Confirmation of Tick Bite by Detection of Antibody to *Ixodes* Calreticulin Salivary Protein

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Ticks introduce a variety of pharmacologically active molecules into their host during attachment and feeding in order to obtain a blood meal. People who are repeatedly exposed to ticks may develop an immune response to tick salivary proteins. Despite this response, people usually are unaware of having been bitten, especially if they are not repeatedly exposed to ticks. In order to develop a laboratory marker of tick exposure that would be useful in understanding the epidemiology of tick-borne infection and the immune response to tick bite, we developed an enzyme-linked immunosorbent assay (ELISA) to detect antibody to a recombinant form of calreticulin protein found in the salivary glands of *Ixodes scapularis*, a member of a complex of *Ixodes* ticks that serve as the vectors for Lyme disease, human babesiosis, and human granulocytic anaplasmosis. Using this assay, we tested sera obtained from C3H/HeN and BALB/c mice before and after experimental deer tick infestation. These mice developed antibody to *Ixodes* calreticulin antigen after infestation. We then used the same assay to test sera obtained from people before and after they experienced deer tick bite(s). People experiencing deer tick bite(s) developed *Ixodes* calreticulin-specific antibody responses that persisted for up to 17 months. This *Ixodes* recombinant calreticulin ELISA provides objective evidence of deer tick exposure in people.

Ticks introduce a variety of pharmacologically active molecules into their host during feeding in order to successfully obtain a blood meal (29). An array of proteins inhibit hemostasis, block pain and itch responses, reduce inflammation, and suppress or modulate innate and specific acquired immune defenses (5, 32). Tick-transmitted pathogens are transferred to their hosts during feeding, and the actions of tick salivary proteins are essential for both tick feeding and pathogen transmission (15, 17, 22, 30, 32). Hosts may develop an immune response to tick salivary proteins following repeated tick exposure that may impair tick and pathogen viability, including cutaneous inflammation that may result in itch and an increased awareness of infesting ticks (30, 32). Experiments with laboratory animals suggest that host immune reactivity against *Ixodes scapularis* (also known as *Ixodes dammini*) protects against transmission of *Borrelia burgdorferi*, the causative agent of Lyme disease (14, 36). Hypersensitivity against *I. scapularis* bites in people also may protect against the acquisition of Lyme disease (6).

Although the human response to tick bite may include intense cutaneous inflammation with accompanying histological changes, people often are unaware of having been bitten (1, 5, 9, 20, 24–26). Quantitative biologic markers of tick exposure are needed to better understand the epidemiology, pathogenesis, immunology, and clinical manifestations of the human tick bite response. One such marker may be host antibody directed against tick antigen. The frequency of exposure to *Ixodes* ticks can be determined using whole salivary gland extract derived from *I. scapularis* and a recombinant calreticulin antigen derived from *Amblyomma americanum* (20, 24–26). No previous studies have used an *Ixodes* recombinant antigen to test deer tick exposure or examined people whose antibody status could be measured before and more than a few months after tick exposure in order to determine antibody kinetics. Accordingly, we determined whether *Ixodes* recombinant calreticulin salivary protein in an enzyme-linked immunosorbent assay (ELISA) may serve as a useful marker of deer tick exposure. In particular, we used an ELISA for detecting human antibody against *Ixodes* recombinant calreticulin salivary protein in people with defined histories of exposure to deer ticks, including some whose sera were available prior to and more than a year following tick bite.

**MATERIALS AND METHODS**

*I. scapularis* infestation of C3H/HeN and BALB/c mice. Pathogen-free *I. scapularis* ticks were obtained from a colony maintained at the University of Connecticut Health Center. Ticks were maintained at 22°C in 97% relative humidity and oversaturated potassium sulfate and with a 16-h:8-h light-dark cycle. Ticks were placed on female C3H/HeN or BALB/c mice. Larvae (200 to 300 per mouse) or nymphs (10 to 40 per mouse) were applied to the entire body of a mouse. Ticks
were then left to feed to completion over 3 to 7 days, and the engorged ticks were collected (4). In some instances, a second infestation was performed after mice were housed for 14 days. Two to three months after the last infestation, mouse blood was collected by retro-orbital bleeding, allowed to clot, and centrifuged at 150 \times 10^3 \text{ g} \text{ for } 10 \text{ min at } 4^\circ\text{C to collect sera prior to storage at } -30^\circ\text{C for further use. All animal experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health Center.}

**Human study population.** The first study group consisted of residents of Block Island, Rhode Island, who developed Lyme disease, babesiosis, or human granulocytic anaplasmosis (HGA) and enrolled in our tick-borne illness study between 1995 and 2000 as previously described (8). These subjects agreed to a history and physical examination and submission of an acute-phase and a convalescent-phase blood sample. For the purposes of this study, we included only the 10 subjects who reported no tick bite prior to illness and who had enrolled in a biannual serosurvey on Block Island for determination of antibodies to the agents of Lyme disease, babesiosis, and HGA prior to development of tick-borne illness. Thus, we were able to test serum samples for antibody against tick salivary protein before, during, and after development of tick-borne illness in these subjects. The second study group consisted of 234 Block Island residents who enrolled in our 2004 serosurvey but did not experience symptomatic Lyme disease, babesiosis, or HGA. They were asked to complete a questionnaire that included information on tick bite within the previous year and tick-associated itch, an indication of the intensity of tick exposure, as well as provide a blood sample for antibody to tick salivary protein.

Finally, we enrolled seven subjects from the Mansfield Family Practice in Mansfield, Connecticut, who had experienced an *I. scapularis* tick bite within the previous 2 days of enrollment during the summer of 2005 and who submitted the tick that bit them for identification and estimate of engorgement level. They were loaded onto a Ni^{2+}-affinity column (Qiagen, Valencia, CA) and eluted with 50 mM NaH_2PO_4, 500 mM NaCl, and 10 mM imidazole (pH 8.0). The optical density of the positive control minus that for the nonspecific binding well was less than 0.04 at 405 nm. Plates were coated with 50 μg of each recombinant antigen per well. In the intervening wells, 50 μg of BSA (Sigma) and washed five times with PBS-Tween 20. Sera from patients with a previous history of tick exposure (diluted from 1:40 to 1:320) were added to matching wells and incubated for 1 h at 37°C and washed as previously described. After incubation for 1 h with peroxidase-labeled anti-human IgG, plates were washed again as described above, and the reactions were visualized with 2,2'-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt/tetramethyl benzidine (ABTS/TMB) as a substrate. Optical density readings at 414 nm were taken on a ΔM Quant plate reader (BioTek, Winooski, VT). The optical density of the positive control minus that for the nonspecific binding well was standardized to 1.0 for IgG. A sample was considered as reactive if the net absorbance (antigen well minus the nonspecific binding well) was 3 standard deviations or more than the mean absorbance of the PBS-containing control wells. A reactive serum was defined as one reacting at a dilution equal to or greater than 1.90 for IgG.

**Western blot assays for detection of antibody against *Ixodes* recombinant calreticulin antigen.** For immunoblot analysis, tick salivary gland extract protein or purified recombinant calreticulin protein was separated, along with molecular mass standards (Bio-Rad, Hercules, CA), by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions using 12% gels (28) and transferred to nitrocellulose membranes using a Mini Trans-Blot electrophoretic

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**Insect cell culture and media for preparation of recombinant *I. scapularis* calreticulin antigens, *Trichoplusia ni* (IgG) antibody to recombinant calreticulin purified from insect cells.** A cell line was used to produce recombinant calreticulin (20). Cell culture and media for preparation of recombinant calreticulin, a recombinant salivary gland protein. Cells were started as adherent cultures and then used to inoculate 65-ml to 7-liter flasks of 293T cells (Invitrogen, Carlsbad, CA) with a 250-ml stirred cell (Millipore, Billerica, MA) fitted with low-protein-binding regenerated cellulose membranes (10-kDa molecular mass cutoff). For purification by immobilized-metal affinity chromatography, concentrates of recombinant calreticulin were loaded onto a Ni^{2+}-nitrilotriacetic acid (Qiagen) column that was pre-equilibrated and washed with 50 mM NaH_2PO_4, 500 mM NaCl, and 10 mM imidazole (pH 8.0). His-tagged protein was eluted with 50 mM NaH_2PO_4, 500 mM NaCl, and 250 mM imidazole (pH 8.0). Eluted fractions were dia lyzed against PBS and concentrated using Amicon Ultra 15 concentrators (10-kDa molecular mass cutoff, Millipore). Proteins were quantified by means of the biarchunchonic acid assay (27), and purity was assessed by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10) and immuno blot analysis (3).
TABLE 1. Development of antibody against *Ixodes* recombinant calreticulin antigen in C3H/HeN and BALB/c mice following *I. scapularis* feeding

| Mouse       | Tick stage | No. of ticks | Antibody titer |
|-------------|------------|--------------|----------------|
| C3H/HeN     | Nymph      | 30–40        | 1:80           |
| 1           | Nymph      | 30–40        | 1:160          |
| 2           | Nymph      | 30–40        | 1:160          |
| 3           | Larva      | 200–300      | 1:80           |
| 4           | Larva      | 200–300      | 1:80           |
| 5           | Larva      | 200–300      | 1:160          |
| 6           | Larva      | 200–300      | 1:160          |
| 7           | Larva      | 200–300      | 1:160          |
| 8           | Larva      | 100–200 (×2)*| 1:80           |
| 9           | Larva      | 100–200 (×2)*| 1:80           |
| 10          | None       | None         | 1:80           |
| 11          | None       | None         | 1:80           |
| BALB/c      | Nymph      | 10 (×2)      | 1:80           |
| 1           | Nymph      | 10 (×2)      | 1:80           |
| 2           | None       | None         | <1:80          |
| 3           | None       | None         | <1:80          |
| 4           | None       | None         | <1:80          |

* ×2, 2 weeks between double infestations.

RESULTS

Antibody against *Ixodes* recombinant calreticulin protein using a mouse model. C3H/HeN and BALB/c mice were allowed to feed to repletion on mice, and in some instances, the infestation was repeated 2 weeks later. Blood was obtained 2 to 3 months after the last infestation for determination of serum calreticulin antibody by ELISA. Sera from mice exposed to deer ticks contained anticalreticulin antibody at concentrations of 1:80 to 1:160, while sera from tick-naive mice were nonreactive (Table 1). Mice exposed to deer ticks develop antibody against *I. scapularis* recombinant calreticulin.

Development of antibody against *Ixodes* calreticulin protein in people exposed to deer ticks who develop tick-borne infection. We tested the sera of 10 Block Island subjects who had experienced Lyme disease, babesiosis, and/or HGA; reported no tick bite prior to infection; and submitted a serum sample before (as part of the biannual serosurvey), during, and within 3 months after the onset of illness. Seven of the 10 subjects had no detectable *I. scapularis* calreticulin ELISA antibody prior to development of infection (Table 2). All of these subjects seroconverted during or within 3 months of acute infection. Among the three subjects from whom long-term follow-up sera were available, calreticulin-specific antibody was detectable at 17 months but not at 29 months after infection in one person and present at 3 to 4 months but absent at 15 and 27 months after infection in the other two. Three of the 10 subjects had detectable calreticulin antibody prior to developing infection despite no recollection of a previous tick bite. As residents of Block Island, these subjects were very likely to have had previous tick bites. One of the three experienced a twofold rise in antibody during the interval before and after infection, while the titer did not change in the other two subjects. Positive control sera derived from pooled acute-phase sera of several patients experiencing Lyme disease were reactive against *Ixodes* recombinant calreticulin antigen, while no such antibodies were detected in the sera of two subjects who lived in Iceland, where no deer ticks are found, and in three children 1 to 2 years of age whose sera were obtained for serum electrolyte testing (Table 2). Antibody against *Ixodes* calreticulin is detectable by ELISA in people following a deer tick bite(s) but may fall to undetectable levels as early as 15 months following tick exposure.

TABLE 2. Anti-*Ixodes* calreticulin ELISA antibody in subjects before and after Lyme disease, Lyme disease and babesiosis, or HGA

| Subject | Illness          | Prior to illness | Following illness | Result: |
|---------|------------------|-----------------|------------------|--------|
| 1       | Lyme disease     | 26 <1:40        | 1 1:40 4 1:160   | 27 <1:40 |
| 2       | Lyme disease     | 26 <1:40        | 1 1:40 3 1:80    | 15 <1:40 |
| 3       | Lyme disease     | 31 <1:40        | 1 1:40 17 1:80   | 29 <1:40 |
| 4       | Lyme disease     | 44 <1:40        | 1 1:40 2 1:160   |       |
| 5       | Lyme disease     | 21 <1:40        | 1 1:40 2 1:40    |       |
| 6       | Lyme disease-babesiosis | 72 <1:40 | 1 1:40 3 1:160 |       |
| 7       | Lyme disease-babesiosis | 3 <1:40 | 1 <1:40 3 1:80 |       |
| 8       | Lyme disease     | 13 1:640        | 1 1:640 2 1:640  |       |
| 9       | Lyme disease-babesiosis | 11 1:160 | 1 1:160 7 1:160 |       |
| 10      | HGA              | 13 1:320        | 1 1:640          |       |

Controls
Positive Lyme disease 1:160
Negative None <1:40
Confirmation that antibody detected by *Ixodes* recombinant calreticulin ELISA is directed at calreticulin. We used a Western blot assay with *Ixodes* recombinant calreticulin as the antigen to test the sera of five of the same Lyme disease subjects who had developed antibody against *Ixodes* calreticulin as measured by ELISA. In each of five Lyme disease serum pairs, a reactive calreticulin band was absent in sera collected before the development of Lyme disease and present in sera obtained after the development of Lyme disease (Fig. 1). A sample of positive control sera from pooled acute-phase sera of several patients experiencing Lyme disease also was reactive against the *Ixodes* calreticulin protein band. In contrast, sera obtained from a control subject with no history of tick bites who lived in Iceland failed to react to the *Ixodes* calreticulin protein band.

Development of antibody against *Ixodes* calreticulin protein in people exposed to deer ticks who do not develop tick-borne infection. We determined whether anticalreticulin antibody forms in people who were bitten by *I. scapularis* ticks but who removed these ticks before transmission of tick-borne infection could develop. Seven subjects who experienced a tick bite within the previous 2 days submitted the *I. scapularis* tick that bit them and a blood sample. The sera from each of these subjects reacted against *Ixodes* calreticulin antigen within 1 to 3 days after exposure. Serum samples were obtained from four of these people 4 weeks after exposure, and all were reactive, although only one increased above the previous titer (Table 3). The same positive and negative control sera used in previous experiments (as depicted in Table 2) were reactive and non-reactive, respectively. People who experience a deer tick bite but no tick-borne infection develop antibody against *Ixodes* calreticulin antigen.

**Association between self-reports of tick bite and the presence of anti-*Ixodes* calreticulin antibody.** We tested the sera of 234 residents of Block Island who participated in the 2004 Block Island serosurvey for antibody against *Ixodes* recombinant calreticulin. We compared their antibody response with a history of tick bite within the previous year, tick bite-associated itch, and the presence of antibody against *B. burgdorferi* and *Babesia microti* (Table 4). None had experienced symptomatic tick-borne illness within the previous year, and most provided a complete history and sufficient sera for testing, although some residents did not respond to questions about tick bite or tick-associated itch. Only 6% (*n = 14*) had detectable antibody against *Ixodes* calreticulin. A significant association was found

### TABLE 3. Anti-*Ixodes* calreticulin antibody in subjects 1 to 2 days and 4 weeks after *I. scapularis* tick bite

| Case | Description of attached tick | Stage | Feeding status | No. of bites in past year | Antibody titer at: |
|------|-----------------------------|-------|----------------|----------------------------|-------------------|
|      |                             |       |                |                            | 1–3 titer | 4 wk |
| 1    | Larva                       | Unfed | ~60            | 2                          | 1:80     | 1:160 |
| 2    | Nymph                       | Fully fed | 1          | 2                          | 1:80     | 1:80  |
| 3    | Nymph                       | Partially fed | 1        | 5                          | 1:80     | 1:80  |
| 4    | Nymph                       | Partially fed | 3        | Uncertain                  | 1:80     | 1:80  |
| 5    | Nymph                       | Partially fed | 2        | 2                          | 1:80     |       |
| 6    | Larva                       | Fully fed | 1          | Uncertain                  | 1:160    |       |
| 7    | Adult                       | Partially fed | 2        | 1                          | 1:640    |       |

Controls
Positive 1:160
Negative <1:40
between seroreactivity to *Ixodes* recombinant calreticulin and the presence of antibody against *B. burgdorferi* and *Babesia microti*, but not with a history of tick bite or tick-associated itch. The sera from about half of the subjects whose sera contained antibody against *B. burgdorferi* and *Babesia microti* were nonreactive against calreticulin antigen, presumably because these subjects had experienced Lyme disease or babesiosis a year or more before their sera were tested for anticalreticulin antibody. The persistence of anticalreticulin antibody generally appears to be less than that for antibody directed against either the Lyme disease or babesial pathogens. The presence of detectable anti-*Ixodes* calreticulin antibody is associated with previous *B. burgdorferi* and *B. microti* infection but not with self-reports of previous tick bite or tick-associated itch.

### DISCUSSION

We found that people experiencing a deer tick bite develop antibody against recombinant *Ixodes* calreticulin protein detectable by ELISA. Anti-*Ixodes* calreticulin antibody is present in the sera of some people within 2 days of tick exposure and may persist for as long as a year and a half. The development of antibody in some people after brief tick attachment suggests previous tick exposure and an amnestic immune response, consistent with the frequent tick exposure encountered by people living where deer ticks are highly endemic. Western blot analysis confirmed that antibody detected by ELISA is directed against calreticulin antigen. No antibody against recombinant *Ixodes* calreticulin was detected in three subjects from Iceland and two subjects less than 2 years of age who were very unlikely to have experienced tick bite. This *Ixodes* recombinant calreticulin ELISA appears to provide a reliable method for detecting IgG antibody against *Ixodes* calreticulin protein following recent *Ixodes* tick exposure.

In a previous study, antibodies to tick salivary gland sonicate were shown to be a potential biological marker of exposure to tick bites among outdoor workers (23). Rabbits experimentally infested with adult *Amblyomma americanum* or *Dermacentor variabilis* (50 females and 20 males) developed antibodies to recombinant calreticulin derived from cDNA prepared from partially fed *A. americanum* females; however, gerbils exposed to the bites of *Aedes aegypti* mosquitoes did not develop calreticulin-specific antibodies (21). Recombinant *A. americanum* salivary gland calreticulin was used to screen sera of military personnel stationed in an area where *A. americanum* is endemic. Personnel exposed to natural tick infestation developed antibodies to the salivary gland recombinant protein (21). Subjects with a recent history of exposure to bites of *I. scapularis* nymphs or adults developed increasing amounts of antibody to *A. americanum* salivary gland recombinant calreticulin over an approximately 6-week period after tick bite (20). A tick that became engorged was a risk factor for development of antibodies to *A. americanum* salivary gland recombinant calreticulin. These studies indicated that people exposed to *I. scapularis* ticks develop antibody to *A. americanum* calreticulin antigen. Calreticulin is a highly conserved protein among tick species (36). Unlike previous reports, we used *Ixodes* recombinant calreticulin antigen and studied a population whose tick-borne disease and tick exposure history were well defined and whose sera were available before and more than a year after tick bite exposure. Consequently, we were able to derive information about the kinetics of antibody persistence in the apparent absence of reexposure to ticks and to rule out any possible cross-reactivity between *B. burgdorferi* and *Ixodes* calreticulin antigen.

Variations in antibody responses to tick bite of participants in this study likely reflect the level of exposure to tick antigens and the balance between the ability of the subject to mount a response and tick modulation of those host responses. The number and frequency of tick bites determine the development of acquired resistance and cutaneous reactivity to tick feeding, which is mediated in part by circulating and homocytotropic antibodies and cell-mediated immune responses (5, 32, 33). Tick feeding has been shown to reduce the ability of the host to develop an antibody response (7, 13, 31). While it was not possible to document all previous arthropod bites that might have altered the calreticulin antibody status of our subjects, any such effect would have been mitigated by testing each subject following a well-defined tick exposure.

Although our *Ixodes* recombinant calreticulin ELISA appears to provide a reliable method for detection of recent exposure to deer ticks, the results of this assay do not correlate well with self-reported tick bite or tick-associated itch in residents living in areas where deer tick-borne disease is endemic. Several possible explanations may account for this apparent contradiction. Tick bites usually go unnoticed. Only about a third of people who experience Lyme disease report being bitten by a tick, in part because saliva of *I. scapularis* contains kininase activity and histamine binding proteins that reduce host pain and itch responses (16, 18, 35). This would weaken the observed association between anticalreticulin antibody and self-reports of tick bite due to misclassification and thus lead to a decreased chance of detecting such an association. The duration of deer tick attachment may be insufficient to allow for adequate amount of saliva to elicit a detectable antibody response, several tick exposures may be required in some people before the antibody response is strong enough to be detected by ELISA, or people may confuse the bite of non-*Ixodes* ticks or other arthropods with that of a deer tick and such bites might not elicit calreticulin antibody. Finally, our data indicate that serum antibody concentration decreases with time after tick bite and may become undetectable in people who have

### TABLE 4. Relationship between anti-*Ixodes* calreticulin antibody in 234 Block Island residents and a history of tick bite within the previous year, tick-associated itch, and concurrent antibody against *B. burgdorferi* and *B. microti*

| Indicator of tick exposure | No. of subjects with antibody |  \( P \) value |
|---------------------------|-------------------------------|-------------|
|                           | Anti-*Ixodes* calreticulin    | No anti-*Ixodes* calreticulin |
|                           | antibody status/no. with indicator | antibody |
| Report of tick bite       | 2/11                          | 48/200      | 1.000 |
| Report of tick-associated itch | 1/14                      | 47/214      | 0.311 |
| *B. burgdorferi* antibody (total) | 8/14                       | 64/220      | 0.029 |
| *B. microti* antibody     |                               |             |
| IgM                       | 5/14                          | 13/220      | 0.002 |
| IgG                       | 5/14                          | 0/220       | <0.001 |
experienced a deer tick bite more than 2 years prior to testing. Any one of these events would weaken the association between self-reported tick bites and anticalreticulin titers.

The most important practical value of this Ixodes calreticulin ELISA is to confirm recent deer tick exposure. Anticalreticulin antibody may be detectable as early as a few days after tick bite in people who have been preexposed to ticks or as long as 2 to 3 months after tick exposure. Epidemiologic and clinical studies of tick-borne disease are complicated by the poor reliability of tick exposure history because tick bites generally are not noticed and people may mistake the bite of another arthropod as that of a tick. The Ixodes calreticulin ELISA also may increase our understanding of the human immune response to tick bite and help confirm whether hypersensitivity to Ixodes bites protects people against infection by the agent of Lyme disease. Feeding of ticks and other blood-feeding arthropods is facilitated by saliva that contains mixtures of pharmacologically active molecules capable of inhibiting host pain/itch responses, hemostasis, and immune defenses (5, 12, 22, 32). Tick pathogen transmission is enhanced because lack of awareness of tick attachment allows sufficient time for transmission to occur and perhaps because pathogen survival is improved by the immunosuppressive action of tick salivary proteins at the site of skin attachment (5). Repeated exposure to the bites of pathogen-free ticks in animals elicits host responses that protect against subsequent tick transmission of infectious agents (2, 34). These immune responses neutralize tick countermeasures against host defenses and lead to rejection of feeding ticks (5, 32). The Ixodes calreticulin ELISA appears to be a useful diagnostic indicator of exposure to Ixodes ticks and may be useful in developing a vaccine against tick-borne infections based on tick salivary protein.

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