Mechanisms of Immunity in Typhus Infection: Some Characteristics of Rickettsia mooseri Infection of Guinea Pigs

JAMES R. MURPHY,† CHARLES L. WISSEMAN, JR.,* AND PAUL FISET

Department of Microbiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

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Rickettsia mooseri infection has been studied in syngeneic guinea pigs inoculated intradermally with the objective of developing a model for the study of immune mechanisms. Characterization of infection included the following: a study of replication, dissemination, and clearance of rickettsiae; measurement of the antibody response with different rickettsial antigens and tests; and attempts to measure the cell-mediated immune response using the correlate of delayed-type hypersensitivity skin reactions. Following intradermal inoculation, rickettsiae replicate locally and then spread to the draining lymph nodes and subsequently cause systemic infection. Spread to draining lymph nodes occurred before the appearance of circulating antibody, whereas systemic infection occurred afterwards. Two distinct patterns of acquired resistance developed. The first was marked by a cessation of rickettsial growth within a given organ and the second by a clearance of rickettsiae. The duration of each of these phases differed markedly from one organ to another. Delayed-type hypersensitivity was not demonstrated by skin testing.

Intraperitoneal (i.p.) inoculation of guinea pigs with blood collected from patients with typhus fever may produce a typhus-like febrile disease in this animal (33). This susceptibility to infection and the similarities between the histopathology of experimental typhus in guinea pigs and typhus fever in humans (17, 20, 21, 33) led early investigators to employ guinea pigs to propagate rickettsiae (38) and to study treatment (40–43) and immunity (7, 8, 37, 39, 43). Thus, the guinea pig is a long-standing host for experimental studies of typhus. One of the results of these early studies was the differentiation of the agent of epidemic typhus fever, Rickettsia prowazekii, from the agent of murine (endemic) typhus fever, Rickettsia mooseri (Rickettsia typhi) (16). Because R. mooseri produces a more severe disease in guinea pigs than R. prowazekii (36) (the converse of their relative virulence for humans), R. mooseri infection in this laboratory animal is often studied as a model of typhus infections.

Following i.p. inoculation of male guinea pigs with an appropriate dose of R. mooseri, a febrile response and inflammation of the scrotum have been used as criteria of susceptibility, whereas the absence of these signs has been considered as indicative of immunity. These criteria, however, are not always reliable, since febrile reactions may be modified by intercurrent fungal (23) or bacterial (9) infections, and scrotal reactions are, at times, observed in immune animals (36; J. R. Murphy, unpublished data). Also, changes in ambient temperature (8, 15) and irritation of the peritoneal cavity (23) are capable of altering these expressions of infection. Further, the results of studies which employ these indirect measures of resistance are often difficult to interpret.

In this report, R. mooseri infections of guinea pigs initiated by i.p., intradermal (i.d.), and intravenous (i.v.) inoculations are evaluated to determine the optimal dose and route of infection for the study of acquired immunity to R. mooseri infection. The i.d. inoculation of relatively small numbers of rickettsiae makes it possible to determine characteristics of the infection, i.e., the dissemination, proliferation, and clearance of rickettsiae, the antibody response, the delayed-type hypersensitivity (DTH) skin reactions, and the development of resistance to a second homologous challenge.

MATERIALS AND METHODS

Animals. Strain 13 guinea pigs, weighing 350 to 500 g, were purchased from a commercial supplier (R.C. Rosecrans, Hamilton, Mont.). The animals were housed individually, maintained on Guinea Pig Chow (Ralston Purina Co., Saint Louis, Mo.), and provided with water ad libitum.

Rickettsiae. The R. mooseri (Wilmington) seed used had a history of 12 egg, 15 guinea pig, and 5 egg passages (12EP/15GP/5EP). It was stored at −70°C...
as a 50% (wt/vol) yolk sac homogenate. Some characteristics of this seed have been published (18).

**Antigens.** R. mooseri (Wilmington) and R. prowazekii (Breinl) soluble (group) antigens were prepared by ether treatment of Formalized 20% yolk sac homogenates and standardized by complement fixation (CF; see below).

Highly purified particulate ("specific") antigens were prepared according to the procedure of Fiset and Silberman (11), treated with aqueous-ethyl ether, washed, made up to a final concentration of 1 mg/ml, and further standardized by CF (5).

Serological procedures. In the CF test, the microtiter adaption of the Laboratory Branch Complement Fixation procedure was used throughout (5). Soluble and particulate antigens were used at concentrations of 2 and 8 U. In the microagglutination test of Fiset et al. (10), the particulate antigens were used at a concentration of 333 μg/ml.

**Plaque assay procedure.** A modification (18) of the primary chicken embryo tissue culture plaque assay technique of Wike et al. (27, 28) was used.

**Skin tests.** Skin tests for DTH were performed by i.d. inoculation of 0.1 ml of soluble or particulate R. mooseri antigen. The particulate antigens were used in amounts of 100, 10, 1.0, or 0.1 μg (dry weight) per test site. The soluble antigen, which had a CF titer of 1:64 when tested in block titration against a reference antiserum, was used undiluted and diluted 1:10, 1:100, and 1:1,000. Controls consisted of the standard diluent, SPG (4), and normal yolk sac antigen prepared from noninfected yolk sacs. The normal yolk sac antigen was used at a concentration greater than the estimated yolk sac material content of the rickettsial antigen. A given animal was tested with one dilution of one of the antigens in three different sites.

**RESULTS**

**Selection of a model.** Experiments were performed to compare and evaluate infections resulting from inoculation of doses of R. mooseri ranging from 10^1 through 10^6 plaque-forming units (PFU) by i.p., i.d., and i.v. routes.

The i.p. inoculation of R. mooseri into male guinea pigs produced an acute febrile illness (rectal temperature > 39.5°C) and grossly observable scrotal lesions (i.e., the scrotum became inflamed; with the higher doses of rickettsiae, the testes became fixed within the scrotum and an exudate covered the inflamed tunica vaginalis). The incubation period ranged from 3 to 9 days. The period of incubation and the duration of febrile periods were inversely and directly, respectively, related to the number of viable organisms inoculated, as had been observed previously (W. Wood, Ph.D. thesis, University of Maryland, Baltimore, 1965).

In contrast, after i.v. or i.d. inoculation, no febrile response, scrotal inflammation, or general state of unthriftiness was seen regardless of dose. However, following i.d. inoculation, an easily observable local lesion developed in the skin at the site of infection, and its size was dose related.

Large doses (>10^6 PFU) introduced by any of the three routes resulted in a rapid antibody response, with high titers against the various antigens developing almost simultaneously. As the number of PFU injected was decreased to below 10^6, the appearance of antibodies was delayed for several days, and antibodies measurable with the different rickettsial antigens and different tests appeared in a sequential manner (agglutinating antibodies, antibodies fixing complement with soluble antigens, and then antibodies fixing complement with particulate antigens; see Table 1).

On the basis of these preliminary tests, the infection resulting from the i.d. inoculation of a relatively small dose (8.2 × 10^5 PFU or approximately 3.4 × 10^8 guinea pig i.d. 50% infectious doses) of R. mooseri in 0.1 ml into the outer aspect of the guinea pig right thigh was selected for the following study.

**Dynamics of the infection and of the host immune response following i.d. inoculation of 8.2 × 10^5 PFU of R. mooseri.** (i) **Recovery of rickettsiae.** After inoculation of 8.2 × 10^5 PFU of R. mooseri into the skin of a hind limb, rickettsiae were recovered from skin at the site of inoculation, whole blood, spleen, and kidney in the following sequence.

Rickettsiae were first recovered from skin at the site of inoculation on day 2 (Fig. 1). From day 2 through day 4 the number of rickettsiae increased, but by day 7 no rickettsiae could be recovered.

A complete quantitative study of viable rickettsiae at the inoculation site on the thigh could not be conducted because of frequent bacterial contamination. However, bacterial colonies which formed within the agar overlay of some of the plaque assays destroyed only the subjacent monolayer and, therefore, estimates of rickettsial content could be made from the monolayer distant from bacterial colonies. The numbers of rickettsiae recorded as recovered from the site of inoculation (Fig. 1) represent such estimates.

Rickettsiae distant from the site of inoculation were first detected in the draining inguinal lymph node on day 6 after infection. Rickettsiae in these nodes decreased in number by day 9 and fell below detectable levels by day 15.

Rickettsiae were first detected in the blood on day 9, as the number of recoverable PFU was decreasing in the regional lymph nodes. The number of rickettsiae in whole blood increased slightly through day 12. As in the case of the lymph nodes draining the site of inoculation, no rickettsiae were recovered from whole blood on or after day 15. No rickettsiae were recovered from plasma samples obtained by low-speed cen-
trifugation of blood (500 × g, 10 min) which were collected 3, 6, 9, 12, 15, 21, and 28 days after infection.

Rickettsiae were first detected in selected deep tissues, viz., spleen and kidney, on day 9, the first day of detectable rickettsemia. In the spleen, rickettsial content increased slightly through day 12, declined slightly by day 15, and then fell below detectable levels by day 21.

In contrast, rickettsial content of the kidney was near maximum when first detected on day 9 and remained on a plateau near this value throughout the remainder of the 28-day observation period.

(ii) Gross changes. During the course of the infection detailed in Fig. 1 certain gross changes were observed. Thus, the bleb raised by inoculation resolved by 6 h but, beginning at 48 or 72 h, erythema developed about the site of inoculation, progressed to maximal size on about day 5, and thereafter waned. Induration at the inoculation site appeared somewhat later, reached a peak by day 6, and persisted at reduced levels subsequently.

At 24 h after infection, red streaks were observed in skin extending proximally from the site of inoculation. These streaks, interpreted as lymphangitis, suggested an early spread of infection.

Additional gross changes included an increase in mass of the inguinal nodes draining the site of infection from 114 mg per group of nodes on day 3 to 228 mg per group on day 10. Subsequently these nodes receded, falling to a value of 159 mg per group by day 29. On day 6 after infection, but not on day 3 or 9 or after day 9, numerous punctate hemorrhagic lesions were observed in the fat surrounding the draining inguinal nodes. Enlargement of distant lymph nodes was recorded somewhat later, on day 12 or 16 (observations restricted to axillary and contralateral inguinal lymph nodes).

The spleen became enlarged from a mean of 580 mg (standard error, ±100 mg) to a mean of
1,100 mg (standard error, ±200 mg) on day 12. Subsequently, the splenic mass receded to 700 mg (standard error, ±100 mg) on day 28.

(iii) Immune response. An attempt was made to correlate the development of elements of the host immune response measured as serum antibody and as DTH skin reactions with infection. Thus, guinea pigs were infected as in the preceding experiment (8.2 × 10^2 PFU of R. mooseri i.d. into a hind limb), and aspects of the immune response were measured during the course of infection.

(a) Antibody response. Table 1 presents the antibody response as measured with selected R. mooseri or R. prowazeki antigens. An antibody response was first demonstrated on day 8 using R. mooseri particulate antigen in the microagglutination test. After the initial detection of agglutinins, the titers rose but did not reach peak values until between days 18 and 26. The CF test, using R. mooseri soluble antigen in high (8 U) concentration, detected antibody on day 10, and the CF test, using R. mooseri particulate antigen (8 U), detected antibody on day 12. In contrast, the low (2 U) antigen concentrations detected antibody on days 14 and 26, respectively. Cross-reacting antibodies were not detected until day 14. All of the tests used showed that antibody titers were highest between 26 and 30 days after infection and then fell slowly.

The battery of tests used to characterize the serological response (Table 1) was found capable of differentiating between the two closely related rickettsiae, R. mooseri and R. prowazeki, from day 8 (microagglutination) and day 12 (CF) through at least 240 days after infection. Moreover, CF tests employing low particulate antigen concentrations were found to be more capable of discriminating between R. mooseri and R. prowazeki than CF tests using high antigen concentrations.

However, in the CF test with both soluble (group) and particulate ("specific") antigens, the high antigen dose detected antibodies earlier in the course of infection than did the low antigen dose. This latter observation is similar to the reaction pattern originally demonstrated in guinea pigs by Hersey et al. (14) and is consistent with the variation in serological reactivity that is associated with a shift in antibody class from immunoglobulin M to immunoglobulin G, as observed following infection of humans with R. prowazeki, as recorded by Murray et al. (19).

(b) Skin tests. Particulate and soluble antigens were employed for skin tests on days 3, 6, 9, 12, 15, 21, and 28 after infection. No DTH reactions were observed at any time with any of the antigen doses used. After day 9, transient erythematous flares were observed with rickettsial antigens. However, although normal yolk sac suspensions gave negative results through day 9, they also produced there after a transient erythematous flare which was maximal at about 6 h and had faded by 24 h. The diluent control (SPG) was consistently negative. Thus, although no DTH response (induration at the inoculation sites attaining a maximum between 24 and 48 h after skin testing) was observed, a transient erythematous skin reaction which attained a maximum about 6 h after skin test was detectable after day 9 with both rickettsial and normal yolk sac antigens. This very likely was an antibody-mediated reaction to yolk sac components in both instances and was not specific for R. mooseri.

Sequential evolution of infection at the i.d. site of inoculation, infection in spleen, and antibody response. The preceding experiments (Fig. 1 and Table 1) demonstrated that R. mooseri infection in skin at the site of inoculation progressed and waned prior to the demonstration of systemic infection. Further, systemic infection occurred at about the time serum antibody was detected and progressed for a time in the face of rising antibody titers. However, our failure to recover R. mooseri quantitatively

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<th>Table 1. Antibody response to i.d. infection of strain 13 guinea pigs with 8.2 × 10^2 PFU of R. mooseri</th>
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<td><strong>Test</strong></td>
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a RM, R. mooseri antigens; RP, R. prowazeki antigens.

b Titters measured on days 0 through 120 represent geometric mean of five animals per point; day 180 and 240 titers are from one animal.
from the site of inoculation left some doubt as to the precise dynamics and interrelationship of local infection, systemic infection, and immune response. Modifications in the procedures employed to establish infection produced a clear definition of this sequence (Fig. 2).

Thus, guinea pigs were infected i.d. in three sites on the back, each site receiving $8.2 \times 10^4$ PFU of R. mooseri. The dynamics of this infection confirmed a sequential evolution and resolution of infection in skin prior to the onset of systemic infection and showed that systemic infection progressed after serum antibody was demonstrable.

**Development of resistance to a second homologous i.d. challenge.** Groups of five guinea pigs each, infected by i.d. inoculation of $8.2 \times 10^2$ PFU into a hind limb, were challenged 3, 5, 7, 9, or 21 days after primary infection by a i.d. inoculation of $8.2 \times 10^6$ PFU into each of three sites in the cephalad half of the back. At each test interval, five normal guinea pigs were also challenged. The relative dynamics of infection were assessed by following the development of lesions (measured as induration) at the sites of i.d. challenge (Fig. 3).

In contrast to the guinea pigs challenged 3 days after the initiation of the primary infection, guinea pigs challenged on day 5, 6, 9, or 21 displayed a marked capacity to resist the effects of the second i.d. R. mooseri challenge. It is probable that this resistance to second challenge reflects the development of immunity.

**DISCUSSION**

Pathogenesis and immunity in rickettsial infections are poorly understood. For example, although the major signs of these infections in vertebrate hosts are well characterized, it is not known whether these are manifestations of rickettsial replication within the affected tissue, rickettsial toxic actions, or, alternatively, immunopathological processes associated with the host response. Similarly, the host defense mechanisms that provide functional immunity have been but partially characterized, and their mech-

![Fig. 2. Dynamics of infection in skin at sites of inoculation, infection in spleen, and serum antibody response after i.d. inoculation of $8.2 \times 10^4$ PFU of R. mooseri into each of three sites on the back. Symbols: (○) PFU per biopsy of inoculation site; (□) PFU per spleen; and (△) reciprocal of microagglutination antibody titer. At each interval, tissues from three guinea pigs were pooled for rickettsial determinations, and three individual serum samples were employed for serology.](http://iai.asm.org/ on October 26, 2017 by guest)
organisms of action are not fully known. This study was initiated because we believe that definition of the infectious process with respect to proliferation and clearance of rickettsiae will be required for the elucidation of the mechanisms of pathogenesis and of immunity.

After i.d. inoculation, rickettsiae were recovered first from the site of inoculation, then from the draining lymph nodes, and subsequently from deep organs (spleen and kidney). This suggests that infection of lymphatic tissues may be important in the pathogenesis of infection. Because the blood-borne rickettsiae identified in this study were cell associated and because a previous study has demonstrated that it is the leukocytes that carry the rickettsiae (Y. A. El Batawi, Ph.D. thesis, University of Maryland, Baltimore, 1964), it is possible that the early infection of lymphatic tissues allows these organisms to establish residence in a cell population within which they subsequently achieve systemic distribution.

Functional immunity, the capacity to control or eliminate rickettsiae, was manifested in a complex pattern. Thus, normal animals infected with *R. mooseri* did not develop an immunity that corresponded to a relatively synchronous elimination of rickettsiae from tissues, but, rather, infections within different tissues appeared to progress and wane in independent patterns. This asynchronous expression of functional immunity raised the question of whether clearance of the agent was effected by the acquisition of a systemic immune capacity or, alternatively, by some local nonspecific component. Because immunity, manifested as clear-

**Fig. 3.** Development of resistance to skin lesion formation at sites of a second i.d. *R. mooseri* challenge (8.2 x 10^6 PFU per site) delivered at the indicated time after primary infection. Relative protection = log_{10} (Ac/At), where Ac = area of duration at sites of *R. mooseri* inoculation of normal guinea pigs (controls) and At = area of induration at sites of *R. mooseri* challenge of *R. mooseri*-infected guinea pigs. Five *R. mooseri*-infected and five normal control animals were employed per point. Points marked with solid symbols differ from controls where *P* ≤ 0.01 (t test).
ance of rickettsiae from skin at the site of primary infection, corresponded to the development of a capacity to resist homologous i.d. challenge at sites distant from that of primary infection, we suggest that functional systemic immunity developed early in the course of infection. Therefore, an explanation must be provided for (i) the development of systemic infection after the development of this immunity and (ii) the differences in the capacity of the host to clear rickettsiae from different organs. Two groups of explanations are offered. First, different immunological effector mechanisms which mature at different rates may have contributed to the protective response. Second, conditions that are unique to the microenvironment of each focus of infection may have to be established before immunity to these obligate intracellular (possibly sequestered) parasites can be expressed, i.e., concentration of available (exposed) antigen, stage of rickettsia growth cycle (29, 32), or degree of "nonspecific" inflammatory response.

The pattern of infection observed in these studies, i.e., the rapid progression and resolution of infection at the site of i.d. rickettsial introduction and the development of aspects of the functional immunity before the occurrence of systemic infection, may be representative of the pattern of some human rickettsial infections. Thus, humans infected with *R. prowazekii* (?) (Combesco as reported by Baltazard [2], *R. conorii* [2], *R. mooseri* [2], or *R. tsutsugamushi* [25]) developed skin lesions at the sites of rickettsial entrance, but these lesions were resolving at the time systemic disease occurred.

It has been established that antibody, phagocytic cells, and thymus-dependent lymphocytes (24) can provide differing levels of protection from *R. tsutsugamushi*. Known protective capacities of antibody include (i) neutralization of rickettsial toxic actions (13), (ii) opsonization (3, 12, 30, 31) and subsequent destruction of rickettsiae by "professional" phagocytic cells (1, 12), (iii) clearance of rickettsiae from the plasma fraction of blood (El Batawi, Ph.D. thesis), and (iv) a capacity demonstrated by adoptive transfer to protect from disease (22, 26, 34, 35, 42). In the present studies, no direct evidence of antibody-mediated protection was obtained. Thus, rickettsial proliferation in the cutaneous inoculation site was controlled before serum antibodies were detectable, whereas the infection cycle in certain distant organs, e.g., spleen, was initiated and completed in the face of substantial quantities of circulating antibody. However, it is suggested that antibody may have contributed to the failure to observe rickettsiae in the plasma fraction of whole blood.

A thymus-dependent immune response was not detected in *R. mooseri*-infected guinea pigs by one of its correlates, i.e., the DTH reaction. However, our failure to demonstrate DTH does not preclude a function for cell-mediated mechanisms in the control of *R. mooseri* infection. Indeed, other studies (J. R. Murphy and C. L. Wiseman, Jr., Fed. Proc. 34:1026, 1975) demonstrated that splenic cells, but not antibody, collected from guinea pigs that had recovered from *R. mooseri* infection possessed capacities to protect, by adoptive immunization, normal syngeneic guinea pigs from i.d. *R. mooseri* infection. This study suggests that the thymus-dependent immunity is effected in the absence of a DTH response.

Evidence from numerous experimental systems suggests that functional immunity to rickettsial infections is effected by more than one element of the host defense capacities. The present study suggests that the determination of the relative importance to protection from infection, and the mode of action of the various defense mechanisms, may be approached directly by determining their effect on the growth or clearance of rickettsiae.

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