INFECTION AND IMMUNITY, Mar. 1976, p. 861-875
Vol. 13, No. 3
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Host Defenses in Experimental Scrub Typhus: Histopathological Correlates

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Received for publication 31 July 1975

Intraperitoneal (i.p.) infection of BALB/c mice with 1,000 50% mouse lethal doses of the Karp strain of *Rickettsia tsutsugamushi* was inevitably lethal, and associated pathological alterations were confined to the peritoneal cavity. These included: (i) continuous proliferation of rickettsial organisms in peritoneal macrophages until death; (ii) hepatic granulomas appearing 6 days after infection and increasing in size and number until death; (iii) splenomegaly, resulting principally from proliferation of lymphoid tissue, and (iv) terminal peritonitis. Under two circumstances, i.p. infections with *R. tsutsugamushi* were not lethal: (i) infection with 100 50% mouse infectious doses of the Gilliam strain, which, in fact, resulted in immune protection against otherwise lethal Karp challenge; and (ii) Karp infection of animals immunized with the Gilliam strain. In both cases, the associated pathological abnormalities were, as with primary Karp infection, restricted to the peritoneal cavity. Also similar was the striking splenomegaly due to lymphoid proliferation, which was particularly prominent in immunized animals. In contrast to primary and lethal Karp infection, however, these infections were characterized by: (i) minimal and transient proliferation of rickettsial organisms in peritoneal macrophages; (ii) disappearance of hepatic granulomas; and (iii) absence of peritonitis. It was concluded that the survival of an animal bearing an i.p. infection of scrub typhus depended on its ability to concentrate a sufficiently vigorous immune response in the peritoneal cavity, resulting in the evolution of rickettsiacidal macrophages capable of suppressing the infection.

In other studies (A. Shirai, P. J. Catanzaro, S. M. Phillips, and J. V. Osterman, submitted for publication) we have verified previous observations that the virulence of the Karp strain of *Rickettsia tsutsugamushi* was far greater than that of the Gilliam strain in mice (6) and that pre-inoculation with an infectious dose of a less virulent strain of scrub typhus protected mice from subsequent challenge with a lethal strain (1). Finally and most importantly, it was observed that cell-mediated immunity induced by Gilliam immunization was principally responsible for the initial stages of heterologous protection.

The ultimate goal of studies such as these is an understanding of the mechanisms of immune protection. An essential prerequisite for achieving this goal is a thorough knowledge of the pathobiology of rickettsial infection. Although others have studied the pathology of scrub typhus (7, 8), such reports have not focused on the pathological consequences of infection with a sublethal dose of Gilliam strain nor on the modifications in lethal Karp pathology effected by prior heterologous immunization with Gilliam. Therefore, in this article, we compare and contrast the pathology of sublethal, immunizing Gilliam infection with lethal Karp infection and also Karp infection of Gilliam-immunized mice.

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MATERIALS AND METHODS

Animals. Female BALB/c mice (Flow Laboratories, Dublin, Va.), 18 to 22 g, were used throughout the study.

Rickettsial strains. The virulent Karp strain (egg passage 47) and the less virulent (for mice) Gilliam strain (egg passage 136) were grown in 6- to 7-day-old embryonated eggs from leukosis-free flocks (Spafas, Inc., Norwich, Conn.). Eggs were inoculated with a dilution of rickettsial suspension that killed half the embryos by post-inoculation day 10. Yolk sacs of the live eggs were harvested aseptically and Giemsa-stained smears were prepared from each membrane. Those yolk sacs with the largest

number of rickettsiae were pooled and homogenized with Snyder I diluent (3) to produce a 20% (wt/vol) suspension that was divided into aliquots, quick-frozen in a dry ice-alcohol bath, and stored at −70 C. The 50% mouse infectious dose (MID_{50}) was determined by the method of Jackson and Smadel (6), except that the challenge dose of Karp strain was 1,000 times the 50% mouse lethal dose (MLD_{50}). Rickettsial suspensions were quantitated by titration in BALB/c mice, and the MLD_{50} and MID_{50} values were calculated by the method of Reed and Muench (10). All inoculations of rickettsiae were made by an intraperitoneal injection in a standard volume of 0.2 ml.

Pathology. At various times after infection (indicated in text), mice were sacrificed by cervical dislocation and subjected to postmortem examination. This included gross and microscopy inspection of the thoracoabdominal contents and the central nervous system. Representative samples of these organs, as well as of skin and skeletal muscle, were promptly fixed in buffered neutral 10% Formalin and processed in the usual manner for light microscopy. Sections approximately 6 μm thick were obtained and stained with either hematoxylin and eosin or Giemsa stain (4). Peritoneal surfaces were scraped with a scalpel blade; the scrapings were spread on glass slides, air-dried, fixed in absolute methanol, and stained by the Giemsa technique.

RESULTS

General. Sequential pathological alterations were observed after intraperitoneal inoculation of *R. tsutsugamushi*. There were three experimental groups: (i) animals infected with a lethal dose (1,000 MLD_{50}) of the Karp strain; (ii) animals infected with a nonlethal dose (100 MID_{50}) of the Gilliam strain, which, in fact, resulted in immune protection against otherwise lethal Karp challenge (A. Shirai et al., submitted for publication); and (iii) animals immunized with the Gilliam strain, as above, 3 days before challenge with 1,000 MLD_{50} of the Karp strain. Groups of three mice were sacrificed on the first and third days postinfection and every third day subsequently. At each time point, a comparable control group was also sacrificed. Regardless of the lethality of the infecting strain, the elapsed time postinfection, or the state of immunity, the histopathological changes were confined to the peritoneal cavity, the peritoneal surfaces of abdominal organs, and the spleen and liver. Lesions outside the peritoneal cavity (excluding the spleen and liver) were not observed in these experiments at the level of resolution of the light microscope. Pathological differences between the three groups of infected mice were usually of a quantitative rather than a qualitative nature, and similar classes of lesions differing only in severity could be demonstrated in each group.

Quantitation of rickettsial strains. Titration of the stock suspension of Karp in BALB/c mice indicated identical values of $10^{9.0}$ MLD_{50}/ml and $10^{9.0}$ MID_{50}/ml. The stock suspension of Gilliam was similarly titrated and found to contain $10^{5.0}$ MLD_{50}/ml and $10^{8.2}$ MID_{50}/ml of rickettsiae. The similarity of MLD_{50} and MID_{50} values for Karp confirmed its lethality for this strain of mice. The difference between MLD_{50} and MID_{50} values for Gilliam reflects its reduced virulence for mice and was the basis for selection of the 100 MID_{50} immunizing dose. Since the MLD_{50} and MID_{50} values were identical for Karp, the inoculum of 1,000 MLD_{50} contained approximately 10-fold more infectious doses of rickettsiae than the inoculum of 100 MID_{50} of Gilliam used for immunization.

Peritoneal cavity. The peritoneal fluid of a normal mouse was clear, straw-colored, and contained cells free in suspension. These cells were mostly mononuclear, consisting of both lymphocytes and macrophages, but occasional polymorphonuclear leukocytes (PMNs) and mast cells were also observed. During the first 6 days after infection with either strain of *R. tsutsugamushi*, there was a definite increase in the number of peritoneal cells observed, particularly mononuclear cells (Fig. 1). At this time, macrophages were principally of the monocytoid type (5). They were of medium size and did not contain prominent granules, vacuoles, or projections (Fig. 2). Rickettsial organisms were not identifiable during this period, but there were numerous examples of lymphocyte-macrophage interaction with occasional lymphoid cell rosette formation around macrophages (Fig. 3). During the initial 6-day period, peritoneal scrapings from the three groups of mice were indistinguishable, but differences began to appear 9 days after infection. At that time, peritoneal fluid from mice bearing lethal Karp infections was thick, tenacious, and pale reddish-gray, suggesting a fibrinous peritonitis. Microscopically, numerous macrophages containing large numbers of intracellular coccobacillary organisms identical in morphology to scrub typhus rickettsiae were observed (Fig. 4). Further, a sharp increase in PMNs also occurred, but no rickettsiae were observed in these cells. In sharp contrast to lethal Karp challenge, rickettsial organisms were rarely observed in either the group infected with a nonlethal dose of Gilliam strain or the group immunized with Gilliam and subsequently challenged with Karp. The animals from these groups both exhibited an increased number of peritoneal macrophages with large empty vacuoles (Fig. 5), in contrast to the organism-filled vacuoles demonstrated in Fig. 4. This morphological appearance suggested that these macrophages were

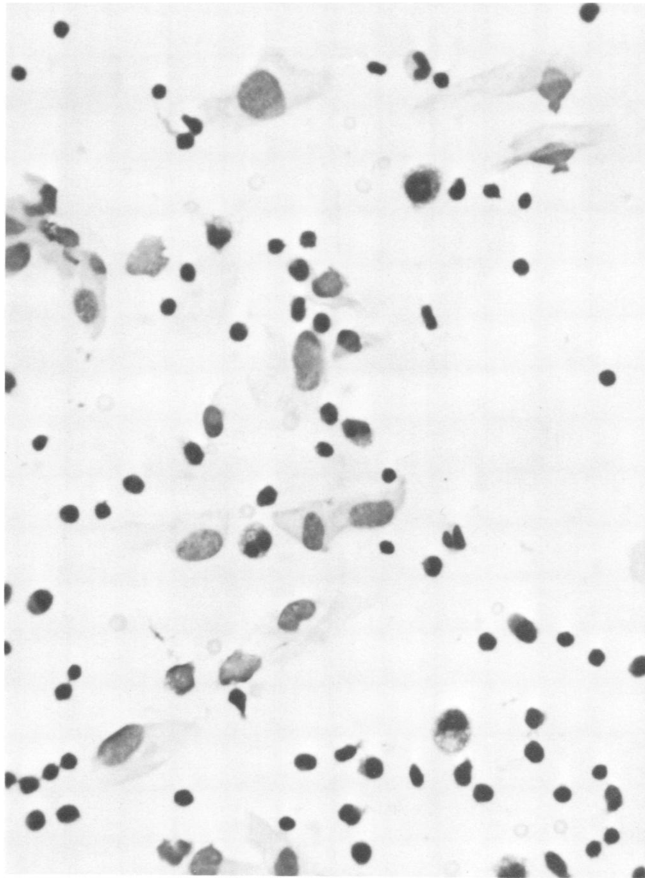

FIG. 1. *Peritoneal scraping prepared 6 days after Gilliam infection. Cellular composition is predominantly mononuclear, consisting of darker-staining small lymphocytes and larger macrophages with pale cytoplasm. Organisms were not identified. (Giemsa, ×400)*

able to destroy the intracellular rickettsiae. Again in contrast to lethal Karp infection, no comparable increase in PMNs was observed, and fibrinous peritonitis did not occur.

Spleen. Until 3 days after infection, spleens of infected mice were indistinguishable from those of control animals (Fig. 6). After that time, infection with either Karp or Gilliam resulted in a marked increase in spleen size due principally to an enlargement of the white pulp (Fig. 7). Also evident at this time was an increased prominence of germinal center formation. This trend of splenomegaly due to increase in white pulp continued with time and reached its peak at 12 days in mice infected with Gilliam and in Karp-infected animals, some of which were moribund. The group of mice immunized with Gilliam and subsequently challenged with Karp showed the most striking development of white pulp (Fig. 8), which was first evident at 3 days after Karp challenge. Rickettsial organisms were not observed in histological sections of the spleen in any of the three groups.

Liver. Infection with either Karp or Gilliam led to a generalized prominence of Kupffer cells during the first 6 days. At low magnification, the prominent nuclei of the usually flattened Kupffer cells were readily identifiable. After day 6, definite nodular aggregates of cells could be identified, scattered randomly throughout the hepatic parenchyma (Fig. 9). At higher magnification, it was observed that these nodules lacked PMNs and were composed of mononuclear cells, mainly Kupffer cells (Fig. 10, 11). After day 9, these mononuclear aggregates or "granulomas" decreased in size and number in animals infected with Gilliam and in animals immunized with Gilliam and subsequently challenged with Karp. However, the converse occurred in Karp-infected animals—the number and size of such granulomas in-

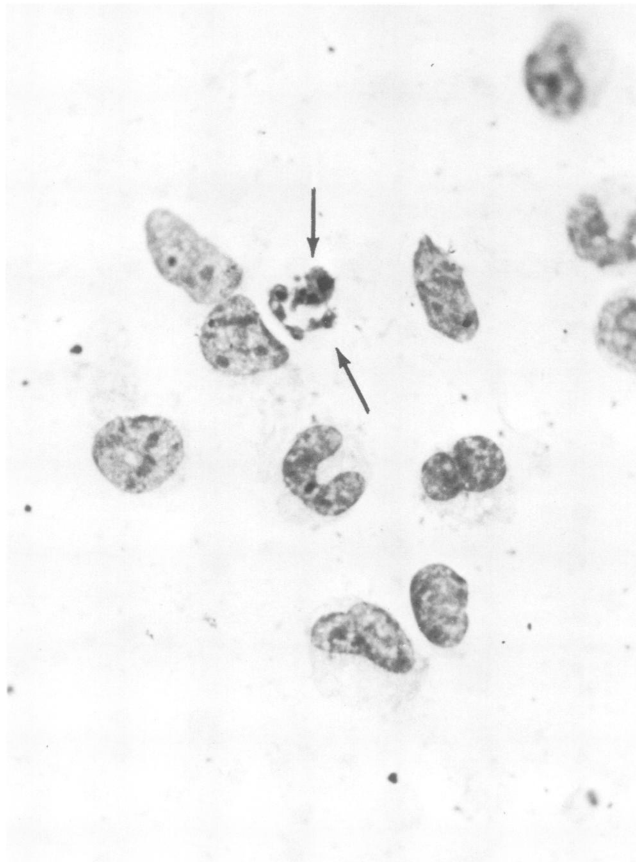

FIG. 2. *Peritoneal scraping prepared 6 days after Karp infection. The cells are predominantly monocytoid macrophages lacking prominent vacuolization. Organisms were not identified. Arrow indicates degenerating PMN. (Giemsa, ×1,000)*

creased until time of death. At no time were rickettsial organisms observed by light microscopy in these granulomas or elsewhere in the hepatic parenchyma.

Peritoneal surfaces. Only Karp-infected animals eventually evidenced a severe fibrinous peritonitis (Fig. 12).

DISCUSSION

Our results have shown a clear difference between the pathobiology of a nonlethal immunizing infection with Gilliam strain and lethal infection with Karp, as well as the moderating effect of prior Gilliam immunization on lethal Karp challenge. It must be understood, however, that we have purposely selected a small (100 MID_{50}) nonlethal dose of Gilliam for study because it provides effective heterologous immunization and allows the study of cross-immunity between strains of scrub typhus. If we had used a larger, lethal dose of Gilliam, it is possible that the pathological manifestations of infection would more closely resemble those seen with Karp.

The results presented confirmed the reports of others (7, 8) that intraperitoneal scrub typhus infection remains largely confined within the peritoneal cavity. However, two histological observations deserve mention before further discussion of the particular results: (i) the lymphocyte-macrophage interactions depicted in Fig. 3 have been previously described in vitro (peripolesis) (12) and in vivo (11) and are thought to represent afferent events in an immune response, probably related to the presentation of antigen by macrophages to immunocompetent lymphocytes (11); (ii) although organisms were not demonstrable within hepatic granulomas, it seemed likely that their appearance and growth were due to the presence of rickettsiae or their products. Such local proliferation of Kupffer cells has been observed in

the livers of mice stimulated by *Corynebacterium parvum* vaccine (2). However, in the absence of radioautographic and immunofluorescent confirmation, this remains speculative.

In animals infected with *R. tsutsugamushi*, two phenomena occurred in parallel: (i) those related to proliferation of rickettsia in macrophages and presumably in hepatic granulomas; and (ii) those related to concomitant immunization and splenic lymphoid hyperplasia. In the case of Gilliam-infected animals, the phenomena relating to rickettsial proliferation were minimal and transient. Thus, hepatic granulomas appeared briefly and regressed, and there was minimal and finite proliferation of rickettsia in peritoneal macrophages (Fig. 5). These findings were consistent with the host's ability to mount a sufficiently vigorous immune response to deal with the infectious burden. The cellular site of rickettsiacidal activity appeared to be the macrophage. This cell undergoes extensive morphological alterations during infection from the "inactive" monocytoid state (Fig. 2) to the "angry" macrophage (Fig. 5) (9), capable of destroying rickettsial organisms in its lysosomal complex.

Similarly, an immune response seems to be initiated in a lethal Karp infection, as is evidenced by splenic lymphoid hyperplasia. In contrast to the Gilliam infection, however, rickettsial proliferative phenomena in peritoneal macrophages (Fig. 4) and the number and size of hepatic granulomata (Fig. 9 to 12) continued to increase until death. These findings are consistent with a host immune response inadequate to deal with the proliferative effects of a highly lethal infection.

It is suggested that survival in scrub typhus infections in mice is the result of a delicate balance between the proliferation of the organism and the intensity of host's immune response. In support of this concept is the experi-

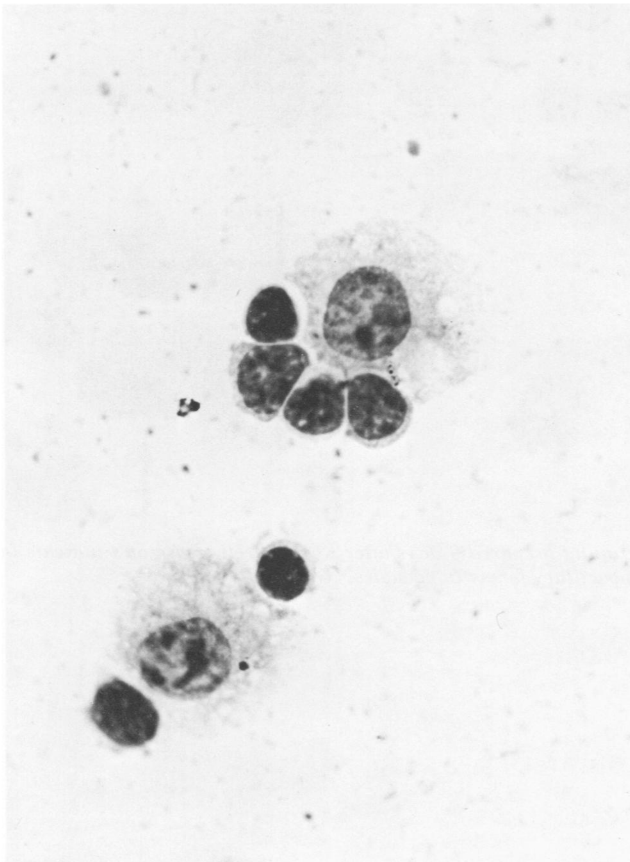

FIG. 3. *Peritoneal scraping prepared 3 days after Karp infection. Early in the infectious period with either Karp or Gilliam, intimate contact between lymphocytes and macrophages was observed but was not seen at later times. (Giemsa, ×1,000)*

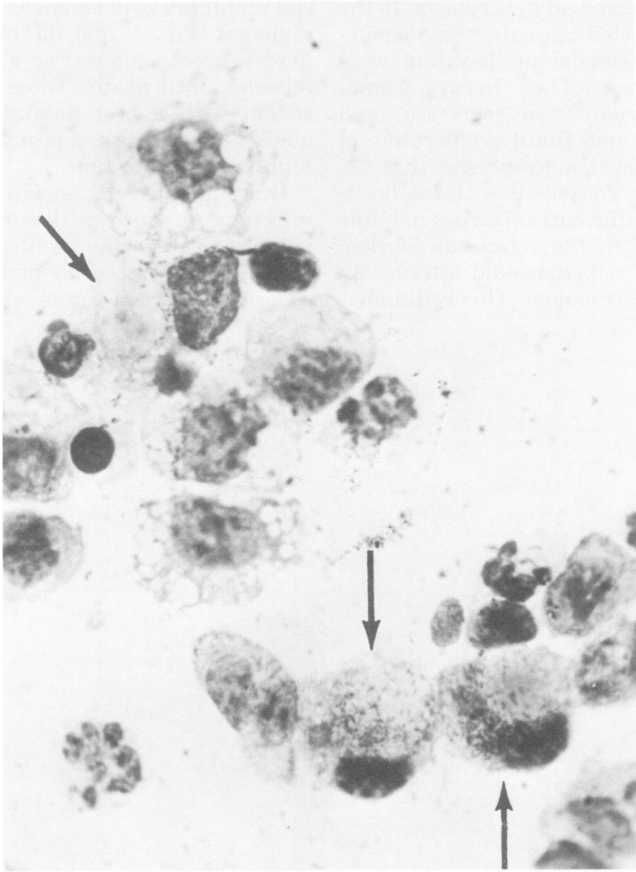

FIG. 4. *Peritoneal scraping prepared 9 days after Karp infection. Arrows indicate large activated macrophages containing coccobacillary forms in vacuoles. (Giemsa, ×1,000)*

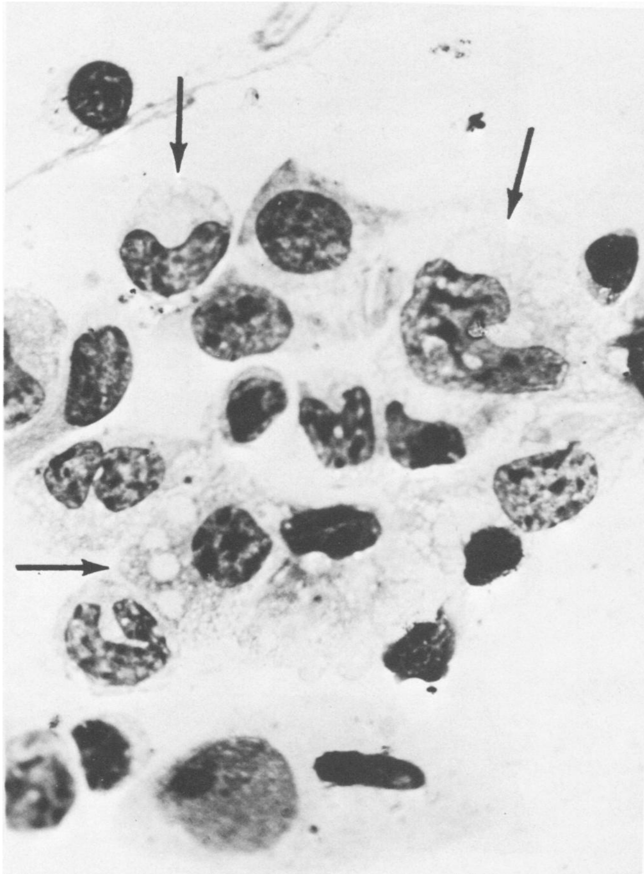

FIG. 5. *Peritoneal scraping prepared 13 days after Karp challenge of a Gilliam-immunized animal. Although organisms are not seen, the exudate contains large, highly vacuolated macrophages (arrows). (Giemsa, ×1,000)*

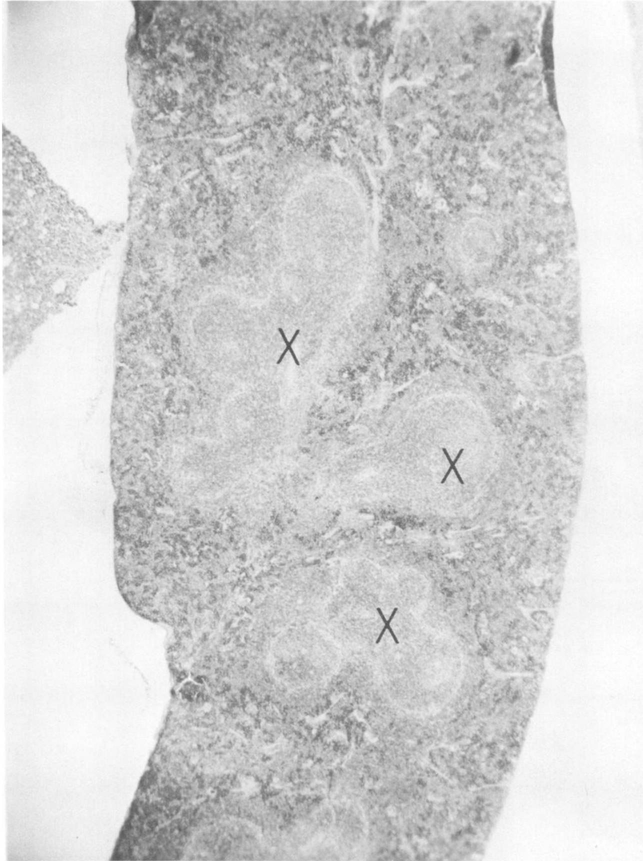

FIG. 6. *Low-power view of a spleen 1 day after Gilliam infection. White pulp area is marked by X. This histological appearance is indistinguishable from uninfected animals. (Hematoxylin and eosin, ×35)*

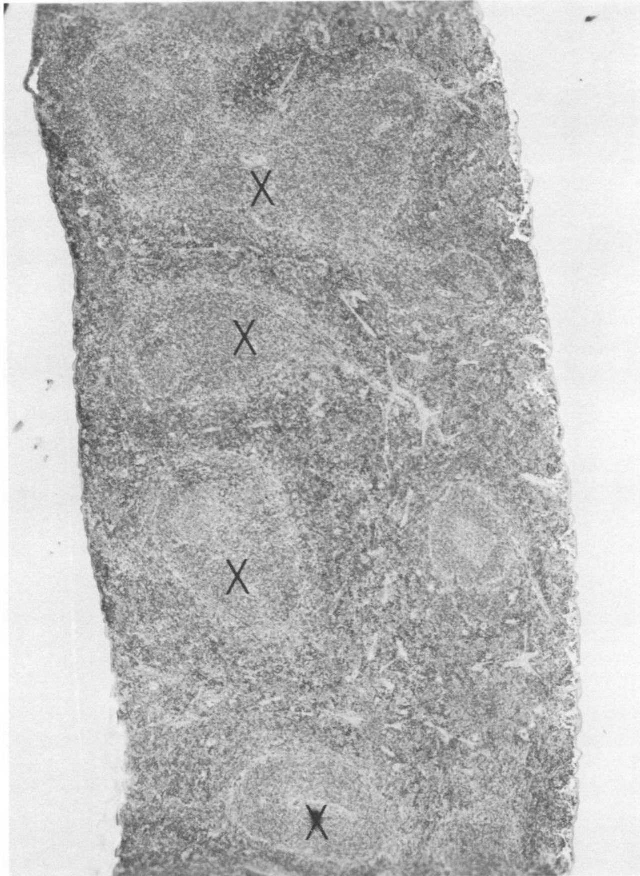

FIG. 7. *Low-power view of a spleen 3 days after Gilliam infection. Note the development of the white pulp (X) at the expense of the red pulp (compare with Fig. 6). (Hematoxylin and eosin, ×40)*

FIG. 8. *Low-power view of a spleen 6 days after Karp challenge of a Gilliam-immunized animal. The spleen is composed almost completely of white pulp. (Hematoxylin and eosin, ×40)*

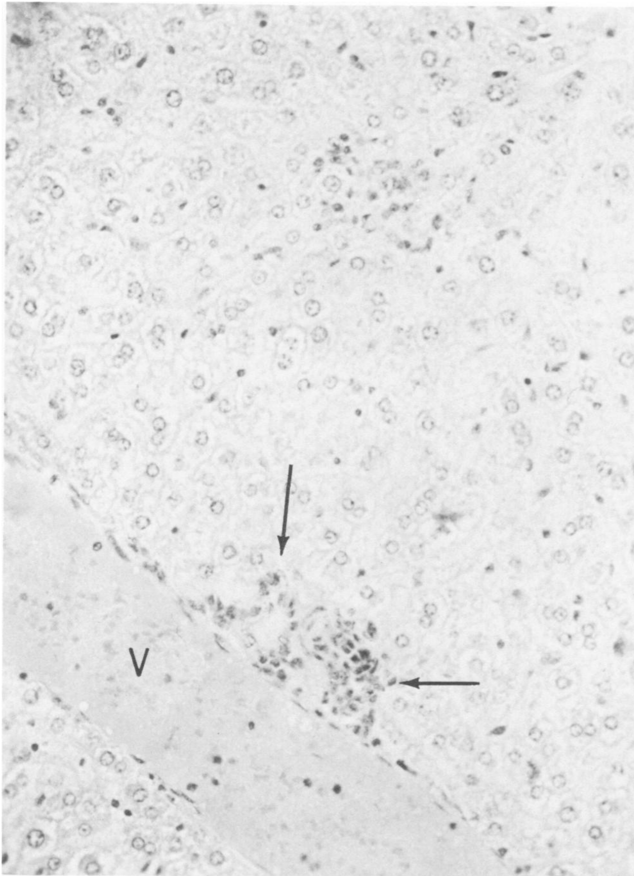

FIG. 9. *Liver 9 days after Gilliam infection. Arrows indicate relationship of "granuloma" to large hepatic vein (V). (Hematoxylin and eosin, ×250)*

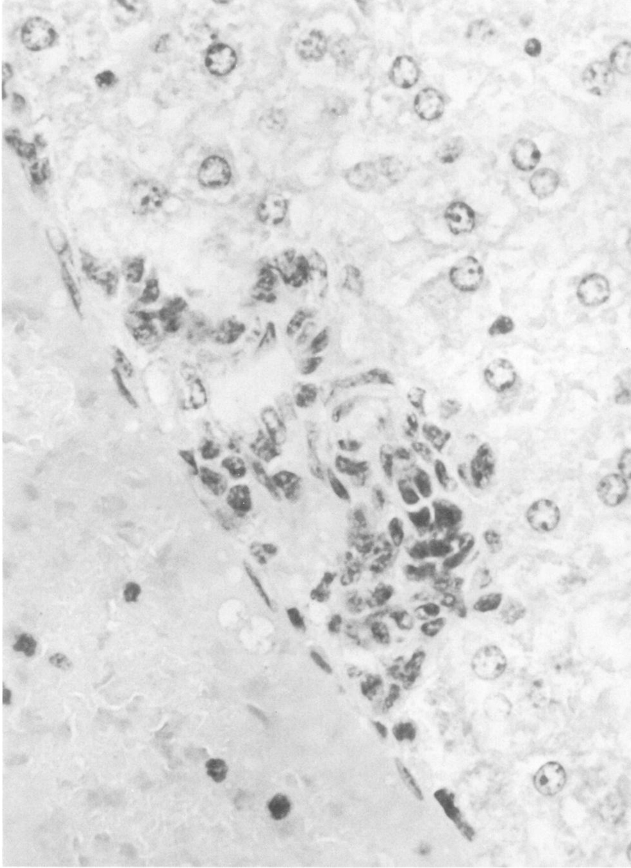

FIG. 10. *Higher-power view of Fig. 9. Granuloma consists of mononuclear cells, many of which appear to be Kupffer cells. Organisms were not seen in such lesions. (Hematoxylin and eosin, ×620)*

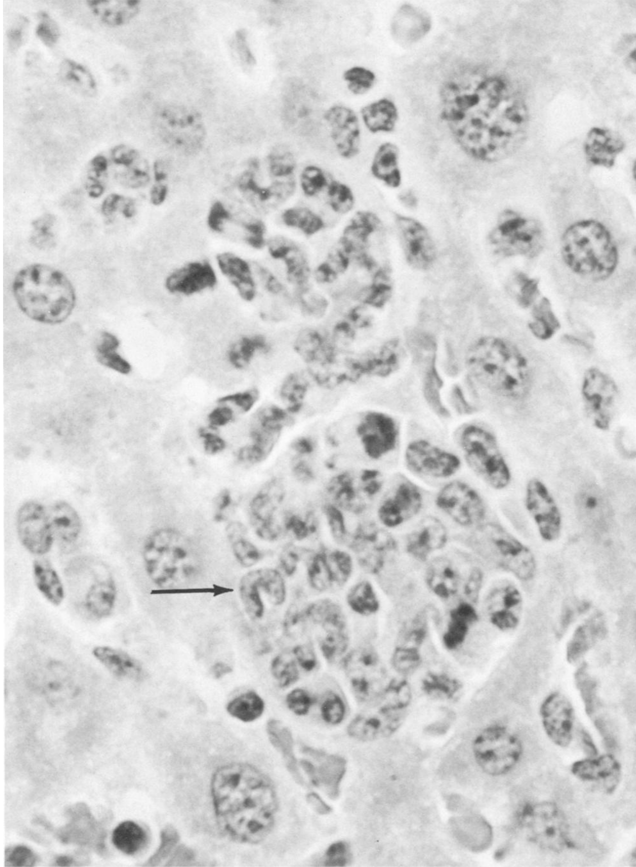

FIG. 11. *High-power view of typical hepatic granuloma 9 days after Karp infection. Note the conspicuous absence of PMNs. Some of the cells appear to be Kupffer cells, whereas others appear to be typical monocytoid macrophages (arrow). Organisms could not be demonstrated histologically. (Hematoxylin and eosin, ×1,020)*

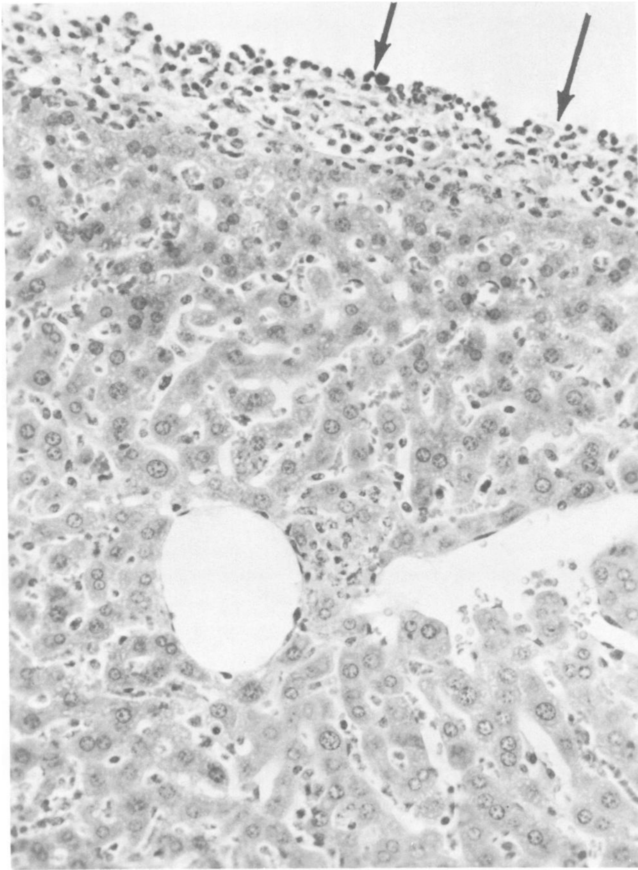

FIG. 12. *Peritonitis (arrows) is quite prominent on the surface of the liver 13 days after Karp infection. Such peritonitis is absent from animals infected with Gilliam and those immunized with Gilliam and subsequently challenged with Karp. (Hematoxylin and eosin, ×250)*

ment in which Gilliam-immunized mice were infected with Karp. Although an equally lethal dose of Karp was given to immune as well as to nonimmune animals, the immune animals displayed a rickettsial proliferative component similar to that observed with a nonlethal Gilliam infection. These immune animals, however, displayed the most striking splenic lymphoid hyperplasia observed in this series of experiments. This suggested that an extremely vigorous host immune response overcame the proliferative capacity of Karp. In this context, preimmunization with Gilliam seems to "prime" the immune system to produce and mobilize sufficient effector cells to deal with the lethal Karp challenge.

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