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Reduced Immune Responsiveness and Lymphoid Depletion in Mice Infected with *Ehrlichia risticii*

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The histopathology of the thymus and spleen and the response of spleen cells to mitogenic stimuli were evaluated in Sprague-Dawley CF-1 mice infected with *Ehrlichia risticii*. Intraperitoneal injection of 10^4 or 10^6 *E. risticii*-infected U-937 cells into mice resulted in 100% morbidity and partial mortality. Thymic atrophy became significant between 1 and 2 weeks postinfection and remained for the duration of the study. The atrophy appeared associated with antecedent destruction and rarefaction of lymphocytes, resulting in the loss of corticomedullary demarcation. Splenomegaly was prominent; significantly increased weights were detected 7 days postinfection. Histopathologic examination revealed rarefaction of lymphocytes around central arteries, the presence of necrotic debris in histiocytes, and replacement of erythropoiesis by granulopoiesis in the red pulp. Marked and acute reduction of in vitro proliferative responses of spleen cells to concanavalin A (ConA) and phytohemagglutinin were observed in mice infected with 10^4 or 10^6 *E. risticii*-infected U-937 cells. Interleukin-2 activity in the supernatant of ConA-stimulated spleen cells was also severely reduced. Both changes were time- and dose-dependent and were not associated with decreased spleen cell viability. Neither morbidity nor mortality occurred in mice infected with 10^2 *E. risticii*-infected U-937 cells. Although there was temporal reduction in phytohemagglutinin-driven lymphocyte proliferation, reduction in neither ConA-driven lymphocyte proliferation nor interleukin-2 activity was observed with this dosage. All *E. risticii*-inoculated mice seroconverted between days 18 and 25, as detected by the indirect fluorescent-antibody procedure. The findings indicate for the first time the hypoimmune responsiveness and histopathologic changes in lymphoid organs associated with *E. risticii* infection.

Potomac horse fever (equine monocytic ehrlichiosis) is a newly recognized disease of equids characterized by fever, anorexia, leukopenia, and sometimes by acute, explosive diarrhea (9, 13, 16, 22). *Ehrlichia risticii*, isolated from blood monocytes of a clinically affected horse, was proved to be the etiologic agent of the disease (13, 16, 21). Serologic procedures (20, 23) have demonstrated that the infection is widely distributed throughout North America (23). *E. risticii* has antigenic determinants in common with *Ehrlichia sennetsu* (16). *E. sennetsu*, the etiologic agent of human sennetsu fever, produces morbidity and mortality in laboratory mice (14). Recently, *E. risticii*-infected mice were also reported to be moribund after injection of blood from a pony exhibiting clinical symptoms of Potomac horse fever, and the organism was found in the spleen by electron microscopy (17). Because of the proclivity of ehrlichial organisms to infect leukocytes (24), they cause disorders such as pancyto penia (*E. canis* infection), plasmacytosis (*E. canis* infection), lymphadenopathy (*E. sennetsu*, *E. canis*, and *E. equi* infections), thrombocytopenia (*E. equi* and *E. canis* infections), and leukopenia (*E. risticii* infection) (9). However, very little information is available regarding the functional or morphological response of lymphoid organs to *E. risticii* infection. This study examined the effects of *E. risticii* infection on the structure and function of the murine spleen and thymus.

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MATERIALS AND METHODS

Mice. Female Sprague-Dawley CF-1 mice were obtained from Harlan Sprague Dawley, Inc., Indianapolis, Ind. Mice were 8 weeks old and weighed 25.7 ± 2.0 g at the time of inoculation. The animals were housed at 25°C and were given antibiotic-free commercial laboratory chow and water ad libitum.

Ehrlichiae. *E. risticii* was isolated from a pony infected by whole-blood transfusion from a naturally infected horse (21). The organism has been maintained by passage in a human monocytelike histiocytic lymphoma cell line, U-937 (CRL 1593; American Type Culture Collection, Rockville, Md.). Cells were maintained in RPMI 1640 medium (pH 7.4; GIBCO Laboratories, Grand Island, N.Y.)–10% fetal bovine serum (GIBCO) at 37°C in a humidified 5% CO_2 atmosphere (21). The infectivity and absence of bacterial contamination were monitored by Diff-Quik staining (American Scientific Products, Obetz, Ohio); indirect fluorescent-antibody (IFA) labeling with convalescent pony serum and electron microscopy established the percentage of *Ehrlichia*-infected cells (21). The U-937 cells used for the experiment were more than 90% infected.

***Ehrlichia* infection of mice.** Each group of 25 mice were inoculated intraperitoneally with 0.5 ml containing 10^2, 10^4, or 10^6 infected U-937 cells in RPMI 1640 medium. As controls, 30 other mice were injected with 10^6 uninfected U-937 cells in the same medium. At day 0, five control mice were sacrificed. Five mice from each group were sacrificed on days 7, 14, 18, 25, and 32. Because of the death of some of the mice, the group inoculated with 10^6 cells had four and three mice sacrificed on days 18 and 25, respectively. The

group inoculated with 10^4 cells had four mice sacrificed on day 25. On day 32, there were no mice in the groups inoculated with 10^4 and 10^6 cells. Blood was collected from the axillary blood vessels and pooled for each group on each day. At necropsy, the whole animal, spleen, and thymus were weighed. Tissue specimens of the spleen and thymus were prepared for light microscopic study. Spleens were also processed for immunologic assay.

Light microscopy. Sections of spleen and thymus were fixed by immersion in 10% neutral-buffered Formalin, dehydrated, cleared, and embedded in paraffin. Sections (5 μm) were deparaffinized and stained with hematoxylin and eosin and by the Steiner silver impregnation procedure for demonstrating ehrlichiae (26).

IFA titration. *E. risticii*-infected U-937 cells coated on 12-well slides were used as the antigen (20). Each batch of prepared slides was tested with known positive and negative horse sera before use. The antigen-coated wells were incubated with twofold serial dilutions of mouse sera and then with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin G (Organon Teknika, Inc., Malvern, Pa.) and observed under a Nikon Optiphoto epifluorescence microscope equipped with a 485-nm interference excitation filter and a 515-nm barrier filter. As a negative control, slides coated with uninfected U-937 cells were incubated with mouse sera in the same manner.

Preparation of splenocyte suspensions. Mice were sacrificed by anesthesia in N_2, followed by exsanguination. The spleens were removed aseptically, and cell suspensions were prepared by pressing the tissues through 50-mesh stainless steel screens into Hanks balanced salt solution (GIBCO). The suspensions were treated with 0.85% (wt/vol) NH_4Cl in water for 5 min to remove erythrocytes and then washed twice. Pelleted cells were reconstituted with RPMI 1640 supplemented medium and counted. The supplemented medium consisted of powdered RPMI 1640 medium with 2 mM glutamine (GIBCO); penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO); 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer; 2 g of $NaHCO_3$ per liter; 4×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, Mo.); and 10% heat-inactivated fetal bovine serum (GIBCO).

Lymphocyte culture and mitogen stimulation. Spleen cells were cultured in flat-bottom microculture plates (Linbro Scientific, Inc., Hamden, Conn.). A suspension of 2×10^5 cells was delivered in 0.05 ml of supplemented medium to each culture well. The optimal concentrations of phytohemagglutinin (10 μl/ml; PHA-P; Difco Laboratories, Detroit, Mich.) and concanavalin A (ConA; 16 μg/ml; Difco) were determined and added to test wells, also in a 0.05-ml volume. All wells, including those with control cultures (which received no mitogens), were made up to a final volume of 0.2 ml per culture well by the addition of medium. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for a total of 72 h. Six hours before assay termination, 1 μCi of [^{3}H]thymidine (specific activity, 5 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added to each well. Samples were harvested with a Multiple Automated Sample Harvester (mini-MASH II; M.A. Bioproducts, Walkersville, Md.) by washing the cells with distilled water onto strips of Whatman 934 glass fiber paper strips (Microbiological Associates, Bethesda, Md.). After drying for 45 min at 75°C, filter disks containing sample well constituents were cut from the glass fiber strip and placed in mini-scintillation counting vials (A. H. Thomas Co., Philadelphia, Pa.). Econofluor (New England Nuclear Corp., Boston, Mass.) was added to the vials, and the radioactivity in the vials was counted by liquid scintillation spectrometry (Liquid Scintillation Counter LS-8100; Beckman Instruments, Inc., Fullerton, Calif.). Mitogen-induced DNA synthesis was expressed as the difference between control (no mitogen) and test (with mitogen) lymphocyte incorporation of [^{3}H]thymidine as measured by counts per minute.

Data presentation. Results of a typical experiment are expressed as mean counts per minute for six culture well replicates containing pooled mouse spleen cells. The standard error of the mean is indicated in figures when it exceeded 10%. In all other instances, the standard error did not exceed 10%.

Measurement of IL-2 activity. Interleukin-2 (IL-2) activity was generated in 2.0-ml splenocyte cultures (10^7/ml) in 24-well culture plates (Costar, Cambridge, Mass.) by adding 2 μg of ConA. Supernatants were harvested after a 24-h incubation (37°C, 5% CO_2). After being filtered through a membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.), these crude IL-2-containing supernatants were stored at −70°C. IL-2 activity was quantitated by culturing $\log_2$ serial dilutions of control and test supernatants with the cloned IL-2-dependent murine cell line HT-2. Supplemented medium (0.1 ml) containing 2×10^4 HT-2 cells was added to 0.1-ml supernatant dilutions in a 96-well microculture plate (Linbro Scientific) in triplicate. The assay mixtures were incubated (18 h, 37°C, 5% CO_2), pulsed with 1 μCi of [^{3}H]thymidine (specific activity, 5 Ci/mmol) per well for 6 h, and harvested onto glass fiber filter strips. The amount of radioactive thymidine incorporated into the DNA of the proliferating cells was determined by liquid scintillation spectroscopy (LS-8100; Beckman). In each experiment, a standard ConA-induced mouse splenocyte culture supernatant was titrated to determine the maximum counts per minute. A linear regression line was computer generated by using $\log_{10}$ transformation of the counts per minute and the reciprocal of the dilution value. The dilution giving 50% maximum response was determined. Titrations were done on each sample, and regression lines were used to find the dilution corresponding to 50% maximum counts per minute (cpm 50% max). IL-2 units in each sample were calculated by using the following equation: IL-2 U/ml = [(sample dilution cpm 50% max)/(standard dilution cpm 50% max)] × 100. By using a specially designed computer program which incorporates linear regression, analysis of variance, and the parallel line assay (3), IL-2 units per milliliter were compared among samples. When 95% confidence intervals did not overlap, the samples are reported as significantly different.

RESULTS

Morbidity and mortality. When mice were injected intraperitoneally (i.p.) with 10^4 or 10^6 infected U-937 cells, morbidity, characterized by inactivity and ruffling of fur, usually preceded death by 1 or 2 days (Fig. 1). All mice in the 10^6 and 10^4 inoculum groups were ill by days 13 and 15 postinfection (p.i.), respectively. They remained ill until death or termination of the experiment. Death occurred beginning on day 14 in the 10^6 inoculum group (which could have suffered 51% cumulative mortality if they had not been sacrificed) and on day 16 in the 10^4 inoculum group (34% cumulative mortality if they had not been sacrificed). Mice inoculated with uninfected U-937 cells, mice given 10^2 infected cells, and uninoculated mice remained well. Body weight, which progressively decreased in the 10^4 and 10^6

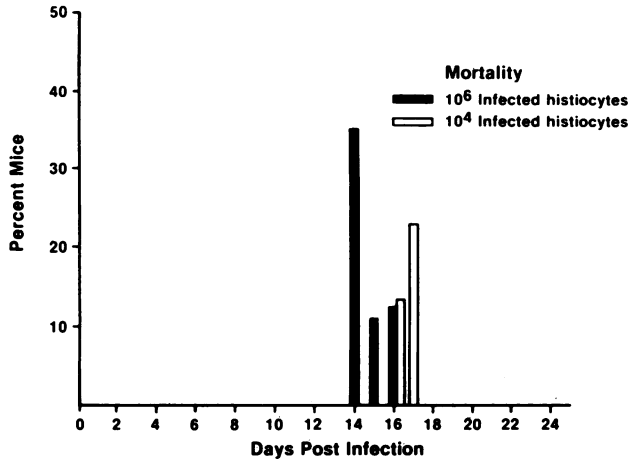

FIG. 1. Mortality of Sprague-Dawley CF-1 mice after i.p. inoculation of *E. risticii*. Mice were inoculated with 10^2, 10^4, or 10^6 infected U-937 cells. The percentages of mice which died on the indicated days are shown. No mice injected with 10^2 infected U-937 cells or 10^6 noninfected U-937 cells died.

inoculum groups beginning on day 18, returned to normal in the surviving mice by 25 days p.i. (Fig. 2).

Morphologic changes in lymphoid tissues. Microscopic changes in the thymus and spleen were correlated with decreased relative weight of the thymus and increased relative weight of the spleen. The thymic and periarteriolar splenic lymphoid populations underwent severe, dose-dependent degeneration and atrophy, and the dominance of erythropoietic cells in the splenic red pulp was replaced by extensive myelopoiesis, contributing to the increased weight.

Thymic atrophy, as assessed by reduced relative thymic weight, became evident from day 14 p.i. in mice given 10^4 or 10^6 *E. risticii*-infected cells (Fig. 3). By day 18 p.i., the mean thymic weights decreased to 55 and 30% of normal in these two inoculum groups, respectively, and remained significantly below control values.

Whereas sham-infected and uninoculated age- and sex-matched control mice had well developed thymic architecture, densely populated by thymocytes and possessing a distinct corticomedullary boundary (Fig. 4A), the thymuses of *E. risticii*-infected mice were atrophied (Fig. 4B). Atrophic thymuses, seen in mice given 10^4 or 10^6 infected cells, were characterized by a reduced cortical thickness, a loss of corticomedullary demarcation (Table 1), extensive loss of cortical and medullary thymocytes, and coincident relative increases in the number of thymic stromal cells. Although mild lesions were evident at 7 days in the thymic medulla of mice given 10^6 infected cells, widespread severe lesions were present in the 10^4 and 10^6 inoculum groups by 14 days and thereafter. Nuclear pyknosis and fragmentation were widespread in all thymuses of the 10^4 inoculum group at day 14 p.i. (Fig. 4, inset), and scattered intrahistiocytic debris was evident in many of the atrophic thymuses. For the duration of the experiment, thymic architecture remained loosely organized and depleted of lymphocytes. Islands of medullary plasma cell infiltration were present in some thymuses, beginning 18 days p.i. Ehrlichial organisms were rarely detected in the thymuses of infected mice by silver impregnation procedures.

Striking, progressive splenomegaly was first indicated by significantly increased relative splenic weights 7 days after infection of mice with 10^4 or 10^6 *E. risticii*-infected cells (Fig. 5). Maximum spleen-body weight ratios were achieved by 14 days p.i. (420% of normal) and remained elevated for the duration of the experiment. Some but not all mice given 10^2 infected cells showed milder splenomegaly, and the relative splenic weights for mice given uninfected U-937 cells did not differ from those of the uninoculated controls.

Extensive histologic changes were apparent in the spleens of *E. risticii*-infected mice. Uninoculated and sham-inoculated mice had lymphocyte-rich splenic white pulp and cellular, predominantly erythropoietic red pulp (Fig. 6A). Destruction and depletion of lymphocytes, particularly in regions adjacent to the central arteriole of the white pulp, first became evident 7 days after injection of 10^4 or 10^6 infected U-937 cells (Fig. 6B). Decreased lymphocyte density remained apparent for the duration of the experiment,

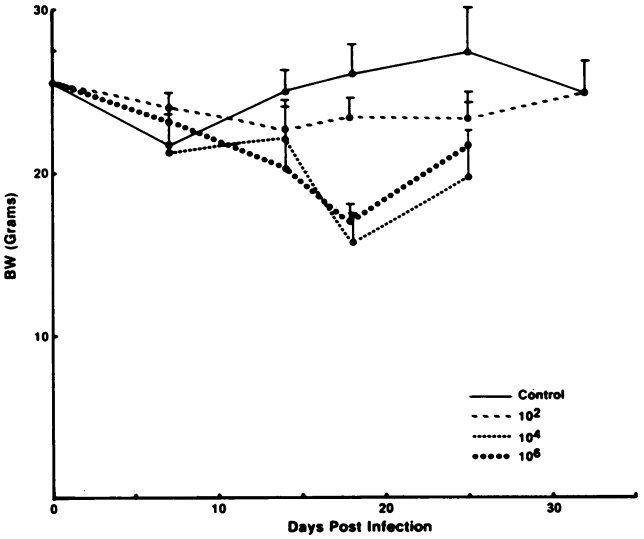

FIG. 2. Body weights (BW) of mice inoculated with *E. risticii*-infected U-937 cells. There was significant loss of body weight of mice inoculated with 10^4 or 10^6 infected U-937 cells on day 18 p.i. On day 25, the surviving mice recovered from weight loss. The values are means of the body weights ± standard deviations (n = 3 to 5).

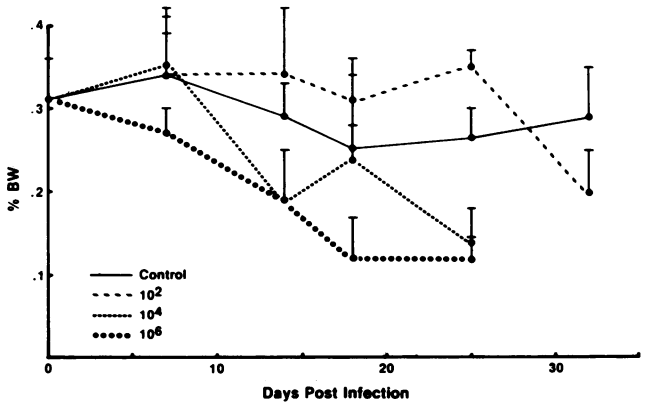

FIG. 3. Thymus weight as a percentage of body weight (BW) for mice inoculated with *E. risticii*-infected U-937 cells. There was significant thymic atrophy evident among mice inoculated with 10^4 or 10^6 infected U-937 cells from day 14 and day 7 p.i., respectively. With 10^2 infected U-937 cells, thymus weight was not significantly different compared with that for the control.

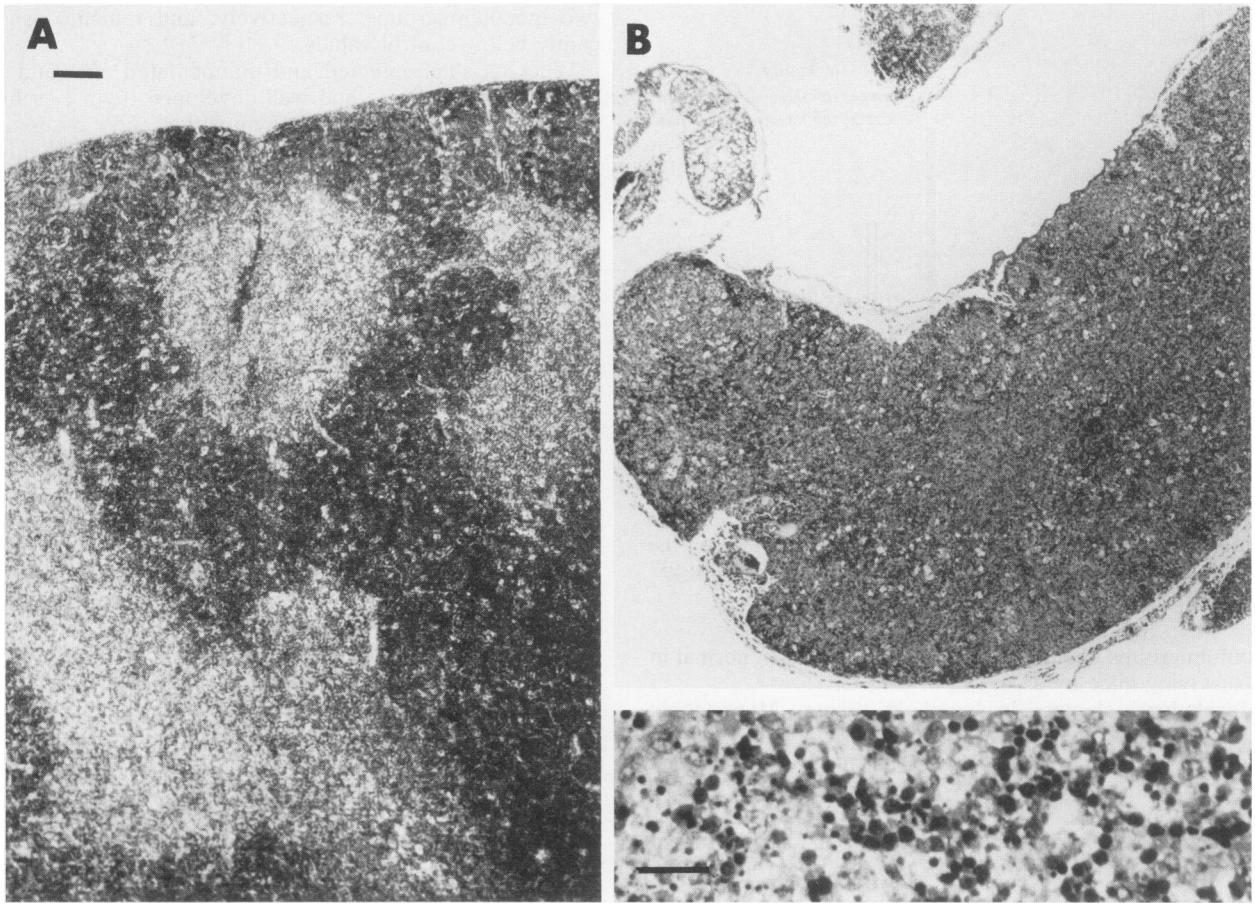

FIG. 4. Light microscopic appearance of thymuses from uninfected (A) and *E. risticii*-infected (B) mice (10^6 uninfected or infected U-937 cells per mouse). Mice were killed on day 25 p.i. Bar (same for panels A and B), 200 μm. The inset shows a higher magnification of thymic tissue from an infected mouse at day 14 p.i. (10^4 infected cells). Bar, 25 μm.

and phagocytized debris commonly filled the cytoplasm of periarteriolar phagocytic reticular cells. One of five mice given 10^2 infected cells 7 days previously had mild lymphoid depletion. Follicular lymphocytes were spared from degenerative changes which affected periarteriolar cells. Few ehrlichial organisms were detected in splenic macrophages with the silver staining procedure.

Concomitant with periarteriolar lymphoid depletion at day 7, there was transient reduction of red pulp erythropoietic cells in the 10^4 and 10^6 inoculum groups. The number and morphology of red pulp megakaryocytes remained similar to that of controls. At day 14 and thereafter, red pulp cellularity progressively increased, but myelopoietic rather than erythropoietic cells predominated (Fig. 7B). Moderately increased myelopoiesis was noted by day 32 in mice inoculated with 10^2 infected cells.

TABLE 1. Thymic atrophy and loss of corticomedullary demarcation in mice after i.p. inoculation of *E. risticii*

No. of infected histiocytes	Response at day postinoculation[a]:					
	0	7	14	18	25	32
0	−	−	−	−	−	−
10^2	−	−	−	−	−	−
10^4	−	−	+	+	+	ND
10^6	−	+/−	+	+	+	ND

[a] +, All mice showed both atrophy and loss of corticomedullary demarcation in hematoxylin-plus-eosin-stained paraffin-embedded thymus; −, all mice showed no thymic atrophy, and corticomedullary demarcation was present; +/−, one thymus showed atrophy and loss of corticomedullary demarcation; ND, not done.

Immune responses. When lymphocyte blastogenic assays using pan-T-cell mitogens (ConA and PHA; 2) were done on unseparated splenocytes, significantly reduced stimulation occurred in mice previously injected with 10^4 and 10^6 infected cells. Progressively reduced mitogenesis occurred in these groups until minimum values were reached beween 14 and 18 days p.i., although stimulation remained reduced throughout the experiment (Fig. 8). Mice given 10^2 infected cells had no decrease in proliferative response to ConA compared to that of the control but had a transient early decrease in proliferation, with later partial recovery, with PHA (Fig. 8).

IL-2 activity in the supernatants of ConA-stimulated mouse splenocytes was decreased in a dose- and time-dependent manner (Table 2). Significant departures from control values became evident in the 10^4 and 10^6 inoculum groups at days 18 and 14 p.i., respectively, whereas IL-2 activity in the supernatants of splenocytes was not significantly reduced for mice which received 10^2 infected cells. Lymphocyte viability in mitogenesis and IL-2 assays was greater than 80%.

IFA titration of mouse sera revealed that all groups given *E. risticii*-infected cells made antibody to the organism by

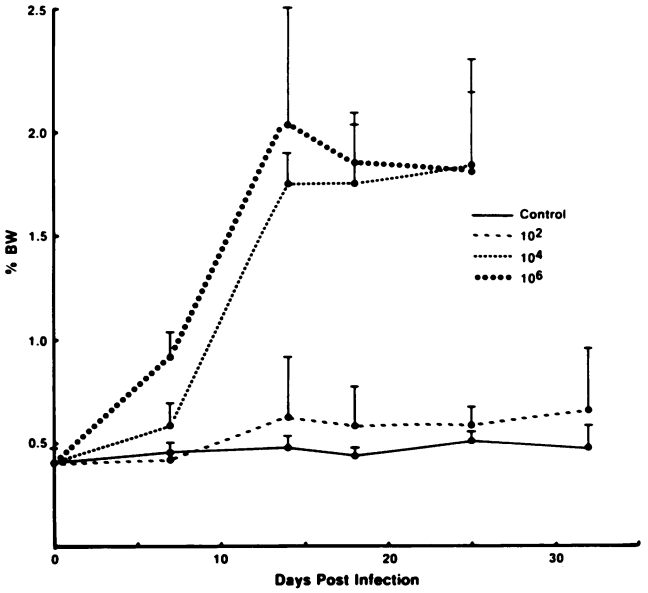

FIG. 5. Spleen weight as a percentage of body weight (BW) for mice inoculated with *E. risticii*-infected U-937 cells. There was significant splenomegaly evident among mice inoculated with 10^4 or 10^6 infected U-937 cells from day 7. With 10^2 infected U-937 cells, there was no splenomegaly.

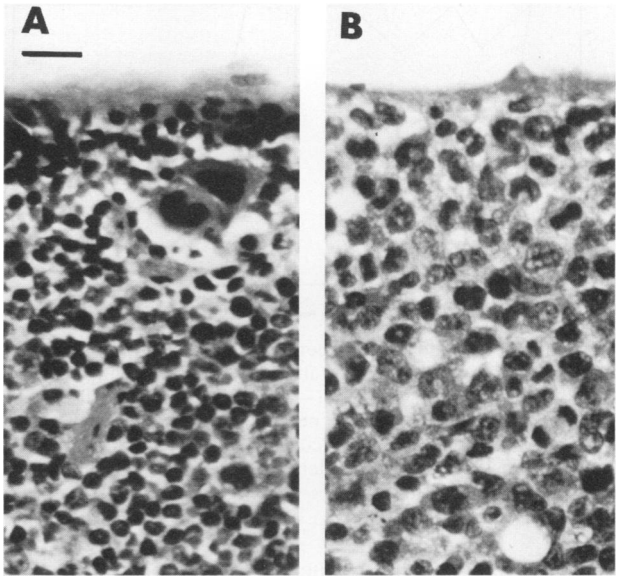

FIG. 7. Splenic red pulp near capsule in control (A) and infected (B) mice. Bar, 25 μm.

the termination of the experiment. Mice given 10^4 or 10^6 infected cells developed a significantly positive titer (>1:1,280) at 18 days p.i. Antibody titers remained at high levels until the termination of the study. At day 25 p.i., antibody titers became positive in the 10^2 inoculum group (Table 3) and progressively increased during the remaining period. The mice inoculated with 10^6 uninfected U-937 cells or control mice did not have IFA-detectable antibodies against *E. risticii* at a 1:20 dilution.

In summary, *E. risticii* infection caused profound morphological and functional changes in the immune system of

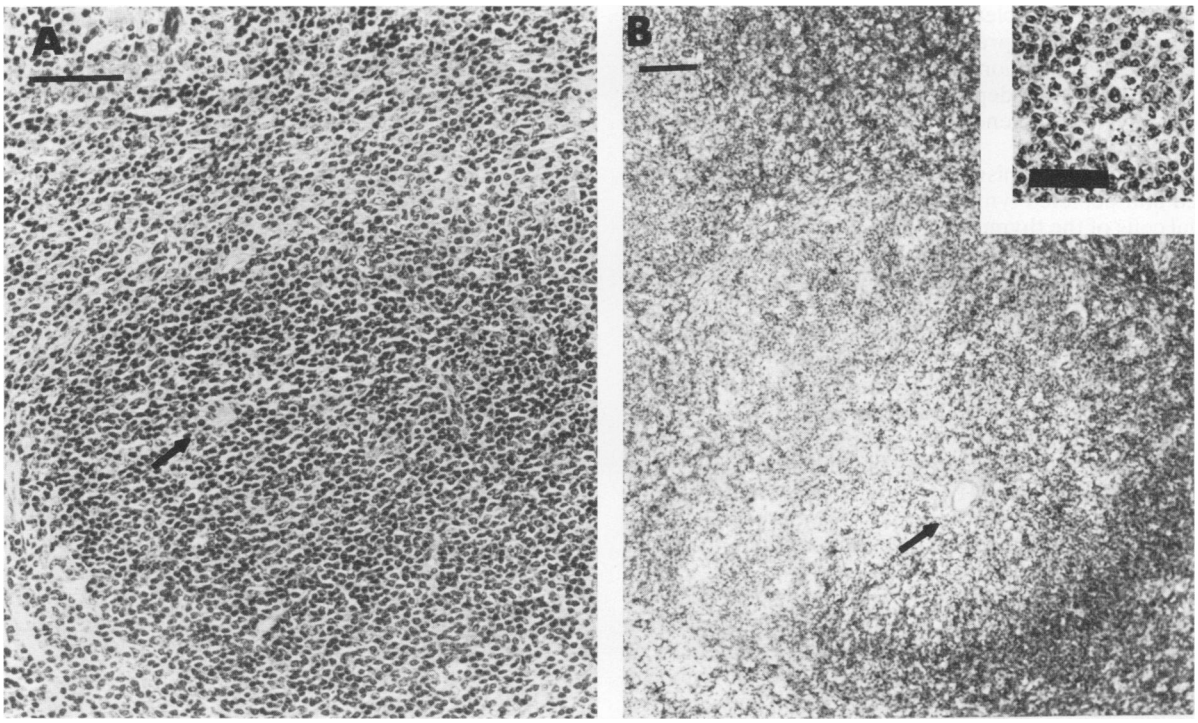

FIG. 6. Splenic white pulp of uninfected (A) and infected (B) mice (10^6 uninfected or infected U-937 cells per mouse). Mice were killed on day 25 p.i. The arrow indicates a sheathed artery. Bars, 100 μm (panel A) and 200 μm (panel B). The inset shows debris in histiocytes. Bar, 50 μm.

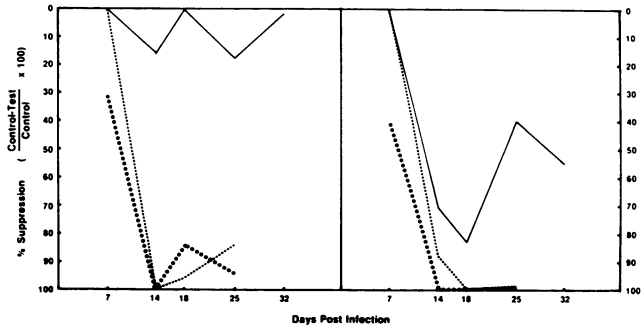

FIG. 8. Dose- and time-dependent suppression of mitogen-induced splenocyte proliferation in mice inoculated with *E. risticii*. Mice were inoculated with 10^2 (———), 10^4 (·····), or 10^6 (•••••) *E. risticii*-infected U-937 cells. The percent suppression of response to ConA or PHA is expressed as [(control − infected)/control] × 100. Note the time- and dose-dependent suppression of the blastogenic response to ConA or PHA.

infected mice, causing reduction of thymic and splenic periarteriolar lymphoid cells, decreased mitogen-induced lymphocyte blastogenesis, and decreased IL-2 activity in the supernatants of splenocyte cultures. Changes were generally dose- and time-dependent and were associated with significant morbidity and mortality in the affected groups.

DISCUSSION

A previous study has shown that i.p. injection of mice (Sprague-Dawley CF-1) with plasma and buffy coat from a pony exhibiting clinical symptoms of Potomac horse fever induces lethargy, squinty eyes, rough coats, and hunched backs at 10 to 12 days postinoculation (17). Ehrlichial organisms were demonstrated in the cytoplasmic vacuoles of mouse spleen mononuclear cells by electron microscopy (17). The results of the present study extend this finding by showing that i.p. injection of cultured *E. risticii* into mice caused dose- and time-dependent morbidity and mortality. Thymic atrophy and splenomegaly were noticed for the first time in this disease.

Whatever the mechanism of injury, *E. risticii* selectively damaged and depleted lymphocytes but not the reticular or epithelial cells of the thymus. Thymic lymphoid atrophy may result from a variety of insults, including stress and the administration of such exogenous agents as corticosteroids, epinephrine, norepinephrine, and endotoxin (12, 19). Although stress may have contributed to the thymic atrophy seen in *E. risticii*-infected mice, several histopathologic features differed from those of murine thymic atrophy caused by corticosteroid administration. In *E. risticii*-induced thymic atrophy, severe lymphocyte degeneration and necrosis were evident in the medulla as well as the cortex. Medullary T cells (1, 5, 8) are resistant to in vivo administration of exogenous steroids, which preferentially destroy the immunologically incompetent, immature cells of the cortex (1, 8).

Plasma cells were observed in thymuses from infected mice at 18 days p.i. and after. It is unclear whether these cells represented a true increase in thymic plasma cells or whether resident cells are merely more visible in lymphocyte-depleted tissue. Plasma cells have been observed in the regenerative phase of hydrocortisone-induced involution (10). This may be due to a breach of the blood-thymus barrier. Plasma cell sequestration attributed to changes in the splenic filtration bed has been reported for *Plasmodium yoelii*-infected mice (27). Although the thymus was not mentioned, perivascular infiltration of plasma cells in various organs occurs in *E. canis* infection (15). The specificity and significance of thymic plasmacytosis are not known.

TABLE 2. IL-2 production in *E. risticii*-infected mouse splenocytes

No. of infected histiocytes/mouse	Amt of IL-2 (U/ml) on day p.i.[a]:			
	7	14	18	25
10^2	93.9*	98.7*	96.9*	85.1*
10^4	111.3*	96.7*	11.9†	0††
10^6	83.4*	22.5**	0††	0††

[a] To assess IL-2 production capabilities, splenocytes were cultured with ConA for 24 h. Log_2 serial dilutions of the supernatants were cultured with the IL-2-dependent cell line HT-2. IL-2 activity was determined by using a computer program which combines the parallel line assay with analysis of variance and generates the number of IL-2 units per milliliter with 95% confidence intervals. Normal mouse splenocyte supernatant was assigned a value of 100 U of IL-2 per ml. Numbers with the same superscript symbol are not significantly different from one another at the 95% confidence level.

Splenomegaly reached the maximum level at day 14 in the 10^4 and 10^6 inoculum groups, and the timing coincided with a nearly 100% inhibition of in vitro lymphocyte proliferative response. The development and duration of this hyporesponsiveness correlated with periarteriolar lymphoid depletion, seen during histopathologic examination of the spleen. Splenomegaly accompanied by persistent lymphocyte hyporesponsiveness was observed in experimental *Coxiella burnetii* infection, as well as with its cell wall vaccine (11, 28).

Splenic lesions seen in murine *E. risticii* infection differ from those seen after endotoxin injection or corticosteroid administration in mice (19). Endotoxin treatment reduced splenic weight and produced lymphocyte depletion and polymorphonuclear cell infiltration of red pulp and splenic follicles. The characteristics of endotoxin treatment (19) were not observed. Splenic histopathology also differed from the splenic atrophy resulting from corticosteroid injections (5) in that *E. risticii* infection resulted in splenic enlargement and development of splenic follicles was unimpaired. Moreover, production of antibody to *E. risticii* appeared unhindered. Thus, degeneration or destruction of lymphocytes during an *E. risticii* infection seems to be selectively directed toward some or all T-cell populations rather than general lymphocyte populations.

Similar alterations in lymphoid tissue have not been observed in horses infected with *E. risticii*. The reasons for this are uncertain; most infected horses are mature at the time of infection, the thymus is already involuted, and atrophy is not evident. Rather, the target organ in the horse is the intestinal wall (22). Finally, any superimposed or contributory effects

TABLE 3. Titer of mouse sera against *E. risticii* determined by IFA test

No. of infected histiocytes	Titer on day postinoculation[a]:					
	0	7	14	18	25	32
0	–	–	–	–	–	–
10^2	ND	–	ND	–	40	1,280
10^4	ND	–	ND	1,280	2,560	ND
10^6	ND	–	ND	2,560	1,280	ND

[a] –, Sera negative at 1:20 dilution against *E. risticii* by IFA test; ND, samples not analyzed.

of stress may be less severe in horses than in mice because of the relative steroid insensitivity of horses (18).

Splenic hematopoietic tissue of *E. risticii*-infected mice was characterized by a profound shift from erythro- to myelopoiesis. Diminished erythropoiesis in the red pulp of infected mice may have correlated with development of anemia: 40% of experimentally infected ponies became anemic (29), but differences in the amounts of extramedullary hematopoiesis between the two species would dictate that this splenic change would occur only in the murine disease. The granulopoietic response is of equally uncertain genesis: both naturally and experimentally infected ponies develop neutropenia with subsequent leukocytosis (9, 29), but coincident bone marrow changes were not examined.

A marked and persistent reduction of mitogen-induced lymphocyte proliferative responses and diminished IL-2 activity were present in vitro after *E. risticii* infection. The responses differed from those after in vivo corticosteroid injection. Steroids fail to suppress the in vitro mature T-cell response (6, 7). Early degeneration of T cell-dependent periarterial areas of the spleen and thymus and dilution of remaining T cells by non-T cell populations in the hypertrophic spleens of *E. risticii*-infected mice may contribute to the hyporesponsiveness of the spleen cell mixtures to lectins. However, significant reduction of IL-2 activity with 10^6 but not 10^4 infected U-937 cells despite the same degree of splenic hypertrophy (approximately four times that of the control) and thymic atrophy (approximately two-thirds that of the control) at day 14 p.i. suggests a more specific primary mechanism for this hyporesponsiveness.

The difference in proliferative responses to PHA and ConA in the spleens of mice inoculated with 10^2 *E. risticii*-infected U-937 cells may have been due to a difference in the subpopulations of lymphocytes responding to these two mitogens. ConA stimulates immature and mature murine thymocytes, whereas PHA stimulates only the mature subpopulation (2).

IL-2 is a T-cell growth factor which causes clonal expansion (25). However, impaired IL-2 production is not the primary cause of the reduced lectin-induced lymphocyte proliferation in *E. risticii* infection. At day 14 p.i., lectin-induced lymphocyte proliferation in the 10^4 inoculum group was almost completely inhibited despite the presence of IL-2 activity similar to that in control splenocyte cultures. Rather, the ability of T lymphocytes to respond to IL-2 is impaired first in *E. risticii* infection. This is not always the case. In *Taenia taeniaeformis*-induced immunosuppression, the rat splenocyte mitogenic response occurs at the same time as or later than the significant inhibition of IL-2 production (4).

The means by which *E. risticii* evades host defenses and eventually kills the host is not known. However, the ability of *E. risticii* to somehow depress lymphocyte responsiveness may possibly serve as a mechanism of survival in an otherwise immunocompetent host. The similarity beween the form of immunosuppression seen in our experiment and in *C. burnetii* infection (11, 28) suggests that the model may be of value in pursuing the nature of the pathogenetic process in rickettsiosis. There may be a common fundamental regulatory defect in all these syndromes.

Although, antigenically, *E. risticii* and *E. sennetsu* are closely related (16), the responses of mice to these agents are slightly different. Characteristics of *E. sennetsu* pathology in mice are lymphadenopathy, lethargy, anorexia, peritonitis, occasional splenomegaly, and light diarrhea (14). In contrast, with *E. risticii*, splenomegaly was the most consistent and rapid obvious pathologic change and peritonitis was not evident.

The 50% lethal dose (LD_{50}) or 50% infective dose (ID_{50}) has not been determined for any ehrlichial organisms. Experimental infection of animals with ehrlichial organisms has been carried out by injection with whole blood or spleen homogenates of other infected animals (14, 15, 29). Thus, neither the number of infected host cells nor the number of ehrlichiae in the blood or spleen is reported for such studies. Neither the methods to purify viable *E. risticii* nor to assess the viability of purified ehrlichiae, such as the plaque-forming assay used for other kinds of rickettsiae, have been developed for determination of an accurate LD_{50} or ID_{50}. One of the reasons for this is that culturing a large quantity of ehrlichial organisms in vitro is difficult or impossible (24). In fact, *E. risticii* is the first ehrlichia effectively cultured in continuous cell lines (13, 21).

Based on our studies, the LD_{50} for mice was approximately 10^6 *E. risticii*-infected U-937 cells and the ID_{50} was approximately 10^3 *E. risticii*-infected U-937 cells. Although the infection of circulating blood monocytes is hardly demonstrable on blood smears from infected horses even during the acute phase of infection, transfusions of 0.5 ml of blood by i.p. injection can cause mice to become sick and die (17; N. K. Jones, personal communication). The exact ID_{50} or LD_{50} of cell-free *E. risticii* is unknown, as is the reason why transfer of cells infected in tissue culture is less effective in establishing infection than transfer of infected pony blood.

The present techniques for identification or isolation of organisms from infected animals are too insensitive to be used as indicators of established infection with *E. risticii*. Furthermore, the development of an immunoglobulin G antibody response to *E. risticii* is not a selective indicator of established infection, since heat-killed or Formalin-killed *E. risticii* could induce an immunoglobulin G antibody response (data not shown). These spleen cell responses are more sensitive and semiquantitative parameters for assessing the relative virulence of different isolates of *E. risticii* or *E. risticii* cultured under different conditions or for assessing the susceptibility of different strains of mice.

The characterization of a murine immune organ response to different doses of an organism which is pathogenic to horses provides a means for relatively inexpensive investigations of immunity, chemotherapy, and pathogenesis in Potomac horse fever.

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