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Original article

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Abstract**Background:**

Alpha gal syndrome is a hypersensitivity reaction to red meat mediated by specific IgE antibody to galactose- α -1,3-galactose carbohydrate (alpha gal). *Amblyomma* tick bites are associated with this condition although the pathophysiology is not understood.

Objective:

To clarify the mechanism of development of alpha gal syndrome after tick bites.

Methods:

We compared alpha gal antibodies between patients with and without a history of tick bites and examined the histology of tick bite lesions between patients with and without detectable anti-alpha-gal IgE antibody.

Results:

Patients who had ≥ 2 tick bites had higher levels of anti-alpha-gal IgE antibody compared with those with 1 tick bite or healthy individuals. On histological investigation, greater numbers of infiltrating basophils and eosinophils, but not mast cells, were observed in the lesions of patients with ≥ 2 tick bites compared with those with 1 tick bite. Type 2 cytokine-producing T cell infiltration was ~~also~~ predominantly observed in such patients.

Limitation:

The study was conducted at a single institution in Japan.

Conclusions:

In *Amblyomma* tick bites, basophils, eosinophils and type 2 cytokine-producing T cells infiltrate the skin, and anti-alpha gal IgE antibodies are produced, which provides a potential mechanistic connection to red meat hypersensitivity.

65 **Abbreviations:** alpha gal, galactose- α -1,3-galactose.

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Introduction

Tick bites give rise to local inflammation at the lesion site and there is a risk of infection by pathogenic microorganisms^{1, 2}. However, it has recently been recognized that tick bites by *Amblyomma* species may also be closely associated with the development of alpha gal syndrome, which is mediated by specific IgE antibody against galactose- α -1,3-galactose (alpha gal) causing immediate hypersensitivity against red meat^{3, 4, 5}. Recent findings of basophil infiltration in the skin lesions of mice bitten repeatedly by ticks⁶ and their antigen presenting function⁷ indicate that basophils present in human skin after tick bites may function as antigen-presenting cells to produce anti-alpha-gal IgE antibody. Using immunohistologic techniques, we investigated basophil and eosinophil infiltration of tick bite skin lesions of patients who had experienced ≥ 2 tick bites in their lifetime. Our findings offer insight into the mechanism of development of alpha gal syndrome after repeated tick bites.

Materials and Methods

Twenty-one healthy volunteers (male: 13, female: 8; age 40.0 ± 11.7 years; range, 26–59 years) and 115 patients (male: 58, female: 57; age, 64.4 ± 17.7 years; range, 4–91 years), who visited our hospital department suffering from tick bites, were enrolled into this study after providing their informed consent. In total, 53 skin specimens were obtained from the patients and were subjected to histological analysis; 34 of these specimens were subjected to immunostaining. Skin specimens that were too small to assess dermal infiltrates were excluded from the immunostaining analysis. The study was performed according to the Declaration of Helsinki, and the study protocol was approved by the Shimada Municipal Hospital Ethical Committee (Accession number 2016-03). Routine laboratory blood examinations for hematology, chemistry and levels of specific IgE antibodies to alpha gal and red meat (beef, sheep, pork and chicken) were performed.

Reagents and antibodies

Purified, FITC-conjugated and PE-conjugated monoclonal antibodies to CD3, CD4, CD8 and CD68, and FITC-conjugated and PE-conjugated monoclonal antibodies against cytokines (interferon (IFN)- γ , interleukin (IL)-4, IL-5, IL-13, IL-17 and IL-22) were purchased from BD PharMingen (San Diego, CA, USA). Human basophil-specific antibody (BB1, anti-basogranulin antibody) was obtained from University of Southampton, UK.

Identification of tick species

For those patients who arrived at our department with ticks still attached to the skin, we

removed the ticks using a tick remover device known as the O'Tom Tick twister® (ProColler, France), which ensures that the mouthparts are not left in the tissue. These ticks were then sent to an expert in the field (Prof. Ai Takano, Yamaguchi University, Japan) to identify the species.

Preparation of skin specimens

Tick bite skin lesions were biopsied using a 4-mm trepan and these skin specimens were subjected to routine HE, toluidine blue and immunohistochemical staining.

Histological analysis

Formalin-fixed, paraffin-embedded sections were treated with antigen-retrieve solution after deparaffinization and were stained with antibodies to CD4, CD8, CD68 and basophil granules (BB1), as previously described⁹. The degrees of cellular infiltration in the epidermis and dermis of the skin lesions were semi-quantitatively evaluated into 1 of 5 categories (0, none; 1, few; 2, mild; 3, moderate and 4, severe) by microscopic analysis by trained inspectors. The number of infiltrating basophils, eosinophils and mast cells were enumerated in eight high-power fields that were randomly selected in each of the lesions, and the mean values were calculated.

Cytokine production by skin-infiltrating T cells

In the patients who had experienced 1 tick bite, 2 tick bites or ≥ 3 tick bites in their lifetime, skin-infiltrating T cells were propagated from the skin specimens using our previously established method⁸. Cytokine production was investigated by intracytoplasmic cytokine staining of the cells after stimulation with phorbol myristate

acetate.

Statistical analysis

Pair-matched differences were analyzed by the Wilcoxon signed rank test, comparisons among multiple samples were analyzed by the Fisher's exact test and the Mann–Whitney U-test or the Kruskal–Wallis test and subsequently the Dunn's multiple comparison test, and correlations were analyzed by the Spearman's rank test, using the software GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). Values of $p < 0.05$ were considered to indicate statistical significance.

Results

Tick species

Thirty-eight ticks were obtained from the infested sites of the patients with a tick twister. Of these, 33 ticks (86.8%) were identified as *Amblyomma testudinarium*, which were mostly nymphs (71.0%). In this study, the patients for whom anti-alpha-gal IgE was detected had all been bitten by *A. testudinarium*. Other tick species identified were *Haemaphysalis* (4/38, 10.5%) and *Ixodes monospinosus* (1/38, 2.6%). The population of tick species detected in our study area differed from that reported for other areas of Japan, such as the Shimane area¹⁰. These geographical differences seem to correlate with the animals populating these areas.

Detection of specific IgE antibody to alpha gal in patients with tick bites

Recent observations suggest that the historical number of tick bites experienced by an individual in their lifetime is a crucial factor in the production of specific antibody^{11, 12}. To address this issue, we investigated the rate of detection of anti-alpha-gal IgE antibody among the patients categorized according to the number of historical tick bites: 1 tick bite; 2 tick bites; 3 tick bites and ≥ 4 tick bites, in addition to 21 healthy control individuals that had never been bitten by ticks (0 tick bites). The specific antibody was not detected (0/21) among the individuals with 0 tick bites, whereas 4% (2/45) of the patients with 1 tick bite were positive for the antibody; however, there was no statistically significant difference between these two groups (Fig 1). By contrast, the patients with ≥ 2 tick bites showed higher detection of the antibody, which was statistically significant compared with the patients with 0 or with 1 tick bite (Fig 1, Table I), with ≥ 3.94 at relative risk (Table IB). The rates of anti-alpha-gal IgE antibody

positivity obtained among the patients with 2 tick bites, 3 tick bites and ≥ 4 historical tick bites were comparable to each other, except for a significant difference between the patients with 2 tick bites and 3 tick bites with 1.89 at relative risk (Table IB). We observed a close positive correlation between the serum levels of anti-alpha gal IgE antibody and specific IgE antibodies to red meat, but not to chicken (SFig 1), indicating that these antibodies reacted to shared molecules, such as alpha gal. However, we also detected a small number of patients who were positive for anti-alpha-gal IgE antibody and negative for anti-red meat IgE antibody (SFig 1), warning of the limitations of anti-red meat IgE antibody for the detection of alpha gal syndrome.

Histological features of tick bites in skin

In total, 53 skin specimens were available from patients with tick bites who were treated by punch biopsy prior to the introduction to our practice of the tick twister, which removes the tick without the need for a punch biopsy. No control specimens from healthy volunteers were available. Because we found a statistical difference in the positive detection of anti-alpha-gal IgE antibody between patients with 1 tick bite and patients with ≥ 2 tick bites, we investigated the histological differences in skin lesions between these groups. Marked lymphocytic and neutrophilic infiltrations around dermal vessels, interspersed with various degrees of eosinophils and vasculitis were common features in both groups. We found denser cellular infiltration in the deep dermis in the group with ≥ 2 tick bites than in the group with 1 tick bite; however, this difference was not statistically significant ($p=0.095$, Fig. 2A). We individually compared skin specimens from a single patient with several tick bites. Skin specimens from eight tick bite sites from four patients (which included the 1st tick bite and a subsequent tick bite

in each patient) were examined (Fig 2B). We found that the degree of cellular infiltration increased in both the epidermis and the dermis along with the historical number of tick bites (the 1st tick bite vs. subsequent tick bites).

Basophil infiltration of tick bite sites

By immunohistochemical analysis with BB1, the number of basophils in the tick bite lesions between the group with 1 tick bite (n=13) and ≥ 2 tick bites (n=20) in their lifetime was compared. Low levels of basophil infiltration were detected in the tick bite lesions of the group with 1 tick bite (Fig 3A and B, left) despite significant cellular infiltration, as previously reported^{9,13}. However, significant levels of basophil infiltration were detected in the tick bite lesions of the groups with ≥ 2 tick bites (Fig 3A and B, right; p=0.0028, Mann–Whitney test), which was consistent with a previous murine study⁶. In the single tick bite lesions, the number of infiltrating basophils (<50 cells/HPF) was marginal (SFig 2A). By contrast, the number of infiltrating basophils increased from 2 days after the tick bites in the two tick bite lesions and from 1 day after the tick bites in the ≥ 3 tick bite lesions (SFig 2B,C), suggesting that the kinetics of basophil infiltration depend on the tick bite history. In accordance with these results, we also found a difference in antibody production between the patients with 2 and ≥ 3 tick bite lesions (SFig 3B,C). Higher numbers of infiltrating eosinophils were detected in the tick bite lesions of the groups with ≥ 2 tick bites compared with the group with 1 tick bite and these cell numbers were positively associated with the basophil number (data not shown). By contrast, the number of mast cells, which are morphologically and functionally similar to basophils, was not associated with the infiltration of basophils (data not shown). This suggests that basophils may be specifically involved in the

pathophysiology of tick bites.

Phenotype of skin-infiltrating T cells at the tick-infested sites

We performed immunohistochemical staining of skin specimens with antibodies to CD4, CD8 and CD68, and evaluated the degree of infiltration as described in the Materials and Methods (Fig 4A and B). Interestingly, CD4⁺ cell infiltration in the dermis was significantly greater in the skin lesions of the groups with ≥ 2 tick bites than in the group with 1 tick bite (Mann–Whitney U-test, $p < 0.05$). Conversely, CD68⁺ cell infiltration in the dermis was significantly lower in the skin lesions of the group with ≥ 2 tick bites than in the group with 1 tick bite (Mann–Whitney U-test, $p < 0.05$). We also found that the levels of infiltration of CD4⁺ and CD8⁺ T cells were higher, and those of CD68⁺ cells were lower in the skin lesions of patients with ≥ 2 tick bites than those of patients with one tick bite when skin biopsies were taken at the same time point (data not shown). There was no difference in epidermal infiltration of CD4⁺ or CD68⁺ cells. CD8⁺ cell infiltration was comparable at all sites between the groups. This finding indicated that cellular infiltration into the skin lesions differed qualitatively between the patients with 1 tick bite and ≥ 2 tick bites.

Cytokine production by infiltrating T cells in skin lesions

To characterize the skin-infiltrating T cells, the cells isolated from the skin samples in 10 cases (four patients with 1 tick bite; three patients with 2 tick bites; three patients with ≥ 3 tick bites) were expanded without artificial distortion of their function by a previously reported method that involved IL-2 and anti-CD3/CD28 antibody-coated microbeads⁸. In the group with 1 tick bite, CD4⁺ cells produced large quantities of

IFN- γ as well as the type 2 cytokines IL-4, IL-5 and IL-13, and small quantities of IL-17 and IL-22 were detected (data not shown). The number of IL-4-producing cells to IFN- γ -producing cells (the Type 2/Type 1 ratio) was approximately 1 (mean $\pm$ SD, 0.9 ± 0.3). In the groups with 2 and ≥ 3 tick bites, CD4⁺ cells produced extremely high quantities of IL4, IL5 and IL13 but a low quantity of IFN- γ , and the Type 2/Type 1 ratio was high (mean $\pm$ SD, 3.0 ± 1.0 ; 22.5 ± 27.3 , respectively) in this group. We observed a similar change in the Type 2/Type 1 ratio when the number of CD8⁺ cells to CD4⁺ cells was analyzed (data not shown). The Type 2/Type 1 ratio in the CD4⁺ and CD8⁺ populations in the patients with ≥ 2 tick bites suggested preferential type 2 T cell infiltration compared with the patients with 1 tick bite.

Discussion

The human immune system recognizes alpha gal, a carbohydrate that is expressed in the cell membrane of many organisms, with the exception of primates, avian species and humans, and produces IgM antibody against this molecule¹⁴. However, under specific situations, anti-alpha-gal antibody-producing B cells undergo isotype class switching to produce IgE antibody that induces immediate hypersensitivity reactions against the entry of alpha gal. Importantly, several observations indicate a close association between the production of anti-alpha-gal IgE antibody and tick bites that contain abundant alpha gal^{15, 16}, suggesting that immune reactions following the entry of alpha gal into the skin via a tick bite triggers anti-alpha-gal IgE antibody production.

Basophils, comprising less than 1% of circulating white blood cells¹⁷, are recruited to the skin, and play a critical role in acquired immunity against ticks in mice bitten repeatedly⁶; however, no significant basophilic infiltration has ever been reported in the limited studies of tick bite lesions in humans^{10,11}. This prompted us to examine a larger sample of skin lesions histologically, and thus, significant basophilic infiltration was detected in human lesions. This disparity with previous reports may reflect different patient profiles. The infiltration of basophils has been reported for other skin disorders.

The production of the specific IgE antibody is associated with an underlying Th2-shifted immune condition; however, there was some dispute regarding which cell type is the source of early IL-4 that is responsible for Th2 cell differentiation. Activated basophils are one source of this cytokine¹⁸; however, controversy surrounded whether these cells act as antigen presenting cells driving Th2 cell differentiation¹⁹. Recently, it

has been clearly demonstrated in mice and humans that basophils can act as antigen presenting cells using MHC sequestered from dendritic cells via trogocytosis for driving type 2 cell differentiation⁷. This observation is consistent with our finding that type 2 T cells infiltrated the skin lesions of patients with ≥ 2 tick bites along with basophils, but not the lesions of patients with 1 tick bite. Eosinophil infiltration was also detected in these patients, as seen in the murine study, and this might be promoted by basophils in the skin along with skin fibroblasts²⁰. Interestingly, we also found fewer CD68+ cells in the skin of patients with ≥ 2 tick bites compared with patients with 1 tick bite. Our observations may shed light on the immunological processes that occur in the development of immediate hypersensitivity induced by repeated entries of allergens into the skin, where basophils function as antigen presenting cells driving type 2 differentiation and the production of specific IgE antibody. Our observations will provide new insight into the mechanism responsible for the correlation between tick bites and anti-alpha-gal IgE antibody production.

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Table 1. Rates of anti-alpha gal IgE positivity among the participants**A.** Rates of anti-alpha gal IgE positivity among the groups with different numbers of tick bites

	Number of tick bites	Anti-alpha-gal IgE	
		Positive*	Negative*
Healthy individuals	0	0/21 (0%)	21/21 (100%)
Tick bite patients	1	2/45 (4.4%)	43/45 (95.6%)
	2	9/26(34.6%)	17/26 (65.4%)
	3	16/24 (66.7%)	8/24 (33.3%)
	≥4	13/20 (65.0%)	7/20 (35.0%)
	≥2	38/70 (54.3%)	32/70 (45.7%)

*Numbers in parentheses indicate the percentages of the total number of cases.

B. Comparison between the groups with different numbers of tick bites

Tick bites	0	1	2	3	≥4	≥2
0		n.s.	0.0025 (-)	<0.0001 (-)	<0.0001 (-)	<0.0001 (-)
1			0.0013 (3.94)	<0.0001 (7.59)	<0.0001 (6.45)	<0.0001 (11.47)
2				0.042 (1.89)	n.s.	
3					n.s.	

Numbers indicate the P values for the relative risk between the groups (as determined by the Fisher's exact test). n.s., not significant. (-), incalculable.

Figure legends

Fig 1. Anti-alpha-gal IgE antibody levels (U_A/ml) in the patients with different numbers of historical tick bites

Fig 2. Histological analysis of tick bite lesions

A. Degree of cell infiltration into the epidermis and dermis of the skin lesions. **B.**

Representative histologic features at different time points.

Fig 3. Basophil infiltration of tick bite lesions

A. Number of infiltrating basophils in tick bite lesions. *, $p=0.0028$ Mann–Whitney

U-test. **B.** Representative basophil staining (red) of the primary (left) and second (right) tick bite lesions in a single patient.

Fig 4. Infiltration of CD4+, CD8+ and CD68+ cells in tick bite lesions

A. Representative CD4, CD8 and CD68 cell staining of the primary (upper) and the second (lower) lesions. **B.** Degree of infiltrating CD4+, CD8+ and CD68+ cells in the skin lesions of the groups with 1 tick bite and the ≥ 2 tick bites over their lifetime. *, $p=0.0022$; **, $p=0.0035$, Mann–Whitney U-test.

Capsule summary

- Alpha gal syndrome has been reported after *Amblyomma* tick bites.
- Patients who had ≥ 2 tick bites had higher levels of anti-alpha-gal IgE antibody than those with 0-1 tick bites.
- Patients with multiple *Amblyomma* tick bites may be at higher risk of developing allergy to red meat.

Key words: alpha gal syndrome, basophil, eosinophil, interferon-gamma, interleukin-4, tick bite, type 2 T cell

Legends for supplementary figures

SFig 1. Correlation between serum concentrations of anti-alpha-gal IgE antibody and anti-beef (white circle) and –pork (black circle) meat IgE antibodies.

SFig 2. Number of basophils in the 1 (1°), 2 (2°) and ≥ 3 ($\geq 3^\circ$) tick bite lesions and the timing (days after tick bites) of the skin biopsies

SFig 3. Levels of anti-alpha gal IgE antibody and the timing (days after tick bites) of the blood tests in the groups with 1 (1°), 2 (2°) and ≥ 3 ($\geq 3^\circ$) tick bite lesions













