TUBERCULOSIS INDUCED BY DROPLET NUCLEI INFECTION

Its Developmental Pattern in Guinea Pigs and Rats in Relation to Dietary Protein *

H. L. Ratcliffe, Sc.D., and John V. Merrick, M.D.

From the Penrose Research Laboratory, Zoological Society of Philadelphia, and the Department of Pathology, University of Pennsylvania, Philadelphia, Pa.

Tuberculosis, as induced in mice, rats, hamsters, guinea pigs, and rabbits by droplet nuclei infection, passes through two distinct phases that apparently reflect stages in developing resistance. The first phase is completed within about 3 weeks. During this time the rate and pattern of tubercle formation are the same for each species of animal irrespective of the human or bovine origin of the *Bacillus tuberculosis* used to infect them. The second phase, which follows immediately upon the first, is distinctly different. In this phase the rate and pattern of tubercle formation quickly become characteristic of the host-parasite combination. Nevertheless, the rate and pattern of tubercle formation in any one host-parasite combination are highly uniform for a period, the duration of which differs with the combination.[1-4]

A considerable amount of evidence suggests that the level of dietary protein is a factor in developing resistance to tuberculosis.[5-10] Such a factor should become apparent during or soon after the second phase of the disease, especially when this is relatively prolonged. The following host-parasite combinations seemed to be the most convenient for the purposes of the present series of experiments to test this hypothesis: hamsters and guinea pigs with bacilli of human origin, and rats with bacilli of bovine origin.[3,4] The periods of uniform reaction with these combinations have ranged from about 50 days for the guinea pigs to more than 150 days for the rats.

The results of the experiments on hamsters have been published.[10] The present report describes the results of concurrent studies on guinea pigs and rats.

Material and Methods

The three isocaloric diets used in these experiments have been found to be about equally acceptable to hamsters, rats, and guinea pigs, and to induce similar responses in these animals. These diets were desig-

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nated by the letters A, B, and C, and their respective composition was stated fully in the paper on hamsters.[10] Protein comprised approximately 30, 17, and 6 per cent of the respective diets. Ascorbic acid was added to the food for guinea pigs in amounts sufficient to supply about 20 mg. per day to each animal. In addition, each guinea pig was given about 25 gm. of kale per day.

Short-haired guinea pigs (300 gm.) and rats (100 gm.) of Long-Evans strain were purchased as immature animals. Animals of each sex were caged separately in small groups. Weights were recorded biweekly. A stock diet was fed until the guinea pigs attained weights of at least 500 gm. and the rats, 150 to 200 gm. Test diets were then fed for at least 6 weeks before infections were induced, at which time the weights of guinea pigs ranged from 550 to 800 gm., and of rats from 180 to 300 gm.

Infections were induced by means of the Wells apparatus and techniques.[11-13] A single aqueous suspension of tubercle bacilli was used to infect the groups of animals on each of the three diets. Thus all animals in any one experiment inhaled organisms of highly uniform virulence and viability.[3] Infections were induced at approximately minimal level, mean numbers of initial tubercles in animals of the several experimental groups ranging from four to eight.

Guinea pigs were infected by *B. tuberculosis* of the H37Rv strain, and rats by bacilli of the Ravenel strain. Cultures were supplied by the Standard Culture Depot of the National Tuberculosis Association, Trudeau, New York, and were maintained on solid and liquid media.[12]

During the experiments one culture of the H37Rv strain became reduced in virulence, but before this change was recognized both hamsters and guinea pigs had been infected with these organisms. The reaction of three diet-groups of hamsters to this organism have been described previously.[10] Following an account of the effects of the diets upon tuberculosis induced by the virulent bacilli of this strain, a description of the reactions of three diet-groups of guinea pigs to the attenuated variety will be given. Bacilli of the Ravenel strain did not change in virulence during this study, as measured by the rate of tubercle formation in rabbits.[14]

From 4 to 14 guinea pigs of each diet-group were sacrificed for study at intervals of from 50 to 180 days after infection; and, similarly, 3 to 12 rats from each group were sacrificed 75 to 260 days after infection. Tissues were prepared for study by the methods previously described for hamsters.[10]

Tuberculosis of Guinea Pigs Induced by Bacilli of High Virulence

Significant differences in the appearances of the lesions in these animals became evident about 75 days after infection and increased rapidly thereafter. Deaths from tuberculosis occurred in animals on diet C within 140 days after infection, and none of the guinea pigs infected by the more virulent organisms was observed beyond 150 days.

Macroscopic Appearances

Figures 1 and 2 illustrate the essential differences in tuberculosis of animals on diets A and C 100 days after infection. At this time the disease in animals on diet B corresponded closely to that in animals on diet A. Thus a majority of the initial tubercles of animals on diets A and B were well defined, solid nodules about 5 mm. in diameter, which was the size of the lesions at 50 days in guinea pigs of all diet-groups. In contrast, a majority of initial tubercles in animals on diet C were not well defined at 100 days, apparently having expanded irregularly from the spherical nodules found earlier. As a rule this change was associated with increased numbers of secondary tubercles in the lungs.

The tubercles of the thoracic lymph nodes usually enlarged between 75 and 100 days after infection, irrespective of the diet, but occasionally were no larger than at 50 days. Liquefaction had developed in many of these lesions within 100 days, irrespective of their size. This change usually was most prominent in animals on diet C. The tubercles in the spleens of animals on diet C sometimes increased in size between 75 and 100 days, whereas in animals on diets A and B, tubercles in this organ apparently regressed after 75 days, and sometimes disappeared within 100 days (Figs. 1 and 2). The livers did not contain macroscopic tubercles at this time.

At 150 days after infection some of the tubercles of animals on diets A and B still were as well circumscribed as at 50 days and had not changed in size. More often, however, the lesions were smaller and not so well defined. The tubercles of the thoracic lymph nodes of animals on these diets usually were smaller than at 100 days, and their spleens did not contain active tubercles. However, the livers of several of them contained small scars and occasional tubercles (Figs. 3 and 4).

In contrast to the state of the disease in animals on diets A and B, a majority of the animals on diet C had developed relatively large cavities, which were associated with extensive local secondary spread

of the infection in the lungs. In others of this group the disease was less extensive in the lungs, but apparently was progressing. Tubercles of the thoracic lymph nodes of the group on diet C ranged as widely in size as at 100 days and possessed conspicuous centers of liquefaction.

The livers of all animals on diet C were more or less distorted throughout by tiny scars which sometimes were associated with small hemorrhagic foci. However, macroscopic tubercles were not found in this organ, and were present in the spleens of only three of this group.

Microscopic Appearances

Figure 7 illustrates the characteristic features of initial tubercles of animals of all diet-groups 50 days after infection, at which time the effects of diets had not become evident. Approximately the central half of each of the tubercles was necrotic and densely infiltrated by polymorphonuclear leukocytes. The periphery was formed by densely massed monocytes. These cells had infiltrated the alveolar walls and spaces to produce expanding zones of consolidation, and had accumulated cords and masses in perivascular and peribronchial lymph spaces to give irregular extensions to the lesions. Bacilli were demonstrated only among the partially degenerated monocytes in the necrotic centers of the tubercles. Usually no more than two or three organisms were found within the outlines of a cell at this time.

The state of the secondary tubercles in the thoracic lymph nodes corresponded closely to that of the initial lesions. The lungs, spleens, and livers of all animals also contained scattered, small, secondary tubercles. Usually these were circumscribed masses of monocytes.

About 75 days after infection differences in the histologic pattern of tuberculosis, that could be related to the diets, became evident. These differences increased with time and were developed most strikingly in the initial tubercles and their associated lesions in the lungs (Figs. 8, 10, 11, and 12).

One hundred days after infection the initial tubercles of guinea pigs on diet C were, to a large degree, merely expansions of those seen at 50 days. At the same time their perivascular and peribronchial extensions were increased in size, expanding into the surrounding alveolar tissues and through the walls and mucosa of bronchi and bronchioles (Figs. 8 and 12). Endovascular reactions were not conspicuous at this time.

Bacilli could be demonstrated throughout the necrotic portions of these tubercles at 100 days, but were most numerous among the fragmented leukocytes. In these sites the microorganisms, present in small, dense clumps, showed a distinct increase over the numbers that had

been found at 50 or 75 days. This evidence of unchecked expansion of lesions in the lungs and increased numbers of bacilli were not found at 75 days in animals on diet A, nor at 100 days in animals on either diet A or B.

At this time, the initial tubercles of all animals on diet A and of a majority on diet B were enclosed by fibrous tissue in which there were calcium deposits. Now, too, the peripheral extensions of these lesions about the blood vessels and bronchi were reduced to inconspicuous fibrous scars (Figs. 10 and 11). As a rule, the miliary tubercles seen at 50 days had disappeared from the lungs of these animals by 100 days, or were represented by scattered, small, dense clusters of monocytes and lymphocytes.

The necrotic centers of the initial tubercles of animals on diets A and B were occupied more or less completely by polymorphonuclear leukocytes, which seemed to be suspended in a faintly eosinophilic matrix. Apparently the tubercles in these animals had liquefied without bronchial spread of the infection. Bacilli still could be demonstrated among the leukocytes in the necrotic centers, but only in small numbers, and not in all lesions.

At 100 days, tubercles of thoracic lymph nodes of animals on diets A and B contained relatively small liquid centers, enclosed in loosely arranged granulation tissue, in which lymphocytes now formed conspicuous groups. Calcium deposits had formed in the granulation tissue adjacent to the necrotic centers. Small cellular tubercles were found in occasional spleens and livers of these animals.

In the guinea pigs on diet C at 100 days, tubercles of the thoracic lymph nodes contained relatively larger centers of liquefaction enclosed in densely cellular granulation tissue. Calcium deposits were not seen in these lesions.

Tubercles, usually appearing as solid cellular masses, were relatively numerous in the spleens and livers of animals on diet C at 100 days. In the liver the tubercles sometimes were associated with small infarcts. These apparently developed when the tubercles, frequently located in the portal regions of the lobules, encroached upon afferent blood vessels to cause angiitis.

One hundred and fifty days after infection the initial tubercles of animals on diets A and B ranged from solid fibrous masses that contained few inflammatory cells and scattered calcium deposits to sharply circumscribed, thick-walled spaces, the small, apparently liquid centers of which were occupied largely by polymorphonuclear leukocytes (Figs. 10 and 11). These centers were surrounded by zones of proliferating granulation tissue that contained calcium deposits. Exudate

from the liquefying centers was not found in adjacent bronchioles, nor were bacilli demonstrated in sections of these tubercles.

The structure of the more active tubercles of animals on diet A was reproduced fairly well in the corresponding lesions of two guinea pigs on diet C. However, in these instances, bacilli were relatively abundant among the cells of liquefying centers and among the dense masses of inflammatory cells, largely polymorphonuclear leukocytes, found within bronchioles near the tubercles. The only source of this exudate seemed to have been the centers of the tubercles, the walls of which were continuous with small but apparently expanding perivascular and peribronchial lesions.

The changes in the lungs of other animals on diet C were confused at this time. Cavities were continuous with relatively large segments of consolidated parenchyma that seemed to be direct extensions of the perivascular and peribronchial foci of infection. Evidence of endobronchial spread of the infection was uncertain, even in the presence of the obviously active cavities. The cavities and adjacent consolidated foci in three animals were continuous with partially calcified, fibrotic initial tubercles. These older lesions still contained active foci of infection and many bacilli, but had not liquefied. Evidently, cavity formation in these animals did not depend primarily upon liquefaction of the initial tubercles. The contribution of the tubercles to a cavity was not so great as that of the secondary lesions about them.

Large numbers of bacilli were found in all active foci of infection in the lungs of animals on diet C at 150 days, especially in foci of liquefaction.

The state of the secondary tubercles of the thoracic lymph nodes could not be related to diet at this time. Centers of liquefaction apparently had developed in all of them, but either they were undergoing fibrosis or had been replaced by fibrous tissue in which there were deposits of calcium. The spleens of only two animals on diet C contained small cellular tubercles. However, such lesions were present in the livers of all animals on diets B and C, and of one on diet A. Bacilli were demonstrated with difficulty in sections of the secondary tubercles in the livers and spleens of animals on diet C, but were not found in these lesions of animals on other diets.

Summary

The development pattern of tuberculosis, induced in guinea pigs by inhalation of virulent bacilli of human origin, was not influenced by levels of protein in diets containing 30, 17, and 6 per cent, respectively,

for about 75 days. Beyond this time the effects of dietary protein were evident chiefly in the lesions of the lungs, which continued to progress only in the animals receiving 6 per cent protein. Development and regression of secondary lesions were not so clearly related to the diets.

Tuberculosis of Guinea Pigs Induced by Bacilli of Lower Virulence

Three diet-groups of 25 to 31 guinea pigs were infected, and several from each group were killed 75, 125, and 180 days later. Counts of initial tubercles at 75 and 125 days indicated that guinea pigs were quite as susceptible to infection by these organisms as to the more virulent bacilli. However, the size of initial tubercles and the state of the infection at 125 days corresponded approximately to those found 50 days after infection by the more highly virulent bacilli.

Figures 5 and 6 illustrate the stages of lesions found in animals on diets A and C 180 days after infection. At this time the infection seemed to be about equally advanced in the lungs of all animals, irrespective of the diet. Outlines of initial tubercles were ill defined and continuous with irregular peribronchial and perivascular lesions. In addition, the lungs of many animals contained large numbers of apparently isolated, secondary tubercles.

Secondary tubercles of the thoracic lymph nodes were advanced about equally in all diet-groups at 180 days, and in approximately the state found at 100 days after infection by the more virulent bacilli. The livers of all animals were free of lesions, as were the spleens of all animals on diets A and B. In five of the ten animals on diet C, the spleens were enlarged, although macroscopic tubercles were not recognized.

Microscopic examination disclosed that the state of these initial tubercles 75 days after infection was closely similar to the stages found at 50 days in guinea pigs that had inhaled the more virulent bacilli. Between 75 and 125 days the necrotic centers of a majority of the tubercles became calcified and surrounded by fibrous tissue. At the same time the outer borders of the tubercles apparently continued to expand irregularly (Fig. 9) and 180 days after infection the appearances of the initial tubercles suggested that the irregular peripheral expansion had continued much the same in each of the diet-groups. However, ulcers in the bronchi were always small, and seemed to be relatively inactive.

Tubercles in the thoracic lymph nodes of animals on diets A and B had progressed to partial liquefaction and focal calcification within

125 days, and by 180 days had been replaced by dense fibrous tissue. Liquefaction seemed to have occurred later and to a greater extent in the thoracic lymph nodes of animals on diet C which, at 180 days, contained relatively larger centers of liquefaction.

In animals on diets A and B, tubercles in the spleen had been replaced by fibrous tissue within 125 days, and in one half of those on diet C this occurred within 180 days. In others of this group the spleen was found to have been irregularly expanded by rather large collections of epithelioid cells in which one or two bacilli per cell were sometimes demonstrated. In the other foci of infection, bacilli were difficult to demonstrate until 180 days when their numbers were about the same in animals of all diet-groups, and about equal to the numbers found at 50 days in the initial tubercles of guinea pigs that had inhaled the more virulent bacilli.

Summary

The developmental pattern of tuberculosis, induced in guinea pigs by the inhalation of attenuated bacilli of human origin, was not influenced by levels of dietary protein for about 100 days. Beyond this time the effects of dietary protein were evident chiefly in the secondary lesions of thoracic lymph nodes and spleen which, in animals on the higher levels of dietary protein (17 to 30 per cent) began regression within 125 days. Tuberculosis of the lungs continued slowly progressive, irrespective of the diets, throughout the 180 days of the experiment.

Tuberculosis of Rats

Levels of dietary protein neither influenced the susceptibility of rats to infection by bacilli of the Ravenel strain nor modified the progress of the initial tubercles of these animals within 260 days. Secondary tubercles did not develop to macroscopic size.

Microscopic examinations demonstrated that the initial tubercles of rats retained one form throughout the period of the study. This form was established within 5 weeks after infection and changed neither with time nor with the level of dietary protein.

These lesions were irregularly outlined foci in the lungs, in which the interstitial tissues were thickened by accumulations of lymphocytes and monocytes. Monocytes also accumulated in the alveolar walls and spaces within the foci, but the most conspicuous cells of the initial tubercles of rats were much larger and more lightly stained, and their cytoplasm was finely vacuolated. A majority of the cells were uninucleate but occasional ones contained two to four nuclei. Probably they

should be called monocytes too, but monocytes that had undergone a change after having ingested bacilli, for these were the only cells in which bacilli were found. However, the number of bacilli even at 260 days was never more than four to six per cell.

With the exception of a brief episode during the third week, the tubercles induced in rats by inhalation of droplet nuclei have not been found to undergo necrosis. Peripheries never were limited by fibrous tissue, and enlargement always seemed to have been across alveolar walls.

Secondary tubercles were found only in the thoracic lymph nodes and at no time were larger than a mass of about ten of the large vacuolated phagocytes, closely pressed together. Such lesions were present in small numbers in rats of all groups at 75 days, and continued so through 260 days.

Discussion

These experiments demonstrate that levels of dietary protein can be critical factors in the development of resistance to tuberculosis. This demonstration was possible only when infections were induced by bacilli that were moderately virulent for the animals. For example, bovine bacilli of the Ravenel strain, which are highly virulent for hamsters and guinea pigs, overwhelm the animals so rapidly that effective resistance cannot develop.[4] Conversely, when hamsters and guinea pigs were infected by relatively avirulent bacilli, or rats infected by bacilli of the Ravenel strain, levels of dietary protein had no demonstrable effects upon the pattern of tuberculosis (especially in the lungs) within the periods of the experiments. The evidence suggests that these organisms grew so slowly in the lesions that antigens were inadequate for the production of effective levels of antibodies.[10]

Failure to demonstrate an effect of protein and of other dietary factors upon the development of resistance to tuberculosis has been reported many times.[15] The present series of experiments suggest that these failures may reflect the choice of an unsuitable host-parasite combination, composition of the diets, or length of observation period.

For example, Metcoff and associates[15] compared the effects of protein at levels of 2, 8, and 18 per cent upon tuberculosis of rats during a period of 58 days following intravenous inoculation. The results were negative, as those of the present experiments on guinea pigs would have been, had observations ended within 58 days. On the other hand, Koerner, Getz, and Long[16] found distinctive differences in the patterns of tuberculosis in rats when protein constituted 15 to

40 per cent of the diet. Here, too, infections were induced by intravenous injection of relatively large numbers of bacilli. Differences, however, did not become evident until the disease had developed for about 150 days. This suggests that if the rats in the present experiments had inhaled much larger numbers of bacilli, the disease might have been altered by the diets. However, inhalation of only a small fraction of the number of bacilli estimated to have been injected into these rats probably would have led to death within 4 to 5 weeks, as the developing tubercles expanded to fill the lungs.[1,2] Thus the rat is not a suitable host for studies of the relation of nutrition to tuberculosis induced by inhalation. The mouse probably is little better.[4] The results of these studies on rats, however, support the opinion that in tuberculosis, resistance develops as a function of time and the growth rate of the bacilli, i.e., the rate at which antigen becomes available.[10] The development of resistance also must depend upon adequate levels of dietary protein,[17] which may differ with the species of animal. In this connection it should be noted that on 17 per cent protein hamsters usually failed to develop evidence of resistance, while guinea pigs on this level developed resistance almost as rapidly and effectively as on 30 per cent protein.

Summary and Conclusions

Six or more weeks before infection, groups of young adult guinea pigs and rats were started on isocaloric diets containing approximately 30, 17, and 6 per cent protein, and were continued on these diets until they were sacrificed for study at stated intervals from 50 to 260 days after infection. Tuberculosis was induced by inhalation at approximately minimal levels. Guinea pigs were infected by bacilli of human origin, and two strains with different levels of virulence were used. Rats were infected by bovine bacilli of the Ravenel strain.

Levels of dietary protein did not influence susceptibility of either rats or guinea pigs to infection by inhaled bacilli nor modify the progress of tuberculosis of rats within 260 days. In guinea pigs which inhaled the less virulent bacilli the developmental pattern of pulmonary lesions did not change within 180 days. However, secondary lesions at sites beyond the lungs healed more rapidly in animals on the higher levels of dietary protein.

When guinea pigs inhaled the more highly virulent bacilli the progress of the disease was not influenced by the diets within 50 days. At 75 days and beyond, all lesions in guinea pigs on the higher levels of die-

tary protein seemed to be healing, and even in animals on 6 per cent protein only the lesions in the lungs continued to be active.

It is concluded that the level of dietary protein can be a critical factor in the development by guinea pigs of effective resistance to tuberculosis. This requires relatively prolonged interaction of host and virulent parasite, and apparently reflects the combined actions of monocytes and other factors which, presumably, are antibodies.

It is concluded also that the highly uniform response of guinea pigs for upward of 50 days after infection is further evidence against the concept of native or hereditary resistance.

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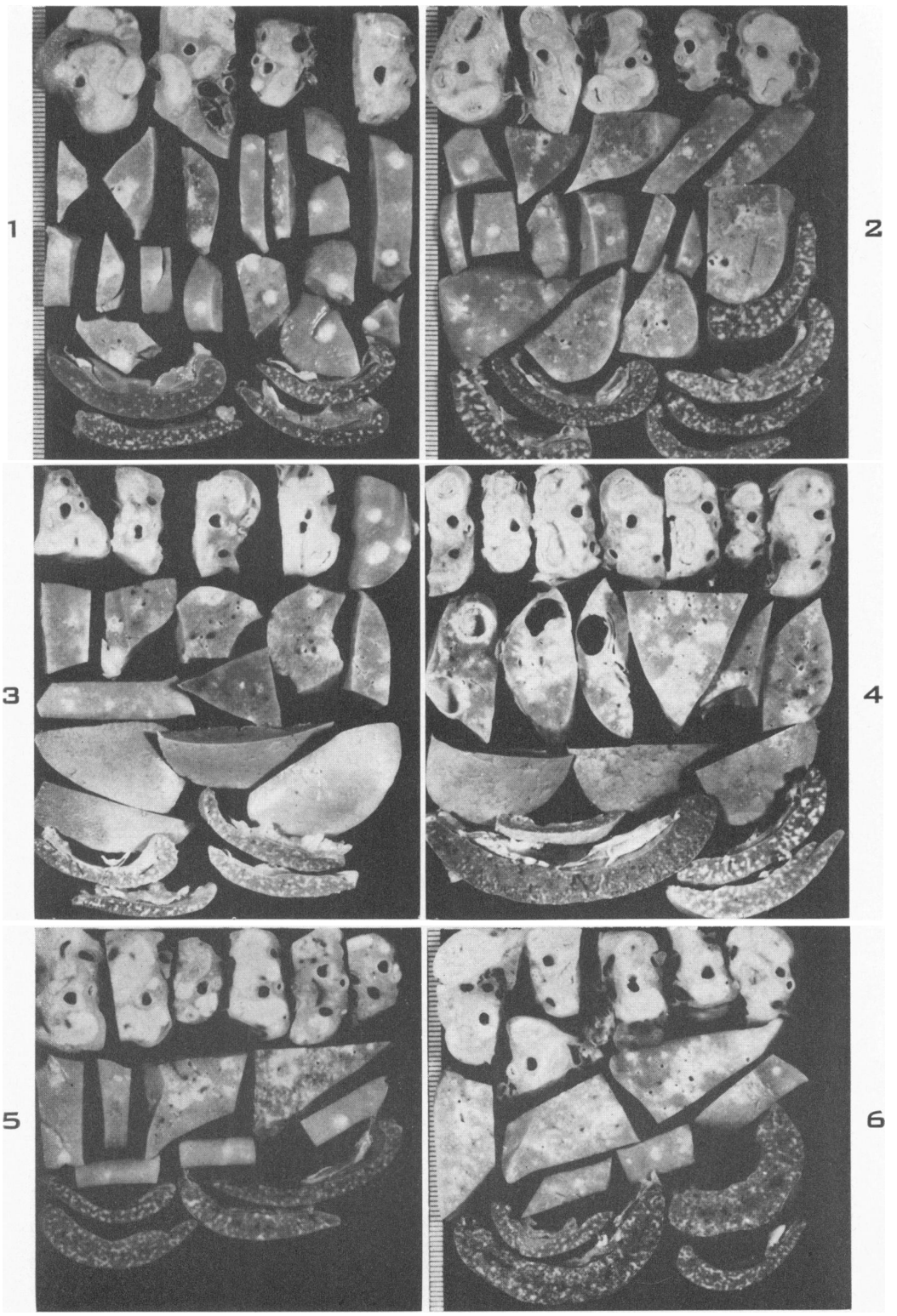
LEGENDS FOR FIGURES

FIGS. 1 (diet A) and 2 (diet C) from guinea pigs 100 days after infection with bacilli of high virulence. Specimens in photographs are arranged in the following order: *upper*—thoracic lymph nodes cut vertically to long axis of body and including sections of trachea, esophagus, and aorta with the nodes; *center*—segments of the lungs of each animal; *lower*—longitudinal sections through spleens. In animals on diet A, tubercles in thoracic lymph nodes and lungs were undergoing fibrosis and tubercles in the spleens were completely fibrotic at this time. In animals on diet C, the thoracic lymph nodes contained large semiliquid foci. Tubercles in the lungs were poorly circumscribed, and secondary tubercles were abundant in the spleens.

FIGS. 3 (diet A) and 4 (diet C) from guinea pigs 150 days after infection with bacilli of high virulence. Specimens arranged as in Figures 1 and 2, but with bits of the free edge of the liver between lungs and spleens. Differences that may be attributed to the development of resistance on diet A and failing resistance on diet C are clearly evident in the lungs and lymph nodes. The enlarged spleen in Figure 4 was associated with extensive scarring in the liver; it was not tuberculous.

FIGS. 5 (diet A) and 6 (diet C) from guinea pigs 180 days after infection with bacilli of low virulence; for comparison with Figures 3 and 4. Lesions of the lymph nodes and spleens of animals on diet A seemed to be healing at this time, but apparently were progressive in the lungs of all animals, and in the spleens of about half on diet C.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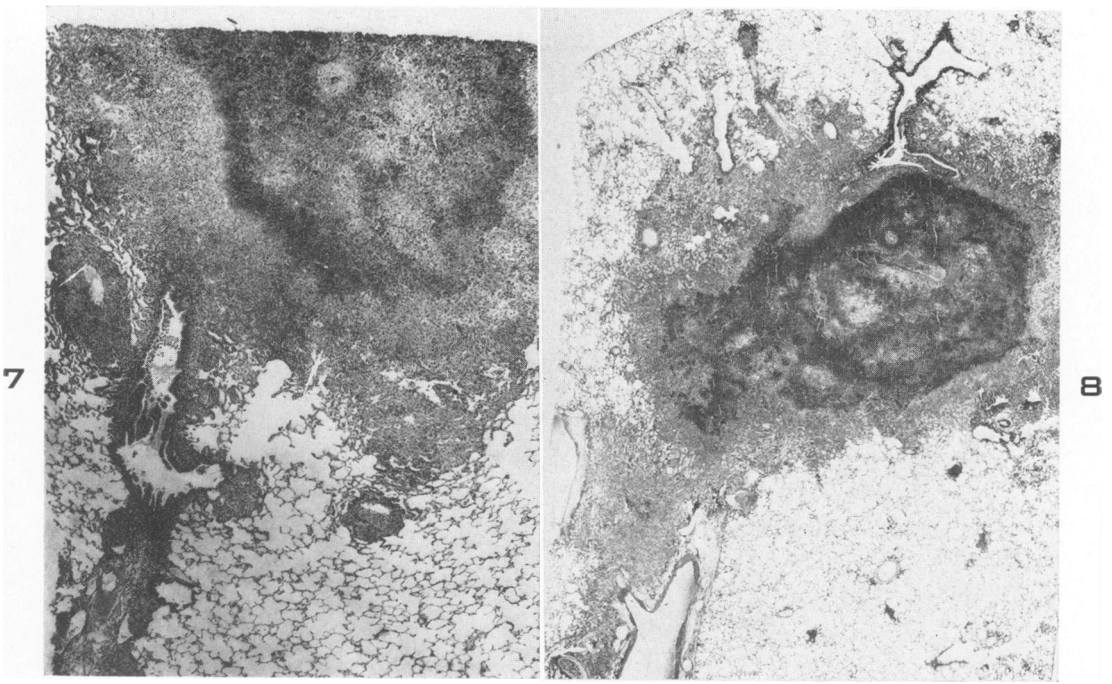

FIG. 7. Initial tubercle of guinea pig 50 days after infection by the more virulent bacilli. The upper half of the lesion was trimmed away, then sectioned at right angles to this cut. The extent of the necrotic center of the tubercle, the irregular periphery, and the peribronchial and perivascular extensions may be noted. Hematoxylin and eosin stain. × 30.

FIG. 8. Initial tubercle of guinea pig on diet C, 75 days after infection by the more virulent bacilli. Of note are the irregular peripheral extension, and exudate in a bronchus adjacent to the necrotic center, and irregular expansion of the necrotic center. Hematoxylin and eosin stain. × 10.

FIG. 9. Initial tubercle of a guinea pig on diet A, 125 days after infection by bacilli of low virulence. A sharply defined necrotic center with dark (calcified) masses is surrounded by a zone of granulation tissue. The periphery is irregular and, in the upper left, has ulcerated into a bronchus. Hematoxylin and eosin stain. × 12.

FIG. 10. Initial tubercle of a guinea pig on diet A, 150 days after infection by bacilli of high virulence. Of note are the remnants of liquefied material, upper left, the well developed, partially calcified fibrous tissue of the wall, and absence of disease in the wall of the adjacent bronchus. Hematoxylin and eosin stain. × 30.

FIG. 11. A segment of the wall of an initial tubercle of a guinea pig on diet A, 100 days after infection by bacilli of high virulence. A thin wall of fibrous tissue outlines this lesion. There is a zone of calcium, with inflammatory cells within this zone. Hematoxylin and eosin stain. × 120.

FIG. 12. A part of a peribronchial extension of an initial tubercle of a guinea pig on diet C, 100 days after infection by bacilli of high virulence. An eccentric mass of tuberculous granulation tissue occupies the wall and replaces the mucosa. Hematoxylin and eosin stain. × 120.

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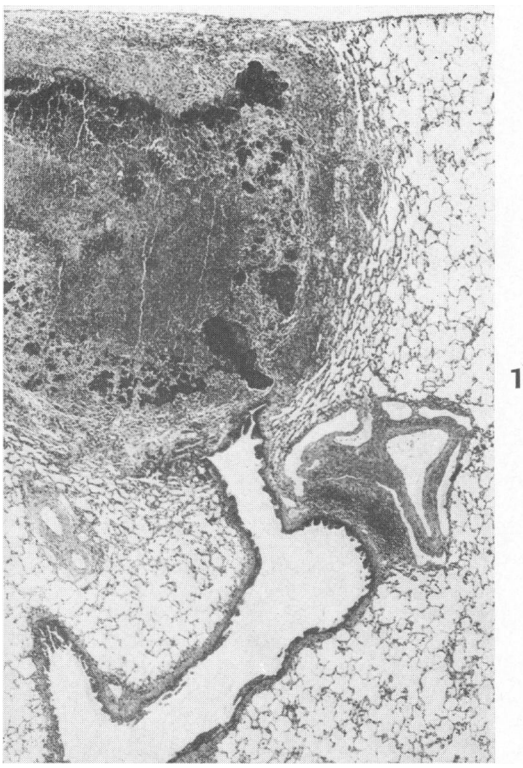

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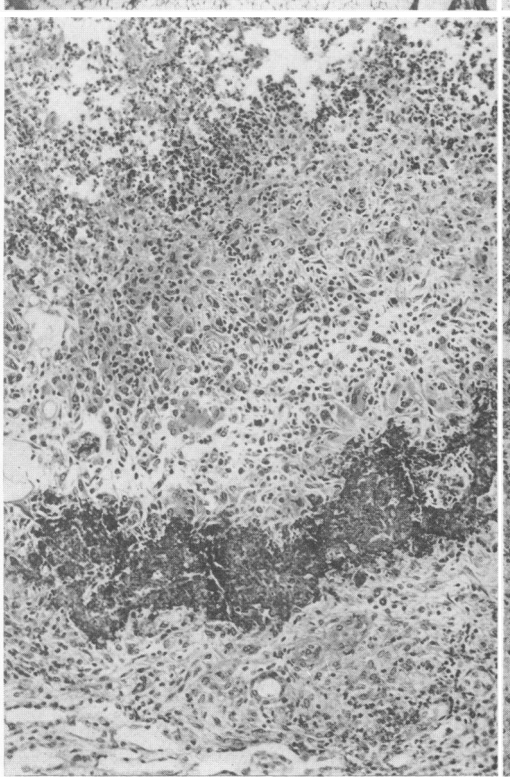

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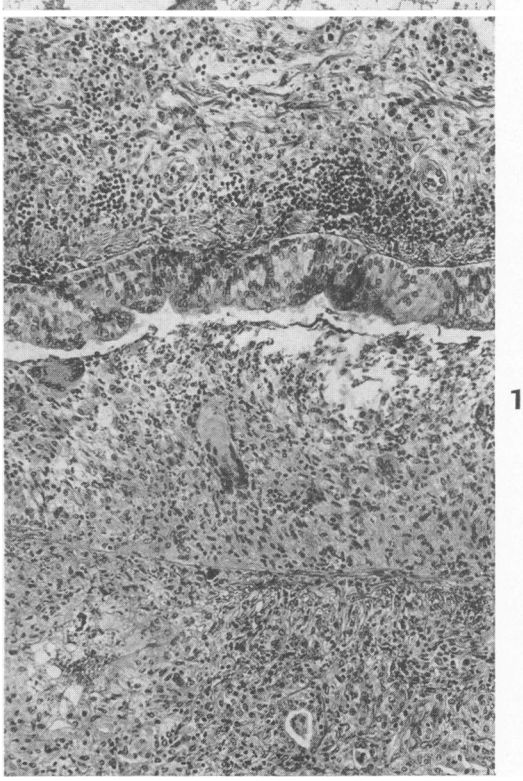

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