THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XLI SEPTEMBER, 1962 NUMBER 3

MORPHOLOGIC AND IMMUNOHISTOCHEMICAL STUDIES OF THE PATHOGENESIS OF INFECTION AND ANTIBODY FORMATION SUBSEQUENT TO VACCINATION OF MACACA IRUS WITH AN ATTENUATED STRAIN OF PASTEURELLA TULARENSIS

I. INTRACUTANEOUS VACCINATION

CAPTAIN MALCOLM H. MCGAVRAN, M.C., USAR*; JOHN D. WHITE, PH.D.; HENRY T. EIGELSBACH, PH.D., AND ROBERT W. KERPSACK, D.V.M.

From the United States Army Chemical Corps, Fort Detrick, Frederick, Md.

In 1930 Foshay used a formalin-killed preparation of *Pasteurella tularensis* in the first attempt at vaccinal prophylaxis against tularemia.[1] Thereafter, various methods of inactivating the bacteria, with retention of antigenic properties, have been used.[2-5] These variations of Foshay's vaccine were not too effective in protecting experimental animals challenged with virulent *P. tularensis*. Much of the information relative to the protection produced in man by similar vaccines was derived from uncontrolled experiments.[2,6] The impression was that although such vaccination did not prevent infection, it did ameliorate the disease. Saslaw's controlled experiments substantiated this supposition.[7,8] Eigelsbach and Downs[9] have reviewed the research with the "avirulent" immunogenic strains of *P. tularensis*. They also described the selection and characteristics of a hypovirulent, immunogenic strain they called "the live vaccine strain (LVS)." LVS was derived by Eigelsbach from a lyophilized Russian vaccine and produced a distinctly more effective immunity, both in experimental animals and man, than the Foshay types.[7-9]

The sites and cells involved in the production of antibody are reasonably well defined, particularly with regard to "simple," nonviable anti-

Accepted for publication, March 12, 1962.

* Present address: Department of Anatomy, Washington University School of Medicine, St. Louis, Mo.

gens.[10] The mechanisms whereby a "complex," viable antigen accomplishes the same end are very probably of a similar nature. As the LVS of *P. tularensis* is among the few effective living bacterial vaccines, a study of the pathogenesis of immunization appeared warranted. We wished to know the sites and extent of antigenic dissemination, the amount of multiplication, the duration of bacterial persistence, the character, course, and residua of the inflammatory response, the sites and cells in which antibody was formed, the time of its appearance and duration of detectability, and, finally, what differences existed between intracutaneous and aerogenic (respiratory) vaccinees.

Materials and Methods

Vaccine. The LVS of *P. tularensis* was cultured and assayed according to the methods described by Eigelsbach and Downs.[9]

Animals. Thirty-four *Macaca irus* (cynomolgus monkeys) were conditioned for 8 to 12 months prior to use. They were caged in pairs and fed Purina Monkey Chow and water *ad libitum*. Their body weights were between 1.9 and 4.5 kg. All monkeys were observed morning and evening throughout the experiment.

Inoculation. A group of 24 monkeys was vaccinated by intracutaneous injection with 0.1 ml. of gelatin-saline containing 100,000 viable LVS cells. The hair of the interscapular area was clipped and the inoculum placed in the midline as superficially as possible through a 27-gauge needle. A group of 10 monkeys was vaccinated on the volar surface of each forearm, 3 to 4 mm. above a previously applied tattoo mark. This was done to avoid sampling errors. Each of these 10 monkeys received 160,000 viable LVS cells in each forearm, a total dose of 320,000 cells per monkey.

Necropsy and Biopsy Procedures

Pairs from the group of 24 monkeys were anesthetized with Pentobarbital® (Abbott), killed by exsangination from the heart, and necropsied at the following times after vaccination: 1, 6, and 12 hours; 1, 2, 3, 5, 7, 10, 14, 28, and 90 days. Three separate harvests were made; the first for quantitative bacteriologic cultures, the second for fluorescent antibody studies, and the third for morphologic observations.

The tissues and organs cultured from each monkey were the blood, lymph nodes from the right and left inguinal, axillary, and deep cervical chains and from the tracheobronchial and celiac groups, portions of the right apical and diaphragmatic lobes of the lungs, the liver, the spleen, and the femoral bone marrow, the entire site of vaccination from one monkey of each pair and one half from the other. All samples, as well as the entire lung, liver, and spleen were weighed. The samples were triturated in glass grinders, serially diluted, and 0.2 ml. aliquots spread on glucose-cysteine-blood-agar plates. Counts were made at 72 and 96 hours and total concentrations calculated.

Equivalent samples of tissues, plus a portion of the ileum, were frozen in isopentane at −70° C. and stored at −20° C. The necropsies were completed with an extended sampling of the viscera, lymph nodes, and the upper respiratory tract. These tissues were fixed in 10 per cent formalin buffered to neutrality. The brain and spinal cord were not examined.

Excisional biopsy specimens from the volar skin, at the site of vaccination, and axillary lymph node dissections were carried out in one of the group of 10 monkeys on each of the following days after inoculation: 1, 2, 3, 5, 7, 10, 14, 22, 28, and 36. The samples from the right arm and axilla were fixed in formalin, and those from the

left were frozen in isopentane. No cultures were made. These monkeys were bled for serologic studies 22 days after vaccination.

The tissues fixed in formalin were processed through paraffin, cut at 4 μ and stained with hematoxylin and eosin and a modified Giemsa method. Selected tissues—lymph nodes, skin, and spleen—were stained with methyl green and pyronine, according to the method of Kurnick.[11] The frozen tissues were cut at 4 μ in a cryostat at $-15°$ C.

Conjugates

Immune monkey serums, with agglutinin titers of 1/1280 and 1/2560 for *P. tularensis,* were fractionated with ammonium sulfate and the crude globulin fractions conjugated with fluorescein isothiocyanate according to the methods of Riggs and associates.[12] The conjugate was adsorbed twice with acetone-extracted rabbit liver powder (100 mg. per ml.) and twice with spleen-marrow powder (50 mg. per ml.) before use. A portion of the conjugate was further adsorbed with washed, formalin-killed *P. tularensis* to remove homologous antibody. This reagent, used for control, did not stain *P. tularensis.*

Demonstration of P. tularensis Antigen in Tissue

Sections of frozen tissues were placed in acetone for 30 minutes, dried in air, and stained for 30 minutes with a 1/50 dilution of conjugate. A companion section was treated similarly but stained with the conjugate adsorbed with tularensis cells.

Demonstration of Anti-tularensis Gamma Globulin (ATGG) in Tissue

Sections of frozen tissue were fixed in 95 per cent ethyl alcohol for 15 minutes and dried in air. To demonstrate ATGG the sections were first covered with a 1/10 dilution of a sonic lysate of *P. tularensis* strain SCHU. After 1 hour the slides were washed and then stained for 30 minutes with a 1/40 dilution of conjugate. Control slides were prepared by using the conjugate adsorbed with tularensis cells or omitting the sonic lysate.

Fluorescence Microscopy

All slides were examined with a Zeiss fluorescence microscope equipped with a 200-watt Osram lamp, Schott UG-2 and UG-5 transmitting filters, and a Schott GG-4 barrier filter.

RESULTS

Cultural Recovery of LVS

LVS was recovered from the site of inoculation through the 14th day, but not at the 28th or 90th days. Quantitative calculations indicated that substantial multiplication of LVS occurred at this site and reached a maximum of 600,000 on the seventh day. The axillary lymph nodes sampled were positive at 1 hour and then sterile until the seventh day. Thereafter LVS persisted in these nodes through the 28th day. Recovery from the deep cervical lymph nodes was sporadic, being positive on the first, third and tenth days. The tracheobronchial lymph nodes contained LVS on the seventh, 14th, and 28th days. Dissemination to the liver and spleen occurred with recovery from the first through the 14th days. LVS was not cultured at any time from the blood, the lungs or the inguinal or celiac lymph nodes.

Gross Observations

The macroscopic alterations in these animals were limited to the changes at the site of vaccination and the regional lymph nodes. In the group of monkeys vaccinated on the midback, the multiplicity of drainage routes resulted in minimal changes in the axillary and deep cervical lymph nodes. In the group vaccinated on the forearms the changes in the axillary nodes were more readily detected.

A 3 to 5 mm. bleb formed as the inoculum was injected intracutaneously. This disappeared within 2 hours, and only the needle wound remained. One day later no change was seen. At 2 days, an area of erythema measuring 3 to 6 mm. in diameter was present. At 3 days a 1 by 0.5 cm. area of pallor surrounded by erythema had developed. The site became increasingly indurated during the next 2 days. By the seventh day distinct elevation and induration, measuring 1.5 by 1 by 0.3 cm., was present. During the next week, approximately two thirds of the sites developed small, 0.2 to 0.4 cm. ulcers. These re-epithelialized during the third week, and the induration and erythema gradually subsided. By the 28th day small (0.2 to 0.4 cm.) areas of brown discoloration remained.

The axillary lymph nodes enlarged slightly during the latter part of the first week and were firm and readily detectable by palpation. The gross architecture of these nodes was not altered. A few petechiae were seen in the cortices during the first and second weeks, but no foci of frank necrosis were ever seen. By the end of the fourth week, we were unable to distinguish gross alterations.

Microscopic Observations

At 24 hours distinct perivascular accumulations of lymphocytes, histiocytes and a few neutrophils were present in the dermis. Leukostasis and transmigration were apparent in the small veins. LVS cells were diffusely distributed between the dermal collagen bundles with some concentration about small veins.* At 2 days the inflammatory exudate had increased and spread to involve the septums in the islands of intradermal fat. The adventitia of small arteries and the media of the small veins were inflamed. LVS cells were more plentiful (Fig. 5). At 3 days the entire dermis and the superficial subcutis were affected (Fig. 1). The character of the exudate remained dominantly histiocytic and lymphocytic, although small aggregates of neutrophils appeared in minute areas of necrosis associated with extravasated erythrocytes. Venous and capillary endothelial proliferation was seen, but no necrosis of the vessel walls

* All references to LVS in this description are based on observations of specifically stained bacteria seen with the fluorescence microscope.

or thrombi were found. The maximal extent and density of the inflammatory reaction occurred by the tenth day. Increasing numbers of LVS cells were found through the seventh day. Thereafter multiplication, as judged by direct observation, diminished, and by the 14th day no bacteria were seen. Amorphous material that stained specifically was present in the dermis on the tenth and 14th days. We interpreted this as soluble bacterial antigen. By the tenth day foci of epidermal necrosis resulted in small ulcers, the bases of which were acutely inflamed and colonized by gram-positive cocci. As the dermal exudate resolved, reepithelialization took place. The small foci of dermal necrosis never developed the characteristics of the granuloma described in tularemia. They were resolved by fibroblastic and capillary ingrowth, and fine dermal scars resulted.

Readily recognizable plasma cells did not appear in significant numbers until the 14th day. It was possible to formulate a transition in cell types from the dominant histiocyte of the inflammatory exudate to the mature plasma cell. The plasma cell precursors had coarse nuclear chromatin patterns that finally clumped to form the typical cartwheel structure. The cytoplasm of these cells stained with increasing intensity with pyronine, indicating an increase in ribonucleoprotein. By the 22nd and 28th days the inflammatory residues in the dermis were minimal, focal, perivascular, and dominantly histiocytic, plasmocytic, and lymphocytic (Fig. 2). A moderate increase in epidermal melanogenesis and dermal melanophages accounted for the aforementioned gross discoloration.

The changes in the axillary lymph nodes were rather subtle. As the initial inflammatory response to LVS was histiocytic and lymphocytic and remained so, differentiation of reactive foci from normal lymphoid elements was difficult. By the second and third days cortical foci of reaction were found in which a few neutrophils were seen (Fig. 3). LVS was not observed in the axillary nodes through the first day. On the second day phagocytized LVS cells were readily found (Fig. 6). They were detected through the seventh day, although in gradually diminishing numbers, both in the cortex and medulla of the nodes. The cortical foci of reaction to LVS disappeared without necrosis. A medullary hyperplasia occurred during the fifth through tenth days, characterized by an increase in histiocytes and littoral cells. Thereafter recognizable plasma cells appeared singly and in clusters and increased in number through the 14th day (Fig. 4). A coincident increase in cytoplasmic pyroninophilia was found in these cells. No increase in number, size, mitotic activity or pyroninophilia was found in the cortical lymphoid centers.

Changes in the spleen were minimal. There was no significant increase in weight, expressed as per cent of body weight, nor any increase in size

of the follicular centers, rims, mitotic activity or pyroninophilia. Scrutiny of the pulp showed very occasional aggregates of neutrophils during the first week. During the latter half of the second week and the third week, clones of plasma cells were found in the pulp, and as before, these cells and what we assumed to be their precursors had pyroninophilic cytoplasm. LVS cells were never identified in the spleen, and the total concentration in the spleen, as determined by culture, did not exceed 18,000 on the fifth day. Generally the concentrations in the entire spleen were between 100 and 1,000 bacteria. No evidence of miliary granulomas was found in the liver or spleen. An equivocal increase in hepatic portal plasmocytic population occurred. The remaining lymph nodes and organs appeared unaltered.

Anti-tularensis gamma globulin (ATGG) was not identified at the site of vaccination until the 14th day. Small droplets of green-staining material were found in cells that were concentrated in the area of maximal inflammation. By the 22nd day intracellular ATGG had increased, and these cells were found throughout the vaccination site and laterally beyond the limits of the inflammatory exudate (Fig. 7). In contrast, ATGG was found in the medullary portions of the axillary lymph nodes on the third day after vaccination (Fig. 8). When first seen, ATGG was in the form of small cytoplasmic droplets. These droplets became more numerous and finally fused so that the entire cytoplasm of the plasma cells stained green. We observed no ATGG within nuclei. In areas where many cells containing ATGG were present we occasionally, by dark-field examination, found droplets and smudges of a specifically stained substance outside of what could be defined as cells. By the tenth and 14th days many plasma cells containing ATGG were present in the axillary lymph nodes (Fig. 9). In the monkeys vaccinated on the back, ATGG was found in the inguinal as well as the axillary and deep cervical nodes, although in lesser amounts and in an inconsistent time sequence. ATGG was present in the splenic pulp on the fifth day, and by the 14th the numbers of plasma cells containing ATGG was remarkable (Fig. 10). ATGG persisted in the spleen and in a few peripheral lymph nodes to the 90th day. Other sites in which ATGG was found, although in lesser amounts, were the liver, the Peyer's patches, the celiac and tracheobronchial lymph nodes.

The serum agglutinin titers for *P. tularensis* became positive on the seventh day, 1/40, reached a maximum of 1/1280 on the 30th day, and by the 90th day, were at levels of 1/40. Titers for the group of 10 monkeys vaccinated on each forearm were determined on the 22nd day and ranged from 1/640 to 1/10,240 (Table I). Excision of the vaccination site, as well as partial extirpation of the regional lymph nodes, had little effect on the serologic response.

Discussion

Following intracutaneous vaccination with LVS, the bacteria multiplied and persisted at the local site for 2 weeks. Almost immediate lymphatic permeation, as well as subsequent escape from the local site, gave rise to infection of the regional (axillary) lymph nodes. Dissemination to the liver and spleen, of necessity hematogenous, followed. In all

Table I

THE EFFECT OF EXCISION OF THE SITE OF VACCINATION ON THE SERUM AGGLUTININ TITER

Monkey no.	Day of excision	Titer on the 22nd day
X_{11}	1	1/5120
X_{30}	2	1/1280
X 6	3	1/640
X 7	5	1/640
X_{12}	7	1/640
X_{77} *	10	
X_{73}	14	1/1280
X_{54}	22	1/10,240
X_{51}	28	1/5120
X_{70}	36	1/1280

* This monkey died of an overdose of anesthetic.

these secondary sites the bacteria produced minimal inflammatory changes. Three to 5 days after vaccination ATGG was detectable in the regional lymph nodes and spleen. ATGG was not found at the site of vaccination until the 14th day. This delay was probably more apparent than real if we invoke the possibility that what antibody was formed at the local site was rapidly fixed by the bacteria and that only as the bacteria disappeared did antibody become manifest. Large numbers of plasma cells containing ATGG were found on the 22nd and 28th days. This was in contrast to the description of White, Coons, and Connolly [13] who, using ovalbumin and adjuvants, found a paucity of cellular antibody and significant amounts of extracellular antibody. Our findings, at the site of inoculation, were more like those described in the alum granuloma by White, Coons and Connolly.[14]

The late involvement of the tracheobronchial lymph nodes in these vaccinees necessitates some speculation regarding the mechanisms by which LVS got there. Retrograde spread from the axillary-supraclavicular-cervical chain seems unlikely. Although LVS was never recovered from the blood or the lungs, the amount of blood cultured was suboptimal. Thus it may well be that a bacteremia, intermittent and of low concentration, occurred. This would allow LVS to get to the lungs, be

filtered out and concentrated in sufficient numbers to be detected in the tracheobronchial lymph nodes.

ATGG was found in the medullary portions of the lymph nodes and the spleen in cells that eventually had the cytologic characteristics of plasma cells. The absence of ATGG in the splenic and lymphoid follicles was similar to the findings reported by Leduc, Coons, and Connolly[15] in the secondary response to diphtheria toxoid in the rabbit. This absence of antibody and the paucity of cytologic changes in the follicles was in contrast to the work of Ward, Johnson and Abell.[16] They injected bovine gamma globulin into rabbits with and without *E. coli* endotoxin and observed a marked stimulation in the splenic follicles and an absence of plasmocytic participation.

Apparently the lymphocytes and their variously named precursors do not participate to a detectable level, if at all, in antibody production following vaccination with LVS. It will, of course, be of interest to study the secondary response using either virulent or attenuated strains of *P. tularensis* and various routes of challenge.

Summary

Monkeys were vaccinated intracutaneously with 100,000 viable cells of the living vaccine strain (LVS) of *P. tularensis*. The bacteria multiplied locally, disseminated via the lymphatics to the regional lymph nodes and systemically to involve the liver and spleen. The bacteria evoked a mild, nongranulomatous and readily resolved inflammatory response. They disappeared from all the sites except the axillary and tracheobronchial lymph nodes between the 14th and 28th days. They were present in these lymph nodes on the 28th but not on the 90th day. Anti-tularensis gamma globulin (ATGG) appeared in plasma cell precursors in the regional lymph nodes on the third day, in the spleen on the fifth, and in the dermis at the site of inoculation on the 14th day. ATGG persisted in the spleen and peripheral (regional) lymph nodes through the 90th day.

References

1. Foshay, L. Prophylactic vaccination against tularemia. *Am. J. Clin. Path.*, 1932, **2**, 7–10.
2. Foshay, L.; Hesselbrock, W. H.; Wittenberg, H. J., and Rodenberg, A. H. Vaccine prophylaxis against tularemia in man. *Am. J. Pub. Health*, 1942, **32**, 1131–1145.
3. Downs, C. M. Immunologic studies on tularemia in rabbits. *J. Infect. Dis.*, 1932, **51**, 315–323.
4. Downs, C. M.; Coriell, L. L.; Eigelsbach, H. T.; Plitt, K. F.; Pinchot, G. B., and Owen, B. J. Studies on tularemia. II. Immunization of white rats. *J. Immunol.*, 1947, **56**, 229–243.

5. BELL, J. F.; LARSON, C. L.; WICHT, W. C., and RITTER, S. S. Studies on the immunization of white mice against infections with *Bacterium tularense*. *J. Immunol.*, 1952, **69**, 515–524.
6. KADULL, P. J.; REAMES, H. R.; CORIELL, L. L., and FOSHAY, L. Studies on tularemia. V. Immunization of man. *J. Immunol.*, 1950, **65**, 425–435.
7. SASLAW, S.; EIGELSBACH, H. T.; WILSON, H. E.; PRIOR, J. A., and CARHART, S. Tularemia vaccine study. I. Intracutaneous challenge. *Arch. Int. Med.*, 1961, **107**, 689–701.
8. SASLAW, S.; EIGELSBACH, H. T.; PRIOR, J. A.; WILSON, H. E., and CARHART, S. Tularemia vaccine study. II. Respiratory challenge. *Arch. Int. Med.*, 1961, **107**, 702–714.
9. EIGELSBACH, H. T., and DOWNS, C. M. Prophylactic effectiveness of live and killed tularemia vaccines. I. Production of vaccine and evaluation in the white mouse and guinea pig. *J. Immunol.*, 1961, **87**, 415–425.
10. COONS, A. H.; LEDUC, E. H., and CONNOLLY, J. M. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exper. Med.*, 1955, **102**, 49–60.
11. KURNICK, N. B. Pyronin Y in the methyl-green-pyronin histological stain. *Stain Technol.*, 1955, **30**, 213–230.
12. RIGGS, J. L.; SEIWALD, R. J.; BURCKHALTER, J. H.; DOWNS, C. M., and METCALF, T. G. Isothiocyanate compounds as fluorescent labeling agents for immune serum. *Am. J. Path.*, 1958, **34**, 1081–1097.
13. WHITE, R. G.; COONS, A. H., and CONNOLLY, J. M. Studies on antibody production. IV. The role of a wax fraction of *Mycobacterium tuberculosis* in adjuvant emulsions on the production of antibody to egg albumin. *J. Exper. Med.*, 1955, **102**, 83–104.
14. WHITE, R. G.; COONS, A. H., and CONNOLLY, M. J. Studies on antibody production. III. The alum granuloma. *J. Exper. Med.*, 1955, **102**, 73–82.
15. LEDUC, E. H.; COONS, A. H., and CONNOLLY, J. M. Studies on antibody production. II. The primary and secondary responses in the popliteal lymph node of the rabbit. *J. Exper. Med.*, 1955, **102**, 61–72.
16. WARD, P. A.; JOHNSON, A. G., and ABELL, M. R. Studies on the adjuvant action of bacterial endotoxins on antibody formation. III. Histologic response of the rabbit spleen to a single injection of a purified protein antigen. *J. Exper. Med.*, 1959, **109**, 463–474.

[*Illustrations follow*]

LEGENDS FOR FIGURES

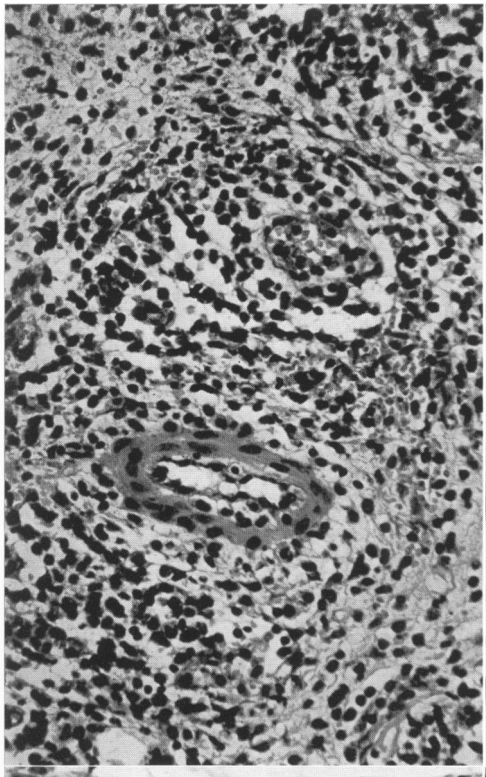
All photomicrogaphs on this plate are of hematoxylin and eosin stained preparations.

FIG. 1. The dermis 72 hours after inoculation with LVS. The dominantly histiocytic and lymphocytic infiltrate is concentrated about the vessels. Small hemorrhages are present but there is no frank necrosis. × 275.

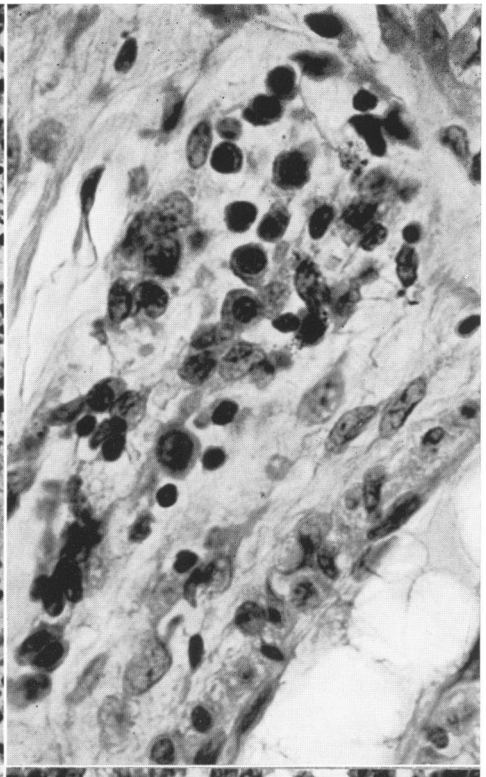
FIG. 2. The residua of the dermal inflammatory reaction at 28 days. Small clusters of plasma cells and macrophages are present. In comparable locations and cells ATGG is readily found. × 600.

FIG. 3. The mild acute inflammatory reaction found in the cortex of an axillary lymph node 72 hours after vaccination. *P. tularensis* was readily identified in comparable lesions. × 275.

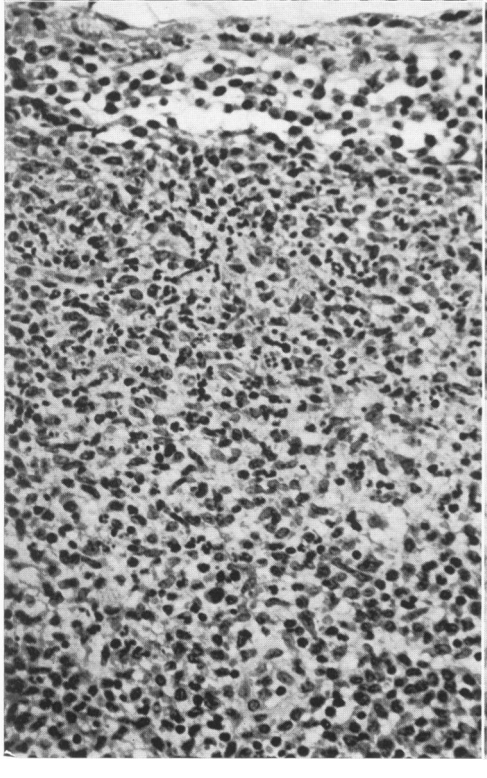
FIG. 4. A clone of plasma cells in a medullary cord of an axillary lymph node 28 days after vaccination. × 600.



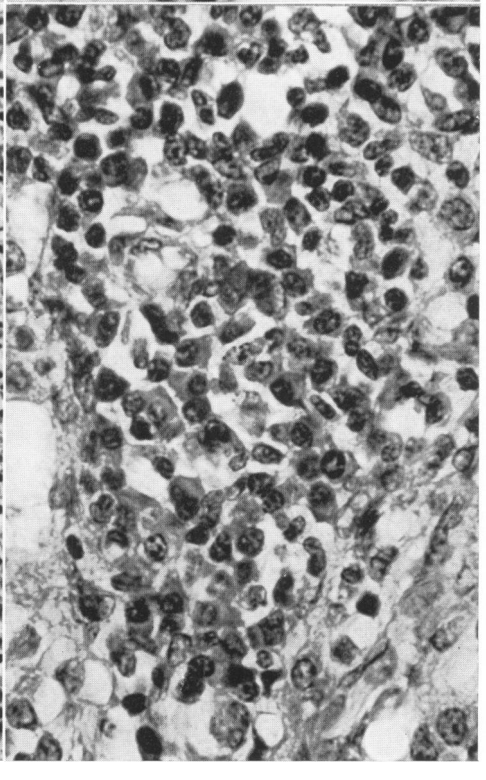
1



2



3



4

All photomicrographs on this plate are of fluorescent antibody stained preparations.

FIG. 5. The dermis 48 hours after inoculation with LVS. Numerous organisms, stained green, are interspersed among the collagen bundles which exhibit blue autofluorescence. The blood vessel in the center of the photomicrograph is minimally involved. × 100.

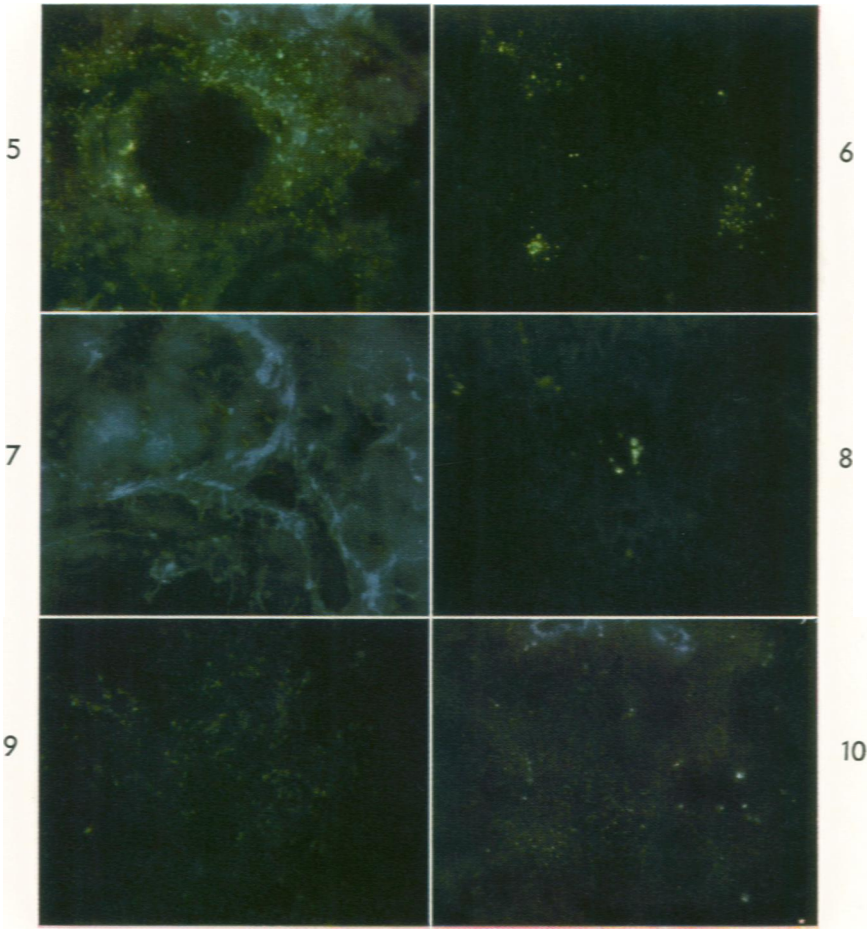
FIG. 6. An axillary lymph node 48 hours after intracutaneous vaccination. LVS cells, stained green, have been phagocytized. × 100.

FIG. 7. ATGG in the dermis 22 days after intracutaneous vaccination. Numerous plasma cells are interspersed throughout the collagenous tissue. × 100.

FIG. 8. An axillary lymph node 3 days after vaccination. The globular appearance of the stained ATGG is apparent. × 380.

FIG. 9. An axillary lymph node 10 days after vaccination. Numerous ATGG-containing cells are manifest. × 100.

FIG. 10. Spleen, 14 days after vaccination. × 100.


5
6
7
8
9
10