*Br. J. exp. Path.* (1978) **59**, 615

# THE PATHOLOGY OF UNTREATED AND ANTIBIOTIC-TREATED EXPERIMENTAL TULARAEMIA IN MONKEYS

A. BASKERVILLE, P. HAMBLETON AND A. B. DOWSETT

*From the Microbiological Research Establishment, Porton, Salisbury, Wilts.*

Received for publication July 5, 1978

**Summary.—Grivet monkeys were infected intranasally with the virulent Schu-S4 strain of *F. tularensis*. One group of animals remained untreated and two other groups received a 7-day course of kanamycin therapy starting on either the third or fourth day after infection. Untreated monkeys developed pyrexia and mucopurulent oculonasal discharge and died 5–7 days after infection. All had pyogranulomatous lesions in the liver, spleen, respiratory tract and lymph nodes. Electron microscopy of liver and spleen showed phagocytosis of *F. tularensis* organisms by macrophages and polymorphonuclear leucocytes, but many bacteria survived phagocytosis and were released on destruction of the cells. Kanamycin therapy enabled most monkeys to survive the disease, but it did not prevent the development of persistent lesions in all animals. Caseous nodules were larger and more widespread in the organs of monkeys in which treatment was delayed until the fourth day of infection.**

TULARAEMIA, caused by infection with the Gram-negative cocco-bacillus *Francisella tularensis*, occurs in man and animals throughout Europe, Russia, North America and the Far East. Though the disease is often fatal if untreated (Lillie and Francis, 1937; Blackford and Casey, 1941; Pullen and Stuart, 1945), it can be treated successfully with several antibiotics (McCrumb, Snyder and Woodward, 1957; Overholt *et al.*, 1961), but the outcome depends on early diagnosis and prolonged treatment, and relapses frequently occur. The experiments described here were part of a study of the effect of antibiotic therapy on clinical and biochemical changes in experimental tularaemia in monkeys. This paper describes the pathological changes in a group of infected monkeys and in two groups treated with kanamycin at different stages of infection.

## MATERIALS AND METHODS

For the untreated tularaemia infection a group of 9 grivet monkeys (*Cercopithecus aethiops*) of either sex and weighing 2·1–4·5 kg was used. For infection and all sampling procedures the animals were anaesthetized by i.m. injection of ketamine hydrochloride (Vetalar—Parke, Davis). Infection was by instillation into the nostrils of a 0·5 ml suspension of cysteine broth containing $5 \times 10^4$ viable *F. tularensis* (virulent Schu-S4 strain). Blood samples for haematology and biochemistry, and nasal swabs for bacteriology, were taken at intervals and the animals were examined clinically and body temperatures recorded at the same time. One additional monkey was not infected and served as a normal control animal.

The kanamycin therapy group consisted of 7 grivet monkeys of either sex, weighing 2·3–3·0 kg. They were infected in the same way as the above group with $5 \times 10^4$ organisms. All monkeys were given 70 mg kanamycin acid sulphate (Kannasyn, Winthrop) daily by i.m. injection for 7 consecutive days. Three animals received the first dose on Day 3 after infection, and the treatment of the remaining 4 began on Day 4.

Monkeys were killed by i.v. injection of pentobarbitone sodium. Necropsy was carried out immediately or soon after death on animals killed or which died, and the following tissues were taken and fixed in 10% buffered neutral formalin: nasal turbinate bones and nasal septum, pharyngeal mucosa, soft palate, trachea, lungs, myocardium; submandibular, cervical and mesenteric lymph nodes, liver, spleen, pancreas, adrenal glands, kidneys, ileum and skeletal muscle. The brain was also taken from 2 monkeys. The nasal bones were decalcified in modified Gooding and Stewart's fluid. All tissues were processed by standard

methods, embedded in paraffin wax, and sections 5 μm thick were cut. Sections were stained with haematoxylin and eosin and selected sections were also stained by a combined Gram–methyl-green–pyronin–light green (Gram–MGPLG) technique (Sowter and McGee, 1976) to demonstrate bacteria, by the periodic acid–Schiff (PAS) method, by Gordon and Sweet's method for reticulin, and by Verhoeff–van Gieson.

For electron microscopy, liver and spleen samples were taken from monkeys which were killed. Portions of tissue approximately 1 $mm^3$ in size were fixed for 2 h at 4° in 4% phosphate-buffered glutaraldehyde at pH 7·2. The blocks were then post-fixed in 1% osmium tetroxide (phosphate-buffered, pH 7·2) for 2 h and processed through a series of alcohols and propylene oxide and embedded in epoxy resin. Semi-thin sections (0·5–1 μm) stained with toluidine blue were examined by light microscopy to locate lesions in these blocks. Ultra-thin sections of suitable fields were stained with uranyl acetate and lead citrate.

## RESULTS

### *Clinical findings*

A detailed report of the clinical signs, together with the haematological and serum biochemical findings appear separately (Hambleton *et al.*, 1978). By Day 3 all monkeys in the untreated tularaemia group developed pyrexia, were anorexic, and had dyspnoea and copious mucopurulent oculonasal discharges. Viable *F. tularensis* organisms were isolated from nasal swabs from all animals. The condition of the monkeys rapidly deteriorated and they became moribund between the fourth and seventh day after infection. One animal was killed *in extremis* on Day 4, others on Days 5, 6 and 7, and a total of 4 monkeys died on Days 5, 6 and 7.

In the other groups pyrexia was also present on Days 3 and 4 when kanamycin treatment began, but other clinical signs were absent until the fifth day after infection, when a mucopurulent oculonasal discharge appeared. The monkeys were still dull and ate little on Day 7. Only 3 animals had pyrexia after the fourth day, one in the early treatment group and 2 in the later treatment group. One monkey in the late treatment group gradually declined in condition, showed respiratory distress and died on Day 16. The others continued to improve from Day 10 and ate normally. After 2 and 3 weeks two monkeys each developed a large abscess in a submandibular lymph node, the swelling extending between the mandibular rami. These abscesses were drained surgically and a further i.m. injection of 70 mg kanamycin was given to each monkey. One of these animals also had a facial abscess which burst on to the skin surface. Viable *F. tularensis* was recovered from the pus of all abscesses. The lesions later healed and all surviving monkeys appeared healthy when they were killed 6 weeks after infection.

### *Necropsy findings*

*Untreated tularaemia group.*—In the monkey killed on the fourth day lesions were present in the spleen, consisting of numerous white foci 1 mm in diameter on the capsular and cut surfaces. The nasal cavity contained mucopus. In monkeys dying or killed at 5 days the surfaces and substance of the liver and spleen were covered with numerous white nodules up to 1 mm in diameter. One of the monkeys had localized peritonitis, with adhesions from the cranial pole of the spleen to the stomach. The nasal cavity was filled with mucopus, and the submandibular, cervical and mesenteric lymph nodes were enlarged and oedematous. Monkeys dying at 6 and 7 days had coalescing nodules up to 2 mm in size in the liver and spleen. In two of the animals there were fibrous adhesions from the surface of the spleen to the cranial pole of the left kidney. Pus was present in the nasal cavity and on the soft palate of all animals, and the submandibular, cervical, bronchial and mesenteric lymph nodes were greatly enlarged. There were no other macroscopic changes.

*Kanamycin-treated tularaemia group.*—The monkey which died on Day 16 had gross splenomegaly. The spleen measured 6·5 × 5 × 4 cm, approximately a five-fold increase in size. There were large areas of

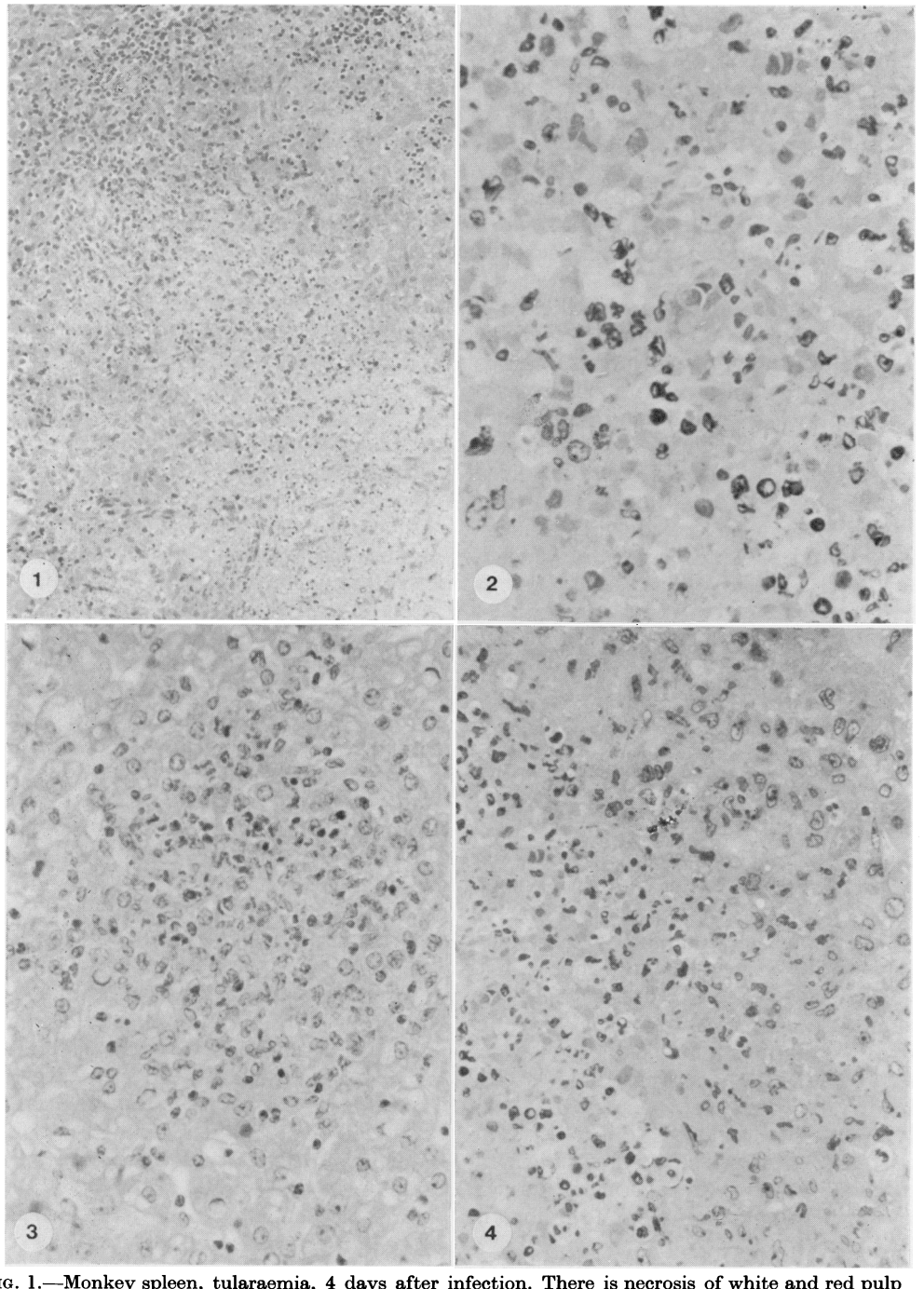



FIG. 1.—Monkey spleen, tularaemia, 4 days after infection. There is necrosis of white and red pulp tissue. H. & E. × 140.

FIG. 2.—Spleen after 4 days' tularaemia, showing areas of necrosis but little cellular reaction. 1 μm section. Toluidine blue. H. & E. × 375

FIG. 3.—Typical nodule in liver, fifth day. H. & E. × 350.

FIG. 4.—Margin of liver nodule 5 days after infection, showing destruction of hepatic parenchyma and activation of Kupffer cells and macrophages. 1 μm section. Toluidine blue. × 350.

necrosis and abscess formation in the splenic substance and fibrous adhesions to the stomach, left kidney and abdominal wall. The liver and lungs contained a large number of abscesses up to 2 mm in size. Pus was present in the nasal cavity and the palatine tonsils, and submandibular, cervical, and bronchial lymph nodes were enlarged and contained caseous nodules.

The lesions in the monkeys killed 5–6 weeks after infection varied in severity and distribution, but included caseous cervical lymphadenitis and tonsillitis, caseous abscesses up to 1·5 cm in diameter projecting from the surface of the spleen, liver and lungs, and occasionally localized peritonitis. In two animals the lung abscesses had formed cavities. The lungs also exhibited varying degrees of consolidation and pleuritis. The subcutaneous abscesses which had been treated surgically in two monkeys had healed to leave thick scar tissue. Lesions were larger and more widespread in the later treatment group. Macroscopical lesions were not present in other organs.

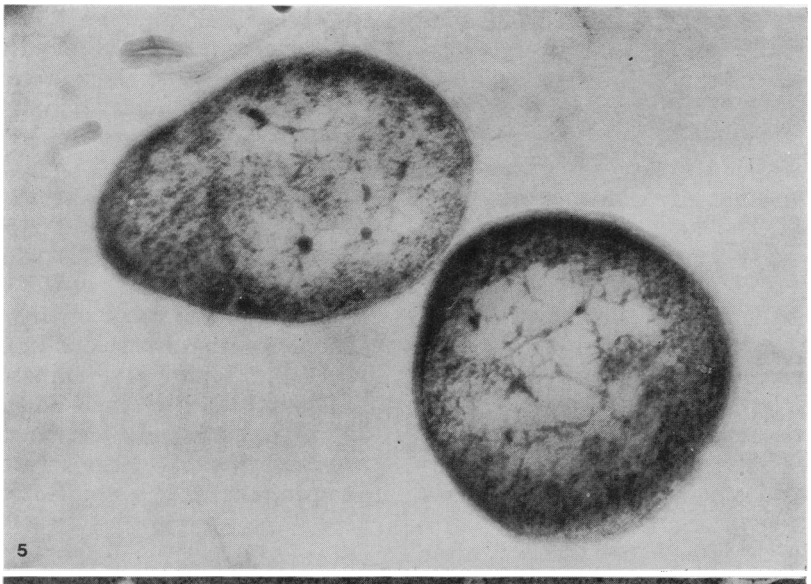
*Histopathological findings*

*Untreated tularaemia group.— Four days*: The liver contained numerous scattered foci of necrosis up to 0·5 mm in size. They were predominantly mid-zonal in distribution and consisted of necrosis of hepatocytes, activated Kupffer cells with elongated nuclei, macrophages and occasional polymorphonuclear leucocytes (PMN). The spleen contained coalescing foci (up to 1 mm) of necrosis of both white and red pulp (Figs. 1 & 2). The nasal mucosa showed early necrotizing rhinitis with some polymorphs in the nasal epithelium. There were no lesions in other tissues.

*Five days:* Liver lesions in all animals were larger and more numerous than at 4 days. The nodules consisted of amorphous debris together with degenerating hepatocytes, Kupffer cells and macrophages (Figs. 3 & 4). Splenic lesions were as on Day 4. Foci of mucosal necrosis extending deep into the wall were present in the pharynx, soft palate and larynx, and there was extensive necrotizing rhinitis. Mesenteric lymph nodes exhibited destruction of many cortical lymphoid follicles and the medulla was oedematous and hyperaemic, but there was no cellular inflammatory response. There were no lesions in other organs.

*Six days*: Liver lesions were the same as at 5 days. In the spleen the changes had progressed to form large areas of necrosis. The lungs contained areas of alveolar necrosis, and alveoli at the periphery of the lesions were distended by oedema fluid. A few airways had focal mucosal necrosis. There was no evidence of cellular response to the damage in any region. All monkeys also had extensive necrotizing pharyngitis and rhinitis, with large numbers of polymorphs in the surface exudate, and one monkey had extensive necrotizing tracheitis. Necrotizing lymphadenitis affected the submandibular, cervical, bronchial and mesenteric nodes. At this and later stages *F. tularensis* organisms were evident in some areas in sections stained by the Gram–MGPLG method. The best tissues for this were the liver, spleen and lymph nodes, where there was a high concentration of organisms. There were no changes in other tissues.

*Seven days:* The liver nodules in all monkeys were at this stage taking on the appearance of granulomas. They were larger and surrounded by a variable-sized zone of Kupffer cells, macrophages and lymphocytes of all sizes, but polymorphs were scarce. There was also diffuse infiltration of sinusoids and portal tracts by lymphocytes and a few polymorphs. The spleens contained large areas of complete necrosis and coalescing granulomas of white and red pulp tissue. These zones were also surrounded by a layer of large macrophages with abundant foamy cytoplasm and outside this a zone of large lymphocytes. Groups of tularaemia organsims were demonstrable in some



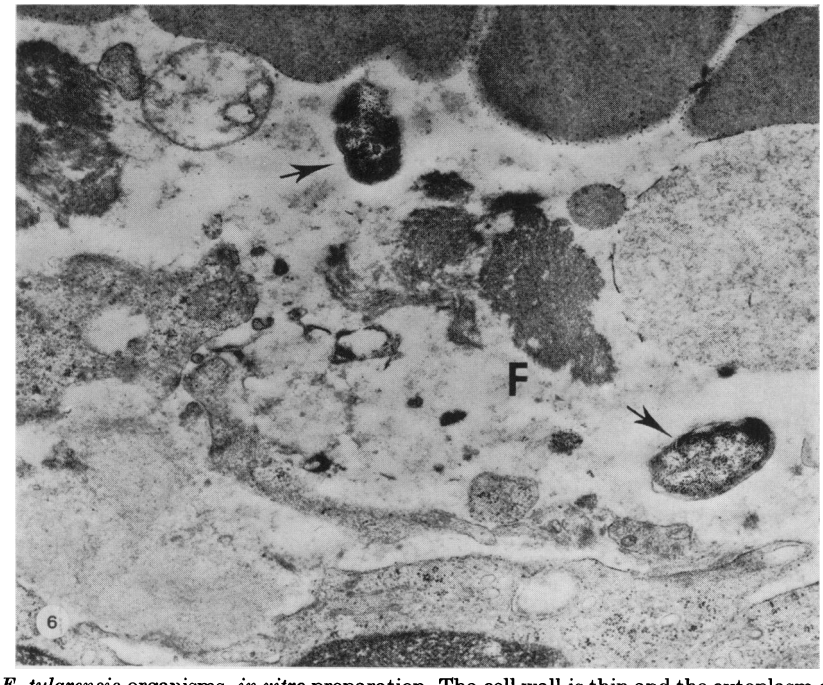




FIG. 5.—*F. tularensis* organisms, *in vitro* preparation. The cell wall is thin and the cytoplasm contains DNA strands and ribosomal material. × 57,500.

FIG. 6.—Spleen, sixth day of infection. Extracellular *F. tularensis* organisms (arrows) are present amongst cellular debris and fibrin (F). A macrophage is at the bottom of the picture. × 18,300.

macrophages and in the central debris. The lungs of all except one animal contained granulomas, most of which apparently originated from airway walls. Submandibular, cervical, bronchial and mesenteric nodes were consistently affected by granulomas. There was purulent pharyngitis and rhinitis in all animals and one monkey had extensive tracheitis. Other organs were normal.

*Kanamycin-treated tularaemia monkeys.*—One animal died on the sixteenth day after infection and the changes in its tissues differed from those in the other 6 killed at 5–6 weeks. The liver and spleen contained massive areas of necrosis and caseation without any evidence of cellular response. The lungs had miliary foci of necrosis up to 2 mm, with destruction of bronchiolar and alveolar walls, and proteinaceous oedema fluid in alveoli at the periphery of lesions. Submandibular, cervical and bronchial nodes showed caseous lymphadenitis, and the abscesses in subcutaneous tissues and muscles of the neck contained caseous pus poorly circumscribed by a small amount of fibrous tissue.

The animals killed 5–6 weeks after infection had variable involvement of organs by caseating pyogranulomatous lesions. Those in the liver, spleen, lungs and lymph nodes were usually very large. Typically they consisted of an extensive centre of caseous debris and degenerating polymorphs, outside this a zone of macrophages with abundant cytoplasm, and externally a layer of large, medium and small lymphocytes. All spleens contained a few multinucleate giant cells in the macrophage layer. In only a few instances were the granulomas surrounded by fibroblasts and a fibrous capsule. White pulp tissue distant from lesions was greatly enlarged (to 1·5 mm in diameter) and their germinal centres consisted of dense sheets of lymphocytes and macrophages in which there was a high mitotic rate. The narrow outer layer was formed by small lymphocytes. In the spleens of 4 monkeys a fine branching network of new trabeculae had developed in the red pulp, on which rows of lymphocytes were present. This gave an acinar appearance and replaced much of the red pulp.

In addition to the granulomas in the lungs there was also localized lymphoid infiltration of interalveolar septa and around blood vessels and airways, and fibrosis obliterated many small airways and alveoli. There were also areas of emphysema and healed pleurisy. In the liver of all animals, there were numerous healing foci of fibrosis, lymphocytes and macrophages, accompanied by local distortion of hepatic plates and distention of sinusoids. The tracheal mucosa of 2 monkeys was heavily infiltrated by lymphocytes and plasma cells which caused considerable thickening of the wall. There were no lesions in other organs.

The large caseous lesions in organs and nodes were found predominantly in the animals not treated with antibiotic until the fourth day after infection.

*Control animal.*—Lesions were not found in the organs of the uninfected monkey.

*Electron microscopy*

*Spleen.*—The morphology of *F. tularensis* organisms in the tissues was similar to that seen in thin sections prepared after growth *in vitro* (Fig. 5). Depending on the plane of section, they appeared as irregularly oval or pear-shaped structures which varied in size from 0·3–0·6 $\mu$m × 0·8–1·2 $\mu$m (Figs. 6–10). Occasional organisms were elongated and measured up to 1·5 $\mu$m. All the bacteria had a thin and apparently flexible cell wall which allowed for some variation in shape. The cytoplasm contained an outer denser region of RNA granules and a less dense central zone within which were prominent strands of DNA material (Figs. 5–10). Some *F. tularensis* organisms were found which had an outer enveloping membrane of irregular thickness and slight electron opacity (Fig. 9).

The changes in the spleen were similar from Days 4 to 7, except that at the later stages there was more necrosis. There was extensive destruction of both white and red pulp tissue and *F. tularensis* organisms were present in groups in the cellular debris (Figs. 6 and 7). The walls of capillaries and venules exhibited varying degrees of damage, from vacuolation of endothelium to complete necrosis, and large quantities of fibrin were deposited

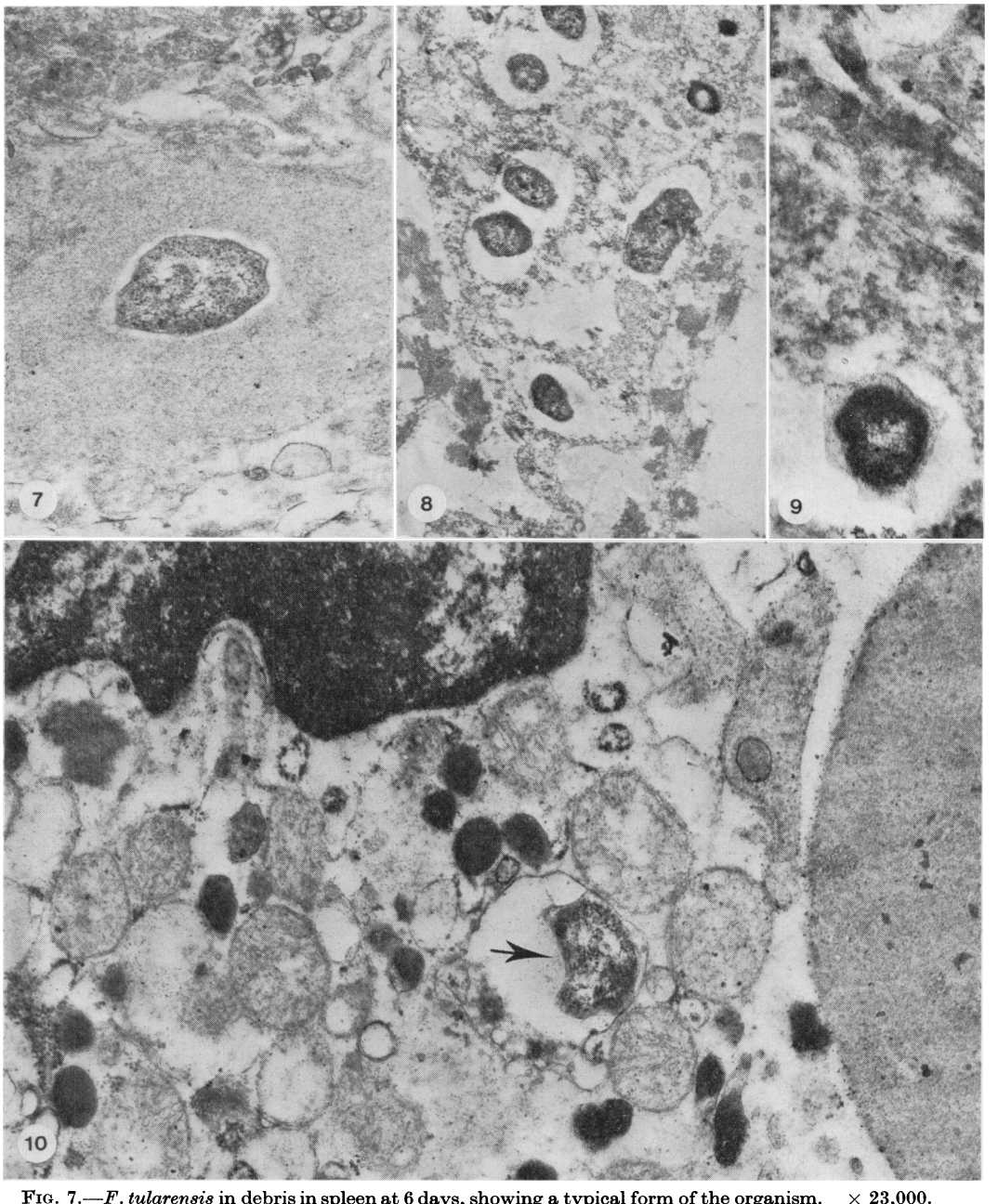



FIG. 7.—*F. tularensis* in debris in spleen at 6 days, showing a typical form of the organism. × 23,000.
FIG. 8.—Destruction of a macrophage containing *F. tularensis* within disintegrating phagosomes. Cell organelles and plasma membrane have been lost. Spleen, 7 days. × 14,000.
FIG. 9.—*F. tularensis* organism bearing the outer irregular enveloping membrane seen in some forms. Spleen, 6 days. × 35,000.
FIG. 10.—Necrosis of a phagocytic PMN in spleen, 7 days. An organism (arrow) is present within a phagosome. The plasma membrane has degenerated. × 22,000.

in all areas (Fig. 6). Groups of bacteria and single organisms were within phagosomes of macrophages and polymorphs (Figs. 8–10). Although some of the bacteria within the phagosomes of macrophages and polymorphs were being digested by the cell, many cells containing the bacteria were themselves degenerating, as shown by extreme cytoplasmic vacuolation, disintegration of mitochondria and other organelles, and dissolution of the plasma membrane (Figs. 8–10). Necrosis of phagocytic cells was accompanied by release of bacteria.

*Liver*.—The degree of tissue damage was greater on Days 6 and 7 than at earlier stages. In affected foci there were large amounts of cellular debris, fibrin and free bacteria. *F. tularensis* organisms were frequently seen within hepatocytes, but the mechanism of penetration of the cells could not be determined. Their presence caused progressive necrosis of affected cells. Bacteria were also present in sinusoids and in bile canaliculi. They were also phagocytosed by macrophages, Kupffer cells and polymorphs. As in the spleen, many of these phagocytes were destroyed by the bacteria, which were then released.

## DISCUSSION

Infection of monkeys with the virulent Schu-S4 strain of *F. tularensis* by the intranasal route produced an acute disease which was consistently fatal. The course and outcome of the infection were thus more severe than in human tularaemia, though the distribution of pathological lesions was similar (Blackford and Casey, 1941; Pullen and Stuart, 1945). In man tularaemia may develop after ingestion and inhalation of the organisms or by skin contamination, and the disease takes typhoidal, pleuropulmonary and ulceroglandular forms which reflect these routes of entry to the body. The mortality in untreated human tularaemia is generally low, 8% being recorded in one series of 225 cases of all forms of the disease (Pullen and Stuart, 1945). However, the pleuropulmonary form is very serious, and in patients not treated with antibacterial agents a mortality of 30–60% has been observed (Overholt and Tigertt, 1960; McCrumb, 1961). Even though in this study bacteria were introduced as droplets into the nose and pharynx, none of the monkeys developed the typhoidal form of the disease. Some organisms must have been swallowed and entered the intestinal tract, since lesions were present in the mesenteric lymph nodes.

There was considerable involvement of the upper respiratory tract, as might be expected after intranasal infection. This contrasts with the findings of White *et al.* (1964), who infected Rhesus monkeys with small-particle-size aerosols of the same strain of *F. tularensis*. Their animals had no lesions in the upper respiratory tract and the pulmonary disease spread from foci which developed at sites of deposition of organisms in respiratory bronchioli and alveoli. Baskerville and Hambleton (1976) showed that infection of rabbits with a small-particle-size aerosol of *F. tularensis* did cause lesions in the upper respiratory tract, but they developed later than the initial foci in the lungs. Upper respiratory tract lesions do occur in a proportion of cases of tularaemia, in which the mode of infection is thought to be by inhalation.

The target organs in the monkeys in the current study, as in man, were the liver, spleen, lungs and lymph nodes. The characteristic lesions in untreated monkeys were foci of parenchymal cell necrosis and macrophage accumulation, with minimal response from other cell types. Lesions in kanamycin-treated animals were not only much larger, probably because they were active for several weeks longer, but they also had caseous centres and zones of intense macrophage, polymorph, plasma cell and lymphoreticular activity. This inflammatory response, together with stimulation of splenic white pulp and lymph node cortical tissue, was

responsible for the production of high levels of circulating antibody to *F. tularensis*. In most monkeys the titres exceeded 1:8000.

The results of antibiotic treatment of infected monkeys illustrates the difficulties of therapy in human cases of this disease. The infection is not always completely eliminated, even by early treatment, probably because of the intracellular location of many bacteria, and relapse may therefore occur if the regime is discontinued too soon. Streptomycin is the drug of choice in tularaemia, but streptomycin-resistant strains of *F. tularensis* do exist and are often responsible for human disease, particularly for laboratory-acquired infections. Kanamycin was chosen for this study because it has been shown to have good bactericidal activity against *F. tularensis in vivo* (Sawyer *et al.*, 1966). Despite its higher toxicity than that of the other group of drugs used in tularaemia, the tetracyclines, kanamycin is preferable because it does not give rise to the relapses which may occur after tetracycline therapy. To simulate clinical conditions as closely as possible, the course of injections was not started until pyrexia first appeared, on the third day of infection. In order to assess the effect of a 24 h delay, treatment of the other group of animals was not started until the fourth day. In these animals tissue damage was greater and the caseating granulomas were much larger, more numerous and more widespread. A delay of 24 h allowed massive multiplication and dissemination of the bacteria, and in two monkeys in this group permanent lesions resulted. For the third animal therapy was ineffective and it died.

Electron microscopy of tissue from untreated monkeys showed that in the liver and spleen *F. tularensis* organisms were phagocytosed by macrophages and polymorphs. However, many of the bacteria survived phagocytosis and a proportion of both types of phagocytic cells was destroyed, with the release and further dissemination of the organisms. This sequence of events is similar to that which occurs in systemic salmonellosis (Baskerville *et al.*, 1972) and suggests that the virulence of *F. tularensis* is dependent on the organism's capacity to survive and multiply within phagocytes, but not on its resistance to phagocytosis.

We are grateful for the skilled assistance of Mrs P. Harris-Smith, Mr N. Bailey, Miss M. E. Orman, Mrs E. Elphick, Mrs I. Francis and Miss D. E. Harwood.

## REFERENCES

BASKERVILLE, A., DOW, C., CURRAN, W. L. & HANNA, J. (1972) Ultrastructure of Phagocytosis of *Salmonella cholerae-suis* by Pulmonary Macrophages *in vivo*. *Br. J. exp. Path.*, **53**, 641.

BASKERVILLE, A. & HAMBLETON, P. (1976) Pathogenesis and Pathology of Respiratory Tularaemia in the Rabbit. *Br. J. exp. Path.*, **57**, 339.

BLACKFORD, S. D. & CASEY, C. J. (1941) Pleuropulmonary Tularaemia. *Archs Intern. Med.*, **67**, 43.

HAMBLETON, P., BASKERVILLE, A., HARRIS-SMITH, P. W. & BAILEY, N. E. (1978) Changes in Whole Blood and Serum Components of Grivet Monkeys with Experimental Respiratory *Francisella tularensis* Infection. *Br. J. exp. Path.*, **59**, 630.

LILLIE, R. D. & FRANCIS, E. (1937) The Pathology of Tularaemia in Man. In *The Pathology of Tularaemia*. Washington, D. C.: National Institutes of Health Bulletin No. 167 p. 1.

MCCRUMB, F. R., SNYDER, M. J. & WOODWARD, T. E. (1957) Studies on Human Infection with *Pasteurella tularensis:* Comparison of Streptomycin and Chloramphenicol in the Prophylaxis of Clinical Disease. *Trans. Ass. Amer. Physicians* **70**, 74.

MCCRUMB, F. R. (1961) Aerosol Infection of Man with *Pasteurella tularensis*. *Bact. Revs*, **25**, 262.

OVERHOLT, E. L. & TIGGERT, W. D. (1960) Roentgenographic Manifestations of Pulmonary Tularaemia. *Radiology*, **74**, 758.

OVERHOLT, E. L., TIGERTT, W. D., KADULL, P. J. & WARD, M. K. (1961) An Analysis of Forty-two Cases of Laboratory-acquired Tularaemia. *Amer. J. Med.*, **30**, 785.

PULLEN, R. L. & STUART, B. M. (1945) Tularaemia: Analysis of 225 Cases. *J. Amer. Med. Ass.*, **129**, 495.

SAWYER, W. D., DANGERFIELD, H. G., HOGGE, A. L. & CROZIER, D. (1966) Antibiotic Prophylaxis and Therapy of Airborne Tularaemia. *Bact. Revs*, **30**, 542.

SOWTER, C. & MCGEE, Z. A. (1976) Evaluation of a New Technique for the Demonstration of Gonococci and Other Micro-organisms in Host Cells. *J. clin. Path.*, **29**, 433.

WHITE, J. D., ROONEY, J. R., PRICKETT, P. A., DERRENBACHER, E. B., BEARD, C. W. & GRIFFITH, W. R. (1964) Pathogenesis of Experimental Respiratory Tularaemia in Monkeys. *J. infect. Dis.*, **114**, 277.