Host Defenses in Experimental Scrub Typhus: Role of Spleen and Peritoneal Exudate Lymphocytes in Cellular Immunity

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Lymphocytes obtained from spleens or peritoneal exudates of immune donor mice were evaluated for their ability to passively confer protection on recipients subsequently challenged with virulent scrub typhus rickettsiae. Peritoneal exudate lymphocytes (PELs) injected intraperitoneally were able to transfer complete protection against rickettsial challenge by 5 days after immunization, whereas splenic lymphocytes (Spl's) required 15 days to exhibit similar resistance. When immune lymphocytes were transferred intravenously, cells from both anatomical compartments required 15 days after immunization before they were able to completely protect recipients. PELs maintained this protective capacity for 2 weeks, but the passive immunity induced by intravenously transferred Spl's rapidly diminished to insignificant levels. It was particularly interesting that the protective effect of Spl's could be dramatically reduced by the concomitant presence of a mineral oil-induced peritoneal exudate. Almost total abrogation of resistance was observed when Spl's obtained from exudate-bearing mice were transferred intravenously. The protective capacity of both PELs and Spl's was resistant to 1,200 rads of gamma radiation at 7 to 10 days after immunization, but resistance was transient and by 3 weeks was undetectable. It was not possible to determine from this study whether the transferred lymphocytes were proximate mediators of protection in scrub typhus infection of mice or whether they served to recruit the host's own defenses, or both. However, it was possible to conclude that PEL's and Spl's exhibited functional heterogeneity and that PELs were more efficient mediators of protection.

In a recent report, we assessed the relative contributions of splenic and humoral immunity in experimental scrub typhus infection of mice (7) by employing Rickettsia tsutsugamushi strains differing in virulence for mice (2). Infection with the less virulent Gilliam strain resulted in heterologous protection against an otherwise lethal challenge of the virulent Karp strain. Furthermore, it was shown that significant protection was afforded by the passive transfer of spleen cells and that the protective lymphoid cell in the splenic material transferred was a thymus-dependent lymphocyte (7).

During these transfer studies, both the immune cells and challenge infection were given intraperitoneally (i.p.). Consequently, the study established only that the spleen contained antigen-reactive cells capable of conferring protection and did not provide information as to whether the spleen cells were, in fact, the proximate or direct mediators of cellular immunity in the physiological situation. Exhaustive studies by McGregor and his colleagues (3-6) have indicated that peritoneal exudate lymphocytes are the physiological mediators of cellular immunity in other bacterial diseases. These studies led us to explore the possibility that in mice peritoneal exudate lymphocytes are also the physiological mediators responsible for immunity to heterologous scrub typhus infection. Specifically, we examined the protection elicited against heterologous rickettsial challenge by both spleen and peritoneal exudate lymphocytes administered either i.p. or intravenously (i.v.); assessed the temporal resistance to gamma radiation of lymphocytes obtained from both spleen and peritoneum; and finally, determined the effect of peritoneal exudate induction on the protective capacity of lymphocytes resident in the spleen.

MATERIALS AND METHODS

Animals. Female BALB/c mice (Flow Laboratories, Dublin, Va.), 18 to 22 g, were used throughout the study.
Scrub typhus strains. The Karp and Gilliam strains of *R. tsutsugamushi* were propagated and stored, and the 50% mouse infective dose (MLD<sub>50</sub>) and the 50% mouse lethal dose (MLD<sub>50</sub>) were determined as previously described (1).

Preparation of spleen cells. Donor mice to be used for cell transfer studies were killed by cervical dislocation. Spleens were removed aseptically and minced. Fragments were pressed through a stainless-steel, 60-mesh screen into a plastic petri dish (60 by 15 mm; Falcon Plastics, Oxnard, Calif.) containing L-15 medium (Microbiological Associates, Bethesda, Md.) supplemented with 10% heat-inactivated fetal bovine serum. Cell suspensions were washed twice with Earle balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.). Unless otherwise stated, spleens were not obtained from animals bearing a peritoneal exudate.

Preparation of peritoneal exudate cells. Peritoneal exudate cells were harvested 3 days after a 5-ml i.p. injection of sterile light mineral oil. Mice were killed by cervical dislocation and injected i.p. with RPMI 1640 (Grand Island Biological Co.) containing 0.005 M HEPES buffer (N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid; Calbiochem, San Diego, Calif.) supplemented with 2% heat-inactivated fetal bovine serum and heparin (20 U/ml). The abdomen was gently massaged for 1 min, and the cell-rich fluid was aspirated aseptically with a Pasteur pipette. The pooled cell suspension was washed three times with Earle balanced salt solution and then suspended in RPMI 1640 containing 10% heat-inactivated fetal bovine serum.

Separation of adherent and nonadherent cells. Freshly harvested and washed cells were incubated in a plastic tissue culture flask (75 cm<sup>2</sup>; Falcon Plastics, Oxnard, Calif.) for 1 h at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> in air. The criteria for identification of lymphocytes included nonadherence to the plastic surface, failure to phagocytize 0.8-μm latex spheres, peroxidase negativity (8), and conventional morphological appraisal. These criteria indicated that the nonadherent leukocytes consisted of more than 95% lymphocytes. Viability of the cell populations was monitored by trypan blue exclusion. The resultant splenic lymphocytes and peritoneal exudate lymphocytes hereinafter will be designated as SpL's and PELs, respectively.

Irradiation of lymphocytes. When required, cells were irradiated by exposure to 1,200 rads in a 60Co gamma irradiator (Gammacell 220; Atomic Energy of Canada, Ltd., Commercial Products, Ottawa, Canada).

Injection of mice. Mice received rickettsiae by i.p. injection and spleen or peritoneal exudate lymphocytes by i.p. or i.v. injection, depending on the experiment. All inoculations were given in a standard volume of 0.2 ml.

RESULTS

Experimental design. The contribution of various lymphoid cell populations to host protection was evaluated by a series of cell transfer experiments in which the following protocol was used. Mice were immunized by a single i.p. inoculation of 100 MLD<sub>50</sub> of *R. tsutsugamushi* Gilliam. As reported previously, animals tolerated this procedure well and few mice died (7). At various days after inoculation, 5 to 50 animals were sacrificed, their spleens and/or peritoneal exudate cells were removed aseptically, and appropriate single-cell, lymphocyte-rich suspensions were prepared as detailed above. The cell concentration was adjusted to 30 × 10<sup>6</sup> lymphocytes per 0.2 ml, since i.p. inoculation of this number of splenic lymphocytes had previously been shown to provide complete protection against i.p. challenge (7). Cells were injected either i.p. or i.v. as required by the experiment, and recipient animals were challenged i.p. 8 h later with 1,000 MLD<sub>50</sub> of the Karp strain. The results were expressed as the percentage of ultimate survivors in each group of 10 or more animals.

Comparison of the protection achieved by i.p. transfer of immune SpL's and PELs. In general, the protection afforded by transfer of SpL's was quite similar to that previously reported (7) and is summarized in Fig. 1. Not until 7 days after strain Gilliam immunization were SpL's able to transfer partial (80%) protection against challenge with strain Karp. Furthermore, all of these survivors evidenced some form of illness such as lethargy and ruffling of fur. Complete protection against both illness and death was achieved with transfer material consisting of 14- to 28-day immune SpL's. This protective capacity of immune SpL's declined slightly on days 35 and 42, when experiments were terminated.

The protection afforded by PEL transfer differed from that achieved with SpL's (Fig. 1). Partial protection (20%) from mortality, but not morbidity, was achieved with PEL transfer as early as day 3. Complete protection against mortality and morbidity was achieved with PELs obtained from animals 7 days after immunization. In addition, this protective capacity of PELs persisted until termination of the experiment and did not show the decline exhibited by SpL's at 35 and 42 days postimmunization. However, at these later times (35 and 42 days), all challenged animals exhibited transient signs of illness.

Also summarized in Fig. 1 is the protection afforded by immune SpL's obtained from animals bearing a mineral oil-induced peritoneal exudate. The net effect of such an exudate was to reduce the effectiveness of SpL transfers. Complete protection was never achieved, and the limited protective capacity dropped rapidly to nonprotective levels by 28 days.

Comparison of the protection achieved by i.v. transfer of immune SpL's and PELs. In contrast to i.p. transfer, immune SpL's given
i.v. were much less effective at early times in protecting against lethal challenge (Fig. 2). Thus, whereas an i.p. transfer of 7-day immune SpL's achieved 80% protection (Fig. 1), a comparable i.v. transfer of these cells resulted in only 20% protection (Fig. 2). At 14 days postimmunization, total protection could be achieved by the transfer of immune SpL's regardless of the route (Fig. 1 and 2). However, whereas the protection afforded by i.p. transfers of immune SpL's plateaued at these high levels for a few weeks (Fig. 1), that afforded by SpL's given i.v. rapidly declined to low levels after day 14 (Fig. 2).

The weak protective response elicited by the i.v. transfer of immune SpL's could be further reduced by inducing a mineral oil exudate in the peritoneal cavity of donor mice. In this instance, partial protection could only be shown by i.v. transfer of 14-day immune SpL's (Fig. 2), and surviving mice all showed signs of illness.

There were differences in protection between i.v. and i.p. PEL transfers. However, these were less striking than the comparable experiments with SpL's. The complete protection seen with i.p. transfer of PELs at 7 days (Fig. 1) was delayed 1 week when transfers of similar immune lymphocytes were given i.v. (Fig. 2). Complete protection, once achieved at day 14, plateaued until 28 days (Fig. 2). Unlike i.p. protec-

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**Fig. 1.** Comparison of the protective effects of Gilliam immune PELs (○) and SpL's (×) transferred i.p. before Karp challenge of recipients. In some experiments, immune SpL's were harvested from donors bearing a mineral oil-induced peritoneal exudate (Δ). Ordinate indicates percent survival of recipients.

**Fig. 2.** Comparison of the protective effects of Gilliam immune PELs (○) and SpL's (×) transferred i.v. before Karp challenge of recipients. In some experiments, immune SpL's were harvested from donors bearing a mineral oil-induced peritoneal exudate (Δ).
tion, which persisted until day 42 (Fig. 1), i.v. protection declined at day 35, at which time the experiment was terminated (Fig. 2).

**Radiation sensitivity of the protective effects of immune SpL's and PELs given by i.p. transfer.** The effect of in vitro irradiation (1,200 rads) on the protection offered by passively transferred (i.p.) SpL's was studied (Fig. 3). In this experiment the protective effects of $30 \times 10^6$ untreated or in vitro irradiated immune SpL's were compared. Early in the course of immunization (i.e., at 7 and 10 days), relatively radiation-resistant populations of SpL's affording 90 and 50% survival, respectively, were demonstrated. At all other times, exposure to gamma radiation in vitro before transfer abolished the protective capacity of immune SpL's. It was interesting that the peak of radiation-resistant protection occurred before nonirradiated SpL's (given i.p.) were able to afford complete protection from mortality and morbidity (Fig. 3).

A similar early (8- to 14-day) radiation-resistant population was seen with gamma-irradiated PELs (Fig. 4). In contrast to the SpL's, this radiation resistance occurred after the time at which unirradiated PELs were able to afford complete protection.

**DISCUSSION**

Previous experiments from this laboratory have shown that immune spleens contain cells capable of conferring protection when given i.p.

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**Fig. 3.** Effect of gamma radiation (1,200 rads) given in vitro to Gilliam-immune SpL's before i.p. transfer to recipients subsequently challenged with Karp. Symbols: $\times$, SpL; $\blacksquare$, irradiated SpL.

**Fig. 4.** Effect of gamma radiation (1,200 rads) given in vitro to Gilliam-immune PELs before i.p. transfer to recipients subsequently challenged with Karp. Symbols: $\bigcirc$, PEL; $\blacksquare$, irradiated PEL.
and followed with an i.p. lethal infectious challenge of scrub typhus rickettsiae (7). It was not clear whether SpL’s participated in the immune response in vivo, particularly in view of histological evidence implicating PELs as mediators of cellular protection against scrub typhus rickettsiae (1) and of considerable data supporting the role of PELs as mediators of cellular immunity in other bacterial infections (3–6).

This study suggests that, whereas SpL transfer represents a valid assay for cell-mediated immunity against *R. tsutsugamushi*, PELs are more likely to represent the physiological protective cell population. It was clear that PELs represented a more efficient source of immune cells for the following reasons: (i) partial protection appeared 4 days earlier and complete protection appeared 1 week earlier than with SpL’s after i.p. transfer, and (ii) the duration of complete protection after i.p. transfer lasted at least 2 weeks longer than that observed with SpL’s. However, these observations could be explained by quantitative differences in immunocompetent lymphocytes. Evidence of qualitative differences was clearly demonstrated in i.v. cell transfer experiments. Whereas PELs administered by this route were able to confer complete protection over a 3-week period, similarly transferred SpL’s evidenced complete protection only on day 14. Further evidence of a qualitative difference between PELs and SpL’s was the fact that protective SpL’s resistant to gamma radiation appeared earlier than comparable resistant PELs but were incapable of conferring complete protection against challenge with virulent rickettsiae. On the other hand, the later-appearing radiation-resistant PELs evidenced a short period of complete protection. These irradiation experiments were not comparable to the vinblastine studies described by Lefford et al. (5) and McGregor and Logie (6) concerning the protective capacity of lymphoid cells. In these latter experiments, the antimitotic drug was given in vivo and clearly affected such important aspects of cell-mediated protection as the ability of immune cells to enter an infectious focus. The fact that we administered irradiated cells i.p. circumvented any impairment in cellular circulation. A possible explanation for radiation resistance is the existence at that time of a large number of antigen-reactive cells, which obviate the need for division or clonal expansion. Other explanations, including radiation-resistant small lymphocytes capable of recruiting the host’s immune response, must also be considered. Our data are insufficient for further speculation on the nature of this phenomenon, and the experiments were designed solely to compare the functional properties of the two populations.

The data presented here also caution against considering either protective PELs or SpL’s as being functionally equivalent during all periods of rickettsial infection. Clearly, the splenic population is temporally heterogeneous with respect to ability to transfer protection by the i.v. route, and lymphocytes from both spleen and peritoneal exudate show temporal differences in radiation resistance. Further evidence for heterogeneity among immunocompetent lymphocytes was seen in experiments in which SpL’s used for i.p. transfer were obtained from animals in which a peritoneal exudate was induced by injection of mineral oil. Early in the course of infection only a slight reduction in the protection transferred by SpLs was noted, but at later times (28 days) the induction of such an exudate totally abolished the protective capacity of SpLs. Even more striking results were observed with i.v. transfer, since only a brief and weakly protective capacity of SpL’s withstood the concomitant peritoneal exudate. These data indicated that a portion of the spleen cells mediating protection were “mobilizable.” The mechanism for depletion of lymphocytes from the spleen by a remote exudate is not well understood. Perhaps a portion of the protective cells are mobilized into the exudate itself and, therefore, are identical with PELs. Alternatively, a phenomenon of negative lymphoid trapping, as described by Zatz and Gershon (9), may have been operative in excluding immunocompetent lymphocytes from the spleen.

We recognize the possibility that induction of suppressor cells may have influenced the differences in protection observed after transfer of immune PELs or SpL’s, and this phenomenon is currently under investigation in our laboratory. The function of the protective cell, whether PEL or SpL, was not defined in this study. It may be that these cells represent the immediate effector cells that interact with macrophages or they may function to recruit the host’s own immune response. Whatever the mechanism, the data presented here clearly indicate the heterogeneity of such cells and accentuate the need for further studies to more clearly define the physiological role of immunocompetent lymphocytes in rickettsial infection.

**LITERATURE CITED**


