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Mechanisms of Immunity in Typhus Infections

V. Demonstration of *Rickettsia mooseri*-Specific Antibodies in Convalescent Mouse and Human Serum Cytophilic for Mouse Peritoneal Macrophages

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Antibodies in both mouse and human *Rickettsia mooseri* (*Rickettsia typhi*) convalescent serum that were cytophilic for mouse macrophages were demonstrated by the rosette technique. Mouse peritoneal macrophages, passively sensitized with early and late serum from mice with a sublethal infection of *R. mooseri*, were washed and exposed to rickettsiae. Rosettes of rickettsiae were found around macrophages, maintained at 4°C, which had been sensitized with immune serum (direct sensitization of macrophages), but no rosettes were found around macrophages sensitized with serum from normal mice. When the macrophages were maintained at 34°C after addition of the rickettsiae, phagocytosis of rickettsiae occurred, indicating one probable role for cytophilic antibodies in typhus infections. If the rickettsiae were mixed with serum from infected mice, washed, and then added to macrophages (indirect sensitization of macrophages), more rosettes were found around the macrophages than around directly sensitized macrophages. The presence of mouse immunoglobulin G on the macrophage surface was also shown by staining living sensitized macrophages with rabbit fluorescein-conjugated anti-mouse immuunoglobulin G.

Early in vitro studies involving incubation of tissue explants from typhus-infected animals in the presence of serum from immune animals demonstrated that humoral factors alone did not obliterate the infectivity of these tissue explants for nonimmune animals (8). Though advanced for their time, these studies did not necessarily assure contact between antibodies and rickettsiae, nor did they control complement content. Moreover, they did not elaborate on the types of cells involved or present quantitative information. Nevertheless, they did raise doubts about the capacity of antibodies alone to account for immunity.

More recently it has been found that typhus-immune serum, with or without complement, has no direct rickettsiacidal action on *Rickettsia prowazeki* or *Rickettsia mooseri* (*Rickettsia typhi*) and does not prevent infection of, and growth in, chicken embryo cells in culture (19). However, immune serum enhanced the uptake of *R. mooseri* by human peripheral blood monocyte-derived macrophages and, although phagocytized *R. mooseri* grew readily in human monocyte-derived macrophages in vitro in the absence of immune serum, antibody-sensitized rickettsiae were rapidly destroyed (5).

Macrophages bind, possibly by two different classes of receptor sites on the plasma membrane, (i) certain free immunoglobulins or (ii) certain antigen-antibody complexes by the Fc portion of the antibody molecule (3, 4, 12, 15). In view of the unique role of antibody in determining the fate of *R. mooseri* in macrophages, studies were undertaken to determine whether macrophage cytophilic antibodies develop in the course of typhus infections and, if so, to assess their influence in the rickettsia-macrophage interaction. In this report, antibody was demonstrated in mouse and human typhus convalescent serum which was cytophilic for mouse peritoneal macrophages, and which enhanced the uptake of rickettsiae by macrophages. A subsequent paper will report on antibody cytophilic for human monocyte-derived macrophages and the effect of this antibody on the fate of rickettsiae in macrophages (1).

MATERIALS AND METHODS

Animals and preparation of macrophages. Peritoneal macrophages were obtained from random-bred white female Swiss-Webster mice, weighing approximately 18 to 20 g, by injecting them intraperitone-

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ally with 2.5 ml of tissue culture medium M-199 containing 25 mM HEPES (*N*-2-hydroxethylpiperazine-*N'*-2-ethanesulfonic acid) buffer with 10% fetal calf serum and 1,000 U of heparin per ml (Grand Island Biological Co., Grand Island, N.Y.). The animals then were killed with chloroform and, finally, peritoneal fluid was removed aseptically. Cells in the peritoneal fluid were washed once in fresh medium by centrifugation (400 × *g* for 10 min), resuspended in M-199 medium with HEPES and 30% fetal calf serum (growth medium [GM], to a concentration of 7.5 × 10^5 cells per ml and placed in Lab-Tek eight-chambered culture slides (0.3 ml per chamber, Lab-Tek Products Inc., Westmont, Ill.) at 34°C for 48 h before use.

Rickettsiae. The Wilmington strain of *R. mooseri* was harvested from infected yolk sacs and partially purified as previously described (5). The preparation had a mouse toxin mean lethal dose of 1:41 (2) and a particle count of 7.5 % 10^9 rickettsia-like bodies (RLB) per ml using the method of Silberman and Fiset (14). The rickettsiae were diluted 1:200 in GM for mouse infection and 1:100 for rosette formation.

Antiserum. Mice were injected intraperitoneally with a sublethal dose of *R. mooseri* (see above). One, two, and three weeks after infection, blood was removed from 15 mice. The serum was separated, pooled, and filtered. Typhus-immune human serum was obtained from a donor with a history of infection and vaccination with both *Rickettsia prowazeki* and *R. mooseri* (5). All serum was heated to 56°C for 60 min before use.

Sensitization of macrophages, rosette formation, and infection of cells. Two methods were employed: (i) direct sensitization of macrophages and (ii) an indirect method (3). For direct sensitization, macrophages were exposed to HEPES-buffered M-199 containing 30% immune serum for 30 min at 20°C. The macrophages were then washed six times with HEPES-buffered M-199 before addition of rickettsiae. For rosette formation, the cells were maintained at 4°C for 24 h after addition of rickettsiae, washed, air-dried, and stained by the Giménez method (6). For detection of phagocytosis, the cells were incubated at 34°C for 2 h after addition of rickettsiae, washed, air-dried, and stained by the Giménez method. For indirect sensitization of macrophages, rickettsiae were mixed with immune serum for 30 min at 20°C, centrifuged for 30 min at 12,000 × *g*, and then resuspended in GM. They were then added to the macrophage cultures and incubated and stained as above.

When examining macrophages that had been incubated with rickettsiae at 4°C, any RLB in apparent contact with or within the visible macrophage cytoplasmic border was counted. When examining macrophages that had been incubated with rickettsiae at 34°C, any RLB within the visible macrophage cytoplasmic border was counted as phagocytized. The data were presented in the following forms: (i) as the percentage of rosettes or phagocytosis (the percentage of cells with rosettes at 4°C or the percentage of cells phagocytizing rickettsiae at 34°C); (ii) as the average number of RLB per cell or the total number of observed rickettsiae divided by the total number of cells counted; or (iii) as the total number of observed rickettsiae divided by the total number of rosettes or infected cells. The mean and standard deviation were calculated from counts of 800 cells.

Fluorescent-antibody technique. The macrophages were mixed with mouse or human serum at 20°C for 30 min and then washed six times in phosphate-buffered saline (pH 7.2). Fluorescein-conjugated rabbit anti-mouse immunoglobulin G (IgG) or rabbit anti-human IgG was added for 60 min at 20°C (Microbiological Associates, Bethesda, Md.; Cappel Laboratories, Dowingtown, Pa.). The cells were then washed, overlaid with a 9:1 glycerol-phosphate-buffered saline mixture (pH 7.2), and observed under a cover slip with a fluorescence microscope (Zeiss) equipped with a mercury vapor burner (HBO 200), an exciter filter (BG-12), barrier filter (OG 4), and a dark-field condenser.

RESULTS

Direct sensitization with mouse serum. When mouse peritoneal macrophages, previously exposed to normal mouse serum, were incubated with a suspension of *R. mooseri* at 4°C, only rarely was a rickettsia found associated with a macrophage (Table 1). This very low frequency suggests the possibility of a chance association, i.e., the level of experimental error, approximately that found with *R. prowazeki* and nonphagocytic chicken embryo cells in culture (C. L. Wisseman, Jr., and A. D. Waddell, manuscript in preparation).

In contrast, when mouse peritoneal cells, sensitized with serum taken from mice 7, 14, and 21 days after infection with *R. mooseri*, were incubated with an *R. mooseri* suspension at 4°C, the majority of macrophages had multiple rickettsiae associated with them. The highest percentage of cells showing associated rick-

TABLE 1. *Rosette formation of R. mooseri after direct sensitization of macrophages with normal or typhus convalescent mouse serum*

Incubation temp	Mouse serum	Days after infection	% Rosettes or phagocytosis	Avg no. of RLB/rosette or infected cell[a]
4°C	Normal		3 ± 1	1
	Immune	7	80 ± 8	8 ± 2
		14	94 ± 9	23 ± 4
		21	61 ± 6	6 ± 2
34°C	Normal		41 ± 4	1 ± 0.1
	Immune	7	97 ± 10	11 ± 1
		14	96 ± 10	16 ± 2
		21	97 ± 10	10 ± 1

[a] A total of 800 cells was counted from two experiments; rosettes were determined with rickettsiae maintained at 4°C, and phagocytosis was determined with rickettsiae maintained at 34°C.

ettsiae and the highest number of rickettsiae associated with each cell were found with serum drawn 14 days after inoculation, suggesting that cytophilic antibodies capable of direct sensitization of macrophages reach a peak titer at or near 14 days after infection. The minimum average number of RLB associated with a sensitized macrophage (six to eight) is of the same order of magnitude as is commonly accepted as a minimum for rosette formation with sheep erythrocytes (9). Thus, sera from *R. mooseri*-infected mice prepare mouse macrophages so that they exhibit a phenomenon equivalent to rosette formation at 4°C. These findings indicate the presence of cytophilic antibodies specific for *R. mooseri* in the sera of *R. mooseri*-infected mice but not in normal mouse serum.

Somewhat different results were obtained with mouse macrophages sensitized with normal mouse serum and then incubated in the presence of viable *R. mooseri* at 34°C. Thus, approximately 41% of the macrophages had an average of one RLB within their cytoplasmic borders. The conditions of the experiment did not permit discrimination between phagocytosis and active penetration. However, in similar experiments done with viable or nonviable rickettsiae, little difference in uptake or rickettsiae by mouse macrophages was noted. Macrophages sensitized with serum from *R. mooseri*-infected mice showed a substantially higher (96 to 97%) proportion of cells with an average of 10 to 16 RLB within the cytoplasmic borders. The differences among sera drawn at 7, 14, and 21 days seen in cells incubated at 4°C were not seen in experiments at 34°C, possibly because the latter system was at or near saturation even with the least potent serum. In any case, it is clear that the phagocytic capacity of macrophages sensitized with serum from *R. mooseri*-infected mice greatly exceeds that of macrophages sensitized with normal mouse serum. The contrast in appearance of the macrophages with rickettsiae at 4°C and 34°C is illustrated in Fig. 1a, c, and d.

Indirect sensitization with mouse serum. The indirect procedure, which entails preincubation of the rickettsiae with test serum, followed by washing and subsequent exposure to macrophages, differs little from conventional tests for immune opsonization. When such tests were carried out with normal mouse serum, *R. mooseri*, and mouse peritoneal macrophages at 4°C, the same low values of association of rickettsiae with macrophages were obtained as in the direct procedure (Table 2) and are considered to be within the limits of experimental error. However, when a serum sample taken from mice 21 days after *R. mooseri* infection was tested at 4°C in this system, most of the macrophages were surrounded by large numbers of rickettsiae, much higher than in the direct test with this serum as described above.

At 34°C, rickettsiae incubated with normal mouse serum presented the same dilemma as in the comparable situation in the direct test—namely, a high proportion of macrophages containing an average of one RLB per cell under conditions that do not discriminate between active penetration and phagocytosis. In the presence of immune (21-day) mouse serum, however, the great majority of macrophages bound a large number of rickettsiae. On incubation at 34°C, approximately the same proportion of macrophages appeared to ingest a number of rickettsiae equivalent to the number found associated with the cell surface at 4°C, suggesting a high efficiency of phagocytosis even though the conditions (incubation time) were different in the two types of experiments.

Sensitization with heterologous serum. Since mouse peritoneal macrophages are easier to obtain than human macrophages, it would be of value if mouse macrophages could be used to study some properties of human typhus-immune serum. When tested by either direct or indirect methods at 4°C, human typhus convalescent serum led to rosette formation with *R. mooseri* (Table 3). As with typhus immune mouse serum, the indirect method yielded higher values. Specificity of the reaction was indicated by the low values, again of the order of experimental error, obtained with normal human serum by both methods. The heterologous system was not tested at 34°C for enhanced phagocytosis.

Detection of IgG on macrophage surface with fluorescent antibody. Staining with fluorescein-conjugated rabbit anti-mouse IgG or rabbit anti-human IgG demonstrated a definite ring of fluorescence with capping on mouse peritoneal macrophages that had previously been incubated with normal mouse (Fig. 1b) or human serum, respectively. Control cells incubated in fetal calf serum showed no fluorescence when stained with the rabbit anti-mouse or anti-human IgG conjugate.

DISCUSSION

Berkin and Benacerraf (3) included, in their definition of cytophilic antibody, both antibody that binds directly to the macrophage (direct sensitization) and antibody that binds to macrophages only after combination with antigen (indirect sensitization). Some investigators (12, 16) believe that the two techniques do not detect

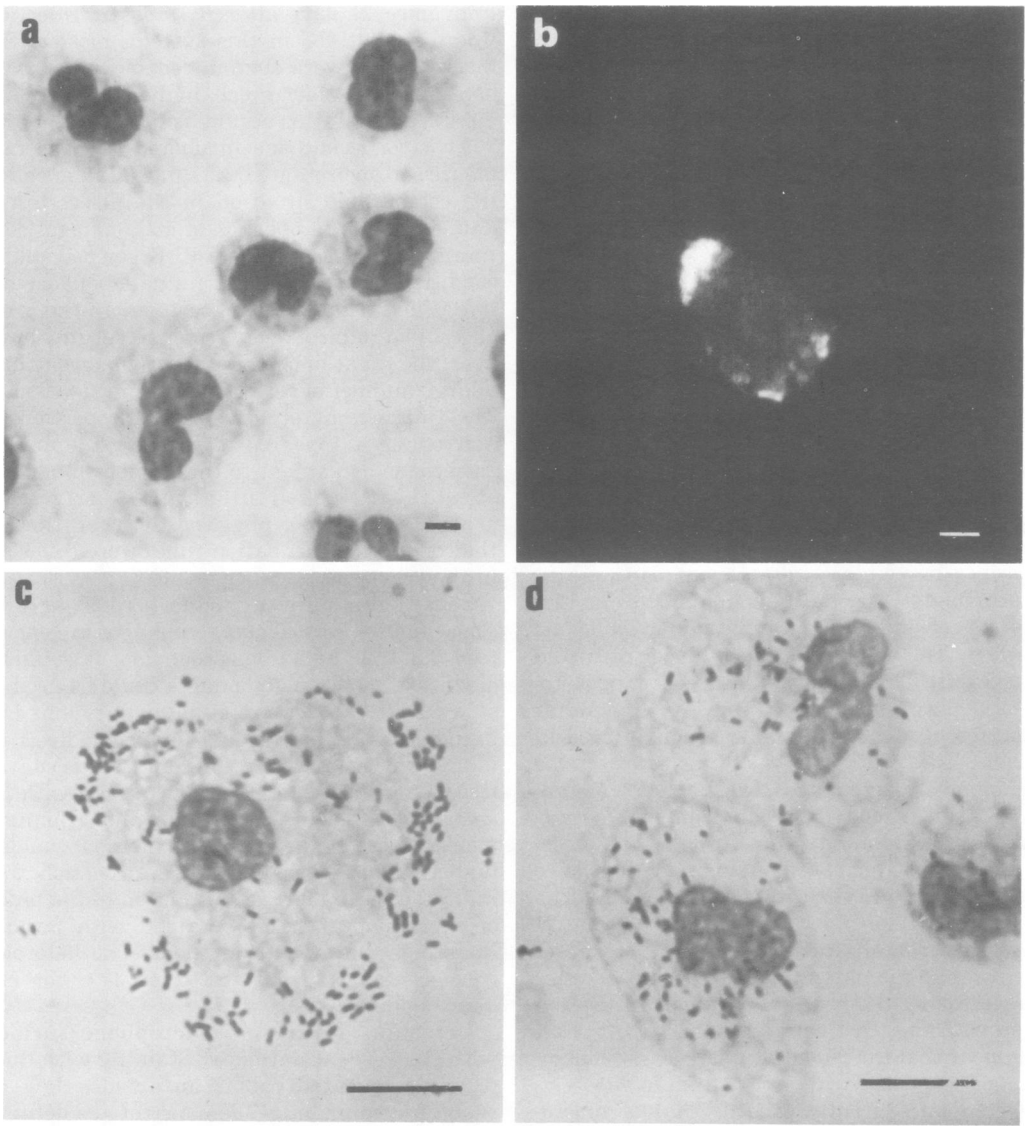

FIG. 1. *(a) Mouse macrophages at 4°C after exposure to normal mouse serum before addition of rickettsiae. Bar corresponds to 10 μm. (b) Mouse macrophages coated with serum and stained with fluorescein-conjugated rabbit anti-mouse IgG. (c) Rosette formation of R. mooseri after direct sensitization of mouse macrophages with immune mouse serum. (d) Phagocytosis of R. mooseri after direct sensitization of mouse macrophages with immune mouse serum.*

the same type of antibody and that antibody detected by indirect sensitization is not cytophilic until complexed with antigen. In any case, cytophilic antibodies are thought to bind by a portion of their Fc fragment with specific receptor sites on the macrophage cell membrane (3).

At physiological temperatures, one end result of the combination of cytophilic antibodies detectable by either method is phagocytosis of the particulate antigen (3). The phenomenon of phagocytosis is thought to occur in two stages: (i) attachment of the particle to the phagocyte surface through receptor sites, either for some surface component of the particle or for the Fc portion of opsonizing antibodies coating the particle (3, 13) and (ii) engulfment of the particle by the phagocyte. It appears that the phenomena of macrophage cytophilic antibodies and of phagocytosis and immune opsonization

TABLE 2. *Rosette formation or phagocytosis of R. mooseri after direct or indirect sensitization of macrophages with normal or convalescent mouse serum (3 weeks)*

Sensitization	Incubation temp	Mouse serum	% Rosettes or phagocytosis[b]	Avg no. of RLB/rosette or infected cell
Direct[a]	4°C	Normal	3 ± 1	1
		Immune	61 ± 6	6 ± 2
	34°C	Normal	41 ± 4	1 ± 0.2
		Immune	97 ± 10	10 ± 1
Indirect	4°C	Normal	5 ± 1	1
		Immune	98 ± 10	38 ± 8
	34°C	Normal	35 ± 4	1 ± 0.2
		Immune	90 ± 9	35 ± 4

[a] Data from Table 1 for comparison.
[b] Rosettes at 4°C; phagocytosis at 34°C.

overlap and in some instances may represent different approaches to, or facets of, the same biological process. This would appear to be the case with typhus rickettsial infections.

Thus, we were able to demonstrate with fluorescein-conjugated anti-mouse immunoglobulin sera that, in the direct method, mouse IgG binds to the surface of mouse peritoneal macrophages and, by the technique of rosette formation at 4°C, mice infected with *R. mooseri* develop specific antirickettsial cytophilic factors detectable by both the direct and indirect methods. As in the case with erythrocytes (3), the indirect method was more sensitive. The low rate of rosette formation with *R. mooseri* in the absence of antibody suggests that mouse peritoneal macrophages have few receptors for surface components of *R. mooseri*, an observation consistent with the low rate of phagocytosis observed previously for typhus rickettsiae by mononuclear phagocytes in the absence of antibodies (18). In this study, it was clearly shown that phagocytosis was markedly enhanced by specific antibody by both the antibody population that attached first to the macrophage membrane (direct method) and by the antibody that attached to the macrophage membrane after combination with the rickettsiae (indirect method).

The indirect method appeared to be more sensitive. This may be due to the fact that more rickettsia-specific antibody was present on the rickettsial surface in this situation than in the case of nonselective attachment of cytophilic antibodies of any specificity in the direct method. However, there were also some technical considerations. Agglutination of rickettsiae may have increased the number of organisms that adhered to the macrophage surface. Nevertheless, regardless of the uncertainties of the relative quantitative aspects, both methods detected cytophilic antibodies and both methods demonstrated enhanced phagocytosis in the presence of antibodies.

TABLE 3. *Rosette formation of R. mooseri after direct or indirect sensitization of macrophages with normal or convalescent human serum at 4°C*

Sensitization	Type of human serum	% Rosettes	Avg no. of RLB/rosette
Direct	Normal	7 ± 1	1
	Convalescent	57 ± 6	7 ± 8
Indirect	Normal	9 ± 1	1 ± 0.1
	Convalescent	98 ± 10	46 ± 12

Because human macrophages are more difficult to obtain in the laboratory than mouse peritoneal macrophages, it is of interest from a practical viewpoint that human serum contains antibodies that are cytophilic for mouse peritoneal macrophages. It has previously been shown that human myeloma immunoglobulins will adhere to guinea pig peritoneal macrophages (7). Since it is not clear if the same or different receptor sites on the macrophages bind homologous and heterologous immunoglobulins, it remains to be determined whether the mouse peritoneal macrophages can substitute for human macrophages in the study of human typhus-specific antibodies cytophilic for macrophages.

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