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In Vitro Evaluation of the Role of Humoral Immunity against *Bartonella henselae*

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The contribution of humoral immunity against *Bartonella henselae* was evaluated by examining the in vitro bactericidal activity of sera and the ability of these microorganisms to activate complement and stimulate phagocytosis and an oxidative burst in polymorphonuclear leukocytes. The organism was killed by complement-mediated cytolysis. Complement activation preferentially proceeded by the alternative pathway. The presence of specific antibodies did not increase the serum bactericidal activity or complement activation. However, phagocytosis and the subsequent production of oxygen radicals, evaluated by flow cytometry, were significantly enhanced in the presence of bacteria previously opsonized with immune sera.

Bartonella henselae, formerly *Rochalimaea henselae*, is a recently identified human pathogen that has been recognized as the cause of bacillary angiomatosis, bacillary peliosis, a prolonged febrile bacteremic syndrome, and cat scratch disease (1, 4, 13, 15, 17–19, 22). Bacillary angiomatosis, a vascular proliferative disease presenting primarily with cutaneous lesions but also with systemic manifestations and multiorgan involvement (1, 7, 15, 17); bacillary peliosis (11, 19, 22); its parenchymal variant; and persistent bacteremia (13, 18) have been described mainly in immunosuppressed individuals, including AIDS patients and transplant recipients receiving immunosuppressive therapy. Immunocompetent subjects infected with *B. henselae* have usually presented with a bacteremic febrile syndrome that generally (18), but not always (10), responded to a short course of antibiotics. Others have had localized bacillary angiomatosis (3, 21) or a clinical and pathologic syndrome consistent with cat scratch disease (4).

Little is known about the interactions of the organism and/or the host that determine the broad range of clinical manifestations of *B. henselae* infection. It is clear that the most severe manifestations occur in individuals with a depressed cellular immune response. The potential contribution of humoral immunity as a host defense mechanism against this agent has not been studied in either immunosuppressed or immunocompetent individuals.

Our objective was to evaluate, in vitro, the role of humoral immune responses against infection caused by *B. henselae*, including the potential bactericidal activity of sera from uninfected donors, and the ability of these organisms to activate complement and stimulate phagocytosis and reactive oxygen production by polymorphonuclear (PMN) leukocytes.

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B. henselae Houston-1 was originally isolated at the Veterans Affairs Medical Center, Houston, Tex., from blood cultures obtained from a 40-year-old human immunodeficiency virus (HIV)-infected man with a history of persistent fever (13). Passages were done on tryptic soy agar supplemented with 5% sheep blood (BBL) and incubated at 37°C in a candle jar. For each assay, 5 to 7-day-old colonies were scraped from the plates, washed three times in Hanks balanced salt solution, sonicated for 5 min to disrupt aggregates, and resuspended in Hanks balanced salt solution to the desired concentration. The bacterial concentration was estimated with a hemocytometer and optical density readings and confirmed by plating duplicate serial 10-fold dilutions on trypic soy agar–5% sheep blood plates and incubating them as described above. After 5 days, CFU per milliliter were estimated by counting colonies.

Antibodies against *B. henselae* were measured by Russell Regnery (Centers for Disease Control and Prevention, Atlanta, Ga.) using an immunofluorescence assay (IFA) (14). A titer of greater than or equal to 64 IFA units is considered positive (14, 20).

All subjects gave informed consent prior to donating blood for this research.

Sera were obtained from two healthy volunteers who had no history of infection with *B. henselae* and whose antibody titers were <64 IFA units. These sera are referred to as nonimmune sera and were utilized for the bactericidal and complement assays, as well as for opsonization.

Sera were obtained from the patient from whom *B. henselae* Houston-1 was originally isolated and from another HIV-infected patient with biopsy-proven bacillary angiomatosis. From the first patient, serum was obtained after two distinct episodes of bacteremia 4 months apart; the antibody titer was 2,048 after each bacteremic episode, falling to 512 eight months after the last episode. The patient with bacillary angiomatosis had serum drawn 8 months after excision of the lesion; his antibody titer was 512. For the opsonization and complement activation assays described below, the convalescent-phase sera (antibody titer, 512 IFA units), referred to from now on as immune sera, were used. Sera were also obtained from rabbits ($n = 3$) immunized with four weekly subcutaneous injections of 10^9 killed organisms emulsified in complete Freund's adjuvant (first dose) and incomplete Freund's adjuvant (subsequent doses). Rabbits were bled 1 week after the last immunization. The

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mean serum antibody titer of the immunized rabbit was 32,768 IFA units.

Serum bactericidal activity was studied with 20, 10, and 2% (in Hanks balanced salt solution) fresh sera from the two donors lacking antibody to *B. henselae*. Twenty percent heat-inactivated sera from the respective donors were used as negative controls. Sera were incubated with 10^7 to 10^9 CFU of organisms per ml at 37°C in a shaking water bath. Ten-microliter aliquots were removed at 30-min intervals up to 120 min, diluted serially in sterile water, and plated in duplicate onto tryptic soy agar–5% sheep blood plates. CFU were counted after 5 days of incubation.

Activation of the complement cascade by *B. henselae* was evaluated by measurement of the Bb and C4d fragments, split products resulting from the activation of the alternative and classical pathways, respectively, by using an enzyme-linked immunosorbent assay (QUIDEL, San Diego, Calif.) and conditions previously described in studies of the complement-activating properties of *Streptococcus pneumoniae* (16). Nonimmune and immune sera were incubated at 37°C with different dilutions of the bacteria and for different time periods, after which the suspensions were placed on ice to stop the reaction and then centrifuged (15,000 × *g* for 5 min at 4°C). Ten microliters of the supernatant was removed, and the assay was performed as indicated by the manufacturer's instructions. To provide a positive control, sera were also incubated with zymosan (2 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) and heat-aggregated human immunoglobulin G (2 mg/ml) (Sigma) to activate, respectively, the alternative and classical pathways. Hanks balanced salt solution was added as a negative control. In addition, *S. pneumoniae* serotype 3 and *Escherichia coli* HB101 (provided by David Watson), organisms well known to activate the alternative pathway, were used as positive controls for that assay.

Phagocytosis and oxidative burst were evaluated by flow cytometry, as previously described (2). The use of flow cytometry has the advantage of allowing the study of the phagocytic capacity of leukocytes in their normal microenvironment. That this method measures ingested, as opposed to cell membrane-associated, bacteria has been demonstrated in previous studies (2) and is supported by the following observations: (i) uptake is temperature dependent and does not occur at 4°C; (ii) unlabeled antifluorescein antibody does not quench the cell-associated fluorescence of leukocytes previously incubated with labeled bacteria, indicating that the labeled bacteria had been internalized; and (iii) for *Staphylococcus aureus*, the addition of lysostaphin to leukocytes that had been previously incubated with fluorescein-conjugated *S. aureus* does not reduce cell-associated fluorescence (2).

For the experiments reported here, heparinized blood was obtained from three healthy subjects known to lack detectable antibodies to *B. henselae*, from three asymptomatic HIV-infected individuals with CD4 counts greater than 400 cells per mm^3, and from three patients with AIDS (AIDS-defining illnesses were Kaposi's sarcoma, cryptococcal meningitis, and *Pneumocystis carinii* pneumonia). None of the HIV-infected subjects had any evidence of *B. henselae* or any other intercurrent infection at the time of the study. Bacteria were labeled with Texas red, and 15 μl of opsonized bacteria was added to 100 μl of whole blood previously incubated with DCFHDA (Molecular Probes, Eugene, Oreg.) for 30 min at 37°C to yield an approximate ratio of bacteria to leukocytes of at least 10:1. Mixtures of the blood and bacteria were incubated in a 37°C horizontal shaking water bath for 45 min, after which the reaction was stopped with cold phosphate-buffered saline (PBS). The cells were washed once with 3 ml of PBS, and the erythrocytes were lysed with 2 ml of fluorescence-activated cell sorter lysing solution (Becton Dickinson, San Jose, Calif.). The leukocytes were washed again with PBS and fixed in 2% paraformaldehyde buffered at pH 7.2, prior to flow cytometric analysis. Prior to incubating with blood, *B. henselae* was opsonized with either 20% heat-inactivated human immune sera, 20% heat-inactivated human nonimmune sera, or 2% heat-inactivated immune rabbit sera on a rocking platform for 30 min.

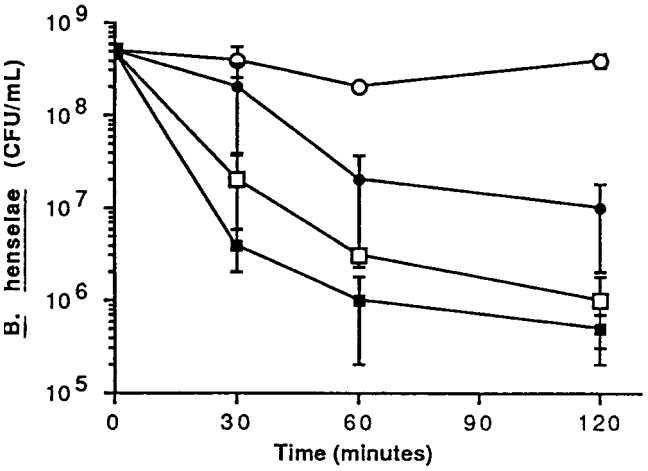


FIG. 1. Bactericidal effects of different concentrations of nonimmune freshly obtained sera on *B. henselae* after different time periods. Each point represents the mean ± standard deviation from two different assays. Concentrations used were 20% (closed squares), 10% (open squares), and 2% (closed circles). Open circles show the lack of bactericidal activity when 20% heat-inactivated serum was used.

Flow cytometry was performed on an Epics Profile I cytometer (Coulter Cytometry, Hialeah, Fla.) with an argon laser tuned to 488 nm. Green fluorescence was obtained through a 525-nm band-pass filter and compensated for spectral overlap by 25% of the signal from the red fluorescence. Red fluorescence was obtained through a 635-nm band-pass filter and compensated for spectral overlap by 10% of the signal from the green fluorescence. PMN leukocytes were selected by the combination of forward-angle light scatter and side-angle light scatter. Mean intensity of fluorescence was converted to a mean channel number based on a 256-channel histogram. Standard curves relating the mean intensity of fluorescence generated by known numbers of labeled bacteria were used to estimate the average number of bacteria per leukocyte from the mean channel number.

Results of serum bactericidal assays are summarized in Fig. 1. Nonimmune human serum had a concentration- and time-dependent bactericidal effect on *B. henselae*. Starting with an inoculum of 5×10^8 CFU/ml, 20% nonimmune human serum killed more than 99% of the organisms after 30 min of incubation. The bactericidal effect was lost by heat inactivation of sera at 56°C for 30 min, suggesting that it is complement mediated. To study if specific antibodies would potentiate the bactericidal effect already present in nonimmune sera, organisms were incubated with either 20% heat-inactivated immune or nonimmune sera prior to the bactericidal assay. Results are shown in Fig. 2. The bactericidal activity of 20% nonimmune sera against bacteria opsonized with immune sera was almost identical at 60 and 120 min to that seen against bacteria opsonized with nonimmune sera, indicating that under these conditions, specific antibodies did not enhance the bactericidal activity of the nonimmune serum.

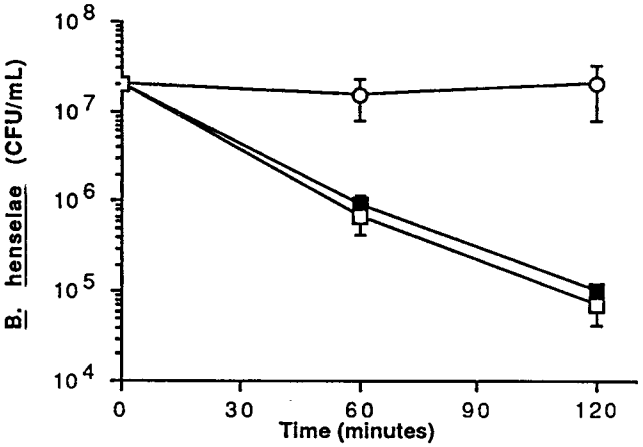
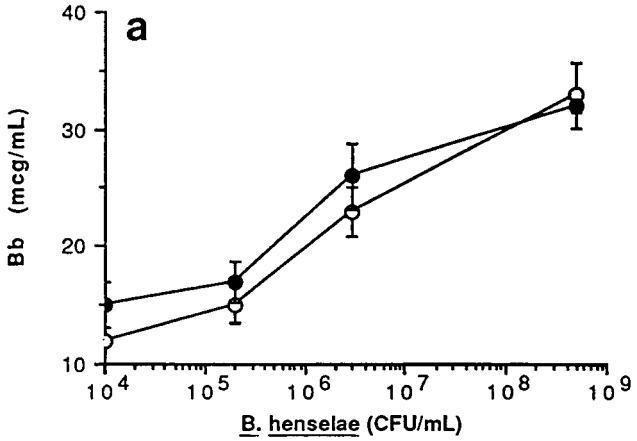


FIG. 2. Bactericidal effects of 20% human nonimmune sera on *B. henselae* previously opsonized with nonimmune or immune heat-inactivated sera. Each point represents the mean ± standard deviation from two different assays. Closed squares, organisms opsonized with 20% nonimmune sera (antibody titer of <64 IFA units); open squares, organisms opsonized with 20% immune sera (antibody titer of 512 IFA units). Twenty percent heat inactivated normal human sera was used as a negative control (circles).



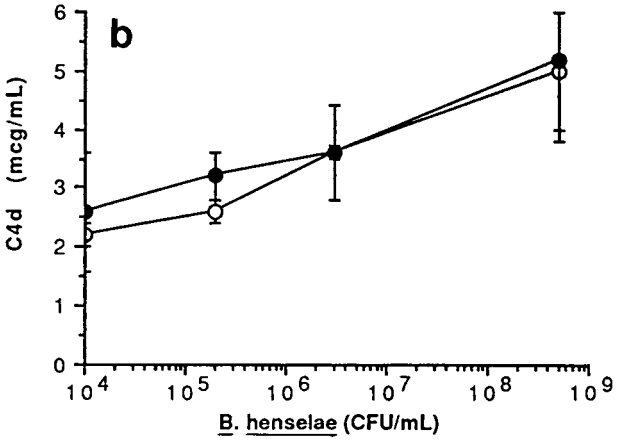


FIG. 3. Bb fragment (a) and C4d fragment (b) generation by nonimmune or immune sera after incubation for 30 min with increasing concentrations of *B. henselae*. Each point represents the mean ± standard deviation from two different assays. Open circles, nonimmune sera; closed circles, immune sera.

B. henselae incubated with nonimmune human serum activated the alternative and classical pathways in a dose-dependent fashion (Fig. 3). The magnitude of Bb and C4d generation after incubation with different concentrations of *B. henselae* was similar for both immune and nonimmune sera. The generation of Bb fragments by 5×10^8 CFU of *B. henselae* per ml was 79% ± 10%, 87% ± 2%, and 89% ± 5% of that after activation by zymosan (2 mg/ml), *S. pneumoniae* (1×10^9 CFU/ml), and *E. coli* (1×10^9 CFU/mL), respectively. The generation of C4d fragments by *B. henselae* (5×10^8 CFU/ml) was 40% ± 10% of that after activation by heat-aggregated immunoglobulin G (2 mg/ml). Blocking the classical pathway by chelation of Ca^{2+} with 10 mM magnesium–ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (MgEGTA) did not diminish the generation of Bb fragments by addition of the bacteria, demonstrating that the alternative pathway activation was independent of the classical pathway. No generation of C4d or Bb occurred in the presence of EDTA (data not shown).

On the other hand, antibody significantly enhanced phagocytosis of *B. henselae* and the generation of reactive oxygen products subsequent to internalization of the bacteria (Table 1). Leukocytes from asymptomatic HIV-infected individuals and healthy donors responded similarly in assays measuring phagocytosis and oxidative burst and when bacteria were opsonized with either immune or nonimmune sera. The leukocytes from AIDS patients were significantly defective in their ability to phagocytose *B. henselae* and generate reactive oxygen products; however, even with these defective PMN leukocytes, opsonization with immune serum effectively enhanced those leukocyte functions (the difference was significant only for phagocytosis after opsonization with 2% rabbit immune serum).

In the present study we have evaluated, in vitro, the contribution of the humoral immune response in the host defense against *B. henselae*. This organism proved to be very sensitive to serum- or complement-mediated cytolysis. Nonimmune sera killed more than 99.5% of an inoculum of 10^7 to 10^9 CFU/ml within 2 h of incubation. This result suggests that specific antibodies do not contribute to the considerable bactericidal activity of serum. Complement activation proceeded by both classical and alternative pathways. The results obtained regarding the alternative pathway are not surprising, since lipopolysaccharides of gram-negative bacteria bind and activate C3. Results of the classical pathway assays suggest that *B. henselae* activates this pathway independent of the presence of specific antibodies (9). It is possible that antibodies present in nonimmune sera bind to this organism, activating complement to initiate the bactericidal process. This possibility is supported by Western blot (immunoblot) analysis of *B. henselae* antigen preparations of sonicated whole-cell organisms and outer membrane proteins indicating that nonimmune sera contain antibodies that react with several of the components of *B. henselae* (unpublished observations). The presence of cross-reactive antibodies in subjects without evidence of *B. henselae* infection has also been noted by others (18). Other nonspecific host defense mechanisms, such as mannan-binding proteins (6), might also play a role in the recognition and killing of *B.*

TABLE 1. Phagocytosis and oxidative burst by *B. henselae*-stimulated PMN leukocytes from HIV-infected and healthy subjects

Serum[a]	Phagocytosis [mean no. of bacteria/leukocyte ± SD (% of cells ± SD)] in PMN leukocytes from[b]:			Oxidative burst [mean channel no. ± SD (% of cells ± SD)] in PMN leukocytes from:		
	Healthy	HIV asymptomatic	AIDS	Healthy	HIV asymptomatic	AIDS
20% Human nonimmune	11.6 ± 1.8 (94 ± 1)	11.6 ± 2.1 (89 ± 4)	9.3 ± 1.2 (88 ± 4)	156 ± 12 (88 ± 5)	175 ± 30 (75 ± 10)	54 ± 24[c] (58 ± 16)
20% Human immune	20.3 ± 1.6[d] (93 ± 3)	18 ± 2.2[d] (93 ± 3)	12.2 ± 0.8[e] (92 ± 4)	191 ± 18[d] (86 ± 1)	204 ± 42[d] (94 ± 2)	69 ± 36[e] (55 ± 17[e])
2% Rabbit immune	33 ± 7.4[d] (95 ± 1)	27.3 ± 3.1[d] (93 ± 5)	16.2 ± 1.7[d,e] (94 ± 2)	222 (96)[f]	222 ± 42[d] (95 ± 3)	82 ± 48[c] (68 ± 13[c])

[a] Bacteria were opsonized with the indicated serum.
[b] Healthy, HIV antibody-negative healthy donor; HIV asymptomatic, asymptomatic HIV-infected patient; AIDS, patient with AIDS.
[c] Significantly different from HIV asymptomatic ($P < 0.05$).
[d] Significantly different from human nonimmune ($P < 0.05$).
[e] Significantly different from healthy and HIV asymptomatic ($P < 0.05$).
[f] Results available for only one set of experiments.

henselae. The polysaccharide composition of the cell wall of *Bartonella* species has not been studied, and we are not able to specify which components could be responsible for the activation of the complement pathways.

Although the presence of specific antibodies did not modify serum bactericidal activity or facilitate complement activation over and above that seen with normal donor serum, phagocytosis of *B. henselae* and generation of oxygen radicals by PMN leukocytes were significantly enhanced by preincubating bacteria in immune sera. As previously described, leukocytes from patients with AIDS showed both diminished phagocytosis and generation of oxygen radicals (5, 8, 12).

Despite the effective serum bactericidal activity conferred by complement and the appropriately enhanced phagocytosis by PMN leukocytes and subsequent adequate stimulation of oxidative radical production conferred by specific antibodies, persistent bacteremia with *B. henselae* has been described in immunocompetent (10) and most frequently in immunodeficient (13, 18) subjects. This raises the possibility that a reservoir persists intracellularly. This hypothesis is supported by the need to treat this infection with antibiotics that exhibit good intracellular penetration, the tendency to relapse, and the need to use lysis centrifugation systems to increase the yield from blood cultures (1, 10, 13, 18). The observed defective phagocytosis and oxidative burst by PMN leukocytes of patients with AIDS against *B. henselae* may be one explanation for the higher susceptibility of these patients to disseminated and persistent *B. henselae* infection.

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