Mechanisms of Immunity in Typhus Infection: Adoptive Transfer of Immunity to *Rickettsia mooseri*

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Received for publication 5 October 1979

When nonimmune guinea pigs are inoculated intradermally (i.d.) with *Rickettsia mooseri* (*R. typhi*), the rickettsiae replicate at the site of inoculation, leading to the development of a grossly observable lesion. In contrast, guinea pigs which have recovered from *R. mooseri* infection are resistant to challenge and prevent both rickettsial growth and the formation of lesions. To study the mechanisms of this immunity, sera or splenic cells collected from nonimmune or immune guinea pigs were inoculated separately into nonimmune recipients. Splenic cells collected from immune donors protected *R. mooseri*-naive recipients from i.d. challenge as measured by control of rickettsial growth and by prevention of development of lesions at i.d. sites of inoculation. In contrast, serum from immune and nonimmune donors failed to protect nonimmune recipients by either criterion.

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When nonimmune guinea pigs are inoculated intradermally (i.d.) with *Rickettsia mooseri* (*R. typhi*), the organisms replicate at the site of inoculation for 3 to 5 days, depending on the size of the inoculum, after which they are rapidly eliminated (8, 9). Despite the fact that rickettsiae disappear from the site of inoculation at about the same time as antibodies become detectable in the serum, the local cellular reaction, rather than antibodies, seems to be correlated more closely with their clearance (9). Although the local reaction strongly suggests a cell-mediated immune mechanism, the infected animals fail to demonstrate delayed-type hypersensitivity when skin tested with *R. mooseri* antigen. Furthermore, systemic dissemination of the rickettsiae occurs only after they have been eliminated from the site of inoculation, at a time when high antibody levels exist (8).

The results of the study reported here establish the immunological basis of rickettsial clearance from the i.d. sites of infection by adoptive transfer of immunity with spleen cells from immune animals to nonimmune recipients.

**MATERIALS AND METHODS**

*Rickettsiae*. Two seed lots of *R. mooseri*, Wilmington strain, were employed. Seed 1 was prepared from monkey kidney cell culture (12EP/15GP/5EP/1GP/4BSC-1) and was used for immunizing infections (see below). Seed 2, prepared from embryonated chicken eggs (12EP/15GP/5EP), was used for challenge infections (see below). Some characteristics of these seed preparations and the method employed for their production and characterization have been published (8, 10). Guinea pigs infected with the seed prepared from monkey kidney cell cultures did not develop hypersensitivity to normal yolk sac components.

**Animals.** Randomly bred Hartley strain guinea pigs were employed for 50% infectious dose (ID50) titrations, and syngeneic strain 13 guinea pigs were employed for all other experiments. Animals of both strains were purchased (R. C. Roscrans, Hamilton, Mont.), housed individually, fed Purina Guinea Pig Chow (Ralston Purina Co., St. Louis, Mo.), and provided water ad libitum.

**Serological techniques.** The microagglutination (MA) test of Fiset et al. (4) was used with ether-treated, "specific" particulate *R. mooseri* antigens at a concentration of 333 μg/ml. The diluent for antigen and serum was 0.9% NaCl, which contained 0.01% merthiolate and 1% normal guinea pig serum.

**Diluent.** In experiments which involved the inoculation of guinea pigs with spleen cells, the diluent, hereinafter designated as D/2 medium, was a half-strength Dulbecco modification of Eagle minimal essential medium with Earle’s salts containing 0.1% glucose (Grand Island Biological Co., Grand Island, N. Y.) (14) without any added serum or antibiotics. With the exception of one experiment in which D/2 diluent was used, the diluent for the yolk sac *R. mooseri* was sucrose-phosphate-glutamate (2). The diluent for the kidney cell culture seed was 3.7% brain-heart infusion (Baltimore Biological Laboratories, Baltimore, Md.).

**Quantitation of rickettsiae.** (i) Plaque assay. The chicken embryo cell plaque assay, described in detail elsewhere (10), was used.

(ii) Guinea pig subcutaneous ID50 determinations. Male Hartley guinea pigs of about 500 g were inoculated subcutaneously (s.c.) in the nape with decimal dilutions of *R. mooseri* seed in D/2 medium, using two animals per dilution. Infection was determined by measuring serological conversion with the MA test. All preinoculation samples displayed MA titers of <1:2. A titer of 1:4 or greater in serum collected 28 days after inoculation was considered posi-
tive and indicative of infection. The ID₉₀ was calculated by the method of Reed and Muench (12).

Preparation of serum and spleen cells. Blood was collected by cardiac puncture. The separated sera were stored at −20°C in sterile rubber-stoppered vaccine bottles.

By a sterile technique, spleens were collected, minced with scissors, gently pressed through 80-mesh stainless steel screen into cold (4°C) D/2 medium, passed through five thicknesses of sterile surgical gauze, washed by low-speed centrifugation, and suspended in 0.85% NH₄Cl to lyse erythrocytes. After 1 min, the NH₄Cl solution was diluted by the addition of 5 volumes of D/2 medium and, after a low-speed centrifugation, the washed cells were suspended to the desired concentration in D/2 medium. Over 80% of the cells prepared by these procedures were viable as measured by trypan blue exclusion.

Sera and splenic cells were delivered to the recipients by s.c. inoculation in the nape.

Experimental model. (i) Immunizing infection. On the basis of a previous report (8), i.d. inoculation of 3.5 × 10⁶ plaque-forming units (PFU) of R. mooseri (kidney cell seed) into the outer aspect of the thigh was used to initiate immunizing infections. This inoculum and route of infection were selected because the resulting infection and aspects of the host immune response to infection occur in a reproducible sequence (8, 9).

(ii) Challenge infection. The challenge infection was selected on the basis of previous observations and consisted of i.d. inoculation of 8.2 × 10⁴ PFU of R. mooseri (egg seed) into each of three sites along the central portion of the shaved back lateral to the mid-line. This challenge produces readily observable and characteristic reactions in the skin of nonimmune animals at the sites of inoculation (9).

(iii) Assays for protection. Experimentally treated and normal guinea pigs were infected with the same R. mooseri inoculum. Differences in the progress of these infections provided the basis of the protection assays.

For one series of experiments, the progress of infection was determined directly by rickettsial titration. At intervals after challenge, biopsies of the skin taken at the sites of i.d. inoculation were titrated in tissue cultures for viable rickettsial content, as previously described (9, 10).

For the other experiments, the progress of infection was estimated indirectly by the magnitude of the indurated lesions at the sites of i.d. inoculation. The area of induration was calculated from measurements of the diameter of the lesions, and relative protection was determined as follows: relative protection = (Ic – It)/Ic where \( I_c \) was the mean area of induration recorded for controls. \( I_t \) was the mean area of induration observed for treated animals. With this formula, an index of 0 indicates no protection, whereas an index of 1 indicates complete protection. A negative value ("negative protection") indicates that treated animals developed larger lesions than did controls. For one series of experiments, the levels of relative protection observed over a 6-day interval were summarized as a single value. This was accomplished by determining the arithmetic mean of the levels of relative protection recorded for days 2 through 6 after infection. This value is referred to as the mean relative protection.

RESULTS

Demonstration that immune splenic cells adoptively transfer immunity to R. mooseri. Figure 1 presents a summary of four experiments which were conducted to determine whether transferred serum or splenic cells, collected from donors at intervals after primary R. mooseri infection, were capable of protecting R. mooseri-naive recipients from homologous challenge, using the indirect measure of suppression of skin lesion formation at the inoculation sites as an index of immunity. Recipients were inoculated with either 10 ml of serum or 2 × 10⁶ viable splenic cells from R. mooseri-immune donors. At 6 h after transfer, recipient animals, as well as immune and nonimmune animals from the same groups that provided donors for serum and spleen cells, were challenged with R. mooseri.

Challenge i.d. of guinea pigs of the donor group before and at intervals after primary infection, corresponding to the times when serum and splenic cells were harvested from other members of the group for the adoptive transfer experiments, showed that the donor animals developed immunity to lesion development in accordance with previous studies of the kinetics of development of immunity (8, 9). Thus, nonimmune control guinea pigs challenged with R. mooseri developed large lesions at sites of i.d. inoculation (Fig. 1). In contrast, similar doses of rickettsiae did not raise lesions if delivered 10, 12, or 21 days after the initiation of the primary infection. These results confirm that active systemic immunity, i.e., a capacity to resist i.d. challenge at a site distant from that of primary infection, had developed in donor animals by day 10 and persisted at least through day 21 after primary infection.

Sera were collected from nonimmune guinea pigs and from animals 10, 12, and 21 days after primary R. mooseri infection. A previous study demonstrated that sera collected at these intervals exhibit serological reactivity patterns consistent with immunoglobulin M antibodies (day 10 and day 12 sera) or immunoglobulin G antibodies (day 21 sera) (8). In the present study, recipients of these sera were not protected against i.d. R. mooseri challenge. Indeed, those animals which received sera containing antibodies developed slightly larger lesions than untreated normal guinea pigs.

In contrast to serum, splenic cells collected from donors immune to R. mooseri challenge conferred substantial immunity upon their adop-
IMMUNITY TO R. MOOSERI

Donors

Non-immune Guinea Pigs

Collected from

Recipient of

Sera

Cells

Collected 10 Days After

Infection

Cells

Collected 12 Days After

Infection

Cells

Collected 21 Days After

Infection

Status of Animals Challenged with 8.2 x 10⁶ PFU R. mooseri / ID Site

Fig. 1. Demonstration that immune splenic cells adoptively immunize naïve recipients against i.d. R. mooseri challenge. The immune status of donor animals was determined before R. mooseri infection and at days 10, 12, and 21 after infection by i.d. challenge. Sera or splenic cells collected at similar intervals were delivered to recipients, 10 ml of serum or 2 x 10⁶ cells; 6 h later, recipients, donors, and normal animals were challenged with R. mooseri, 8.2 x 10⁶ PFU, inoculated into each of three i.d. sites. The development of lesions on experimental animals was compared with controls inoculated with the same inoculum.

tive hosts. Splenic cells collected from normal nonimmune guinea pigs did not transfer protection.

Inhibition of growth of R. mooseri by immune spleen cells. To confirm that lesion development or suppression in these experiments corresponds to susceptibility or immunity as reflected by rickettsial multiplication or inhibition, respectively, a series of three experiments was conducted to determine directly the effect of serum or cell transfer on the growth of R. mooseri at sites of i.d. challenge. Sera and cells collected 21 days after donor infection were transferred to nonimmune animals. Rickettsial growth was determined from plaque counts of inoculation site biopsies.

Figure 2 presents the patterns of R. mooseri growth for one of these experiments and is rep-
representative of the series. Recipients of immune serum were not protected. Thus, the rickettsiae in skin of serum recipients attained high titers by day 3 after challenge, as did rickettsiae inoculated into the skin on nonimmune control animals. In contrast, *R. mooseri* inoculated into the skin of recipients of immune cells did not grow to high titer. Rather, an apparent stasis of rickettsiae was observed, with neither rapid growth nor clearance of organisms. It appears, therefore, that the capacity of immune cell recipients to resist lesion formation at sites of i.d. inoculation of rickettsiae resides in the capacity of these animals to control rickettsial growth. Moreover, since a direct relationship between lesion development and rickettsial growth was established in the system under study, all subsequent experiments relied upon the more convenient observation of lesion development.

**Requirements for the transfer of protection.** Immune splenic cells collected 21 days after donor infection were used for the following experiments.

(i) **Titration of splenic cells.** Figure 3 shows that adoptive immunization with $2 \times 10^5$ or $2 \times 10^6$ immune splenic cells protected recipients from *R. mooseri*, whereas doses of $2 \times 10^7$ and $2 \times 10^5$ cells did not. The protection was better with $2 \times 10^6$ cells than with $2 \times 10^5$ cells; significant protection was detected sooner after challenge with the larger dose.

Recipients of $2 \times 10^7$ and $2 \times 10^6$ cells developed lesions at the site of challenge which were larger than those of the control animals. The reason for this enhancement is not known.

(ii) **Effect of inactivation of immune spleen cells.** In one experiment immune spleen cells were heated to 56°C for 30 min, and another the cells were frozen and thawed. In each experiment untreated immune spleen cells from the same lot, which served as positive controls, were held at 4°C in vitro during the period of treatment of the other portion. Treated and control cells were administered in a dose of $1 \times 10^6$ cells per nonimmune recipient before challenge.

Figure 4 shows that killing of immune spleen cells by heating at 56°C or by freezing and thawing substantially diminished, but did not abolish completely, their capacity to protect recipients from the development of lesions at sites of i.d. inoculation with *R. mooseri*. Thus, recipients of live cells showed a capacity to resist lesion formation which progressively increased through day 6 (Fig. 4). In contrast, recipients of killed cells showed a lesser degree of protection which did not increase with interval after transfer. The difference in the dynamics of the protection transferred with live as compared with dead cells suggests that replication of immune cells or their continued production of a factor may be required for the maintenance of a high level of immunity.

**Rickettsiae transferred with spleen cells.** It was considered likely that splenic cells col-
lected 21 days after *R. mooseri* infection contained small numbers of live rickettsiae even though we were unable to detect rickettsiae when such spleen cell suspensions were titrated in the chicken embryo cell plaque assay. However, a guinea pig titration showed that there were about $1 \times 10^7$ guinea pig s.c. ID₅₀ of *R. mooseri* per $2 \times 10^9$ spleen cells.

To determine the effect of viable rickettsiae transferred with splenic cells on the development of lesions at sites of i.d. challenge, $7 \times 10^2$ ID₅₀ of *R. mooseri* suspended in D/2 medium were inoculated s.c. into the nape of 5 guinea pigs. At 6 h after inoculation these animals and five untreated animals were given the standard i.d. *R. mooseri* challenge. Figure 5 shows that s.c. inoculation of this number of rickettsiae did not prevent lesion formation at the sites of subsequent i.d. challenge. However, the animals which had both s.c. and i.d. inoculations of *R. mooseri* showed larger lesions on days 1 and 2 than did controls. The reason for this early enhancement of lesion formation is not known.

Adoptive immunity is expressed in the absence of humoral antibody. Recipients of 10 ml of immune serum or $2 \times 10^8$ immune splenic cells and normal animals were bled by cardiac puncture before and at intervals after *R. mooseri* challenge. The recipient animals were bled 6 h after treatment and immediately thereafter were challenged with *R. mooseri*.

Table 1 shows significant titers of humoral antibody, as measured by the MA test, for the serum recipients from 6 h through 5 days. Thus, serum antibody inoculated s.c. rapidly enters the blood compartment and maintains high titers for at least 3 days.

In contrast, sera collected from spleen cell recipients and control animals did not contain anti-*R. mooseri* MA antibody until 5 days after infection. It is clear, therefore, that (i) immune

![Diagram](http://iai.asm.org/)

**Fig. 4.** Demonstration that killing of immune spleen cells ablates their capacity to convey protection to recipients. Cells collected 21 days after donor infections were either heated at 56°C for 45 min or frozen and thawed through three cycles. Points with (□) above them represent values which differ from the corresponding control with a significance of $P \leq 0.05$.

![Diagram](http://iai.asm.org/)

**Fig. 5.** Effect of the subcutaneous inoculation of $7 \times 10^3$ ID₅₀ of *R. mooseri* on lesion formation at sites of subsequent i.d. inoculation of this agent. The i.d. challenge was delivered 6 h after the subcutaneous infection.
spleen cells do not provide adoptive recipients with an accelerated capacity to produce humoral antibody and (ii) adoptive immunity is expressed in the absence of humoral antibody detectable by this test (Fig. 2).

**DISCUSSION**

This study suggests that immunity to *R. mooseri* as expressed at i.d. sites of infection resides in a population of specifically sensitized cells. Adoptive immunization revealed that such cells protect recipients from both the growth of rickettsiae and the development of skin lesions. On the other hand, recipients of immune serum were not protected against rickettsial growth or lesion development. Thus, this study suggests that acquired immunity to i.d. *R. mooseri* infection is cell mediated and probably independent of humoral antibody. However, the possible production of local antibody at sites of local i.d. lesions has not been excluded.

The results of the present studies extend and, in part, provide explanations for observations reported previously. For example, we have demonstrated that the in vitro incubation of *R. mooseri* with antibody does not effect a reduction in the growth of the rickettsiae as determined after i.d. inoculation of the rickettsiae-antibody mixture. Further, infiltration of skin with antibody before inoculation of rickettsiae failed to effect control of infection (J. R. Murphy and C. L. Wiseman, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, D52, p. 60). The available evidence, therefore, does not support a measurable role for antibody in the immunological control of *R. mooseri* infection of skin. It is suggested that antibody-sensitized rickettsiae are capable of entering and replicating within the guinea pig's endothelial cells. This possibility is supported by the demonstration that antibodies which prepare *R. prowazekii* for destruction by macrophages (1, 5) do not prevent this parasite from entering or replicating within chicken embryonic cells (16), cells which are not professional phagocytes.

A previous study demonstrated that *R. mooseri* infection in skin is controlled in parallel with the formation of organized granulomatous lesions (9), suggesting that a cell-mediated host defense may have participated in rickettsial control. The present study provides direct evidence in support of this hypothesis. It appears probable, therefore, that a thymus-dependent lymphocyte-mediated host defense effects control of i.d. *R. mooseri* infection. This interpretation receives indirect support from the demonstration by Shirai et al. (13) that sensitized thymus-dependent lymphocytes are capable of protecting mice from an otherwise lethal challenge with *R. tsutsugamushi*. Taken together, these results suggest that a thymus-dependent lymphocyte-mediated immunological effector mechanism may be a common element in the vertebrate defense against rickettsial infection.

The relationship between the cellular mechanisms which control local infection in the skin and those which control systemic infection remains to be determined. This question is raised by the demonstration in previous studies that immunity to i.d. infection, measured as clearance of rickettsiae from the primary site of i.d. infection and as a capacity to resist second homologous challenge delivered at skin sites distant from that of primary infection, develops well before the onset of systemic infection (8). It appears, therefore, that systemic infection develops in the presence of the immunological control of the local skin infection.

These observations can be interpreted to suggest: (i) that *R. mooseri*, in its systemic spread, possesses a capacity to evade the kind of cellular immune response which appears to operate in the skin as described herein and previously (8, 9); (ii) that different kinds of immunological effector mechanisms which mature independently are operative in different organs and tissues; or (iii) that similar effector mechanisms are operative in all tissues and organs but that they require a specific signal, such as antigen which is present only after infection is established in a given site, to initiate the recruitment sequence of the various effector cells, in a manner perhaps analogous to that described by Jungi and McGregor (7) for *Listeria monocytogenes* and *Francisella tularensis* without necessarily assuming an identical final effector pathway for *R. mooseri* inhibition. Another study (J. R. Murphy et al., manuscript in preparation) addresses some of these considerations.
The results of this study do not preclude a role for antibody in the defense mechanism of guinea pigs against *R. mooseri* infection. First, adoptive immunization with immune spleen cells provided recipients with a capacity to control *R. mooseri* infection at sites of i.d. challenge, but not with a capacity to eliminate rickettsiae rapidly from this site. In contrast, animals, which have recovered from one *R. mooseri* infection (similar to the donors employed for these experiments) and which presumably possess both immune lymphocytes and antibody, exhibit an immunity of higher quality. They rapidly clear an i.d. challenge (8, 9). It is possible, therefore, that a synergistic interaction of antibody and sensitized cells is responsible for this higher quality of immunity, a possibility which was not tested by these experiments. Second, it is clear that specific antibody can effect some protection to typhus group rickettsiae. In guinea pigs, antibody appears capable of clearing serum rickettsemia (Y. A. El Batawi, Ph.D. thesis, University of Maryland, Baltimore, 1964) and can protect recipients from the *R. prowazekii* and *R. mooseri* infection-associated febrile responses (19, 20). Further, in other experimental systems, antibody has been demonstrated to protect mice against toxic death and lethal infection (6; C. L. Wisseman, Jr., unpublished data), to protect humans from disease (3, 11, 15, 17, 18) and to sensitize rickettsiae in a manner which prepares them for destruction by macrophages (1, 5).

ACKNOWLEDGMENTS

We thank Lillian Snyder for her excellent assistance.

This research was supported in part by contract DADA 17-71-C-1007 with the U.S. Army Medical Research and Development Command, Office of the Surgeon General, Department of the Army, and by Public Health Service Training Grant AI 00016 from the National Institute of Allergy and Infectious Diseases. Preparation of the manuscript was supported in part by the Trudeau Institute.

J. R. Murphy received partial support from the training grant as a predoctoral trainee.

LITERATURE CITED


