Borrelia burgdorferi Complement Regulator-Acquiring Surface Protein 1 of the Lyme Disease Spirochetes Is Expressed in Humans and Induces Antibody Responses Restricted to Nondenatured Structural Determinants[▽]

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***Borrelia burgdorferi* complement regulator-acquiring surface protein 1 (CRASP-1), the dominant factor H and FHL-1-binding protein of the Lyme disease spirochete *B. burgdorferi*, is implicated in pathogen persistence and was recently reported to be nonimmunogenic in humans. Here we show that serum samples from Lyme disease patients contain antibodies with exclusive specificity for nondenatured structural determinants of CRASP-1.**

Pathogenic spirochetes of the *Borrelia burgdorferi* sensu lato complex express complement regulator-acquiring surface proteins (CRASP) that bind human serum factor H (FH) and FHL-1 (2, 8, 12, 13, 19, 27). Both plasma proteins control the alternative pathway of complement activation at the level of C3b by competing with factor B for binding of C3b. In addition, FH and FHL-1 accelerate the decay of the C3 convertase, C3bBb, and act as cofactor for factor I-mediated degradation of C3b (15, 21, 29). To date, five surface-exposed *B. burgdorferi* CRASPs have been identified in complement-resistant *B. burgdorferi* isolates, including proteins that bind either both, FH and FHL-1 (CRASP-1 and CRASP-2) or factor H alone (CRASP-3, -4, and -5 proteins and Erp proteins) (1, 2, 8, 10, 11, 14, 19, 20, 23). Among CRASPs, *B. burgdorferi* CRASP-1 is the dominant FH and FHL-1 binding protein conferring complement resistance to in vitro-cultivated spirochetes (10). Recent studies showed that inactivation of the CRASP-1 gene in *Borrelia burgdorferi* results in a serum-sensitive phenotype and that complementation of the mutant strain with a CRASP-1 shuttle vector restores its resistance to complement-mediated lysis (5). These data suggest that CRASP-1 contributes to evasion and/or survival of spirochetes in humans. However, it is still debated whether spirochetes express CRASP-1 during infection in humans (4, 24, 26). In fact, a recent study showed that sera from Lyme disease patients did not contain antibodies specific for denatured recombinant CRASP-1 when tested by Western blot analysis (18).

According to its known atomic structure, native CRASP-1

represents a homodimer with a previously unknown complex protein fold (6). Since antibodies generated to in vivo-expressed CRASP-1 are expected to be directed mainly to physiological, nondenatured CRASP-1 determinants, which may be lost during denaturation in sodium dodecyl sulfate (SDS), we have now reevaluated sera from Lyme disease patients for the respective antibodies by using nondenatured recombinant CRASP-1 as a target in two independent assays.

In the present study we report that sera from patients with Lyme disease were immunoreactive with nondenatured CRASP-1, as revealed by appropriate line blot immunoassay and enzyme-linked immunosorbent assay (ELISA) test systems, but not with denatured CRASP-1 on Western blots. These data demonstrate that spirochetes express CRASP-1 in an immunogenic form during infection in humans and suggest the involvement of CRASP-1 in FH/FHL-1-mediated immune evasion strategies of *B. burgdorferi* in humans.

All patients in the present study were diagnosed and treated at the Borreliosis Clinic of the Dermatology Department, Technical University of Munich. Five Lyme patients from the Bavarian area (ACA31, ACA32, ACA54, AH17, and HAS633) with late disease manifestation, namely, acrodermatitis chronica atrophicans, were originally enrolled in the present study. We characterized immunoreactivity to CRASP-1, VlsE, and OspC in 99 patients with predominantly late Lyme disease symptoms by using ELISA on nondenatured proteins. The healthy control group consisted of blood donors from the Heidelberg Blood Bank. For SDS-polyacrylamide gel electrophoresis, 10 µg of *B. burgdorferi* ZS7 lysate or 1 µg of recombinant protein was loaded per lane. Gels were either stained with Coomassie brilliant blue R-250 or processed for Western blotting as previously outlined (28). Nitrocellulose filters were incubated with human sera (1:500) diluted in phosphate-buffered saline–3% nonfat dry milk. After a washing step, the filters were incubated with horseradish peroxidase-conjugated

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FIG. 1. Detection of antibodies specific for denatured *B. burgdorferi* proteins or for denatured recombinant Osps, CRASP-1, VlsE, and OspC in four sera derived from Lyme disease patients or from a healthy individual. The loading of cell lysates of *B. burgdorferi* ZS7 and of recombinant spirochetal proteins (1 μ g/lane), as well as the integrity of the latter, was assessed by Coomassie staining and by screening with peroxidase-conjugated anti-human antibodies. Immunoblots were screened with sera from human Lyme disease patients (ACA54, ACA31, AH17, and HAS633) and with a healthy control serum.

goat anti-human immunoglobulin G (IgG) serum (1:5,000; Dianova, Hamburg, Germany). Immunoreactive bands were visualized by addition of DAB buffer (Roche Diagnostics, Mannheim, Germany) as the substrate.

For the line immunoassay, a highly sensitive method for detecting specific antibodies to native determinants, the recombinant proteins CRASP-1 (2 ng), VlsE (6 ng), and OspC (6 ng) were transferred to the nitrocellulose membrane by a mi-

FIG. 2. Representative line immunoassays of five Lyme disease patients and a healthy control serum using nondenatured protein preparations of CRASP-1, VlsE, and OspC as targets. CRASP-1, VlsE, or OspC were also tested for factor H binding by using an affinity ligand binding immunoblot assay (factor H). Bound proteins were visualized by using monoclonal antibodies specific for CRASP-1 (RH-1) and OspC (LA97). The “ø” symbol indicates alkaline phosphatase-conjugated goat anti-mouse antibodies (1:2,000; Dianova) alone.

FIG. 3. Detection of antibodies specific for nondenatured preparations of CRASP-1, VlsE, and OspC in sera from five Lyme patients and from one healthy individual by ELISA. Plates were coated with CRASP-1, VlsE, and OspC and screened with the indicated sera, as described in Materials and Methods.

crodispensing method, followed by incubation with human sera (1:100) diluted in PBS–0.1% Tween. Binding of specific antibodies was detected by using alkaline phosphatase-conjugated goat anti-human IgG serum (1:5,000; Dianova) or anti-mouse antibodies (1:2,000; Dianova). Immunoreactive bands were visualized by the addition of 3 ml of diethanolamine buffer supplemented with BCIP (5-bromo-4-chloro-3-indolylphosphate; Sigma) at 165 μ g/ml and nitroblue tetrazolium (Sigma) at 330 μ g/ml as a substrate.

For the respective ELISA test system, nondenatured CRASP-1 was coated at a concentration of 1 μ g/ml (Maxisorp; Nunc) overnight. After washing and blocking of nonspecific binding sites, human sera were added at a dilution of 1:100. For the detection of specific antibodies, peroxidase-labeled goat anti-human IgG antibodies (1:2,000) were used as conjugates. A substrate reaction was performed with *o*-phenylendiamin dihydrochloride (Sigma) at room temperature.

Expression and enrichment of recombinant CRASP-1 have previously been described (27). For the production of recom-

binant *B. burgdorferi* OspC, the encoding gene lacking the leader peptide was cloned into the pGEX-2T vector (Amersham). The *B. burgdorferi* GST-OspC fusion protein was expressed in *Escherichia coli* JM109 and affinity purified, and recombinant OspC without glutathione *S*-transferase was obtained by thrombin cleavage (25). Recombinant *B. burgdorferi* VlsE protein was expressed and purified according to the method of Lawrenz et al. (16).

To date, antibody responses to *B. burgdorferi*, including those to outer surface proteins VlsE and OspC, have been mainly evaluated by using denatured protein preparations in Western blots (18, 26). Therefore, as expected from previous studies, sera obtained from Lyme disease patients (e.g., ACA54, ACA31, AH17, and HAS633) but not from healthy controls (one representative shown) readily reacted with whole spirochetal cell lysates using conventional Western blotting (Fig. 1). In addition, all four human sera reacted with VlsE, the most sensitive borrelial antigen for the detection of IgG antibodies, and one of four sera reacted with OspC under similar

conditions (3). However, none of these human serum samples reacted with denatured CRASP-1 in a Western blot, even after prolonged exposure, a finding that supports previous results (18). Similar results were obtained when recombinant CRASP-1 was subjected without boiling and reduction to SDS-PAGE (data not shown), indicating that SDS treatment might be the critical factor responsible for the loss of the immunogenic epitopes of CRASP-1.

In order to reveal whether sera from Lyme disease patients contain antibodies with specificities to nondenatured determinants of CRASP-1, we have used two appropriate test systems, i.e., a line immunoassay (7, 17) and an ELISA, in which nondenatured forms of recombinant *Borrelia* proteins serve as target molecules. The integrity of the three recombinant spirochetal proteins used, i.e., CRASP-1, VlsE, and OspC, was verified by Coomassie staining (Fig. 1). The monoclonal antibodies RH-1 and LA97 to CRASP-1 and OspC, respectively, were shown to be specific for both nondenatured and denatured forms of the respective proteins (Fig. 1 and 2).

As shown in Fig. 2, all five sera from selected Lyme disease patients, but not that of a healthy control, strongly reacted to nondenatured CRASP-1 in the line immunoassay. The same preparation of nondenatured CRASP-1 also reacted with the monospecific RH-1 antibody, as well as FH, as revealed by ligand affinity blotting under similar conditions. The latter finding suggests that CRASP-1 represents the functional active protein. In addition, all five human sera, but not the control sample, were shown to react to nondenatured forms of VlsE and OspC. Together, these data clearly indicate that, like VlsE and OspC, CRASP-1 is produced by *B. burgdorferi* during infection and that antibodies contained in human sera are directed exclusively to native structural determinants, most probably distinct from that of the FH binding site.

In a second approach, the four sera described above and one additional sample from another Lyme disease patient were screened on nondenatured CRASP-1 by ELISA. As shown in Fig. 3 significant amounts of antibodies to CRASP-1 were detected in sera from patients ACA31, ACA32, ACA54, AH17, and HAS633 but not in the control serum. Moreover, all five human sera also reacted with VlsE and OspC, although at highly variable levels, under these conditions. Thus, as before with the line immunoassay, the ELISA data emphasize the notion that *B. burgdorferi* express CRASP-1 in an immunogenic form during infection in humans.

Next, sera from 99 Lyme disease patients were subjected to the CRASP-1 ELISA and compared to those of healthy controls ($n = 94$). All patients met CDC criteria of Lyme disease and were treated at the Dermatology Clinic of the Technical University of Munich. As shown in Fig. 4 the vast majority of sera from Lyme disease patients, but not from healthy controls, reacted positively with nondenatured CRASP-1 in the ELISA. The mean optical density $\pm$ the standard error of the mean in 99 patients was 0.802 ± 0.025 and significantly exceeds that of the healthy control group with a calculated optical density of 0.209 ± 0.019 ($P < 0.0001$). Screening healthy humans for antibodies to *B. burgdorferi* from high-incidence areas both in the United States and in Europe has demonstrated a high rate of seropositivity, ranging from 5 to 10%, which could indicate asymptomatic *Borrelia* infections (9, 22). Therefore, the

FIG. 4. Comparison of the levels of serum IgG antibodies with specificity for nondenatured CRASP-1 in sera of 99 patients with Lyme disease and in those of 94 healthy individuals by ELISA. The statistical significance between the two groups was evaluated by the Mann-Whitney U test.

present finding that $\sim 10\%$ of sera from healthy individuals are reactive to CRASP-1 under these conditions is not unexpected.

The differential reactivity of human antibodies to nondenatured (line immunoassay and ELISA) versus denatured (Western blot) CRASP-1 may be due to the particular dimeric structure of the functionally competent CRASP-1 (6). In fact, our recent studies have shown that truncation of the C-terminal 10 residues (i.e., residues 241 to 250) of CRASP-1 resulted in destabilization of the biologically relevant dimeric structure of CRASP-1 and completely abolished binding to both immune regulators FH and FHL-1 (10). However, treatment of CRASP-1 with SDS alone does not seem to interfere with its potential to interact with FH, although it critically effects the binding of infection-induced human antibodies. Such a restricted antibody response to particular complex nondenatured epitopes of a *B. burgdorferi* protein has not been described before for other outer surface lipoproteins. These data also suggest that the CRASP-1 determinants involved in binding of FH are distinct from those relevant for the induction of human antibodies.

The expression of CRASP-1 by spirochetes during infection in humans, which are irrelevant for the zoonotic cycle of *B. burgdorferi*, may be fortuitous. One could thus speculate that *B. burgdorferi* has evolved a whole array of genes encoding CRASP-like molecules in order to guarantee its survival/persistence in the multiple reservoir host species and that one

paralog of the 14-member protein family 54 (The Institute for Genomic Research designation), i.e., CRASP-1, accidentally exhibits cross-binding potential for FH and FHL-1 from humans. However, the evolutionary significance of the phenomenon is elusive.

In summary, the data presented here show that *B. burgdorferi* expresses CRASP-1 during infection in humans and suggest that the antibodies generated are restricted to nondenatured structural determinants of the functionally active protein. The results also indicate that the antibodies produced do not interfere with the binding of human FH to CRASP-1, which may be relevant for the persistence of spirochetes in infected humans. However, further studies are required to settle the issue of whether the immunogenic epitope of CRASP-1 is distinct from that critical for factor H binding. The present findings are relevant for the analysis of humoral immune responses to proteins in general—be they of pathogen origin or any other origin—in emphasizing the need to use nondenatured recombinant proteins, in addition to denatured ones, in order to elucidate the entire array of antibody specificities generated to the respective antigen.

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