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Characterization of a 17-Kilodalton Antigen of *Bartonella henselae* Reactive with Sera from Patients with Cat Scratch Disease

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A library of *Bartonella* (*Rochalimaea*) *henselae* DNA was constructed in the cloning vector lambda ZAPII and screened for expression of antigenic proteins by using a pool of sera from patients who had been diagnosed with cat scratch disease (CSD) and had antibodies to *Bartonella* spp., as determined by indirect fluorescent-antibody (IFA) assay. Ten immunoreactive phages were subcloned as recombinant plasmids by in vivo excision. All 10 recombinants expressed a protein of approximately 17 kDa when they were examined by immunoblot with the pool of human sera. Restriction endonuclease digestion of each recombinant plasmid indicated seven profiles, suggesting that cloning bias was not the reason for repeated isolation of clones expressing the 17-kDa antigen. The gene coding for the 17-kDa antigen was sequenced and shown to code for an open reading frame of 148 amino acids with a predicted molecular mass of 16,893 Da. The amino terminus of the deduced amino acid sequence was hydrophobic in nature and similar in size and composition to signal peptides found in gram-negative bacteria. The remainder of the deduced amino acid sequence was more hydrophilic and may represent surface-exposed epitopes. Further subcloning of the 17-kDa antigen as a biotinylated fusion protein in the expression vector PinPoint Xa-2 resulted in a 30-kDa protein that was highly reactive on immunoblots with individual serum samples from patients with CSD. The agreement between reactivity with the 30-kDa fusion protein on immunoblot analysis and the results obtained by IFA assay was 92% for IFA-positive sera and 88% for IFA-negative sera. The recombinant-expressed 17-kDa protein should be of value as an antigen for serologic diagnosis of CSD and *Bartonella* infections and warrants further study in attempts to develop a subunit vaccine to prevent long-term *Bartonella* infection in cats and the potential for further spread of these organisms to humans.

Cat scratch disease (CSD) is a common cause of regional lymphadenitis in children and young adults; approximately 22,000 cases occur each year in the United States (18). Annual health care costs attributed to CSD have been estimated to be more than $12 million (18). CSD was originally described by Debre et al. in 1950 (10) and is manifested by lymphadenopathy, which is often preceded by a primary papule at the inoculation site that is associated with a cat scratch or cat exposure (25). Although CSD usually resolves spontaneously within a few months, complications involving the central nervous system, liver, spleen, lungs, bones, skin, and eyes arise in a small percentage of patients (8, 25). The affected lymph nodes characteristically exhibit granulomas, microabscesses, and follicular hyperplasia (8). Specialized staining by the Warthin-Starry method may reveal bacilli. The identity of these bacilli has only recently been determined.

In 1988, an organism termed the cat scratch disease bacillus was isolated from the lymph node tissues of 10 patients with CSD (13). This organism, which was later named *Afipia felis* (6), has not subsequently been associated with the majority of CSD cases. However, recent data have provided compelling evidence that *Bartonella* (formerly *Rochalimaea*) *henselae* is the major etiologic agent of CSD. *B. henselae* is a newly described organism that was first associated with bacillary angiomatosis (BA) (33), a disease affecting primarily immunocompromised patients and described originally by Stoler et al. (37). *B. henselae* has also been associated with a variety of other clinical syndromes in human immunodeficiency virus-infected patients (29, 36, 39). Similarities between the clinical presentations of BA and CSD and their respective etiologic agents led to speculation that BA may be a manifestation of CSD in the immunocompromised host (16, 20, 22). This initial hypothesis has been supported by recent serology (31, 40), culture (11), and PCR (2, 4) studies that have shown *B. henselae* to be the primary etiologic agent of CSD. These new data have resolved many of the questions about the etiology of CSD (see reference 1 for a review).

In light of recent findings associating *B. henselae* with CSD and opportunistic infections among immunocompromised individuals, we attempted to identify individual antigens of *B. henselae* that elicit strong antibody responses in patients with CSD. Here, we report a 17-kDa antigen that elicits a strong immune response in patients with CSD. The nucleotide sequence of the gene encoding this antigen and the deduced amino acid sequence for this antigen are also described. The gene coding for the 17-kDa antigen has been overexpressed as a fusion protein, and preliminary studies designed to evaluate a potential role for this protein as a diagnostic reagent are described.

MATERIALS AND METHODS

Cultivation of *Bartonella* spp. *Bartonella* strains were grown on heart infusion agar supplemented with 5% defibrinated rabbit blood (BBL, Cockeysville, Md.). The Houston-1 strain of *B. henselae* and the Fuller strain of *B. quintana* were used for all experiments described in this report. Cultures were incubated at 34°C in the presence of 5% CO_2. After 3 to 4 days of growth, bacterial cells were harvested with sterile applicators and suspended in TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA) for extraction of DNA or phosphate-buffered saline (PBS)

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for immunoblot analysis. Bacteria were concentrated by centrifugation as needed. Bacterial cells were then frozen and stored for subsequent immunoblot analysis, or DNA was extracted as outlined below.

Patient sera. For this study, human sera were selected from samples submitted to the reference laboratory at the Centers for Disease Control and Prevention for confirmation of clinically diagnosed, suspect CSD cases. Serologic confirmation of *Bartonella* infection was performed by indirect fluorescent-antibody (IFA) assay as previously described (31). Control serum samples were from non-CSD cases with no titers to *Bartonella* spp., as determined by IFA assay. Sera from a total of 23 CSD cases were used in this study; 10 were pooled for immunoscreening of the *B. henselae* DNA library, and 13 were used to determine reactivity to the recombinant fusion protein expressed in *Escherichia coli.*

Construction of *B. henselae* DNA library. A lambda phage library of *B. henselae* DNA was prepared with the lambda ZAPII vector (Stratagene, Torrey Pines, Calif.) by a novel method developed specifically for constructing DNA libraries of AT-rich organisms (3). Total (chromosomal and extrachromosomal) DNA was isolated from the Houston-1 strain of *B. henselae* by lysis of bacterial cells with 1.0% sodium dodecyl sulfate (SDS) in the presence of proteinase K (100 ng/μl) at 55°C for 90 min. The resulting lysate was extracted four times with a 50:50 mixture of phenol and chloroform. Nucleic acids were precipitated from the aqueous phase by the addition of sodium acetate to 0.3 M and 2.5 volumes of absolute ethanol. The precipitate was collected by centrifugation and resuspended in TE buffer with 10 ng of RNase A per μl. The extracted DNA was digested with the restriction endonuclease *Eco*RI under conditions that promote star activity. This enzymatic activity (star activity) is less specific and the cleavage is more random than the cleavage with *Eco*RI under normal conditions. *Eco*RI star activity generated DNA fragments that are efficiently cloned into *Eco*RI-cleaved vectors (3). Selection for 3- to 8-kbp DNA fragments was carried out by electrophoresis through a 0.7% agarose gel and purification by adsorption to glass milk (GeneClean; Bio 101, Vista, Calif.). The purified and sized DNA was ligated to *Eco*RI-digested, alkaline phosphatase-treated lambda ZAPII vector. Then the recombinant phage concatamers were packaged into lambda particles by using commercially available packaging extracts (Gigapack; Stratagene). After packaging, the titer of an aliquot of the library was determined; the percentage of recombinants was assayed by plating with *E. coli* XL1 (Stratagene) on NZY agar plates. The library was amplified by plating the entire packaged ligation reaction and collected by overnight diffusion of recombinants into phage dilution buffer (10 mM Tris [pH 8.0], 10 mM $MgCl_2$, 0.1% gelatin).

Immunoscreening of recombinant clones. For screening of recombinant phage for immunoreactivity with human sera, lambda phage clones were plated at a density of approximately 30,000/150-mm-diameter plate of NZY agar. After incubation for 3 to 4 h at 42°C (until the plaques were barely visible), plates were overlaid with nitrocellulose filters impregnated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and incubation continued for an additional 3 h at 37°C. Then the resulting filters were washed twice in Tris-buffered saline containing 0.1% Tween 20 (pH 8.0) (TBST) and blocked with TBST containing 5.0% dehydrated skim milk (Difco, Detroit, Mich.) for 1 h at room temperature. Then filters were reacted for 2 h with a pool of serum samples (diluted 1:300 in TBST with 5% skim milk) from patients clinically diagnosed with CSD. This pool of serum samples consisted of 10 individual sera from patients shown to have IFA titers of 1:1,024 or greater. Then filters were washed four times with TBST, and bound antibody was detected by reacting the filters with goat anti-human immunoglobulin G conjugated with horseradish peroxidase (diluted 1:3,000 in TBST with 5% skim milk) for 1 h at room temperature. The filters were washed four times in TBST, and the color was developed by using TMB (3,3′,5,5′ tetramethyl benzidine) membrane substrate (Kirkegaard and Perry, Gaithersburg, Md.).

Rescue and analysis of recombinant phagemids. After several rounds of plaque purification of immunoreactive recombinant phage, the isolated phage plaques were subcloned from the lambda phage recombinants into pBluescript-derived plasmids according to the directions of the manufacturer (Stratagene). Briefly, by superinfecting lambda recombinant-infected *E. coli* XL1 MRF′ cells with R408 helper phage, the pBluescript portion of the lambda ZAPII vector containing the inserted *Bartonella* DNA was rescued. Then these recombinant phagemids were used to infect *E. coli* SOLR cells (Stratagene), in which they replicate as plasmids upon selection with ampicillin. This procedure results in colonies that harbor pBluescript-derived recombinant plasmids with *Bartonella* DNA inserts. These recombinant plasmids were used for further analysis and sequencing of DNA and for immunoblot analysis of plasmid-encoded *Bartonella* proteins expressed in *E. coli.*

Immunoblot analysis. To analyze human antibody responses to individual antigens, whole-cell lysates of *Bartonella* and *E. coli* recombinants were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis. *Bartonella* strains were grown as described above. Growth was harvested from plates, washed once in PBS, and resuspended in water. *E. coli* recombinants were grown in Luria-Bertani broth containing 100 μg of ampicillin per ml at 37°C with shaking to mid-log phase, and 1 mM IPTG was added. Incubation was continued for an additional 2 h at 37°C with shaking, and cells were collected by centrifugation and resuspended in water. Proteins were solubilized in sample buffer (2% SDS, 50 mM Tris [pH 8.0]) for SDS-PAGE for 5 min at 100°C.

Solubilized *Bartonella* and *E. coli* recombinant cell proteins (15 μg per well) were electrophoresed through SDS-PAGE (14% or 8 to 16% gradient polyacrylamide) minigels (Novex, San Diego, Calif.). The resulting cell proteins were stained directly with Coomassie brilliant blue or transferred to nitrocellulose by using a mini-transfer apparatus (Bio-Rad, Richmond, Calif.). After electrotransfer of proteins to nitrocellulose, the resulting filters were reacted with antisera and treated as described above.

Sequencing of phagemid DNA. Plasmid DNAs from recombinants were prepared from minipreps by alkaline lysis. The resulting plasmid DNAs were used for restriction endonuclease digestion and directly for double-stranded plasmid sequencing by the dideoxy chain termination method. Plasmids were sequenced by alkaline denaturation by the method of Zhang et al. (41), with subsequent chain extension and termination with T7 DNA polymerase (Sequenase; U.S. Biochemical) and ^{35}S-dATP. Sequenced DNA was electrophoresed through a denaturing 6% acrylamide–urea gel, and the resulting gel was fixed with 10% acetic acid–5% methanol. After vacuum drying, the gel was exposed to X-ray film for approximately 12 to 24 h and developed. Plasmids were sequenced with the M13 forward or reverse primers or primers that were synthesized from the *B. henselae* insert as the sequencing progressed. Sequence data were analyzed by using version 8.0 of the Genetics Computer Group software package (14). To further localize the 17-kDa antigen gene and for additional sequencing, restriction fragments were subcloned into pUC19 by standard methods (23) and examined for expression by immunoblot analysis.

Construction of the fusion protein. Gene fusion techniques were used to confirm the identity of the putative 17-kDa antigen gene and allow optimal expression in *E. coli*. PCR primers from the putative 17-kDa antigen gene sequences were designed so that they contained a *Hin*dIII site on the 5′ end (5′ AAAAGCTTGAAAAAATATAGCTTAGTCAC 3′) and a *Bam*HI site on the 3′ end (5′ AAGGATCCAGAAATGCTCTCAAAC 3′). These unique restriction sites facilitate directional and in-frame cloning. DNA from the Houston-1 strain of *B. henselae* was amplified through 35 cycles of 94°C for 1 min, 48°C for 2 min, and 68°C for 1.5 min by using GeneAmp reagents, *Taq* polymerase, and a thermal cycler (Perkin-Elmer, Norwalk, Conn.). The resulting PCR product was cleaved with *Hin*dIII and *Bam*HI, purified on a gel, and ligated to the expression vector PinPoint Xa-2 to produce a fusion of the antigen with the 13-kDa biotinylation tag sequence of the vector (Promega, Madison, Wis.). This tag sequence has been engineered by the manufacturer to permit rapid purification of biotinylated fusion proteins from other *E. coli* cell proteins. There are also endoprotease cleavage sites to permit the cleavage of proteins from the tag sequence. The ligation mixture was used to transform *E. coli* XL1 MRF′ (Stratagene). Potential clones were examined by restriction endonuclease analysis to confirm the presence of an insert of the correct size. Clones containing inserts of the correct size were grown to early log phase in Luria-Bertani broth and induced with 1 mM IPTG, and growth was allowed to continue for an additional 2 h. *E. coli* cells were collected by centrifugation and examined for the expression of biotinylated fusion proteins by immunoblot analysis with streptavidin conjugated to horseradish peroxidase. Clones expressing the biotinylated fusion protein were examined for reactivity with the serum pool from patients with CSD as well as with individual serum samples from patients with CSD and from controls. For this purpose, the clone which expressed the fusion protein was solubilized and resolved on a single preparative well of an 8 to 16% gradient minigel. Proteins were transferred to nitrocellulose, and the resulting filter was cut into individual strips and reacted with individual serum samples.

Nucleotide sequence accession number. The nucleotide sequence for the 17-kDa antigen of *B. henselae* has been deposited in the GenBank database under accession no. U23447.

RESULTS

Identification and characterization of immunoreactive clones. Approximately 1.2×10^6 recombinant phage clones were obtained. The quality of the resulting library was previously examined by assaying the cloning efficiency of a marker gene (3). For the purpose of that report, the 16S rRNA gene was chosen, and it was shown to hybridize with 0.125% of clones. These results approach those predicted for random cloning and suggest that all DNA fragments are represented in the *B. henselae* library. Approximately 0.3% of the 300,000 *B. henselae* recombinant plaques screened with the pool of sera from patients diagnosed with CSD were immunoreactive. Ten of these immunoreactive recombinants were selected for further study and isolated by three or four rounds of plaque purification. All 10 were subcloned as pBluescript-based phagemids and used to infect *E. coli* SOLR cells (Stratagene). Each of these subclones appears to direct synthesis of the 17-kDa antigen that is highly reactive on immunoblots with the pool of sera from patients with CSD. Five of these clones are depicted in Fig. 1A (lanes C through G). A control strain of *E. coli* SOLR, which harbored only the plasmid vector pBlue-

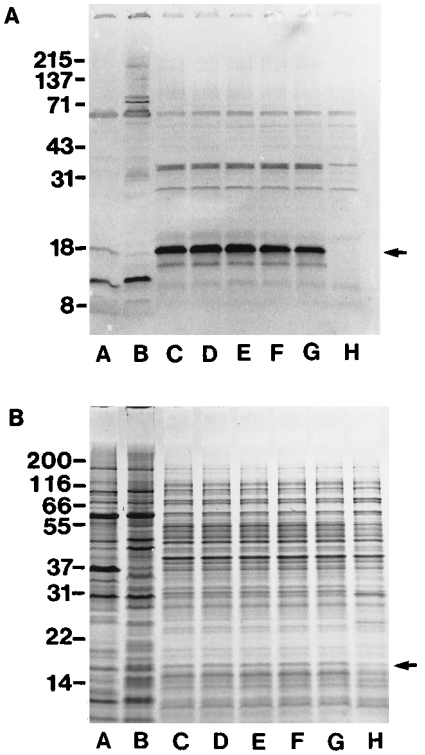

FIG. 1. (A) Immunoblot of antigen-expressing subclones reactive with serum pool from patients with CSD. Whole-cell protein preparations were electrophoresed through a 14% acrylamide minigel and transferred to nitrocellulose. Then the filter was blocked with 5% BLOTTO and reacted with a 1:300 dilution of a serum pool from patients with CSD and subsequently with a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-human antibody. The filter was developed with a commercially available chromogenic peroxidase substrate. Proteins from the following bacteria are shown: *B. quintana* Fuller strain (lane A), *B. henselae* Houston-1 strain (lane B), five *E. coli* (SOLR) recombinants (lanes C through G), and *E. coli* (SOLR) containing the pBluescript control (lane H). (B) Duplicate gel stained with Coomassie brilliant blue. The positions of molecular size standards (in kilodaltons) are shown on the left. The position of the 17-kDa antigen is indicated by an arrow in each panel.

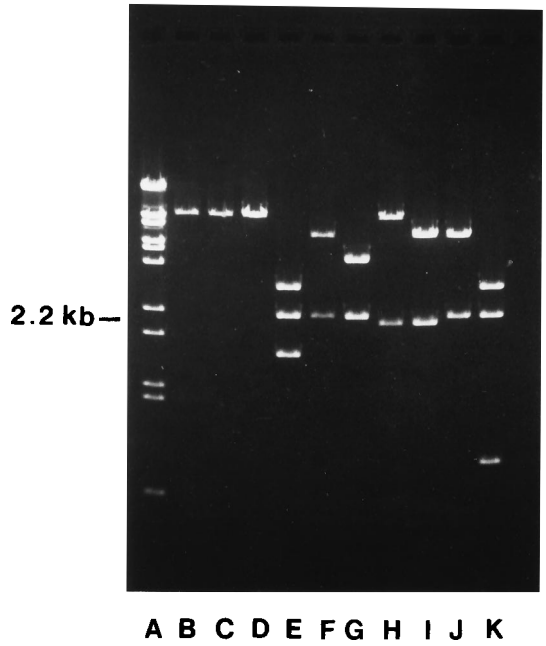

FIG. 2. Agarose gel electrophoresis of 10 isolated recombinant plasmids which direct synthesis of the 17-kDa antigen. Recombinant plasmids were digested with *Eco*RI and resolved on a 1.0% agarose gel (lanes B through K). Lane A, molecular size standards (lambda phage DNA cleaved with *Bst*EII). The 17-kDa antigen gene was localized to a region within the 2.2-kb *Eco*RI fragment common to several clones.

script SK, did not express the same immunoreactive 17-kDa antigen (Fig. 1A, lane H). *B. henselae* appeared to express the 17-kDa antigen weakly (Fig. 1A, lane B). *B. quintana* also expressed the 17-kDa antigen that reacted with the pool of sera from patients with CSD (Fig. 1A, lane A), suggesting that this antigen (or portions of the antigen) is shared by *B. henselae* and *B. quintana*. The 17-kDa antigen expressed in *E. coli* (Fig. 1A, lanes C through G) appears to react with the serum pool more strongly than does the protein expressed in *B. henselae* (lane B). The 17-kDa antigen expressed by *B. henselae* and by *E. coli* clones did not react with the control human serum pool from patients with no history of CSD (data not shown). The antibody response from the serum pool did not appear to be directed against a variety of *B. henselae* antigens. Major immunoreactive antigens with approximate molecular masses of 14, 62, 74, and 80 kDa are seen with *B. henselae* (Fig. 1A, lane B), in addition to the minor bands seen at 17, 32, 58, and 190 kDa. Clones expressing the 14-, 62-, 74-, and 80-kDa antigens were not among the first 10 selected for further study.

To correlate the immunoreactivity levels of the 17-kDa antigen with expression of total protein, whole-cell lysates were subjected to SDS-PAGE and stained with Coomassie brilliant blue (Fig. 1B). A protein migrating to 17 kDa was observed for *B. henselae* (Fig. 1B, lane B) and *B. quintana* (lane A) stained with Coomassie brilliant blue. A protein with a similar molecular mass can also be seen for the immunoreactive *E. coli* clones (Fig. 1B, lanes C through G). The 17-kDa protein expressed by clones was not a major band detected by the staining of total proteins (Fig. 1B), but it was the most intensely reactive band seen by immunostaining (Fig. 1A). Thus, the 17-kDa antigen appears to be highly antigenic (at least when it is expressed in *E. coli*), even though it is expressed at low or moderate levels in cells. These results imply that the 17-kDa antigen is highly immunoreactive but is not expressed at high levels in *B. henselae*. However, the immunoblotting results indicate that patients with CSD have a strong antibody response to this antigen relative to those to other proteins expressed by *B. henselae.*

Analysis of the gene encoding the 17-kDa antigen. To determine if cloning bias was an explanation as to why clones expressing the 17-kDa antigen were isolated with such frequency, restriction endonuclease analysis was performed. *Eco*RI digestion of the plasmids from all 10 recombinants expressing the 17-kDa antigen revealed seven profiles (Fig. 2). Thus, even though 17-kDa-antigen-expressing clones represent the first 10 clones isolated, at least seven DNA fragments contain the entire gene. These results indicate that cloning bias is not responsible for isolating the same antigen-expressing clone by overrepresentation of a particular fragment of DNA in the library. Previous immunoscreening of the same library with mouse monoclonal antibodies and hyperimmune anti-*B. henselae* rabbit serum revealed proteins of 14 and 21 kDa and of 18, 44, and 60 kDa, respectively (3a). Accordingly, these results, together with the results showing few immunoreactive bands for *B. henselae* (Fig. 1A, lane B), suggest that the 17-kDa antigen is one of a few antigenic proteins recognized by the humoral immune response of a patient with CSD.

The gene for the 17-kDa antigen reactive with the pool of

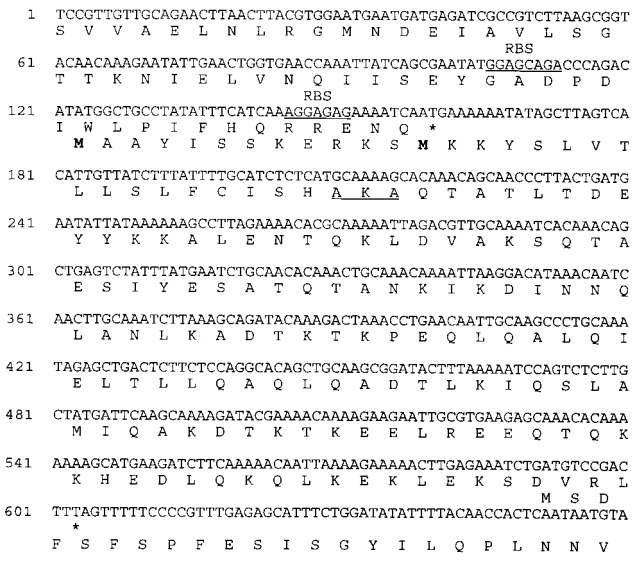

FIG. 3. Nucleotide sequence of the 17-kDa antigen gene of *B. henselae*. The deduced amino acid sequence is shown below the nucleotide sequence. The open reading frame for the 17-kDa antigen begins at nucleotide 123 and ends at the stop codon at nucleotide 603. Two potential initiator methionines for the 17-kDa antigen gene are in boldfaced type. The potential ribosome binding site (RBS) for each putative initiator methionine is underlined. An amino acid sequence with homology to the signal peptidase cleavage site of *E. coli* (A-X-A) is also underlined. The deduced amino acid sequences of two partial open reading frames preceding (nucleotides 1 to 159) and following (nucleotides 591 to 660) the 17-kDa antigen gene are also shown. Stop codons are indicated by asterisks.

sera from patients with CSD has been localized within a 2.2-kb *Eco*RI fragment (Fig. 2), and the entire fragment has been sequenced. The gene coding for the 17-kDa antigen was localized to a single open reading frame by subcloning and immunoblot analysis with the serum pool from patients with CSD. This gene directed synthesis of the 17-kDa antigen in *E. coli* only when it was subcloned into pUC19 in the direction of the *lacZ* alpha peptide. Thus, a *B. henselae* promoter is either absent upstream of this gene or nonfunctional in *E. coli*. The open reading frame consisted of either 160 or 148 amino acids, depending on which of the two putative initiator methionines identified is functional (Fig. 3). The first potential initiator methionine (Fig. 3; nucleotides 123 to 125) is preceded by a polypurine-rich sequence which is similar to the consensus ribosome binding site in *E. coli* (15). The second potential initiator methionine (Fig. 3; nucleotides 159 to 161) is preceded (by 8 bases) by a sequence which is identical to the consensus *E. coli* ribosome binding site. Assuming the second potential initiator methionine is used for the start of translation, then a deduced protein of 148 amino acids with a predicted molecular mass of 16,893 Da is obtained. This is closer to the apparent molecular size of 17 kDa observed on immunoblots than the molecular mass predicted (18,245 Da) when the first potential initiator methionine is used. The deduced amino acid sequence of the 17-kDa antigen gene does not share significant homology with any other bacterial protein in the GenBank database. Assuming the second potential initiator methionine is used, the deduced amino acid sequence has several similarities to bacterial membrane-associated proteins. The first 18 amino acids are hydrophobic in nature and contain two lysine residues at the immediate amino terminus (residues 2 and 3). This type of motif is typical of bacterial outer surface proteins, in which the lysine residues interact with the phospholipids of the membrane and the following hydrophobic residues form a membrane-spanning or membrane anchor sequence (35). An amino acid sequence identical to the *E. coli* consensus signal peptidase cleavage site (A-X-A) is present at residues 18 to 20 after the second putative initiator codon (Fig. 3). It is not known if this site is cleaved by *B. henselae* signal peptidases to yield a mature form of the 17-kDa antigen. The remaining 130 amino acid residues deduced from the 17-kDa antigen gene sequence are predominantly hydrophilic or neutral, with no long hydrophobic domains. The deduced amino acid sequence for the 17-kDa antigen is lysine rich (14.9 mol%) and has a predicted isoelectric point of 9.24.

Regardless of which potential initiator methionine is used for the start of translation, there is a short overlap with another long open reading frame. The reading frame upstream of the 17-kDa antigen gene continues for at least an additional 380 nucleotides upstream of what is shown (Fig. 3). The entire sequence of this open reading frame has not yet been obtained. It is likely that the 17-kDa antigen gene is part of an operon that is transcribed from a promoter upstream of this unidentified open reading frame. A second unidentified open reading frame overlaps the 3′ end of the 17-kDa antigen gene and continues for at least an additional 700 nucleotides. No sequences capable of forming the stable loop- and stem-type secondary structures that are characteristic of bacterial transcription terminators are seen immediately downstream of the 17-kDa antigen gene. This third open reading frame may be a gene that is cotranscribed from a single promoter along with the 17-kDa antigen gene and the potential gene upstream. There are no regions upstream of the 17-kDa antigen gene with correct spacing and homology to the *E. coli* consensus −35 (TTGACA) and −10 (TATAAT) promoter sequences (17). It is possible that the *Bartonella* RNA polymerase recognizes promoter sequences that differ significantly from those of *E. coli*. The lack of potential promoter sequences and expression of the 17-kDa antigen only when it is cloned in the direction of the vector *lacZ* promoter provide further data that this gene is cotranscribed with an upstream gene.

Gene fusion construction. To create a highly expressed fusion protein, the portion of the gene coding for amino acid residues 2 to 148 was synthesized by PCR amplification with the primers described in Materials and Methods. PCR primers that permit in-frame fusion of the entire protein (assuming that the second methionine is the start of translation) minus the methionine residue of this gene to the 13-kDa biotinylation tag sequence of expression vector PinPoint Xa-2 were selected. This results in a fusion protein which consists of the tag sequence (amino-terminal portion) fused to the antigen (carboxy-terminal portion) under the control of the *lacZ* promoter of vector pXa-2. The 30-kDa fusion protein is expressed efficiently in *E. coli*, as shown by immunoblot analysis with streptavidin conjugated to horseradish peroxidase (Fig. 4). Two clones that contain the antigen gene sequence successfully fused to the biotinylation tag sequence are depicted in Fig. 4 (lanes B and C). A control consisting of an 8-kDa portion of an unrelated gene fused to the Xa-2 vector in *E. coli* XL1 MRF′ expresses a biotinylated protein of approximately 21 kDa (Fig. 4, lane A). A naturally occurring biotinylated protein that has an apparent molecular mass of approximately 26 kDa is found in all three clones (Fig. 4). This naturally occurring biotinylated protein has been observed in all K-12 strains of *E. coli* used for recombinant DNA purposes by the vector manufacturer (Promega).

Immunoreactivity of the fusion protein. To examine the reactivities of the 30-kDa fusion protein with individual serum samples from patients with CSD as well as controls, immunoblot analyses were performed. When individual serum samples

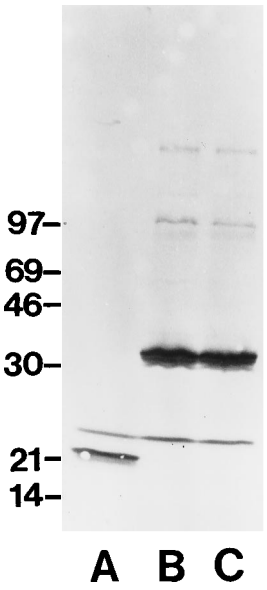

FIG. 4. Immunoblot of biotinylated fusion proteins. Whole-cell protein preparations from *E. coli* containing the 17-kDa antigen fused to the biotinylation tag sequence of vector pXa-2 were electrophoresed through an 8 to 16% gradient minigel and transferred to nitrocellulose. Then biotinylated proteins were detected as described in the legend to Fig. 1, except that the filter was reacted with streptavidin conjugated to horseradish peroxidase. Potential fusion proteins of the *B. henselae* 17-kDa antigen with the biotinylation tag sequence of plasmid vector pXa-2 are shown (lanes B and C). Lane A, fusion protein consisting of a portion of an unrelated gene and the biotinylation tag sequence of vector Xa-2. The positions of molecular size standards (in kilodaltons) are shown on the left.

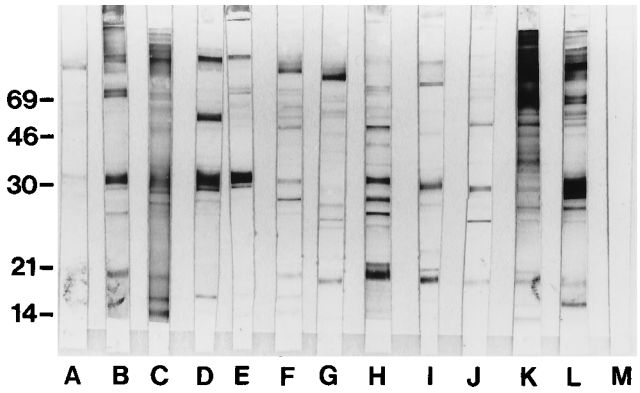

FIG. 5. Immunoblot of the 30-kDa fusion protein reacted with individual serum samples from patients with CSD and controls. Whole-cell protein preparations from *E. coli* containing the 17-kDa antigen fused to the biotinylation tag sequence of vector pXa-2 were electrophoresed through an 8 to 16% gradient minigel and transferred to nitrocellulose. Then the filter was cut into strips and reacted with individual serum samples from patients with CSD and controls (diluted 1:300) as described in the legend to Fig. 1. Lanes B through E and H through J, serum samples from patients demonstrating positive IFA titers; lanes A, F, G, and K, serum samples from patients with negative IFA titers; lane L, the pool of sera from patients diagnosed with CSD that was used to screen the *B. henselae* DNA library; lane M, not reacted with serum. The positions of molecular size standards (in kilodaltons) are shown on the left.

were reacted with the proteins expressed by the clone containing the fusion protein, 12 of 13 serum samples that had been positive for antibody to *Bartonella* spp. by IFA assay were also reactive with the 30-kDa recombinant fusion protein. Likewise, eight of nine serum samples that had been negative by IFA assay did not react by immunoblot with the 30-kDa fusion protein. Figure 5 depicts the results for 11 serum samples from patients with CSD or controls. The samples shown in lanes B through E and H through J are serum samples that were positive by IFA assay (titer equal to or greater than 1:64). Samples A, F, G, and K are serum samples that were negative by IFA assay (titer of 1:32 or less). When immunoblot strips are examined only for the reactivities of the 30-kDa fusion protein, there is good correlation with a clinical diagnosis of CSD and the IFA titer. Some IFA-negative samples have weak reactivities with the 30-kDa fusion protein (Fig. 5, lanes A, F, and K). However, the reactivities of IFA-positive samples appear to be much stronger. It is possible that an *E. coli* protein of the same molecular mass is cross-reactive with some serum samples. This appears to be the case with samples D and E, for which a band slightly smaller than the 30-kDa fusion protein is evident (Fig. 5). These results also indicate that a dominant epitope of the 17-kDa antigen recognized by serum samples from patients with CSD is retained in the fusion protein.

DISCUSSION

Members of the genus *Bartonella* are small gram-negative rods that include *B. henselae*, *B. quintana*, *B. elizabethae*, and *B. vinsonii*, which have recently been reclassified from the genus *Rochalimaea*, as well as the type species, *B. bacilliformis* (7, 29, 32). Both *B. henselae* and *B. quintana* have been associated with BA and a variety of clinical syndromes resulting from opportunistic infections in patients with AIDS (21, 29, 33, 39). *B. elizabethae* has recently been added to the genus; it was isolated from the blood of an immunocompetent patient with endocarditis (9). *B. vinsonii* has not been associated with human disease. *B. bacilliformis* is the etiologic agent of Carrion's disease, which is found only in South America. To date, *B. henselae* is the only *Bartonella* species that has been associated with CSD and appears to be the major etiologic agent of this disease.

Current methods for laboratory confirmation of BA, CSD, and other *Bartonella* infections include serology (5, 28, 31), PCR amplification of *Bartonella* DNA (4, 19, 33), and isolation and identification of the organism (11, 29, 39). While PCR has shown promise for rapid detection and identification of *Bartonella* spp. in clinical samples, it may not yet be practical for most clinical laboratories. Likewise, the isolation of *Bartonella* organisms depends on lengthy incubation of primary cultures. The resulting isolates must still be identified by biochemical or genetic techniques. Accordingly, serology remains the most convenient means for laboratory confirmation of *Bartonella* infection. Both enzyme immunoassays (5, 28) and the IFA assay (31) for detecting anti-*Bartonella* antibodies in patient sera have been developed and reported in the literature. However, only the IFA assay has received extensive evaluation for the diagnosis of CSD (40). While it is instrumental in providing serologic association between *Bartonella* spp. and CSD, the IFA assay depends to some degree on subjective interpretation of the results. In addition, tests are performed with a whole-bacterial-cell antigen preparation that contains a number of individual antigens. The specificities of antibody responses to individual antigens have yet to be determined. The expression of recombinant *Bartonella* antigens in *E. coli* vector systems should allow rapid purification and preparation of precisely defined diagnostic reagents. The identification and characterization of individual antigens should facilitate the development of a more specific enzyme immunoassay that can be readily automated, avoiding some of the drawbacks associated with some current serologic assays for *Bartonella* spp. Moreover, the utilization of such recombinant-expressed *B. henselae*

antigens in a rapid serologic assay (such as latex agglutination) may result in a diagnostic test that is practical in any clinical setting.

Repeated isolation of different cloned fragments of *B. henselae* DNA, all of which direct synthesis of the same 17-kDa antigen, is puzzling. Previous immunoscreening of the same *B. henselae* DNA library with mouse monoclonal antibodies and rabbit anti-*B. henselae* serum resulted in the isolation of clones expressing five antigenic proteins. These clones were weakly reactive or nonreactive with human serum samples from patients with CSD (3a). Clones expressing the 17-kDa antigen were not isolated in those experiments. Hybridization of the *B. henselae* library with a marker gene indicated the predicted cloning frequency for a representative library (3). Although it is possible that a gene encoding a small protein (such as the 17-kDa antigen) would be more efficiently cloned than a longer gene, the repeated isolation of different clones expressing the same 17-kDa antigen is not likely due to cloning bias. Alternatively, it is possible that many of the antigenic proteins (other than the 17-kDa antigen) of *B. henselae* lose antigenicity when they are expressed in *E. coli*. Perhaps the most plausible explanation of the repeated isolation of clones expressing the 17-kDa antigen is that this protein is immunodominant in terms of eliciting a human antibody response. This is supported by strong antibody reactivity with the cloned 17-kDa antigen despite the low to moderate levels of expression of this protein in *E. coli*.

Few antigens of *B. henselae* appear to elicit a strong antibody response in patients diagnosed with CSD (Fig. 1A, lane B). The cloned version of the 17-kDa antigen reacted more strongly with the pool of sera from patients diagnosed with CSD than did the 17-kDa antigen expressed in *B. henselae*. These results suggest that expression is higher in *E. coli* than in *B. henselae*, although the 17-kDa antigen does not appear to be the major protein in *E. coli* clones (Fig. 1B, lanes C through G). It is also possible that the 17-kDa antigen gene is downregulated in *B. henselae* under conditions that are not yet understood and is poorly expressed when *B. henselae* is cultivated in the laboratory. Clearly, the antigen must be expressed at significant levels during infection with *B. henselae*, since patients with CSD mount significant antibody responses to this protein. Coordinate regulation of bacterial genes involved in pathogenesis during infection of the host has been well described (see reference 26 for a review). Similar activation of certain *Bartonella* genes might be possible only when they are exposed to a human host. This hypothesis is supported by the recent results of Drancourt et al. who performed IFA assays of sera from patients with *B. quintana* bacteremia (12). They found dramatically higher antibody titers to *B. quintana* cocultivated with human endothelial cells than to *B. quintana* grown on agar plates. Selective expression of the 17-kDa antigen during human infection might explain the prevalence of antibodies to the cloned antigen in the sera of patients with CSD, despite the weak reactions of these sera with agar-grown *Bartonella* organisms. In addition, differential expression of surface proteins of *B. henselae* may explain the colony morphology changes seen upon repeated subculture of fresh isolates on agar medium (29). It is possible that selective expression of the 17-kDa antigen is responsible for the changes observed in both colony morphology and antigenic composition.

The 17-kDa antigen appears to be expressed in *B. quintana* as well as in *B. henselae*. Thus, the 17-kDa antigen, or a related version of it, appears to be shared by these two bacteria. We have not observed the presence of an immunoreactive antigen of this size in *B. elizabethae* or *B. vinsonii*. However, we have not yet examined the genomes of these two bacteria for the presence of a 17-kDa antigen gene homolog. This (or other) antigen(s) shared by *B. henselae* and *B. quintana* may help to explain the high level of serologic cross-reactivity between these two bacteria that is also seen by IFA assay. Initial IFA results for the Fuller strain of *B. quintana* indicated that the human antibody response seen during CSD was specific to *B. henselae* and not cross-reactive with *B. quintana* (31). More recent data indicate that other isolates of *B. quintana* are strongly cross-reactive with antibodies from patients known to be infected with *B. henselae* (38). These results suggest that a major epitope, differentially expressed in certain strains of *B. quintana* and observed by IFA assay, is shared by *B. henselae* and *B. quintana*. The properties of the 17-kDa antigen are consistent with such a differentially expressed antigen that is shared by *B. henselae* and *B. quintana*. It is possible that the 17-kDa antigen contains a major cross-reactive epitope that is detected by the IFA procedure. Epitope mapping of various domains of the 17-kDa antigen may serve to identify the shared domains of *B. henselae* and *B. quintana*. Likewise, the 17-kDa antigen may also contain species-specific epitopes that can be identified in a similar manner, allowing development of a more specific assay.

The initial results obtained by immunoblot analysis of *E. coli* recombinants expressing the 30-kDa fusion protein indicate good agreement with the results obtained by IFA assay. Twenty-two serum samples were tested by both IFA assay and immunoblotting for reactivity with the 30-kDa fusion protein. This included 13 samples that were IFA positive and 9 samples that were IFA negative. One of the IFA-positive serum samples, from a patient clinically diagnosed with CSD, failed to react with the 30-kDa fusion protein on an immunoblot. Conversely, one of the IFA-negative serum samples (from a non-CSD control patient) demonstrated strong reactivity with the 30-kDa fusion protein. Additional serum samples need to be tested for reactivity with the 30-kDa fusion protein to properly assess its potential as a diagnostic reagent. Although these initial results are encouraging, it is possible that an occasional patient with CSD does not mount an antibody response to the 17-kDa antigen. It is difficult to explain the serum sample that reacted with the 30-kDa fusion protein on an immunoblot but was negative by IFA assay. The 17-kDa antigen or the 13-kDa peptide tag of the plasmid vector may contain a cross-reactive epitope previously seen by this patient's immune system. We are in the process of purifying the fusion protein from *E. coli* lysates and proteolytically cleaving the antigenic peptide from the tag-peptide to further assess the reactivity of this protein with additional sera from well-defined CSD cases and controls.

The fact that the 17-kDa antigen and the 30-kDa fusion protein derivative reacted well on immunoblots suggests that the predominant antibody response is directed to an epitope that is not irreversibly altered by denaturation. Solubilization at either 37 or 100°C had no effect on immunoblot reactivity. Thus, a sequential epitope is likely recognized by patients with CSD. Epitope mapping of individual domains of the 17-kDa antigen should allow identification of the short amino acid sequences that retain reactivity with human sera. This synthetic peptide(s) or purified 30-kDa fusion protein may be a useful antigen for a recombinant-derived antigen enzyme immunoassay or other serologic assay. A recombinant-derived antigen should allow development of a diagnostic serologic test devoid of the subjective interpretation and technical difficulties of existing serologic tests. Alternatively, recombinant-expressed proteins could be used as antigens in an agglutination test that permits the development of a simpler serologic test. Such a test

is needed to allow serologic diagnosis of CSD on a widespread and routine basis.

We have begun a study designed to identify the major antigenic proteins of *Bartonella* spp. Since *B. henselae* has only recently been associated with CSD and other opportunistic infections seen primarily among patients with AIDS, little is known about the immune responses to individual *B. henselae* antigens. Utilizing recombinant DNA techniques, we have isolated clones expressing an immunoreactive 17-kDa antigen of *B. henselae*. This antigen is highly reactive with sera from patients with CSD and may be of value as a diagnostic reagent. Rapid and objective serologic diagnosis will allow the efficient diagnosis and treatment of *Bartonella* infection. Although the efficacy of antibiotic treatment for uncomplicated CSD remains uncertain (24), the successful antibiotic treatment of CSD in immunocompromised patients has been well documented (27, 34). The role of the 17-kDa antigen in eliciting a protective immune response is not yet known, but it will be the focus of additional studies. The identification of antigenic components that elicit protective immunity will be of value in preventing infection in cats. Long-term infection of cats with *B. henselae* has previously been reported (30). Hence, a vaccine that prevents long-term infection of cats may remove the reservoir of infection (19) from the environment of patients at risk for CSD. This is especially relevant for human immunodeficiency virus-infected patients, who are at high risk for opportunistic *Bartonella* infection.

REFERENCES

1. **Adal, K. A., C. J. Cockerell, and W. A. Petri.** 1994. Cat scratch disease, bacillary angiomatosis, and other infections due to *Rochalimaea*. N. Engl. J. Med. **330:**1509–1515.
2. **Anderson, B., C. Kelly, R. Threlkel, and K. Edwards.** 1993. Detection of *Rochalimaea henselae* in cat-scratch disease skin test antigens. J. Infect. Dis. **168:**1034–1036.
3. **Anderson, B., and G. McDonald.** 1993. Construction of DNA libraries of A-T rich organisms using *Eco*RI activity. Anal. Biochem. **211:**325–327.

3a. **Anderson, B., K. Sims, D. Jones, W. Dewitt, and W. Bibb.** 1993. Molecular cloning of *Rochalimaea henselae* antigens, abstr. D-90, p. 111. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology 1993. American Society for Microbiology, Washington, D.C.

4. **Anderson, B., K. Sims, R. Regnery, L. Robinson, M. J. Schmidt, S. Goral, C. Hager, and K. Edwards.** 1994. Detection of *Rochalimaea henselae* DNA in specimens from cat scratch disease patients by PCR. J. Clin. Microbiol. **32:**942–948.
5. **Barka, N. E., T. Hadfield, M. Patnaik, W. A. Schwartzman, and J. B. Peter.** 1993. EIA for detection of *Rochalimaea henselae*-reactive IgG, IgM, and IgA antibodies in patients with suspected cat-scratch disease. J. Infect. Dis. **167:** 1503–1504.
6. **Brenner, D. J., D. G. Hollis, C. W. Moss, C. K. English, G. S. Hall, J. Vincent, J. Radosevic, K. A. Birkness, W. F. Bibb, F. D. Quinn, B. Swaminathan, R. E. Weaver, M. W. Reeves, S. P. O'Connor, P. S. Hayes, F. C. Tenover, A. G. Steigerwalt, B. A. Perkins, M. I. Daneshvar, B. C. Hill, J. A. Washington, T. C. Woods, S. B. Hunter, T. L. Hadfield, G. W. Ajello, A. F. Kaufman, D. J. Wear, and J. D. Wenger.** 1991. Proposal of *Afipia* gen. nov., with *Afipia felis* sp. nov. (formerly the cat scratch disease bacillus), *Afipia clevelandensis* sp. nov. (formerly the Cleveland Clinic Foundation strain), *Afipia broomeae* sp. nov., and three unnamed genospecies. J. Clin. Microbiol. **29:**2450–2460.
7. **Brenner, D. J., S. P. O'Connor, H. H. Winkler, and A. G. Steigerwalt.** 1993. Proposals to unify the genera *Bartonella* and *Rochalimaea*, with descriptions of *Bartonella quintana* comb. nov., *Bartonella vinsonii* comb. nov., *Bartonella henselae* comb. nov., and *Bartonella elizabethae* comb. nov., and to remove the family *Bartonellaceae* from the order *Rickettsiales*. Int. J. Syst. Bacteriol. **43:**777–786.
8. **Carithers, H. A.** 1985. Cat-scratch disease: an overview based on a study 1,200 patients. Am. J. Dis. Child. **139:**1124–1133.
9. **Daly, J. S., M. G. Worthington, D. J. Brenner, C. W. Moss, D. G. Hollis, R. S. Weyant, A. G. Steigerwalt, R. E. Weaver, M. I. Daneshvar, and S. P. O'Connor.** 1993. *Rochalimaea elizabethae* sp. nov. isolated from a patient with endocarditis. J. Clin. Microbiol. **31:**872–881.
10. **Debre, R., M. Lamy, M.-L. Jammet, L. Costil, and P. Mozziconacci.** 1950. La maladie des griffes de chat. Bull. Mem. Soc. Med. Hop. Paris **66:**76–79.
11. **Dolan, M. J., M. T. Wong, R. L. Regnery, J. H. Jorgensen, M. Garcia, J. Peters, and D. Drehner.** 1993. Syndrome of *Rochalimaea henselae* adenitis suggesting cat-scratch disease. Ann. Intern. Med. **118:**331–336.
12. **Drancourt, M., J. L. Mainardi, P. Brouqui, F. Vandenesch, A. Carta, F. Lehnert, J. Etienne, F. Goldstein, J. Acar, and D. Raoult.** 1995. *Bartonella* (*Rochalimaea*) *quintana* endocarditis in three homeless men. N. Engl. J. Med. **332:**419–423.
13. **English, C. K., D. J. Wear, A. M. Margileth, C. R. Lissner, and G. P. Walsh.** 1988. Cat-scratch disease isolation and culture of the bacterial agent. JAMA **259:**1347–1352.
14. **Genetics Computer Group.** 1994. Program manual for the Wisconsin package, version 8. Genetics Computer Group, Madison, Wis.
15. **Gold, L., D. Pribnow, T. Schneider, S. Shinedling, B. Singer, and G. Stormo.** 1981. Translation initiation in prokaryotes. Annu. Rev. Microbiol. **35:**365–407.
16. **Hall, A. V., C. M. Roberts, P. D. Maurice, K. A. McLean, and S. Shousha.** 1988. Cat-scratch disease in patients with AIDS: atypical skin manifestation. Lancet **ii:**453–454.
17. **Hawley, D. K., and W. R. McClure.** 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. **11:**2237–2255.
18. **Jackson, L. A., B. A. Perkins, and J. D. Wenger.** 1993. Cat scratch disease in the United States: an analysis of three national databases. Am. J. Public Health **83:**1707–1711.
19. **Koehler, J. E., C. E. Glaser, and J. W. Tappero.** 1994. *Rochalimaea henselae* infection: new zoonosis with the domestic cat as reservoir. JAMA **271:**531–535.
20. **Koehler, J. E., P. E. LeBoit, B. M. Egbert, and T. G. Berger.** 1988. Cutaneous vascular lesions and disseminated cat-scratch disease in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. Ann. Intern. Med. **109:**449–455.
21. **Koehler, J. E., F. D. Quinn, T. G. Berger, P. E. LeBoit, and J. W. Tappero.** 1992. Isolation of *Rochalimaea* species from cutaneous and osseous lesions of bacillary angiomatosis. N. Engl. J. Med. **327:**1625–1631.
22. **LeBoit, P. E., T. G. Berger, B. M. Egbert, S. B. Yen, M. H. Stoler, T. A. Bonfiglio, J. A. Strauchen, C. K. English, and D. J. Wear.** 1988. Epithelioid haemangioma-like vascular proliferation in AIDS: manifestation of cat scratch disease bacillus infection. Lancet **i:**960–963.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Margileth, A. M.** 1992. Antibiotic therapy for cat-scratch disease: clinical study of therapeutic outcome in 268 patients and a review of the literature. Pediatric. Infect. Dis. J. **11:**474–478.
25. **Margileth, A. M.** 1992. Sorting out the causes of lymphadenopathy. Contemp. Pediatr. **12:**23–40.
26. **Miller, J. F., J. F. Mekalanos, and S. Falkow.** 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science **243:** 916–922.
27. **Mui, B. S. K., M. E. Mulligan, and W. L. George.** 1990. Response of HIV-associated disseminated cat-scratch disease to treatment with doxycycline. Am. J. Med. **89:**229–231.
28. **Patnaik, M., W. A. Schwartzman, N. E. Barka, and J. B. Peter.** 1992. Possible role of *Rochalimaea henselae* in pathogenesis of AIDS encephalopathy. Lancet **340:**971.
29. **Regnery, R. L., B. E. Anderson, J. E. Clarridge III, M. C. Rodriguez-Barradas, D. C. Jones, and J. H. Carr.** 1992. Characterization of a novel *Rochalimaea* species, *R. henselae* sp. nov., isolated from blood of a febrile, human immunodeficiency virus-positive patient. J. Clin. Microbiol. **30:**265–274.
30. **Regnery, R. L., M. Martin, and J. Olson.** 1992. Naturally occurring "*Rochalimaea henselae*" infection in domestic cat. Lancet **340:**557–558.
31. **Regnery, R. L., J. G. Olson, B. A. Perkins, and W. Bibb.** 1992. Serologic to "*Rochalimaea henselae*" antigen in suspected cat scratch disease. Lancet **339:**1443–1445.
32. **Relman, D. A., P. W. Lepp, K. N. Sadler, and T. M. Schmidt.** 1992. Phylogenetic relationships among the agent of bacillary angiomatosis, *Bartonella bacilliformis*, and other alpha-proteobacteria. Mol. Microbiol. **6:**1801–1807.
33. **Relman, D. A., J. S. Loutit, T. M. Schmidt, S. Falkow, and L. S. Tompkins.** 1990. The agent of bacillary angiomatosis: an approach to the identification of uncultured pathogens. N. Engl. J. Med. **23:**1573–1580.
34. **Rudikoff, D., R. G. Phelps, R. E. Gordon, and E. J. Bottone.** 1989. Acquired immunodeficiency syndrome-related bacillary vascular proliferation (epithelioid angiomatosis): rapid response to erythromycin therapy. Arch. Dermatol. **125:**706–707.
35. **Silhavy, T. J., S. A. Benson, and S. D. Emr.** 1983. Mechanisms of protein localization. Microbiol. Rev. **47:**313–344.
36. **Slater, L. N., D. F. Welch, D. Hensel, and D. W. Coody.** 1990. A newly recognized fastidious gram-negative pathogen as a cause of fever and bacteremia. N. Engl. J. Med. **23:**1587–1593.
37. **Stoler, M. H., T. A. Bonfiglio, R. T. Steigbigel, and M. Pereira.** 1983. An

atypical subcutaneous infection associated with acquired immune deficiency syndrome. Am. J. Clin. Pathol. **80:**714–718.
38. **Waldvogel, K., R. L. Regnery, B. E. Anderson, R. Caduff, J. Caduff, and D. Nadal.** 1995. Disseminated cat-scratch disease: detection of *Rochalimaea henselae* in affected tissues. Eur. J. Pediatr. **153:**23–27.
39. **Welch, D. F., D. A. Pickett, L. N. Slater, A. G. Steigerwalt, and D. J. Brenner.** 1992. *Rochalimaea henselae* sp. nov., a cause of septicemia, bacillary angiomatosis, and parenchymal bacillary peliosis. J. Clin. Microbiol. **30:**275–280.
40. **Zangwill, K. M., D. H. Hamilton, B. A. Perkins, R. L. Regnery, B. Plikaytis, J. L. Hadler, M. L. Cartter, and J. D. Wenger.** 1993. Cat scratch disease in Connecticut: epidemiology, risk factors and evaluation of a new diagnostic test. N. Engl. J. Med. **329:**8–13.
41. **Zhang, H., R. Scholl, J. Browse, and C. Somerville.** 1988. Double stranded DNA as a choice for DNA sequencing. Nucleic Acids Res. **16:**1220.