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Cross-Reactive Lymphocyte Responses and Protective Immunity against Other Spotted Fever Group Rickettsiae in Mice Immunized with *Rickettsia conorii*

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Lymphocyte proliferation in response to antigens on spotted fever group rickettsiae was used as a method to investigate the group-specific protective immunity to rechallenge characteristic of this group of rickettsiae at the T-cell receptor level. Spleen cells from *Rickettsia conorii*-immune C3H/HeJ mice proliferated in response to *R. rickettsii* Sheila Smith, *R. sibirica* 246, *R. australis*, and all tested strains of *R. conorii* (Casablanca, Moroccan, and Malish). Spleen cells from these mice, however, responded poorly or not at all to antigens prepared from the Kaplan or Hartford strain of *R. akari*. Proliferation of immune T cells maintained as in vitro cell lines showed a similar pattern of reactivity to these antigens; however, response to *R. akari* was consistently demonstrable. Spleen cells from C3H/HeJ mice immunized with *R. akari* responded to *R. akari* and *R. conorii* antigens as well as antigens from the other spotted fever group rickettsiae. Lymphocytes obtained from lymph nodes draining foot pads infected with *R. conorii* or *R. akari* demonstrated cross-reactivity similar to that found with immune spleen cells. If immunization was accomplished with *R. conorii* antigen emulsified in Freund complete adjuvant, the resulting lymph node cells were able to respond to *R. akari* antigens. These data suggest that infection with *R. conorii* induces a population of T lymphocytes that recognize an antigen(s) that also is found on other spotted fever rickettsiae and that may be responsible for cross-protective immunity. This antigen probably is not a major antigen on *R. akari*.

In experimental animal model systems, recovery from sublethal infections with spotted fever group rickettsiae has been shown to produce immunity to rechallenge not only with the homologous rickettsia but also with other members of the spotted fever group (4, 7, 27). In the guinea pig model, it has been shown that infections with the relatively nonpathogenic *Rickettsia conorii* or *R. montana* induce protective immunity against the more pathogenic *R. rickettsii* (7, 27). It has also been shown in recent studies that the C3H/HeJ mouse is susceptible to lethal infection with *R. conorii* Malish and that infection of these animals with spotted fever group rickettsiae that do not produce lethal infections, such as *R. rickettsii*, induces protective immunity against infection with *R. conorii* (4).

Immunity to members of the genus *Rickettsia* is thought to be mediated primarily by cellular mechanisms. Although the mechanisms of immunity to members of the spotted fever group have not been well studied, the importance of cell-mediated immunity in protection against these organisms has been suggested by a number of studies. Resistance to *R. conorii* can be passively transferred to naive mice with immune thymus-derived lymphocytes (T cells) (15), and macrophages activated as a result of infection have been suggested as important effector cells (14). Transfer of immunity was not possible with immune cells relatively late after immunization, although this was at a time when the donor animals were still resistant to infection (15), suggesting the possibility of other mechanisms. The presence of cellular immunity in human and experimental animals convalescent from spotted fever group rickettsial infections has been suggested on the basis of well-established in vitro parameters of cell-mediated immunity, including lymphocyte proliferation in response to specific antigenic stimulation (3, 11, 19). The necessity of T cells in resistance to spotted fever group rickettsiae was also suggested by the studies of Kenyon and Pedersen (12) on congenitally athymic (*nu/nu*) mice infected with *R. akari*. The animals that lacked a T-cell response died from a fulminant rickettsial infection. Interestingly, these animals produced a marked antibody response which apparently had no effect on the ultimate outcome of the infection. In further studies, it was shown that athymic mice were susceptible to lethal infections with *R. akari* and *R. conorii* and that the antibody produced was cross-reactive with other rickettsiae in the spotted fever group (9; T. Jerrells and C. Eisemann, unpublished observation). Taken together, these data suggest that recovery from a primary infection with a spotted fever group rickettsia and the resulting group-specific immunity to rechallenge is probably a T-cell-dependent event and therefore requires antigens that stimulate T-cell response directly or T-cell-dependent antibody production.

The purpose of the present study was to use the recently described C3H/HeJ mouse model for *R. conorii* to determine whether cellular immunity to the group- and species-specific antigens present on the members of this group (5, 18, 20–23, 26) results from infection with this rickettsia and to characterize partially the specificity of this response.

MATERIALS AND METHODS

Rickettsiae. The following rickettsiae were used in this study as yolk sac preparations: *R. conorii* Malish (egg pass 12), *R. conorii* Casablanca (egg pass 40), and *R. conorii* Moroccan (egg pass 287); *R. rickettsii* Sheila Smith (egg pass 17); *R. sibirica* 246 (egg pass 20); *R. australis* Phillip (egg pass 121); and *R. akari* Kaplan (egg pass 21) and *R. akari* Hartford (egg pass 15). Rickettsial seed stocks were prepared from

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infected yolk sacs of embryonated eggs (SPAFAS, Inc., Norwich, Conn.) as previously described (6) and stored at −70°C. Rickettsiae were quantitated in all preparations by a plaque assay as described by Oaks et al. (17) and titers were expressed as PFU per ml. Rickettsial antigens used in vitro were prepared from tissue culture-grown rickettsiae as previously described (5, 10), and after purification and irradiation, antigen preparations were standardized by total protein determined by the procedure of Lowry et al. (16). In some experiments, tissue culture-grown rickettsiae were further purified with Renografin gradients (8, 28), and no difference was detected in the ability of these further purified preparations to act as an antigen in the assays used.

Mice and infections. Female C3H/HeJ mice were obtained from Jackson Laboratory, Bar Harbor, Maine, and used at 4 to 8 weeks of age. Immunizing infections were established by inoculating mice subcutaneously (s.c.) in the inguinal region with 10^4 PFU in 0.2 ml of cold brain heart infusion broth. To assess protection, we infected immune or naive mice with 10^5 PFU given intraperitoneally. To produce immune lymph node lymphocytes, we administered a total of 10^4 PFU s.c. in the hind foot pads and inguinal regions of mice. To investigate the response of lymph node cells to antigen administered in Freund complete adjuvant (Difco Laboratories, Detroit, Mich.), we gave each animal a total of 100 μg of rickettsial antigen emulsified in adjuvant administered in the hind foot pads and the base of the tail.

Cross-protection studies. Mice immunized by an s.c. infection were challenged 28 days after immunization with 10^5 PFU of *R. conorii* Malish, *R. akari* Kaplan, or *R. sibirica*, which have been previously shown to produce lethal infections in naive C3H/HeJ mice (4). In all experiments, naive mice were infected to ensure potency of the inocula. Infected animals were observed for 21 days, and deaths were recorded.

Cell preparation. At the appropriate interval after immunization, as indicated in Results, either spleens or draining lymph nodes (inguinal, popliteal, and periaortic) were obtained, and single-cell suspensions were prepared by mincing each organ through stainless steel screens. The resulting single-cell suspensions were washed with Hanks balanced salt solution and finally adjusted to 5×10^6 viable cells per ml in RPMI 1640 containing 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, 1% fresh glutamine, 50 μg of gentamicin sulfate per ml, 5×10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum, all obtained from MA BioProducts, Walkersville, Md.

Production and maintenance of immune T-cell lines. Spleen cells obtained from *R. conorii*-immune mice were stimulated in bulk culture with the immunizing antigen for 96 h. Blast cells were enriched by centrifugation of the cultured cells over a Ficoll-Hypaque gradient (lymphocyte separation medium; Litton Bionetics, Kensington, Md.) at 750 × *g* for 20 min. The resulting cells were maintained in vitro by alternating periods of rest for 10 days and antigen stimulation for 4 days as described by Kimoto and Fathman (13), except that the T-cell lines were stimulated with antigen in the presence of 5% supernatant of concanavalin A-stimulated rat spleen cells as a source of interleukin-2. Proliferative responses of the cell line to antigens or mitogens were evaluated with rested cells after isolation of viable cells with Ficoll-Hypaque gradients as described above. The resulting cells were suspended in RPMI 1640 supplemented as described above.

Lymphocyte proliferation assay. The ability of isolated spleen and lymph node lymphocytes to proliferate in response to rickettsial antigens was measured with a microculture assay system described previously (10). Briefly, 0.1-ml samples of cells containing 5×10^5 cells were placed in wells of 96-well microtiter plates (Costar, Cambridge, Mass.), and quadruplicate wells received either medium (control) or dilutions of rickettsial antigens. Cells maintained as in vitro lines were cultured at 2×10^4 cells per microtiter well along with 5×10^5 irradiated (2,500 rad) spleen cells from noninfected C3H/HeJ mice as a source of antigen-presenting cells. The cultures were incubated at 37°C in a 5% CO_2 atmosphere for 72 h. One microcurie of tritiated thymidine (5 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added to each well for the final 6 h of culture, and incorporated radioactivity was determined after cells were harvested onto glass fiber strips with a multiple harvesting system (PHD Cell Harvester; Cambridge Technology, Inc., Cambridge, Mass.). Data were expressed as net counts per minute calculated by subtracting the counts per minute of cultures receiving only medium from the counts per minute of antigen-stimulated cultures or as stimulation indexes (SI) calculated by dividing the counts per minute of antigen-stimulated cultures by the counts per minute of cultures that received only media. Statistical analysis was accomplished with the Student *t* test.

RESULTS

Cross-protection of mice immunized with spotted fever group rickettsiae. As shown by us and others (4, 7, 27), recovery from a sublethal infection with rickettsiae of the spotted fever group produces a broad spectrum of immunity to other members of this group. In Table 1, the data from a representative challenge experiment are presented. Infection of C3H/HeJ mice with rickettsiae which are virulent for this host (e.g., *R. conorii* Malish) or rickettsiae that do not produce overt infections (e.g., *R. rickettsii*) produced immunity to challenge with all spotted fever group rickettsiae that would otherwise have produced a lethal infection in naive mice. That this immunity is not completely nonspecific was suggested by the finding that mice immune to spotted fever group rickettsiae were not protected from challenge with *R. tsutsugamushi* Gilliam, and infections in these mice were indistinguishable from infections of naive mice with *R. tsutsugamushi* (data not presented).

Lymphocyte-proliferative responses of *R. conorii*-immune mice. To determine whether the cross-reactive protective immunity is reflected in a population of lymphocytes with cross-reactive specificities, we stimulated spleen cells from mice 28 to 35 days after immunization with antigens prepared from various members of the spotted fever group. A representative experiment with spleen cells from *R. conorii*-immune mice is presented in Fig. 1a. As can be seen from these data, a strong proliferative response was elicited by the homologous *R. conorii* antigen, and nearly identical results were obtained with the *R. rickettsii* antigen. When the data from all experiments were considered, the responses of spleen cells from *R. conorii*-immune mice to *R. conorii* and *R. rickettsii* were not statistically different. Similar responses were obtained with antigens prepared from *R. sibirica* and *R. australis*. As a general rule, the responses of *R. conorii*-immune spleen cells to these antigens were less than to *R. conorii* or *R. rickettsii* antigens. Antigens prepared from any of the three strains of *R. conorii* available (Malish, Casablanca, or Moroccan) were equally effective in stimulating spleen cells from mice immunized with the Malish strain (data not shown). Interestingly, in all the

experiments performed to date with *R. conorii*-immune spleen cells, little or no response was elicited with antigen prepared from the Kaplan or Hartford strain of *R. akari* at any dose of antigen used. In all experiments performed, a complete dose range of antigen from a maximum of 250 μg/ml was used. Optimal responses were generally in the range of 50 to 100 μg/ml, and the data presented in Fig. 1 were obtained with 100 μg of each antigen per ml.

To determine whether the *R. akari* antigen was capable of eliciting a response, we evaluated spleen cells from mice immunized with *R. akari* Kaplan for their ability to respond to the *R. akari* antigen as well as to the other spotted fever group antigens. These data (Fig. 1b) demonstrated that the *R. akari* antigen used in this study was highly stimulatory for spleen cells obtained from *R. akari*-immune mice. When the other spotted fever group antigen responses were evaluated, an interesting pattern of responsiveness was noticed. In the experiment presented in Fig. 1, spleen cells from *R. akari*-immune mice were as responsive to antigens of *R. rickettsii* as to the homologous antigen. This was a reproducible finding and extended throughout the dose range of antigen used (data not presented). In the majority of experiments performed, the responses of spleen cells from *R. akari*-immune mice to *R. conorii* and *R. rickettsii* were indistinguishable and of greater magnitude than the response to *R. akari*.

The response of long-term T-cell lines established from spleen cells responsive to antigens of *R. conorii* was also evaluated for responsiveness to antigens of various spotted fever group rickettsiae. A representative experiment is presented in Table 2. Essentially the same pattern of responses was noted with these cells as with bulk spleen cell cultures. The most notable exception was the response of these cells to *R. akari*. This cell line, as well as a comparable line independently established, have been tested on multiple occasions, and the pattern presented in Table 2 has been a reproducible finding.

The specificity of immune lymphocytes developing at a more localized site of rickettsial infection was evaluated by testing lymphocytes obtained from the lymph nodes draining the site of an s.c. infection. Nodes obtained from animals immunized with *R. conorii* Malish and *R. akari* Kaplan were studied. The data from a representative experiment are presented in Fig. 2a and b, respectively. Both sets of data were obtained with lymph node cells 10 days after infection, a time which was shown to be optimal in another system (22)

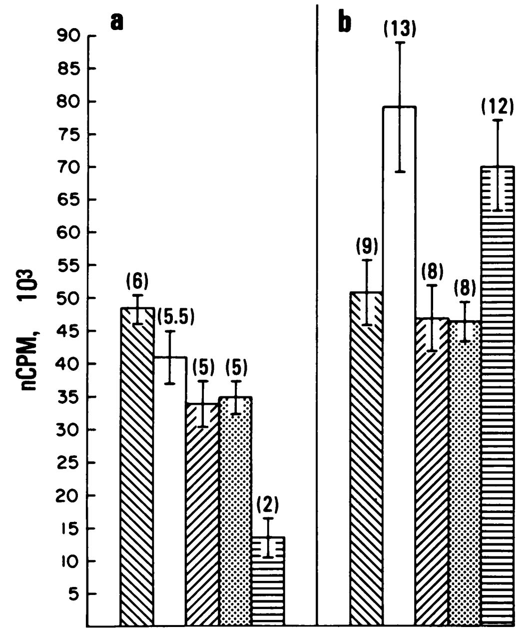

FIG. 1. Proliferative responses of spleen cells obtained 28 days after immunization of C3H/HeJ mice with 10^4 PFU of *R. conorii* Malish (a) or *R. akari* Kaplan (b). The mean net counts per minute ± 1 standard deviation of quadruplicate cultures stimulated with *R. conorii* Malish (▧), *R. rickettsii* (□), *R. sibirica* (▨), *R. australis* (▩), or *R. akari* Kaplan (▤) are presented. Values in parentheses are SIs.

TABLE 1. Protection against homologous and heterologous challenge as a result of sublethal infection of C3H/HeJ mice with various spotted fever group rickettsiae

Immunizing strain[a]	Challenge strain[b]	Survival[c]
None	*R. conorii* Malish	0/5
None	*R. akari* Kaplan	0/5
None	*R. sibirica*	0/5
None	*R. tsutsugamushi* Gilliam	0/5
R. conorii Malish	*R. conorii* Malish	10/10
R. conorii Malish	*R. akari* Kaplan	8/10
R. conorii Malish	*R. sibirica*	10/10
R. conorii Malish	*R. tsutsugamushi* Gilliam	0/5
R. akari Kaplan	*R. conorii* Malish	9/10
R. akari Kaplan	*R. akari* Kaplan	5/5
R. akari Kaplan	*R. sibirica*	8/10
R. akari Kaplan	*R. tsutsugamushi* Gilliam	0/5
R. sibirica	*R. conorii* Malish	4/5
R. sibirica	*R. akari* Kaplan	5/5
R. sibirica	*R. sibirica*	5/5
R. sibirica	*R. tsutsugamushi* Gilliam	0/5
R. rickettsii Sheila Smith	*R. conorii* Malish	5/5
R. rickettsii Sheila Smith	*R. akari* Kaplan	5/5
R. rickettsii Sheila Smith	*R. sibirica*	5/5
R. rickettsii Sheila Smith	*R. tsutsugamushi* Gilliam	0/5

[a] Mice were immunized s.c. with 10^4 PFU of the indicated rickettsiae or with diluent.

[b] Twenty-eight days after immunization, animals were challenged intraperitoneally with 10^5 PFU of the appropriate spotted fever group rickettsiae or 1,000 50% minimal lethal doses of the Gilliam strain of *R. tsutsugamushi*.

[c] Number of surviving animals 21 days after challenge/total number of animals challenged.

TABLE 2. Responses of an *R. conorii*-immune T-cell line to antigens of spotted fever group rickettsiae

Antigen	cpm ± SD (SI)[a]
None	549 ± 170
R. conorii Malish	35,631 ± 8,007 (65)
R. conorii Casablanca	21,219 ± 8,040 (39)
R. conorii Moroccan	33,832 ± 8,579 (62)
R. rickettsii	29,836 ± 6,273 (54)
R. sibirica	10,715 ± 1,155 (20)
R. australis	6,725 ± 1,421 (12)
R. akari	7,211 ± 1,793 (13)
R. tsutsugamushi	1,018 ± 241 (2)

[a] Average counts per minute ± 1 standard deviation of quadruplicate cultures stimulated with the optimal concentration of each antigen. Values in parentheses indicate SIs calculated by dividing experimental counts per minute by control counts per minute.

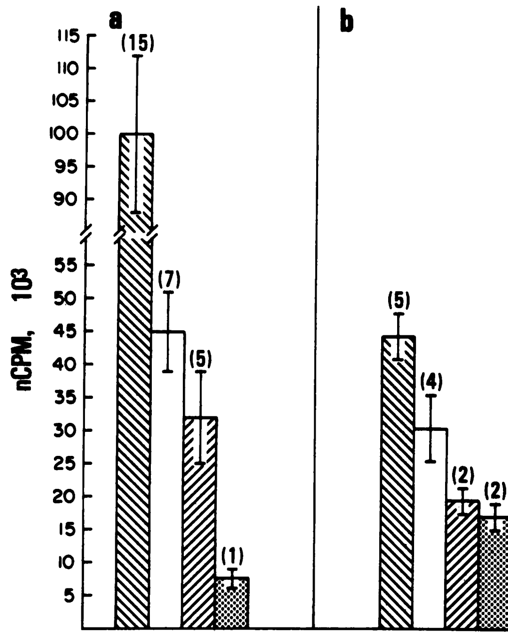

FIG. 2. Proliferative responses of lymph node cells obtained 10 days after infection of C3H/HeJ mice in the foot pads with *R. conorii* Malish (a) or *R. akari* Kaplan (b). The mean net counts per minute ± 1 standard deviation of quadruplicate cultures stimulated with *R. conorii* Malish (▧), *R. rickettsii* (□), *R. sibirica* (▨), or *R. akari* Kaplan (▩) are presented. Values in parentheses are SIs.

and in preliminary studies with these rickettsiae. Lymph node cells obtained from *R. conorii*-infected mice (Fig. 2a) reacted highly with homologous antigen, indicating a strong local immune response to infection with this rickettsia. Although proliferative responses were noted with antigens of the other spotted fever group rickettsiae, a response was not detected with *R. akari* antigen. When lymph node cells obtained from *R. akari*-immunized mice were stimulated in vitro with rickettsial antigens (Fig. 2b), the strongest proliferation was induced by the *R. conorii* Malish and *R. rickettsii* antigens, and a relatively weak response was elicited with *R. akari* Kaplan and *R. sibirica* antigens. In some experiments (data not shown), no response to the *R. akari* antigen was detected with lymph node cells from animals infected with *R. akari* Kaplan.

To study lymph node cell response to minor antigens on *R. conorii* and *R. akari*, we immunized mice in the foot pads and at the base of the tail with antigen emulsified in Freund complete adjuvant and collected draining nodes 10 days after immunization. Figure 3a and b present a representative experiment with nodes obtained from mice immunized with *R. conorii* Malish (3a) or *R. akari* Kaplan (3b). Animals immunized with rickettsial antigens in this way showed responses to both rickettsial antigens, although the homologous antigen was generally the most stimulatory.

Since *R. conorii*-immune mice were resistant to challenge with *R. akari* Kaplan (Table 1) but did not develop lymphocytes responsive to *R. akari* antigens in the spleen or draining lymph nodes, we examined the development of *R. akari*-antigen-responsive spleen lymphocytes in *R. conorii*-immune mice after infection with *R. akari*. The results of these experiments (Fig. 4) show that responses to *R. conorii* and *R. akari* were initially depressed at 3 and 7 days after the challenge infection, but prechallenge levels were regained by 14 days after infection. The response to *R. akari* antigen developed relatively slowly and peaked 14 to 21 days after challenge. The kinetics of lymphocyte responsiveness resembled that seen in naive mice (data not shown). The responsiveness of spleen cells from these mice to the homologous *R. conorii* antigen also increased somewhat, although the responses of 21 and 28 days after challenge were essentially the same as that on day 0.

DISCUSSION

It is clear from the data presented in this paper that spleens obtained from mice immune to *R. conorii* contain lymphocytes that recognize many but not all of the spotted fever group rickettsiae used in this study. It is interesting that lymphocytes from these mice assayed in bulk cultures did not recognize antigens of *R. akari* as assayed with lymphocyte proliferation. In the study by Anacker et al. (1), *R. akari* was shown to lack a major protein shared by other rickettsiae in this group (23). Although polyclonal antisera produced to spotted fever group rickettsiae clearly cross-react with *R. akari* and the other members of this group (1, 15, 18), the data presented in this study show that the T-lymphocyte response has different specificities. To rule out the possibility that the *R. akari* antigen used in the study was simply unable to stimulate lymphocytes, we immunized C3H/HeJ mice with viable *R. akari* and assessed spleen cell- and lymph node cell-proliferative responses to the antigens used in this study. The results of these studies clearly ruled out the possibility that the antigen was not functional and provided surprising results. It was shown that spleen cells from mice immune to *R. akari* responded to *R. conorii* and *R. rickettsii* antigens consistently more than to the immuniz-

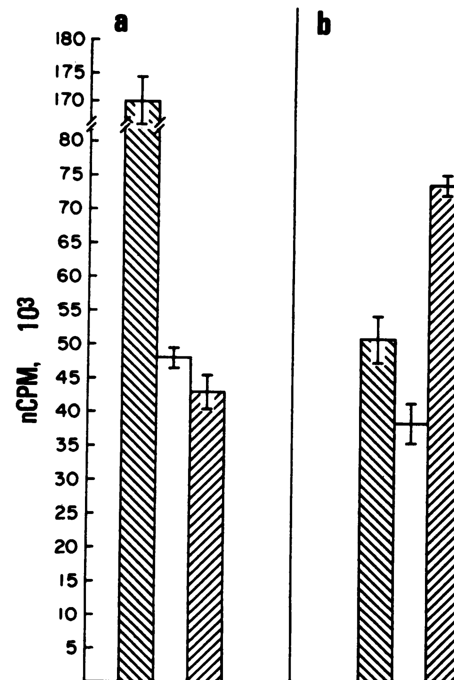

FIG. 3. Proliferative responses of lymph node cells obtained 10 days after immunization of C3H/HeJ mice with *R. conorii* Malish (a) or *R. akari* Kaplan (b) in Freund complete adjuvant. The mean net counts per minute ± 1 standard deviation of quadruplicate cultures stimulated with *R. conorii* Malish (▧), *R. akari* Kaplan (□), or purified protein derivative (▨) are presented.

ing antigen. This heteroclitic response (24) was a reproducible finding in this study, but no data are available to address the mechanism of this phenomenon.

R. conorii and *R. rickettsii* are closely related organisms genetically and antigenically (26). Thus, it was not surprising that immune lymphocytes from *R. conorii*-immune mice did not distinguish between these two rickettsiae. Since bulk lymphocyte cultures would be expected to represent all specificities, it is impossible to determine from the present study if these two rickettsiae contain unique antigens, and this question can be approached only by using monoclonal antibodies and cloned T-cell lines or T-cell hybridomas.

Infection of experimental animals with spotted fever group rickettsiae has been shown to lead to a suppression of cellular immune response that extends to both rickettsial and unrelated antigens (19). It is possible that the development of antigen-specific suppressor cells influenced the in vitro responses in this study. This suggestion is strengthened by the finding that lymph node lymphocytes obtained from mice infected with *R. akari* responded relatively poorly to the *R. akari* antigen and more vigorously to the other antigens used (Fig. 2b). The presence of an antigen-specific suppressor cell in bulk spleen or lymph node cell cultures might also explain the appearance of responsiveness to *R. akari* antigens in cells maintained in vitro, as repeated antigen stimulation would tend to select responsive cells away from a suppressor cell population. These interesting questions are being actively pursued in this laboratory.

The patterns of responsiveness noted in lymphocytes obtained from mice immunized with *R. conorii* or *R. akari* might also be due to the presence of a major antigen on *R. conorii* that also is expressed on *R. rickettsii*, *R. sibirica*, and *R. australis* and that is recognized by immune T lymphocytes from an animal immunized by an active infection. Animals immunized with *R. conorii* may direct their major cellular immune response to this antigen that is expressed in small quantities physically or functionally absent from *R. akari*; thus, spleen or lymph cells from *R. conorii*-immune animals fail to respond to *R. akari* antigens. This antigen may be a minor component of *R. akari* and may be capable of inducing a detectable population of antigen-responsive T lymphocytes only if *R. akari* is presented to the animal in adjuvant or if the antigen mass was expanded sufficiently by rickettsial replication. In animals immunized with *R. akari*, especially in animals that are highly susceptible to *R. akari*, such as C3H/HeJ mice (2), rickettsiae may replicate to sufficient numbers to generate an immune T-cell population that responds both to this minor antigen and to the major spotted fever group antigens. Previous studies have shown that the magnitude of the immune response to *R. akari* and other spotted fever group rickettsiae depends on the immunocompetency of the host (9, 12, 25), the relative susceptibility of the host (4), and factors that allow the rickettsiae to replicate freely as evidenced by a markedly higher antibody response (4, 9, 12). It would be expected that a similar increase in the response of T cells to minor antigens would be seen in these situations. If this is a major antigen on *R. rickettsii* and *R. conorii*, these organisms would be expected to be better stimulants in vitro. The immune response to this minor antigen of *R. akari* could also be magnified by immunization of animals with adjuvant.

As would be expected, T-cell hybridomas derived from mice immune to *R. conorii* have shown that cross-reactive epitopes exist among spotted fever group rickettsiae and, to date, no hybridoma has been found that reacts with *R. akari* (D. L. Jarboe, C. S. Eisemann, and T. R. Jerrells, submitted for publication). The resolution of this complex interrelationship will require the use of monoclonal T-cell reagents as well as isolated rickettsial antigens, and these projects are currently in progress.

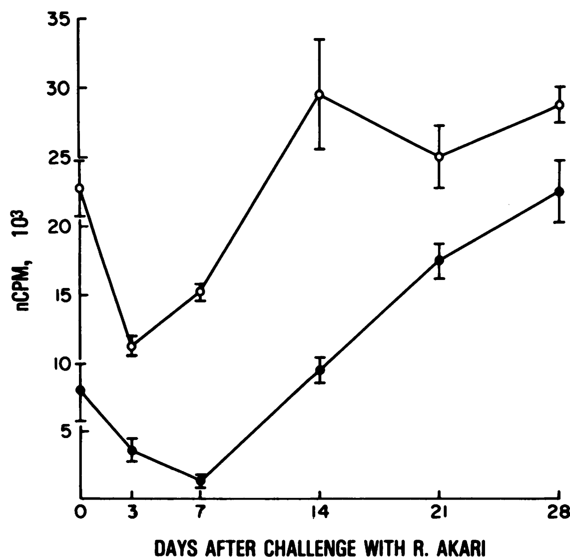

FIG. 4. Development of antigen-specific lymphocyte-proliferative responses in C3H/HeJ mice immune to *R. conorii* after challenge with 10^5 PFU of *R. akari*. Each point represents the mean net counts per minute ± 1 standard deviation of quadruplicate spleen cell cultures stimulated with 100 μg of *R. conorii* (○) or *R. akari* (●) per ml.

ACKNOWLEDGMENTS

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