ANTIBODY TO A cDNA-DERIVED CALRETICULIN PROTEIN FROM AMBLYOMMA AMERICANUM AS A BIOMARKER OF TICK EXPOSURE IN HUMANS

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Abstract. The antibody responses of human and animal hosts were studied to determine the utility of antibody against recombinant tick calreticulin (rTC), a cDNA-derived protein isolated from salivary glands of Amblyomma americanum L., as a biologic marker of tick exposure. Rabbits fed upon by either A. americanum or Dermacentor variabilis Say developed significant anti-rTC antibody responses, as measured by both ELISA and immunoblot assay. In contrast, gerbils exposed to Aedes aegypti did not develop anti-rTC antibodies, as measured by ELISA or immunoblot assay. The utility of the assay was next evaluated in humans at high risk for tick exposure. During April through September 1990, 192 military personnel who originated from either Fort Chaffee, Arkansas or Fort Wainwright, Alaska were studied during maneuvers in tick infested areas at Fort Chaffee. Study subjects completed a questionnaire and had pre- and post-maneuvers serum specimens analyzed for antibodies to rTC. In adjusted analysis (controlling for age, fort of origin, attached tick during maneuvers, and bed netting use), the use of bed netting and home station were associated with post-maneuvers anti-rTC antibody seropositivity by ELISA. Subjects from Fort Wainwright were more likely to be seropositive for anti-rTC antibody (adjusted odds ratio = 5.3, 95% confidence interval [CI] = 1.1–25.6). Personnel who did not report the use of bed netting were more likely to be anti-rTC seropositive (adjusted odds ratio = 6.8, 95% CI = 1.4–32.4). Immunoblot assays showed that humans had specific anti-rTC antibody responses. The animal experiments demonstrate that hosts exposed to naturally feeding ticks develop anti-rTC antibodies. The data also indicate that hosts exposed to Ae. aegypti saliva may not develop antibodies against rTC. Observations in tick-exposed humans support the hypothesis that anti-rTC antibody seropositivity is a biologic marker of tick exposure.

The measurement of human antibody response to infectious agents is common in epidemiologic research to assess exposure and the risk of disease. Humans and animals produce antibodies to salivary proteins from various blood feeding arthropods, including mosquitoes,1 Sarcoptes scabiei (scabies),2 biting flies,3 and ticks.4, 5 Since 1991, investigations have suggested that anti-tick saliva antibodies are biologic markers of exposure to ticks.6–9 For example, levels of anti-tick saliva antibody (ATSA), using sonicated whole Ixodes scapularis Say salivary glands as antigen, are associated with self-reported tick exposure, Lyme disease seroprevalence, and an index of tick engorgement.7, 10 The ATSA levels also decrease during periods of decreased tick exposure.11

Prior studies of ATSA have used whole salivary glands from two species of ticks, Ixodes scapularis and Amblyomma americanum (L.), 7, 10, 12 In a study of military personnel on maneuvers in areas of Arkansas heavily infested by A. americanum, ATSA seropositivity was associated with self-reported tick exposure, age, and military grade, and negatively associated with self-reported personal preventive behaviors. Western blots of post-maneuvers sera revealed large differences among individuals in the tick salivary gland proteins recognized by human subjects.5 In a study of tick bite subjects in Westchester County, New York,7 ATSA serologic testing was shown to have a sensitivity of 0.81 and a specificity of 0.56 for a bite of I. scapularis that became engorged. It was hypothesized that the sensitivity and specificity of ATSA as a biomarker of tick exposure may be improved by measuring the antibody response to a limited number of specific salivary proteins rather than the antibody response to whole sonicated glands.

Tick saliva contains many distinct proteins that facilitate tick feeding and affect the host immune response, and the proteins expressed in the tick salivary glands change during the course of feeding.6, 7, 13 Recently, a component of tick saliva was cloned and expressed as a recombinant protein.14 This protein, calreticulin, is a major calcium-binding protein of the endoplasmic reticulum, and appears to be secreted in the course of feeding. Calreticulin is not detectable in the saliva of unfed ticks, but begins to be secreted around the third day of feeding.14

Herein, we present evidence that the antibody response of humans and animal hosts against recombinant tick calreticulin (rTC) may be a biologic marker of tick exposure. We show that rabbits experimentally exposed to feeding ticks (both A. americanum and D. variabilis) develop antibodies that bind to recombinant tick calreticulin, and that the anti-calreticulin response increases upon secondary exposure to feeding ticks. We present indirect evidence that secreted calreticulin can be found in the saliva of D. variabilis, and that hosts exposed to the saliva of Aedes aegypti do not appear to develop antibodies against rTC. Finally, we report the use of a cDNA-derived calreticulin to measure human antibody response to this component of tick saliva in a group of mil-
itary personnel in Arkansas who had previously had ATSA measured using sonicated whole glands as antigen.

MATERIALS AND METHODS

Ticks. Adult A. americanum and D. variabilis were acquired from laboratory colonies at the Acarology Laboratory at Oklahoma State University (Stillwater, OK). Mosquitoes. Adult Ae. aegypti were laboratory reared at Johns Hopkins University according to a previously described protocol.15

Rabbits and gerbils. Female New Zealand white rabbits (3 kg) and gerbils, obtained from a commercial source (Gingrich Animal Supply, Fredericksburg, PA), were used. The animals were certified pathogen free and had no previous contact with blood-feeding arthropods. The animals were housed in a climate/light controlled facility and all procedures followed an approved animal protocol. The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals.

Antigen preparation. Recombinant tick calreticulin was prepared as described previously.14 Briefly, a truncated A. americanum calreticulin DNA clone (missing 90 basepairs of the 5′ end) was subcloned into the pRSETB (Invitrogen, San Diego, CA) expression vector and transformed into TOPP 5 cells (Stratagene, La Jolla, CA). Expression of the fusion protein was accomplished by the protocols provided by Invitrogen. Briefly, a single colony was grown in selective medium, induced with isopropyl β-D-thiogalactopyranoside (IPTG) and infected with M13/T7 phage. Cells were harvested by centrifugation and lysed in guanidinium buffer. Fusion protein was used directly after the removal of the guanidinium using a Centriprep 3 (Amicon, Beverly, MA).

Study population. The study population was part of a larger group of 1,194 Department of Defense personnel who worked or trained at Fort Chaffee, Arkansas from May 2 through September 27, 1990 and have been previously described.9,16 The population was based either at Fort Chaffee or at Fort Wainwright, Alaska and traveled to Fort Chaffee for a training exercise. Anti-rTC antibody testing was performed in 192 individuals with approximately 50 subjects randomly selected from each of four groups characterized by home fort location and self-reported tick bite status during maneuvers. Sera from subjects in Westchester County, New York who denied ever having a tick bite, and who have had low ATSA levels in prior studies using whole tick salivary glands as antigen7 were used as negative controls. Informed consent was obtained from all human participants and the appropriate institutional review boards approved the project.

Tick challenge. Three rabbits each were exposed to D. variabilis or A. americanum (50 females and 20 males) placed in ear bags as described previously.12 The ticks were allowed to feed to repletion. The number and date of tick detachment was recorded. Blood was collected from the rabbits by venipuncture at 0 (pre-exposure), 5, 10, and 15 days post-median tick removal (5, 10, and 15 days after the 25th female tick detached). The day after the last bleed, the rabbits were reinfested with the same number and species of ticks and the protocol was repeated. The blood was allowed to clot overnight at 4°C, centrifuged at 2,500 × g for 10 min, and the serum fraction was collected and stored at −70°C.

Rabbit anti-rTC ELISA. The ELISA plates were coated with 0.2 mg of rTC (in 100 ml of phosphate-buffered saline [PBS]) per well overnight at 4°C. The plates were blocked with blotto (2% nonfat milk in PBS) and sera were then added after a 1:100 dilution in blotto and incubated overnight at 4°C. The plates were then washed with PBS and a 1:1,000 dilution of goat anti-rabbit IgG (gamma chain specific) conjugated to horseradish peroxidase (The Binding Site, San Diego, CA) in blotto was added to the plates and incubated for 1 hr at 37°C. The plates were developed with ABTS (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) and the optical density (OD) was measured at 405 nm. The rabbit anti-rTC antibody OD used in the figures and results was calculated as the mean OD of two wells coated with blotto (background) subtracted from the mean OD of two wells coated with rTC antigen.

Gerbil anti-rTC ELISA. Three gerbils were anesthetized and fed upon by approximately 50 female Ae. aegypti twice, two weeks apart. The gerbils were bled four days after the second exposure. Sera were harvested as described for the rabbits. The ELISA analysis was performed as described for the rabbits, except that gerbil sera were used as the primary antibody (1:100 dilution). A 1:500 dilution of rabbit anti-gerbil IgG in blotto was added to the plates and incubated overnight at 4°C. The plates were then washed with PBS and a 1:1,000 dilution of goat anti-rabbit IgG (gamma chain specific) conjugated to horseradish peroxidase (The Binding Site) in blotto was added to the plates and incubated for 1 hr at 37°C. Gerbil sera at a 1:5,000 dilution in PBS were used to coat ELISA wells as a positive control.

Gel electrophoresis. Gel electrophoresis of the tick salivary gland preparations, using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under reducing conditions.17 Approximately 4 mg of rTC preparation was run on a precast, 4–20% polyacrylamide gradient denaturing minigel (Jules Biotechnology, New Haven, CT) along with broad range molecular weight markers (Bio-Rad Laboratories, Hercules, CA). The gel was then stained with Coomassie blue to visualize protein bands.

Immunoblotting. Approximately 40 mg of rTC was subjected to SDS-PAGE on a precast, 4–20% polyacrylamide gradient denaturing minigel (Jules Biotechnology). Proteins were transferred to a nitrocellulose (NC) membrane (TransBlot; Bio-Rad Laboratories) in a Mini-Protean II Electrophoresis/Transfer Unit (Bio-Rad Laboratories) containing 192 mM glycine, 89 mM Tris, and 20% (v/v) methanol in distilled water at 4°C for 3.5 hr at 170 mA.

Following transfer, the NC membrane was cut into 4 mm-wide strips and blocked with blotto. The NC strips were incubated separately with human sera (diluted 1:100 in blotto), pooled sera from rabbits exposed to either A. americanum or D. variabilis ticks (diluted 1:100 in blotto, sera from 5 days post secondary tick exposure was used), or pooled sera from gerbils exposed to adult Ae. aegypti (diluted 1:100 in blotto) overnight at 4°C with rocking. The strips were incubated with gerbil sera at room temperature for 3 hr, washed with PBS, 0.5% Tween-20 (PBST), and incubated for 3 hr with rabbit anti-gerbil IgG (diluted 1:500 in blotto)
with rocking. The NC strips were washed with PBST and incubated for 1 hr with rocking at room temperature with either goat anti-rabbit IgG (gamma chain specific) or goat anti-human IgG (gamma chain specific) labeled with horseradish peroxidase at a 1:1,000 dilution. The NC strips were washed with PBST and PBS, and then developed with 4-chloro-1-naphthol (4-CN Peroxidase Substrate; Kirkegaard & Perry Laboratories, Inc.).

Human sera collection. Pre-maneuvers serum specimens were obtained from Fort Wainwright personnel in late April 1990 and from Fort Chaffee personnel in late May 1990. Fort Wainwright personnel then had assignment to tick-infested areas from May 2 to 21, 1990 and Fort Chaffee personnel from May 29 to September 27, 1990. Post-maneuvers specimens were obtained from Fort Wainwright personnel in late June 1990 and from Fort Chaffee personnel in late September 1990. A questionnaire was administered at the time of the post-maneuvers serum specimen collection and included demographic data, work duties at Fort Chaffee, signs and symptoms during and immediately after assignment to Fort Chaffee, self-reported arthropod exposure, self-reported preventive activities, recreational activities in 1990, and animal contact.16

Human anti-rTC ELISA. All serologic testing was performed without knowledge of the questionnaire results. The ELISA plates were coated with 0.2 mg of rTC (in 100 ml of PBS) per well overnight at 4°C. The plates were blocked with 1% fetal bovine serum (FBS) in PBS with PBST overnight at 4°C. Sera were diluted 1:100 in PBST with 1% FBS and 100 ml/well was incubated for 1 hr at 37°C. The plates were then washed with PBS and a 1:4,000 dilution of goat anti-human IgG conjugated to horseradish peroxidase (The Binding Site) in PBST with 1% FBS was added to the plates and incubated for 1 hr at 37°C. The plates were developed with ABTS (Kirkegaard & Perry Laboratories, Inc.) and the OD was measured at 405 nm. The human anti-rTC antibody OD used in the figures and results was calculated as described for the rabbit anti-rTC ELISA, except that FBS was used instead of blotto for determining background levels.

Statistical analysis. Statistical analyses were performed with BMDP Statistical Software programs.18 In the analysis of animal data, Student’s t-tests were used to compare antibody response levels against rTC. Associations were examined between the study variables and pre- and post-maneuvers anti-rTC levels in the analysis of the human data. Associations between pairs of dichotomous variables were examined by contingency tables. Crude odds ratios (ORs) with 95% exact confidence intervals and chi-square values were calculated. All reported P values are for two-tailed tests of significance. Stratification and logistic regression were used to control for confounding variables and adjusted ORs with 95% confidence intervals are reported.

RESULTS

Rabbit anti-rTC ELISA. The amount of rabbit anti-rTC antibody in the sera of rabbits exposed to ticks was measured in order to demonstrate that hosts exposed to feeding ticks develop specific anti-rTC antibodies. Rabbit anti-rTC antibody levels are shown in Figure 1. Mean anti-tick calreticulin antibody levels in the sera collected at 5, 10, and 15 days after primary exposure were not significantly different from the mean of day 0 (pre-exposure) levels in rabbits exposed to either tick species (all P values > 0.10, by Student’s t-test). Significant anti-rTC IgG responses developed in rabbits after a second exposure to either A. americanum or D. variabilis ticks (Figure 1). The mean OD values (± SE) of the three rabbits exposed to A. americanum ticks for the three post-challenge bleeds at days 41, 46, and 51 were 0.535 ± 0.039, 0.677 ± 0.058, and 0.615 ± 0.057, respectively. The mean OD values (± SE) of the three rabbits exposed to D. variabilis ticks for the three post-challenge bleeds at days 41, 46, and 51 were 0.428 ± 0.058, 0.46 ± 0.041, and 0.457 ± 0.049, respectively. These were all significantly greater than mean prebleed levels (all P values < 0.01, by Student’s t-test). When comparing the level of anti-rTC antibody response between the two tick species, ELISA OD levels in the A. americanum exposed rabbits after challenge were significantly higher at each time point than those found for D. variabilis exposed rabbits (all P values < 0.05, by Student’s t-test). Exposure to other blood-feeding arthropods is a potential source of cross-reactivity for the anti-rTC antibody assay. However, gerbils exposed repeatedly to Ae. aegypti developed no significant increase in anti-rTC IgG levels (P > 0.1, by Student’s t-test).

Human anti-rTC ELISA. Subjects from Fort Chaffee represented a group of older military instructors and Department of Defense civilian employees who lived and worked at Fort Chaffee (Table 1). Pre- and post-maneuvers anti-rTC antibody levels were dichotomized at an ELISA OD of 0.305, which was the 25th percentile in the distribution of anti-rTC antibody levels in the 192 military per-
sonnel, and three standard deviations above the mean ELISA OD in five subjects from Westchester County, New York who denied ever having a tick bite. These subjects have had ATSA levels measured in previous studies using whole sonicated *I. scapularis* or *A. americanum* salivary glands as antigen, and have been found to have low levels of antibodies to tick salivary gland antigens.

The mean (± SD) anti-rTC antibody levels in the pre- and post-maneuvers specimens were 0.474 ± 0.077 and 0.466 ± 0.082 (post–pre) difference (5) (Table 1). These results were highly correlated (r = 0.93, P < 0.01). Pre- and post-maneuvers specimens had levels that were not significantly different (P > 0.05). Pre- and post-maneuvers specimens were therefore pooled for subsequent analyses. There were no significant changes in pre- to post-maneuvers antibody levels in either fort of origin group (P > 0.10). Pre- and post-maneuvers anti-rTC antibody levels were highly correlated (r = 0.93, P < 0.01). Anti-rTC antibody levels were poorly correlated with ELISA OD levels using whole, three day–fed *A. americanum* salivary gland antigen5 (pre-maneuvers specimens, r = 0.08, P = 0.28; post-maneuvers specimens, r = 0.14, P = 0.05).

In crude and adjusted analyses (controlling for age, fort of origin, attached tick during maneuvers, and bed netting use), fort of origin and bed netting use were both associated with anti-rTC seropositivity. Subjects from Fort Wainwright and subjects who reported not using bed netting were more likely to be anti-rTC seropositive (adjusted ORs = 5.3 and 6.8, respectively) (Table 2).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting. The SDS-PAGE procedure was done to ascertain the purity of the rTC sample. Coomassie blue staining of the partially purified rTC preparation showed a prominent band at approximately 64 kD, the reported molecular weight of the rTC fusion protein14 (lane 1, Figure 2). The tick calreticulin comprised a majority of the protein present in the preparation.

Immunoblot analysis using rTC as antigen was performed to determine the specificity of the antibody response detected by ELISA. When the rTC was used in immunoblot analysis, no bands were visualized using the gerbil sera from either before or after mosquito feeding (lanes 2 and 3, respectively, Figure 2). No bands were visualized using the sera from either *A. americanum*-exposed rabbits (lane 4, Figure 2) or *D. variabilis*-exposed rabbits (lane 6, Figure 2) before tick feeding. Sera from both *A. americanum*-exposed rabbits and *D. variabilis*-exposed rabbits contained IgG that recognized the 64-kD rTC (lanes 5 and 7, respectively, Figure 2) after tick feeding.

All of the tested human serum samples had IgG that recognized a band of approximately 64 kD using both pre- and post-maneuvers serum samples (lanes 8 through 14, Figure 2). The post-maneuvers serum from the subject from Fort Chaffee who reported a bite (lane 9, Figure 2), as well as the post-maneuvers serum from the Fort Wainwright subject who did not report a bite (lane 15, Figure 2), showed three additional bands at approximately 45, 50, and 55 kD. The same triplet of bands was observed on the pre- and post-maneuvers sera from the subject from Fort Chaffee who did not report a tick bite (lanes 10 and 11, respectively, Figure 2).

**DISCUSSION**

The present study examined associations between anti-rTC antibody levels and several risk and preventive factors.

### Table 1

Demographic characteristics of military personnel on maneuvers at Fort Chaffee, Arkansas, 1990

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Fort Chaffee</th>
<th>Fort Wainwright</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>96</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>Age, years (mean ± SD)</td>
<td>31.2 ± 8.4</td>
<td>25.3 ± 5.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Military grade/rank, %</td>
<td></td>
<td></td>
<td>0.01</td>
</tr>
<tr>
<td>Civilian</td>
<td>4%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>Enlisted</td>
<td>79%</td>
<td>54%</td>
<td></td>
</tr>
<tr>
<td>Officer</td>
<td>17%</td>
<td>27%</td>
<td></td>
</tr>
<tr>
<td>Male gender, %</td>
<td>99%</td>
<td>87%</td>
<td>0.001</td>
</tr>
<tr>
<td>Self-reported attached tick bite, %</td>
<td>51%</td>
<td>49%</td>
<td>0.83</td>
</tr>
</tbody>
</table>

**Table 2**

Associations (crude and adjusted* odds ratios [ORs] with 95% confidence intervals [CIs]†) of selected study variables with post-maneuvers anti–recombinant tick calreticulin antibody seropositivity, 192 military personnel, Arkansas, June–September 1990

<table>
<thead>
<tr>
<th>Study variable</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)‡</td>
<td>1.0§</td>
<td>0.0§</td>
</tr>
<tr>
<td>22–26</td>
<td>2.4 (0.8–7.4)</td>
<td>1.4 (0.5–3.6)</td>
</tr>
<tr>
<td>27–33</td>
<td>1.5 (0.6–4.0)</td>
<td>1.3 (0.5–3.4)</td>
</tr>
<tr>
<td>&gt;33</td>
<td>1.5 (0.6–4.0)</td>
<td>1.4 (0.5–3.9)</td>
</tr>
<tr>
<td>Fort Wainwright vs. Fort Chaffee</td>
<td>1.0 (0.5–1.9)</td>
<td>5.3 (1.1–25.6)</td>
</tr>
<tr>
<td>Attached tick bite, yes vs. no</td>
<td>0.6 (0.3–1.2)</td>
<td>1.5 (0.7–3.0)</td>
</tr>
<tr>
<td>Bed netting use, no vs. yes</td>
<td>3.1 (1.5–6.5)</td>
<td>6.8 (1.4–32.4)</td>
</tr>
<tr>
<td>Permanone use, no vs. yes</td>
<td>0.5 (0.2–1.0)</td>
<td>NS¶</td>
</tr>
<tr>
<td>Skin rash during maneuvers, no vs. yes</td>
<td>3.3 (1.3–10.0)</td>
<td>NS</td>
</tr>
<tr>
<td>In subjects without tick bite#</td>
<td>13.5 (1.9–149)</td>
<td>NS</td>
</tr>
<tr>
<td>In subjects with tick bite#</td>
<td>1.6 (0.4–6.2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

* Adjusted for age, fort, attached tick bite during maneuvers, and bed netting use.
† Exact confidence intervals are given where appropriate (expected cell sizes less than 5).
‡ Differences between age groups were not significant (P = 0.35).
§ Reference group.
¶ NS = not significant.
# P for difference between ORs in subjects with and without tick bites is 0.05 (chi-square test for homogeneity).
during assignment of military personnel to tick-infested areas of Arkansas. The anti-rTC antibody responses of rabbits experimentally exposed to feeding ticks, as well as that of gerbils exposed to mosquitoes, were also examined.

Little change was observed between paired pre- and post-maneuvers anti-rTC levels in human subjects from either study group. A similar result was obtained in a previous study involving this group of study subjects using whole *A. americanum* salivary gland antigen.8 The presence of anti-rTC antibodies in pre-exposure specimens may represent previous exposure to feeding ticks. Evidence suggests that people do not recognize tick bites very well and that self-reported measures of long-term past exposure to ticks are not valid.6,11 It has been demonstrated that tick calreticulin is not detectable in the saliva of feeding ticks until three days after attachment,19 so another explanation for the lack of change is that military personnel only received tick bites of short duration. Tick calreticulin is only one of many proteins found in tick saliva. A single tick bite would involve only a small dose of tick calreticulin, which would explain the lack of change in anti-rTC antibody levels.

Immunoblots of individuals reveal that humans make a specific anti-rTC antibody response. Little change is seen between pre- and post-maneuvers specimens, which is consistent with the ELISA results. The triplet of bands observed in several subjects may represent host antibody recognition of calreticulin proteolytic products resulting from normal degradation of the recombinant protein. They may also represent shared epitopes between the full size tick calreticulin and calreticulin products with shortened termination sequences formed during expression.

Using data obtained from a prior study of this population,9 anti-rTC antibody levels and ATSA levels were found to be poorly correlated. Tick saliva consists of numerous proteins, and the content of tick saliva changes during the course of attachment.4 Anti-tick calreticulin antibodies are increased in subjects from Fort Chaffee. This could explain the lack of correlation between the two measurements. The lack of correlation may also reflect differential IgM or other isotype-mediated blocking of IgG binding to rTC.

Different associations were observed between ATSA, anti-rTC antibody, and risk and preventive behaviors of the military personnel. The prevalence of ATSA seropositivity increased with age,9 but anti-rTC antibody seroprevalence was not associated with age in the present study. This may be due to the extent to which cross-reactivity plays a role in the total ATSA measurement. Many tick saliva proteins have been found to be present in the saliva of other blood-feeding arthropods19 and are also shared between the saliva of different tick species.7,20,21 Anti-tick saliva antibody may therefore include significant cross-reactivity from previous exposure to other blood-feeding arthropods and ticks; as subject age increases, so does cumulative exposure to these hematophagous arthropods. Calreticulin has also been found in the saliva of fleas.22 No history of flea infestation was obtained from study subjects, but flea exposure could account for higher anti-rTC antibody levels in pre-exposure samples.

Individuals who originated at Fort Wainwright were found to be 5.3 times more likely to be seropositive for anti-rTC antibody compared with subjects from Fort Chaffee, when controlling for age, fort of origin, self-reported tick bite during maneuvers, and self-reported use of bed netting during maneuvers. A small number of Fort Chaffee personnel reported using bed netting during maneuvers (n = 3), so this may be an unstable estimate of the association. This finding contrasts with a previous observation regarding the association between fort of origin and seropositivity to ATSA;9 in that study, the risk of seropositivity to ATSA was found to be increased in subjects from Fort Chaffee.

The ATSA seropositivity may be indicative of general tick exposure, while anti-rTC seropositivity may reflect exposure to ticks feeding for three days or longer. Anti-tick saliva antibody is a more general response against numerous tick salivary gland proteins that are delivered into the bite site from the moment of attachment. Fort Wainwright personnel
would likely be at increased risk of having longer duration tick bites due to a lack of familiarity with tick prevention protocols because of origin from an area without significant human biting tick infestation (Alaska) and without *A. americanum*. Tick bites of longer duration in Fort Wainwright personnel could explain this contrasting observation in anti-rTC and ATSA levels in personnel from the two forts.

Individuals who denied the use of bed netting were found to be 6.8 times more likely to be seropositive for anti-rTC antibodies than those subjects who used bed nets. A prior study of this population revealed that bed netting, along with other personal protective behaviors, decreased the risk of ATSA seropositivity. *Amblyomma americanum* commonly quest for blood meals from sundown to late evening. As previously discussed, the untreated, 27-mesh nylon bed nets could provide protection from tick bites to subjects sleeping on the ground during the hours of active tick feeding.

To our knowledge, the immunodynamics of rTC have not been described in hosts exposed to feeding ticks. Previous work has identified and characterized the *A. americanum* calreticulin clone using sera from rabbits immunized by injection of nitrocellulose membrane containing the fusion protein. In the present study, rabbits exposed to either *A. americanum* or *D. variabilis* ticks produced measurable amounts of anti-rTC antibodies. Despite the large number of ticks placed on the rabbits, significant increases in anti-rTC antibody levels were not seen until after a second tick feeding period. Immunoblots revealed this was a specific antibody response against rTC.

Both *A. americanum*- and *D. variabilis*-exposed rabbits visualized a 64-kD band, which is the reported size of the recombinant tick calreticulin. In contrast, gerbils repeatedly exposed to *Ae. aegypti* do not appear to produce a specific anti-rTC response. While the gerbil experiment is not an ideal control for the rabbit experiment, it demonstrates that rTC may not be present in the saliva of mosquitoes. More data concerning the immunodynamics of calreticulin in animal models must be collected before any definitive conclusions can be drawn.

The use of anti-rTC antibody responses as a marker of tick exposure has several current and potential advantages over the use of whole salivary glands as antigen in an assay. First, it is more convenient to use a cDNA-derived protein (i.e., rTC) rather than obtaining antigen by dissecting three-day fed adult ticks. A cDNA-derived protein can be produced in large amounts and the consistency from batch to batch would be easier to control. Second, focusing on individual proteins in tick saliva offers the possibility of improved specificity of the ATSA assay because of the reduced risk of cross-reactivity to other arthropod saliva proteins. Finally, a protein like calreticulin, which is not detectable in tick saliva until the third day of feeding, may be a marker for longer duration tick bites, and perhaps more relevant to disease risk.

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