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Rickettsia australis Infection: A Murine Model of a Highly Invasive Vasculopathic Rickettsiosis

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A mouse model of spotted fever group rickettsiosis, in which disease results from disseminated rickettsial infection of endothelial cells and vascular damage, was developed by intravenous inoculation of 6- to 8-week-old, male, Balb/c mice with* Rickettsia australis. *Animals developed progressively severe vasculitis, interstitial pneumonia, and multifocal hepatic necrosis. These lesions correlated with early disseminated infection of endothelial cells followed by growth and invasion of rickettsiae into perivascular cells. The dose of 2 × 10^6 organisms was uniformly lethal. Serum interleukin- (IL) 1, IL-6, and interferon (IFN) increased by day 3 and tumor necrosis factor (TNF) on day 5. TNF, IL-6, and IFN declined on day 7. Spleen cells responded to* Rickettsia australis *antigen by producing IFN, TNF, IL-1, and IL-6 on day 5, followed by lower quantities of these cytokines on day 7. Despite the production of antibodies, IFN, TNF, IL-1, and IL-6, a lethal outcome occurred frequently. A decreased ability to secrete IL-2 suggests an element of infection-associated immunosuppression. (Am J Pathol 1993, 142:1471–1482)

Acute vascular injury, the proposed pathological consequence of many diseases, is exemplified in rickettsial infections. Disseminated endothelial infection and damage are the hallmarks of rickettsioses of the spotted fever and typhus groups.[1–4] The etiological microorganisms are small, genetically related, obligate intracellular bacteria, which are transmitted by ticks, mites, fleas, and lice.[5] Among these agents, *Rickettsia australis*, the cause of Queensland tick typhus, is one of the least studied. Recent recognition that this disease has increased in incidence, has a wider geographic distribution than previously known, and is capable of causing lethal human illness has focused enhanced attention on *R. australis*.[6–12]

Animal models for human spotted fever group and typhus group rickettsioses have major deficiencies, including utilization of mammalian species for which few immunological reagents are available (e.g., guinea pig, cotton rat), incorrect target cells (i.e., macrophages instead of endothelial cells), and incorrect pathological lesions (i.e., peritonitis instead of vasculitis).[13–16] Most animal models are based upon intraperitoneal inoculation of guinea pigs or inbred mice. The ensuing events are principally the results of rickettsial infection of peritoneal lining cells. Exceptionally, *R. rickettsii* causes significant vascular invasion from the peritoneal cavity of guinea pigs with hematogenous dissemination to establish many distant foci of endothelial infection. Inoculation of *R. conorii* into the dermis of guinea pigs causes dermal endothelial infection and the formation of a typical eschar with minimal dissemination.[17] Reports of attempts to develop models of experimental rickettsial infections in mice have been limited to illness associated with intraperitoneal infections of mice of selected inbred strains or under specific conditions (e.g., newborn, weanling, or vitamin-deficient animals) with *R. conorii*, *R. akari*, *R. sibirica*, and *R. australis*.[18–22] Because intracutaneous inoculation did not cause fatal disease in preliminary studies, the dermal events were bypassed by direct intravenous inoculation of *R. australis*.

In this study, *R. australis* established disseminated intracellular endothelial infection and caused pathological lesions in the testis, lung, and liver similar to what is observed in human Rocky Mountain spotted fever and boutonneuse fever. It provides an attractive model of rickettsial invasiveness, cytokine responses in rickettsial infection, and progressive acute vascular injury. The availability of commercial reagents for mouse immunological assays makes this species a particularly useful model.

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Materials and Methods

Rickettsiae

R. australis (Cutlack strain) was obtained from C. Pretzman (Department of Health Laboratory, Columbus, OH) and was passaged twice in Vero cells and twice in specific pathogen-free embryonated chicken egg yolk sac in our laboratory. The rickettsial stock was mycoplasma-free.

Experimental Design

Thirty-six 6- to 8-week-old male Balb/c mice (Harlan Sprague-Dawley, Indianapolis, IN) were randomly divided into three groups. Groups 1 and 2 were inoculated intravenously with 1×10^5 pfu and 2×10^6 pfu/mouse, respectively, of *R. australis*. Group 3 served as uninoculated controls. On days 3, 5, and 7 after inoculation, four mice from each group were exsanguinated, and on day 7, surviving mice were sacrificed. The organs from the animals that were sacrificed or died, including spleen, liver, kidney, heart, lung, testis, and epididymis, were removed and divided into portions. A portion of each tissue was fixed in 4% neutral buffered formaldehyde for paraffin embedding and histopathological examination. A portion of testis was fixed in 2% paraformaldehyde and 2% glutaraldehyde for electron microscopy. Half of the spleen was placed in Dulbecco's minimal essential medium for the immunological tests. The blood samples from each group of mice were pooled, and the sera were separated, aliquoted, and stored at −80 C until used.

Histology and Immunoperoxidase Studies

Organs fixed in 4% neutral buffered formaldehyde solution were infiltrated with paraffin, sectioned at 4 μ, stained by hematoxylin and eosin, and examined by light microscopy. Simultaneous demonstration of histopathology and immunohistological detection of rickettsiae by light microscopy was performed using a modified immunoperoxidase method.[23] Briefly, rehydrated 4-μ paraffin tissue sections were digested with pronase E (1 mg/ml, Sigma Chemical Co., St. Louis, MO) for 30 minutes at 37 C, and proteolysis was terminated by immersion in 95% ethanol. Endogenous peroxidase activity was quenched by immersion of slides into 3% H_2O_2 solution in phosphate-buffered saline (PBS) for 15 minutes. After washing in PBS and blocking in PBS with 1% bovine serum albumin and 5% normal goat serum (blocking buffer) for 10 minutes, slides were incubated for 1 hour at room temperature in rabbit hyperimmune anti-spotted fever group rickettsial serum diluted 1:32,000 in blocking buffer. Preimmune rabbit serum was used in parallel for random tissue sections. After washing, tissue sections were then incubated in biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), washed, treated with avidin-horseradish peroxidase solution (Dako, Carpinteria, CA), and washed again. Bound enzymatic activity was revealed by a 10-minute incubation in either diaminobenzidine (0.5 mg/ml) or aminoethylcarbazol (0.25 mg/ml) substrate, followed by a water wash, Mayer's hematoxylin counterstain, and final water wash. Sections were mounted with crystal mount (Biomeda, Foster City, CA), and when dry, they were coverslipped.

Electron Microscopy

The fresh specimens were fixed immediately in a cacodylate-buffered solution containing 2% paraformaldehyde and 2% glutaraldehyde and post-fixed in 1% osmium tetroxide. After dehydration with a series of graded ethanol concentrations, the specimens were embedded in Polybed 812 resin. Thin sections were cut with a Sorvall MT-6000 ultramicrotome and poststained with uranyl acetate and lead citrate. The specimens were examined and photographed on Philips 201 electron microscope.

Spleen Cell Stimulation

The spleens of each group were pooled, and single cell suspensions were prepared after washing twice at 400*g* for 10 minutes at 4 C. The concentration of the spleen cells was adjusted to 5×10^6/ml, and they were stimulated by incubation with 100 μg/ml of *R. australis* antigen, 5 μg/ml of concanavalin A (Con A), 100 μg/ml of Vero cell antigen, or medium. The spleen cell supernatants were harvested at 24 or 48 hours (after Con A stimulation) or 24 and 72 hours (after medium control and antigen stimulation), aliquoted, and stored at −80 C until used.

Rickettsial Antigen

Vero cell monolayers were inoculated with infected yolk sac suspension diluted in sucrose-phosphate-glutamate buffer (SPG; 0.218 mol/L sucrose, 0.0038 mol/L KH_2PO_4, 0.0072 mol/L K_2HPO_4, 0.0049 mol/L monosodium L-glutamic acid, pH 7.0) containing 3×10^5 pfu/ml of *R. australis*. After incubation at 34

C for 7 to 10 days, the cells were removed by scraping and centrifuged at 25,900*g* for 30 minutes at 4 C. The pellet was resuspended in SPG, and the suspension was centrifuged at 400*g* for 10 minutes, after which the supernatant was removed and saved. The pellet was resuspended in SPG and sonicated in an ice-bath (Braun-sonic 2000, output 28 watts) for 50 seconds, followed by centrifugation at 400*g* for 10 minutes. The two supernatants were combined and centrifuged at 25,900*g* for 30 minutes, and the supernatant was discarded. The subsequent pellet was resuspended in SPG, and the protein concentration was measured by bicinchoninic acid (BCA) protein assay method (Pierce, Rockford, IL). Vero cell antigen was prepared in the same manner as for the rickettsial antigen except that uninfected Vero cells were used.

Cell Lines

The interleukin- (IL) 2-dependent murine cell line, CTLL-2, IL-2- and IL-4-dependent cell line, HT-2, interferon- (IFN) γ sensitive B cell lymphoma cell line, WEHI 279, IL-1 reactive cell line, LBRM-TG6, IL-6-dependent cell line, 7TD1, and Vero cells (African green monkey kidney) were obtained from American Type Culture Collection.

IL-1 Assay

The assay for IL-1 was performed as described by Larrick et al.[24] Briefly, 1×10^5 LBRM-TG6 cells and 1.5×10^4 HT-2 cells and 1.0 µg phytohemagglutinin in 100 µl of medium containing Dulbecco's minimal essential medium, 5% fetal calf serum, 10 mmol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, and 50 µg/ml gentamicin were mixed with serial threefold dilutions of human recombinant IL-1 standards (Boehringer Mannheim Corp., Indianapolis, IN) or test samples beginning with a 1:3 dilution of stimulated spleen cell supernatant and a 1:1 dilution of serum and incubated overnight. On the next day, hypoxanthine (10^{-4} mol/L) and azaserine (10 µg/ml, final concentration) were added. After 4 to 6 hours of incubation, each well was pulsed with 1 µci [^{3}H]thymidine/well. Four hours later the cells were harvested, and [^{3}H]thymidine incorporation was measured.[24]

IL-2 Assay

The IL-2 assay was based upon the proliferation of the IL-2-dependent murine cell line, CTLL-2.[25] Briefly, 2×10^4 CTLL-2 cells/well were added to serial threefold dilutions of recombinant IL-2 standards or test samples beginning with a 1:1 dilution of stimulated spleen cell supernatant and a 1:3 dilution of serum. After incubation for 48 hours, 0.5 µci of [^{3}H]-thymidine was added to each well, and 6 hours later the cells were harvested and counted with a β counter.

IL-6 Assay

The IL-6-dependent cell line, 7TD1, was used for quantification of IL-6.[26] Serial threefold dilutions of samples (50 µl/well), beginning with 1:10 dilutions of spleen cell supernatant and serum, were mixed with 3×10^3 7TD1 cells/well, and the mixtures were incubated at 37 C in 5% CO_2 for 90 hours and then pulsed with 0.5 µci of [^{3}H]thymidine/well. After an additional 6 hours, the cells were harvested and counted in a β counter.

Tumor Necrosis Factor (TNF) Assay

An enzyme-linked immunosorbent assay (ELISA) and a bioassay were used. The ELISA test was performed according to the manufacturer's recommendations (Mouse Tumor Necrosis Factor ELISA Kit, Genzyme Corp., Boston, MA). The bioassay for TNF was performed as previously described.[27] Briefly, a confluent monolayer of L-929 cells was established by overnight incubation of 2×10^4 cells per well in a 96-well plate. Twofold dilutions of test samples beginning with a 1:2 dilution of serum and a 1:1 dilution of stimulated spleen cell supernatant or mouse recombinant TNF-α (Genzyme) were added to the monolayer (0.1 ml/well), and each well was adjusted to contain 0.25 µg of actinomycin D (Sigma). The plates were incubated at 37 C in a 5% CO_2 atmosphere for 24 hours, then washed three times with warm serum-free medium, fixed with 10% neutral buffered formalin, and stained with 1% crystal violet. The optical density of the wells was measured at 540 nm in an ELISA reader after adding 100 µl of 100% methanol.

IFN Assay

The microtiter assay of cytopathic effect was used to quantitate IFN.[28] A confluent L-929 cell monolayer was prepared as for the TNF assay. One hundred µl of each sample that was serially diluted, beginning with 1:3 dilutions of stimulated spleen cell supernatant and of serum, were incubated with a

monolayer of cells overnight; 100 $TCID_{50}$ of vesicular stomatitis virus (a gift of Dr. Judith Aronson, University of Texas Medical Branch at Galveston) were added to each monolayer in 0.1 ml. The cytopathic effect was monitored microscopically until the virus control showed 50% cytopathic effect in the 100-fold dilution of virus used for that experiment (generally about 48 hours after the virus was added). To ascertain that the activity represented that of IFN-γ, the samples were heated at 56 C for 30 minutes or were dialyzed in 0.2 mol/L glycine adjusted to pH 2 with HCl, 0.15 mol/L NaCl final concentration, at 4 C overnight, and then dialyzed against PBS (pH 7.4) for 4 hours.[29] These treated samples were filtered through 0.22-μ filter membranes before analysis of acid-stable IFN activity.

Calculations

All of the samples were tested in duplicate or triplicate. One unit of IL-1 and IL-6 was defined as the reciprocal of the dilution of samples containing 50% of the maximum stimulatory activity of the standard. One unit of IFN activity was defined as the concentration that caused 50% inhibition of the cytopathic effect induced by vesicular stomatitis virus.

Results

Course of Disease

All mice appeared mildly ill with somewhat ruffled fur on day 3 after inoculation. During the ensuing three days, all animals became progressively more severely ill with markedly ruffled fur, a hunched posture, and partially closed eyelids. The four remaining animals in group 1 survived until sacrificed on day 7. All the animals in group 2 that had not been sacrificed by day 5 died of the infection on day 6. None of the control animals became ill.

Immunohistological Localization of *R. australis*

The time course of rickettsial and pathological events, the intensity of infection, and severity of lesions differed between the groups of animals that were inoculated with lower and higher doses of *R. australis*. Among the mice inoculated with 10^5 pfu of *R. australis*, organisms were detected on day 3 of infection in multiple foci in moderate quantities in hepatic sinusoidal lining cells and in splenic macrophages, particularly in the sinusoids adjacent to the periarteriolar lymphocytic sheaths. Rickettsiae were present in small quantities in the pulmonary alveolar septa and in foci containing greater quantities of rickettsiae in endothelial cells and perivascular interstitial cells of the testis (Figure 1). Rickettsiae were also observed in endothelial cells of blood vessels in the peritesticular adnexal fat and in focal renal glomeruli.

On day 5 in mice inoculated with the lower dose, the number of foci containing rickettsiae and the quantity of rickettsiae in the foci in the liver and spleen diminished moderately compared with day 3. Some of the rickettsial antigen seemed to be in large cytoplasmic vacuoles of macrophages. In contrast, the quantity of rickettsiae increased moderately in the lungs and kidneys and increased substantially in the testis and its adnexal adipose tissue. The invasion of rickettsiae into the testicular perivascular and interstitial cells was remarkable.

On day 7 after inoculation, the hepatic and splenic infection was markedly reduced with less rickettsial antigen and few morphologically distinct rickettsiae. Infection persisted in pulmonary alveolar septa but was not detected in the kidneys. Further increase in the quantity of rickettsiae was observed in the testis and surrounding tissues with invasion of rickettsiae into the submucosa of the vas deferens, the seminiferous tubules, blood vessels in the cremaster muscle, and adnexal adipose tissue.

The mice inoculated with the higher dose of *R. australis* had a similar distribution of rickettsiae on day 3 after inoculation but with a greater number of foci of infection in the testis, lung, spleen, and liver and more extensive perivascular invasion in the testis than with the lower dose. No rickettsiae were detected in the kidney. On day 5, these mice had extensive rickettsial infection of the pulmonary alveolar septa (Figure 2) and exhibited multifocal infection of the renal glomeruli and interstitium. Focal infection was observed in the capillaries of the myocardium. In contrast with arrest of the infection in the reticuloendothelial organs of animals infected with the lower dose of rickettsiae, the mice infected with the higher dose showed progression of the splenic and hepatic infection with organisms predominantly in macrophages. Rickettsial invasion and proliferation was most pronounced in the testis and its adnexae. The testicular interstitium appeared to be virtually replaced by rickettsiae (Figure 3), and infection of adnexal blood vessels (Figure 4) and invasion of the submucosal cells of the vas deferens were prominent.

In the mice that died on day 6, overwhelming rickettsial proliferation and invasion was observed,

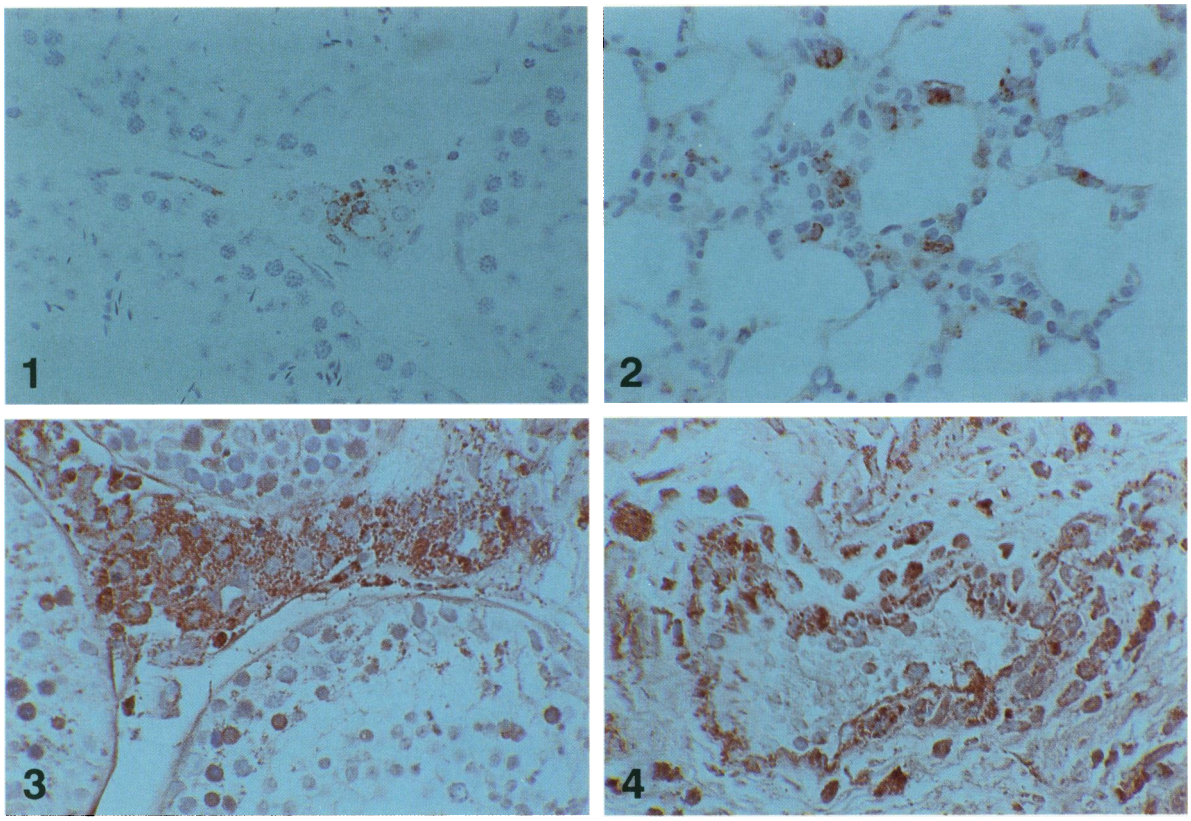

Figure 1. (top, left): *Photomicrograph of* R. australis *demonstrated by immunoperoxidase in the endothelium and perivascular interstitial cells of the mouse testis 3 days after inoculation. Anti–*R. australis *immunoperoxidase with hematoxylin counterstain. × 128.*
Figure 2. (top, right): *Photomicrograph of* R. australis *demonstrated by immunoperoxidase in the alveolar capillaries of mouse lung 5 days after inoculation. Anti–*R. australis *immunoperoxidase with hematoxylin counterstain. × 160.*
Figure 3. (bottom, left): *Photomicrograph of numerous* R. australis *demonstrated by immunoperoxidase in the interstitium of mouse testis 5 days after inoculation. Anti–*R. australis *immunoperoxidase with hematoxylin counterstain.*
Figure 4. (bottom, right): *Photomicrograph of numerous* R. australis *demonstrated by immunoperoxidase in the endothelium, vascular wall, and perivascular space of a blood vessel in the peritesticular adnexa of a mouse 5 days after inoculation. Anti–*R. australis *immunoperoxidase with hematoxylin counterstain. × 160.× 128.*

including marked infection of the pulmonary interstitium, splenic red pulp, hepatic sinusoids, and testicular interstitium. Multifocal infection was also detected in renal and cardiac interstitial capillaries. No rickettsial antigen was detected in uninfected control animals. Infected tissue sections examined by immunoperoxidase using normal rabbit serum contained no reaction product.

Histopathology

The pathological lesions and their time course in the mice infected with both doses of *R. australis* were qualitatively similar although somewhat more severe with the higher rickettsial dose of inoculum. Multifocal interstitial vasculitis and perivasculitis containing both polymorphonuclear and mononuclear leukocytes were present in the testis on day 3 and progressed on day 5 to severe necrotizing vasculitis with a predominance of polymorphonuclear leukocytes (Figure 5) and occasionally the presence of focal nonocclusive thrombi. On days 6 (high dose) and 7 (low dose), there were perivascular fibrinous exudates and more vascular thrombi. A similar progression of events was observed in the blood vessels of the peritesticular adnexae.

In the liver on day 3, there were multifocal lobular accumulations of mononuclear cells and polymorphonuclear leukocytes that were occasionally associated with adjacent hepatocellular necrosis. Microvesicular hepatocellular fatty change was also present. On days 5, 6, and 7, the multifocal hepatic inflammatory lesions had become granulomalike accumulations of mononuclear cells accompanied by smaller quantities of polymorphonuclear leukocytes (Figure 6). Focal areas of coagulative necrosis seemed to represent infarctions, and hepatocellular microvesicular fatty change was prominent. Splenic lesions included increased sinusoidal polymorphonuclear leukocytes on days 3 and 5 and

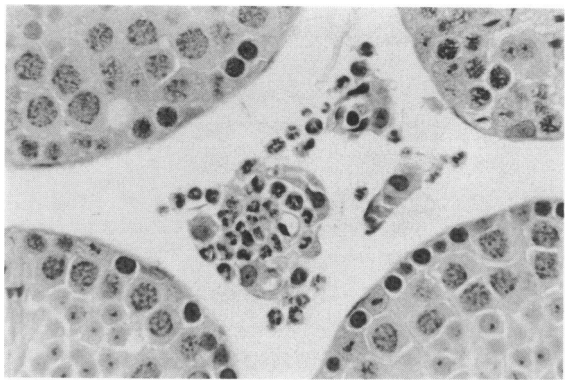

Figure 5. *Photomicrograph of vasculitis in the interstitium of the testis of a Balb/c mouse inoculated intravenously 3 days previously with* 2×10^6 R. australis. *Hematoxylin-eosin.* ×365.

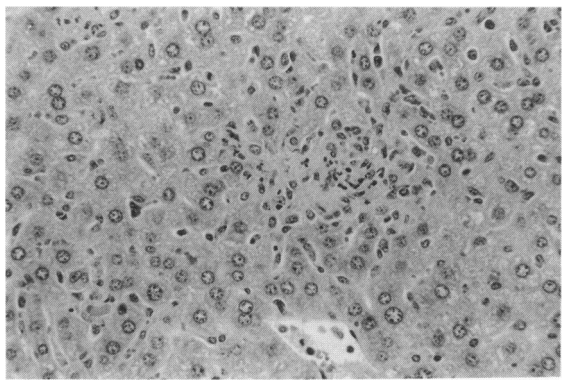

Figure 6. *Photomicrograph of a granulomalike lesion comprising an aggregate of enlarged macrophages admixed with polymorphonuclear leukocytes in the liver of a Balb/c mouse inoculated 5 days previously with* 10^5 R. australis. *Hematoxylin-eosin.* ×180.

increased prominence of sinusoidal macrophages on day 5. The spleens of mice receiving the high dose of rickettsiae contained sinusoidal fibrin and polymorphonuclear leukocytes at the time of death on day 6. In contrast, the spleens of the mice receiving the lower dose of rickettsiae showed lymphoid hyperplasia on day 7. Pulmonary lesions included mononuclear leukocytic margination on day 5 and frank interstitial pneumonia on days 6 and 7. No lesions were detected in the kidneys of any of the animals.

Ultrastructural Observations

R. australis organisms were observed in the cytosol of endothelial cells (Figure 7), spindle-shaped pericytes, and Leydig cells on day 3 after inoculation. On days 5 and 7, the architecture of the testicular tissue was markedly distorted by extensive fibrinous exudates and infiltration by macrophages and polymorphonuclear leukocytes (Figure 8). Rickettsiae were present in large quantities mainly in the cytosol of interstitial cells but also in the cytosol of endothelial cells, within the nuclei of unidentified cells, in cytoplasmic vacuoles of macrophages, and in the extracellular space (Figures 8 and 9). Rickettsiae undergoing binary fission were observed only intracellularly.

Host Antibody and Cytokine Responses to Infection

The level of serum antibody against *R. australis* measured by indirect immunofluorescence assay was detectable at a titer of 40 on day 3 and was markedly elevated at titers of 1280 and 2560 on days 5 and 7, respectively. There was no difference in the antibody titers between groups 1 and 2. No antibody to *R. australis* was detected in sera from uninfected control mice. The serum levels of IL-1, IL-6, TNF, and IFN in group 1 animals increased significantly during the period of observation, but each differed in the day of appearance, day of peak concentration, and subsequent rise or fall in concentration (Figure 10). TNF was detected only on day 5. The highest titer of IL-1 was on day 7, whereas the

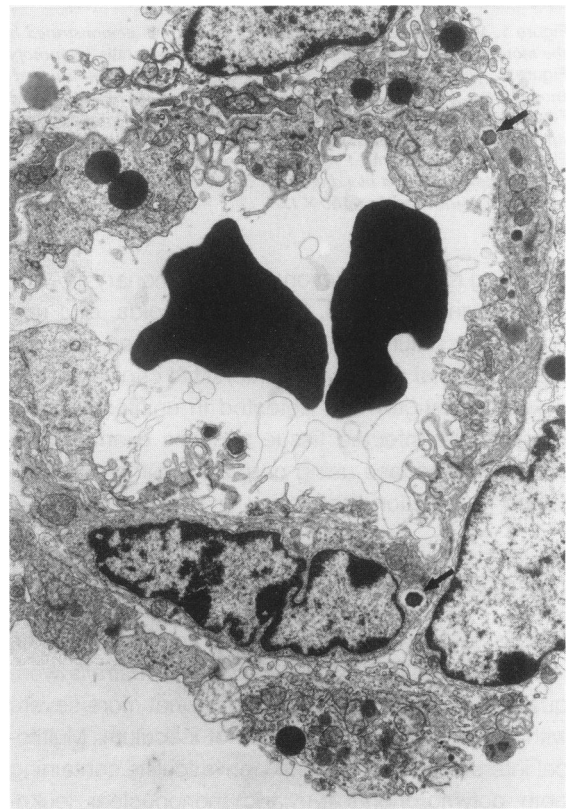

Figure 7. *Electron photomicrograph of a capillary in the testis of a mouse 3 days after intravenous infection with* 2×10^6 R. australis. *Rickettsiae are present in the cytoplasm of vascular endothelium (arrows).* ×9,000.

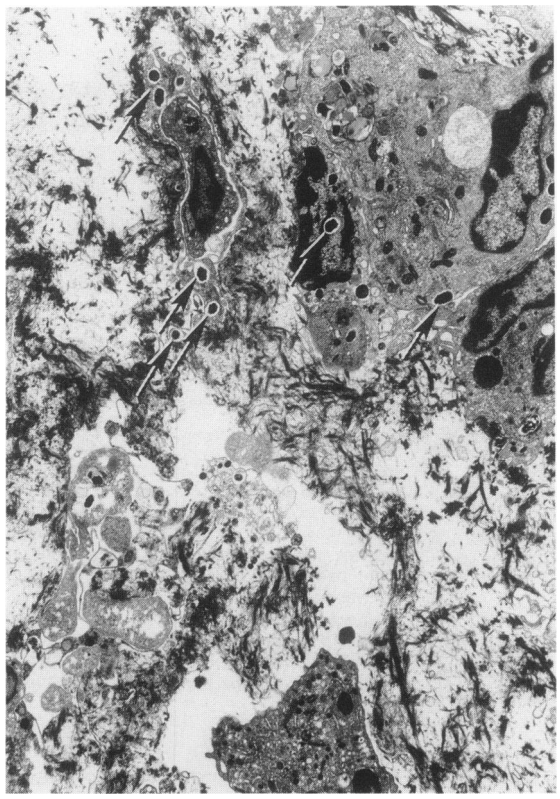

Figure 8. *Electron photomicrograph of the interstitium of the testis of a mouse 5 days after intravenous infection with 2 × 10⁶* R. australis. *Numerous cytoplasmic and intranuclear rickettsiae (arrows) are present in an area with abundant fibrinous exudate.* ×5,250.

peak of IL-6 was on day 5. Because the mice were moribund on day 7, an insufficient quantity of serum was obtained for analysis of IFN. The IFN level was significantly increased on days 3 and 5, the only time points tested.

The cytokines, IL-1, IL-6, TNF, and IFN, were secreted into the supernatant of spleen cells after stimulation with rickettsial antigen or Con A (Figure 11). The IFN level in the supernatant of the spleen cells after stimulation with rickettsial antigen increased significantly on day 5 after infection and had decreased remarkably on day 7. This IFN was sensitive to acid (pH 2) and heat (56 C for 30 minutes). The peak levels of TNF, IL-1, and IL-6 secreted by rickettsial antigen-stimulated spleen cells were observed on day 5. As the disease progressed to the moribund state, the IL-1, IL-6, and TNF concentrations decreased.

The spleen cells of mice infected with *R. australis* 3, 5, and 7 days earlier had a reduced capability to secrete IL-2 when stimulated with Con A when compared with spleen cells from either uninfected mice or immune mice (Figure 12). Although the spleen cells of infected and naive mice did not respond to *R. australis* antigen by secretion of the key immunoregulatory lymphokine, IL-2, spleen cells from immune mice secreted significant quantities of IL-2. These immune mice had recovered from *R. australis* infection and had been rechallenged without morbidity. Supernatant fluid from spleen cells of mice with reduced responses to Con A did not contain humoral factors that were capable of suppressing IL-2 production by normal spleen cells stimulated with Con A.

Discussion

The development and description of this experimental spotted fever group rickettsiosis provides the first, well-characterized, disseminated, endothelial-target mouse model of rickettsial infection. It offers several specific opportunities for the investigation of rickettsial pathogenic mechanisms and immunity to intracellular bacteria as well as possible study of general acute vascular injury. In

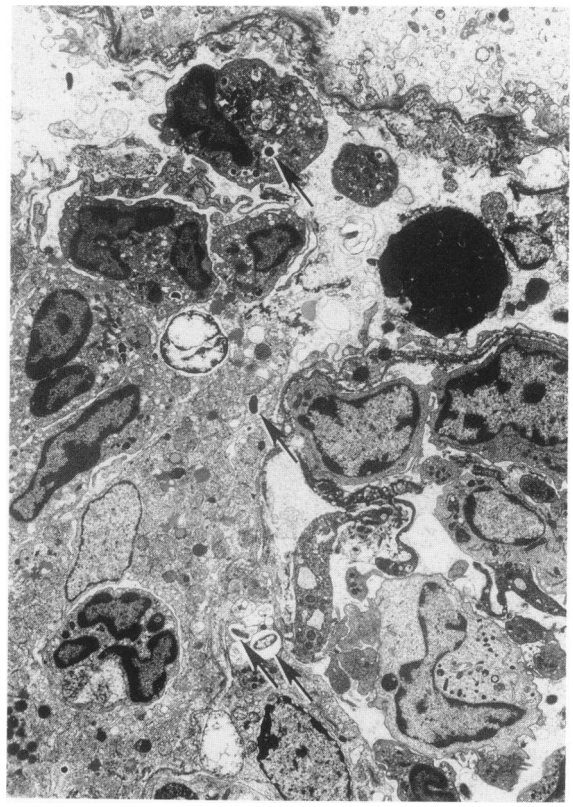

Figure 9. *Electron photomicrograph of the interstitium of the testis of a mouse 5 days after intravenous infection with 2 × 10⁶* R. australis. *Rickettsiae (arrows) are present in the cytosol and membrane-bound cytoplasmic vacuoles of phagocytes and in the cytosol of endothelial and interstitial cells.* ×3,750.

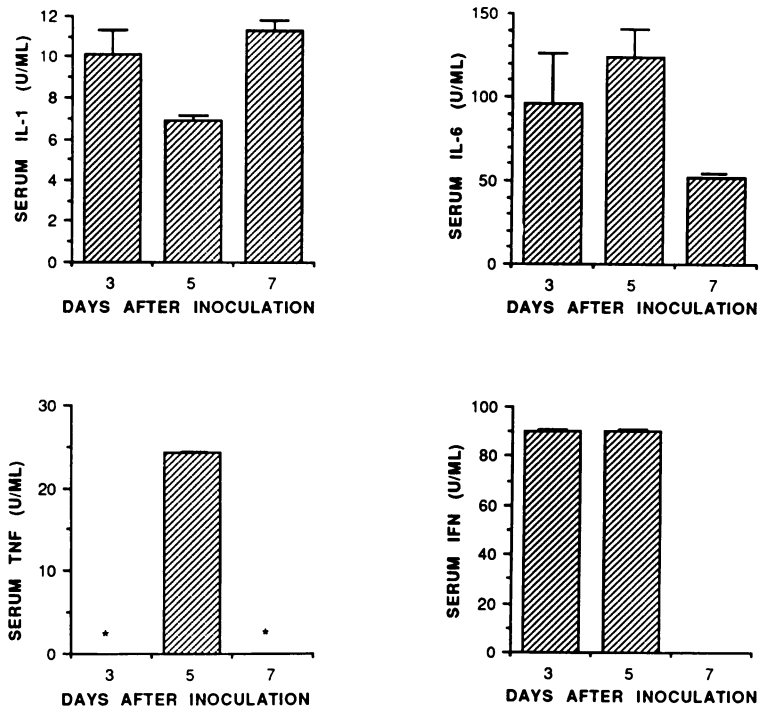

Figure 10. *Cytokine concentration in the serum of Balb/c mice infected with* R. australis *on days 3, 5, and 7 after inoculation.* Left upper panel: *serum concentration of IL-1.* Right upper panel: *serum concentration of IL-6.* Left lower panel: *serum concentration of TNF (asterisk: undetectable on days 3 and 7).* Right lower panel: *serum concentration of IFN (not tested on day 7). IL-1, IL-6, TNF, and IFN were undetectable in the sera of uninfected control mice.*

this report, endothelial infection is clearly demonstrated by identification of intraendothelial cell rickettsiae by immunohistology and electron microscopy from the earliest time points studied. It should also be noted that infection of macrophages and other extravascular cell types was also clearly demonstrated in this model. These mononuclear phagocytes are the likely important sources of IL-1 and TNF. IL-6 possibly originates in infected macrophages, endothelial cells, or perivascular fibroblasts. These three cytokines are the likely effectors of the acute-phase response in this animal model of dis-

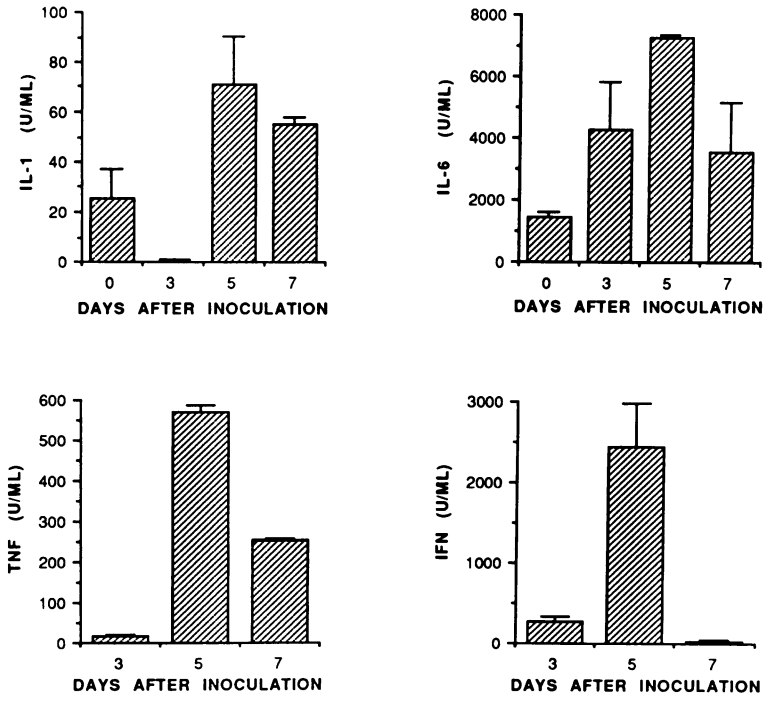

Figure 11. *Cytokine concentration in supernatant of spleen cells of Balb/c mice stimulated by* R. australis *antigen.* Left upper panel: *IL-1 concentration secreted by uninfected control spleen cells (O) and by cells from mice on days 3, 5, and 7 after infection with* R. australis. Left lower panel: *TNF concentration secreted by antigen-stimulated spleen cells on days 3, 5, and 7 of infection.* Right upper panel: *IL-6 concentration secreted by antigen-stimulated spleen cells from uninfected controls and mice on days 3, 5, and 7 of infection.* Right lower panel: *IFN concentration secreted by antigen-stimulated spleen cells on days 3, 5, and 7 of infection. TNF and IFN were not detected in the supernatant of unstimulated spleen cells.*

seminated rickettsiosis. In experimental animal infections previously used in research on rickettsial diseases, the target cells have not been identified or the animal species has limited reagents available for further dissection of immune mechanisms. The importance of the endothelial target is emphasized in the fundamental question concerning immunity to rickettsiae: how are these obligate intracellular bacteria cleared from their location in the cytosol of endothelial cells? Animal models that result in demonstrable infection only in peritoneal, splenic, and hepatic macrophages cannot address the role of such effectors as IFN-γ, TNF-α, or cytotoxic T lymphocytes in clearance of rickettsiae from the endothelium, the major target of spotted fever and typhus group rickettsioses. Experimental manipulations which enable macrophages to destroy rickettsiae, such as opsonization by antibody[30–32] or activation by cytokines,[33] are unlikely to exert a similar effect on rickettsiae established within endothelial cytosolic loci. The two principal candidates for effectors of immunity to rickettsiae, T lymphocytes, and IFN-γ[34] have never been critically evaluated in an animal model characterized as having endothelial cells as target cells of the infection.[33,35,36] This model in an inbred strain of mice offers the opportunity to utilize adoptive transfer of T lymphocytes to examine the immune effector mechanisms. The availability of specific immunological reagents for the species *Mus musculus* also allows for study of the role of T-lymphocyte subsets by depletion or by reconstitution via adoptive transfer. The roles of IFN-γ and TNF-α[37] could also be investigated *in vivo* by depletion with specific neutralizing monoclonal antibodies and by treatment with these recombinant murine cytokines.

The lower dose infection with *R. australis* in this study, which was shown in preliminary studies to result in survival of the minority of infected mice, provided evidence for active host defenses. These mice had reduction in the rickettsial infection of the spleen on days 5 and 7 compared with day 3. The lower dose mice also showed morphological evidence of a prominent immune response with splenic lymphoid hyperplasia on day 7. In contrast, the animals inoculated with the higher dose showed neither control of the rickettsial infection in the liver and spleen nor a splenic lymphoid response. That the animals infected with the lower dose lacked sufficient immunity to recover in most instances (Feng HM and Walker DH, unpublished data) is demonstrated by an actual decrease in the ability of the spleen cells to secrete IL-2 when stimulated by either Con A or rickettsial antigen although none of the IL-2 levels assayed were significant. These data suggest that *R. australis* infection might have a suppressive effect on cell-mediated immunity. This hypothesis was supported by demonstration by flow cytometry that the spleens of infected mice contained remarkably fewer CD4+ and CD8+ T lymphocytes on day 6 than the spleens of uninfected mice (Feng HM, unpublished data).

Cytokines have been hypothesized previously to play a role in the pathogenesis of some of the disease manifestations of vasculopathic rickettsioses, including fever and the acute phase reaction,[38] as well as playing a role in immunity to rickettsiae.[37] The description in this report of elevated serum concentrations of IL-1, IL-6, TNF, and IFN and the enhanced ability of stimulated spleen cells to elaborate these cytokines support their possible roles as pathogenic mechanisms or immune effectors. It is likely that T-helper-1 lymphocytes and NK cells are the important sources of IFN-γ, although experiments with purified cell types were not performed in this study. The decline in the ability of the infected animals to secrete IFN and TNF in the late stage of infection suggests that the host defenses were overwhelmed terminally. Future definitive experiments will be required to test the hypothetical roles of the various cytokines.

This new experimental model also has distinct advantages for the investigation of rickettsial pathogenic mechanisms. Disseminated rickettsial infec-

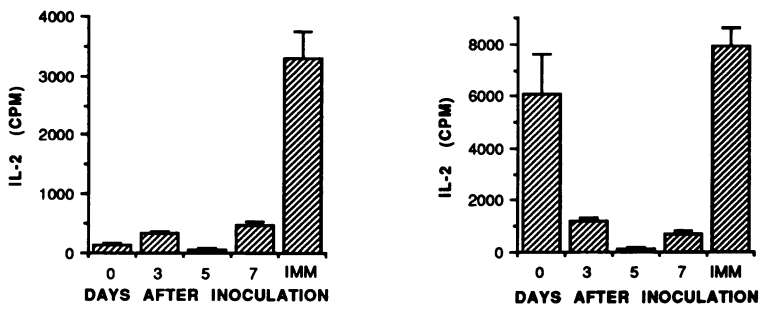

Figure 12. *IL-2 concentration in the supernatant of spleen cells of uninfected Balb/c control mice (O), mice on days 3, 5, and 7 of infection, and immune mice (Imm).* Left panel: R. australis–*stimulated spleen cells.* Right panel: *Con A-stimulated spleen cells.*

tion of endothelium and other cells of the blood vessel wall results in multisystem acute vascular injury and the pathological consequences similar to human Rocky Mountain spotted fever, particularly interstitial pneumonia and vasculitis in the testis and surrounding tissues.[38–40] This pathological model would seem to be far superior to the rickettsial peritonitis, which is the principal effect of intraperitoneal inoculation of guinea pigs with *R. typhi* or *R. conorii*.[14,15]

The most prominent feature of this mouse–*R. australis* interaction is the rickettsial invasiveness. *R. rickettsii* in Rocky Mountain spotted fever is more highly invasive than *R. prowazekii* in louse-borne typhus fever. *R. rickettsii* spreads from the endothelium to invade the adjacent vascular smooth muscle cells, in contrast with *R. prowazekii* which remains confined to the endothelium.[2,41,42] The distribution of *R. australis* has not been described in human cases of Queensland tick typhus. The virulence trait of invasiveness has not been elucidated. *In vitro* correlates include more rapid release of spotted fever group rickettsiae from host cells and more extensive spread to other host cells than epidemic typhus rickettsiae.[43,44] The basis for the invasiveness or spread of *R. australis* through the blood vessel wall might be hypothesized to be related to phospholipase A activity or protease activity.[45–47] This model provides the best *in vivo* expression of rickettsial invasiveness in an experimental animal for investigation of the cellular and molecular mechanisms involved. The expression of this pathological consequence is observed most vividly in the testis, presumably because the lower temperature of this organ is most favorable to rickettsial growth.[15]

It is important to recognize the differences between this *R. australis* model and the so-called mouse toxin phenomenon. Intravenous inoculation of very high doses of viable spotted fever or typhus group rickettsiae results in the development of diffusely increased vascular permeability and death within 1 to 24 hours.[48,49] The mechanism is unknown but does not involve any identified toxin. In fact, there is no evidence for the existence of an exotoxin in the genus *Rickettsia*[50] and the lipopolysaccharides are not significantly endotoxic.[51] The lethal toxicity usually occurs before the passage of 10 hours, the average rickettsial generation time.[43,44] Thus, this lethal toxicity model does not even represent a true infection. In contrast, this *R. australis* mouse model includes entry of rickettsiae into and growth within target cells, rickettsial escape from these cells and spread to other target cells, and injury to the infected cells and the blood vessels that they comprise. Consequently, this experimental model is substantially superior to the mouse toxicity phenomenon for the evaluation of mechanisms of immunity to rickettsiae and pathogenicity of rickettsiae.

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