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

Running title: *Ixodes* Calreticulin Antibody Assay

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ABSTRACT

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 ELISA assay 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. We tested sera obtained from C3H/HeN and BALB/C mice before and after experimental deer tick infestation using this assay. 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 antibody assay 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 (9, 12, 14). 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, 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 ELISA assay may serve as a

useful marker of deer tick exposure. In particular, we used an ELISA assay for detecting human antibody against *Ixodes* recombinant calreticulin salivary protein in people with defined histories of exposure to deer ticks, including some whose sera was 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, 97% relative humidity and over saturated potassium sulfate, and a 16:8 hr 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 retroorbital bleeding, allowed to clot, and centrifuged at 150 x g for 10 minutes at 4°C to collect sera prior to storage at -30°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 Block Island, Rhode Island residents 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 and convalescent blood sample. For the purposes of this study, we only included 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 two 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 asked to provide a history, undergo a physical examination, provide a blood sample for determining their antibody against tick salivary protein and to return four to six weeks later for a clinical examination and for blood testing for antibody against tick salivary protein.

Positive control sera consisted of pooled acute sera from Connecticut residents who had experienced Lyme disease within the previous three months and whose sera contained anti-*B. burgdorferi* antibody. Negative control sera were obtained from two residents of Iceland where no vector ticks are found and from three children between one to two years of age living in Connecticut whose sera were obtained for routine serum electrolyte testing. Sera was extracted immediately after blood drawing and maintained frozen at -80°C until testing. Negative control

sera were individually tested in all experiments. Written informed consent was obtained from study participants in accordance with human experimentation guidelines approved by the institutional review boards at Connecticut Children's Medical Center and the Harvard School of Public Health.

Insect cell culture and media for preparation of recombinant *I. scapularis* calreticulin antigen. *Trichoplusia ni* cells (BTI-TN-5B1-4, High 5^{TM}, Invitrogen, Carlsbad, CA) used in protein expression were grown at 27°C in Express 5 serum-free medium (Invitrogen), supplemented with 17 mM L-glutamine, 10 µg/ml gentamicin (GIBCO, Carlsbad, CA), and 10 µg/ml Blasticidin (Invitrogen). Cells were started as adherent cultures and then used to inoculate 65-ml to 125-ml spinner flask suspension cultures at an initial density of 1.0 x 10^6 cells/ml. Once a density of 6-7 x 10^6 cells/ml was reached, cells were harvested for processing.

Rapid amplification of cDNA ends (RACE) synthesis for preparation of recombinant *Ixodes* calreticulin antigen. Prior to dissection, partially fed female *I. scapularis* ticks were washed with sterile 0.1 M phosphate-buffered saline (PBS; pH 7.4). Salivary glands were then removed, rinsed into 500 ul of sterile EDTA-free PBS (Roche, Indianapolis, IN) and transferred immediately into a 2.0-ml cryovial containing 1.0 ml RNALater RNA storage solution (Ambion, Austin, TX). The suspensions of glands were then stored at -80°C until use.

Poly A+ mRNA was isolated from tick salivary glands with the Oligotex Direct mRNA Micro Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. First-strand cDNA was isolated from tick salivary gland poly A+ mRNA and used directly in 5'- and 3'-RACE polymerase chain reaction (PCR), using the SMART RACE cDNA Amplification kit (Clontech, Mountain View, CA) according to manufacturer instructions. Calreticulin-specific oligonucleotide primers were designed from the consensus sequence resulting after aligning

published calreticulin sequences from the ticks *Amblyomma americanum* (U07708) and *Boophilus microplus* (AF420211). Sequences were as follows: sense primer Calret3 5' - ACTCGGGCTTGTCCCAGTCCTCGGG - 3' and antisense primer Calret5 5'- AAGCACGAGCAGAACATCGACTGCG- 3'. These primers created overlapping 5'- and 3'-RACE products, respectively, that were joined by restriction digestion and ligation using a restriction site *Xho*I in the region of the overlap to create the full-length cDNA. The resulting 1,548 bp cDNA was then used as template to generate a PCR product containing the entire 5'-end of the gene and the 3'-end up to the stop codon. Oligonucleotide primers used for this purpose were CalretFFL 5'- GGCTTCTAATACGACTCACTATAGGG -3' and CalretRFL 5'-CACAAGTTCCTCGTGGTCGTGCTTG -3'. The stop codon was eliminated in order to allow fusion of the C-terminal region of the expressed protein to a 6X His tag to facilitate protein purification. The 1,349-bp PCR product was then cloned into the pIB/V5-His-TOPO expression vector (Invitrogen), which contains the blasticidin resistance gene for selection of cells that are stably transfected. Transcription of the calreticulin insert was driven by the baculovirus *Orgyia pseudotsugata* immediate-early 2 promoter *(OpIE2)*(28). The resulting construct, pIB/Calreticulin, was then sequenced in both directions using standard dideoxynucleotide sequencing procedures (19). After aligning published calreticulin protein sequences from various *Ixodes* tick species (*Ixodes woodi*, *Ixodes ricinus*, *Ixodes persulcatus*, *Ixodes pararicinus*, *Ixodes pacificus*, *Ixodes pavlovskyi*, *Ixodes ovatus*, *Ixodes nipponensis*, *Ixodes muris*, *Ixodes minor*, *Ixodes jellisoni*, *Ixodes affinis*) with the *I. scapularis* used in this study, we found that the difference in amino acid sequences between species was negligible with an average percent identity of approximately 98% between all sequences. These data indicate that antibody response

177 to recombinant *Ixodes scapularis* calreticulin antigen would be the same as that against any other
178 recombinant *Ixodes* calreticulin antigen.

179 Endotoxin-free pIB/Calreticulin recombinant plasmid was purified using the EndoFree
180 Plasmid Maxi Kit (Qiagen) and transfected into High 5^{TM} cells according to protocols supplied
181 by the manufacturer. Stably transfected cells were maintained routinely in medium containing
182 blasticidin at a final concentration of 10 μg/ml. For media collection, cells were grown to a
183 density of greater than 6-7 x 10^6 cells/ml, at which point media were collected and centrifuged at
184 6,000 x g to remove cells and particulate matter. Cell culture supernatants were then buffer-
185 exchanged with PBS, pH 7.2, and concentrated at least 10X with a 250-ml stirred cell (Millipore,
186 Billerica, MA) fitted with low protein-binding regenerated cellulose membranes (10 kDa
187 MWCO).

188 For purification by immobilized-metal affinity chromatography (IMAC), concentrates were
189 loaded onto a Ni^{+2}-Nitriloacetic acid (NiNTA, Qiagen) column that was pre-equilibrated and
190 washed with 50 mM NaH_2PO_4, 500 mM NaCl, 10 mM Imidazole, pH 8.0. His-tagged protein
191 was eluted with 50 mM NaH_2PO_4, 500 mM NaCl, and 250 mM Imidazole, pH 8.0. Eluted
192 fractions were dialyzed against PBS and concentrated, using Amicon Ultra 15 concentrators (10
193 kDa MWCO, Millipore). Proteins were quantified by means of the bicinchoninic acid (BCA)
194 assay (27) and purity was assessed by discontinuous sodium dodecyl sulfate-polyacrylamide gel
195 electrophoresis (SDS-PAGE) (10) and immunoblot analysis (3).

196 **ELISA for detecting antibody against *Ixodes* recombinant calreticulin antigen.** The
197 ELISA assay is a quantitative microtiter method for detecting IgG antibody to recombinant
198 *Ixodes* calreticulin purified from High 5 cells. This ELISA method is a modification of that
199 described by Magnarelli et. al. (11). Recombinant calreticulin was added to alternate wells of

flat-bottom microdilution plates at a final concentration of 5 μg per well. In the intervening wells, 50 μL of PBS (1.54 mM KH_2PO_4, 2.7 mM Na_2HPO_4.7H2O, 154 mM NaCl, pH 7.2) was added to test for nonspecific binding. The plates were blocked with PBS (200 uL) containing five percent horse serum (JR Scientific, Woodland, CA) and 0.01 percent dextran sulfate (Sigma) and washed 5X with PBS-Tween 20. Sera from selected patients with a previous history of tick exposure (diluted from 1:40 to 1:320) were added to matching wells and incubated for one hour at 37ºC and washed as previously described. After incubation for one hour with peroxidase-labeled anti-human IgG, plates were washed again as described above and the reactions visualized with 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt/Tetramethyl benzidine (ABTS/TMB) as substrate. Optical density readings at 414 nm were taken on a μQuant plate reader (BioTek, Winooski, VT). The optical density of the positive control minus that for the non-specific binding well was standardized to 1.0 for IgG. A sample was considered as reactive if the net absorbance (antigen well minus the non-specific binding well) was three standard deviations or more than the mean absorbance of the PBS-containing comparison wells. A reactive serum was defined as one reacting at a dilution equal to or greater than 1:80 for IgG.

Western blot assays for detecting antibody against *Ixodes* recombinant calreticulin antigen. For immunoblot analysis, tick salivary gland extract protein or purified recombinant calreticulin protein were separated, along with molecular mass standards (BioRad, Hercules, CA) by SDS-PAGE under reducing conditions using 12% gels (28) and transferred to nitrocellulose membranes using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Human sera were diluted 1:2000 in blocking buffer (10 mm Tris-HCl, 300 mM NaCl, pH 7·4, 5% non-fat dry milk) and incubated for 1 hour at room temperature (11). Antigen binding was detected with

horseradish peroxidase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:10,000 in blocking buffer and reacted for 1 hour at room temperature. Antigen-antibody complexes were visualized by chemiluminescence (Supersignal, Pierce, Rockford, IL) on radiography film (Kodak, Rochester, NY).

Statistical analysis. Fisher's exact test was used to compare two proportions (2x2 contingency tables). Exact binomial confidence intervals were calculated for individual proportions. SAS 9 for Windows (SAS Institute Inc., Cary, NC) was used for statistical analysis.

RESULTS

Antibody against *Ixodes* recombinant calreticulin protein using a mouse model. C3H/HeN and BALB/C mice were exposed to deer ticks and tested for antibody against *Ixodes* recombinant calreticulin. Larval or nymphal *I. scapularis* ticks were allowed to feed to repletion on mice and in some instances the infestation was repeated two weeks later. Blood was obtained two to three months after the last infestation for determination of serum calreticulin antibody by ELISA. Sera from mice exposed to deer ticks contained anti-calreticulin antibody at concentrations of 1:80 to 1:160 while sera from tick naïve mice were non-reactive (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 three 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 three months of acute infection. Among the three subjects from whom long term follow-up sera was 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 two-fold 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 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 one to two years of age whose sera was 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.

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 sera 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 (Figure 1). A sample of positive control sera from pooled acute sera of several patients experiencing Lyme disease also were reactive against the *Ixodes* calreticulin protein band. In contrast, sera obtained from a

control subject who lived in Iceland with no history of tick bites, 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 anti-calreticulin 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 two 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 one to three days after exposure. Serum samples were obtained from four of these people four 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.

Correlation of 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 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 non-reactive against calreticulin antigen, presumably because these subjects had experienced Lyme disease or babesiosis a year or more before their sera was tested for anti-calreticulin antibody. The persistence of anti-calreticulin antibody generally appears to be less than that 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 two 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 two 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 endemic for *A. americanum*. 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 six 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 absence of re-exposure 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 determines the development of acquired resistance and cutaneous reactivity to tick feeding, which is mediated in part by circulating and homocytotropic antibodies and cell mediated

337 immune responses (5, 32-33). Tick feeding has been shown to reduce the ability of the host to
338 develop an antibody response (7, 13, 31). While it was not possible to document all previous
339 arthropod bites that might have altered the calreticulin antibody status of our subjects, any such
340 effect would have been mitigated by testing each subject following a well defined tick exposure.
341 Although our *Ixodes* recombinant calreticulin ELISA appears to provide a reliable method
342 for detecting recent exposure to deer tick, the results of this assay do not correlate well with self-
343 reported tick bite or tick-associated itch in residents living in areas where deer tick-borne disease
344 is endemic. Several possible explanations may account for this apparent contradiction. Tick bites
345 usually go unnoticed. Only about a third of people who experience Lyme disease report being
346 bitten by a tick, in part because saliva of *I. scapularis* contains kininase activity and histamine
347 binding proteins that reduce host pain and itch responses (16, 18, 35). This would weaken the
348 observed association between anti-calreticulin antibody and self-reports of tick bite due to
349 misclassification and thus lead to a decreased chance of detecting such an association**.** The
350 duration of deer tick attachment may be insufficient to allow for adequate amount of saliva to
351 elicit a detectable antibody response, several tick exposures may be required in some people
352 before the antibody response is strong enough to be detected by ELISA, or people may confuse
353 the bite of non-*Ixodes* ticks or other arthropods with that of a deer tick and such bites might not
354 elicit calreticulin antibody. Finally, our data indicates that serum antibody concentration
355 decreases with time after tick bite and may become undetectable in people who have experienced
356 a deer tick bite more than two years prior to testing. Any one of these events would weaken the
357 association between self-reported tick bites and anti-calreticulin titers.
358 The most important practical value of this *Ixodes* calreticulin ELISA is to confirm recent deer
359 tick exposure. Anti-calreticulin antibody may be detectable as early as a few days after tick bite

in people who have been prexposed 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 protect people against infection by the agent of Lyme disease. Feeding of ticks and other blood feeding arthropods are facilitated by saliva that contain 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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509 TABLE 1. Development of antibody against *Ixodes* recombinant calreticulin antigen in

510 C3H/HeN and BALB/C mice following *I. scapularis* feeding

511

Mouse	Tick stage	Number of ticks	Antibody titer
C3H/HeN			
1	Nymph	30-40	1:80
2	Nymph	30-40	1:160
3	Nymph	30-40	1:160
4	Larvae	200-300	1:80
5	Larvae	200-300	1:160
6	Larvae	200-300	1:160
7	Larvae	200-300	1:160
8	Larvae	100-200 x 2[a]	1:80
9	Larvae	100-200 x 2	1:160
10	none	none	<1:80
11	none	none	<1:80
BALB/C			
1	Nymph	10 x 2	1:80
2	Nymph	10 x 2	1:80
3	none	none	<1:80
4	none	none	<1:80

512 [a] two weeks between double infestations

513

514 TABLE 2. Anti-*Ixodes* calreticulin ELISA antibody in subjects before and after Lyme disease,
515 Lyme disease and babesiosis, or human granulocytic anaplasmosis (HGA)
516

Subject	Illness	Prior to illness		Following illness					
		Month	Antibody titer	Month	Antibody titer	Month	Antibody titer	Month	Antibody titer
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	160	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	Lyme disease				1:160				
- controls	None		<1:40						

517
518

519 TABLE 3. Anti-*Ixodes* calreticulin antibody in subjects one to two days and
520 four weeks after *I. scapularis* tick bite
521

Case	Description of attached tick			Bites in past year	Antibody titer	
	Stage	Feeding status	No. attached		1-3 days	4 weeks
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					1:160	
- controls					<1:40	

522

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

Indicator of tick exposure	Anti-*Ixodes* calreticulin antibody	No anti-*Ixodes* calreticulin antibody	P value
Report of tick bite	2/11	48/200	1.000
Report of tick 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
B. microti antibody (IgG)	5/14	0/220	<0.001

526

FIG 1. Western blots of sera against *Ixodes* recombinant calreticulin (10µg/strip) from people before (B) and within three months after (A) development of Lyme disease. The ELISA antibody results of these subjects are shown in Table 2. Positive control consists of pooled acute sera from patients experiencing Lyme disease. Negative control consists of serum from a resident of Iceland.