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Cavitary Tuberculosis Produced in Rabbits by Aerosolized Virulent Tubercle Bacilli

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Liquefaction of solid caseous tuberculous lesions and the subsequent cavity formation are probably the most dangerous processes in the pathogenesis of human pulmonary tuberculosis. In liquefied caseum, the tubercle bacilli grow extracellularly for the first time since the onset of the disease and can reach such large numbers that mutants with antimicrobial resistance may develop. From a cavity, the bacilli enter the bronchial tree and spread to other parts of the lung and also to other people. Of the commonly used laboratory animals, the rabbit is the only one in which cavitary tuberculosis can be readily produced. This report is the first to describe and analyze the complete course of cavitary tuberculosis, produced by aerosolized virulent bovine-type tubercle bacilli in commercially available New Zealand white rabbits. After the inhalation of 220 to 880 bacillary units, all of the rabbits were overtly well until they were sacrificed at 33 weeks. After the inhalation of 3,900 to 5,800 bacillary units, half of the rabbits died of progressive tuberculosis between 5 and 9 weeks and the other half lived until they were sacrificed at 18 weeks. Pulmonary cavities developed in both low- and high-dose groups, some beginning as early as 6 weeks. Bacilli from primary cavities sometimes caused nearby secondary cavities, but more frequently, they ascended the bronchial escalator, were swallowed, and caused secondary tubercles in the lymphoid tissue of the appendix and ileocecal junction. Histologically, and by culture, the number of bacilli found in the liquefied caseum varied from many to comparatively few. Strong tuberculin reactions at 4 weeks after infection were associated with fewer primary lesions, while strong tuberculin reactions at 33 weeks were associated with more cavitary lesions. In the tuberculous granulation tissue surrounding caseous and liquefied pulmonary foci and cavities, we found many mature epithelioid macrophages that contained high levels of the proteinase cathepsin D. Therefore, cathepsin D probably plays a major role in the liquefaction of solid caseous material and in the subsequent cavity formation.

Liquefaction followed by cavity formation perpetuates tuberculosis in humans (3, 14, 18, 21, 30) because coughing aerosolizes tubercle bacilli growing on a cavity wall, thereby enabling them to infect other people. The extracellular multiplication of the bacilli in liquefied and cavitary lesions may be tremendous. Such multiplication increases the likelihood that mutant bacilli possessing antimicrobial resistance will develop. Multidrug-resistant tubercle bacilli are becoming a major public health problem in the world today. This extracellular growth of tubercle bacilli in liquefied and cavitary lesions occurs irrespective of the immune status of the host. In fact, hosts with cavitary tuberculosis usually show a highly effective immune response against progressive exogenous and endogenous infection with low numbers of bacilli (21). In other words, the extracellular multiplication of tubercle bacilli in the liquefied caseum of tuberculous lesions (including cavities) is not affected by the immune status of the host: The host's defense cells cannot survive in such sites.

Tubercle bacilli do not multiply well in the cytoplasm of macrophages that are activated by the immune response (1), and they do not seem to multiply at all in the solid caseous centers of tuberculous lesions (3, 21). However, in liquefied caseum, the bacilli, unreachable by the forces of formerly effective acquired resistance of the host, often multiply profusely (3, 7–9, 12, 14, 18, 21).

There are only a few studies on the causes of liquefaction (reviewed in reference 13). In brief, Yamamura et al. (37, 38) found that liquefaction and cavity formation in rabbits could be inhibited by various immunosuppressive drugs and by desensitizing these animals to tuberculoprotein. Our laboratory showed that nucleases (11, 13) and proteinases (13, 31, 34) (derived from macrophages) were involved in the liquefaction process of dermal tuberculous lesions produced by *Mycobacterium bovis* BCG. The present communication shows that the proteinase cathepsin D is involved in the liquefaction of pulmonary caseous foci.

The present report is the first to (i) make a thorough study of pulmonary tuberculosis produced by aerosolized bovine-type tubercle bacilli over long periods of time, (ii) present details on fibrocaseous tuberculosis (which is so common in the chronic forms of the human disease), and (iii) show the effects of high and low doses of inhaled virulent bacilli on the course of the disease. Enzyme- and immunohistochemical techniques, as well as thin glycol methacrylate-embedding techniques, were used to study the chronic cavitary tuberculosis that we produced.

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MATERIALS AND METHODS

Rabbits and the number of bacillary units they inhaled. We exposed *Pasteurella*-free New Zealand White rabbits to high and low doses of *M. bovis* (Ravenel

TABLE 1. Summary of necropsy data

Expt and rabbit no.	No. of wk after infection (no. of inhaled bacilli)	No. of primary caseous or liquefied lesions in entire lung	No. of cavities in entire lung	No. of TB[a] lesions in appendix/no. in ileocecal junction	Comments	No. of viable bacilli in ~10 μl of liquefied caseum[b]
Low dose						
Expt I						
1	22 (240)	5	ND[c]	0/0	Numerous 2° lesions[a] in both lungs, several of which were liquefied	
3	22 (220)	2	ND	Many/many	A few 2° lesions in lungs	
10	19 (240)	1	0	0/0	Primary lesion (3 mm); scattered 2° lesions	
2	22 (940)	11	ND	150/100		
4	22 (880)	16	ND	3/1	Occasional 2° lesions in lungs	
8	19 (620)	5	1	Many/many	A cavity (14 mm) contained 4 or 5 smaller compartments (Fig. 1A); scattered 2° lesions; one kidney had two 3- to 4-mm TB lesions	
Expt III						
1	33 (490)	12	3	400/100		85,000
2	33 (560)	8	17	0	Several cavities had multiple locules	A:450 B:300
3	33 (540)	4	0	0	Almost healed lesions	<10
4	33 (300)	10	4	80/40	TB laryngitis (Fig. 2A) originating from an 8-by-6-mm cavitary lesion; shortness of breath	A:160 B:600,000
5	33 (340)	ND	Many	150/100	Honeycombed cavities in right upper and right lower lung (Fig. 1B); about 55 well-developed lesions were present in the right lung; most were probably secondary lesions from the bronchial spread of bacilli from the local cavity(ies); much normal tissue present	
6	33 (340)	2 (healed)	ND	0/0	No active TB present but OT[a] was definitely positive	
7	33 (330)	6 (small)	1	0/12	The 6 small lesions seemed nonprogressive; whole left nostril full of pus destroying turbinates on that side (probably *Pasteurella* infection); spleen very large	
8	33 (440)	8	1	0	Well-controlled disease	550
9	33 (420)	1	5	100/10	Two large lesions (20 to 30 mm); secondary lesions near left lung cavity; multilocular pus pockets	
10	33 (420)	10	0	60/80	Several liquefied lesions but no cavities	<1
11	33 (410)	9	2	0/2	Very little TB	300
12	30 (420)	3	~3	~100/~200	Labored breathing; tracheal and esophageal TB with partial obstructions of each (Fig. 2B); 450 secondary pulmonary lesions, not liquefied; 40 (probably tuberculous) lesions in kidneys	
High dose (expt II)						
1	18 (4,400)	192	18	±110/±80		
3	18 (5,300)	218	33	800/400	Emaciated	
5	18 (5,800)	228	7	55/55		
6	18 (4,900)	217	5	150/120	Turned blue when held	
7	18 (5,700)	313	39	90/120		
8	18 (5,500)	335	35	220/150	One tubercle in left kidney and one in left testicle, both ~2 mm	

[a] TB, tuberculous or tuberculosis. OT, skin test with Old Tuberculin; 2°, secondary lesions.

[b] In experiment III (low-dose infection), nine liquefied (but not cavitary) lesions were cultured on L-J medium (see last column). Note the extreme variability in the number of viable bacilli present in the liquefied caseous centers of noncavitary tuberculous lesions. In experiment II (high-dose infection), one to two small abortive tubercles (0.5 to 1.0 mm in diameter) were seen in the tracheobronchial lymph nodes of several rabbits. Secondary pulmonary lesions were common, but the numerous primary lesions caused most of the pulmonary pathology.

[c] ND, not determined.

S) in the excellent aerosol facilities at the United States Army Medical Research Institute for Infectious Diseases at Fort Detrick, Frederick, Md. The rabbits were cared for in adherence to the *Guide for the Care and Use of Laboratory Animals*, prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (4a).

In the two low-dose experiments (I and III), *Pasteurella*-free New Zealand White female rabbits from Hazelton Laboratories, Vienna, Va., were used. In experiment I, six rabbits each inhaled 220 to 280 viable bacillary units, each containing 1 to 3 bacilli, and another six female rabbits each inhaled 620 to 880 bacillary units (Table 1). In experiment III, 12 rabbits inhaled 300 to 560 bacillary units (Table 1).

In experiment II (the high-dose experiment), 11 male inbred New Zealand White rabbits, race IIIVO/JU, each inhaled about 3,910 to 5,800 bacillary units (Table 1). The IIIVO strain was developed by Paul B. Sawin and Richard R. Fox at the Jackson Laboratory in Bar Harbor, Maine (15). It was further inbred by L. F. M. van Zutphen (Faculty of Veterinary Medicine, University of Utrecht, The Netherlands) because of its resistance to dietary-induced hypercholesterolemia (2, 25, 26). Max B. Lurie's tuberculosis-resistant race III rabbits originated from the same Jackson Laboratory lineage (21, 22).

Preparation of bacillary suspensions. Virulent bovine-type tubercle bacilli, strain Ravenel S, obtained lyophilized from the American Type Culture Collection (catalog no. 35720) were subcultured on the surface of solid BBL Lowenstein-Jensen (L-J) medium slants (Becton-Dickinson Co., Cockeysville, Md.). Judging from the number of primary tubercles produced in rabbits from an inhaled human dose of bacilli, we concluded that the virulence of this strain of Ravenel S is about one-third that of a fully virulent strain. With fully virulent strains, 3 bacillary units must be inhaled to produce one primary pulmonary tubercle (21). With this Ravenel strain, about 10 such units were required.

Several platinum loopfuls of these bacilli (subcultured for 10 days) were ground (in a laminar flow hood) for 10 min with an agate mortar and pestle with about 40 μl of Tween 80. (HEPA-filtered half-face masks were worn.) The bacilli were then suspended in 4 ml of a 1:10 dilution (in 0.9% NaCl) of the BBL oleic albumin complex (Dubos) (Becton-Dickinson Co.). After the few bacillary clumps settled out for 10 min in a test tube (1.0-cm diameter), the optical density of the bacillary suspension was read at 540 nm in a Bausch and Lomb model Spectronic 20 spectrophotometer. A 0.100 optical density reading corresponded to about 10^7 viable bacillary units when 10-fold serial dilutions were cultured on L-J slants.

Head-only bioaerosol exposure system. Aerosolization of the bacillary suspension was performed in a class III stainless steel biological safety cabinet under negative pressure. The exposure chamber (36) was a rectangular Plexiglas 16-liter box. One side of the box had a circular opening closed by a latex dam with a porthole cut in its center. The muzzle (i.e., nose and mouth) of the rabbit to be exposed was inserted through the porthole. During the 10-min exposure, each rabbit was gently hand-held after being placed in a loose-fitting canvas restraining bag (Cat Sack; Four Flags over Aspen, Janesville, Minn.).

The aerosols were generated with a Collison nebulizer (23), containing 8 ml of bacilli suspended in the diluted oleic albumin complex. The small aperture of the nebulizer dispersed clumps of bacilli into units of 1 to 3 bacilli. Air samples were taken through a 1/4-in. (1 in. = 2.5 cm) stainless steel sampling port situated in the center of the side of the exposure chamber at the level of the animal's nose. The samples were taken with an all-glass impinger (AGI-30; Ace Glass, Inc., Vineland, N.J. [5]) during the entire 10 min in which each rabbit was exposed. The impinger contents were cultured on L-J slants, so that the concentration of tubercle bacilli in the air inhaled by each rabbit could be calculated. Prior to aerosol challenge, the volume of air that each rabbit breathed per minute was calculated from its per-minute ventilation, respiratory rate, and tidal volume (determined by plethysmography), according to the principles outlined by Guyton (16), with the Pulmonary Mechanics Computer model no. 6 software from Buxco Electronics, Sharon, Conn.

Tuberculin reactions. Undiluted veterinary tuberculin (Intradermic Tuberculin [mammalian] from human isolates; Coopers Animal Health, Inc., Kansas City, Kans.) was injected intradermally, and 2 days later the resulting skin reactions were measured with calipers (10). The tuberculin reactions in the rabbits of these experiments ranged from 160 to 4,700 mm^3. The mean reaction size and its standard error was 1,660 ± 200 mm^3.

Lesion counts and gross evaluation. After the rabbits were sacrificed by intravenous injection of 2.5 to 3.0 ml of sodium pentobarbital (65 mg/ml), one lung was inflated with 10% formalin (4% HCHO). After several days of fixation, the lesions were counted and representative lesions were prepared for histological studies. The other lung was dissected at necropsy, so that solid and liquefied caseous material could be precisely identified. (Formalin tends to solidify liquefied caseous material.) Primary lesions can frequently be distinguished from secondary lesions by their size.

Histochemical stains for macrophage activation. Our standard procedure for demonstrating β-galactosidase and acid phosphatase activities in glycol methacrylate-embedded tissue sections was employed (27). The tissue sections were incubated at 37°C in either the indolyl substrate for 27 h or the naphthyl substrate for 18 h, respectively. The slides were counterstained with Giemsa (35).

Cathepsin D, the main proteolytic enzyme of rabbit macrophages, was immunostained by a specific antibody (produced in goats) to the purified cathepsin D (31, 33). Rabbit biotinylated anti-goat immunoglobulin G and the avidin-biotin-peroxidase (complex) method were used (ABC kit from Vector Laboratories, Burlingame, Calif.).

TABLE 2. Tuberculin reactions of rabbits in Experiment III[a]

Rabbit no.	Size (mm^3) of tuberculin reaction at:	
	4 wk	33 wk
1	1,890	1,420
2	1,890	1,200
3	4,500	1,660
4	1,040	1,660
5	2,600	2,700
6	3,500	630
7	1,900	650
8	2,700	810
9	4,700	1,050
10	1,850	500
11	2,400	610
12	1,620	ND[b]
Mean ± SEM[c]	2,550 ± 330	1,170 ± 200

[a] All rabbits (except for no. 4 and no. 5 [see the text]) had significant reductions in the sizes of their reactions at 33 weeks ($P < 0.005$). The reduced tuberculin reactions were associated with greater control of the disease, including fibrotic encapsulation of the lesions.
[b] ND, not done.
[c] SEM, standard error of the mean.

Culture and fluorostain for tubercle bacilli. In experiment III, a 10-μl aliquot of the liquefied contents of each of nine pulmonary (noncavitary) lesions was removed. These samples were cultured on L-J slants in serial dilutions to determine the number of bacilli present.

In addition, glycol methacrylate-embedded tissue sections were stained for bacilli with the TB Fluorostain Kit, containing auramine O and rhodamine B, purchased from Polysciences, Inc. (Warrington, Pa.). The bacilli were visualized with a 40× objective of an epifluorescence microscope at wave lengths of 495 nm (excitation) and 535 nm (emission). Compared with the standard carbol-fuchsin method, the fluorescent method seems to be about three times more sensitive in identifying the bacilli.

Statistics. The sizes of the early and late tuberculin reactions (Table 2) were compared by Student's paired *t* test. Linear regression analysis was used for Fig. 8.

RESULTS

Low-dose experiments (experiments I and III). Two rabbits, each, were sacrificed at 5, 8, 10, and 13 weeks after infection. They had relatively few grossly visible or palpable primary pulmonary lesions (1 to 18 in number). Therefore, almost all rabbits remained in good health until the experiment was completed. At 5 weeks, the primary tubercles had solid caseous centers. At 8 weeks, liquefaction of a few of the tubercles had taken place, but with this low bacillary dose, no cavities had formed. However, at 10 and 13 weeks, small cavities were present. Subsequently, over half of the solid caseous foci liquefied, and many of the liquefied foci had formed cavities.

Most of the rabbits sacrificed at 19, 22, or 33 weeks seemed to have their disease under control (Table 1). Their pulmonary lesions had solid, semisolid, or liquefied caseous centers, often surrounded by considerable fibrosis. Cavities were common (Fig. 1). These lesions were slowly progressive or nonprogressive. One rabbit had only four 2- to 3-mm pulmonary lesions that were almost healed.

The tracheobronchial lymph nodes were slightly enlarged and occasionally contained tuberculous granulomas. Similar to immunocompetent adult human beings, rabbits with moderate-to-high native resistance to tuberculosis do not develop progressive lesions in their tracheobronchial nodes (21, 22). Much variation existed among the individual rabbits. Some had

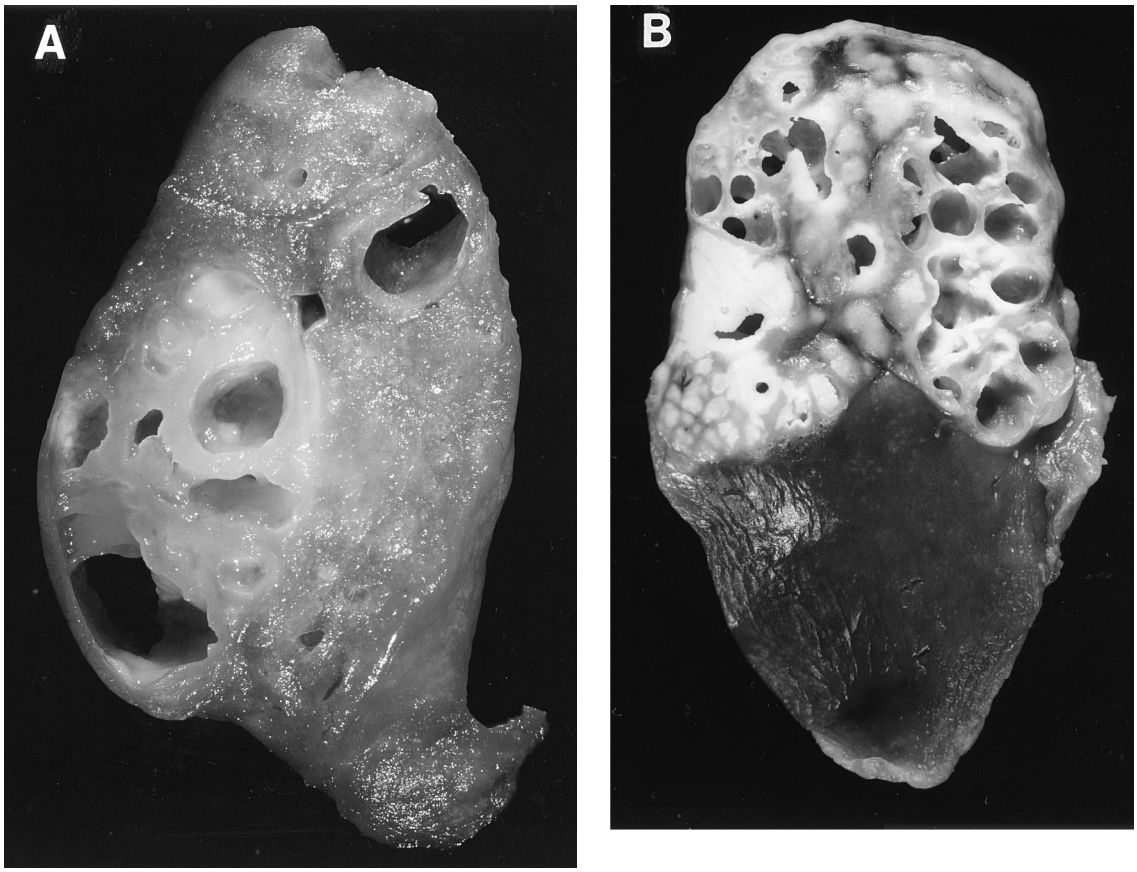


FIG. 1. (A) Multiloculated cavity in the lung of a rabbit (no. 8, experiment I) that had inhaled 620 virulent bovine type tubercle bacilli (Ravenel S) 19 weeks previously (see Table 1). The hole at the upper right side of the photograph is an intact bronchus. The hole at the lower left is part of the loculated cavity. The centers of each are black because the back of the specimen was sliced off to stabilize it for photography. Magnification, ×3.5. (B) Multiple cavities in the upper part of the right lower lobe of the lung of a rabbit (no. 5, experiment III) sacrificed 33 weeks after the inhalation of 340 virulent bovine-type tubercle bacilli. These are mostly secondary cavities that developed locally from bronchogenic spread of large numbers of bacilli from one or two original primary lesions. In spite of the extensive (locally spread) tuberculosis in the upper part of the lobe, no secondary lesions were grossly visible in the lower part of the lobe. In addition, the whole left lung of this rabbit was free of grossly visible tuberculous lesions. Magnification, ×0.9.

hardly any disease, whereas others had local destruction of sometimes half of a pulmonary lobe (see below).

Secondary pulmonary lesions were found in the lungs of several rabbits (Table 1). They probably arose by bronchial spread of the disease from one or more cavities. The secondary lung lesions were usually (but not always) much smaller than the primary lesions, so that each type could usually be identified. Secondary lesions (1 to 2 mm) were often found in the lymphoid tissue of the ileocecal junction and appendix (Table 1). The latter arose when numerous bacilli (growing in cavities) ascended the bronchial escalator and were swallowed. A few rabbits had tubercles (1 to 4 mm in diameter) in their kidneys (probably of hematogenous origin).

Unique events during late disease in low-dose experiments. At 33 weeks, one rabbit (experiment III, no. 5 [Table 1]) showed honeycomb formations, i.e., multiple coalescing cavities in both its right upper and right lower lung lobes (Fig. 1B). Evidently, one or two cavitary lesions had discharged many bacilli locally. These bacilli created new lesions nearby, which in turn cavitated. However, in spite of this extensive destruction of part of these two lung lobes, the rabbit remained in good health, and the remainder of its lungs showed no grossly visible evidence of tuberculosis.

At 33 and 30 weeks, respectively, two rabbits (experiment III, no. 4 and no. 12 [Table 1]) had labored breathing and stopped eating, so they were sacrificed. They would probably have died of anoxia in 1 or 2 days. At necropsy, both rabbits showed airway obstruction from tuberculous lesions. The first rabbit had extensive tuberculous laryngitis with airway obstruction (Fig. 2A). The second had several lesions (5 to 8 mm) in the bronchial tree. The more distal lesion seemed to have started in the hilus of the left upper lobe of the lung and to have formed a cavity that discharged into the left main bronchus. Numerous tubercle bacilli from this cavity apparently seeded the large bronchi and trachea, so that two obstructive caseous lesions developed. The more distal obstructive lesion (Fig. 2B) narrowed the airway near the hilus; the more proximal obstructive lesion (data not shown) narrowed the trachea and esophagus, which accounted for the labored breathing and anorexia. The process must have been ongoing for several weeks because all lobes of both lungs were peppered with numerous caseous secondary lesions (0.5 to 2.0 mm). These secondary lesions probably arose from the large numbers of bacilli present in the airways, but a hematogenous origin cannot be ruled out. This was the only rabbit in the three experiments with such extensive secondary pulmonary lesions, possibly a result of the malnutrition (24) associated with partial obstruction of the esophagus.

High-dose experiment (experiment II). In this experiment with inbred rabbits, the number of primary lesions in the lungs

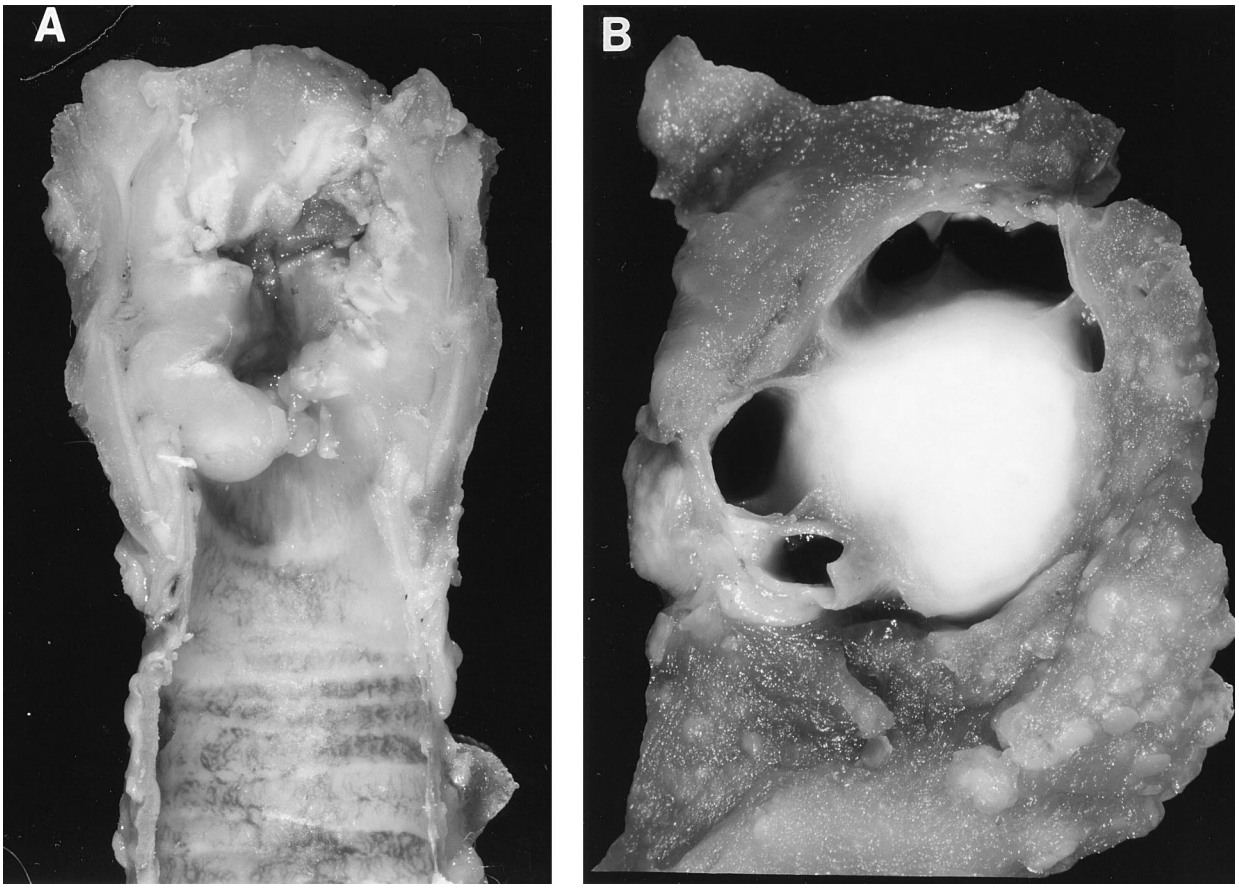


FIG. 2. (A) Tuberculous laryngitis in a rabbit (no. 4, low-dose experiment III) that had inhaled 300 virulent bovine-type tubercle bacilli 33 weeks previously. The lung of this rabbit had 14 lesions, 4 of which had formed cavities. The cavity in the right lower lung apparently discharged so many bacilli into the bronchial tree that the larynx became infected. Eventually, the airway was partly obstructed, so that the animal was breathing with difficulty at the time of sacrifice. Magnification, ×6.3. (B) A caseous tubercle at the bifurcation of a bronchus near the hilus in a rabbit (no. 12, low-dose experiment III) that had such labored breathing that it had to be sacrificed at 30 weeks after the inhalation of 420 virulent bovine-type tubercle bacilli (see text). Magnification, ×6.3.

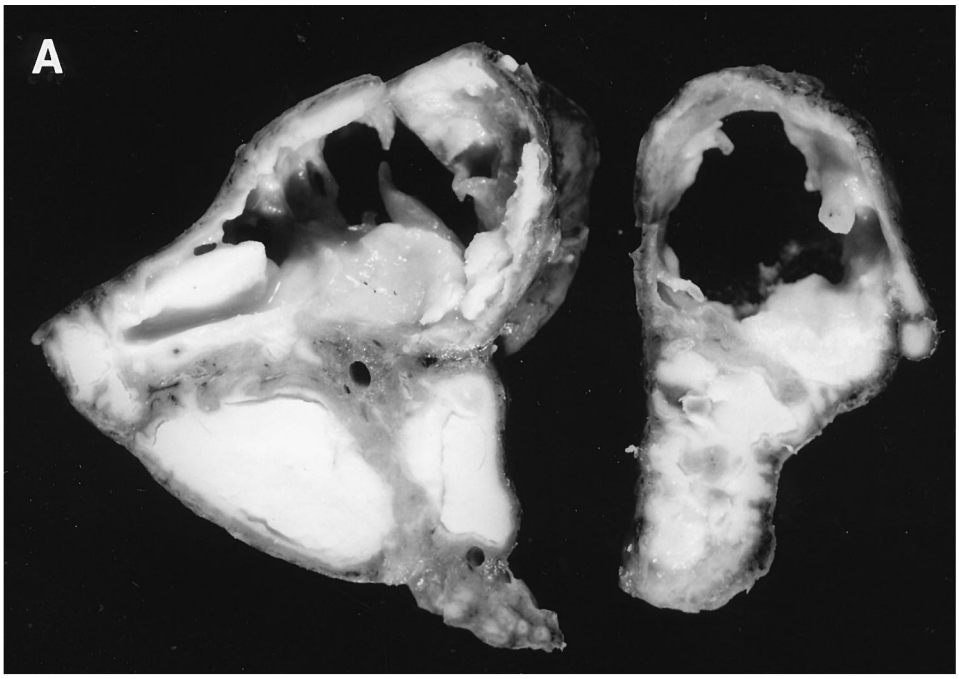
was high: 184 to 550. Therefore, about half of the rabbits had to be sacrificed for impaired breathing before the experiment was terminated at 18 weeks. The first rabbit was sacrificed at 5 weeks. Although it was still eating, it became cyanotic with labored breathing upon exertion. Its lungs contained about 550 primary (3- to 4-mm) tubercles (with small caseous centers), which occupied about two-thirds of the inflated lung. Four other dyspneic rabbits were sacrificed at 6, 7, 8.5, and 9 weeks after infection. Their lungs contained 210, 260, 250, and 500 primary lesions, respectively. Most of these pulmonary lesions had liquefied centers, and variable numbers of cavities were always present.

The other half of this group of rabbits was sacrificed at 18 weeks (Table 1). The various types of lesions found among these rabbits are illustrated in Fig. 3A. Because they survived for 4 months, their pulmonary lesions resembled those of the surviving rabbits in the low-dose experiments, i.e., most were well encapsulated. The rabbits that were given high doses, however, were less active and more emaciated and often had dyspnea upon exertion. Because of the cavities present, all of these rabbits showed secondary lesions in the appendix and ileocecal junction because they swallowed the bacilli ascending the bronchial tree. Due to the large number of primary pulmonary lesions and the long duration of the experiment, we could not differentiate secondary lesions from primary lesions in the lung.

In this high-dose experiment, the tracheobronchial lymph nodes were larger and showed more caseation than the tracheobronchial nodes in the low-dose experiments, apparently because of the increased bacillary load. However, none of these lymph nodes showed the extensive caseous necrosis of Lurie's susceptible rabbits (9, 21, 22). In other words, an adult type rather than a childhood type of tuberculosis was produced (14, 30).

The rabbits in this experiment were inbred by van Zutphen in The Netherlands (see Materials and Methods). As expected, the variation in the number of primary pulmonary tubercles found among these rabbits was less than the variation in the number found among the outbred rabbits. The number of such tubercles in the low-dose experiment (with commercial rabbits, including those that died before 19 weeks) ranged from 1 to 18, an 18-fold increase from lowest to highest, whereas the number in the high-dose experiment (with inbred rabbits) ranged from 184 to 550, only a 3-fold increase from lowest to highest. The existence of other tubercles probably has little effect on whether a primary tubercle becomes grossly visible because each lesion is mainly controlled by local factors (6, 11). However, the larger antigenic dose probably stimulated a more rapid immune response and, probably, earlier liquefaction.

The high dose apparently taxed host resistance to its limits, so that hosts with slightly weaker resistance succumbed to the disease early and those with slightly higher resistance lived



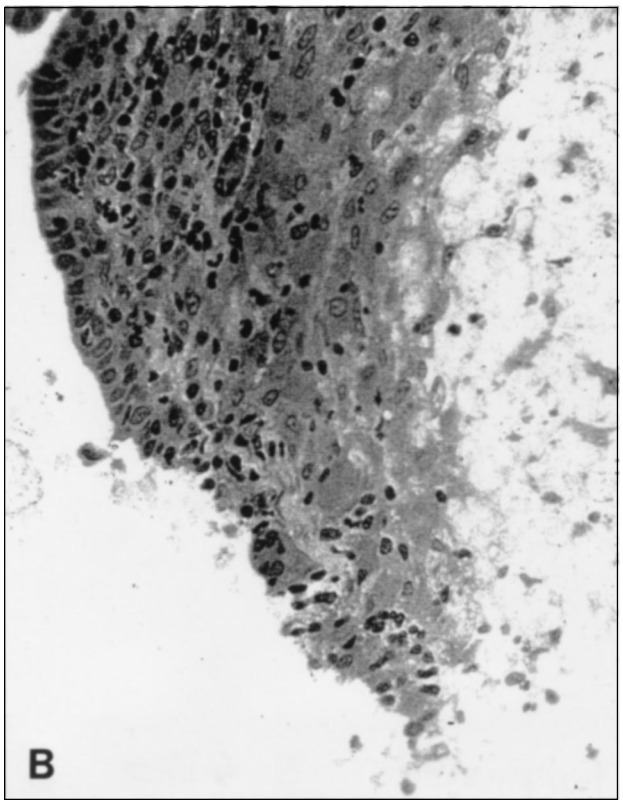


FIG. 3. (A) Tuberculous cavities from the lungs of two rabbits 18 weeks after they had inhaled about 5,000 virulent bovine-type tubercle bacilli (Ravenel S) in high-dose experiment II. Below each cavity are encapsulated (fibrotic) lesions with semisolid caseous centers which had not discharged into bronchi. Magnification, ×2.9. (B) Cathepsin D in the wall of a tuberculous cavity. The cavity was produced in a rabbit (no. 4, experiment I) by inhalation of an aerosol of 880 virulent bovine-type tubercle bacilli (Ravenel S) 22 weeks previously. At the left of the photograph is intact bronchial epithelium. The lower part of this epithelium has ruptured, allowing the liquefied caseum in the cavity (at the right) to be discharged into the bronchus. High levels of cathepsin D are present in the large epithelioid cells next to the liquefied caseum, indicating that cathepsin D is a major cause of liquefaction. Immunostained with polyclonal goat antibody to rabbit cathepsin D and by the avidin-biotin peroxidase technique. In the tissue section, the cathepsin D in epithelioid cell cytoplasm is more easily recognized than in this photograph because it stains brown. Magnification, ×370.

until they were sacrificed. In contrast, none of the rabbits in the low-dose experiment died of tuberculosis before the experiment was ended. (We killed the rabbits when their breathing became labored, so the animals would not suffer. The exact time that they would have died from tuberculosis was therefore not determined.)

Histopathology. (i) Cavity formation. DNases, RNases, proteinases, and probably lipases play major roles in the liquefaction of solid caseous material (13). Immunohistochemical staining showed that cathepsin D, the main proteolytic enzyme of macrophages (31), is present (in high amounts) in the live and dead macrophages that surround caseous and liquefied pulmonary lesions (Fig. 3B). It is probably the major proteinase involved in the liquefaction process.

In clinical tuberculosis, there is frequently blood-tinged sputum and occasionally frank hemoptysis (14). The cause of bloody sputum is the erosion of blood vessels in the cavity wall by the necrotizing process (Fig. 4A).

(ii) Granulated macrophages. Macrophages, containing granules that stained darkly with Giemsa, methylene blue, or azure A, were occasionally found in the intact tuberculous granulation tissue that surrounded the semisolid and liquefied (and cavitary) caseous centers of the pulmonary lesions (Fig. 4B). These macrophages occurred both as isolated cells and as small groups but constituted less than 5% of the macrophage/epithelioid cell population. The dark macrophage granules were autofluorescent and birefringent. They stained darkly with Sudan black B, which has an affinity for both lipids and acidic materials, and some of these granules were even dark in color in unstained tissue sections. They did not stain with

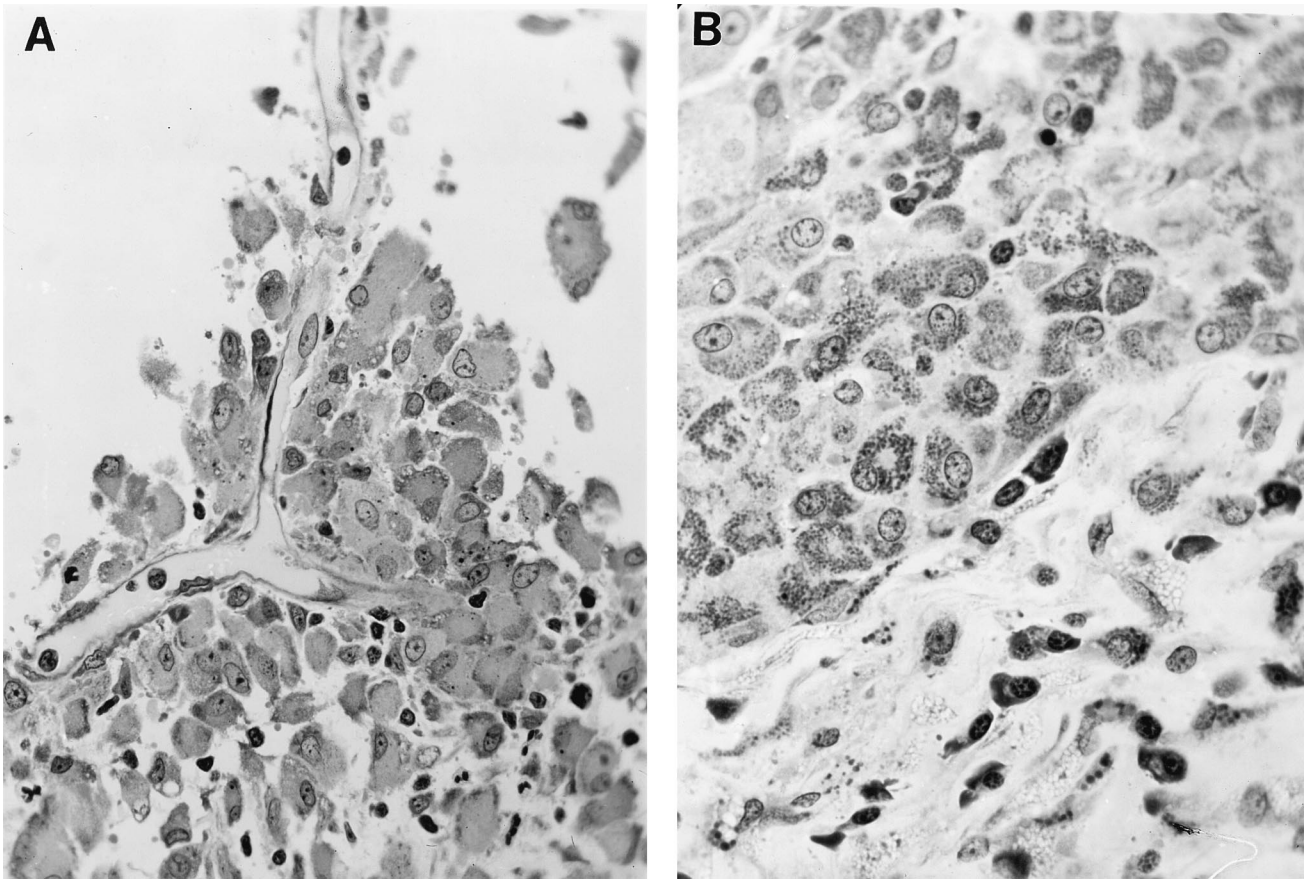


FIG. 4. (A) A small blood vessel extending into the lumen of a cavity. Such exposed vessels are the source of blood in the sputum. If a larger, bare vessel similarly crosses a cavity and ruptures, there may be massive hemoptysis and sometimes fatal hemorrhage. In this area, the inner wall of the cavity contains disintegrating mature epithelioid cells. Lurie (19, 21) showed that such large ovoid cells have destroyed many of the bacilli that they once contained. He postulated that their rather homogenous cytoplasm was due to finely dispersed bacillary lipids (21, 39). Magnification, ×400. (B) An area slightly deeper into the cavity wall than that depicted in panel A. To the left of center are macrophages full of dark-staining granules. Such granulated macrophages occur in some places of the wall, but in most places, nongranulated macrophages predominate. The nature of these granules is unknown (see text). In the lower half are fibroblasts and plasma cells located in the inner layer of the connective tissue capsule which surrounds this cavitary lesion. Many of the fibroblasts contain translucent (probably secretory) granules. Glycol methacrylate-embedded tissue sections stained with Giemsa. Magnification, ×600.

carbol fuchsin (for acid-fast material) or with Alcian blue or the periodic acid Schiff (PAS) reagents (both for carbohydrates). These dark granules may contain lipofuscin or ceroid but most likely contain tissue and possibly bacillary debris.

The immature and mature epithelioid macrophages and alveolar macrophages in these tissue sections often contained granules of a similar size, but they did not stain darkly with Sudan black B or with any of the other stains mentioned. These granules were not autofluorescent or birefringent and showed weak or negative staining with PAS.

Enzyme histochemical staining for β-galactosidase and acid phosphatase (27, 33) enables visualization of activated macrophages (6, 11, 33) that are capable of destroying bacilli (1). The tuberculous granulation tissue that surrounds the caseous and liquefied centers contains many such activated macrophages (Fig. 5).

Large mature epithelioid cells are known to be cells that have destroyed many or all of the tubercle bacilli that they once contained (19, 21). Their histologic appearance is due to the bacillary components being finely dispersed throughout their cytoplasm (21, 39). Such mature epithelioid cells were often found adjacent to the liquefied contents of cavities. At the edge of the liquefied caseum, epithelioid cells were disintegrating (Fig. 4A and 5A). Only some of these viable and dying mature epithelioid cells stain positively for β-galactosidase and acid phosphatase (Fig. 5). In other words, these histochemical stains do not identify all of the macrophages capable of destroying tubercle bacilli. These negative-staining epithelioid cells may be activated for other products, e.g., reactive oxygen and nitrogen intermediates, or they may have already destroyed their intracellular bacilli and have deactivated (4), or they may be dying. Probably all these possibilities occur.

(iii) Fibroblasts. At 19 and 22 weeks, fibroblasts were common in the peripheral regions of the tuberculous granulation tissue, and collagen fibers formed a capsule around the lesion (Fig. 6A). Were it not for the extracellular multiplication of tubercle bacilli in the liquefied caseum, the disease would be arrested.

(iv) Lymphocytes and plasma cells. From 5 weeks on, the disease appeared to be slowly progressing. Many lymphocytes and plasma cells were present in the lesions (Fig. 6). During the later stages of the disease (at 19 and 22 weeks), even occasional lymphoid nodules were found in the granulation tissue surrounding some of the cavities. Since cavities are connected to the bronchial tree, some of these nodules may have

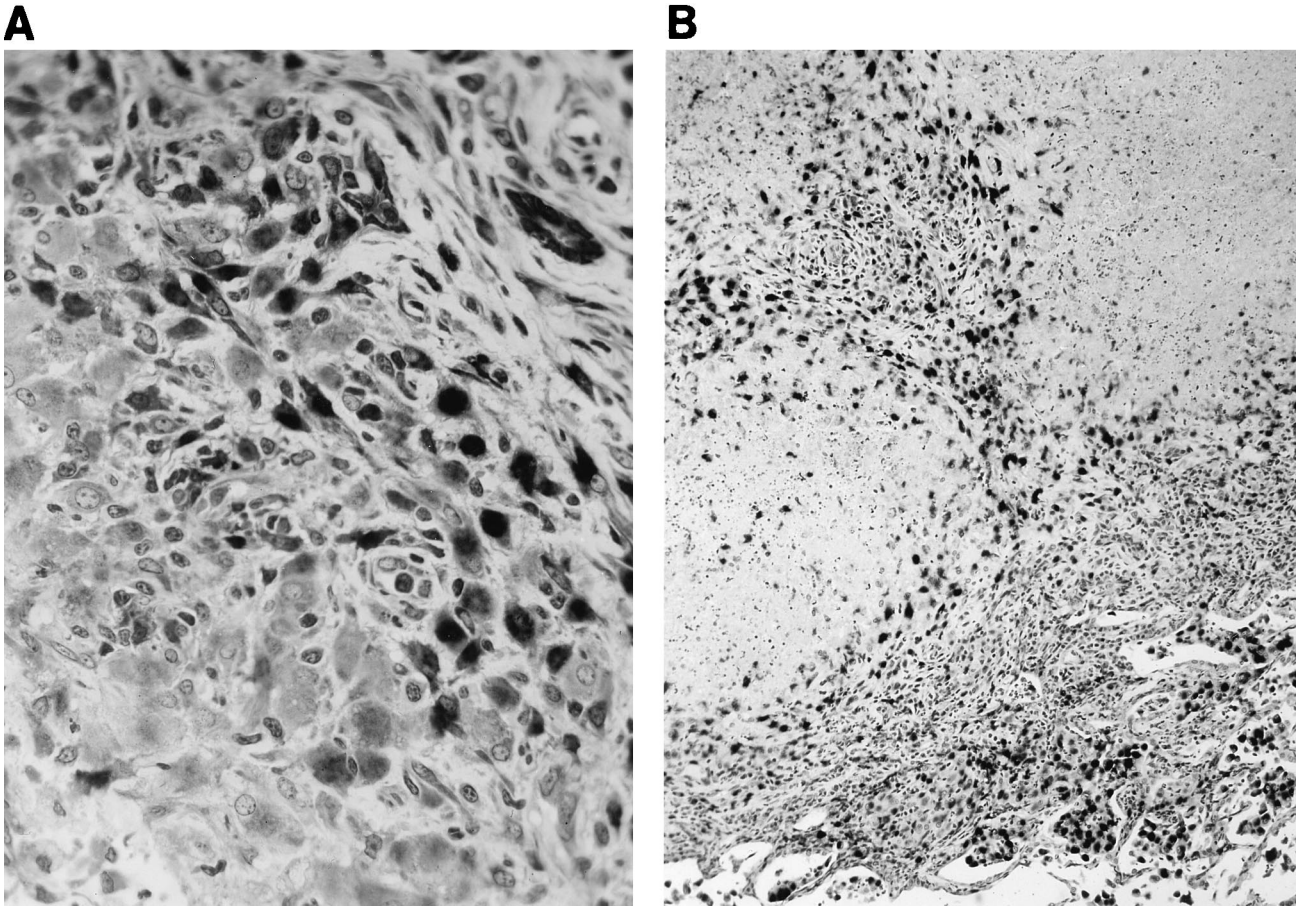


FIG. 5. Glycol methacrylate-embedded tissue sections of tuberculous lesions (counterstained with Giemsa) from a rabbit (no. 8, experiment I) that had inhaled 620 virulent bovine-type tubercle bacilli 19 weeks previously. (A) Section stained for acid phosphatase (a bright red color). The edge of a cavity is at the lower left. Next (proceeding to the upper right) are unstained macrophages (epithelioid cells), some of which are disintegrating. Next comes an area containing many acid phosphatase-positive macrophages, cells with a dark-red cytoplasm (black in this photograph). The outer layer (upper right) shows a metaplastic alveolus within the cavity's connective tissue capsule which contained many fibroblasts (a few of which were also stained for acid phosphatase). Not all of the mature epithelioid cells (large cells with a rounded outline) stained positively for acid phosphatase, indicating heterogeneity in this cell population (33). Magnification, ×400. (B) Section stained for β-galactosidase (a dark blue color). It shows three semisolid caseous centers surrounded by tuberculous granulation tissue containing numerous β-galactosidase-positive macrophages (epithelioid cells). These caseous centers are encapsulated by a region that is rich in macrophages, lymphocytes, plasma cells, and fibroblasts. At the lower right (and bottom) are alveoli with thickened walls. In the lumens of many of these alveoli are accumulations of alveolar macrophages, easily recognized by their dark β-galactosidase-staining cytoplasm. Magnification, ×100.

been bronchus-associated lymphoid tissue, rather than nodules arising de novo from infiltrating lymphocytes.

(v) Metaplastic alveolar epithelium and chemotaxis of alveolar macrophages. In the outer layers of the tuberculous granulation tissue of lesions 10 or more weeks old, the epithelium of what probably were respiratory bronchioles and alveoli became cuboidal and formed air-filled saccules (Fig. 6). Some were composed almost entirely of type II alveolar epithelial cells. Others might be metaplastic type I cells (19) or proliferating type II cells that relined denuded alveoli (32). Alveolar macrophages (AM) seemed to accumulate in these saccules (Fig. 6B), as well as in adjacent normal alveoli. Evidently, chemotactic factors from the lesions caused the migration of AM into the perifocal regions. These perifocal AM occasionally contained an intact tubercle bacillus (see below), apparently from the bacilli-laden liquefied caseum that entered the bronchial tree.

(vi) Number of bacilli. By fluorescent staining, more tubercle bacilli were usually seen in liquefied caseum (Fig. 7A) than in solid caseum. Since we used 1- to 2-μm glycol methacrylate-embedded tissue sections, instead of 7-μm paraffin-embedded sections, the number of bacilli seen microscopically appeared to be less than that described in the literature. Bacilli were also found in tissue spaces (possibly lymphatics), in tissue macrophages, and in the exudate in nearby alveoli (Fig. 7B).

When liquefied lesions were cultured, considerable variability was observed (Table 1). Some lesions (see experiment III, rabbit 4 [Table 1]) had tremendous numbers of bacilli, while other liquefied lesions in the same rabbit (i.e., experiment III, rabbit 4) had relatively few. In other rabbits, no bacilli grew at all (see experiment III, rabbit 10).

Tuberculin reactions. Although every rabbit in these experiments became tuberculin positive, the tuberculin reactions in low-dose experiment III were most informative. At 4 weeks after infection, the size of the tuberculin reactions averaged 2550 ± 330 mm^3 (mean and standard error). At 33 weeks, the size had decreased to about half (1170 ± 200 mm^3) (Table 2). This decrease with time was highly significant ($P < 0.005$ by the paired t test). Note that the exceptions to this trend, rabbits 4 and 5, had rare forms of the disease (Table 1). Rabbit 12 was so sick that it had to be sacrificed before we could administer

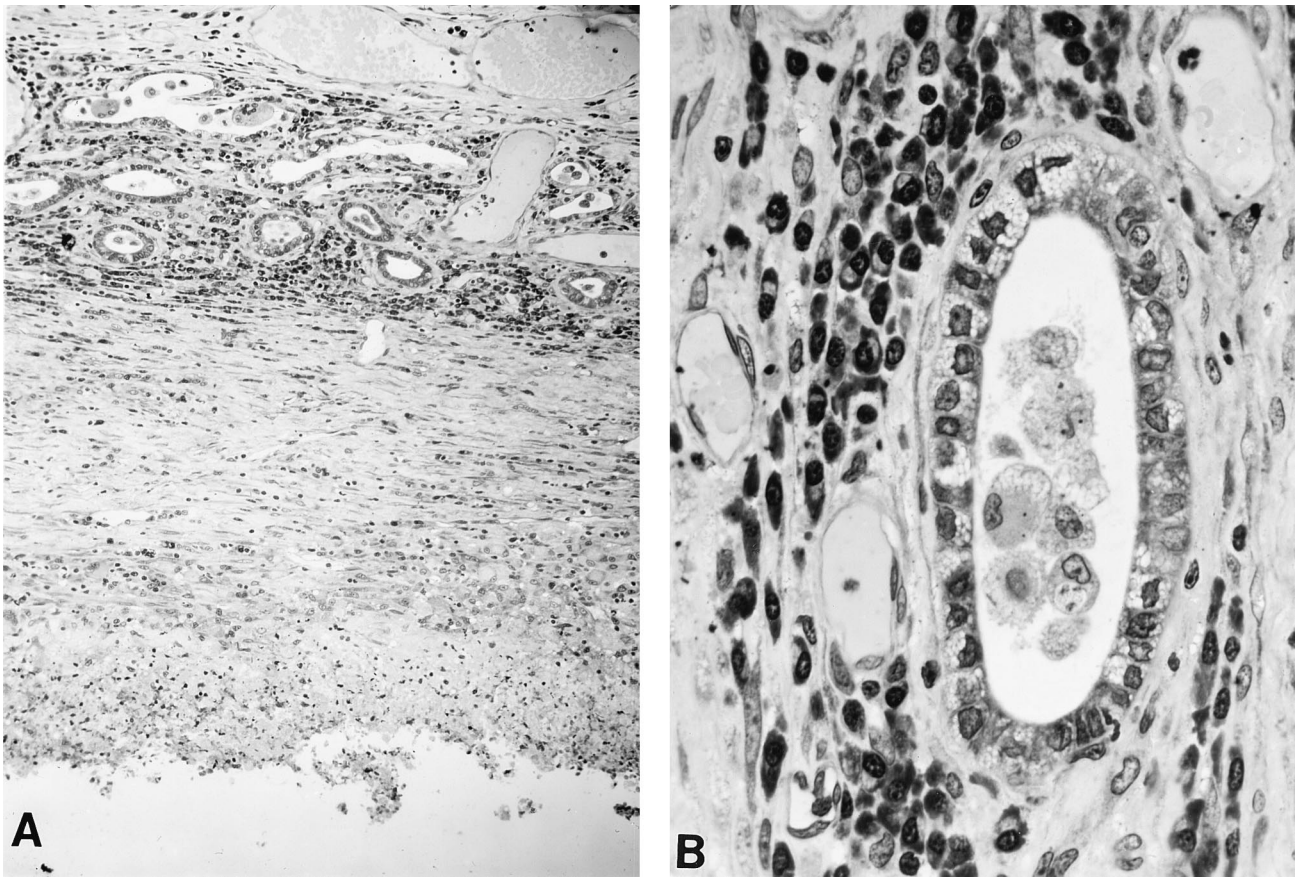


FIG. 6. Glycol methacrylate-embedded tissue sections stained with Giemsa. (A) Wall of a large cavity in the lung of a rabbit 18 weeks after the inhalation of about 5,000 virulent tubercle bacilli (experiment II). The inner layer (below) is liquefied caseum. The next layer is the fibrous capsule. The next layer (top) contains alveoli with metaplastic epithelium and large macrophages in the air spaces. Adjacent to these alveoli are macrophages, plasma cells, lymphocytes, fibroblasts, and dilated microvessels (probably venules). Magnification, ×125. (B) An alveolus with metaplastic epithelium containing alveolar macrophages (AM) in the air space (from the wall of the same cavity shown in panel A). Such alveoli have ceased to function in the oxygenation of blood because of at least partial obstruction of the connecting bronchioles by the adjacent lesion. Many of the epithelial cells are vacuolated, suggesting that they were type II alveolar epithelial cells that had replaced the original type I epithelium. For repair, type II cells are known to replace injured type I cells and then transform into functional type I cells. Therefore, this cuboidal epithelium may be either repairing type II cells (32) or metaplastic type I cells. Type II cells secrete surfactant (mostly phospholipids). Their secretory granules containing this lipid produce the vacuolated appearance shown. AM ingest the surfactant that lines the surface of the alveoli. Thus, AM frequently have the vacuolated appearance shown in this photograph. Surrounding the alveolus is an area rich in lymphocytes and plasma cells containing several dilated capillaries and/or venules. Accumulations of plasma cells and dilated microvessels are frequent components of tuberculous granulation tissue. Magnification, ×600.

the 33-week skin test, but it did have the second weakest tuberculin reaction at 4 weeks (Table 2).

Since tuberculin reactivity is maintained in the host by antigenic stimulation from the bacilli, the decrease in skin test size suggests that the number of bacilli was reduced or that the bacilli were sequestered and therefore no longer provided much antigenic stimulation. At 33 weeks, the pulmonary lesions were fibrotic and the disease seemed at least partly arrested. (Almost all of the rabbits were in good physical condition, so the decrease in tuberculin reactivity was probably not due to a depressed immune response.)

Other correlations also could be made between the amount of tuberculin sensitivity and the amount and type of pulmonary disease in experiment III. At 33 weeks, the time when the rabbits were sacrificed, fewer visible tubercles were present in the lungs of rabbits that had strong tuberculin reactions at 4 weeks than in the lungs of rabbits that had weak tuberculin reactions at 4 weeks (Fig. 8A). This finding indicates that the rabbits which developed a strong early immune response prevented some of the early microscopic tubercles from reaching a grossly visible size. The grossly visible number of primary lesions would not change between 4 and 33 weeks because such lesions would not disappear (21).

Also in experiment III, more cavities were present in the lungs of rabbits with strong tuberculin reactions at 33 weeks than in the lungs of rabbits with weak tuberculin reactions at that time (Fig. 8B). The best explanation of this finding is that the substantial extracellular growth of tubercle bacilli in liquefied and cavitary lesions enhanced the late tuberculin sensitivity of the host because of the large antigenic stimulus produced.

DISCUSSION

These are the first long-term studies of cavitary tuberculosis produced in rabbits by aerosolized virulent *M. bovis*. In previous studies (20, 21, 28, 29) cavities were noted after exposure by various routes but the subject was not explored in any depth. It is surprising how well the rabbits in our experiments controlled this potentially fatal disease when low doses of inhaled bacilli were given. These rabbits had a fibrocaseous form of

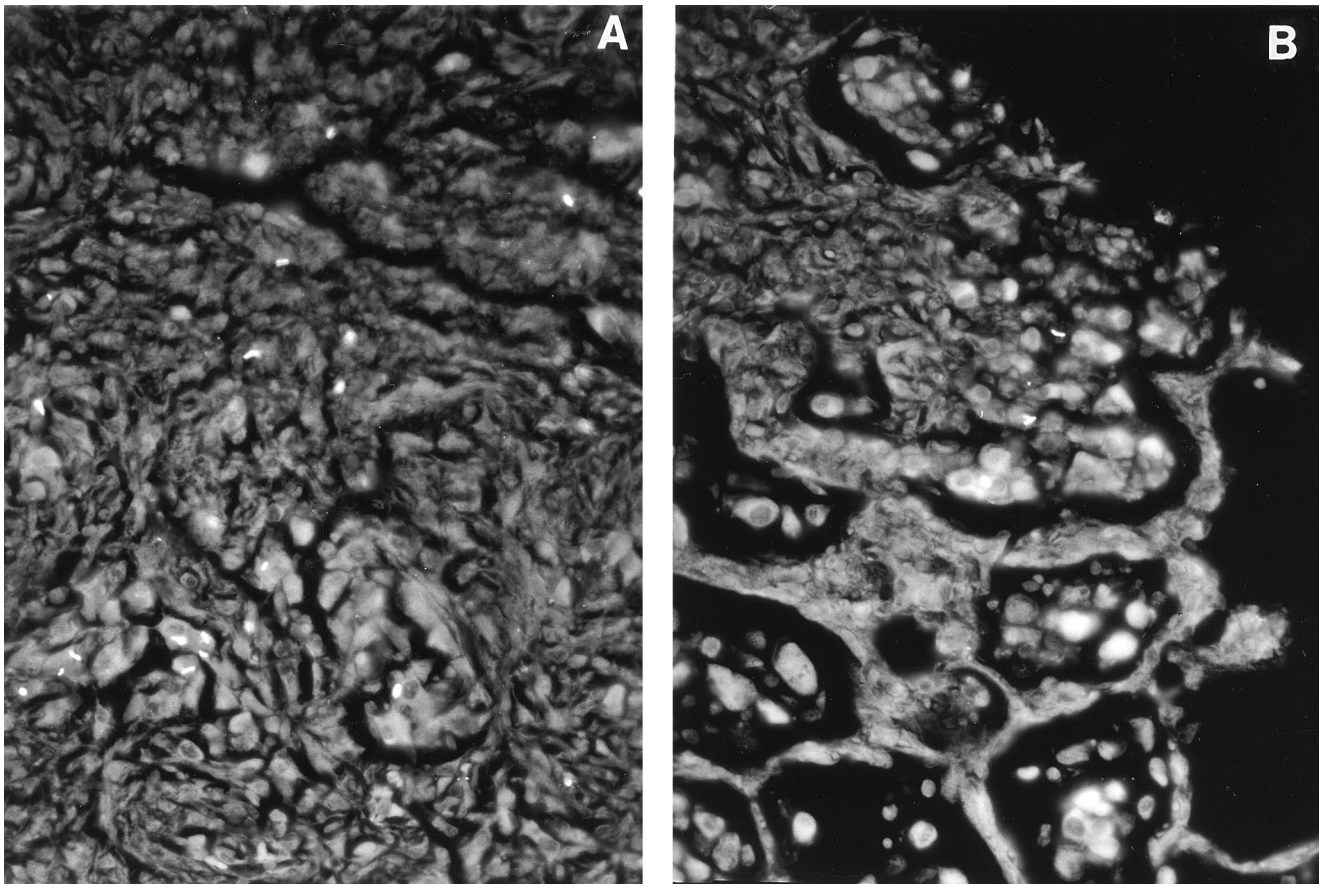


FIG. 7. (A) Tissue section of liquefied caseum in a pulmonary lesion of a rabbit that had inhaled about 5,000 virulent tubercle bacilli 7 weeks previously (experiment II). The bacilli were stained with TB Fluorostain (Polysciences) (see Materials and Methods). The liquefied caseum contained variable numbers of tubercle bacilli, both microscopically in tissue sections and by culture (see the text). Magnification, ×400. (B) Several alveoli just outside a 7-week primary pulmonary tubercle with a liquefied center. The air space of these alveoli contains liquefied caseum from a nearby cavity. In this caseum, near the center of the photograph, a few tubercle bacilli are stained by Polysciences TB Fluorostain (rhodamine B/auramine O). Magnification, ×400.

chronic cavitary tuberculosis, quite similar to the form frequently found in humans (14).

Pathogenesis of tuberculosis. The two basic immune mechanisms that inhibit the growth of tubercle bacilli in the mammalian host have only recently been correlated with the pathogenesis of this disease (7, 8, 12). One mechanism is macrophage activation, a form of protective cell-mediated immunity, caused by interactions of macrophages with T cells and their lymphokines. Such macrophages are able to ingest tubercle bacilli and destroy them. The other immune mechanism is the killing of nonactivated or partly activated macrophages that have allowed tubercle bacilli to grow in their cytoplasm. Such killing eliminates the intracellular environment (within these macrophages) that is so favorable to bacillary growth.

Such macrophage destruction is a major mechanism by which the host controls tuberculosis, but unfortunately it is usually accompanied by destruction of nearby tissues (7, 8, 12). The tissue-destroying mechanism is effective because the bacillus cannot multiply in the solid caseous necrotic tissue produced, apparently because of anoxia, toxic fatty acids, and low pH. This tissue-damaging defense mechanism seems to be due to delayed-type hypersensitivity (DTH) to the tuberculin-like products of the bacillus (37). The tubercle bacillus itself is rather nontoxic (21). The cause of tissue necrosis is the host's DTH reaction wherever the bacillary antigenic load gets high.

After the tissue-damaging DTH develops, the tuberculous lesions consist of a center of dead (caseous) tissue and a mantle of viable tuberculous granulation tissue containing macrophages, lymphocytes, plasma cells, fibroblasts, and a supporting vascular network. In this solid caseous material, many bacilli gradually die (3), while others remain dormant even for years (3, 14). Some bacilli, however, escape into the surrounding perifocal tuberculous granulation tissue, where they may be ingested by activated macrophages, which inhibit and destroy them. If there are numerous activated macrophages present, the disease is arrested. If there are numerous nonactivated macrophages present, the bacilli (when ingested) again multiply intracellularly, and again, the tissue-damaging DTH reaction kills these bacilli-laden macrophages, along with the surrounding tissues. Bacilli may enter the damaged blood vessels and may cause a hematogenously spread childhood-type tuberculosis (14, 30).

An arrested solid caseous lesion may eventually liquefy. In that case, the bacilli multiply extracellularly in the liquefied caseum (Fig. 7A), often reaching tremendous numbers (Table 1) (3, 14, 17, 18, 21, 30). Such large numbers can overwhelm the strong cell-mediated immunity of the host. Cavities form, and bacilli spread in the lung, both locally and more distally via the airways. With coughing, bacilli spread to the patient's environment and may infect other people. Among the large number of bacilli in liquefied caseum, mutants with antimicrobial resistance can develop. Liquefaction (with subsequent cavity

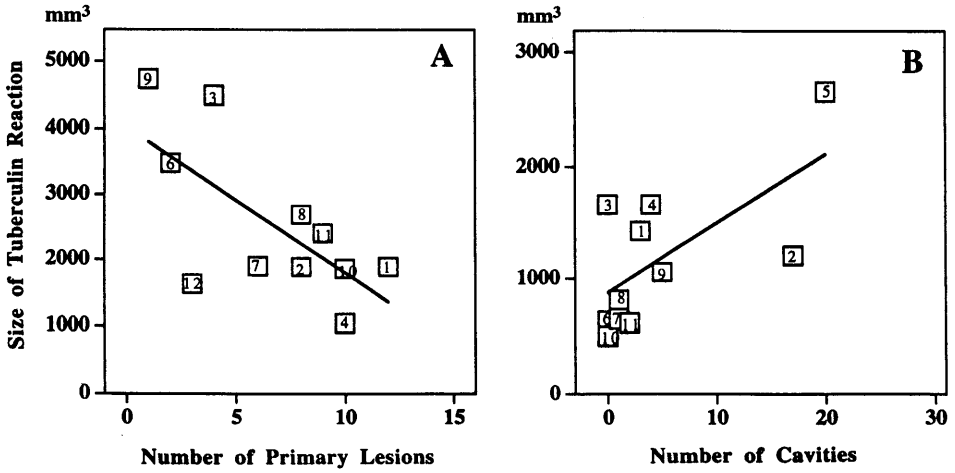


FIG. 8. Tuberculin sensitivity and establishment of primary tubercles. In experiment III, a strong tuberculin reaction at 4 weeks after the inhalation of a low dose of virulent bovine-type tubercle bacilli seemed to be correlated with a decrease in the number of grossly visible primary tubercles ($P < 0.025$). (B) Tuberculin sensitivity and cavity formation. In experiment III, strong tuberculin reactions 33 weeks after the inhalation of a low dose of virulent bovine-type tubercle bacilli seemed to be correlated with an increase in the number of lesions that cavitated ($P < 0.025$). The numbers in boxes refer to individual rabbits as described in Table 1.

formation) is the main cause of both the spread of bacilli to other people and the development of multidrug-resistant organisms (after inappropriate therapy). Therefore, from the perspective of pathogenesis, liquefaction and cavitation are the main causes for the perpetuation of tuberculosis in the world today.

Cavitary tuberculosis. The mechanisms responsible for liquefaction and cavity formation have never been completely determined. Local proteinases (including the cathepsin D herein described), as well as nucleases and probably lipases, evidently hydrolyze solid caseous material (reviewed in references 13 and 18), but what triggers this hydrolysis is unknown. (In vivo experiments employing enzyme inhibitors to prevent such liquefaction remain to be performed.) After these enzymes digest solid caseous material, there is an increase in local osmotic pressure, which causes fluid to be absorbed, creating an ideal culture medium for the extracellular growth of tubercle bacilli (3, 14, 17, 21). Because of the high levels of tuberculin-like products present (and probably many other factors), macrophages cannot survive in the liquefied menstruum. Therefore, the host is powerless to control bacillary growth in such sites. The tuberculous host, however, is highly immunized by the infection and able to control the low doses of tubercle bacilli that seed the lung from endogenous sources. Large numbers of bacilli may, however, be discharged from the cavity into the bronchial tree. If there are many bacilli, the strong host resistance is overwhelmed, and secondary lesions occur. In our rabbit model (and probably in most immunocompetent adult humans), the secondary pulmonary lesions were small and seemed well controlled (Table 1), with two exceptions: (i) secondary lesions produced by the bronchial spread of numerous bacilli to areas adjacent to a cavity, as in Fig. 1B, and (ii) secondary lesions in the lymphoid tissue of the appendix and ileocecal junction produced by the swallowing of liquefied caseum containing numerous bacilli (Table 1).

Our data from bacillary cultures and from fluorescent staining of tissue sections indicate that multiplication of tubercle bacilli in liquefied caseous material is variable and does not always accompany the liquefaction process. Evidently, the composition of (and oxygen tension within) the liquefied menstruum must be precisely right for such extracellular bacillary growth to occur. Alternatively, the percentage of bacilli changing from a state of dormancy to a state of active extracellular multiplication may vary between sites. The fact that tubercle bacilli do not always grow profusely in liquefied caseum gives us hope of finding the reason for such growth and developing new types of therapeutic agents to control it.

Epithelioid cells. On the basis of staining for acid-fast bacilli, Lurie (19, 21) suggested that the mature epithelioid cell, a macrophage that has a rounded or oval appearance, had destroyed the tubercle bacilli that it once contained. We proved this point by using radiolabeled bacilli and autoradiography (1). The presence of many mature epithelioid cells surrounding a solid caseous focus, therefore, indicates that the lesion is not apt to progress (12, 19, 21). Mature epithelioid cells are highly activated macrophages, often staining positively for β-galactosidase and acid phosphatase, our markers for macrophage activation (6, 33). The studies herein reported showed these histochemical markers do not identify all of these microbicidal macrophages: mature epithelioid cells were present in caseous and liquefied lesions that did not stain for β-galactosidase or acid phosphatase. Such stains may only identify certain stages in the development of microbicidal macrophages, or they may be activated for microbicidal activities, e.g., increasing their production of reactive oxygen and nitrogen intermediates, without simultaneously increasing the production of these hydrolytic enzymes (see reference 33).

In summary, we have established that aerosolized virulent bovine-type tubercle bacilli readily produce a slowly progressing cavitary tuberculosis in the lungs of New Zealand White rabbits. This type of tuberculosis closely resembles the form found in immunocompetent humans. We therefore highly recommend the use of this animal model to develop new agents for the prevention and treatment of cavitary tuberculosis.

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Raymond E. Lund, Norman J. Barker, and Rick M. Tracey, registered biological photographers, made many of the photographs. Rena

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ADDENDUM IN PROOF

In other tissue sections of 33-week pulmonary lesions, we recently found truly massive numbers of tubercle bacilli in some, but by no means all, liquefied caseous centers: 500 to 1,000 times the numbers shown in Fig. 7A. These sections were visual confirmation of the 600,000 bacilli cultured from one of the samples of liquefied caseum presented in Table 1. (Bacilli were scarce in solid caseous centers.)

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