood monocytes, obscured or replaced the alveolar macrophages which now were compressed into more or less necrotic masses in the centers of the lesions (Figs. 7–9).

The bacilli were greatly increased over the numbers seen in the 12-day stages and were concentrated in the remains of the centrally placed masses of alveolar macrophages.

18-Day and 21-Day Stages:—In the 18-day tubercles the leucocytes were no longer so abundant as at 15 days, and alveolar macrophages had been largely replaced by monocytes from the blood stream (Figs. 10–12). Also between 15 and 18 days the bacilli were reduced by more than half, as judged by the number that could be demonstrated in the lesions (compare Figs. 7 to 9 with 10 to 12). At 21 days reduction in the numbers of bacilli and leucocytes was even more pronounced. Otherwise the 21-day tubercles were simply expansions of those seen at 18 days.

Between 21 and 28 days after infection, however, features that were characteristic of the particular host-parasite combination became apparent; thereafter the pattern of the reaction and the progress of the disease varied with the species of host and the strain of parasite. Thus it seems evident that the initial response to infection and the early stages of tubercle formation in these eight host-parasite combinations followed one pattern. Clear cut deviations from this pattern did not appear until an acute inflammatory reaction had developed and subsided, more or less, and the number of bacilli had been sharply reduced.

The Development and Progress of the Initial Tubercles after the 4th Week

The 28-day tubercles of rats, mice, and hamsters were indefinitely and irregularly outlined foci within which thickened alveolar walls formed a sort of skeleton. Monocytes and lymphocytes accumulated in the perivascular and interlobular tissues enclosed by the lesions and filled alveolar spaces more or less completely about the periphery. In the guinea pigs, however, the 28-day tubercles were sharply circumscribed with some slight growth of fibrous tissue in the expanding periphery, even in animals infected by the bovine bacilli. The necrotic centers of these tubercles also were well defined and single, in contrast to the multicentric lesions of other animals at this stage.

Rats.—Tubercles of rats developed slowly after the 4th week. Their rate of progress and their histological makeup were the same with both strains of bacilli. Fig. 15 illustrates the essential features of tubercles in the lungs of this species at any time between the 5th and the 15th week. The tubercles slowly expanded as irregularly outlined foci to diameters of 3 to 4 mm. The alveolar walls within the mass were irregularly infiltrated by monocytes and lymphocytes and, within an occasional narrowed alveolus, clusters of larger, finely vacuolated cells (modified monocytes?) carried an occasional bacillus. The infections did not spread to thoracic lymph nodes nor to other organs, in so far as could be determined by macroscopic inspection.

Mice.—At 28 days the tubercles in the lungs of mice closely resembled those of rats, but the number of parasitized monocytes and the number of bacilli per cell were distinctly greater (Fig. 14). Between the start of the 5th and the end of the 7th week tubercles induced in mice by the human strain of bacillus expanded slowly and irregularly, but necrotic centers that communicated with bronchioles had developed within this time. Spread of organisms by way of the bronchi became conspicuous after the 7th week. The bovine tubercles also followed this same pattern, but expanded more rapidly. Death from bronchial spread of this infection occurred before 60 days in this group of mice; the experiment was then terminated. Tubercles did not develop to macroscopic size in extra-pulmonary foci of either group of mice.

Hamsters.—In the 28-day tubercles of hamsters the parasitized monocytes with occasional leucocytes filled alveolar spaces in almost unbroken masses from periphery to center (Fig. 13). Occasional small necrotic foci persisted from the earlier stages and expanded slowly. Expansion of the tubercles during the 4th week had been quite rapid, but from the 5th to the 10th week human tubercles in this species scarcely doubled in size. The histological pattern also was unchanged, although bronchial spread of the infection occurred within 10 weeks. The number of bacilli per parasitized monocyte did not appreciably increase within 10 weeks. Secondary tubercles were seen in the thoracic lymph nodes, the spleen, and kidneys within this period.

Bovine tubercles of hamsters were much more rapidly destructive. Within 5 weeks after infection cavities had formed in the initial tubercles, and bronchial spread of the infection caused deaths within 8 weeks. Macroscopic tubercles had formed in the spleen and lymph nodes within 5 weeks in this group of animals.

Guinea Pigs.—The histological makeup of the initial tubercles in the lungs of guinea pigs at 28 days is illustrated by Fig. 16, which shows part of the necrotic center and of the inflammatory zone within the peripheral layer of young fibrous tissue. Bacilli were relatively numerous in the inner part of the inflammatory zone.

These tubercles had expanded quite rapidly during the 4th week, but there-

after the progress of the human tubercles was surprisingly slow. Within 12 weeks these tubercles hardly had doubled in size, and in some animals actually were reduced, but within this time they formed a wide border of fibrous tissue that contained many epitheloid cells. Also within 12 weeks the necrotic centers of some human tubercles of this species had become compact eosinophilic masses, but in other lesions the inflammatory reaction continued.

Thoracic lymph nodes of this group of guinea pigs contained macroscopic tubercles within 4 weeks; these enlarged rapidly but did not undergo necrosis until about 12 weeks. Within 6 weeks after infection the lungs, spleen, and liver contained many epithelioid tubercles. These secondary tubercles in the lungs did not progress appreciably within 12 weeks, and at that time were overshadowed in some animals by bronchial spread of the infection.

The bovine tubercles of guinea pigs expanded more rapidly than any other lesion during the 4th week, and bronchial spread of the infection was evident at this time. Hence all guinea pigs that had been infected by the bovine bacillus were killed within 28 days after infection.

DISCUSSION

These experiments demonstrate that the initial reaction of rats, mice, hamsters, and guinea pigs to infection by inhalation of virulent tubercle bacilli, whether of bovine or human strains, is highly uniform. This phase of inhaled tuberculosis progressed through three distinct stages: (*a*) growth of the bacilli in alveolar macrophages that collected about the points at which the bacilli had been deposted in the lungs, (*b*) acute inflammatory reactions about these foci, with necrosis of the alveolar macrophages and a sharp reduction in the number of bacilli, and (*c*) transformation of the inflammatory reaction into a less active process that slowly took on features characteristic of the host-parasite combination. Following this third step in the development of the reaction, the initial tubercles in any one of the host-parasite combinations continued to progress at a highly uniform rate for a time. Thereafter the course of the infection varied with the host-parasite combination, presumably reflecting the genetic makeup of the host (10, 11). Thus it seems evident that these animals are equally susceptible to infection by the organisms of human and bovine tuberculosis and differ only in their capacity to acquire resistance.

This assertion is, of course, contrary to current opinion (12, 13). In a large measure, however, current opinion is based upon experiments in which tuberculosis was induced by injecting considerable numbers of bacilli intravenously or into some other particular site in the animal body. Whether infections so induced approximate any of the interrelationships of host and parasite in natural air-borne tuberculosis is problematical. Certainly it is highly unlikely that natural air-borne tuberculosis is ever induced by the deposition of any considerable number of bacilli at any one focus in the lungs. The maximum

dimensions of particles that ordinarily may become air-borne and be inhaled are known to be so limited that rarely can more than single bacilli be contained in them. At the same time the concentration of air-borne bacilli in any atmosphere is extremely low (7, 14). Therefore, it is highly probable that, as in the present experiments, air-borne tuberculosis in man is initiated by single bacilli, deposited separately on the alveolar surfaces of the lungs, and highly improbable that more than one organism ever is deposited at any one site (6, 7, 15–20).

The changes in rate and pattern of tubercle formation during the terminal stage of the homogeneous phase of the reaction, and in all subsequent stages, may be supposed to reflect the development of measurable levels of resistance (2, 21, 22). Apparently however, these changes in the response of the host to the parasite do not depend upon the number of initial tubercles developing in the lungs, that is, these expressions of resistance are not related quantitatively to the amount of antigen. The rate and pattern of tubercle formation seemed to be the same whether less than 50 or more than 500 bacilli were inhaled, unless of course, the developing tubercles replaced so much respiratory surface as to kill the animal.

Evidences of resistance, as manifested by the pattern of tubercle development after the 4th week have, of course, varied with the host-parasite combination, and with the diet (23–25). However, in all instances, developing resistance has been expressed as the capacity to inhibit, more or less, the multiplication of the bacilli and, at least for a time, to reduce the intensity of the inflammatory response in proportion to the number of bacilli in the tissue. These observations are in keeping with the belief that resistance to tuberculosis involves mechanisms other than conventional immune processes (26).

SUMMARY AND CONCLUSIONS

Separate groups of rats, mice, hamsters, and guinea pigs were caused to inhale virulent tubercle bacilli, of human or bovine strains, as single cells in fine droplet nuclei. Members of each of these eight host-parasite combinations were killed for study at stated intervals after infection.

For approximately 3 weeks after the bacilli were deposited in the lungs the progress of the infection, and the reaction of all species to it, followed a highly uniform developmental pattern. During the 4th week the rate and pattern of tubercle formation became distinctive for the species of host and the strain of parasite, but within any host-parasite combination this rate and pattern continued uniform for a time. The duration of this period of homogeneous response after the 4th week varied with the host-parasite combination, ranging from less than 5 weeks to more than 12 weeks after the induction of infection.

It is concluded that the highly uniform initial response is evidence that these animals do not differ in their inherent resistance to inhaled infection by the

bacilli of human or bovine tuberculosis. Instead, they differ widely in their capacity to acquire resistance to these organisms, as shown by the variation in the later stages of the disease.

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EXPLANATION OF PLATES

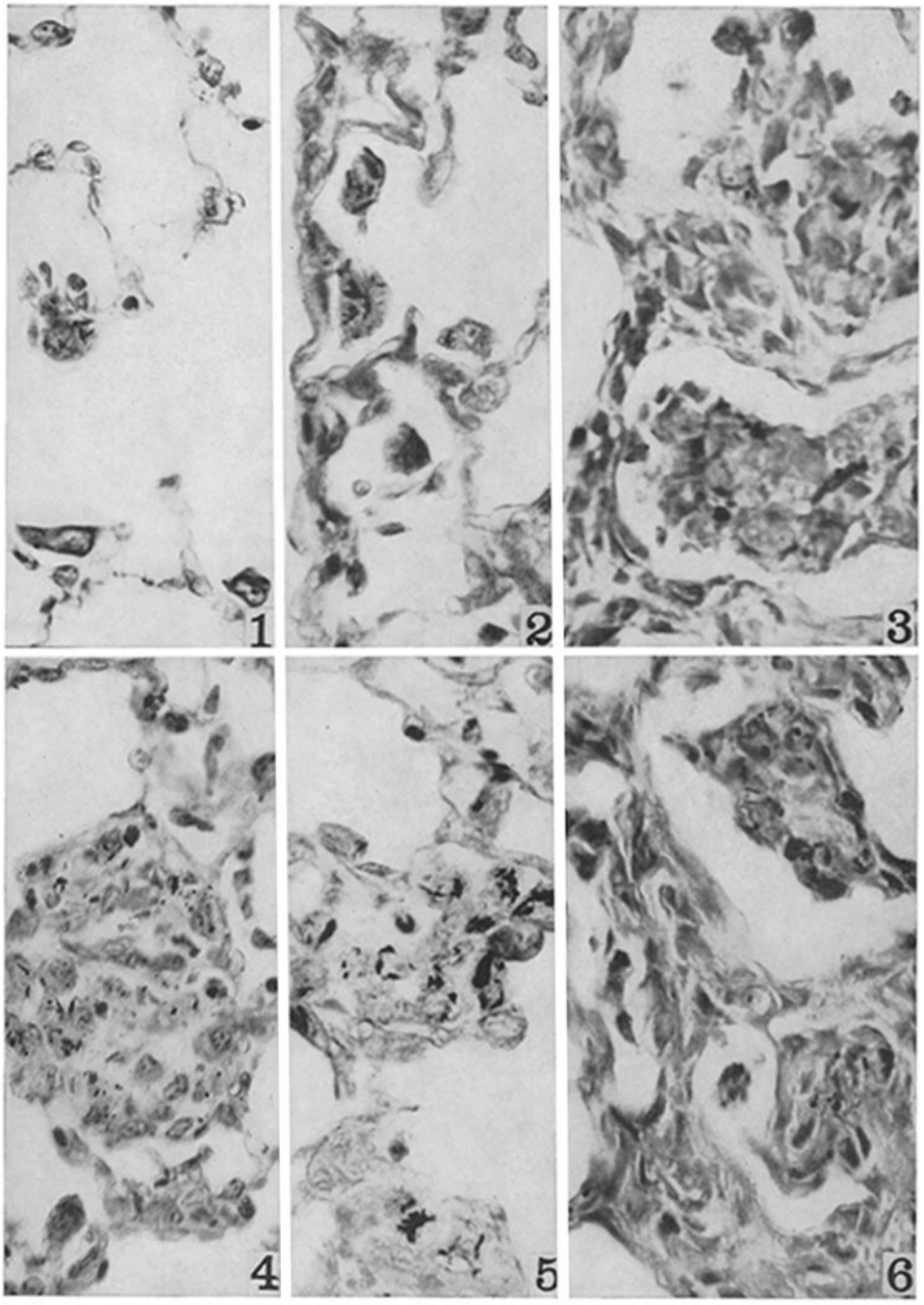
PLATE 1

FIGS. 1 to 3. Tubercle formation 6 to 9 days after infection.

Fig. 1. Hamster–human bacilli 6 days after infection. A single parasitized macrophage on the wall of an alveolar duct. × 650. Fig. 2. Mouse–human bacilli 9 days after infection. Two parasitized cells in an alveolar space just beneath the pleura. The dark masses in the nearby cells are carbon granules. × 800. Fig. 3. Guinea pig–bovine bacillus 9 days after infection. Parasitized macrophages have formed compact masses in 2 adjoining alveolar spaces, the walls of which have been infiltrated by monocytes. × 800.

FIGS. 4 to 6. Tubercle formation 12 days after infection.

Fig. 4. Hamster–human bacilli. Alveolar macrophages, with occasional monocytes and leucocytes compactly massed in an alveolus. × 650. Fig. 5. Mouse–human bacilli. Two adjoining alveolar spaces filled by inflammatory cells, again without appreciable change in adjacent tissues. × 650. Fig. 6. Guinea pig–human bacilli. Alveolar walls about the masses of parasitized cells infiltrated by monocytes. × 800.



(Ratcliffe and Palladino: Tuberculosis induced by droplet nuclei infection)

PLATE 2

FIGS. 7 to 9. Tubercle formation 15 days after infection. × 800.

Fig. 7, hamster–human bacilli; Fig. 8, mouse–bovine bacilli; Fig. 9, mouse–human bacilli. All show the central parts of the developing tubercles with abundant bacilli singly or in masses within the cells. Well stained leucocytes and monocytes can be identified at the periphery of these fields, but within the masses of cells nuclei did not stain well and cell outlines were obscured.

FIGS. 10 to 12. Tubercle formation 18 days after infection. × 800.

Fig. 10, hamster–human bacilli; Fig. 11, rat–bovine bacilli; Fig. 12, guinea pig–human bacilli. All also show the central parts of developing tubercles, with bacilli less numerous than that at 15 days.

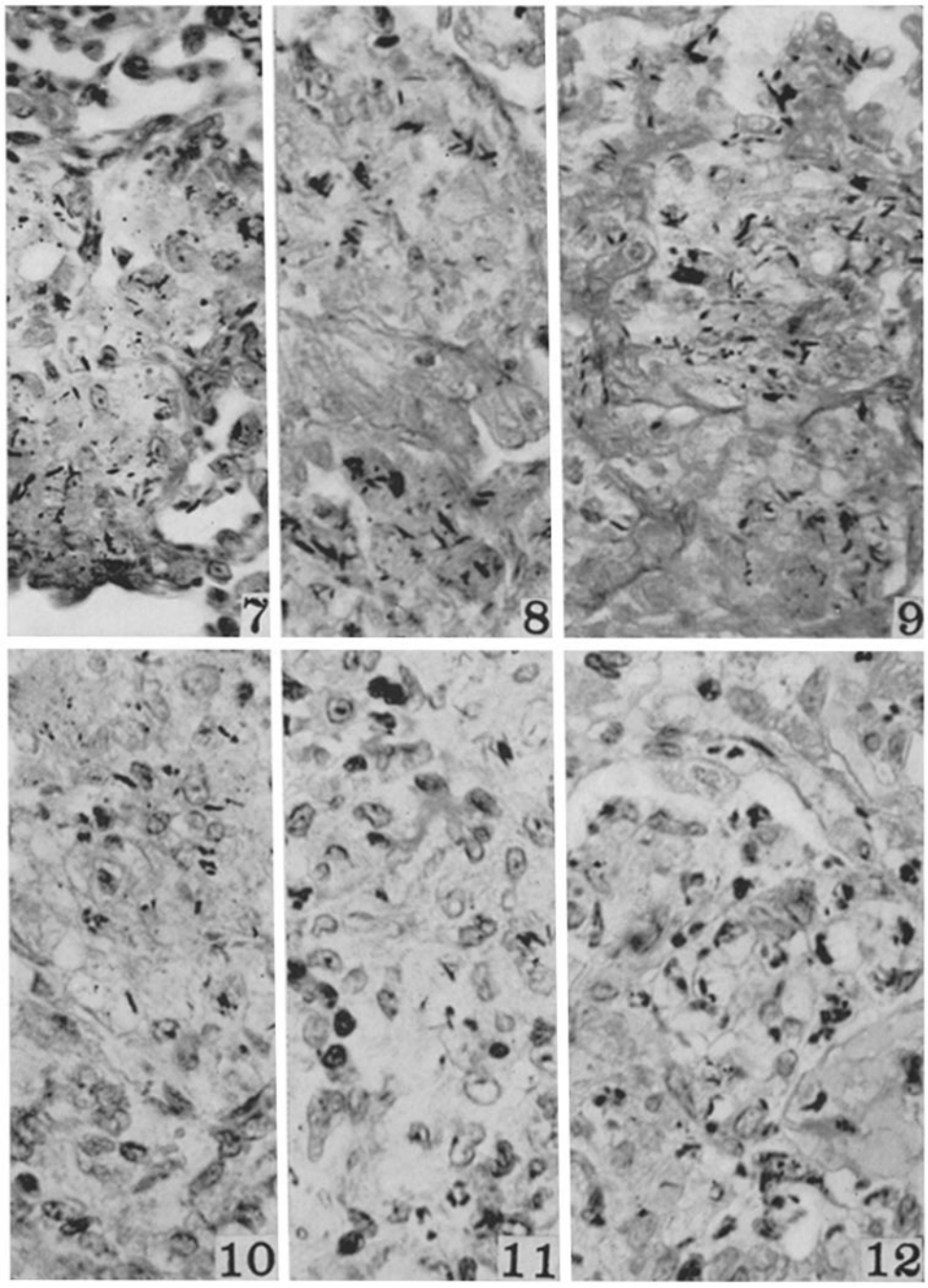


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PLATE 3

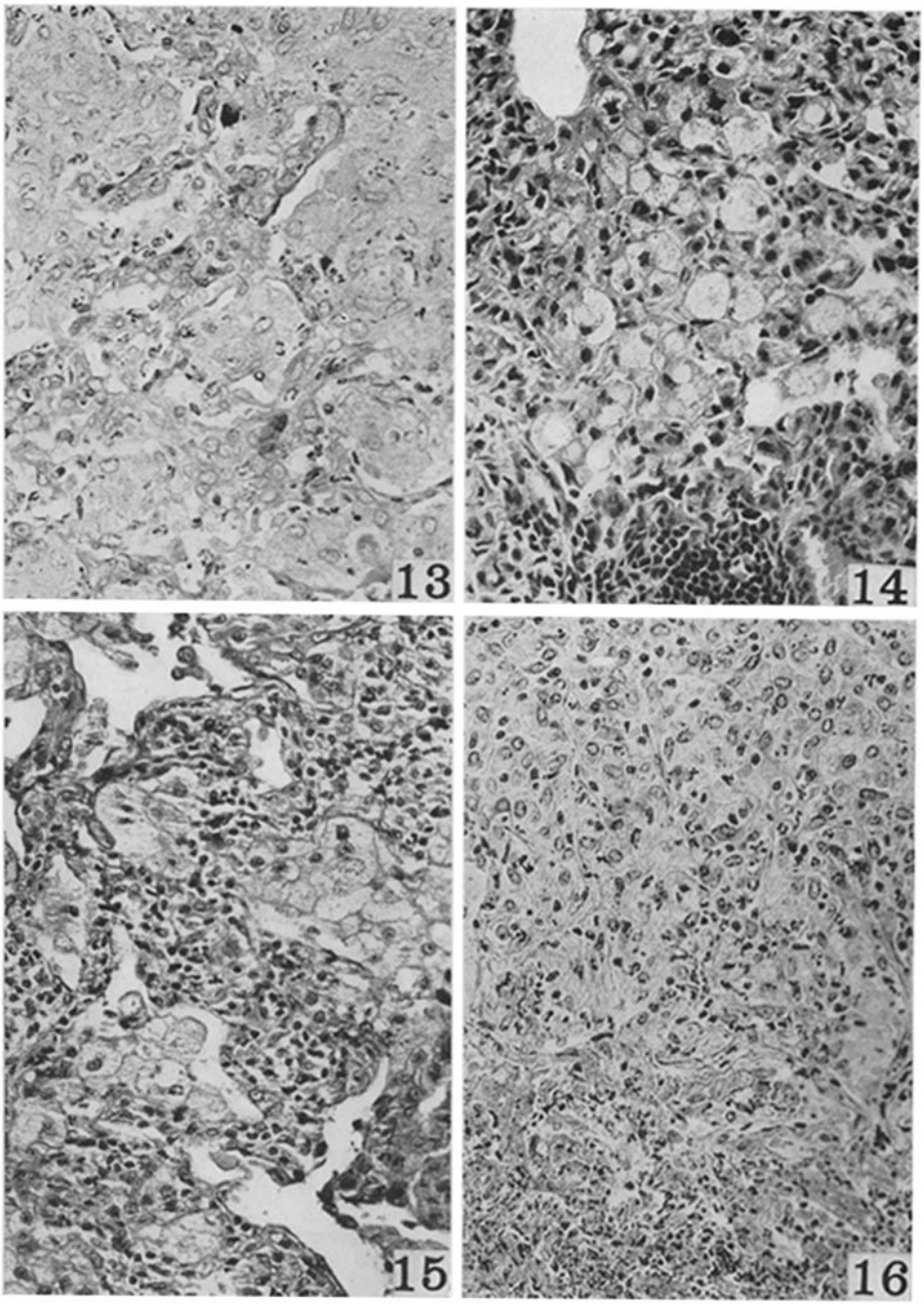
FIGS. 13 to 16. Tubercle formation in rats, mice, guinea pigs, and hamsters during and after the 4th week. × 300.

Fig. 13. Hamster–human bacilli (5 weeks). Masses of parasitized cells with scattered leucocytes enclosed by thickened alveolar walls; bacilli, seen as black rods, are scattered 3 or 4 per alveolar space. This microscopic appearance was unchanged for about 12 weeks. Bovine tubercle bacilli induced a more rapidly progressive disease.

Fig. 14. Mouse–human bacilli (5 weeks). Large vacuolated cells (monocytes?) each containing 1 or 2 bacilli fill alveolar spaces in the center of the tubercle with peribronchial (lower right) and perivascular (upper left) infiltration of monocytes and lymphocytes irregularly subdividing the tubercle. At 7 to 10 weeks necrosis begins in foci of large cells which by that time contain many more bacilli. Leucocytes also again become numerous in these points of necrosis. At 5 weeks bovine tubercles of mice also have approximately this structure, but ulcerate more rapidly.

Fig. 15. Rat–human or bovine tubercle (4 to 15 weeks). The structure of these tubercles was less compact than the 5-week tubercles of mice, but otherwise very similar. Occasional bacilli were contained in the large vacuolated cells within the alveolar spaces. Interstitial tissues were moderately infiltrated by monocytes and leucocytes.

Fig. 16. Guinea pig–human bacillus (5 weeks). A segment of the wall of a developing tubercle and the edge of the necrotic center; bacilli sparsely scattered in cells within the granulation tissue. Bovine tubercles of guinea pigs were much more rapidly destructive, but in them also a thin zone of granulation tissue formed during the 4th week.



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