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Demonstration and Partial Characterization of Antigens of *Rickettsia rhipicephali* That Induce Cross-Reactive Cellular and Humoral Immune Responses to *Rickettsia rickettsii*

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The relatively unrelated spotted fever group rickettsia *Rickettsia rhipicephali* conferred on guinea pigs protective immunity against challenge with virulent *R. rickettsii*. Immunity was conferred at all doses of *R. rhipicephali* used in the study. Because of the serologic unrelatedness of these two rickettsiae, determined by the use of microimmunofluorescence and other serological assays, further studies were performed to define the nature of the immune response elicited by *R. rhipicephali* and the characteristics of the rickettsial antigens that evoke cross-reactive antibody responses. Animals immune to *R. rhipicephali* tested at the time of challenge showed a complete cross-reactive lymphocyte proliferative response to rickettsial antigens prepared from each species. In fact, spleen cells from *R. rhipicephali*-immune animals responded better to *R. rickettsii* antigens than to homologous immunizing antigens. Serum samples were obtained from *R. rhipicephali*-infected animals at various times after infection and tested by the use of Western immunoblot assay for antibodies that were cross-reactive with antigens of *R. rickettsii*. By 10 days after infection with *R. rhipicephali*, antibodies to antigens of both species were noted, and by 37 days after infection, sera from immune animals showed strong reactivity to antigens of *R. rhipicephali* with apparent molecular masses of 107 and 151 kDa. The cross-reactive antibody response to antigens of *R. rickettsii* was relatively strong and involved predominantly the rOmpB protein and the rickettsial lipopolysaccharide. These findings establish the presence of T-cell-dependent epitopes associated with antigens of *R. rhipicephali*, which confer protective immunity against challenge with *R. rickettsii*. Results of Western immunoblot assays support the contention that the *R. rickettsii* rOmpB surface antigen contains important protective epitopes.

The spotted fever group (SFG) of the genus *Rickettsia* consists of a number of species. Some, including *Rickettsia rickettsii*, are recognized human pathogens; others have been referred to collectively as nonpathogenic SFG rickettsiae. The latter organisms have been isolated almost exclusively from ticks and are not considered to be agents of human disease (8, 34, 39). These nonpathogenic rickettsiae presumably are maintained in nature almost exclusively by transovarial and transstadial transmission in ticks, and horizontal transmission, involving infection of ticks by feeding on rickettsemic mammals, is of minor importance. Laboratory study results, however, have demonstrated that at least some of these nonpathogenic rickettsiae can infect various mammals and that infected animals often become ill. For example, in guinea pigs and voles infected with the nonpathogenic rickettsia *R. rhipicephali* typical symptoms of rickettsial infection develop (9). The resulting disease, however, is generally less severe and of shorter duration than that associated with infection with *R. rickettsii*.

An interesting finding in animal susceptibility studies has been that guinea pigs infected with nonpathogenic SFG rickettsiae are at least partially protected against challenge by pathogenic rickettsiae (9, 17). This cross-protective response has not been well characterized and deserves further investigation because identification and characterization of the protective antigens that are shared between nonpathogenic and pathogenic SFG rickettsiae might provide valuable insight into the mechanisms of rickettsial immunity, as well as information useful for the development of an effective subunit vaccine (16).

Previous investigations of rickettsial immunity have documented the primary importance of cell-mediated immunity for successful clearance of rickettsiae from an infected host. These conclusions are based on a number of study findings demonstrating that passive transfer of T cells from an immune host will confer protection to a naive animal (11, 25, 28, 36). The mechanisms involved in immunity to rickettsial infections are supported by those demonstrated with assays of cell-mediated immunity, such as delayed-type hypersensitivity or proliferation of immune T cells in the presence of a specific antigen (6, 13, 22–24, 26). Evidence for the secondary importance of humoral immunity is suggested by the inability to protect susceptible animals from rickettsial infection by passive transfer of immune sera (20, 25). Although passive transfer of immune sera fails to provide complete protection from rickettsial infection, it may reduce the severity of disease and aid in the clearance of rickettsiae from the host (20, 33, 36). Study results obtained with athymic mice infected with the SFG rickettsia *R. akari* also suggest that cell-mediated immunity is critical for immunity to rickettsial infection (21, 27, 28). Although athymic mice produce significant quantities of rickettsia-specific T-cell-independent antibody after infection with *R. akari*, they nonetheless die of fulminating rickettsial disease.

Pretreatment of rickettsiae with antibodies, especially antibodies to surface proteins, apparently neutralizes infec-

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tivity for animals (1, 3). On the basis of this finding, it has been suggested that antibodies to these surface epitopes play an important effector role. It has not been established, however, whether these antibodies are produced in vivo in sufficient quantities to produce the same effects.

The existing body of evidence supports the idea that antigen-responsive T cells, especially those of the helper T-cell subset, play a critical role in antirickettsial immunity through cellular effector mechanisms and, perhaps, production of T-cell-dependent antibodies.

The purpose of our study was to investigate the development of humoral and cell-mediated immune responses in guinea pigs after infection with *R. rhipicephali*. We chose to study *R. rhipicephali* because of the distinct differences between this species and *R. rickettsii* determined by using serological assays, such as complement fixation, microimmunofluorescence, and microagglutination, although it should be noted that sufficient cross-reactivity between these two species exists for both to be placed in the SFG (9). The humoral immune response was analyzed by using the Western immunoblot assay to determine which antigens of *R. rickettsii* react with antibodies from animals previously infected with *R. rhipicephali* and when after infection these cross-reactive antibodies appear. Cross-reactive cell-mediated immune responses of guinea pigs infected with *R. rhipicephali* were analyzed by using lymphocyte proliferation assays. The results of these studies show that both cross-reactive antibodies and T-cell responses result from infection with *R. rhipicephali*, and these responses are associated with protective immunity to challenge with the pathogenic species *R. rickettsii*.

(This report was presented in part at the 38th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Honolulu, Hawaii, December 1989, abstract 226.)

MATERIALS AND METHODS

Animals. Male Hartley guinea pigs were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) and weighed 700 to 800 g at the start of the study.

Cultivation, titration, and purification of rickettsial strains. *R. rickettsii* (Sheila Smith strain) was cultivated in the yolk sacs of 5-day-old, specific-pathogen-free, embryonated chicken eggs (SPAFAS, Norwich, Conn.) as previously described (15). *R. rhipicephali* (3,7-♀6 strain) was grown in Vero cells. Aliquots of the resulting tissue culture of yolk sac harvests were maintained at −70°C in sucrose-phosphate-glutamate buffer (pH 7.0; 218 mM sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2HPO_4, 4.9 mM glutamate) (7). Titers for both rickettsial stocks were determined by using a Vero cell plaque assay, and the resulting values were expressed as PFU (14). Rickettsial antigens for use with the Western immunoblot assay were purified by Renografin density centrifugation (38). Heavy- and light-band rickettsiae were pooled, and the resulting suspension was centrifuged at 16,300 × *g* for 20 min. The resulting rickettsial pellets were resuspended in sucrose-phosphate-glutamate buffer, aliquoted, and stored at −70°C before use.

Rickettsial antigens used in the lymphocyte proliferation assays were partially purified by using a modification of the method of Jerrells and Osterman (23). The frozen rickettsial stocks described above were thawed at 37°C, suspended in sucrose-phosphate-glutamate buffer, thoroughly vortexed, and subjected to low-speed spinning (500 × *g*) for 10 min to remove host cell debris. Next, the supernatants were centrifuged at high speed (16,300 × *g*) for 20 min to remove rickettsiae from the suspension. The resulting pellet was resuspended in distilled water to disrupt the rickettsiae. A volume of concentrated (2×) phosphate-buffered saline equal to the amount of distilled water used to resuspend the pellets was added. The protein content of this suspension was determined by using the method of Lowry et al. (30).

Serologic determinations. The rickettsial antibody titers of guinea pig sera were determined by using the indirect immunofluorescence assay on the basis of standard methods (35). Fluorescein isothiocyanate-labeled goat anti-guinea pig immunoglobulin G was obtained from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, Md.). Antigen slides were prepared from live *R. rickettsii* and *R. rhipicephali* cell culture preparations.

Rickettsial antigen assays. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique was performed by using a modification of the method of Laemmli (29). Antigens were dissolved in Laemmli sample buffer at room temperature. Electrophoresis was conducted at constant wattage (10 W) in running buffer cooled by a refrigerated, circulating water system set at 4°C. Stacking gels contained 4% (wt/vol) acrylamide, and separating gels contained 10% (wt/vol) acrylamide. Approximately 30 μg of rickettsial protein was loaded into each of the appropriate lanes. Proteins separated by using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis technique were transferred to Immobilon polyvinylidene difluoride membranes (Millipore Corp., Bedford, Mass.). Transfers were performed for 2 h at 27 V in a cooled (4°C) reservoir containing 25 mM sodium phosphate (pH 7.4) transfer buffer (5). Immobilon membranes were removed from the blot apparatus and immediately blocked in BLOTTO (5% dried milk dissolved in 50 mM sodium phosphate–150 mM sodium chloride [pH 7.4]) phosphate-buffered saline. Next, the membranes were incubated for 1 h in the appropriate antisera diluted 1:100 in BLOTTO containing 0.5% Tween 20. After incubation, the membranes were subjected to two 30-min rinses in phosphate-buffered saline containing 0.5% Tween 20. After rinsing, membranes were placed in sealed freezer bags containing 15 ml of BLOTTO with 0.5% Tween 20 and 20 μl of ^{125}I-labeled protein A (142 μCi/ml) (New England Nuclear, DuPont, Wilmington, Del.) and incubated overnight at room temperature. After this incubation, the membranes were washed twice in phosphate-buffered saline containing 0.5% Tween 20 and dried at 37°C for 2 h. Autoradiography of the membranes was performed at −70°C in stainless steel exposure cassettes (Sigma Chemical Co., St. Louis, Mo.) by use of X-OMAT AR X-ray film (Kodak, Rochester, N.Y.) and a Cronex Lightning-Plus intensifying screen.

Lymphocyte proliferation assay. Lymphocytes were obtained from the spleens of guinea pigs, and single-cell suspensions were prepared as previously described (24). The resulting suspension of spleen cells was removed from the petri dish, rinsed twice in Hanks' balanced salt solution, counted, and then suspended at the proper cell density in RPMI 1640 containing 5% fetal calf serum, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1 mM L-glutamine (GIBCO, Grand Island, N.Y.), 50 mg of gentamicin per ml, and 5×10^{-5} M 2-mercaptoethanol. This cell suspension was aliquoted into 96-well plates (2.5×10^5 cells per well). Antigen dilutions ranged from 250 to 1 μg/ml, and each concentration was tested in triplicate. The plates were incubated for 72 h in a 37°C incubator with a humidified 5% CO_2 atmosphere. After incubation, 1 μCi of tritiated thymidine was added to each well.

Experimental design. All guinea pigs were seronegative at the beginning of the study, with antibody titers of <1:8 (cutoff for a positive titer, ≥1:32). Animals were divided into four groups: a control group (eight animals) that was not inoculated with *R. rhipicephali* and three experimental groups (seven animals per group) that differed only in the dose of *R. rhipicephali* that was administered by intraperitoneal inoculation. Animals in the low-dose group were infected with 7.5×10^2 PFU of *R. rhipicephali*. Those in the medium- and high-dose groups were infected with 7.5×10^4 and 7.5×10^6 PFU, respectively. Rectal temperatures of all animals were monitored daily by using a telethermometer, and animals were considered febrile if the rectal temperature was 39.8°C or higher. Animals also were observed daily for development of scrotal swelling. Blood was collected by cardiac puncture on days 0, 10, 21, 37, 48, and 67 to obtain sera for indirect immunofluorescence and Western immunoblot assays. On day 37, four of seven animals from each of the experimental groups and three of eight from the control group were challenged with 1.0×10^3 PFU of *R. rickettsii*. Two of eight animals in the control group were used as uninfected controls. These animals were observed daily for signs of illness, including fever and scrotal swelling. In addition to the animals mentioned above, three from each of the experimental groups and the control group were killed on day 37, and the spleens of two animals from each of these groups were used to provide spleen cells for the lymphocyte proliferation assay described earlier. These experiments were repeated completely as described above, and aspects were repeated a minimum of three times.

RESULTS

Antibody titers of animals infected with *R. rhipicephali* varied in a time- and dose-dependent manner (Fig. 1B to D). Kruskal-Wallis one-way analyses of variance were used to make statistical comparisons between titers from different dosage groups and time periods, as well as to compare titers of antibodies against homologous and heterologous antigens within groups, and P values of <0.05 were considered to denote significant differences. All animals in the medium- and the high-dose groups had demonstrable antibody titers of >1:32 by day 10, with the highest titers recorded in the high-dose group. In this representative experiment, six of seven guinea pigs infected with the low dose of *R. rhipicephali* also had high titers (>1:32) by day 10, but the titers were noticeably lower than those in the other two groups. Animals infected with the highest dosage of *R. rhipicephali* had significantly higher anti-*R. rhipicephali* and anti-*R. rickettsii* titers at this time than did animals in the low-dose (P = 0.0013 and P = 0.0019, respectively) or medium-dose (P = 0.0100 and P = 0.0191, respectively) group. The single seronegative animal in the low-dose group became seropositive later (day 21). In each of the three dosage groups, titers for day 10 antisera against the homologous *R. rhipicephali* antigen were not significantly higher than those against the heterologous *R. rickettsii* antigen. By day 21, titers against both rickettsial antigens had risen sharply. Unlike the day 10 results, however, for each serum sample tested, the antibody titers against *R. rhipicephali* antigen were two- to fourfold higher than those against *R. rickettsii* antigen ($P \leq 0.05$). There was also a positive correlation between the antibody titer and the dose of *R. rhipicephali*. Differences between immunofluorescent antibody titers to *R. rhipicephali* and *R. rickettsii* were less on day 37 than on day 21, but the titers to the homologous antigen remained higher than those to the heterologous antigen. Antibody titers against *R. rhipicephali* increased only slightly between days 21 and 37 in the low-dose group and actually decreased somewhat in the medium- and high-dose groups. The titers did not change to any great extent after day 37 and remained relatively stable up to 90 days (data not shown).

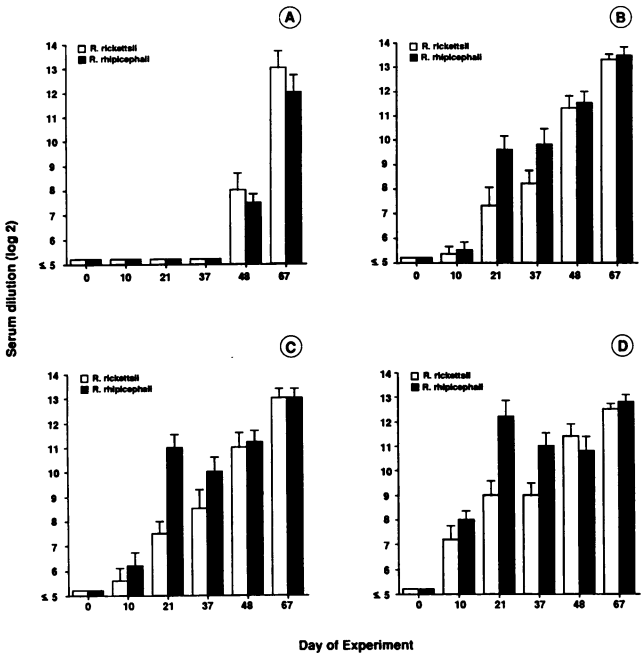

FIG. 1. Geometric mean titers of antibodies to *R. rhipicephali* and *R. rickettsii* in guinea pig sera. (A) Geometric mean titers, as determined by using an indirect immunofluorescence assay, for surviving animals in the control group challenged with *R. rickettsii* (n = 2). (B, C, and D) Geometric mean titers for animals in low-, medium-, and high-dose groups, respectively (n = 7 up to and including day 37; n = 4 on days 48 and 67).

There apparently was no relationship between the inoculating dose of *R. rhipicephali* and the presence of fever in animals. Fever (39.8°C or higher rectal temperature) developed in six of seven animals in both the low- and high-dose groups. Fevers persisted for 1 to 3 days in the high-dose group, compared with 1 to 2 days in the low-dose group. Only two of seven animals in the medium-dose group became febrile, one for 2 days and one for a single day. There was, however, a correlation between the dose of *R. rhipicephali* and the number of days postinoculation that animals in each group first became febrile. In the high-dose group, four of six animals that became febrile had fever within 2 days after infection. In the remaining two animals, fever developed by day 3. In the medium-dose group, both animals that had fever became febrile on day 3. In the low-dose group, only two of six animals that experienced fever were febrile by day 4 and the remaining four animals did not exhibit fever until day 5.

As described earlier, on day 37 four animals from each of the experimental groups and three from the control group (no prior infection with *R. rhipicephali*) were inoculated with 1.0×10^3 PFU of *R. rickettsii*. Immunofluorescent antibody titers of sera taken 11 days after challenge with *R. rickettsii* showed that animals from each of the three dosage groups produced an anamnestic antibody response to the cross-reactive antigens of *R. rickettsii* and *R. rhipicephali*. This

response was especially noticeable in the high-dose group, in which there was little or no increase in titers of antibodies to *R. rickettsii* between days 21 and 37 (Fig. 1D).

The three animals in the control group that were infected with *R. rickettsii* on day 37 showed typical signs of rickettsial illness, with fever and scrotal swelling. One of them was febrile for 4 days before death 7 days after inoculation. The remaining two animals had fever for 5 and 8 days, respectively, after which times they recovered from infection. The latter two animals had low but significant antibody titers when tested 11 days after infection with *R. rickettsii* (Fig. 1A). A less than twofold difference in titers against *R. rickettsii* and *R. rhipicephali* existed when sera from these animals were tested.

The responses of animals in each of the three dosage groups to challenge with *R. rickettsii* differed from those of animals in the control group with respect to appearance of fever or scrotal swelling. None of the animals in the high-dose group experienced fever when challenged with *R. rickettsii*, and only two of four animals from both the low- and medium-dose groups were febrile. The fevers observed in the latter animals lasted only 1 to 2 days, and the highest observed temperatures were lower than those recorded for the naive control animals. Scrotal swelling was not apparent in any of the animals from the three experimental groups, but it was pronounced in *R. rickettsii*-infected animals in the control group.

Spleen cells taken from guinea pigs killed on day 37 were analyzed by using a lymphocyte proliferation assay to determine the relative abilities of *R. rhipicephali* and *R. rickettsii* antigens to serve as sources for stimulating proliferation of *R. rhipicephali*-immune T cells. The spleens were collected on the same day (day 37) on which the remaining guinea pigs were challenged with *R. rickettsii*, which, we hoped, would provide a measure of the cell-mediated immune status of the animals at the time of challenge. The most interesting result of this assay was that lymphocytes from animals previously infected with *R. rhipicephali* proliferated to a much greater extent when stimulated with heterologous *R. rickettsii* antigen than when tested with homologous *R. rhipicephali* antigen (Fig. 2). This heteroclitic response was observed for all dose groups. There was no detectable effect of the dose of *R. rhipicephali* used initially to infect the guinea pigs on the proliferation of spleen cells from these animals. Control animals showed no proliferative response to either antigen.

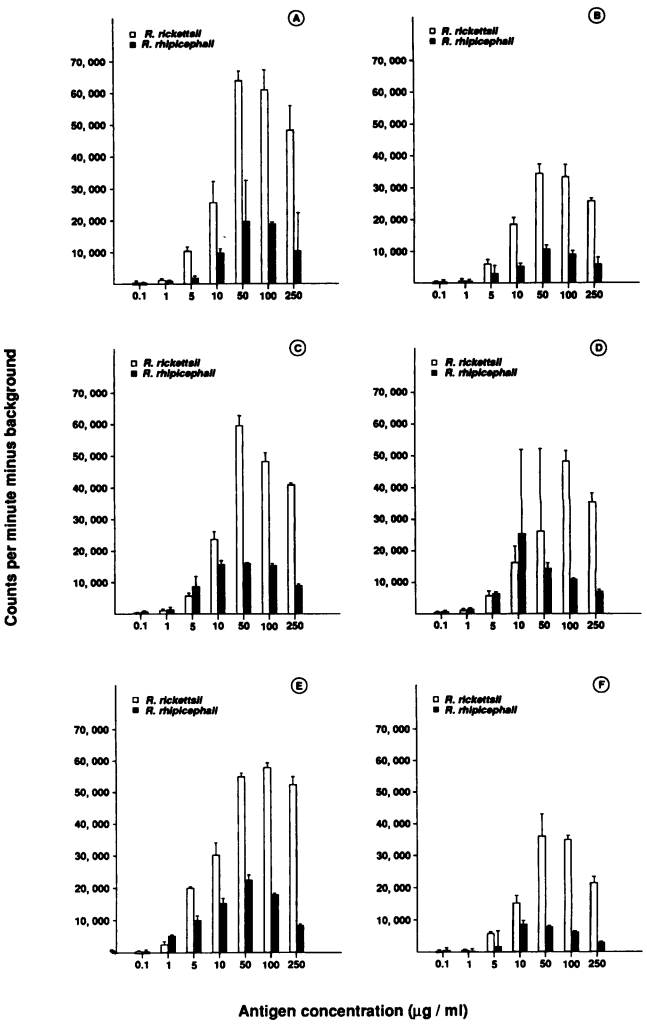

FIG. 2. Development of T-cell responsiveness after infection of guinea pigs with *R. rhipicephali*. A heterologous (*R. rickettsii*) or homologous (*R. rhipicephali*) antigen was used to stimulate proliferation of splenic lymphocytes harvested from guinea pigs that received low (A and B), medium (C and D), and high (E and F) doses of *R. rhipicephali*. Samples were run in triplicate, and standard error bars are given for the values obtained at the different antigen concentrations.

Western immunoblots of guinea pig sera taken just before challenge on day 37 indicated a clear effect of the size of the *R. rhipicephali* inoculum on the reactivity of sera with both *R. rhipicephali* and *R. rickettsii* antigens (Fig. 3). Serum samples did not react by immunoblotting with components of uninfected host cells (Vero cells or yolk sacs) (data not shown). Sera from animals in the low- and medium-dose groups reacted less intensely than did sera from animals in the high-dose group (Fig. 3). The distinctive ladder-like banding pattern at the bottom of the immunoblots was due to rickettsial lipopolysaccharide (LPS) (1). Strong cross-reactivity between the LPS of *R. rhipicephali* and that of *R. rickettsii* was noted for antisera collected from *R. rhipicephali*-immune guinea pigs in each of the three dosage groups. A number of higher-molecular-weight antigens also were visible in the *R. rhipicephali* antigen lanes (Fig. 3, lanes B, D, F, H, and J). The most prominent of these had an apparent molecular mass of 107 kDa (Fig. 3A, lane J). We also noted a high-molecular-mass (125-kDa) cross-reactive antigen in all but one of the *R. rickettsii* antigen lanes probed with day 37 antisera (Fig. 3B, lane I). The only serum sample that failed to react to this antigen was from a guinea pig in the low-dose group (Fig. 3, lane A). Reactivity to this *R. rickettsii* antigen increased with the dose of *R. rhipicephali* present in the original inoculum. This antigen has been referred to conventionally as the 120-kDa antigen, but the molecular mass has been reported to vary considerably, depending on a number of factors discussed elsewhere (12). Recently, the name rOmpB has been proposed to avoid the confusion resulting from the variations in molecular weight reported for this antigen (18, 19). To remain consistent with other reports, we will refer to this protein as the rOmpB antigen of *R. rickettsii* for the remainder of this report.

Western immunoblot analyses of sera obtained throughout the study showed the appearance of antibodies specific for a number of rickettsial antigens. Figure 4 shows a Western immunoblot of a series of sera taken from a guinea pig in the high-dose group. All antigen strips in the figure were processed simultaneously and differed only in the rickettsial

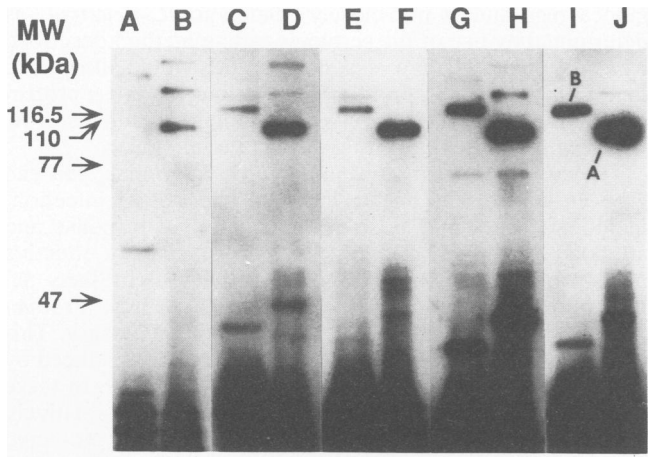

FIG. 3. Effect of an immunizing dose of *R. rhipicephali* on immunoreactivity of antisera in Western immunoblots. Lanes: A, C, E, G, and I, *R. rickettsii* antigen; B, D, F, H, and J, *R. rhipicephali* antigen. Antigen strips A-B and C-D were probed with sera from guinea pigs in the low-dose group. Antigen strip E-F was probed with serum from a guinea pig in the medium-dose group. Antigen strips G-H and I-J were probed with sera from guinea pigs in the high-dose group. All sera were diluted 1:100. Antigen strips were processed simultaneously and exposed to the same sheet of X-ray film for autoradiography. MW, molecular mass.

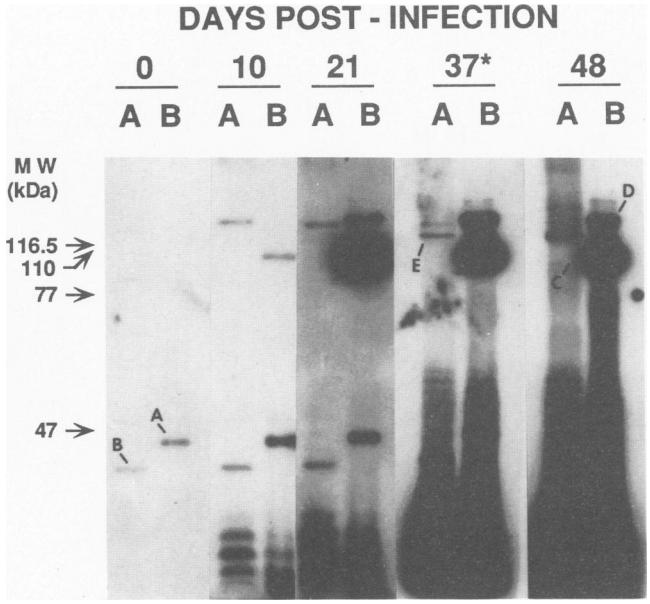

FIG. 4. Development of cross-reactive antibodies after infection of guinea pigs with *R. rhipicephali*. Serum from a guinea pig in the high-dose group was obtained and tested at different times before (day 0) and after (days 10, 21, 37, and 48) inoculation. Lanes: A, *R. rickettsii* antigen; B, *R. rhipicephali* antigen. Anti-*R. rhipicephali* immunofluorescence titers for this animal on days 0, 10, 21, 37, and 48 were <1:32, 1:256, 1:8,192, 1:4,096, and 1:4,096, respectively. Anti-*R. rickettsii* immunofluorescence titers on the same days were <1:32, 1:128, 1:512, 1:1,024, and 1:4,096, respectively. MW, molecular mass.

antisera applied to each. After the strips were incubated in antisera and ^{125}I-labeled protein, they were placed in a film cassette and exposed to the same piece of film for the same amount of time. The intensity of the bands observed on this immunoblot correlated reasonably well with the immunofluorescent antibody titers for the same animal on any given day (Fig. 4, legend). Long exposures, such as those shown in Fig. 4, resulted in slight reactivity of the prebleed sera to a low-molecular-mass band in both the *R. rhipicephali* (46 kDa; band A in lane B) and *R. rickettsii* (44 kDa; band B in lane A) lanes, and the reactivity to these bands increased with time. This reactivity could be due to cross-reactivity of the rickettsial antigen with a similar antigen on some bacterial species or other infectious agent to which the guinea pigs had been exposed.

The immunoblot in Fig. 4 shows that within 10 days after inoculation with *R. rhipicephali*, recognizable rickettsia-specific antibodies were present in the serum of this guinea pig. These early-response antibodies were directed primarily toward the LPS of *R. rhipicephali*, but a high-molecular-mass antigen (107 kDa; band C in lane B) of *R. rhipicephali* was recognized by day 10. Reactivity to LPS continued to increase from day 10 until the last day on which samples were taken (day 48). Recognition of the most reactive high-molecular-mass (107-kDa) antigen of *R. rhipicephali* peaked on day 21 and then decreased somewhat by days 37 and 48. An additional high-molecular-mass (154-kDa; Fig. 4, band D in lane B) antigen of *R. rhipicephali* became apparent by day 21 and was strongly recognized by day 37. Another antigen of approximately 123 kDa first appeared in immunoblots of day 37 sera. This band, which appeared just above and merged with the staining for the 107-kDa band, was clearly discernible on the original X-ray film but was less distinct in the photographic reproduction of this autoradiograph.

The guinea pig whose serum was used for Fig. 4 had cross-reactive antibodies to antigens of *R. rickettsii* within 10 days after inoculation. Most of the cross-reactivity was directed toward the LPS. This anti-LPS reactivity continued to increase throughout the study. Cross-reactive, higher-molecular-weight antigens of *R. rickettsii* also were visible as early as day 10. The most significant of these was the rOmpB antigen. This antigen was almost imperceptible on day 21 but was quite apparent on day 37 (Fig. 4, band E in lane A for days 21 and 37). When the guinea pig whose serum was used in Fig. 3 was challenged with *R. rickettsii* on day 37, it underwent an anamnestic response to the cross-reactive antigens of *R. rhipicephali* and *R. rickettsii* (Fig. 4, day 48, lanes A and B). Although most of this anamnestic response appeared to be directed against the LPS, there was a significant increase in reactivity to the rOmpB *R. rickettsii* antigen.

Cross-reactivity between *R. rickettsii* and *R. rhipicephali* also was demonstrated by Western immunoblot analysis of serum from a control guinea pig that had not been infected with *R. rhipicephali* during the first part of the experiment but was infected with *R. rickettsii* on day 37 and bled 30 days later (day 67). Serum from this guinea pig reacted strongly to three high-molecular-weight antigens of *R. rickettsii* (Fig. 5, lane A). The antigens recognized included the previously mentioned rOmpB (band A) antigen, as well as two antigens of slightly higher molecular masses (142 [band B] and 163 [band C] kDa). In all likelihood, the heavier of these two antigens (163 kDa) is the same as that observed by other investigators. Like the rOmpB antigen of *R. rickettsii*, the molecular masses reported for this larger antigen vary considerably from laboratory to laboratory, and it has been given numerous designations in the literature, including the

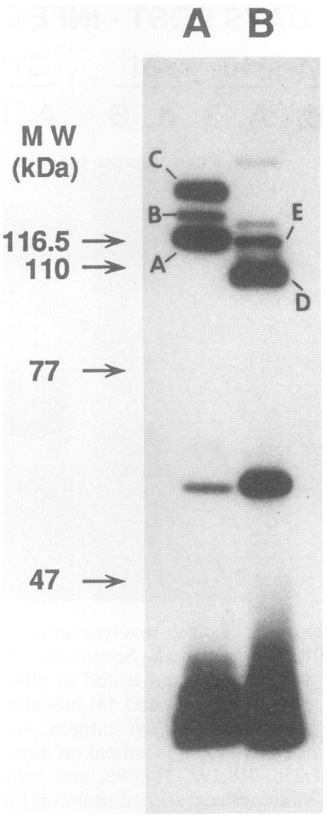

FIG. 5. Appearance of cross-reactive antibodies in the serum of an animal in the control group infected with *R. rickettsii* (no prior infection with *R. rhipicephali*). Lanes: A, *R. rickettsii* antigen; B, *R. rhipicephali* antigen. Immunofluorescence titers for this animal were 1:4,096 against *R. rickettsii* and 1:2,048 against *R. rhipicephali*. MW, molecular mass.

155-kDa antigen, the 190-kDa antigen, and rOmpA of *R. rickettsii* (2, 4, 18, 19). We will refer to this protein as the rOmpA antigen of *R. rickettsii* throughout the remainder of this report. As with the *R. rhipicephali*-immune animals, much of the cross-reactivity between the two rickettsial antigens appeared to be directed against the LPS, but higher-molecular-weight antigens of *R. rhipicephali* also were recognized by serum from the guinea pig whose serum was used for Fig. 5. Interestingly, this serum sample reacted with similar intensity to the LPS and higher-molecular-weight antigens of both *R. rickettsii* and *R. rhipicephali*. This result agrees well with the observed immunofluorescent antibody titers for this animal (Fig. 5, legend). The high-molecular-weight antigens of *R. rhipicephali* that were recognized by serum from this *R. rickettsii*-immune guinea pig appear to be the same as those observed in Western immunoblots of sera from the *R. rhipicephali*-immune animals discussed previously (Fig. 3 and 4). This included reactivity to the 107- and the 123-kDa antigens. No cross-reactivity with the 154-kDa antigen of *R. rhipicephali* was observed.

DISCUSSION

The experiments described in this report were designed to explore the development of immunity to the pathogenic SFG organism *R. rickettsii* after exposure of animals to *R. rhipicephali*. Data in this report confirm the findings of Burgdorfer et al. (9); that is, *R. rhipicephali* is of low virulence for guinea pigs and is not closely related to *R. rickettsii*, as determined by use of the serologic typing method described by Philip et al. (34). We reasoned that this would allow us to define the rickettsial proteins responsible for cross-protection and thus, by inference, the predominant proteins of *R. rickettsii* involved in eliciting protective immunity.

The current study findings show that *R. rhipicephali* can grow in guinea pigs on the basis of evidence of infection, including a challenge dose-dependent febrile response and antibody responses. The development of a highly effective cross-protective immune response in this system likely depends on proliferation of *R. rhipicephali* in the host. Production of clinical illness, however, may not be necessary. This point is made apparent by the level of protection induced by the lowest dose of *R. rhipicephali* (10^3 PFU) used. In these experiments, this dose of rickettsiae produced a relatively short duration of fever and the animals were protected against rechallenge.

A relatively large body of evidence supports the idea that immunity to members of the genus *Rickettsia* critically depends on the development of a population of antigen-responsive T lymphocytes (6, 10, 11, 13, 21–28). Of importance was the finding that in animals immunized with *R. rhipicephali*, the response of lymphocytes to *R. rickettsii* was as good as or better than that to the immunizing antigen. A similar finding has been reported with the use of a murine model of SFG immunity, including the finding of a markedly better response of spleen lymphocytes to heterologous antigens (22). It is not known why such a heteroclitic response occurs, but it may be due to a number of factors, including (i) greater expression of the putative T-cell epitope on a protein of the heterologous rickettsia, (ii) enhanced antigen presentation because of a better ability to associate with the class II major histocompatibility complex, or perhaps (iii) degradation of the rickettsial proteins by the antigen-presenting cell to create the epitope peptide fragments. Regardless, this phenomenon seems to be a general finding in at least two systems and seems worthy of further investigation.

Although it is difficult to quantify proliferation of lymphocytes in response to complex antigens, it is clear from the data presented here that these two members of the SFG, relatively unrelated in terms of antibody cross-reactivity, share similar levels of cross-reactive T-cell epitopes. It is tempting to conclude that this is the basis for the noted cross-protection. Although this is a likely possibility, further studies are required to confirm this conclusion.

Animals can be protected against rickettsial infection by use of cloned antigens obtained from *R. rickettsii* (31, 32) and *R. conorii* (37). In both cases, the antigens used for immunization were the higher-molecular-mass (155 kDa in *R. rickettsii* and 135 kDa in *R. conorii*) immunodominant proteins. Although preliminary data suggest that these proteins contain epitopes recognized by antigen-specific T lymphocytes (22a), the immune mechanisms involved in development of resistance to infection after administration of these recombinant antigens have not been defined.

To begin the initial characterization of the cross-reactive antigen on *R. rhipicephali* and *R. rickettsii*, we used sera from *R. rhipicephali*-immune animals for Western immunoblot analysis of *R. rickettsii* proteins. Our rationale for this approach was to identify the proteins that elicit a T-cell-dependent antibody response and thus proteins that stimulate production of antigen-responsive helper T cells. To increase the likelihood of producing T-cell-dependent antibodies, serum samples were examined relatively late after infection (day 37) and after challenge with *R. rickettsii*.

Surprisingly, one of the predominant cross-reactive antigens was the rickettsial LPS. On the basis of previous work (1, 21, 27, 28), it is unlikely that the antibodies to the LPS are indicative of protective immunity. The strong response to LPS relatively late in the infection might be due to the presence of a chronic infection. More work is required with this system to address this question.

The findings we present support the notion that the rOmpB antigen is important in protective immunity to *R. rickettsii*. We assume that the antigen we describe is the same one noted by other investigators, who have reported slightly different molecular weights. It has been referred to in the literature as the 120-kDa antigen of *R. rickettsii*, although the molecular mass appears to vary considerably, depending on a number of factors discussed elsewhere (12). It is also apparent from the studies of McDonald et al. (31, 32) and Vishwanath et al. (37) that immunization with the higher-molecular-weight protein of *R. rickettsii* or *R. conorii* (rOmpA) also induces protective immunity. On the basis of results of the present study, we predict that the rOmpB antigen would produce stronger immunity. Although it remains to be shown that this antigen is responsible for the lymphocyte proliferative responses noted in our study, it seems likely that this protein is a major factor in stimulation of antigen-responsive helper T lymphocytes. We are currently performing experiments to address this possibility. A high-molecular-weight antigen of the typhus group of rickettsiae has also been suggested to be of importance in the generation of antigen-responsive T cells (10).

In summary, we have described a model system of cross-protective immunity by using two members of the SFG rickettsiae that share relatively few protein antigens that stimulate antibody production. The results of this initial study lead us to suggest that the activity of sensitized T cells plays a major role in protective immunity.

This model system should provide a convenient mechanism to identify antigenic epitopes important for production of the appropriate effector T-cell population(s).

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