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Role of T-Lymphocytes in Production of Antibody to Antigens of *Rickettsia tsutsugamushi* and Other *Rickettsia* Species

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The requirement of thymus-dependent lymphocytes for antibody production to *Rickettsia tsutsugamushi*, *Rickettsia akari*, *Rickettsia conorii*, and *Rickettsia typhi* was investigated by comparing antibody production in athymic (nu/nu) or thymus-bearing BALB/c mice. Athymic BALB/c mice produced antibody after infection with *R. akari*, *R. conorii*, and *R. typhi* as measured by indirect fluorescent antibody titration or radioimmunoassay. Antibody production in these mice was as great or greater than in the thymus-bearing mice and demonstrated similar kinetics. In contrast, athymic BALB/c mice infected either intraperitoneally or subcutaneously with the Gilliam strain of *R. tsutsugamushi* failed to produce demonstrable antibody. The requirement of thymus-dependent lymphocytes for antibody production to *R. tsutsugamushi* was further suggested by the demonstration of antibody production after transfer of immune thymus-dependent lymphocytes to athymic mice and the demonstration of *R. tsutsugamushi*-specific T helper cells in immune thymus-bearing mice. The antibody produced in athymic mice after infection with *R. akari*, *R. conorii*, and *R. typhi* was predominantly immunoglobulin M, based on isotype-specific radioimmunoassays and sucrose gradient fractionation. Furthermore, the antibody produced by athymic mice in response to *R. akari* infection reacted with a carbohydrate-containing outer membrane component.

Infection of laboratory animals or humans with *Rickettsia tsutsugamushi* produces circulating antibodies which demonstrate strain specificity (6, 14, 32, 37, 42). Although the role of antibody in acquired resistance to infection with *R. tsutsugamushi* is unclear, a possible role for antibody in immunity is suggested by data demonstrating a close relationship between peak antibody titers and acquisition of resistance to reinfection (C. S. Eisemann and J. V. Osterman, unpublished data). Furthermore, animals immunized with viable *R. tsutsugamushi* do not develop a rickettsemia after challenge, in contrast to animals immunized with irradiated rickettsiae. The major difference noted between mice immunized with viable rickettsiae or irradiated organisms is the amount of antibody produced after each immunization scheme (10, 22).

More direct evidence for the role of antibody in resistance to infection comes from studies which demonstrated that immune sera could neutralize the infectivity of *R. tsutsugamushi* for animals (6) or tissue culture (4, 32). Furthermore, administration of immune sera to naive mice was shown to protect against challenge (39). The mechanism of this protection might be an enhanced macrophage handling of infectious organisms after rickettsiae-antibody interaction, either in terms of macrophage-mediated killing (31) or by an antibody-mediated adjuvant effect which enhances the development of a cell-mediated immunity as suggested for *Coxiella burnetii* (19).

One argument against antibody as the exclusive mediator of immunity is that athymic mice are capable of producing antibody in response to infection with *Rickettsia akari*, and in spite of a high level of antibody these animals still succumb to the infection (24). Also, in studies with *C. burnetii*, Humphres and Hinrichs (19) showed that passively transferred antibody enhanced rickettsial clearance from the spleens of thymus-bearing mice, but transfer of immune sera to athymic mice had no effect on rickettsial clearance.

The antibody response to an antigen can be classified as either T lymphocyte dependent (TD) or T lymphocyte independent (TI), based on the requirement for T lymphocytes (5). The TI antigens are thought to interact directly with B lymphocytes and will provoke an antibody response in athymic (nu/nu) mice (5, 12, 25, 43, 45). A number of bacterial products, including dextran (12, 40), lipopolysaccharide (25, 43, 45), and pneumococcal polysaccharide (1–3, 18), are considered classical TI antigens. In one report,

the spotted-fever-group rickettsia *R. akari* was shown to produce antibody in athymic mice (24), thus suggesting that this rickettsia possesses TI antigens, in contrast to a report suggesting that athymic mice do not produce antibody after infection with *R. tsutsugamushi* (23). Rickettsiae of the spotted fever and typhus groups have been shown to possess a carbohydrate outer component which is a likely candidate for the TI antigenic component and, importantly, is lacking in scrub typhus rickettsiae (34).

Because of the biochemical difference in terms of surface components among rickettsiae of the three major groups and because of the suspected effector role of antibody in the immunity elicited by rickettsiae, the present study was designed to define the antibody response to representative rickettsiae, in terms of T independency and T dependency. These studies demonstrated that infection of athymic BALB/c mice with *R. akari*, *Rickettsia conorii*, and *Rickettsia typhi* all resulted in the production of antibody of the immunoglobulin M (IgM) type. In contrast, infection of nu/nu BALB/c mice with *R. tsutsugamushi* failed to elicit an antibody response. The TD nature of the antibody response to *R. tsutsugamushi* was further suggested in that passive transfer of immune T lymphocytes to athymic mice allowed production of antibody after infection, and rickettsia-specific T-helper cells were demonstrable in immune thymus-bearing mice.

MATERIALS AND METHODS

Mice. Female BALB/c and C3H/HeDub mice were obtained from Flow Laboratories, Inc. (Dublin, Va.), and used at 8 to 12 weeks of age. Female BALB/c (nu/nu) mice were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.) and also used at 8 to 12 weeks of age.

Rickettsiae. The Gilliam strain of *R. tsutsugamushi* (165th egg passage) was plaque purified (33) and propagated in embryonated hen eggs (11). The Kaplan strain of *R. akari*, the Malish strain of *R. conorii*, and the Wilmington strain of *R. typhi* were also propagated in embryonated hen eggs. Titers of *R. tsutsugamushi* were expressed as the 50% mouse lethal dose (MLD_{50}) based on titration in C3H/HeDub mice and calculated by the method of Spearman and Karber (13). Titers of *R. akari*, *R. conorii*, and *R. typhi* were determined with a plaque assay with irradiated L-929 cells and expressed as PFU.

Infection of mice. Mice were infected with *R. akari*, *R. conorii*, and *R. typhi* by intraperitoneal injection of 10,000 PFU. Mice were infected with *R. tsutsugamushi* by injection of 1,000 MLD_{50} either intraperitoneally or subcutaneously. In some experiments, athymic BALB/c mice and thymus-bearing BALB/c mice were given drinking water containing 2.5 mg of chloramphenicol per ml 5 to 7 days after infection to allow their survival. This regime has been shown to control rickettsial replication but still allow an immune response to develop (38).

Antibody determination. Mice were bled from the axilla at various times after infection after CO_2 anesthesia, and sera were collected and stored frozen (−40°C) until assayed. Antibody titers were determined by using an indirect immunofluorescence assay (IFA) as previously described (37). Fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin and goat anti-mouse IgM (Cappel Laboratories, Cochranville, Pa.) were used as second antibodies in the assay. Antibody titers also were determined by a radioimmunoassay (RIA), using either rickettsial-soluble antigens or French press lysates as the solid-phase RIA antigens. Soluble antigens were extracted from *R. akari*, *R. conorii*, and *R. typhi* organisms as described previously (34) by shaking suspensions of infected yolk sacs with diethyl ether, collecting the aqueous phases, and centrifuging to remove the particulate material. Residual ether was removed from the soluble antigen preparations by dialysis against phosphate-buffered saline (PBS). French press antigens were prepared from yolk sac-grown *R. akari* organisms and from *R. tsutsugamushi* rickettsiae grown in L-929 cells and partially purified by differential centrifugation (9). Organisms were irradiated with 300 krad of gamma irradiation, passed twice through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 18,000 lb/in^2, and then centrifuged at 500 × *g* for 10 min to clarify the solubilized antigens. For each antigen preparation, the dilution chosen for use in the RIA was the highest dilution that resulted in optimal binding of the ^{125}I-conjugated antiglobulin to dilutions of reference 28-day immune mouse serum. The RIA was performed essentially as described by Zollinger et al. (44). Twenty-five microliters of antigen diluted in Dulbecco PBS, pH 7.4, was added to the wells of 96-well, flexible polyvinyl chloride round-bottomed microtiter plates, washed four times with PBS containing 20% newborn calf serum, 0.0025% phenol red, and 0.2% sodium azide (PBS-NCS), and then reacted with 25 μl of test or control sera diluted with PBS-NCS in half $\log_{10}$ (3.3 fold) dilutions. All serum titrations were performed in duplicate. Plates were covered, incubated at 4°C for 18 to 20 h, and then washed four times with PBS-NCS. ^{125}I-labeled antiglobulin, 150,000 cpm in 25 μl, was then added to each well, and the plates were incubated at 37°C for 2 h in a humidified atmosphere. The antiglobulins were obtained as a mixture of rabbit anti-mouse IgG, IgA, and IgM (Calbiochem-Behring Corp., LaJolla, Calif.) and purified by affinity column chromatography by M. K. Gentry, Department of Biochemistry, Walter Reed Army Institute of Research, or were purchased as affinity-purified goat anti-mouse IgG or IgM (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). All antiglobulin preparations were labeled with ^{125}I, using the chloramine T method of Greenwood et al. (16). Excess ^{125}I-labeled antiglobulin was removed from the microtiter plates by washing four times in PBS-NCS and then extensive washing with tepid tap water. Individual wells were cut apart, and bound ^{125}I was measured by counting each well in a Packard PGD auto-gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The antibody titer of each serum was determined by dividing the average counts per minute of the test (T) serum wells by the average counts per minute of the wells containing the corresponding dilution of normal (N) serum. The highest dilution of serum yielding a

TABLE 1. Susceptibility of athymic (nu/nu) and thymus-bearing (+/+) BALB/c mice to the Gilliam strain of *R. tsutsugamushi*

Challenge dose (MLD_{50})	% Mortality	
	nu/nu	+/+
10,000	ND[b]	0 (0/5)
1,000	100 (5/5)	0 (0/5)
100	80 (4/5)	0 (0/5)
10	100 (5/5)	ND
1	0 (0/5)	ND

[a] Rickettsial stocks were diluted to contain the approximate challenge dose based on previous animal titrations with C3H/HeDub mice, which are susceptible to lethal infection, and were administered intraperitoneally.

[b] ND, Not done.

T/N ratio of 2.5 or greater was considered the endpoint.

Transfer of immune T lymphocytes. Thymus-bearing BALB/c mice were immunized by subcutaneous infection with 1,000 MLD_{50} of the Gilliam strain of *R. tsutsugamushi* as previously described (17, 21). After 28 days the animals were sacrificed and their spleens were removed, and a single-cell suspension prepared in RPMI 1640 supplemented with 50 μg of gentamicin per ml, 1% glutamine, and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer. The resulting spleen cells were adjusted to 2×10^8 per ml in RPMI 1640 medium supplemented as above and also containing 10% fetal calf serum and passed over a 5 g nylon wool column. The column-passed cells (>95% Thy 1.2 positive) were adjusted to 5×10^7 per ml in RPMI 1640, and 1-ml portions were injected intravenously into athymic BALB/c mice. Recipient animals were infected with 1,000 MLD_{50} of the Gilliam strain of *R. tsutsugamushi* intraperitoneally 24 h after cell transfer and bled 21 days after infection. As before, mice were given chloramphenicol in their drinking water 7 days after infection.

Demonstration of rickettsia-specific T-helper cells. Thymus-bearing BALB/c mice were immunized with the Gilliam strain of *R. tsutsugamushi* as above, and 28 days after infection, T-helper cells specific for *R. tsutsugamushi* were demonstrated with trinitrophenyl (TNP)-conjugated Gilliam rickettsiae by a technique described by Burgess et al. (7). Briefly, immune mice were challenged with various amounts of TNP-Gilliam strain rickettsiae, TNP-*R. akari*, or TNP-bovine serum albumin (BSA) given intravenously. Five days after administration of TNP-conjugated antigens, animals were sacrificed and spleen cell suspensions were prepared in Hanks balanced salt solution. Cells producing anti-TNP antibody were determined by using a slide modification of the Jerne plaque assay (8), which employs sheep erythrocytes conjugated with TNP as described by Rittenberg and Pratt (36). Results were expressed as plaque-forming cells (PFC) per 10^6 nucleated spleen cells and per spleen.

RESULTS

Previous reports from this laboratory have demonstrated that thymus-bearing BALB/c mice are genetically resistant to lethal infection with the Gilliam strain of *R. tsutsugamushi* (17). In an initial experiment, athymic BALB/c mice were infected intraperitoneally with various doses of the Gilliam strain of *R. tsutsugamushi*. The results of this experiment (Table 1) clearly demonstrated that athymic BALB/c mice, unlike thymus-bearing mice, are susceptible to lethal infection with Gilliam rickettsiae. As we were interested in determining whether the infection in athymic mice progressed to a lethal infection in spite of the development of circulating antibody as previously reported for *R. akari* (24), we infected athymic BALB/c mice subcutaneously and examined sera for the development of IFA antibodies. In preliminary studies, athymic BALB/c mice survived without antibiotic treatment through 21 days after subcutaneous infection, thus allowing antibody determinations through this time. In further experiments, athymic BALB/c mice were infected intraperitoneally or subcutaneously with *R. tsutsugamushi* or intraperitoneally with *R. akari* and placed on water containing antibiotic 5 to 7 days after infection. Regardless of the route of infection or antibiotic intervention, athymic BALB/c mice infected with *R. tsutsugamushi* did not make detectable antibody at the times after infection that these animals were tested (Table 2). In contrast, athymic mice responded as well as thymus-bearing mice in terms of antibody production after infection with *R. akari*. As it was possible that the level of antibody in athymic mice infected with Gilliam rickettsiae was too low to detect with IFA, sera from these mice and from athymic mice infected with *R. akari* were compared with sera from infected thymus-bearing mice by an RIA (Table 3). Even with this sensitive assay, antibody was not detectable in the sera of athymic mice infected with *R. tsutsugamushi*. In contrast, high titers of antibody of both IgM and IgG classes were detectable in thymus-bearing mice from 10 days after infection with *R. tsutsugamushi* and in both athymic and thymus-bearing mice infected with *R. akari* (Table 3). Interestingly, the IgM response in mice infected with *R. akari* appears early (days 3 to 5) relative to the response noted in mice infected with *R. tsutsugamushi*. To determine whether the ability to provoke an antibody response in athymic mice was limited to *R. akari*, we infected athymic and thymus-bearing BALB/c mice with another spotted-fever-group rickettsia, *R. conorii*, and a typhus-group rickettsia, *R. typhi*, and quantitated the antibody produced after infection. Infection with both of these rickettsiae resulted in antibody production in athymic mice as well as in thymus-bearing mice (Table 4). As with *R. akari*, antibody was detected in both groups of mice at the same time

TABLE 2. Comparison by IFA of antibody production by athymic (nu/nu) and thymus-bearing (+/+) BALB/c mice after infection with the Gilliam strain of *R. tsutsugamushi* or *R. akari*

Rickettsia and route of infection	Antibiotic treatment[a]	Days after infection	Antibody titer	
			nu/nu	+/+
R. tsutsugamushi subcutaneously[b]	−	3	<2	<2
		7	<2	40
		14	<2	80
		21	<2	80
R. tsutsugamushi subcutaneously[b]	+	3	ND[c]	ND
		7	<2	20
		14	<2	40
		21	<2	80
R. tsutsugamushi intraperitoneally[b]	+	3	<2	<2
		7	<2	20
		14	<2	80
		21	<2	80
		28	<2	320
R. akari intraperitoneally[d]	+	3	ND	ND
		7	ND	ND
		14	ND	ND
		21	40	80
		28	160	80

[a] Mice were given drinking water containing 2.5 mg of chloramphenicol per ml 5 to 7 days after infection.
[b] A 1,000-MLD_{50} dose of the Gilliam strain of *R. tsutsugamushi* was administered.
[c] ND, Not done.
[d] A 10,000-PFU dose of *R. akari* was administered.

(days 3 to 5 after infection), and the antibody titers were at least as high in athymic mice as they were in thymus-bearing mice. Again, infection with these rickettsiae produced an early IgM response in contrast to *R. tsutsugamushi*.

In further studies, the isotype of the antibody produced in athymic and thymus-bearing mice as well as the nature of the antigen reactive with the antibody were investigated with affinity-purified anti-mouse IgM and anti-mouse IgG and various antigen fractions. Athymic mice (Table 5) produced predominantly an IgM response to *R. akari*. When sera from thymus-bearing animals were assayed, reactivity to the whole rickettsial extract (French press antigen) consisted of both an IgM and IgG response. We also examined sera obtained from athymic and thymus-bearing mice which were infected with *R. conorii* or *R. typhi* and obtained similar results (data not shown). In this series of experiments, we also employed a soluble antigen extract recoverable from *R. akari* as the antigen in the RIA. The levels of reactivity noted with this antigen fraction were similar to those found by using the whole rickettsial preparation, and furthermore, the same pattern of reactivity was noted in that athymic sera contained essentially only IgM antibodies, whereas sera from immune thymus-bearing mice had nearly equal amounts of IgG and IgM (Table 5).

Some experiments suggested that low levels

TABLE 3. Comparison by RIA of antibody production by athymic (nu/nu) and thymus-bearing (+/+) BALB/c mice after infection with the Gilliam strain of *R. tsutsugamushi* or *R. akari*

Rickettsia[a]	Days after infection	Antibody titer[b]		
		nu/nu (IgM + IgG)	+/+	
			IgM	IgG
R. tsutsugamushi	0	<10	<10	<10
	3	<10	<10	<10
	7	<10	<10	<10
	10	ND[c]	100	1,000
	14	<10	1,000	10,000
	21	<10	3,000	30,000
R. akari	0	<10	<10	<10
	1	ND	10	<10
	3	ND	300	<10
	5	300	1,000	<10
	7	1,000	30,000	1,000
	10	10,000	>100,000	10,000
	14	30,000	>100,000	>100,000
	21	1,000	>100,000	>100,000
	28	100	>100,000	>100,000

[a] A 1,000-MLD_{50} dose of the Gilliam strain of *R. tsutsugamushi* or 10,000 PFU of *R. akari* were administered intraperitoneally. Mice were given drinking water containing 2.5 mg of chloramphenicol per ml at 7 days after infection.
[b] Reciprocal of the serum dilution exhibiting a 2.5-fold increase in isotope binding as compared to nonimmune serum control with ^{125}I-labeled anti-mouse IgM or IgG.
[c] ND, Not done.

TABLE 4. Comparison by RIA of antibody production by athymic (nu/nu) and thymus-bearing (+/+) BALB/c mice after infection with *R. conorii* or *R. typhi*

Rickettsia[a]	Days after infection	Antibody titer[b]		
		nu/nu (IgM)	+/+	
			IgM	IgG
R. conorii	0	<10	<10	<10
	1	ND[c]	<10	<10
	3	ND	<10	<10
	5	300	300	<10
	7	10,000	10,000	<10
	10	10,000	10,000	30
	14	10,000	10,000	1,000
	21	30,000	10,000	1,000
	28	30,000	1,000	3,000
R. typhi	0	<10	<10	<10
	1	ND	<10	<10
	3	ND	<10	<10
	5	300	300	<10
	7	1,000	3,000	1,000
	10	1,000	3,000	10,000
	14	ND	3,000	30,000
	21	ND	3,000	10,000
	28	ND	300	30,000

[a] Mice were infected intraperitoneally with 10,000 PFU of *R. conorii* or *R. typhi*. Drinking water containing 2.5 mg of chloramphenicol per ml was given to *R. conorii*-infected animals at 7 days after infection and to *R. typhi*-infected animals at 5 days after infection.

[b] Reciprocal of the serum dilution exhibiting a 2.5-fold increase in isotope binding as compared to nonimmune serum control with ^{125}I-labeled anti-mouse IgM in nu/nu titrations or ^{125}I-labeled anti-mouse IgM or IgG in +/+ titrations.

[c] ND, Not done. Athymic mice infected with *R. typhi* died 12 days after infection.

TABLE 5. Antibody isotype produced by athymic (nu/nu) and thymus-bearing (+/+) BALB/c mice after infection with *R. akari*[a]

Antigen preparation	Isotype specificity of second antibody	Antibody titer[b]	
		nu/nu	+/+
French press	Anti-IgM + anti-IgG[c]	10,000	10,000
	Anti-IgM	30,000	30,000
	Anti-IgG	<10	3,000
Soluble antigen	Anti-IgM + anti-IgG[c]	3,000	3,000
	Anti-IgM	10,000	10,000
	Anti-IgG	<10	30,000

[a] Sera were obtained from athymic and thymus-bearing mice 21 days after intraperitoneal infection with 10,000 PFU of *R. akari*. All mice were given drinking water containing 2.5 mg of chloramphenicol per ml at 7 days after infection.

[b] Reciprocal of the serum dilution exhibiting a 2.5-fold increase in isotope binding as compared to nonimmune serum control.

[c] A mixture of affinity purified anti-mouse IgM and IgG.

of IgG were present in sera from nu/nu mice infected with *R. akari*. As it was possible that this reactivity was in fact due to production of IgG antibodies or perhaps due to nonspecific binding of antiglobulin, we performed sucrose gradient fractionation of one such pooled serum obtained from athymic mice 21 days after intraperitoneal infection with *R. akari*. Each fraction was then reacted with *R. akari* French press antigen and ^{125}I-labeled anti-mouse IgM or anti-mouse IgG. Data presented in Fig. 1 demonstrate that only one major peak of antigen binding activity was detectable after reaction with anti-mouse IgM and it appeared in fractions 7 through 11, where the IgM activity would be expected. When each fraction was reacted with anti-mouse IgG, a minor peak was noted in fraction 18, which was only slightly above the background anti-IgM reactivity. Immune sera from thymus-bearing mice fractionated in a similar manner showed IgM reactivity in fractions 7 through 11 and IgG reactivity in fractions 15 through 20 (data not shown).

As data to this point suggested that a dichotomy existed between rickettsiae of the scrub typhus group and representative members of the spotted fever and typhus groups in terms of the requirement for T cells in antibody formation, further experiments were performed to establish the T lymphocyte dependency of responses to

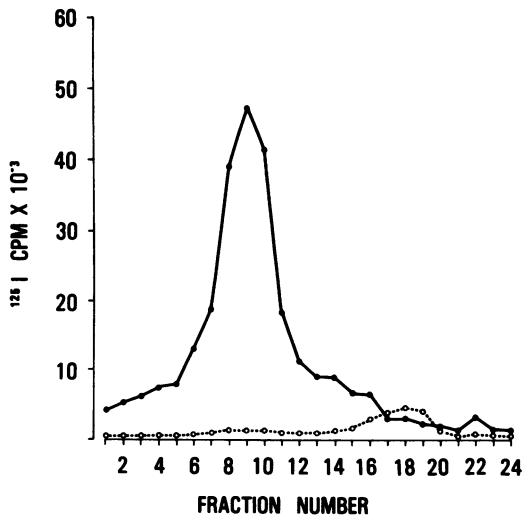

FIG. 1. RIA reactivity of serum obtained 21 days after infection of athymic (nu/nu) BALB/c mice wtih *R. akari* after fractionation on a continuous sucrose gradient. Antibody binding was quantitated by using ^{125}I-labeled anti-mouse IgM (●) or anti-mouse IgG (○).

TABLE 6. Effect of transfer of immune T cells on antibody production of athymic mice after infection with *R. tsutsugamushi*[a]

Cells transferred	Antibody titer	
	IFA[b]	RIA[c]
None	<2	<10
5×10^7 immune T cells	40	100

[a] Mice were infected with 1,000 MLD_{50} of *R. tsutsugamushi* intraperitoneally 24 h after receiving saline or 5×10^7 immune T cells given intravenously.

[b] Reciprocal of the serum dilution exhibiting 2+ fluorescence after development with flouresceinated anti-mouse immunoglobulin.

[c] Reciprocal of the serum dilution exhibiting a 2.5-fold increase in isotope binding as compared to nonimmune serum control.

R. tsutsugamushi. In the first experiment (Table 6), athymic mice were given 5×10^7 immune, nylon-wool-nonadherent spleen cells and infected intraperitoneally with *R. tsutsugamushi*. Sera were obtained from these animals and from untreated control mice 21 days after infection and assayed for antibody. This representative experiment was repeated twice and demonstrated that reconstitution of athymic mice with immune syngeneic T cells provided the capability for antibody production after infection with *R. tsutsugamushi*.

As it has been shown that T-helper cells arising during an infectious process provide carrier-specific help for antibody production to the hapten TNP (7), the next experiments were designed to formally demonstrate T-helper cells in thymus-bearing mice (Table 7). Thymus-bearing mice which were immunized with *R. tsutsugamushi* (28 days after subcutaneous infection) produced an anti-TNP PFC response only after challenge with TNP-*R. tsutsugamushi*. No response was noted in immune animals after challenge with TNP-BSA or TNP-*R. akari*, and nonimmunized mice did not respond to any of the challenges. The lack of response of immune mice to TNP-BSA or to TNP-*R. akari* suggested that the immune animals possessed *R. tsutsugamushi*-specific T-helper cells at the time after infection that these studies were performed.

DISCUSSION

The spotted-fever-group rickettsiae *R. akari* and *R. conorii* as well as the typhus-group rickettsia *R. typhi* were shown to induce antibody production of the IgM class in mice lacking mature T lymphocytes. In contrast, athymic BALB/c mice infected with the Gilliam strain of *R. tsutsugamushi* failed to make antibody detectable with either an IFA or RIA technique at any time point tested, which confirmed the results of a previous study (23). It is possible that levels of antibody below the sensitivity of the RIA, which has been shown to be in the 1 to 10 ng of antibody per ml (41), are produced in athymic mice infected with *R. tsutsugamushi*. As rickettsial replication is apparently unchecked in athymic mice, it is also possible that the failure to detect antibody in athymic mice is due to all or most of the circulating antibody

TABLE 7. Demonstration of *R. tsutsugamushi*-specific T-helper cells in thymus-bearing BALB/c mice immunized with *R. tsutsugamushi*

Primary immunogen[a]	Secondary challenge[b]	Challenge amt (μg)	TNP PFC per:	
			10^6 Spleen cells	Spleen
R. tsutsugamushi	None		0	0
	TNP-BSA	100	1	1,600
	TNP-*R. akari*	100	0	0
	TNP-*R. tsutsugamushi*	1	56	13,664
		10	56	15,456
		100	24	7,416
None	None		0	0
	TNP-BSA	100	0	0
	TNP-*R. akari*	100	0	0
	TNP-*R. tsutsugamushi*	1	0	0
		10	2	3,200
		100	0	0

[a] Thymus-bearing BALB/c mice were infected subcutaneously with 1,000 MLD_{50} of the Gilliam strain of *R. tsutsugamushi* or given an equal volume of saline subcutaneously.

[b] Animals were challenged intravenously 28 days after the primary immunization with the indicated immunogens and assayed for TNP PFC 5 days later. Results presented are the arithmetic mean TNP PFC obtained from 3 to 5 mice.

existing in the form of antigen-antibody complexes because of the resulting large numbers of rickettsiae in the bloodstream. This possibility is considered unlikely, as animals placed on antibiotic-containing water still did not develop an antibody response. Treatment of mice with antibiotics in this manner has been shown to markedly suppress the replication of rickettsiae and does not seem to hamper the development of immunity if given 5 to 7 days after infection (38). The results of the studies performed with athymic BALB/c mice clearly suggest that at least the majority of the antibody response to *R. tsutsugamushi* is dependent on the presence of mature T lymphocytes. This concept is further suggested by the experiments which demonstrated that antibody production occurs in athymic mice if these animals are reconstituted with immune T cells (Table 6) and the demonstration of *R. tsutsugamushi*-specific T helper cells in immune thymus-bearing mice (Table 7). Although immune T cells were successful in reconstituting athymic mice, it is possible that T cells from nonimmune animals could also perform this function, and attempts are currently in progress to further define the cellular requirements for reconstitution of athymic mice.

In direct contrast to *R. tsutsugamushi*, the antibody responses to *R. akari*, *R. conorii*, and *R. typhi* were not dependent on the presence of mature T cells, which confirms the results of a previous study with *R. akari* (24). Furthermore, the antibody produced in response to these organisms was shown to be predominately of the IgM class. It has been shown that thymus-bearing mice produce an isotype-restricted antibody response to carbohydrate antigens consisting of an IgM and IgG3 response (2, 3, 35), and it is generally felt that mice lacking mature T lymphocytes produce predominantly an IgM response, although low levels of IgG production in response to TNP-Ficoll have been reported in athymic mice (28). In the majority of our experiments, only antibody reacting with anti-IgM was detected in sera from infected athymic mice, although it was felt that some sera did display a very low level of IgG reactivity, but this could not be conclusively confirmed by sucrose gradient fractionation (Table 5 and Fig. 1). It is possible that low levels of IgG are produced in athymic mice after infection with *R. akari*, which are lost or diluted by gradient separation, and further studies are in progress to elucidate this point.

It was also shown in this study that antibody produced in thymus-bearing and athymic mice after infection with *R. akari* was reactive not only with a whole rickettsial extract but also with a soluble antigen preparation prepared by ether extraction of purified rickettsiae. This antigen has been shown to be a protein-carbohydrate complex (34), and it is demonstrable in rickettsiae of the spotted fever group and typhus group but lacking in scrub typhus rickettsiae. These data and the reactivity of antibody from athymic mice with this antigen strongly suggest that the TI antigen is within this complex. As the majority of complex carbohydrates of microbial origin have been shown to be TI antigens, such as pneumococcal polysaccharide (1–3, 18), dextran (12, 40), and lipopolysaccharide (26, 43, 45), it is tempting to conclude that the TI portion of the spotted-fever- or typhus-group soluble antigen is the carbohydrate, and studies are in progress to show definitively the nature of the TI antigen(s) in these organisms.

Recent studies from this laboratory (20, 21, 39) and by other workers (19) of cell-mediated immunity produced by infection with rickettsiae have suggested that antibody most likely does not perform a primary effector function in the immune response generated as a result of rickettsial infections. It has been argued that due to the obligate intracellular nature of these organisms and the fact that antibody generally does not penetrate cells antibody plays little or no role in immunity, and thus the cellular immune system is of primary importance. Numerous studies (19–21, 23, 30, 31, 39) as well as data generated in this study (Table 1) support the importance of cell-mediated immunity in immunity to rickettsiae. An indirect role for antibody in either facilitating the development of cell-mediated immunity (19), preparing rickettsiae for destruction by macrophages (30), or facilitating the clearance of rickettsiae from the blood (22; unpublished data) is a distinct possibility. It is possible that these antibody-mediated events occur after the interaction of antibody and extracellular rickettsiae and that these complexes have a predilection for the cells of the reticuloendothelial system. This would result in a rapid clearing of circulating organisms and delivery of the antigen to cells capable of antigen processing, which in effect adjuvantizes the antigen for the development of a cell-mediated immunity.

It is because of these considerations that the findings in this study gain an importance further than simply the demonstration of a biological difference between scrub typhus and other rickettsiae. It has been well documented that TD and TI antigens produce antibody by distinct mechanisms involving a distinct subpopulation of B cells (29) and that responses to these two types of antigens are under different control mechanisms (15, 27). Because of these differences in host responses to TD and TI antigens, it is of practical importance to know the class of antigen responsible for antibody production when designing rickettsial vaccine studies.

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