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Characteristics of Lymphoid Cells That Adoptively Transfer Immunity to *Rickettsia mooseri* Infection in Mice

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The capacity of adoptively transferred immune lymphoid cells or passively transferred immune serum to alter the course of an established *Rickettsia mooseri (R. typhi)* infection in the spleen was evaluated in BALB/c mice. Immune cells, but not immune serum, controlled the established infection. An effective lymphocyte was a T-cell which had to possess a capacity to divide.

Previous studies from this laboratory identified roles for both humoral and cell-mediated immune responses in the in vivo control of *Rickettsia mooseri (R. typhi)* infection in guinea pigs (16–18). Transfer experiments in which intradermally infected recipients were employed showed that immune sera and immune spleen cells were capable of suppressing the systemic spread of infection and that immune spleen cells possessed a unique capacity to restrict *R. mooseri* infection in skin at sites of intradermal inoculation (18, 19). In other experiments (4) with BALB/c mice, the control of splenic *R. mooseri* infection paralleled the systemic expression of enhanced, nonspecific macrophage microbicidal capacity. Taken together, these results suggested that the immunological defense against the obligate intracellular bacterial parasite *R. mooseri* might be mechanistically similar to the well-characterized (1, 8–10, 15, 22–26) T-cell-dependent, macrophage-effected mechanism responsible for the immune control of infections with numerous facultative intracellular bacteria.

This proposition is pursued in this report in which we show that immunity to established *R. mooseri* infection in the spleen is antibody independent and mediated by T lymphocytes.

(The work recorded here was presented by Arthur E. Crist, Jr., in partial fulfillment of the requirements of the Ph.D. degree. University of Maryland, Baltimore, 1980.)

MATERIALS AND METHODS

Animals. Female BALB/c mice, weighing 18 to 22 g, were purchased (Flow Laboratories, Inc., McLean, Va.), fed Purina Rodent Chow (Ralston Purina Co., St. Louis, Mo.), and provided with water ad libitum.

Rickettsiae. Two seeds of *R. mooseri*, Wilmington strain, were used. For the first seed, which was employed for challenge infections, infected yolk sacs were homogenized in 3.7% brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) to produce a 20% (wt/vol) suspension which was dispensed into glass ampoules, flame sealed, quick-frozen in a dry ice-alcohol mixture, and stored at −70°C. This seed with a passage history of 42 embryonated egg, 3 tissue culture, and 2 embryonated egg passages had been plaque purified. By established procedures (reviewed in reference 4), it was shown that the challenge dose contained 2.04×10^5 rickettsial bodies, 1×10^4 conventional PFU, and 4.83×10^4 centrifuged PFU. Sucrose-phosphate-glutamate solution (2) was the diluent.

The second *R. mooseri* seed which was used to initiate immunizing infections was derived from the above yolk sac seed by passage in L cells and has been previously described (4). The diluent was brain heart infusion broth.

The purpose of using the two seeds and the different diluents was to minimize the influence of culture contaminants.

Quantitation of rickettsiae. Rickettsiae in the homogenates of spleens were enumerated by plaque assay (20), the lower limit of sensitivity of which was about 100 PFU/g of spleen tissue.

Serological procedures. Antibody titers of serum were determined by a modification (C. L. Wisseman, Jr., et al., manuscript in preparation) of the indirect fluorescent-antibody test of Elisberg and Bozeman (5).

Cytotoxic sera and complement. Mouse anti-theta (Thy 1.2) gamma globulin (mouse: AKR/J anti-C3H/HeJ), mock mouse anti-theta serum (AKR/Cum anti-C3H/HeJ), and guinea pig complement were purchased from Litton Bionetics, Inc., Kensington, Md. Rabbit anti-mouse immunoglobulin G (IgG; heavy and light chain specific) was purchased from Cappel Laboratories, Cochranville, Pa.

Preparation of spleen cells. Mice were killed by cervical dislocation, and their spleens were removed aseptically, placed in sterile petri dishes, and minced with scissors and forceps. The resulting fragments were pressed through a stainless-steel 80-mesh screen into sterile beakers containing incomplete medium which consisted of RPMI 1640 (Flow Laboratories, Inc.) supplemented with 2 mM glutamine (GIBCO Laboratories, Grand Island, N.Y.) and 15 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (GIBCO Laboratories). The spleen cell suspensions were washed three times in incomplete medium by centrifugation ($250 \times g$) and adjusted to the desired concentration. To remove erythrocytes, spleen cells were suspended in Tris-buffered ammonium chloride for 10 min at 4°C (3), washed three times in incomplete medium, suspended in complete medium (incomplete medium supplemented with 5% fetal bovine serum; GIBCO Laboratories), and then incubated in plastic tissue culture flasks or in glass roller bottles for 1 h at 37°C in a humidified atmosphere containing 5% CO_2 in air. After incubation the leukocytes which were not adherent to the plastic or glass surface were collected.

Treatment of spleen cells with cytotoxic sera. Spleen cells (25×10^6/ml) depleted of erythrocytes and glass-adherent cells were incubated in complete medium with either anti-

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theta (Thy 1.2) gamma globulin or rabbit anti-mouse IgG (final dilutions, 1:5) for 30 min at room temperature or 4°C, respectively, and washed twice in complete medium, and then guinea pig complement (final dilution, 1:10) was added. The cell suspensions were incubated for 30 min at 37°C and then washed twice in incomplete medium. Cell viability was determined by trypan blue exclusion.

Characterization of the spleen cell populations. Several experiments were performed in which one or more of the following treatments were utilized.

(i) Freezing and thawing. Spleen cells (10^6/ml) in complete medium were subjected to three rapid freeze-thaw cycles (−70°C to 37°C), washed twice in incomplete medium, and adjusted to the desired concentration. This resulted in the death of more than 99% of the cells as determined by trypan blue exclusion.

(ii) Irradiation. Spleen cells (10^7/ml) were X-irradiated (2,400 rads) (31) and washed twice with incomplete medium. The viability of these cells after irradiation was greater than 90% as determined by trypan blue exclusion.

(iii) Mitomycin C. Spleen cells (10^7/ml) in complete medium were incubated in the presence of mitomycin C (P-L Biochemicals, Inc., Milwaukee, Wis.) (final concentration, 40 μg/ml) for 1 h in a 37°C water bath (7) with frequent agitation and then washed three times in incomplete medium. Apparent cell viability by trypan blue exclusion was 91%. This DNA-cross-linking agent prevents subsequent cell division even when applied to cells not in mitosis (6).

(iv) Vinblastine. Vinblastine sulfate (Velban, Eli Lilly & Co., Indianapolis, Ind.; 100 μg per mouse) was dissolved in 0.15 M NaCl and administered intravenously (i.v.). This dose should have destroyed cells in mitosis over a period of ca. 15 h (12, 22, 28). The viability of the transferred cells by trypan blue exclusion was 94%.

Experimental design and datum presentation. (i) Immunizing infection. On the basis of a previous study (4), the subcutaneous inoculation of 10^4 PFU of *R. mooseri* (L-cell seed) into the left hind footpad was used to initiate immunizing infections.

(ii) Challenge infection. The challenge infection was by an i.v. injection of 10^4 PFU of *R. mooseri* (egg seed) into the lateral tail vein 6 h before the mice received cells or serum. This allowed assessment of the capacity of the transferred materials to restrict an established systemic infection, as measured in the spleen, rather than their capacity to prevent infection.

(iii) Transfer of spleen cells or serum. Unless otherwise stated, spleen cells (10^8/recipient) or serum (0.5 ml per recipient) was transferred intraperitoneally (i.p.). Unless otherwise indicated, spleen cells from immune donor mice were collected 14 days after infection.

(iv) Assay for protection. Protection was measured by comparing the growth of rickettsiae in the spleens of mice receiving cells or serum with the growth of rickettsiae from the same inoculum delivered to untreated mice.

(v) Data presentation. Data are expressed either directly as the $\log_{10}$ of the geometric mean number of PFU per spleen per group or indirectly as relative protection, an index which was obtained by subtracting the $\log_{10}$ geometric mean number of rickettsiae per spleen of experimentally treated mice from the $\log_{10}$ geometric mean number of rickettsiae recovered from control animals. In experiments in which relative protection was used, spleens were collected on day 5 of the challenge infection.

Statistics. Data were analyzed by the Student *t* test or the analysis of variance and *Q* test.

RESULTS

Adoptive transfer of protection with immune spleen cells. After the i.v. inoculation of 10^4 PFU into naive mice, rickettsiae were detected in the spleen by day 1 and rapidly increased through day 3 (Fig. 1). The peak titer occurred on day 5, and thereafter, the majority of rickettsiae was rapidly cleared.

Adoptive transfer of immune spleen cells, but not passive transfer of immune sera, provided a degree of control of spleen infection (Fig. 2 [a composite of two experiments]).

To determine when, during the course of immunizing infection, cells with anti-*R. mooseri* capacity were generated, spleen cells were collected at 7-day intervals (Fig. 3). Significant immunity was transferred by spleen cells collected from day 14 through at least day 28. Spleen cells collected at day 14 of donor infection were used for all subsequent experiments.

Some requirements for the transfer of protection. (i) Number of cells. Above a threshold, the quality of adoptive immunization was dependent on the dose of immune spleen cells transferred (Fig. 4).

(ii) Effect of X-irradiation or freezing and thawing. Samples of immune spleen cells were subjected to either X-irradiation or three freeze-thaw cycles or were held untreated at room temperature. After identical intervals in vitro, treated and untreated cells were administered to separate groups of infected mice. Killing immune spleen cells completely ablated their capacity to adoptively transfer protection (data not shown).

Characteristics of protective spleen cells. (i) Evidence for a T lymphocyte. Spleen cells which had been depleted of

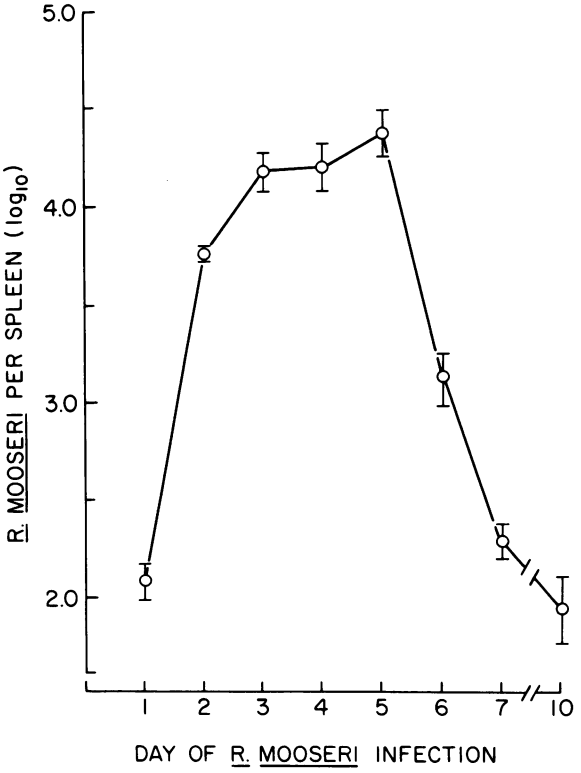


FIG. 1. Course of *R. mooseri* infection in the spleens of nonimmune mice after i.v. inoculation of 10^4 PFU of *R. mooseri*. Geometric mean of four mice per time point ± standard error.

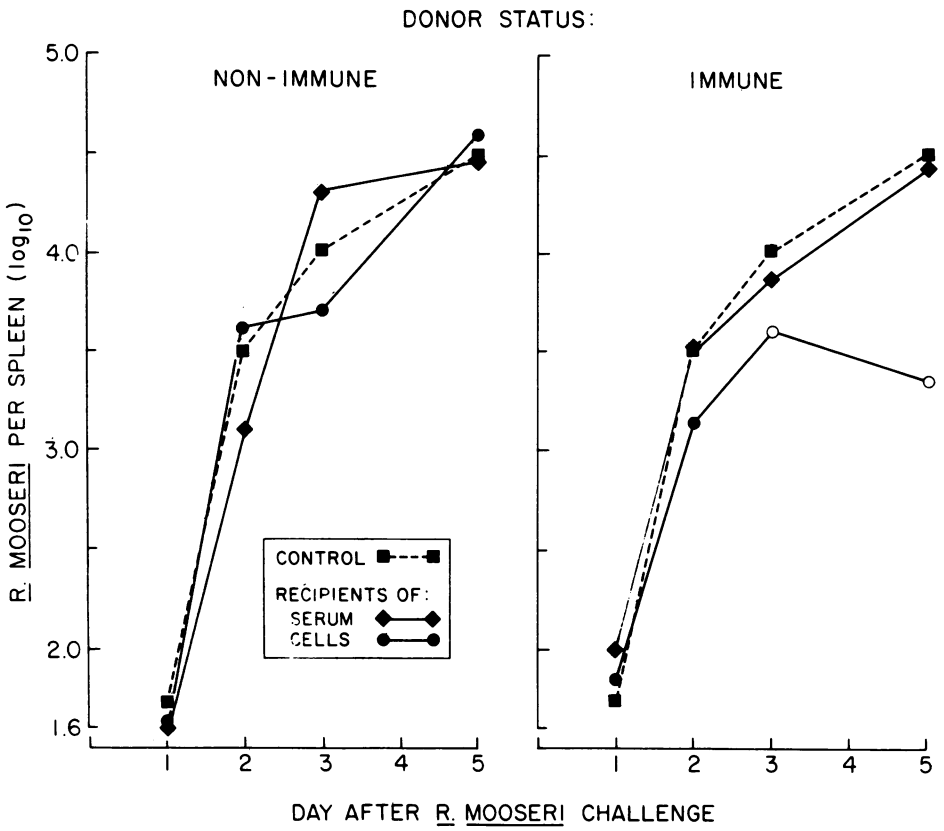


FIG. 2. Demonstration that mice injected with immune lymphoid cells are better equipped than control mice to restrict rickettsial growth in the spleen. Spleen cells or sera collected before (indirect fluorescent-antibody titer, <4) or 28 days after (indirect fluorescent-antibody titer, 2,560) donor infection were transferred to mice which had been infected 6 h previously with *R. mooseri*. There were eight mice per group per time point. Those groups which differed significantly ($P \leq 0.01$) from controls which were infected with *R. mooseri* but not injected with sera or cells are indicated by open symbols.

erythrocytes and glass-adherent cells were treated with either anti-theta gamma globulin and complement or rabbit anti-mouse IgG and complement or were untreated. After identical intervals in vitro, treated and untreated cells (5×10^7 per recipient) were administered to separate groups of mice by the i.p. or i.v. route. Anti-theta gamma globulin and complement, but not anti-mouse IgG and complement, were capable of significantly diminishing protection (Fig. 5).

(ii) Evidence for a requirement for cell division. Reduction of control of rickettsial replication by treatment with vinblastine sulfate or mitomycin C (Fig. 6) showed that mediator lymphocytes must retain a capacity to replicate if they are to transfer protection.

DISCUSSION

Previous studies with guinea pigs (18, 19) provided direct evidence that immune control of the growth of *R. mooseri* within a tissue or organ is mediated by immune lymphoid cells. Immune serum, whether passively transferred (18), injected directly into a skin site before rickettsial inoculation into the same site, or admixed with rickettsiae before intradermal inoculation, does not restrict rickettsial growth in skin (J. R. Murphy and C. L. Wisseman, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, D52, p. 60). However, passively transferred immune serum is capable of modifying the pattern of dissemination from skin (19). The mechanisms and specific mediators of these different types of protection are not known.

In the present study, *R. mooseri* infection in spleen tissue was initiated before cell or serum transfer to allow direct measurement of the effects of humoral or cellular mediators on an established splenic infection. We emphasize that this model was selected to measure mediators capable of modifying established infection in the spleen, not dissemination of infection to the spleen. The results show that the control of established splenic infection is effected by thymus-dependent lymphocytes which must retain a capacity to divide. No evidence of immune serum-mediated restriction of established splenic infection was obtained. Taken together, the results of this study which focused on infection in the spleen and the results of previous studies which focused on infection in skin (16–19) suggest that a cellular defense is the critical component in controlling rickettsiae once they have lodged in a tissue or organ. Therefore, it might seem reasonable to suggest that the lymphocyte mediator of the cellular defense would be the same in skin and the spleen.

Possibly arguing against the concordance of the splenic and skin defense mechanisms is the demonstration that after primary intradermal infection the control of *R. mooseri* infection in skin is expressed by day 5 or 6, whereas splenic infection progresses during this interval and is not controlled until at least day 14 (16). Because replicating lymphocytes preferentially accumulate at sites of inflammation (13, 27), the identification of a replicating T-cell as a mediator of immunity to *R. mooseri* suggests that one explanation for the differences between foci in the expression of immunity to *R.*

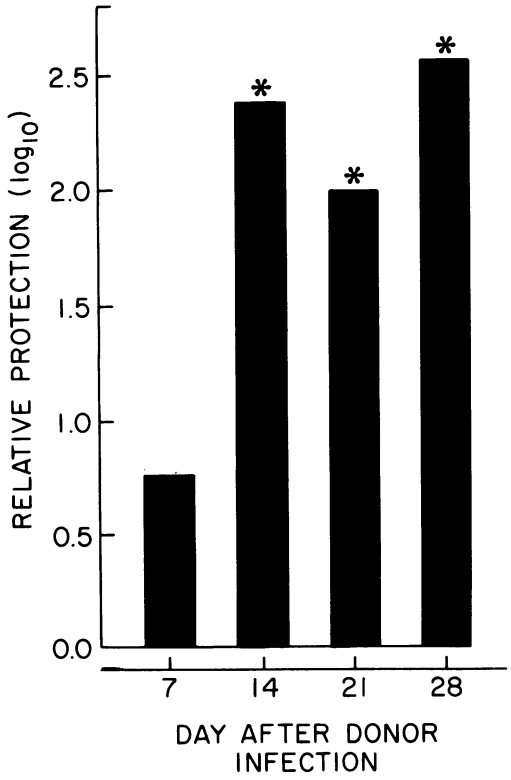
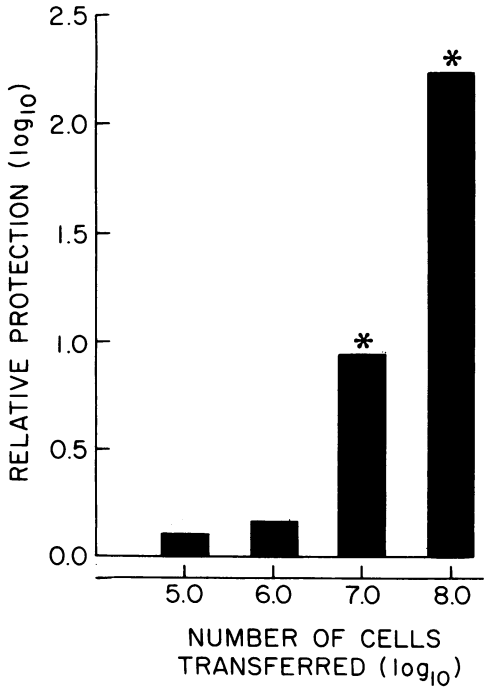


FIG. 3. Dynamics of the generation of protective cells after subcutaneous inoculation of 10^4 PFU of *R. mooseri*. Infected mice were sacrificed at the indicated intervals, and their spleen cells were adoptively transferred to naive recipients. The figure presents the difference in the 5-day growth of *R. mooseri* between normal mice and recipients of cells. Significantly fewer ($P \leq 0.01$, indicated by *) rickettsiae were recovered from recipients of spleen cells collected 14 through 28 days after donor infection than from similarly infected normal mice. There were four mice per group per time point.



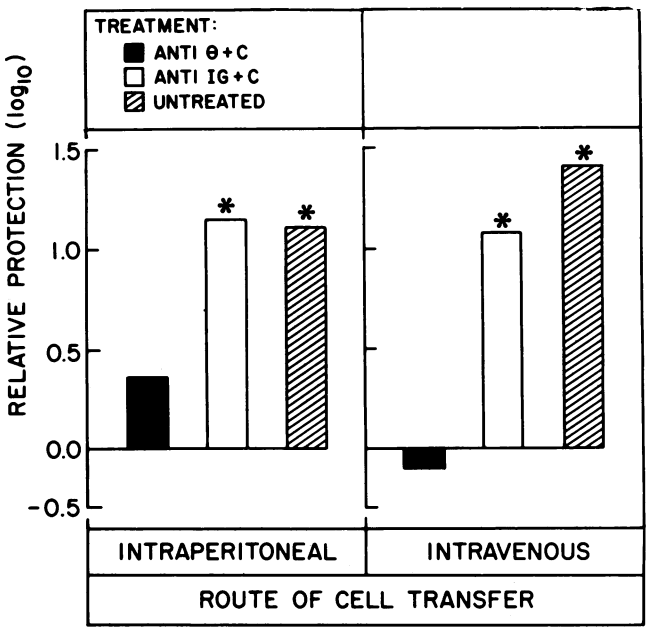


FIG. 5. Evidence that an immune spleen cell which adoptively transfers protection to *R. mooseri* is a T lymphocyte. In each of two experiments which yielded similar results, a pool of spleen cells was divided and either treated with anti-theta gamma globulin plus complement (causing 42 to 45% cell lysis) or anti-mouse IgG plus complement (causing 30 to 32% cell lysis) or untreated (positive control) and maintained in vitro under similar culture conditions. The cells were then inoculated i.p. or i.v. into separate groups of *R. mooseri*-infected mice. The figure, in which the results of one experiment are recorded, shows that recipients of anti-IgG (IG) or untreated immune spleen cells were significantly ($P \leq 0.01$, indicated by *) better equipped to restrict the 5-day growth of *R. mooseri* than were nonimmune untreated mice. In contrast, treatment of immune spleen cells with anti-theta gamma globulin (Θ) plus complement significantly reduced protection. The number of *R. rickettsiae* cells recovered from recipients of anti-theta gamma globulin-treated immune cells was significantly greater ($P \leq 0.05$ or 0.01, i.p. or i.v. experiment) than the number recovered from recipients of anti-IgG-treated or untreated immune spleen cells. There were four mice per group.

FIG. 4. Demonstration that the level of adoptive immunity to *R. mooseri* is directly related to the number of immune spleen cells transferred. Recipients of 10^7 or 10^8 immune spleen cells were significantly ($P \leq 0.01$, indicated by *) better equipped to control the 5-day growth of *R. mooseri* than were recipients of fewer immune cells or normal mice. There were four mice per group per interval.

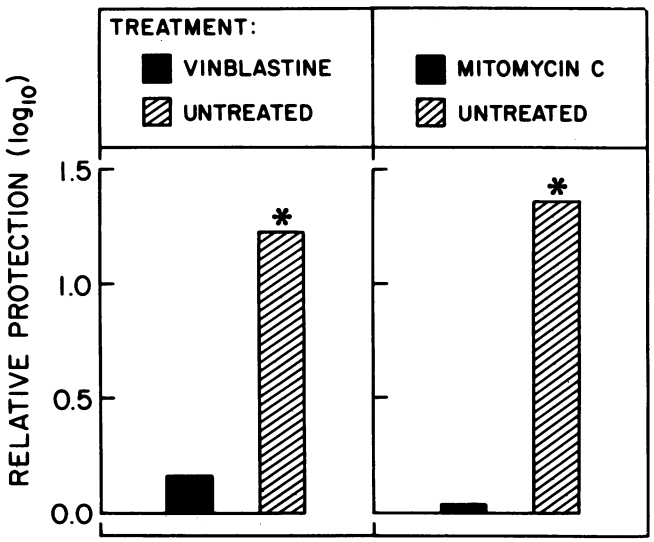


FIG. 6. Evidence that a mediator of adoptive immunity to *R. mooseri* is a dividing population of lymphocytes. For the first experiment immune spleen cells were collected from mice which were either untreated (positive control) or inoculated i.v. with vinblastine 15 h before cell harvest. For the second experiment a pool of immune spleen cells was divided and either treated with mitomycin C or kept untreated (positive control) and maintained in vitro under similar culture conditions. Recipients of immune cells taken from untreated donors restricted significantly ($P \leq 0.01$, indicated by *) the 5-day growth of *R. mooseri* as compared with the growth in normal mice. In contrast, immune spleen cells taken from vinblastine-treated donors or treated in vitro with mitomycin C failed to adoptively transfer protection. The numbers of rickettsiae recovered from recipients of vinblastine- or mitomycin C-treated cells were significantly greater ($P \leq 0.01$) than the number recovered from recipients of untreated immune cells. There were four mice per group.

mooseri may be the dynamics of the rickettsia-caused inflammatory reaction within each site.

The identification of a T-cell which must retain its capacity to divide as a mediator of immunity to *R. mooseri* and the knowledge that this class of lymphocytes can signal macrophages to increase their microbicidal capacities (11, 14, 15, 22–24) suggest that a T-cell-directed, macrophage-mediated defense might be important in controlling *R. mooseri* in vivo. The demonstration that the expression of immunity to *R. mooseri* in the spleen is accompanied by systemic activation of macrophage microbicidal capacities (4) and that in vivo- and in vitro-activated macrophages are better equipped to control *Rickettsia tsutsugamushi* (21; C. A. Nacy, M. S. Meltzer, P. K. Russel, and J. V. Osterman, Fed. Proc. **38**:1078, 1979) supports this proposition. The strongest argument against it is that rickettsiae possess mechanisms which might allow them to avoid contact with macrophages by retreating behind various kinds of host cell membranes (reviewed in references 29 and 30).

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