Effect of Immunosuppression on *Rickettsia rickettsii* Infection in Guinea Pigs

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The role of the immune response in the pathogenesis of *Rickettsia rickettsii* infection in guinea pigs was investigated by immunosuppression, using antilymphocyte serum. Twenty guinea pigs were inoculated with *R. rickettsii*, Sheila Smith strain, on day 0. Fifteen animals received antilymphocyte serum on days −1, 0, 2, 4, and 6. Five animals received normal rabbit serum on the same schedule. At necropsy, specimens were collected for histological examination, rickettsial immunofluorescence, rickettsial titration, and antirickettsial antibody titration. All normal rabbit serum recipients and 12 of 15 antilymphocyte serum recipients developed typical disease. Comparison of animals in terminal stages of disease revealed the same clinical course and gross lesions, but differing rickettsial burden and cellular response. Immunosuppressed animals had higher titers of splenic rickettsiae and greater numbers of immunofluorescent rickettsiae. Thus, although antibody was undetectable in both groups, there appeared to be an inhibition of antirickettsial immunity. Microscopic vasculitis was similar quantitatively, but differed qualitatively, with immunocompetent animals having the typical mononuclear/lymphocytic inflammation and immunosuppressed animals having neutrophilic predominance. This study demonstrates that immunopathological mechanisms are not necessary for the pathogenesis of experimental Rocky Mountain spotted fever. The rickettsiae themselves seem capable of causing cellular and tissue damage.

The clinical symptoms and pathological lesions in human Rocky Mountain spotted fever (RMSF) and experimental infection of guinea pigs with *Rickettsia rickettsii* are primarily manifestations of injury to blood vessels. Immunopathological mechanisms for production of cellular and tissue injury to vessel walls were suggested by morphological analogy of the lesion to immune complex vasculitis, clinical similarity of RMSF and serum sickness (1, 7), presence of circulating rickettsiae and antibodies (8), and demonstration of immunoglobulin and the third component of complements late lesions in experimentally infected guinea pigs (3). The present experiments were performed (i) to test the hypothesis that immune mechanisms (cell mediated and/or antigen-antibody-complement mediated) contribute to the development of the observed vascular lesions and (ii) to begin to delineate the role of cell-mediated immunity in controlling the replication of rickettsiae in the infected host. This paper presents the results achieved with an immunosuppressive regimen of antilymphocyte serum (ALS).

**MATERIALS AND METHODS**

**ALS.** Anti-guinea pig lymphocyte sera produced in rabbits (purchased from Microbiological Associates, Bethesda, Md., lot numbers 13164 and 14270) were tested for immunosuppressive activity. Three guinea pigs were immunized with 2 × 10^6 washed sheep erythrocytes (SRBC) inoculated intraperitoneally on day 0. Each animal received a course of normal rabbit serum (NRS) or one of the lots of ALS given intraperitoneally on days −1, 0, 2, and 4 in 2-ml doses. When the animals were bled and tested on day 6 after SRBC immunization, serum from the NRS recipient had a 1:64 titer of hemolytic antibody to SRBC, and sera from the two ALS recipients had no detectable hemagglutinating or hemolytic antibody to SRBC.

*Rickettsiae.* *R. rickettsii*, Sheila Smith strain, was obtained as an infected yolk sac suspension from Charles C. Shepard, Virology Division, Bureau of Laboratories, Center for Disease Control, Atlanta, Ga. The rickettsial strain was further passed once in guinea pigs, which were sacrificed on day 3 of fever. A 50% blood-spleen suspension was made in sucrose-phosphate-glutamate (2) and frozen at −70°C in multiple portions. A 10^-2 dilution of the original spleen-blood pool (1/50 of 50% suspension) was prepared in sucrose-phosphate-glutamate solution for animal inoculation.

**Experimental design.** Twenty adult male guinea pigs, Hartley strain, 450 to 600 g, were divided as follows (Table 1). Ten animals (group 1) were inoculated intraperitoneally with 1.0 ml of *R. rickettsii* suspension on day 0 and given 2.0 ml of ALS (lot
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13164) intraperitoneally on days −1, 0, 2, 4, and 6. Five animals (group 2) were inoculated with the same rickettsial and ALS (lot 14270) doses and schedule. Five animals (group 3) were inoculated with the same rickettsial dose and 2.0 ml of normal rabbit serum intraperitoneally on days −1, 0, 2, 4, and 6. One control animal received ALS from each lot of ALS with the same dose and schedule as the other animals without rickettsial inoculation (group 4). The animals were observed daily, and rectal temperatures were taken. When moribund, guinea pigs were sacrificed, and specimens were collected for histopathology, demonstration of rickettsiae by immunofluorescence, electron microscopy, rickettsial plaque assay, and rickettsial serology. Two ALS recipients were sacrificed while still febrile prior to entering the moribund state.

**Histology.** Specimens of scrotal skin, lymph node, spleen, liver, heart, lung, kidney, adrenal, brain, epididymis, testis, and cremaster muscle were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned at 6 µm, and stained with hematoxylin-eosin and Giemsa.

**Immunofluorescence.** Specimens of spleen and epididymis were embedded in a polyethylene glycol compound (OCT, Ames Co., Division of Miles Laboratories, Elkhart, Ind.) and were frozen on dry ice. Frozen sections (5 µm) were cut, fixed in two changes of acetone for 10 min each, and stored at −20°C. Fluorescein-conjugated rabbit anti-R. rickettsii globulin fraction (obtained from Theodore Tzianibos, Bureau of Laboratories, Center for Disease Control) was reacted with sections by a direct immunofluorescent technique (15). Tests for conjugate specificity have been documented previously (15).

Sections were washed in phosphate-buffered saline, dipped in distilled water, mounted in a 90% glycerol-10% saline solution, and examined with a Leitz ultraviolet microscope using fluorescein isothiocyanate exciter and barrier filters. Nonspecific fluorescence was not observed in guinea pig tissues, and absorption of conjugate was not required.

**Electron microscopy.** Blocks of epididymis and cremaster muscle (1 mm³) were dissected and fixed in 4% formaldehyde-1% glutaraldehyde. Tissues were washed in 0.1 M Sorensen phosphate buffer, postfixed in 2% osmium tetroxide, dehydrated in graded ethanol series, embedded in a mixture of Epon and Araldite (10), sectioned, stained with uranyl acetate and lead citrate (14), and examined on a Zeiss 10A electron microscope.

**Rickettsial titration.** Rickettsiae were titrated by plaque assay according to the technique of Wike et al. (16). Samples of spleen were ground with mortar and pestle and serially diluted from 1 to 10⁻⁴ in 3.7% brain heart infusion. Suspensions (0.1 ml) were inoculated into 25-cm² flasks containing antibiotic-free primary duck embryo cell monolayers. After absorption for 2 h, monolayers were covered with 5 ml of nutrient overlay containing medium 199, 5% fetal calf serum, and 1% agarose (Sea Kem, Marine Colloids, Inc., Springfield, N.J.). Flasks were incubated at 32°C for 7 days prior to a second overlay with 5 ml of nutrient agar containing 0.01% neutral red. After 24 h of further incubation, plaques were counted.

**Serology.** Specific complement-fixation (CF) titers for antibodies to R. rickettsii were performed by Martha Redus, Leprosy and Rickettsia Branch, Virology Division, Center for Disease Control (13). Indirect fluorescent (IF) antibody testing was performed according to a modification of the microimmunofluorescence test of Phillip et al. (11). The assay employed acetonet-fixed microdots of a 1/20 suspension of yolk sac containing R. rickettsii in phosphate-buffered saline and 30-min washes after serum incubation for 10 min and after incubation for 30 min with anti-guinea pig immunoglobulin conjugate (Wellcome, lot K0623). Serum containing CF antibody at a 1:1,024 titer and serum with no detectable CF antibody demonstrated titers of 1:1,024 and <1:4, respectively, by IF antibody. Conjugate control was negative.

**Guinea pigs deficient in C4.** Five male retired breeders (850 to 1,100 g) deficient in the fourth component of complement (C4) (5, 6) as a congenital autosomal recessive trait were obtained from the animal facility of the National Institutes of Health. They were inoculated intraperitoneally with the same doses and strain of R. rickettsii as the previous animals. Daily observations, rectal temperatures, and postmortem gross and microscopic examinations were performed.

**RESULTS**

**Clinical course and gross pathology of ALS experiment.** Both groups of ALS-treated animals and the NRS-treated animals which were infected with R. rickettsii developed essentially identical clinical onset of fever, lethargy, anorexia, scrotal reaction, and moribund state or death.

| Table 1. Response of guinea pigs to ALS and R. rickettsii |
|---------------------------------|
| **Group** | **Treatment** | **No. of animals** | **No. of typical febrile disease** | **Outcome** |
| 1 | ALS/R. rickettsii | 10 | 8 | Two died on day 9, 3 sacrificed moribund on day 9, 2 sacrificed febrile on day 9, 2 sacrificed moribund on day 10, 2 afebrile course |
| 2 | ALS/R. rickettsii | 5 | 4 | Four sacrificed moribund on day 9, 1 afebrile course |
| 3 | NRS/R. rickettsii | 5 | 5 | One died on day 9, 2 sacrificed moribund on day 9, 2 sacrificed moribund on day 10 |
| 4 | ALS | 2 | 0 | Two afebrile course |
Eight of ten animals in group 1, four of five animals in group 2, and all five NRS recipients developed typical febrile disease (Table 1). At necropsy, gross edema, congestion, and hemorrhage were observed in the scrotal skin and focally in epididymis in all infected animals. Other findings included conjunctivitis, multiple focal hepatic infarcts, and dehydration. These findings appeared identical in all three groups of animals.

**Rickettsial titers, immunofluorescence, and serology of ALS experiment.** Comparison of rickettsial titers in spleens of animals which were sacrificed in a moribund, subthermic state or had died just recently and thus represent the same terminal stage of infection revealed that all five of the ALS recipients' spleens which were assayed contained more rickettsiae than did the spleens of all four NRS recipients which were assayed (Table 2). Immunofluorescent examination of spleen and epididymis from both groups of ALS recipients demonstrated many more rickettsiae in epididymal vessels (Fig. 1) and spleen than in NRS recipients. The rickettsial infection in NRS animals was usually focal and did not contain striking numbers of orga-

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**Table 2. Tilters of R. rickettsii in spleen at terminal stage of infection**

| Animal no. | Treatment | Rickettsial titer\(a\) (PFU/\(g\)) |
|------------|-----------|----------------------------------|
| 1          | NRS\(c\)  | \(2 \times 10^6\)                |
| 2          | NRS       | \(3 \times 10^6\)                |
| 3          | NRS       | \(8 \times 10^6\)                |
| 5          | NRS       | \(2 \times 10^6\)                |
| 7          | ALS\(d\)  | \(9 \times 10^6\)                |
| 9          | ALS       | \(1 \times 10^7\)                |
| 12         | ALS       | \(2 \times 10^7\)                |
| 13         | ALS       | \(4 \times 10^7\)                |
| 14         | ALS       | \(4 \times 10^7\)                |

\(a\) ALS recipients have greater rickettsial titer than NRS recipients by Mann-Whitney two-sample test (\(P = 0.008\)).

\(b\) PFU, Plaque-forming units.

\(c\) Two milliliters on days -1, 0, 2, 4, and 6 (group 3).

\(d\) Two milliliters on days -1, 0, 2, 4, and 6 (group 1).

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**Fig. 1.** Epididymal blood vessels from guinea pig which received ALS and was sacrificed on day 9 after inoculation contained numerous *R. rickettsii* demonstrated by specific direct immunofluorescence. Fluorescein-isothiocyanate conjugate. \(\times280\).
nisms. In contrast, ALS recipients had larger foci of infection, and the infected vessels contained larger quantities of rickettsiae in their walls and endothelium.

Antibody to *R. rickettsii* was not detected in the CF or the IF antibody test in serum samples collected from all groups of animals when sacrificed moribund on day 9 or 10 after inoculation.

**Histopathology of ALS experiment.** Quantitatively, the number of epididymal vessels involved and the degree of vascular damage in all terminal ALS and NRS animals were similar. Vasculitis was characterized by heavy perivascular and moderate intramural cellular infiltration, focal perivascular and intramural fibrin deposition, and areas of intraluminal thrombosis which varied from a thin lining of the luminal surface to total occlusion. The cellular population in the ALS recipients' vasculitis differed strikingly from that of the NRS recipients in cell types. Whereas the NRS animals contained predominantly the typical mononuclear cell component consisting of lymphocytes, macrophages, and unidentifiable mononuclears with few polymorphonuclear leukocytes (Fig. 2A), the cellular element of the vasculitis in the ALS animals was characterized by predominantly polymorphonuclear leukocytes with rare mononuclear inflammatory cells (Fig. 2B). ALS animals which were sacrificed when still febrile prior to the moribund state demonstrated an earlier stage of this process with milder polymorphonuclear leukocytic infiltration of the vessel walls.

Splenic periarteriolar lymphocytic sheaths of animals treated with ALS showed marked depletion of lymphocytes in comparison with spleens of NRS-treated animals.

**Ultrastructural observations of ALS experiment.** The findings in NRS animals were

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**Fig. 2.** Histopathological lesions in guinea pigs infected with *R. rickettsii*. (A) Epididymal venules and artery from control guinea pig which received normal rabbit serum and was sacrificed on day 10 after inoculation with *R. rickettsii*. Typical terminal vasculitis with perivascular and intramural lymphocytes and other mononuclear cells. (B) Epididymal vessel from guinea pig which received ALS and was sacrificed on day 9 after inoculation with *R. rickettsii*. Severe vasculitis with massive infiltration of vascular wall and perivascular tissue with predominantly polymorphonuclear leukocytes virtually effaced architecture of blood vessel. Hematoxylin-eosin. ×240.
Fig. 3. Electron micrograph of epididymal blood vessel from guinea pig which received ALS and was sacrificed on day 9 after inoculation with R. rickettsii. There was vasculitis. Rickettsiae were observed within cytoplasm (c), cytoplasmic invaginations into nucleus (i), and nucleus (n). Deposition of fibrin (F) between cells and a swollen mitochondrion (M) of an infected cell were demonstrated. Lead citrate and uranyl acetate. ×20,000.
similar to those reported previously in the unmodified infection (15). The vasculitis in ALS guinea pigs was similar in most aspects, including intracytoplasmic rickettsial infection, host cell cytopathology, and intramural deposition of fibrin (Fig. 3). Although most apparently intranuclear rickettsiae were actually within cytoplasmic invaginations, examples of true intranuclear rickettsial infection were observed.

Complement-deficiency experiment. All five C4-deficient guinea pigs inoculated with R. rickettsii developed fever and died on day 7 postinoculation. At necropsy, the gross and microscopic pathological lesions were entirely characteristic of infection with R. rickettsii. The vasculitis observed in epididymis demonstrated no unusual microscopic features.

DISCUSSION

Failure to ameliorate the course of experimental RMSF in guinea pigs by abrogation of immunopathological mechanisms of cellular and tissue damage was demonstrated using two different lots of ALS in proven immunosuppressive doses. Furthermore, absence of mononuclear leukocytes (lymphocytes and macrophages) in the lesion of immunosuppressed animals argues against the possibility of cell-mediated immunopathogenesis such as occurs in acute murine lymphocytic choriomeningitis. Failure to detect humoral antibody to R. rickettsii in any group of animals makes antibody-dependent mechanisms of immunopathological damage unlikely. Although CF and IF may not be sensitive enough to detect very small quantities of antibody, inhibition of immune response was clearly demonstrated in the increased numbers of rickettsiae in splenic titrations and in epididymal and splenic rickettsial immunofluorescence. In fact, the morphological anatomy of vasculitis in RMSF is characterized by mononuclear inflammatory reaction; immune complex disease, with its release of chemotactic factors, results in predominantly a polymorphonuclear leukocyte response. Likewise, the course of disease in guinea pigs deficient in C4 confirms that the classic complement pathway is not necessary for the disease process; however, the alternate properdin pathway cannot be excluded. Moreover, the report by Moe et al. of minimal depletion of total hemolytic complement in experimentally infected guinea pigs throughout the course of infection, before and during the period of demonstrable rickettsemia, circulating antirickettsial antibody, and histopathological vasculitis, is further evidence against immune complex-mediated vasculitis (9).

Histopathological and ultrastructural demonstration of severe vasculitis in immunosuppressed animals with deposition of fibrin in vessel walls not only argues against the role of immunopathology in the pathogenesis but also suggests that the leakage of plasma proteins including immunoglobulins and complement into the vessel wall is nonspecific and unrelated to pathogenetic mechanisms.

It would appear that rickettsiae may damage cells without immune mechanisms and that understanding this process may be facilitated by scrutiny of the rickettsia-host cell interaction in vitro. There too, rickettsiae lethally injure cells with no complex host-directed process such as immune response or Shwartzman phenomenon, as shown in the plaque assay for titration. Elucidation of the mechanisms of host cell injury will require an understanding of penetration, metabolic competition, energy parasitism, and release mechanisms at fine structural and biochemical levels.

The demonstration that tissues of ALS-treated R. rickettsii-infected guinea pigs contained more rickettsiae than controls suggests that cell-mediated immunity may play a significant role in control of R. rickettsii infection in guinea pigs; this immune mechanism has been implicated in control of R. tsutsugamushi in mice (4, 12). Further study into the mechanisms of immunity in intracellular infection with R. rickettsii is warranted.

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