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Development of Specific and Cross-Reactive Lymphocyte Proliferative Responses During Chronic Immunizing Infections with *Rickettsia tsutsugamushi*

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The development of antigen-responsive lymphocytes was followed in mice immunized with the Gilliam, Karp, or Kato strains of *Rickettsia tsutsugamushi* by utilizing an in vitro lymphocyte proliferation assay. Subcutaneous immunization with viable rickettsiae of all three strains resulted in the appearance of lymphocytes in the spleen responding to irradiated tissue culture-grown rickettsiae used as stimulating antigens. Although all animals demonstrated antigen-induced proliferation elicited by homologous antigen by 14 days after immunization, the time of peak responsiveness varied, depending on the strain of rickettsiae used for immunization. In all cases, peak proliferative responses occurred at a time after immunization that was after the previously reported time after immunization at which resistance to rechallenge was observed. Reactivity to heterologous strains of *R tsutsugamushi* developed roughly in parallel with homologous reactivity in Karp- and Gilliam-immunized mice, with a marked degree of heterologous reactivity evident. Kato-immunized mice demonstrated greater reactivity to heterologous antigens early in the development of antigen reactivity and demonstrated a somewhat greater degree of cross-reactivity, relative to homologous responses, than the other groups. It was found that nylon wool-nonadherent immune cells, if cultured with antigen and adherent cells obtained from normal spleens or peritoneal exudates, responded in culture. The thymus-derived lymphocyte nature of the responding cell was further suggested when treatment of immune spleen cells with anti-Thy 1.2 serum and complement eliminated antigen response.

A number of studies have suggested that immunity to *Rickettsia tsutsugamushi* in a murine model is dependent on the development of a cell-mediated immunity (CMI), as assessed by cell transfer (27), by production of lymphokines by thymus-derived lymphocytes (T-lymphocytes), which results in the activation of macrophages (20, 21), and by the demonstration of delayed-type hypersensitivity responses after immunization (16).

Although antibody is also produced in response to natural and experimental infections with *R. tsutsugamushi* (27, 31), the role of antibody in immunity to reinfection is unclear. A number of biological activities have been attributed to antibody produced in response to *R. tsutsugamushi*, including neutralization of a toxic effect associated with this organism (11), neutralization of rickettsial infectivity if mixed with rickettsiae in vitro before animal inoculation (2), and neutralization of infectivity for tissue culture (1, 23). In one study the passive administration of specific antibody to naive animals rendered them resistant to lethal challenge with the strain of *R. tsutsugamushi* used to produce the antiserum (28). Although the mechanism(s) of each of these activities is unknown, a recent study with a different rickettsia, *Coxiella burnetii*, has demonstrated that administration of immune sera to naive mice enhanced rickettsial clearance through an adjuvant-like activity, which resulted in an enhancement of the cell-mediated response (13). Studies have demonstrated that antibody produced in response to one strain of *R. tsutsugamushi* reacts in neutralization tests almost exclusively with the homologous strain, and very little cross-reactivity with heterologous strains was noted (1, 2, 11, 22), which is in direct contrast to the strong heterologous resistance to reinfection exhibited by immunized animals (12). It also has been shown that animals immunized with irradiated (thus, nonreplicating) rickettsiae are resistant to homologous and heterologous challenge, although these immunogens produce little or no antibody response (7). These data argue against a direct

role of antibody in protective immunity to *R. tsutsugamushi*, although a possible cooperation between antibody and CMI has not been adequately studied.

A number of studies have demonstrated that natural and experimental rickettsial infections result in the appearance of proliferating lymphocytes (5, 14, 17, 18), which have been shown to be relatively long lived (14) and associated with the presence of a CMI. Lymphocyte proliferation in vitro has been shown to be due to at least the response of antigen-responsive T-lymphocytes with the specific immunizing antigen and has been used to study the specificity of antigen receptors on T-lymphocytes (25, 29).

In the present study, we have examined mice immunized with the Karp, Kato, or Gilliam strains of *R. tsutsugamushi* for the appearance of lymphocytes proliferating in culture to the immunizing antigen as a method to detect the presence of CMI by in vitro techniques. Further, we have employed the lymphocyte proliferation (LP) assay to examine the specificity of antigen receptors on lymphocytes arising as a result of immunization in terms of responsiveness to heterologous strains of *R. tsutsugamushi* in an attempt to correlate T-lymphocyte responsiveness and protective immunity to infection with *R. tsutsugamushi*.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from Flow Laboratories, Inc. (Dublin, Va.) and used at 8 to 12 weeks of age in all experiments.

Rickettsiae and immunization of mice. The Karp (52nd egg passage), Gilliam (165th egg passage), and Kato (162nd egg passage) strains of *R. tsutsugamushi* were plaque purified (23) and propagated in embryonated eggs; infected yolk sac suspensions were prepared and stored at −70°C as described previously (9). Rickettsial titers were expressed as 50% mouse lethal doses (MLD_{50}) calculated by the method of Spearman and Karber as described by Finney (10). Mice were immunized with the appropriate rickettsial strain by subcutaneous (s.c.) injection of 1,000 MLD_{50} of rickettsiae contained in 0.2 ml of brain heart infusion broth.

Antigen preparation. Each strain of *R. tsutsugamushi* was propagated in irradiated L-929 cell cultures as described previously (6). The resulting rickettsiae were liberated from L-929 cells, freed of cell debris, and irradiated with 180 kilorads of gamma irradiation (^{60}Co; Gamma cell 220; Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada) as described previously (16).

LP assay. The ability of spleen lymphocytes to proliferate in response to rickettsial antigens was measured using a modification of a previously described microculture LP assay (15). Briefly, spleens were removed aseptically, and single-cell suspensions were prepared by mincing the spleens into RPMI 1640 supplemented with 50 μg of gentamicin per ml, 1% fresh glutamine, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (MA Bioproducts, Walkersville, Md.). Cells were washed twice at room temperature and were adjusted to 5×10^6 viable cells per ml in RPMI 1640 supplemented as described above (15) and containing 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.). Individual wells of 96-well microtiter plates (Micro Test II; Falcon Plastics, Oxnard, Calif.) received 0.1-ml samples of the cells. Quadruplicate wells received medium, rickettsial antigens, concanavalin A (ConA; Calbiochem, La Jolla, Calif.), or *Escherichia coli* lipopolysaccharide (LPS; O11B4; Difco Laboratories, Detroit, Mich.), all diluted to the desired concentration in RPMI 1640 without added serum, making the final fetal bovine serum concentration 5%. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere for 72 h. One microcurie of tritiated thymidine (specific activity, 6.2 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to each well for the final 6 h of culture. Cells were harvested onto filter strips by using a multiple harvesting system, and incorporated radioactivity was determined by scintillation counting (Prias; Packard Instrument Co., Inc., Downers Grove, Ill.). Data were expressed as net counts per minute (cpm) calculated by subtracting cpm of cultures receiving only media (control cultures) from cpm of cultures receiving antigens or mitogens, and as stimulation indexes (SI) calculated by dividing the cpm of antigen- or mitogen-stimulated cultures by the cpm of control cultures.

Characterization of responding lymphocytes. Spleen cells prepared as described above were suspended in RPMI 1640 supplemented with 10% fetal bovine serum, and 2×10^8 to 5×10^8 cells were added to a 5-g nylon wool column. After incubation for 60 min at 37°C, nonadherent cells were slowly eluted with 30 ml of medium. Nonadherent cells consisted of ≥95% lymphocytes staining with fluorescinated anti-Thy 1.2 serum and ≤2% lymphocytes staining with fluorescinated anti-mouse immunoglobulin G. The resulting cells were cultured as described above either alone or in the presence of spleen-adherent cells (SAC) as a source of antigen-presenting cells. To prepare SAC, spleen cells from normal syngeneic mice were allowed to adhere to 75-cm^2 plastic tissue culture flasks (Falcon) for 60 min at 37°C in RPMI 1640 supplemented with 20% fetal bovine serum. After extensive washing with warm RPMI 1640 to remove nonadherent cells, adherent cells were removed from the plastic by gentle scraping with a rubber policeman. The resulting cells were washed twice and adjusted to the desired concentration in RPMI 1640 supplemented with 10% fetal bovine serum. To deplete lymphocytes with Thy 1.2 surface antigen, immune spleen cells were adjusted to 10^7/ml in RPMI 1640, and monoclonal anti-Thy 1.2 (Cedarlane; Accurate Chemical & Scientific Corp., Westbury, N.Y.) was added to a final concentration of 1:20. Cells were incubated for 60 min at 4°C, washed twice, suspended in medium containing a 1:10 dilution of rabbit serum (Low Tox M; Cedarlane; Accurate Chemical & Scientific Corp.) as a source of complement, and incubated at 37°C for a further 60 min. The resulting cells were washed twice and cultured as described above. Treatment with anti-Thy 1.2 and complement resulted in 39 and 41% cytotoxicity of the nucleated spleen cells, respectively, as assessed by exclusion of trypan blue in the two experiments performed.

RESULTS

Development of LP responsiveness after immunization. At various intervals after SC immunization, spleen cells were obtained and tested for LP reactivity in response to the immunizing antigen. In all experiments, a wide range of antigen dose was employed. Figure 1 shows dose-response data obtained from experiments in which the peak proliferative response to each immunizing strain was observed. In most experiments, regardless of the time after immunization, the optimal proliferative response was elicited by using antigens diluted to contain 10 to 25 μg of protein per ml, and data resulting from stimulation of lymphocytes with 25 μg of protein per ml are presented. Responses of BALB/c mice immunized with Gilliam (Fig. 2A), Karp (Fig. 2B), or Kato (Fig. 2C) strains of *R. tsutsugamushi* are shown. Mice demonstrated responsiveness (SI ≥ 3.0) to the immunizing antigen 14 days after immunization regardless of the strain of rickettsiae used for immunization. Gilliam-immunized animals demonstrated a peak LP responsiveness assessed by net cpm relatively early after immunization, compared with animals immunized with Karp or Kato (28 days versus 42 and 49 days, respectively). Although the time of peak LP response differed, depending on the immunization, the magnitude of the peak was essentially identical in all three groups. After a sharp peak response, the LP reactivity declined, but remained demonstrable for 60 days after immunization. Reactivity to homologous antigens remained in mice up to 90 days after immunization, although at relatively low levels (data not presented). The results presented in Fig. 2 are from a single representative experiment that employed three to five mice for each data point. These experiments have been repeated on two occasions with similar results. In the experiments presented net cpm rather than SI were plotted as it was found that control responses showed a great deal of variability, which affects the SI. We found that net cpm was more reproducible from experiment to experiment. In the majority of experiments, spleen cells from nonimmunized mice were also cultured in the presence of rickettsial antigens. These data (Table 1) demonstrate that only low levels of stimulation are noted in cultures of nonimmune spleen cells. Based on these data, we arbitrarily defined a positive LP response as having SI ≥ 3.0, and the cpm are significantly different ($P \leq 0.05$) than the cpm of control cultures.

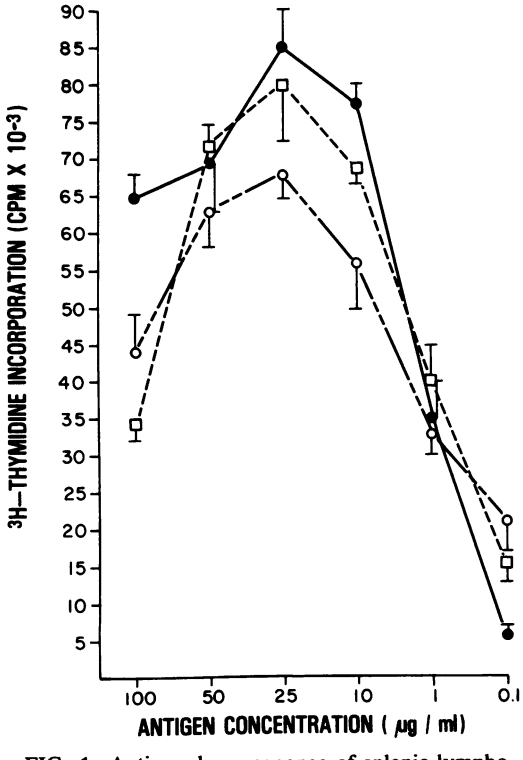

FIG. 1. Antigen dose response of splenic lymphocytes obtained from mice 28 days after Gilliam immunization (●), 42 days after Karp immunization (○), or 49 days after Kato immunization (□). Each point represents mean net cpm of quadruplicate cultures ± 1 standard error obtained from three to five mice.

Development of cross-reactivity to heterologous antigens. Spleen cells from immunized mice also were tested with antigen preparations prepared from rickettsiae other than the immunizing strain to follow the development of lymphocyte populations responsive to shared or cross-reactive antigens. Mice immunized with Gilliam rickettsiae demonstrated LP reactivity in response to both Karp and Kato antigens, and this cross-reactivity peaked at the same time after immunization as the homologous antigen response (Fig. 3). At the time of maximal homologous antigen response of Gilliam-immunized mice, the response to Karp and Kato antigens was 71 and 52%, respectively, of the Gilliam antigen response. This level of cross-reactivity remained fairly constant throughout the remainder of the experiment, with the exception of the response to Kato, which was relatively low at 60 days after Gilliam immunization. A similar pattern of cross-reactivity was evident in mice immunized with Karp rickettsiae, except that the response to Gilliam antigen was slightly greater (114%) than the response to homologous Karp antigen (Fig. 4). Also of note, Karp-immunized mice demonstrated a higher degree of cross-reactivity to Kato antigens than did Gilliam-immunized mice relative to the response to the homologous, immunizing antigen, although the magnitude of

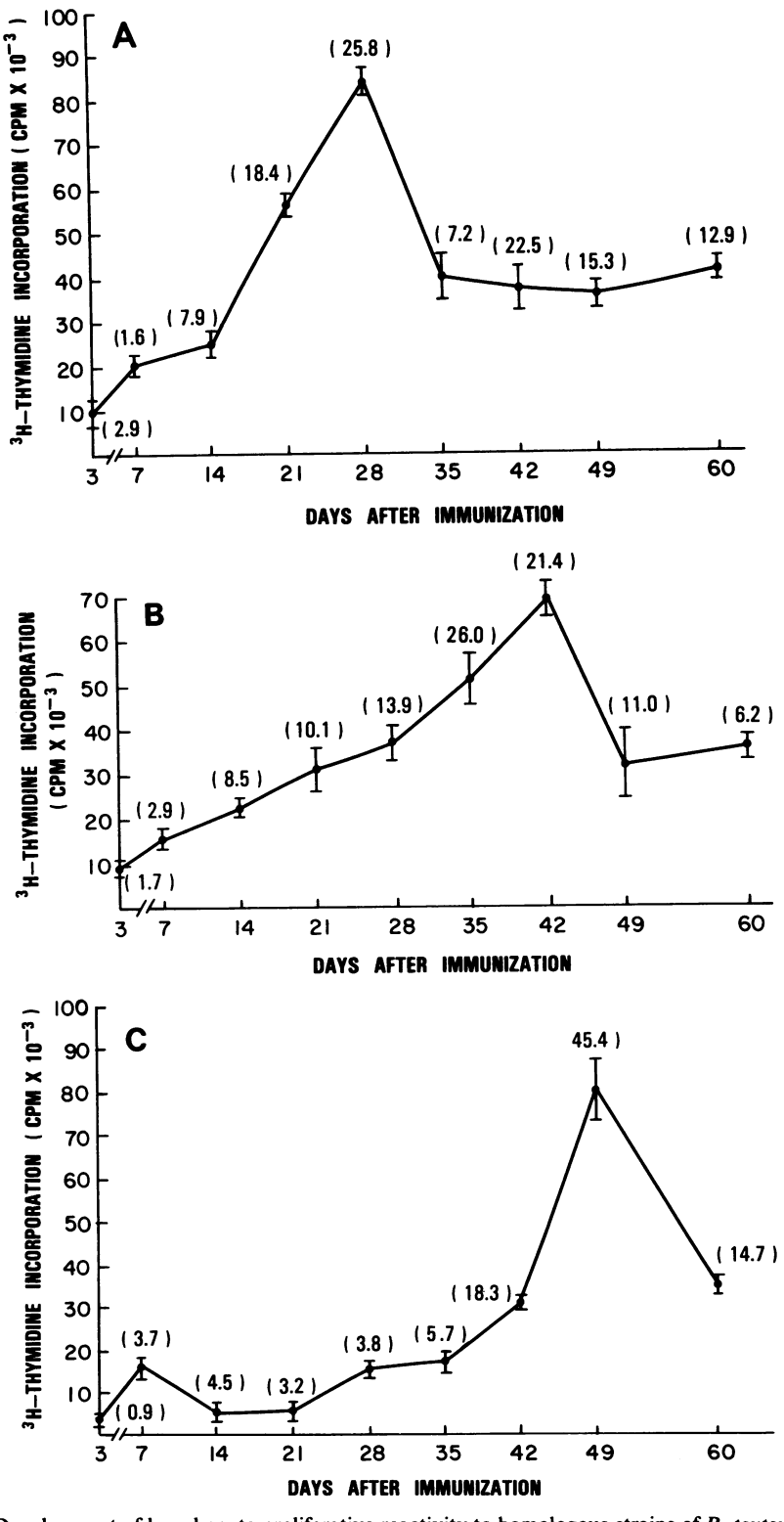

FIG. 2. Development of lymphocyte proliferative reactivity to homologous strains of *R. tsutsugamushi* after s.c. immunization with 1,000 MLD_{50} of the appropriate rickettsiae. (A) Responses of Gilliam-immunized BALB/c mice to Gilliam antigen. (B) Responses of Karp-immunized BALB/c mice to Karp antigen. (C) Responses of Kato-immunized BALB/c mice to Kato antigen. Each point represents mean net cpm of quadruplicate cultures ± 1 standard error with antigen (25 μg of protein per ml) obtained from three to five mice.

TABLE 1. Lymphocyte proliferative responses of spleen cells from nonimmunized BALB/c mice to *R. tsutsugamushi* antigen[a]

Antigen source	Mean cpm ± SE[a] (range)	Mean SI ± SE[b] (range)
Gilliam	4,732 ± 851 (8,026–2,112)	2.1 ± 0.15 (2.7–1.4)
Karp	2,475 ± 747 (5,258–252)	1.5 ± 0.18 (2.5–0.8)
Kato	4,050 ± 471 (7,148–2,688)	2.1 ± 0.14 (2.7–1.6)

[a] The results are from nine experiments.

the response to Kato antigen was similar in both groups of mice through 35 days after immunization. The peak response of Karp-immune mice to Kato antigen, as well as Gilliam antigen, occurred at the same time as the peak response to homologous Karp antigen; also of note, the magnitude of the Kato response at this time was nearly twice the maximal response of Gilliam-immune mice to Kato antigen (48,283 versus 27,803 cpm). When the LP responses of Kato-immunized mice to heterologous antigens are examined (Fig. 5), an interesting pattern is noted. Early in the development of responsiveness, these mice were much more responsive to Gilliam antigen than Karp or homologous antigens, although by 49 days after immunization, when responsiveness to all three antigens was maximal, the greatest response was to the immunizing antigen. At all time points examined, the response to Gilliam antigen was greater than the response to Karp antigen and, except at days 42 and 49, was greater than the response to the immunizing antigen. At these time points, the responses to Gilliam antigen were 79 and 89% of the response to Kato antigen, respectively.

Previous studies from this laboratory have demonstrated that s.c. immunization of mice leads to a protective immunity against homologous intraperitoneal challenge, which is completely effective first at 21 to 28 days after immunization (16). As LP reactivity was relatively low at these times, especially in Kato-immunized mice, we challenged immunized mice with 1,000 MLD_{50} of homologous rickettsiae intraperitoneally and examined LP responsiveness to the immunizing rickettsiae and heterologous strains 3 and 7 days after challenge. In all groups of mice, intraperitoneal challenge with the homologous rickettsiae prompted a marked increase in LP reactivity to homologous antigens and also to heterologous antigens (Table 2). Generally, the relative responses remained constant, which is due to an apparent proportionate increase in all responses. Note that even though the homologous response of Kato-immunized mice was increased from 8,211 to 32,409 (nearly fourfold) by intraperitoneal challenge with Kato rickettsiae, better responses were generated with Karp and Gilliam antigens. A similar situation is evident in the other groups examined.

Characterization of responding lymphocytes. As spleen cells consist of nearly equal proportions of T-lymphocytes and bone marrow-derived lymphocytes (B-lymphocytes), it was of interest to determine the class of lymphocytes responding to *R. tsutsugamushi* antigens. In initial experiments, a T-lymphocyte-enriched population of cells was obtained by passage of spleen cells from Karp-immune mice over nylon wool columns. As described, these cells consisted of ≥95% T-lymphocytes based on Thy 1.2 surface antigen and approximately 2% B-lymphocytes, based on surface immunoglobulin, and ≤1% macrophages, determined by morpho-

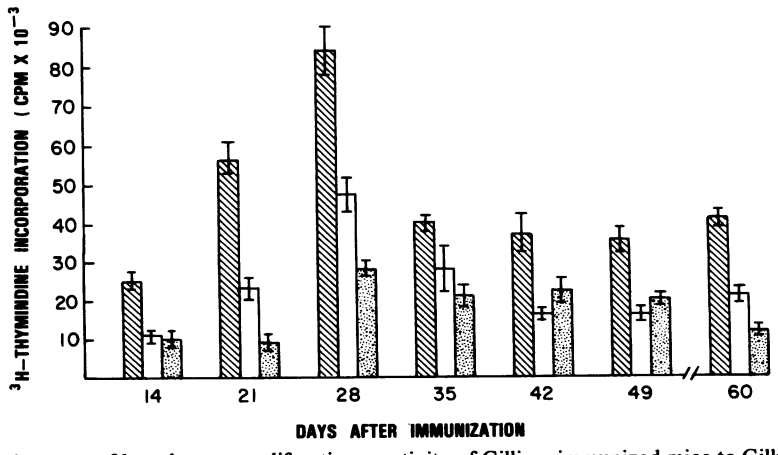

FIG. 3. Development of lymphocyte proliferative reactivity of Gilliam-immunized mice to Gilliam (■), Karp (□), and Kato (▩) antigens. Each point represents mean net cpm of quadruplicate cultures ± 1 standard error with antigen (25 μg of protein per ml) obtained from three to five mice.

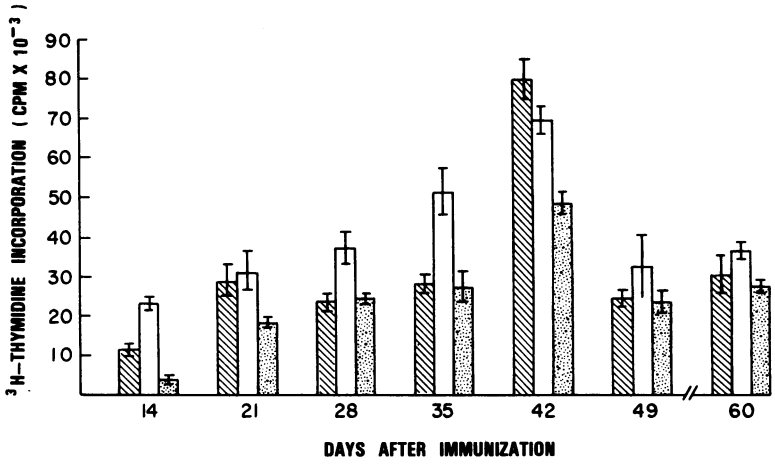

FIG. 4. Development of lymphocyte proliferative reactivity of Karp-immunized mice to Gilliam (■), Karp (□), and Kato (▩) antigens. Each point represents mean net cpm of quadruplicate cultures ± 1 standard error with antigen (25 μg of protein per ml) obtained from three to five mice.

logical criteria. Nylon wool-nonadherent lymphocytes responded well to the T-lymphocyte mitogen ConA, but failed to respond to LPS as well as to Karp antigen (Table 3). When the nylon wool-nonadherent cells were supplemented with 5% normal SAC, responsiveness to Karp antigen was noted; and a small amount of LPS responsiveness also was evident when adherent cells were added. In a second experiment, the nylon wool-nonadherent cells were reconstituted with 10% peritoneal cells, which also restored responsiveness as did SAC, but to a lesser degree. Although responsiveness was detected in nylon wool-nonadherent cells reconstituted with plastic-adherent cells, the response was less than that noted in unfractionated spleen cells. To address the possibility that spleen cells other than T-lymphocytes might be proliferating in response to these antigens, immune spleen cells were selectively depleted of T-lymphocytes by treatment with anti-Thy 1.2 serum and complement. In these experiments (Table 4), Karp-immune spleen cells, obtained 45 days after immunization, and normal spleen cells were tested with Karp antigen after treatment with complement alone or after treatment with anti-Thy 1.2 serum and complement. Treatment of normal and immune spleen cells with anti-Thy 1.2 and complement markedly reduced the response of these cells to ConA without a dramatic decline in LPS response. Depletion of immune spleen cells of Thy 1.2-bearing lymphocytes

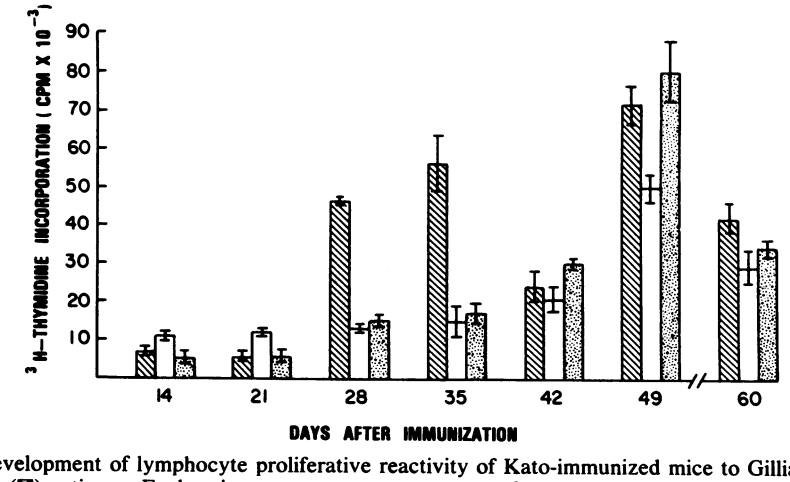

FIG. 5. Development of lymphocyte proliferative reactivity of Kato-immunized mice to Gilliam (■), Karp (□), and Kato (▩) antigens. Each point represents mean net cpm of quadruplicate cultures ± 1 standard error with antigen (25 μg of protein per ml) obtained from three to five mice.

TABLE 2. Effect of homologous rechallenge on specific lymphocyte responses and cross-reactivity to heterologous antigens

Immunization[a]	Days after rechallenge[b]	Net cpm[c]		
		Gilliam	Karp	Kato
Gilliam	0	32,575 ± 3,234	16,939 ± 2,047	18,242 ± 976
	3	126,537 ± 3,082	40,168 ± 4,794	ND[d]
	7	136,544 ± 2,189	70,802 ± 4,323	57,029 ± 6,753
Karp	0	56,527 ± 3,791	47,106 ± 3,649	32,503 ± 4,629
	3	108,357 ± 6,274	123,022 ± 5,634	16,943 ± 1,131
	7	63,210 ± 3,126	64,794 ± 4,918	43,815 ± 2,982
Kato	0	ND	13,712 ± 2,762	8,211 ± 1,511
	3	42,814 ± 5,132	15,240 ± 870	7,352 ± 260
	7	50,058 ± 3,604	43,808 ± 1,846	32,409 ± 2,659

[a] Mice were immunized 28 days before testing with 1,000 MLD_{50} of indicated organism given s.c.
[b] Mice were challenged with 1,000 MLD_{50} of homologous rickettsiae intraperitoneally.
[c] Mean maximum response ± standard error.
[d] ND, Not determined.

resulted in nearly complete elimination of responsiveness to Karp antigen. To ensure that T-lymphocyte depletion would not simply shift the antigen optimum, a full dose-response assay was performed; reactivity to Karp was not detected at any antigen dose tested (data not shown). Interestingly, some Gilliam reactivity was still evident in the T-lymphocyte-depleted cell populations obtained from immune and normal mice, but only at the highest doses tested (data not shown).

DISCUSSION

A number of reports have described the appearance of lymphocytes after rickettsial infections responding in culture to the rickettsial antigens. It has been shown that infection of humans (5, 14) and experimental animals (17, 18) results in lymphocytes that are capable of proliferating in response to specific rickettsial antigens, and that this responsiveness is associated with the development of a CMI. Studies with

TABLE 3. Lymphocyte proliferative responses of nylon wool-passed immune lymphocytes to *R. tsutsugamushi* antigen

Responding cells[a]	Stimulant	Net CPM (maximum ± SE)[b]	SI
Expt I			
Spleen cells	ConA	246,876 ± 35,869	70
	LPS	40,799 ± 3,361	13
	Karp antigen	40,914 ± 2,378	13
Nylon wool passed	ConA	257,314 ± 10,277	382.0
	LPS	923 ± 240	1.4
	Karp antigen	748 ± 338	1.1
Nylon wool passed +5% SAC[c]	ConA	279,724 ± 17,170	600.0
	LPS	3,542 ± 142	7.6
	Karp antigen	11,097 ± 785	23.8
Expt II			
Spleen cells	ConA	226,017 ± 12,708	50.6
	LPS	13,720 ± 2,177	3.0
	Karp antigen	53,741 ± 7,010	12.0
Nylon wool passed	ConA	240,235 ± 15,450	20.5
	LPS	385 ± 50	0.3
	Karp antigen	294 ± 65	0.3
Nylon wool passed + 10% PEC[d]	ConA	220,635 ± 10,702	36.9
	LPS	7,378 + 576	1.2
	Karp antigen	12,134 ± 1,408	2.1

[a] Spleen cells were obtained from mice immunized with 1,000 MLD_{50} of the Karp strain 45 days before testing and treated as indicated.
[b] Results are from quadruplicate cultures.
[c] SAC were obtained from nonimmunized syngeneic mice.
[d] PEC, Peritoneal exudate adherent cells obtained from nonimmunized mice.

TABLE 4. Effect of depletion of Thy 1.2 cells on lymphocyte proliferative responses to *R. tsutsugamushi* antigens

Responding cells	Treatment	Stimulant	Net CPM (maximum ± SE)[a]	SI
Expt I				
Nonimmune spleen cells	None	ConA	231,439 ± 2,907	91.0
		LPS	53,771 ± 1,308	22.0
		Karp antigen (25 μg/ml)	392 ± 238	1.2
Nonimmune spleen cells	Anti-Thy 1.2 and complement	ConA	6,529 ± 201	9.3
		LPS	23,362 ± 1,207	25.0
		Karp antigen (25 μg/ml)	395 ± 36	1.6
Immune spleen cells[b]	None	ConA	266,446 ± 11,715	69.0
		LPS	53,884 ± 2,375	15.0
		Karp antigen (25 μg/ml)	15,602 ± 877	5.0
Immune spleen cells	Anti-Thy 1.2 and complement	ConA	5,062 ± 883	18.0
		LPS	25,061 ± 1,340	87.0
		Karp antigen (25 μg/ml)	189 ± 40	1.6
Expt II[c]				
Immune spleen cells	None	ConA	245,212 ± 3,829	69.0
		LPS	50,490 ± 1,223	15.0
		Karp antigen (25 μg/ml)	22,384 ± 1,173	7.2
Immune spleen cells	Anti-Thy 1.2 and complement	ConA	267 ± 100	1.5
		LPS	12,368 ± 907	24.0
		Karp antigen (25 μg/ml)	533 ± 94	2.0

[a] Results are from quadruplicate cultures.
[b] Spleen cells were obtained from mice immunized with 1,000 MLD_{50} of the Karp strain 45 days before testing.
[c] Spleen cells were treated twice with anti-Thy 1.2 and complement.

synthetic antigens and haptens have clearly shown that T-lymphocyte receptor specificity can be evaluated by examining the proliferation of specifically sensitized T-lymphocytes, and this proliferation is thought to be a reliable reflection of the events leading to the ultimate expression of CMI (25, 29).

In the present study, we have shown that s.c. immunization of inbred mice with viable strains of *R. tsutsugamushi* results in the appearance of splenic lymphocytes that respond in culture to irradiated preparations of the immunizing rickettsiae. This antigen-induced proliferation was evident early in the course of immunization regardless of the strain of rickettsiae used. Previous studies from this laboratory have demonstrated that s.c. immunization of mice results in partial protection against rechallenge approximately 14 days after immunization, and by 21 to 28 days after infection animals are completely resistant to a second challenge (16). Protective immunity has been associated with a population of T-lymphocytes capable of transferring resistance to naive mice (28) as well as the appearance of a specific delayed-type hypersensitivity response (16).

In this study, LP reactivity was first evident 14 days after immunization, which is similar to the time of appearance of delayed-type hypersensitivity (16). Interestingly, the peak of responsiveness differed, depending on the strain of *R. tsutsugamushi* used for immunization. Immunization with Gilliam rickettsiae resulted in peak responses at 28 days after immunization, in contrast to Karp and Kato, with respective peak responses at 42 and 49 days after infection. The early development of LP responses in BALB/c mice immunized with Gilliam rickettsiae may be related in part to the previously observed genetic resistance of this strain of mice to infection with the Gilliam strain of rickettsiae (12). In other model systems of chronic infections, it has been noted that these infections result in perturbations of the immune response, resulting in a loss of responsiveness to a number of antigens (3, 4, 24, 26). It is possible that more severe infections result from s.c. infection with the Karp and Kato strains and result in an immunosuppression that acts to delay the onset of CMI as measured by the LP assay or in some manner acts to limit the expression of LP after interaction with antigen. When the development of LP responsiveness is compared with the development of delayed-type hypersensitivity (16) to these antigens, it is clear that these responses develop at different rates and persist for different periods of time. Whether this is due to different subsets of T-lymphocytes mediating each response or to different regulatory mechanisms is of fundamental importance, and studies are in progress to address these questions. It seems unlikely that an irreversible state of immunosuppression exists in these chronically infected mice, based on their ability to resist rechallenge

at times when relatively low levels of LP responses are noted in Karp- and Kato-immunized mice. It is clear from these studies that LP responsiveness as a general event correlates with the development of resistance to challenge (12, 16), although in all cases the maximal response was noted at a time after resistance to challenge would be observed.

To examine this question further, we infected mice 28 days after immunization and examined their LP reactivity 3 and 7 days after the secondary challenge. In all cases, the challenge resulted in a marked boost in LP reactivity (Table 2), suggesting that these animals were not hampered in their ability to be boosted antigenically. It also is important to note that mice immunized and challenged in this fashion routinely survive the challenge infection (12, 16) and also survived challenge in these experiments (data not presented).

Earlier studies suggested that antibody specificity for various strains of *R. tsutsugamushi* varied, depending on the strain of rickettsiae used to immunize animals, but antisera raised to a number of strains showed only strain specificity in terms of neutralizing ability (2, 11). In light of the data generated to date that suggest that CMI is of primary importance in immunity to *R. tsutsugamushi* (16, 20, 21, 28) as well as the studies that suggest that immunization with a single strain of *R. tsutsugamushi* confers broad group immunity, the possibility is raised that the T-lymphocyte immune mechanism generated by infection exhibits different specificities than the B-lymphocyte antibody, which also arises after immunization. The data generated in this study clearly demonstrate a marked degree of cross-reactivity at the level of the splenic proliferating lymphocyte. In a recent study (32) it was postulated that macrophage-processed antigens, which are recognized by immune T-lymphocytes, consist of sequestered internal bacterial antigens as well as surface antigens. It is possible that a similar situation exists in the rickettsiae, in that T-lymphocyte proliferation is induced by all antigens after macrophage processing, whereas antibody is also produced to all antigens, but only surface antigens are detected with immunofluorescence assays. In some cases, LP responses to heterologous antigens were as great or greater than the response to the immunizing antigen. In the case of animals immunized with Kato rickettsiae, early responses are striking in that responses to both Karp and Gilliam antigens were markedly greater than responses to the Kato antigen. This may simply reflect poorer antigenicity of tissue culture-grown Kato, although the same antigen elicited a strong homologous response late after immunization. Again, it is intriguing to correlate the level of responses elicited by the irradiated antigens and the pathogenicity of the corresponding strains. The least pathogenic Gilliam rickettsiae provide the strongest homologous and heterologous responses when used as an antigen, and the more pathogenic Karp and Kato rickettsiae provide lesser stimulation of immune lymphocytes. Further studies are required to attempt to answer these important questions.

It is important to note that the antigen preparation used in this study consisted of irradiated intact rickettsiae, and the lymphocyte proliferation noted was obviously due to stimulation with numerous antigens. Further studies are required to determine whether the strains of *R. tsutsugamushi* truly contain common, cross-reactive antigens or whether the cross-reactivity is at the level of the lymphocyte receptor.

Numerous studies have suggested that murine B-lymphocytes have the ability to proliferate in culture in response to specific antigenic stimuli (8, 19, 30). To determine the nature of the lymphocyte population responding to antigens of *R. tsutsugamushi*, immune spleen cells were treated either to enrich or to deplete T-lymphocytes. Nylon wool passage of immune spleen cells resulted in a population of cells highly enriched for T-lymphocytes, which responded well to ConA but failed to respond to the appropriate rickettsial antigen. The addition of SAC or peritoneal exudate cells both containing ≥90% macrophages restored responsiveness to the rickettsial antigen, but at a somewhat lower level than that noted in the unfractionated spleen cell population. As it was possible that a portion of the responsiveness in the unfractionated spleen cell population was due to B-lymphocyte proliferation in response to rickettsial antigen, we tested a B-lymphocyte population obtained by treatment with anti-Thy 1.2 serum and complement. Treatment of immune spleen cells to remove T-lymphocytes reduced homologous antigen responses to nonreactive levels at optimal antigen doses. It is clear that at least a portion of the proliferation noted is due to antigen recognition by immune T-lymphocytes. The responses clearly are dependent on adherent cells, and their role remains to be elucidated. The relatively poor responsiveness of nylon wool-passed cells might be due to an inappropriate number of added adherent cells or to disruption of adherent cell function due to technical factors or perhaps to a portion of the antigen responsive T-lymphocytes remaining on the column. In a recent study it was demonstrated that antigen reactive T-lymphocytes could nonspecifically recruit B-lymphocytes to proliferate (30), and that this B-lymphocyte proliferation was dependent on T-lymphocyte recognition of antigen. It is a distinct possibility that a similar

situation is occurring in the proliferative response to *R. tsutsugamushi* antigens. Attempts to prepare more representative populations of accessory macrophages and T-lymphocytes as well as studies to investigate T-lymphocyte recruitment of other cells are in progress and should add insight into the fundamental question concerning the nature of the cell populations contributing to LP in response to *R. tsutsugamushi* antigens.

LITERATURE CITED

1. **Barker, L. F., J. K. Patt, and H. E. Hopps.** 1968. Titration and neutralization of *Rickettsia tsutsugamushi* in tissue culture. J. Immunol. **100**:825–830.
2. **Bennett, B. L., J. E. Smadel, and R. L. Gauld.** 1949. Studies on scrub typhus (tsutsugamushi disease). IV. Heterogeneity of strains of *R. tsutsugamushi* as demonstrated by cross-neutralization tests. J. Immunol. **62**:453–461.
3. **Cheers, C., H. Pavlov, C. Riglar, and E. Madruso.** 1980. Macrophage activation during experimental brucellosis. III. Do macrophages exert feedback control during brucellosis? Cell. Immunol. **49**:168–177.
4. **Collins, F. M., and S. R. Watson.** 1979. Suppressor T-cells in BCG-infected mice. Infect. Immun. **25**:491–496.
5. **Coonrod, J. D., and C. C. Shepard.** 1971. Lymphocyte transformation in rickettsioses. J. Immunol. **106**:209–216.
6. **Eisemann, C. E., and J. V. Osterman.** 1976. Proteins of typhus and spotted fever group rickettsiae. Infect. Immun. **14**:155–162.
7. **Eisenberg, G. H. G., Jr., and J. V. Osterman.** 1977. Experimental scrub typhus immunogens: gamma-irradiated and formalinized rickettsiae. Infect. Immun. **15**:124–131.
8. **Elfenbein, G. J., E. M. Shevach, and I. Green.** 1972. Proliferation by bone marrow derived lymphocytes in response to antigenic stimulation in vitro. J. Immunol. **109**:870–874.
9. **Ewing, E. P., A. Takeuchi, A. Shirai, and J. V. Osterman.** 1978. Experimental infection of mouse peritoneal mesothelium with scrub typhus rickettsiae: an ultrastructural study. Infect. Immun. **19**:1068–1075.
10. **Finney, D. J.** 1971. Statistical methods in biological assay, 2nd ed., p. 524–530. Charles Griffin & Co., Ltd., London.
11. **Fox, J. P.** 1949. The neutralization technique in tsutsugamushi disease (scrub typhus) and the antigenic differentiation of rickettsial strains. J. Immunol. **62**:341–352.
12. **Groves, M. G., and J. V. Osterman.** 1978. Host defenses in experimental scrub typhus: genetics of natural resistance to infection. Infect. Immun. **19**:583–588.
13. **Humphres, R. C., and D. J. Hinrichs.** 1981. Role for antibody in *Coxiella burnetii* infection. Infect. Immun. **31**:641–645.
14. **Jerrells, T. R., L. P. Mallavia, and D. J. Hinrichs.** 1975. Detection of long-term cellular immunity to *Coxiella burnetii* as assayed by lymphocyte transformation. Infect. Immun. **11**:280–286.
15. **Jerrells, T. R., and J. V. Osterman.** 1981. Host defenses in experimental scrub typhus: inflammatory response of congenic C3H mice differing at the *ric* gene. Infect. Immun. **31**:1014–1022.
16. **Jerrells, T. R., and J. V. Osterman.** 1982. Host defenses in experimental scrub typhus: delayed-type hypersensitivity responses of inbred mice. Infect. Immun. **35**:117–123.
17. **Kenyon, R. H., M. S. Ascher, R. A. Kishimoto, and C. E. Pedersen, Jr.** 1977. In vitro guinea pig leukocyte reactions to *Rickettsia rickettsii*. Infect. Immun. **18**:840–846.
18. **Kishimoto, R. A., J. W. Johnson, R. H. Kenyon, M. S. Ascher, E. W. Larson, and C. E. Pedersen, Jr.** 1978. Cell-mediated immune responses of guinea pigs to an inactivated phase I *Coxiella burnetii* vaccine. Infect. Immun. **19**:194–198.
19. **Lipsky, P. E., and A. S. Rosenthal.** 1976. The induction and regulation of guinea pig B-lymphocyte proliferation in vitro. J. Immunol. **117**:1594–1602.
20. **Nacy, C. A., and M. S. Meltzer.** 1979. Macrophages in resistance to rickettsial infection: macrophage activation *in vitro* for killing of *Rickettsia tsutsugamushi*. J. Immunol. **123**:2544–2549.
21. **Nacy, C. A., and J. V. Osterman.** 1979. Host defenses in experimental scrub typhus: role of normal and activated macrophages. Infect. Immun. **26**:744–750.
22. **Oaks, S. C., Jr., F. M. Hetrick, and J. V. Osterman.** 1980. A plaque reduction assay for studying antigenic relationships among strains of *Rickettsia tsutsugamushi*. Am. J. Trop. Med. Hyg. **29**:998–1006.
23. **Oaks, S. C., Jr., J. V. Osterman, and F. M. Hetrick.** 1977. Plaque assay and cloning of scrub typhus rickettsiae in irradiated L-929 cells. J. Clin. Microbiol. **6**:76–80.
24. **Riglar, C., and C. Cheers.** 1980. Macrophage-activation during experimental brucellosis. II. Inhibition of *in vitro* lymphocyte proliferation by Brucella-activated macrophages. Cell. Immunol. **49**:154–167.
25. **Schwartz, R. H., C. L. Horton, and W. E. Paul.** 1977. T-lymphocyte-enriched murine peritoneal exudate cells. IV. Genetic control of cross-stimulation at the T-cell level. J. Exp. Med. **145**:327–343.
26. **Scott, M. T.** 1981. Delayed hypersensitivity to *Trypanosoma cruzi* in mice: specific suppressor cells in chronic infection. Immunology **44**:409–417.
27. **Shirai, A., P. J. Catanzaro, G. H. G. Eisenberg, Jr., and J. V. Osterman.** 1977. Host defenses in experimental scrub typhus: effect of chloramphenicol. Infect. Immun. **18**:324–329.
28. **Shirai, A., P. J. Catanzaro, S. M. Phillips, and J. V. Osterman.** 1976. Host defenses in experimental scrub typhus: role of cellular immunity in heterologous protection. Infect. Immun. **14**:39–46.
29. **Solinger, A. M., and R. H. Schwartz.** 1980. The T-lymphocyte proliferative response to poly-L-Glu-poly-D, L-Ala-poly-L-Lys. J. Immunol. **124**:2485–2490.
30. **Tse, H. Y., J. J. Mond, and W. E. Paul.** 1981. T lymphocyte-dependent B lymphocyte proliferative response to antigen. I. Genetic restriction of the stimulation of B lymphocyte proliferation. J. Exp. Med. **153**:871–882.
31. **Van Peenen, P. F. D., C. M. Ho, and A. L. Bourgeois.** 1977. Indirect immunofluorescence antibodies in natural and acquired *Rickettsia tsutsugamushi* infections of Philippine rodents. Infect. Immun. **15**:813–816.
32. **Ziegler, K., and E. R. Unanue.** 1981. Identification of a macrophage antigen-processing event required for I-region-restricted antigen presentation to T lymphocytes. J. Immunol. **127**:1869–1877.