Murine Antibody Responses Distinguish *Rochalimaea henselae* from *Rochalimaea quintana*

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*Rochalimaea henselae* causes persistent bacteremia, bacillary angiomatosis, and parenchymal bacillary peliosis. Detection of a specific antibody response to *R. henselae* infection may represent an alternative to cultivation as a means of diagnosis. We assessed the specificity of induced murine antibodies for antigens from *R. henselae* and the closely related species *R. quintana*. Groups of CD-1 mice were inoculated with whole organisms of six strains of *R. henselae* and two of *R. quintana*. Pre- and postinoculation blood specimens were collected. Enzyme immunoassay assays were performed by using as antigens preparations of immunogenic proteins from one isolate of *R. henselae* or from the *R. quintana* type strain. These demonstrated high specificity of *R. henselae*-induced antibodies for proteins of *R. henselae* and of *R. quintana*-induced antibodies for proteins of *R. quintana*. Protein preparations extracted from all of the strains were separated electrophoretically. After their transfer to membranes, immunoblots were performed by using 1:1,000 dilutions of all of the pre- and postinoculation sera in combination with proteins from all of the strains. Preinoculation sera had minimal reactivity. All of the *R. henselae*-induced immune sera reacted with numerous proteins of all of the *R. henselae* strains but cross-reacted minimally with proteins of *R. quintana*. Immune sera from *R. quintana*-inoculated mice had similar species specificity. An immunofluorescence assay was developed by using antiserum to one strain of *R. henselae*. A 1:1,000 dilution yielded fluorescence with all strains of *R. henselae* but with none of *R. quintana*, *Bartonella bacilliformis*, or *Afipia felis*. *Acinetobacter calcoaceticus* subspp. *anitratus* was also unreactive with a dilution of 1:500. A 1:10 dilution yielded weak fluorescence with *R. quintana* but none with *Staphylococcus epidermidis*.

*Rochalimaea henselae*, a recently identified fastidious gram-negative bacillus, can cause prolonged fever associated with persistent bacteremia in immunocompromised and immunocompetent persons (28). On the basis of cellular fatty acid content, Slater et al. (28) found this organism to be most similar to the rickettsial pathogen *R. quintana*, the etiologic agent of trench fever (30). It differed from *R. quintana* in its restriction endonuclease digestion pattern of DNA and in its composition of comparably prepared protein extracts. This pathogen has since been recognized as the cause of bacillary angiomatosis and bacillary peliosis hepatitis (25, 29).

An alternative to culture as the basis of diagnosis is needed because *R. henselae* is fastidious and therefore difficult to cultivate. The measurement of host antibody response to infection, as is commonly performed with other rickettsial infections (3, 9, 10, 14), represents one such possibility. A serum specimen obtained from one patient during his convalescence from persistent bacteremia yielded lines of identity between his own and other isolates of *R. henselae* by the immunodiffusion technique (28). This serum was not reactive with *R. quintana*. However, sera from several other patients recovering from bacteremia due to *R. henselae* have not contained such immunoprecipitating antibody activity. We performed studies of mice after inoculation with six strains of *R. henselae* and two strains of *R. quintana* to further detail the extent and specificity of antibody response as a foundation for further studies of human serologic response.

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*MATERIALS AND METHODS*

*Microorganisms.* Six strains of *R. henselae* were used. All had been isolated from the blood of bacteremic patients and identified as previously described (28, 31). Two strains of *R. quintana* were used, i.e., the Fuller type strain (VR358; American Type Culture Collection [ATCC], Rockville, Md.) and a clinical isolate from our laboratory confirmed to be *R. quintana* by DNA hybridization studies (31).

*Animals.* Adult female CD-1 mice (TACSO, Omaha, Nebr.) were selected for immunization on the basis of preliminary studies which had indicated that such animals lack naturally occurring specific antibodies to *R. henselae* and *R. quintana* and also that they tolerate intraperitoneal inoculation of large numbers of viable organisms of either species without apparent morbidity. Eight groups of six mice were inoculated. Immediately prior to the first inoculation, 0.5 ml of blood was obtained from each animal by cardiocentesis after anesthesia. The serum was stored at −75°C until use.

*Production of antiserum.* Each strain was cultivated on five chocolate agar plates at 37°C in 5% CO₂ for a week to yield confluent growth and then suspended in 2 ml of phosphate-buffered saline (PBS) for inoculation. Serial dilutions were plated on agar to calculate inoculum size. Mice received three weekly intraperitoneal inoculations of 0.2 ml each without evidence of morbidity except as directly related to cardiocentesis. A week after the last inoculation, animals were subjected to euthanasia by exsanguination after anesthesia. Sera derived from blood specimens were stored at −75°C until use. For comparison with unabsorbed pre- and postimmunization sera, small aliquots of all of the postim-

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munization sera from *R. henselae*-immunized mice were absorbed with whole *R. quintana* organisms of the ATCC type strain. Likewise, aliquots of all of the postimmunization sera from *R. quintana*-immunized mice were absorbed with whole cells of *R. henselae* 87-66 (deposited with the ATCC as no. 49793).

**Enzyme immuno sorbent assay (EIA).** As has been done in earlier studies (19, 28, 29), proteins were extracted from *R. henselae* 87-66 and from the *R. quintana* type strain with methods originally applied to the isolation of outer membrane proteins of *Haemophilus influenzae* (22). Aliquots of protein preparations (1 μg in 100 μl of carbonate-bicarbonate coating buffer [pH 9.6]) were applied to the flat-bottom wells of microtiter plates. After overnight incubation, the wells were rinsed twice with PBS–0.1% Tween 20 and then blocked with PBS–2% Tween 20 during incubation for 1 h at 37°C. After two further washes, 50-μl aliquots of murine sera were applied in 10-fold dilutions. Sera from animals inoculated with *R. henselae* were tested in dilutions of 10⁻² to 10⁻⁴ against *R. henselae* proteins and in dilutions of 10⁻² to 10⁻⁴ against *R. quintana* proteins. Likewise, sera from animals inoculated with *R. quintana* were tested in dilutions of 10⁻² to 10⁻⁴ against *R. quintana* proteins and in dilutions of 10⁻² to 10⁻⁴ against *R. henselae* proteins. Each antigen-sorbent combination was performed in duplicate. After incubation overnight at 4°C, wells were washed four times and then incubated with 50-μl volumes of a 1:3,000 dilution of goat anti-mouse immunoglobulin G (Fc specific)-alkaline phosphate conjugate (Sigma, St. Louis, Mo.) for 2 h at room temperature. After four additional rinses, p-nitrophenol phosphate (2 mg/ml in diethanolamine buffer; Sigma) was added as the substrate (12) and incubated for 45 min for color development before determination of optical density by using an automated reader (Dynatech). The average optical density of duplicate determinations was calculated.

**ImmunobLOTS.** Proteins from all of the *R. henselae* and *R. quintana* strains were extracted, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electrophoretically transferred to polyvinylidene difluoride immobilizing membranes (Immobilon-P; Millipore, Bedford, Mass.). Outside lanes of each gel contained colored protein molecular weight markers (Rainbow markers; Amersham, Arlington Heights, Ill.) which were also transferred to the membranes and then excised before the blocking step. Following blocking by immersion in 3% skim milk–0.05% Tween 20 in Tris-buffered saline (pH 7.5) overnight at 4°C, the membrane sections containing the *Rochalimaea* proteins were incubated with 1:1,000 dilutions of mouse sera for 1 h at room temperature. After washes, they were incubated with a rabbit anti-mouse immunoglobulin G-alkaline phosphate conjugate (Sigma) for 1 h and then developed in a solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma), as described by Dao (6), to yield color where murine antibodies had bound to protein bands. After the membranes dried, the sections containing molecular weight markers were realigned.

**Immunofluorescence studies.** Whole cells of all *R. henselae* and *R. quintana* strains, *Bartonella bacilliformis* (ATCC 35685), two strains of *Acinetobacter calcoaceticus* subsp. *anitratus* (ATCC 19606 and a clinical isolate from a patient with meningitis), *Staphylococcus aureus*, and *S. epidermidis* were harvested from agar cultures, formalin fixed, washed in PBS, and adjusted to a density of approximately 10⁶ CFU/ml. One-microliter aliquots were air dried in wells of glass slides. Similarly prepared slides of *Afipia felis* (the cat scratch disease bacillus [2]) strains BV and GT were obtained from the Armed Forces Institute of Pathology. To these wells were added postimmunization sera from mice inoculated with *R. henselae* 87-66. After 30 min of incubation at 37°C in a moist chamber and two 5-min PBS washes, goat anti-mouse immunoglobulin G-fluorescein isothiocyanate conjugate (Chemicon, Temecula, Calif.) was added for 30 min of incubation at 37°C. Following additional rinses and air drying, the slides were prepared with mounting medium (buffered glycerol) and coverslips for observation by epifluorescence microscopy using an Olympus BH-2 microscope and mercury light source.

**RESULTS**

Ten mice (20.8%) died within 2 h of the cardiocentesis and first inoculation, and their deaths were attributed to cardico- centesis. However, since the animals had already been inoculated and mortality within each inoculation group varied, the final number of animals studied in each group for antibody production and specificity ranged from three to six, with a mean of 4.75 and a median of 5.

The number of viable organisms inoculated varied despite comparable growing conditions and comparable turbidity of suspensions. The geometric mean inoculum sizes for all strains clustered in the range of 3.9 × 10⁶ to 9.6 × 10⁷ CFU per animal, except for the clinical isolate of *R. quintana*, for which the viable inoculum was substantially smaller (geometric mean, 1.4 × 10⁵ per animal). Total microscopic counts of bacteria were not performed. Nevertheless, the results below demonstrate that antigenic stimulation was adequate and specific regardless of the proportion of inoculated organisms which were viable.

EIA studies demonstrated an absence of significant murine antibody activity against proteins of *R. quintana* or *R. henselae* before inoculation. Sera obtained after the immunization series contained high antibody activity and specificity for the proteins of the inoculated species (*R. henselae* or *R. quintana*), which was not abrogated by absorption with the other species. Figure 1 summarizes the results of assays performed with the sera of each group of mice.

Immunoblot studies confirmed the development of antibodies directed at specific proteins. Preimmunization sera yielded virtually no bands, and postimmunization sera yielded multiple bands. Animals immunized with any strain of *R. henselae* developed antibodies to numerous proteins of all of the *R. henselae* strains while developing minimal activity to those of either *R. quintana* strain. *R. henselae* proteins with a wide range of apparent molecular masses, from 20 to >200 kDa, induced strong antibody responses. Mice immunized with either strain of *R. quintana* developed antibodies to several proteins of both while developing minimal activity to those of any *R. henselae* strain. Only *R. quintana* proteins with a limited range of apparent molecular masses, from roughly 33 to 43 kDa, induced strong antibody responses. Figures 2A and B exemplify these findings.

The results of immunofluorescence studies are detailed in Table 1; Fig. 3 demonstrates a positive assay. Murine antisera raised against *R. henselae* 87-66 yielded immunofluorescence with all of the *R. henselae* strains at a dilution of 1:1,000. At the same dilution, the antisera yielded no fluorescence with either *R. quintana* strain, *B. bacilliformis*, either *A. felis* strain, or *S. epidermidis*. At a dilution of 1:500, no fluorescence occurred with either strain of *A. calcoaceticus* subsp. *anitratus*. At a dilution of 1:10, but not at 1:100, the antisera was weakly cross-reactive with *R. quintana*.  

VOL. 30, NO. 1, JULY 1992

DISTINCTIVE ANTIBODY RESPONSES TO ROCHALIMAEA SPP.

FIG. 2. Immunoblots. The first 12 lanes of each section, illustrating reaction with postimmunization sera from pairs of mice inoculated with six different R. henselae strains, are marked by solid brackets. The last four lanes, demonstrating reaction with postimmunization sera from pairs of mice inoculated with two different R. quintana strains, are marked by dashed brackets. (A) Blots using proteins from R. henselae 87-66 as antigens. (B) Blots using proteins from the R. quintana type strain as antigens. The serum of one R. henselae-immunized mouse displayed low antibody activity. The numbers on the left are molecular sizes in kilodaltons.

At 1:10, there was no reaction with S. epidermidis. Interestingly, the antiserum did react with S. aureus at a 1:1,000 dilution, likely because of nonspecific binding of immunoglobulin G to S. aureus protein A.

FIG. 3. An example of a strongly positive immunofluorescence assay using murine antiserum generated against R. henselae 87-66. Bar, 10 μm. The large arrowhead points to a cluster of bacteria, which often autoagglutinate when suspended in PBS. The small arrowheads point to individual organisms.

DISCUSSION

These studies have demonstrated that in mice without evidence of previous exposure to R. quintana or R. henselae, strong antibody responses to protein antigens can be induced by intraperitoneal inoculation. The antibodies have a high degree of species specificity but intraspecies (strain) cross-reaction. This is demonstrable by EIA and immunoblotting techniques. We have shown that R. henselae-induced murine antibodies can be employed in an immunofluorescence assay which is also highly specific. This assay has diagnostic applications, having been used in our laboratories to establish provisional identification of organisms whose growth characteristics and morphology are consistent with those of Rochalimaea spp. (29). Cross-reaction of the immune sera with S. aureus is of no consequence, since it cannot be mistaken for a small, curved gram-negative bacillus.

The lack of reaction of R. henselae-induced antibodies with two strains of A. felis is very important. Before the cultivation of R. henselae (28) and subsequent demonstration of its association with bacillary angiomatosis and parenchymal bacillary peliosis (25, 29), bacillary angiomatosis was hypothesized to be the consequence of disseminated infection by A. felis. This was because of the finding in lesions of bacillary angiomatosis of Warthin-Starry-staining bacilli which usually eluded cultivation but reacted with an immunoperoxidase staining reagent based upon a hyperimmune rabbit antiserum raised against A. felis (15, 16, 21). However, in other cases with Warthin-Starry-staining bacilli found in tissue, anti-A. felis antibodies failed to bind specifically to the organisms (4, 5). In one case, cultivation of the...
cat scratch disease agent was reported from a patient with AIDS, and this antiserum reacted with the isolate (27). However, in a later report of isolation of a bacterium from the lesions of a patient with bacillary angiomatosis, no reaction with the organism could be demonstrated with immune sera directed against either \textit{A. felis} or \textit{B. bacilliformis} (5). Other reports which have hypothesized or claimed to demonstrate disseminated \textit{A. felis} infection have been based upon nonculture diagnostic techniques which may lack specificity, i.e., tissue Warthin-Starry stains and/or skin testing with \textit{A. felis}-derived antigens (1, 7, 11, 13, 17, 20, 24, 26).

Since \textit{R. henselae} is distinct phenotypically and genetically from \textit{A. felis} (8, 22, 28, 31), the question of whether the techniques or antisera used in early immunoperoxidase studies of bacillary angiomatosis were nonspecific must be raised. It is possible that some antigenic structures are shared by \textit{R. henselae} and \textit{A. felis} despite their clear-cut distinction. In one report (18), hyperimmune rabbit antisera raised to \textit{A. felis} cross-reacted with a genetically distinct bacterium since proven to be \textit{R. henselae} (19). Therefore, development of monoclonal antibodies to species-specific antigens may be an important direction for further investigation.

Demonstration of \textit{R. henselae} proteins which are both antigenically distinct from those of a genetically closely related pathogen (31) and able to induce strong antibody responses through experimental infection of mice lays the foundation for pursuit of evidence of human infection by detection of antibodies with sensitive EIA and immunofluorescence assay techniques. Similar studies have been performed for \textit{R. quintana} (9, 10) and \textit{B. bacilliformis} (14). Such investigations will be of great importance in the effort to understand the prevalence and clinical importance of this newly recognized pathogen.

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