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LETTER TO THE EDITOR

Agranulocytosis associated with voriconazole-induced hypersensitivity syndrome

Dear Editor,

Drug-induced hypersensitivity syndrome (DIHS) is a life-threatening severe cutaneous adverse reaction.[1] Herein, we report severe agranulocytosis in a patient with DIHS induced by voriconazole.

A 68-year-old woman with pulmonary aspergillosis treated with voriconazole for 1 month suddenly developed high-grade fever and generalized rash with mild facial edema (Fig. 1a) and visited us. She had no history of drug allergy or serious diseases except for nail lichen planus, hypertension and hyperlipidemia. She looked healthy and we did not detect any immunological abnormality in the routine laboratory investigation during these treatments. Despite cessation of voriconazole for 1 week, the rash developed over the entire body with exaggeration of facial edema (Fig. 1b) at the second visit. Her body temperature was 39.8°C. Pruritic erythema and purpura with scratching marks on

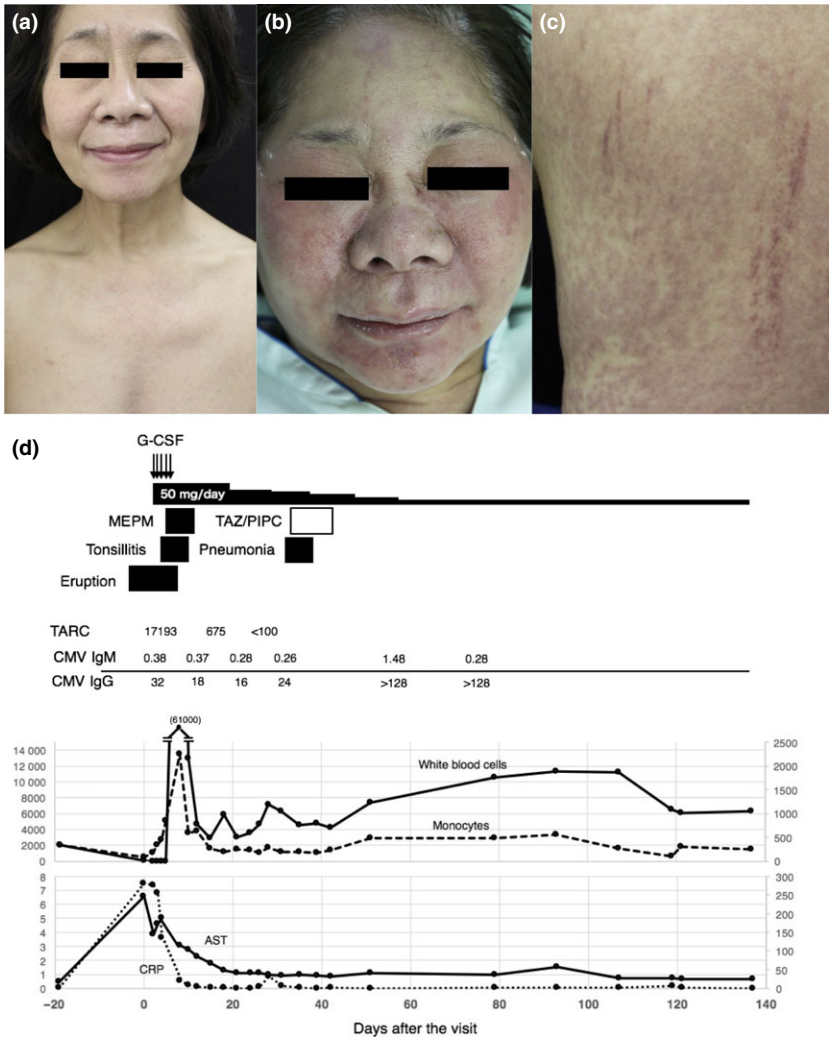

Figure 1. Clinical skin manifestations at the (a) first and (b,c) second visits. (d) Clinical course of this case. CMV, cytomegalovirus; G-CSF, granulocyte colony-stimulating factor; Ig, immunoglobulin; MEPM, meropenem hydrate; PIPC, piperacillin; TARC, thymus and activation regulated chemokine; TAZ, tazobactam.

Correspondence: Hideo Hashizume, M.D., Ph.D., Department of Dermatology, Shimada Municipal Hospital, 1200-5 Noda, Shimada, Shizuoka 427-8502, Japan. Email: hihashiz0001@mac.com

the body were observed in addition to cervical, axillary and inguinal lymphadenopathy (Fig. 1c). Skin histology revealed mild liquefaction and lymphocytic infiltration with a few eosinophils around blood vessels (Fig. S1). The laboratory investigations disclosed a drastic reduction in the white blood cell count (1500/μL) accompanied by agranulocytosis (10/μL), which is not often seen in this disease (Fig. 1d). Increased levels of aspartate aminotransferase (95 IU/mL; normal, 6–30) and C-reactive protein (7.89 mg/dL; normal, <0.3) was noted. Remarkably high serum levels of soluble interleukin-2 receptor (3304 IU/mL; normal range, 0–500) and thymus and activation-regulated chemokine (17193 pg/mL; normal, 0–450) were documented. Antibodies to nuclear DNA or DNA were not detected. Antibody titers to the human herpes viruses were not increased during three time points until 40 days after hospital admission. Although these findings did not fulfill the criteria, the skin manifestation was highly suggestive of DIHS/drug rash with eosinophilia and systemic symptoms (DRESS) to make an early decision of treatment for DIHS/DRESS of prednisolone (50 mg/day p.o.). She was hospitalized in a clean room and administrated meropenem (i.v.) for 7 days and granulocyte-colony stimulating factor (G-CSF, i.c.) for 5 days successively because of oral findings suspicious of bacterial tonsillitis. Seven days after these treatments, fever and rash disappeared completely. The lymphocyte stimulation test with voriconazole revealed a significant proliferative response (stimulation index, 180%), suggesting that it was the culprit.

Voriconazole is a triazole that has been used widely to treat serious fungal infections although several adverse reactions due to voriconazole have been reported.[2,3] Among those reports, no case of DIHS/DRESS induced by voriconazole has been found: this is the first reported case. The clinical and laboratory features specific to this disease appeared sequentially with time to fulfill the criteria of the Japan Severe Cutaneous Adverse Reaction group (atypical DIHS) and RegiSCAR project (definite DRESS) in this case. The most characteristic feature in our case was agranulocytosis that coincided with the emergence of DIHS/DRESS. Although it is induced only by voriconazole as a serious adverse reaction, multiple cases of agranulocytosis in patients with DIHS/DRESS have been reported (Table S1), so agranulocytosis may be a variant feature accompanied by DIHS/DRESS,[4] which should be borne in mind by all dermatologists.

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CONFLICT OF INTEREST: None declared.

Yasuhito KANEKO, Reiko KAGEYAMA, Hideo HASHIZUME
Department of Dermatology, Shimada Municipal Hospital, Shimada, Japan

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Reported cases of agranulocytosis in patients with drug-induced hypersensitivity syndrome/drug rash with eosinophilia and systemic symptoms

Figure S1. Skin histology (hematoxylin–eosin, original magnifications: [a] ×100; [b,c] ×400).

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LETTER TO THE EDITOR

Short course of cyclosporin A as a treatment option for drug-induced hypersensitivity syndrome: Case reports and review of the published work

Dear Editor,

Drug-induced hypersensitivity syndrome (DIHS), alternatively drug rash with eosinophilia and systemic symptoms (DRESS), is a distinct entity of life-threatening severe cutaneous adverse reactions (SCAR) to drugs.[1,2] We herein present two cases of DIHS/DRESS that were successfully treated with a short course of cyclosporin A (CyA).

Table 1. DIHS/DRESS cases treated with CyA

	Our cases		Kirchhof *et al.*[5]		Zuliani *et al.*[3]	Zhang *et al.*[4]
	Case 1	Case 2	Case 1	Case 2	Case	Case
CyA dose/days (mg/d × d)	150 × 7	150 × 7	200 × 5	350 × 3	200 × 5	300 × 7
RegiSCAR criteria						
Body temperature of >38°C	Yes (1)	No (−1)	Yes (1)	No (−1)	Yes (1)	Yes (0)
Lymph nodes (>2 sites, ≥1 cm)	Yes (1)	No (0)	No (0)	No (0)	No (0)	Yes (1)
Atypical lymphocytes	Yes (1)	Yes (1)	No (0)	No (0)	No (0)	No (0)
Eosinophils				No (0)		
0.70–1.49 × 10^9/L	Yes (1)		Yes (1)		Yes (1)	
>1.50 × 10^9/L		Yes (2)				Yes (2)
Rash						
Extent of >50%	Yes (1)	Yes (1)	Yes (1)	Yes (1)	Yes (1)	Yes (1)
Edema, infiltration, purpura, scaling (≥2 instances)	Yes (1)	Yes (1)		Yes (1)		
Biopsy suggestive of DRESS	Yes (0)	Yes (0)	Not done	Yes (0)	Not done	Yes (0)
Internal organ involvement	No (0)					
1		Yes (1)	Yes (1)		Yes (1)	Yes (1)
≥2				Yes (2)		
Resolution in >15 days	No (CyA)	No (CyA)	Yes (1)	No	Yes (1)	No
≥3 investigations negative for other diseases	Yes (1)	Yes (1)	Yes (1)	Yes (1)	Yes (1)	Yes (1)
RegiSCAR score	Definite (7)	Definite (6)	Definite (6)	Probable (4)	Definite (6)	Definite (6)
JSCAR criteria						
1. Rash developing >3 weeks after starting culprit drug	Yes	Yes	Yes	No	Yes	No
2. Prolonged symptoms after stopping the drug	Yes	Yes	Yes	Yes	Yes	Yes
3. Fever of >38°C	Yes	No (37.8°C)	Yes	Yes	Yes	Yes
4. Elevated liver enzymes or other organ involvement	Yes (hepatitis)	Yes (thyroiditis)	Yes	Yes	Yes	Yes
5. Leukocyte abnormality (≥2)	Yes	Yes	Yes	No	Yes	Yes
Leukocytes >11 × 10^9/L	Yes	Yes	Yes	No	Yes	Yes
Atypical lymphocytes >5%	Yes	No	No	No	No	No
Eosinophils >1.5 × 10^9/L	No	Yes	No	No	No	Yes
6. Lymphadenopathy	Yes	No	No	Yes	No	Yes
7. Reactivation of HHV	Yes	Yes	(Not described)	(Not described)	(Not described)	(Not described)
JSCAR definition†	Typical	DIHS-like‡	Atypical	DIHS-like‡	Atypical	Atypical

†The presence of all seven criteria defines a typical case; the presence of one to five criteria defines an atypical case. The cases did not meet the criteria for DIHS according to the JSCAR definition, but the patients' clinical symptoms were suggestive. ALT, alanine transaminase; CyA, cyclosporin A; DIHS, drug-induced hypersensitivity syndrome; DRESS, drug rash and eosinophilia with systemic symptoms; HHV, human herpesvirus; JSCAR, Japanese Research Committee on Severe Cutaneous Adverse Reactions; RegiSCAR, European Registry on Severe Cutaneous Adverse Reactions.

Correspondence: Hideo Hashizume, M.D., Ph.D., Department of Dermatology, Shimada Municipal Hospital, 1200-5 Noda, Shimada, Shizuoka 427-8502, Japan. Email: hihashiz0001@mac.com

Case 1, a 25-year-old woman with bipolar disorder, was treated with lamotrigine for 3 months. She then developed a sudden fever of 40°C with simultaneous emergence of a generalized rash and facial edema (Supporting information Fig. S1a–c). The cervical, axillary and inguinal lymph nodes were palpable with tenderness. Histological examination of the skin lesions on her left arm showed spongiosis of the epidermis with mild liquefaction and lymphocytic infiltration around the vessels (Supporting information Fig. S1e). Laboratory investigation revealed increased leukocytes with eosinophilia, atypical lymphocytosis and a high serum C-reactive protein concentration. The concentrations of soluble interleukin-2 receptor (sIL-2R) and serum thymus and activation-regulated chemokine (sTARC) were remarkably high (4847 U/mL [reference range, 0–500] and 15 489 pg/mL [reference range, 0–250], respectively). The Japanese Research Committee on SCAR (JSCAR) criteria were fulfilled, indicating typical DIHS. Because the patient declined steroid therapy, we chose short-term CyA therapy (p.o. administration at 3 mg/kg per day for 7 days), and the eruption rapidly disappeared (Supporting information Fig. S2). No relapse occurred thereafter.

Case 2, an 88-year-old woman with Alzheimer's disease, developed a generalized rash 15 days after beginning vancomycin for treatment of aspiration pneumonia. Although the vancomycin was discontinued, the eruption progressed. She was afebrile upon presentation. Facial edema and pruritic papules that coalesced into erythema with purpura on the body and extremities were found (Supporting information Fig. S3a). Lymphadenopathy was not obvious. Histological examination of the skin lesions on her left leg showed mild liquefaction and lymphocytic infiltration around the vessels (Supporting information Fig. S3b). Laboratory investigation revealed increased leukocytes with eosinophilia and atypical lymphocytosis and an increased C-reactive protein concentration. Her sIL-2R and sTARC concentrations were remarkably high (3818 U/mL and 15 555 pg/mL, respectively). Although the JSCAR criteria were not completely fulfilled, her RegiSCAR score was 6, indicating definite DRESS. Because the patient's son declined corticosteroid therapy, we decided to administrate short-term CyA therapy (p.o. administration at 3 mg/kg per day for 7 days), which resolved the skin manifestation (Supporting information Fig. S4). No relapse occurred thereafter.

Generally, administration of high-dose corticosteroids is empirically recommended as a standard treatment for DIHS/DRESS;[1] however, no solid evidence of the efficacy has been accumulated. Six DIHS/DRESS cases treated with CyA were reported (Table 1).[3–5] Surprisingly, all patients were administrated a relatively low dose (150–350 mg/day) of CyA for 7 days or less, resulting in complete regression without relapse or sequelae. In both our cases, raising of the specific antibody was suggestive of cytomegalovirus reactivation but no clinical symptom was found. No description about this was found in others. The short CyA therapy was deemed adequately effective although we cannot assess whether it prevents virus reactivation, and further investigation is needed to clarify this issue.

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Hideo HASHIZUME, Reiko KAGEYAMA, Yasuhito KANEKO
Department of Dermatology, Shimada Municipal Hospital, Shimada, Japan

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Clinical and histological features of case 1. (a) Enanthema on the hard palate, (b) papules and erythema on the abdomen, and (c) edema of the face were observed. (d) All symptoms disappeared 7 days after starting cyclosporin A. (e) Histological examination of skin from the left arm (hematoxylin–eosin, original manifestation ×100).

Figure S2. Clinical course of case 1. The concentrations of sIL-R2 and serum TARC during the patient's clinical course are shown. Atyp-Lym, atypical lymphocytes; BT, body temperature; CRP, C-reactive protein; CyA, cyclosporin A; LDH, lactate dehydrogenase; sIL-R2, soluble interleukin-2 receptor; TARC, thymus and activation-regulated chemokine; WBC, white blood cells.

Figure S3. Clinical and histological features of case 2. (a) Papules and erythema on the abdomen. (b) Histological examination of skin from the abdomen (hematoxylin–eosin [HE], original magnification ×100).

Figure S4. Clinical course of case 2. The eruption spontaneously disappeared approximately 10 days after onset, but it then reappeared 15 days after onset. The concentrations of sIL-R2 and serum thymus and activation-regulated chemokine during the patient's clinical course are shown. CRP, C-reactive protein; CyA, cyclosporin A; Eos, eosinophils; LDH, lactate dehydrogenase; sIL-R2, soluble interleukin-2 receptor; TARC, thymus and activation-regulated chemokine; VCM, vancomycin; WBC, white blood cells.

ORIGINAL ARTICLE

Repeated *Amblyomma testudinarium* tick bites are associated with increased galactose-α-1,3-galactose carbohydrate IgE antibody levels: A retrospective cohort study in a single institution

Hideo Hashizume, MD, PhD,[a] Toshiharu Fujiyama, MD,[b] Takatsune Umayahara, MD,[a] Reiko Kageyama, MD,[a] Andrew F. Walls, PhD,[c] and Takahiro Satoh, MD, PhD[d]
Shimada, Hamamatsu, and Tokorozawa, Japan; and Southampton, United Kingdom

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

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

Methods: We compared alpha-gal antibody levels between patients with and without a history of tick bites and examined histologic stainings of tick bite lesions between patients with and without detectable alpha-gal IgE antibody.

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

Limitations: The study was conducted at a single institution in Japan.

Conclusion: In *Amblyomma* tick bite lesions, basophils; eosinophils; and type 2, cytokine-producing T cells infiltrate the skin and alpha-gal IgE antibodies are produced. These findings provide a potential mechanistic connection between *Amblyomma* bites and red meat hypersensitivity. (J Am Acad Dermatol https://doi.org/10.1016/j.jaad.2017.12.028.)

Key words: alpha-gal syndrome; basophil; eosinophil; interferon γ; interleukin 4; tick bite; type 2 T cell.

There is local inflammation and a risk for infection by pathogenic microorganisms at the lesion sites of tick bites,[1,2] and recently, it has been recognized that bites from the *Amblyomma* species tick might be closely associated with the development of alpha-gal syndrome, a disease mediated by IgE antibody specific to galactose-α-1,3-galactose (alpha-gal), which causes immediate hypersensitivity to red meat.[3-5] The recent findings that basophils infiltrate the skin lesions of mice bitten repeatedly by ticks[6] and that basophils can function in antigen presentation[7] indicate that basophils

From the Department of Dermatology, Shimada Municipal Hospital[a]; Department of Dermatology, Hamamatsu University School of Medicine[b]; Department of Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton[c]; and Department of Dermatology, National Defense Medical College, Tokorozawa.[d]

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Correspondence to: Hideo Hashizume, MD, PhD, Department of Dermatology, Shimada Municipal Hospital 1200-5 Noda Shimada, Shizuoka, 427-8502, Japan. E-mail: hihashiz0001@mac.com.

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might be present in skin after tick bites and function as antigen-presenting cells to produce alpha-gal IgE antibody in humans. 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.

CAPSULE SUMMARY

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

MATERIALS AND METHODS

Twenty-one healthy volunteers (13 men, 8 women; 40.0 ± 11.7 [range 26-59] years of age) and 115 patients (58 boys and men, 57 girls and women; 64.4 ± 17.7 [range 4-91] years of age), 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 histologic 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 hematology and chemistry blood examinations and quantification of the levels of IgE antibodies specific to alpha-gal and 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, L-17, and IL-22) were purchased from BD PharMingen (San Diego, CA). Human basophil-specific antibody (BB1, basogranulin antibody) was obtained from the University of Southampton (Southampton, United Kingdom).

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 (Dr Ai Takano, Yamaguchi University, Japan) to identify the species.

Preparation of skin specimens

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

Histologic 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 described.[8] The degrees of cellular infiltration in the epidermis and dermis of the skin lesions were semiquantitatively evaluated and assigned to 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 8 high-power fields that were randomly selected from each of the lesions, and the mean values were calculated.

Cytokine production by skin-infiltrating T cells

Skin-infiltrating T cells from skin specimens of patients who had experienced 1 tick bite, 2 tick bites, or ≥3 tick bites in their lifetime were propagated using our previously established method.[9] 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 multiple comparison test. Correlations were analyzed by the Spearman rank test. GraphPad Prism 7 (GraphPad Software Inc, La Jolla, CA) was used for statistical analyses. P values $<.05$ were considered to indicate statistical significance.

Abbreviations used:

alpha-gal: galactose-α-1,3-galactose
IFN: interferon
IL: interleukin
T_H2: T helper 2

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*, and most were nymphs (71.0%). In this study, the patients for whom 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 area.[10] These geographic differences seem to correlate with the animals populating these areas.

Detection of IgE antibody specific 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 compared the rate of detection of alpha-gal IgE antibody in patients categorized by the number of historical tick bites (1 tick bite, 2 tick bites, 3 tick bites, and ≥4 tick bites) and 21 healthy control individuals that had never been bitten by ticks. The specific antibody was not detected (0/21) among the individuals with 0 tick bites, and 4% (2/45) of the patients with 1 tick bite were positive for the antibody; however, the difference between the antibody concentrations for these 2 groups was not statistically significant (Fig 1). By contrast, the patients with ≥2 tick bites showed higher antibody levels, which were significantly higher when compared with the antibody levels in patients with 0 or 1 tick bite (Fig 1; Table I), and a relative risk for alpha-gal syndrome of ≥11.4 (Table II). The percentage of alpha-gal IgE antibody positivity among patients with 2, 3, and ≥4 historical tick bites were similar, except there was a significant difference between patients with 2 and 3 tick bites, with a relative risk for alpha-gal syndrome of 1.89 (Tables I and II). We observed a close positive correlation between the serum levels of alpha-gal IgE antibody and IgE antibodies specific to red meat (Supplemental Fig 1; http://www.jaad.org) but not to chicken, indicating these antibodies reacted to shared molecules, such as alpha-gal. However, we also detected a small number of patients who were positive for alpha-gal IgE antibody and negative for red meat IgE antibody (Supplemental Fig 1), warning of the limitations of using red meat IgE antibody for diagnosing alpha-gal syndrome.

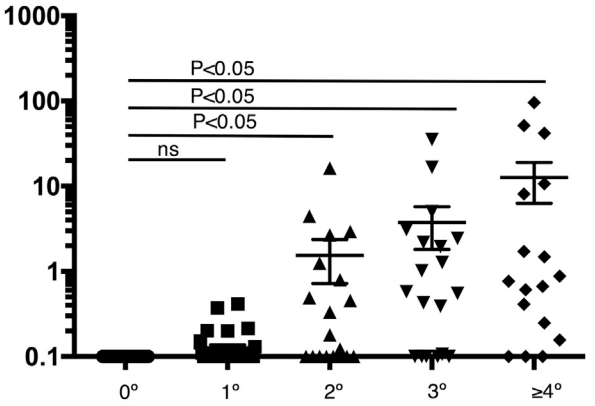

Fig 1. Alpha-gal IgE antibody levels (KU/L) in patients with different numbers of historical tick bites. *ns*, Not significant.

Table I. Alpha-gal IgE positivity among participants with different numbers of tick bites

		Alpha-gal IgE, n/total (%)	
Participants	**Tick bites, n**	**Positive**	**Negative**
Healthy individuals	0	0/21 (0)	21/21 (100)
Patients with tick bites	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)

Histologic features of tick bites in skin

In total, 53 skin specimens were available from patients who had ticks removed by punch biopsy rather than tick twister because these cases occurred before the introduction of the tick twister to our practice. No control specimens from healthy volunteers were available. Because we found a statistical difference in the concentrations of alpha-gal IgE antibody between patients with 1 and ≥2 tick bites, we investigated the histologic 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 infiltrations in the deep dermis in the group with ≥2 tick bites than in the group with

Table II. Comparison between groups with different numbers of tick bites

	P value (relative risk)*					
Tick bites, n	**0**	**1**	**2**	**3**	**≥4**	**≥2**
0		NS	.0025 (—)	<.0001 (—)	<.0001 (—)	<.0001 (—)
1			.0013 (3.94)	<.0001 (7.59)	<.0001 (6.45)	<.0001 (11.47)
2				.042 (1.89)	NS	
3					NS	

NS, Not significant; (—), incalculable.
**P* values for the relative risk between groups as determined by Fisher's exact test is indicated.

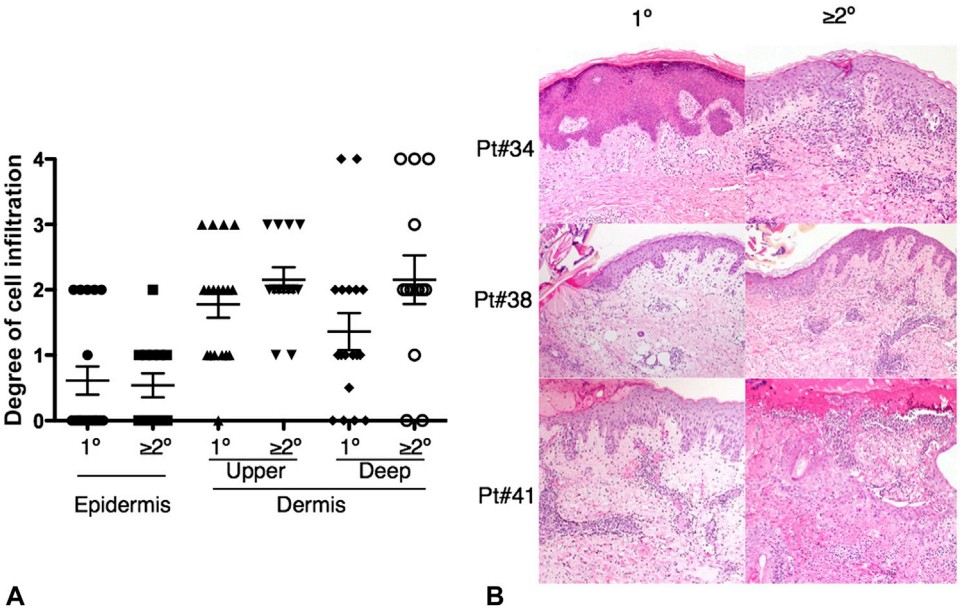

Fig 2. Histologic analysis of tick bite lesions from patients with 1 (1°) and ≥2 (≥2°) tick bites. **A**, Degree of cell infiltration into the epidermis and upper and deep dermis of skin lesions. The degree of infiltration was defined as follows: 0) none, 1) few, 2) mild, 3) moderate, and 4) severe. The medians and standard deviations are indicated. **B**, Histologic features present after 1 (1°) and ≥2 (≥2°) tick bites in representative patients. *Pt*, Patient.

1 tick bite; however, this difference was not statistically significant (P = .095; Fig 2, *A*). We individually compared skin specimens from a single patient with several tick bites. Skin specimens from 8 tick bite sites from 4 patients (which included the first tick bite and a subsequent tick bite in each patient) were examined (Fig 2, *B*). We found that the degree of cellular infiltration increased in both the epidermis and the dermis with the number of 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) was compared. Low levels of basophil infiltration were detected in the tick bite lesions of the group with 1 tick bite (Fig 3, *A* and *B*) despite significant cellular infiltration, as previously reported.[8,13] However, significant levels of basophil infiltration were detected in the tick bite lesions of the groups with ≥2 tick bites (Fig 3, *A* and *B*; P = .0028, Mann—Whitney U test), which was consistent with a previous murine study.[6] In the lesions from individuals with single tick bites, the number of infiltrating basophils (<50 cells/high-power field) was marginal (Supplemental Fig 2, *A*; available at http://www.jaad.org). By contrast, in the lesions from persons with 2 historical tick bites, the number of infiltrating basophils increased starting day 2 after the second tick bite, and in lesions from persons with ≥3 historical tick bite lesions, the number of basophils were high on day 1 after the last tick bite (Supplemental Fig 2, *B* and *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

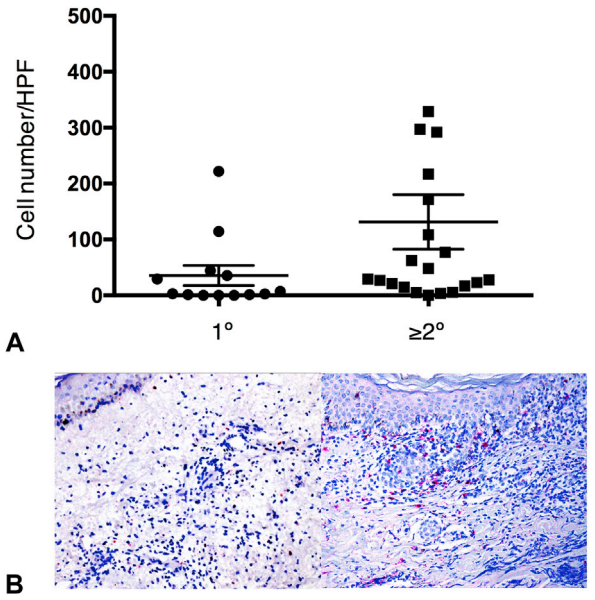

Fig 3. Basophil infiltration of tick bite lesions. **A**, Number of infiltrating basophils in tick bite lesions from patients with 1 (1°) and ≥2 (≥2°) tick bites. The medians and standard deviations are indicated. **B**, Representative basophil staining (*red*) of the first (left) and second (right) tick bite lesions in single patient. *HPF*, High-power field.

production between the patients with 2 and ≥3 tick bite lesions (Supplemental Fig 3, *B* and *C*; available at http://www.jaad.org). Higher numbers of infiltrating eosinophils were detected in the tick bite lesions of the groups with 2 and ≥3 tick bites compared with the group with 1 tick bite (Supplemental Fig 3, *A-C*), 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 might 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 Materials and Methods (Fig 4, *A* 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, $P < .05$). 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, $P < .05$). 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 1 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 those with ≥2 tick bites.

[F4-4/C]

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 (4 patients with 1 tick bite, 3 patients with 2 tick bites, and 3 patients with ≥3 tick bites) were expanded without artificial distortion of their function by a previously reported method that involved IL-2 and CD3 and CD28 antibody–coated microbeads.[9] 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 ~1:1 (mean ± SD: 0.9 ± 0.3:1). In the groups with 2 and ≥3 tick bites, $CD4^+$ cells produced extremely high quantities of IL-4, IL-5, and IL-13 but a low quantity of IFN-γ, and the type 2:type 1 ratios were high (mean ± SD: 3.0 ± 1.0:1 and 22.5 ± 27.3:1, respectively). 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 of the $CD4^+$ and $CD8^+$ populations in the patients with ≥2 tick bites suggested preferential type 2 T cell infiltration in the lesions of patients with ≥2 tick bites compared with those from patients with 1 tick bite.

DISCUSSION

The human immune system recognizes alpha-gal, a carbohydrate that is expressed on the cell membrane of many organisms, with the exception of primates, avian species, and humans. The human immune system produces IgM antibody against this molecule.[14] However, under specific situations, 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, research findings have indicated a close association between the production of alpha-gal IgE antibody and tick bites that contain abundant alpha-gal,[15,16] suggesting that

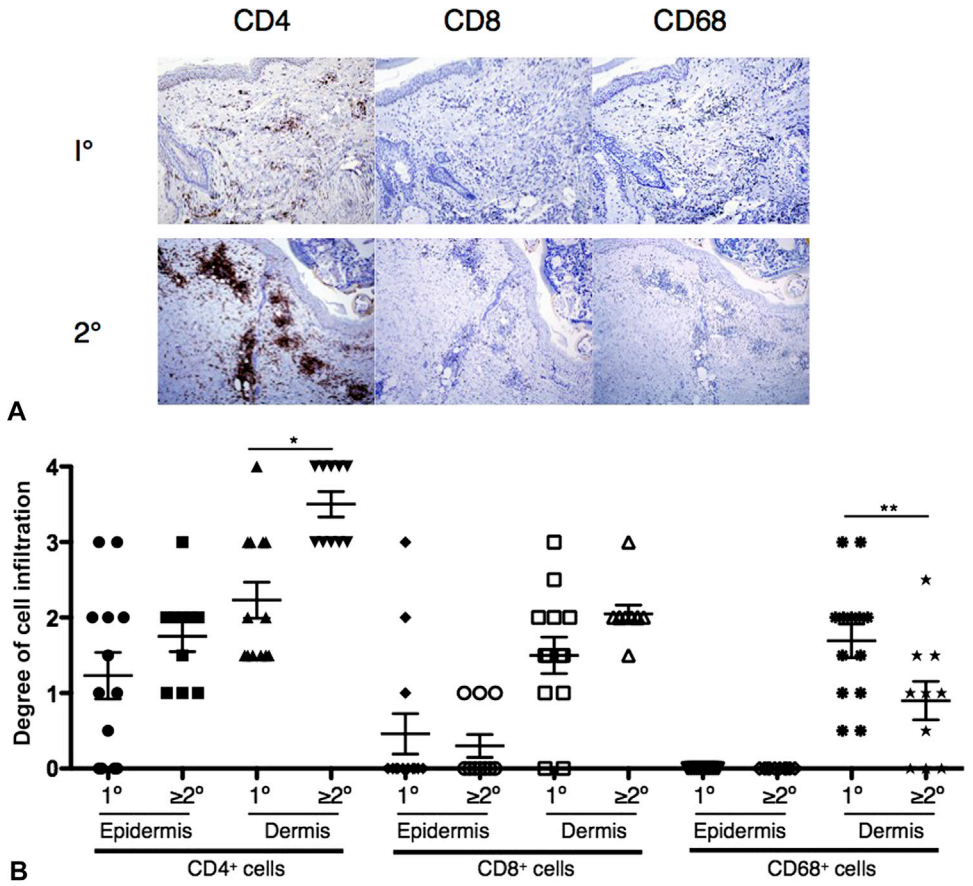

Fig 4. Infiltration of $CD4^+$, $CD8^+$, and $CD68^+$ cells in tick bite lesions of patients with 1 (1°) and ≥2 (≥2°) tick bites. **A**, Representative CD4, CD8, and CD68 cell staining of the first (upper) and the second (lower) lesions. **B**, Degree of infiltrating $CD4^+$, $CD8^+$, and $CD68^+$ cells in skin lesions of patients with 1 and ≥2 tick bites over their lifetimes. The degree of infiltration was defined as follows: 0) none, 1) few, 2) mild, 3) moderate, and 4) severe. $^*P = .0022$; $^{**}P = .0035$ by Mann–Whitney U test.

immune reactions following the entry of alpha-gal into the skin via a tick bite triggers alpha-gal IgE antibody production.

Basophils, comprising <1% of circulating white blood cells,[17] are recruited to the skin, and play a critical role in acquired immunity against ticks in mice bitten repeatedly[6]; 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, which lead to us detecting significant basophilic infiltration in human lesions. This disparity with previous reports might 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 T helper 2 (T_H2)–shifted immune condition; however, there was some dispute regarding the cell type that was the source of early IL-4, which was responsible for T_H2 cell differentiation. Activated basophils are a source of this cytokine[18]; however, there was controversy over whether these cells were acting as the antigen-presenting cells driving T_H2 cell differentiation.[19] Recently, it was clearly demonstrated in mice and humans that basophils can act as antigen-presenting cells using major histocompatibility complexes they sequester from dendritic cells via trogocytosis and drive type 2 cell differentiation.[7] This observation is consistent with our finding that type 2 T cells along with basophils infiltrated the skin lesions of patients with ≥2 tick bites 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, along with fibroblasts, in the skin.[20] Interestingly, we also found fewer $CD68^+$ cells in the skin of patients with ≥2 tick bites compared with the skin of patients with 1 tick bite. Our observations shed light on the immunologic 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 provide new insight into the mechanism responsible for the correlation between tick bites and alpha-gal IgE antibody production.

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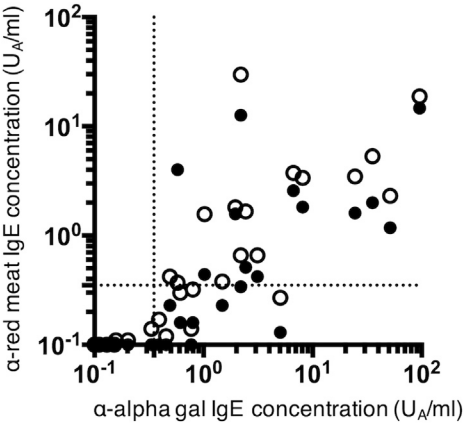
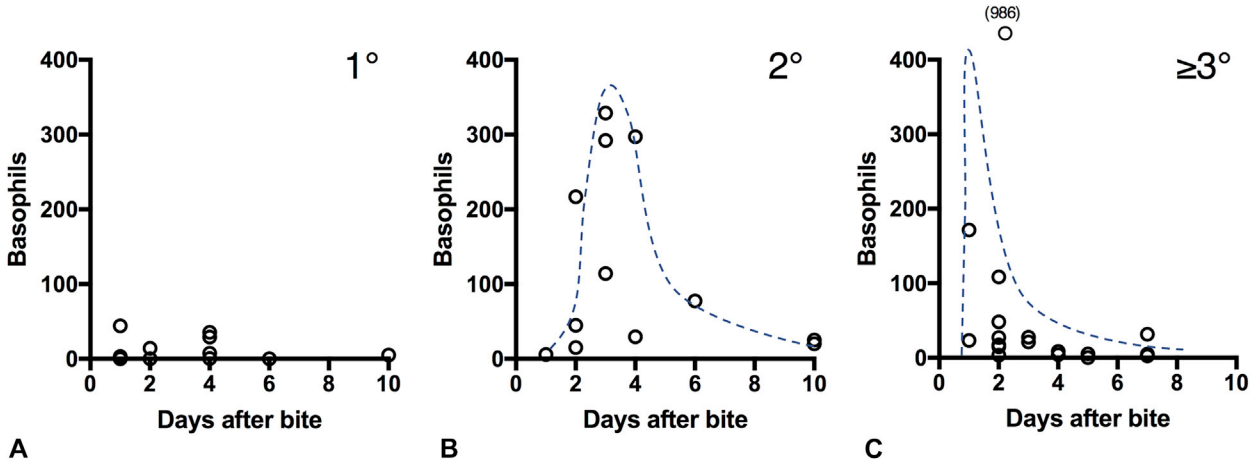
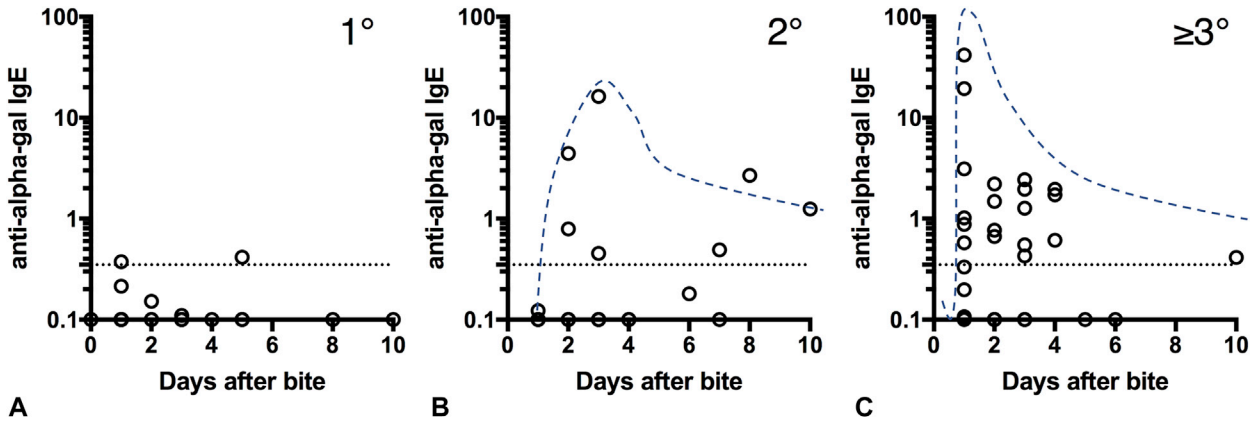


Supplemental Fig 1. Correlation between serum concentrations of alpha-gal IgE antibody and beef (*white circle*) and pork (*black circle*) meat IgE antibodies.



Supplemental Fig 2. A-C, Kinetics of basophil infiltration in skin biopsies from patients with 1 (1°), 2 (2°), and ≥3 (≥3°) tick bites.



Supplemental Fig 3. A-C, Kinetics of alpha-gal IgE antibody production (U_A/mL) in blood after tick bite in patients with a history of 1 (1°), 2 (2°), and ≥3 (≥3°) tick bites.

Non-pigmenting fixed drug eruption with mixed features of acute generalized exanthematous pustulosis induced by pseudoephedrine: a case report

Risa Fukuda, Takeshi Ouchi, Ikuko Hirai, Takeru Funakoshi, Aki Honda, Keiji Tanese, Akiko Tanikawa, Masayuki Amagai and Hayato Takahashi

Department of Dermatology, Keio University School of Medicine, Tokyo 160-8582, Japan

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Key words: acute generalized exanthematous pustulosis; case report; non-pigmenting fixed drug eruption; pseudoephedrine.

Case Report

A 43-year-old Japanese woman was referred to our department because of a widespread painful skin eruption. She had taken Asgen® (Asgen, Nagoya, Japan) containing pseudoephedrine hydrochloride (HCl) (CAS no. 345-78-8) for treatment of a common cold. Six hours after one dose, she developed patches of demarcated erythema with pustules on the chest, neck, lower back, navel,

Correspondence: Hayato Takahashi, Department of Dermatology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku Tokyo 160-8582, Japan. Tel: +81 3 5363 3823; Fax: +81 3 3351 6880. E-mail: hayato_takahashi@keio.jp

Conflicts of interest: There is no conflict of interest for this article.

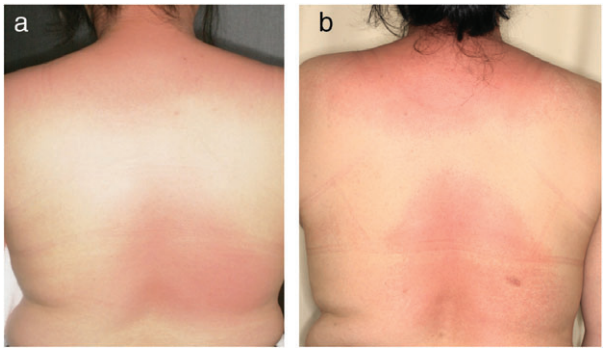

Fig. 1. The eruption developed in the same area during the (a) first episode and (b) second episode.

and cubital fossa; she also developed a fever of 37.8°C (Fig. 1a). Her white blood cell count was 15.8×10^9/l. The medication was discontinued, and the skin lesions disappeared within 1 week without residual pigmentation during a 10-day course of oral prednisolone starting at 60 mg/day. Eight months after this episode, the patient took Pavlon Gold A® (Taisho Pharmaceutical, Tokyo, Japan) containing dimethylephedrine HCl, codeine phosphate and acetaminophen for 3 days for treatment of a common cold. She subsequently also took Shoseiryuto® (Tsumura, Tokyo, Japan) containing ephedrine and pseudoephedrine HCl and acetaminophen (Tatsumi Kagaku, Ishikawa, Japan). Six hours later, she developed extensive erythema with pustules, which recurred at the same sites as in the first episode, with a fever of 37.5°C. However, additional lesions were observed in the groin, and the individual eruptions were larger in size (Fig. 1b). The patient's white blood cell count was 7.4×10^9/l (neutrophils, 73.7%). Eruptions disappeared after cessation of drugs and 10 days of oral prednisolone treatment starting from 40 mg/day within 1 week and without hyperpigmentation; the superficial pustules dried and were covered by fine scales.

Histopathological examination of lesional skin during the first episode showed non-follicular subcorneal pustules with intraepidermal neutrophilic infiltration. No liquefaction degeneration was observed. In the upper dermis, there was mixed perivascular and interstitial infiltration of lymphocytes, eosinophils, and neutrophils (Fig. 2). A biopsy obtained during the second episode showed similar findings, which corresponded to the histological features seen in acute generalized exanthematous pustulosis (AGEP). A lymphocyte transformation test gave positive results for Asgen®, Pavlon Gold A®, and Shoseiryuto®, and a negative result for acetaminophen. Patch testing of the tablets (5% and 10% pet.) on previously affected skin gave positive results for Asgen® and Shoseiryuto®, but negative results for Pavlon Gold A® and acetaminophen. Thus, pseudoephedrine HCl, which is a common component of Asgen® and Shoseiryuto®, was suspected to be the causative agent. Because pseudoephedrine HCl on its own is not available in Japan, owing to legal limitations, we performed patch tests with Allegra® (Sanofi K.K., Tokyo, Japan) and Dellegra® (Sanofi K.K.), an equivalent of Allegra® and pseudoephedrine HCl, on both previously affected and unaffected skin areas. Only the patch tests with Dellegra® gave positive results on both previously affected and unaffected skin areas (Fig. 3, and Fig. S1 in File S1), and the patch test with Allegra® gave a negative result. Thus, pseudoephedrine HCl was diagnosed to be the causative agent (see File S1).

Discussion

Non-pigmenting fixed drug eruption (NPFDE) was first reported as a clinically distinct drug eruption caused by pseudoephedrine HCl and tetrahydrozoline in 1987 by Shelley and Shelley (1). The characteristic clinical features of NPFDE are large, symmetrical erythematous plaques on the axilla, buttocks and inguinal regions that recur at the same sites and resolve without the pigmentation seen in classic fixed drug eruption (1). Although several cases of NPFDE have been reported, its pathology and pathogenesis have not been well established. AGEP is a severe cutaneous adverse reaction characterized by widespread erythema with multiple sterile subcorneal pustules (2). The aetiology, clinical features and histopathology of AGEP have been well described. Currently, NPFDE and AGEP are not considered to belong to the same disease spectrum, and a common pathogenesis of NPFDE and AGEP has not been identified, as cases of AGEP with features of NPFDE, and vice versa, have not been reported.

This is the first reported case of NPFDE with features of AGEP. The clinical features of recurrence at the same sites and remission without residual pigmentation led us to the diagnosis of NPFDE. The more severe patch test reaction observed on the previously affected skin than on the unaffected skin may support our diagnosis. Individual eruptions with multiple pustules and pathological neutrophil infiltration into the epidermis are features of AGEP. Our patient's neutrophil count and body temperature at disease onset were high, but did not fulfil the diagnostic criteria for AGEP (2).

Pseudoephedrine HCl and dimethylephedrine HCl are sympathomimetic drugs with vasoconstricting effects. They are frequently used in commercially available cold medicines. Thirty-two cases of adverse drug reactions to

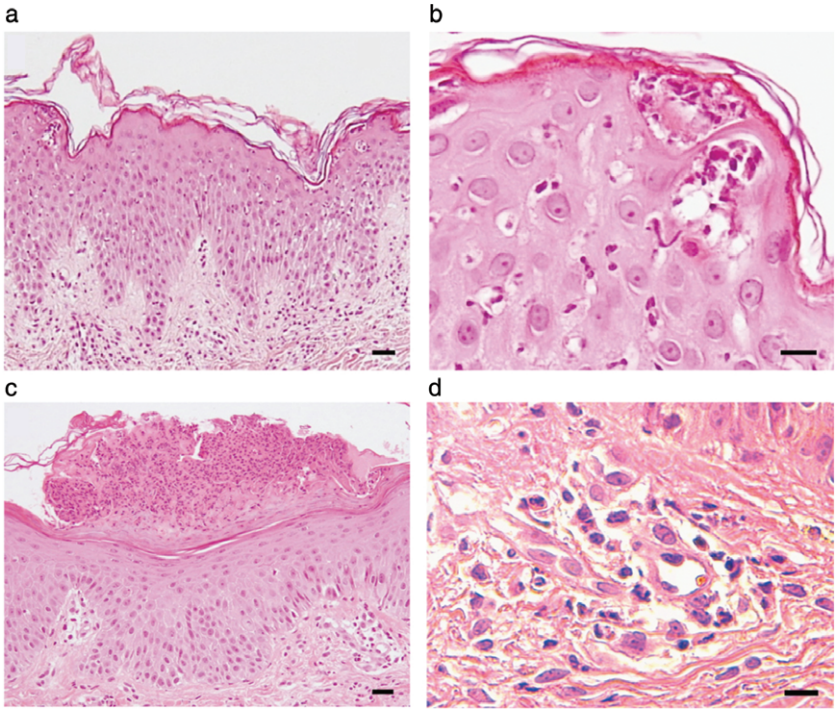

Fig. 2. Histopathology of the lesional skin. (a, b) Pathological findings in the first episode. (a) There was a microabscess under the horny layer, and infiltration of neutrophils into the epidermis. No liquefaction degeneration was observed. (b) The upper dermis showed infiltration of lymphocytes and neutrophils. (c, d) Pathological findings in the second episode. (c) There was an abscess in the horny layer. No liquefaction degeneration was observed. (d) The upper dermis showed infiltration of lymphocytes, eosinophils, and neutrophils. Scale bars: 100 μm.

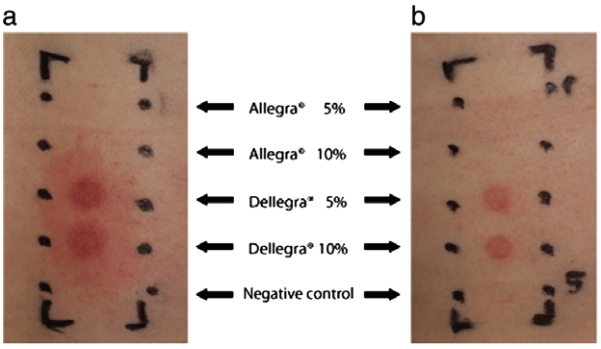

Fig. 3. Results of patch testing on day 3. Patch testing was performed on the (a) previously affected area and (b) unaffected skin area. A positive reaction to Dellegra® (5–10%) was observed at both sites, and the skin reaction was stronger in the affected than in the unaffected skin area.

pseudoephedrine HCl have been reported in the literature (1, 3–32). The clinical types of adverse reactions induced by pseudoephedrine HCl are relatively limited: NPFDE in 11 cases (1, 3, 14, 20–25, 27), AGEP in 3 cases (9, 10, 12), baboon syndrome in 3 cases (7, 13, 19), fixed drug eruption in 4 cases (4, 6, 28, 32), erythema and papules in 5 cases (5, 8, 15–17), and others in 6 cases (11, 18, 26, 29–31). According to our literature search, a skin biopsy was performed in only 5 cases of NPFDE (32–36). We found that the histological examination of NPFDE-affected skin did not show prominent lymphocytic infiltration into the epidermis or obvious epidermal keratinocyte necrosis, in contrast to cases of classic fixed drug eruption, and 2 cases showed neutrophil infiltration into the epidermis or upper dermis, similarly to our case (32, 36). These findings, including our case, suggest that interface dermatitis is not necessarily a typical histological change in NPFDE. The clinical and pathological features commonly seen in NPFDE and AGEP indicate that NPFDE caused by pseudoephedrine HCl may have a pathogenesis similar to that of AGEP.

Acknowledgements

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

File S1. Details to identify pseudoephedrine as a causative drug in this case.

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CONCISE COMMUNICATION

Unexpected recalcitrant course of drug-induced erythema multiforme-like eruption and interstitial pneumonia sequentially occurring after nivolumab therapy

Hisashi NOMURA,[1] Hayato TAKAHASHI,[1] Satsuki SUZUKI,[1] Yuichi KURIHARA,[1] Shotaro CHUBACHI,[2] Ichiro KAWADA,[2] Hiroyuki YASUDA,[2] Tomoko BETSUYAKU,[2] Masayuki AMAGAI,[1] Takeru FUNAKOSHI[1]

[1]*Department of Dermatology,* [2]*Division of Pulmonary Medicine, Department of Medicine, Keio University School of Medicine, Tokyo, Japan*

ABSTRACT

Vemurafenib improves survival of melanoma patients. However, cutaneous side-effects commonly occur in them. Nivolumab and ipilimumab are monoclonal antibodies against programmed death 1 and cytotoxic T-lymphocyte-associated antigen 4, both of which regulate excessive T-cell activation. Although these agents induce antitumor immunity against melanoma, the modified immune condition may result in an unexpected adverse reaction which has not been observed previously. Herein, we report a case who manifested severe erythema multiforme-like eruption with mucosal involvement associated with vemurafenib following nivolumab. The patient also subsequently suffered from ipilimumab-induced interstitial pneumonia with refractory course. Such a case has never been reported. This case suggested that dermatologists should pay special attention to unexpected adverse events of these drugs, and carefully observe cutaneous and respiratory status of patients during the treatment of melanoma.

Key words: erythema multiforme, interstitial pneumonia, ipilimumab, nivolumab, vemurafenib.

INTRODUCTION

Nowadays, the therapeutic strategies against metastatic melanoma are drastically revised with newly available drugs targeting specific molecules. The selective BRAF inhibitor, vemurafenib, improves survival of melanoma patients but skin toxicity commonly occurs in them.[1] Nivolumab and ipilimumab are monoclonal antibodies against programmed death 1 (PD-1) and cytotoxic T-lymphocyte-associated antigen 4, respectively, both of which regulate excessive T-cell activation and are critical to maintain immune homeostasis. Both agents evoke antitumor immunity against melanoma and improve overall patient survival rates; instead, a modified immune condition may result in unexpected adverse reactions which have not been observed previously, especially when combining these reagents.[2,3] Here, we report an exceptional case who manifested extended erythema multiforme (EM)-like eruption with mucosal erosion associated with vemurafenib following nivolumab. The patient also subsequently suffered from ipilimumab-induced interstitial pneumonia (IP) with refractory course. This case suggested that physicians should pay special attention to unexpected side-effects of these drugs, and carefully observe cutaneous and respiratory status of patients during the management of melanoma.

CASE REPORT

A 43-year-old Japanese man with no history of drug eruption or allergy was diagnosed with malignant melanoma of his right cheek. The primary lesion (pT4aN0M0, stage IIB) was resected, but 1-year postoperatively, right cervical lymph node metastasis was detected. Following cervical lymph node dissection, recurrence at the primary surgical site and cervical lymph nodes occurred repeatedly; thus, serial local excisions were performed. Four years after the initial operation, metastasis to the parotid gland was detected and we administrated nivolumab therapy (2 mg/kg bodyweight, every 3 weeks). During the treatment, the patient achieved stable disease. However, after six courses, progressive disease (PD), metastasis to the parotid gland and liver, was confirmed.

The BRAF V600E mutation was found in a tumor of lymph node; thus, nivolumab was switched to oral vemurafenib, with no other concomitant drugs. Ten days later (37 days after nivolumab cessation), high fever (104°F) and integrated multiple

Correspondence: Takeru Funakoshi, M.D., Ph.D., Department of Dermatology, Keio University School of Medicine, 35-banchi, Shinanomachi, Shinjuku-ku, Tokyo 160-0016, Japan. Email: takeruf@a8.keio.jp
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erythemas with tension ridges and central dark color plaques developed over the whole body, with broad erosion of hard palate but no eye involvement (Fig. 1a–d). The skin manifestation of our patient did not include scaling, desquamation, edema or purpura. Histopathology of the eruptions on the trunk revealed vacuolar degeneration of the epidermis and inflammatory lymphocytic infiltrate in the dermis, but no epidermal necrosis (Fig. 1e,f). Laboratory data showed marginally elevated white blood cell count (9600/μL; neutrophils, 92%; eosinophils, 1.5%; lymphocytes, 5.2%; and basophils, 0.1%). The patient did not manifest atypical lymphocytosis. Although liver function enzymes were normal, creatinine (1.46 mg/dL) was elevated. Particle agglutination test for detecting Mycoplasma pneumonia antibody was negative (titer, <40). The test of antinuclear antibody and blood cultures were not conducted. Lymphocyte transformation test (LTT) of vemurafenib showed a stimulation index (SI) of 1.7. Systemic corticosteroid (prednisolone, PSL) was started (1 mg/kg per day) with cessation of vemurafenib. However, the eruptions and fever did not improve. Therefore, i.v. corticosteroid pulse therapy (methyl PSL 1000 mg/day, 3 days) was started, resulting in immediate resolution of the eruptions and fever. The oral corticosteroid dose was tapered over 3 weeks with no recurrence of the eruption. Then, computed tomography (CT) revealed PD of melanoma, namely, new liver metastatic lesions.

Four weeks after the cessation of vemurafenib, ipilimumab was started as an alternative melanoma treatment (3 mg/kg bodyweight, every 3 weeks). After two courses of treatment, 23 days from ipilimumab initiation, severe dyspnea and cough developed. Oxygen saturation was 90% in room air. He had fine crackles in the lower lung field. Chest CT revealed diffuse bilateral ground-glass opacities with predominantly lower lobe distribution (Fig. 2a). Because of the deteriorated respiratory function, bronchoscopy could not be conducted. Laboratory data revealed elevated KL-6 (1065 U/mL) and SP-D (140 mg/mL) levels; thus, ipilimumab-induced IP was suspected. i.v. corticosteroid pulse therapy (methyl PSL 1000 mg/day, 3 days) was started and then switched to oral PSL therapy (70 mg/day). The patient's dyspnea and hypoxemia gradually improved. Repeat chest imaging showed resolution of the infiltrates 18 days later (Fig. 2b). Three weeks after IP onset, he developed maculopapular drug eruption associated with trimethoprim/sulfamethoxazole, which was used as prophylactic agent against *Pneumocystis jiroveci* pneumonia. The eruption disappeared immediately after drug cessation. The LTT for trimethoprim/sulfamethoxazole was positive (SI, 2.6). PSL was tapered to 10 mg/day over 2 months from IP onset. However, 2 months after the initial IP onset, it relapsed, resulting in breathing difficulties. CT confirmed the recurrence of diffuse infiltrative shadows in the lungs; blood test results revealed elevated KL-6 (2083 U/mL) and SP-D (191 mg/mL) levels. Cytopathological and microbiological analyses by bronchoscopy showed no evidence of infection or malignancy, while bronchoalveolar lavage revealed an increased proportion of lymphocytes, suggesting lymphocytic alveolitis (lymphocytes, 78%; neutrophils, 2%; eosinophils, 5.5%; monocytes, 10.5%; and macrophages, 4%). Transbronchial biopsy specimens showed interstitial inflammation, septal thickening and alveoli organization. The PSL dose was increased to 35 mg/day, which gradually improved the respiratory function. PSL was tapered over 3 months. Eight months from IP onset, there was no relapse.

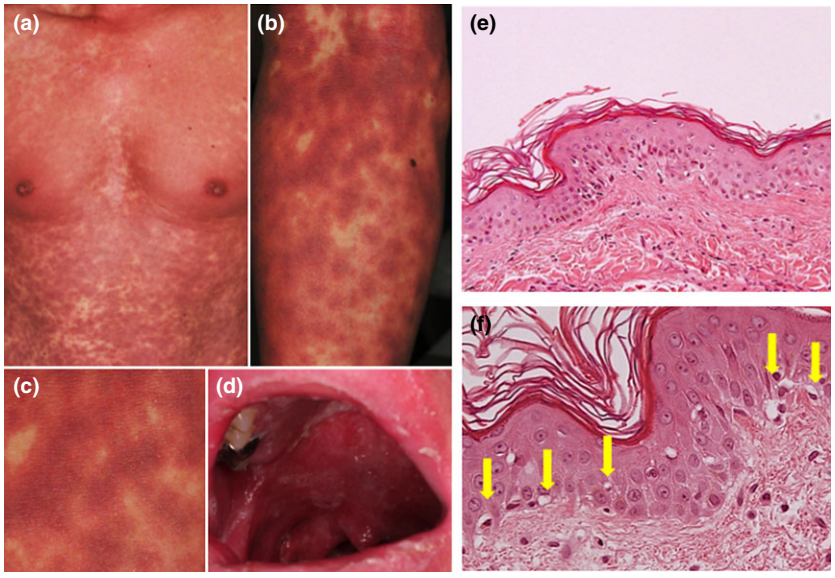

Figure 1. Clinical manifestations of the patient. (a) Lesions on the body. (b,c) Integrated multiple erythemas with tension ridges and central dark color plaques on the right arm and its extended image. (d) Broad erosion of the hard palate. (e,f) Histopathology of the lesion (hematoxylin–eosin [HE], original magnifications: [e] ×100; [f] ×400). Vacuolar degeneration (arrows) of epidermis and no epidermal necrosis.

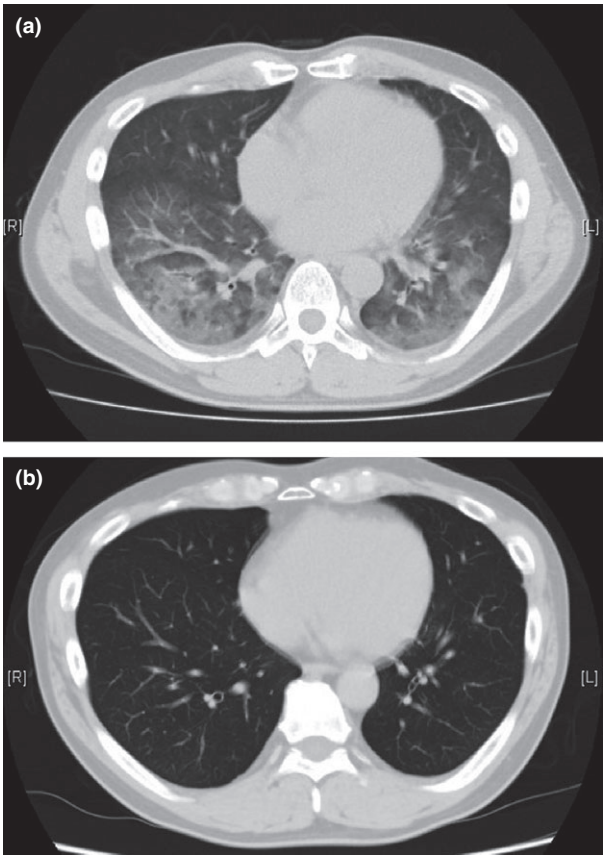

Figure 2. (a) Computed tomography (CT) of the onset of interstitial pneumonia (IP). Bilateral diffuse ground-glass opacities in the posterior parts of the lungs. (b) CT image at the initial resolution of IP. Disappearance of the infiltrates.

DISCUSSION

The published work on vemurafenib-associated EM is lacking, owing to its rarity.[4] Our patient presented with severe and atypical EM-like eruptions. The rash was distributed over the whole body with a high fever and broad erosion of the hard palate. Because the initial corticosteroid therapy was insufficient, high-dose corticosteroid pulse therapy was needed. EM usually does not require corticosteroid pulse therapy. The manifestations of our case mimicked severe drug eruption, as observed with Stevens–Johnson syndrome (SJS). However, there was no epidermal necrosis in histology; thus, the possibility of SJS was excluded. There are also some reports of drug rash with eosinophilia and systemic symptoms (DRESS) cases due to vemurafenib.[5] In the scoring system for classifying DRESS,[6] the score of this case is 0, excluding the possibility of DRESS. Our case also did not meet the criteria of drug-induced hypersensitivity syndrome developed by a Japanese consensus group.[7]

Imafuku *et al.*[8] reported severe rash development with vemurafenib administration following nivolumab and hypothesized that altered immune function by nivolumab worsened the vemurafenib-induced rash. Johnson *et al.*[9] reported severe hypersensitivity drug eruptions with multi-organ injury early in the BRAF inhibitor treatment after the initial anti-PD-1 therapy. In another report, patients treated sequentially with vemurafenib following ipilimumab developed severe eruptions that responded poorly to corticosteroids.[10] In our case, nivolumab use probably influenced immunological status and worsened the subsequent vemurafenib-induced EM-like eruptions.

Although ipilimumab induces various adverse effects of the gastrointestinal, hepatic, cutaneous and endocrine systems,[3] IP is relatively rare.[11] A pathophysiology of drug-induced IP is harmful immune reaction, which may be T-cell mediated.[12] Excessive T-cell activation under ipilimumab therapy might have played a role in the onset of IP. The previously reported cases with ipilimumab-induced IP usually resolved within several weeks after medical intervention, and did not recur.[11] In our case, however, the recurrence was observed while tapering PSL, and it took over 6 months to control disease activity of IP, demonstrating that the IP seen in this case was refractory. In fact, it has recently been reported that nivolumab followed by ipilimumab therapy caused a high frequency of severe adverse effects including pneumonitis and grade 4 respiratory failure,[13] indicating that sequential use of these reagents may cause more severe side-effects than those usually expected. The initial nivolumab treatment might have contributed to the deterioration of ipilimumab-induced IP in this case. Nishino *et al.*[14] described three melanoma patients who developed pneumonitis associated with nivolumab therapy. Interestingly, two of them had been either sequentially or previously treated with ipilimumab, and presented severe clinical courses.

Two agents were administrated after the initial nivolumab therapy, and caused quite severe clinical symptoms. The underlying immune hypersensitivity after the nivolumab use may trigger not only various but severe and unusual manifestations associated with subsequent medications. The approval of nivolumab for first-line treatment of advanced melanoma has been announced.[15] Thus, the number of cases following vemurafenib or ipilimumab administration after nivolumab therapy is expected to increase. Physicians should recognize the possibility of such severe and unusual side-effects after this drug sequence. Careful observation of cutaneous and respiratory function of patients after administration of these agents is recommended.

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CONFLICT OF INTEREST: None declared.

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Novel interferon-γ enzyme-linked immunoSpot assay using activated cells for identifying hypersensitivity-inducing drug culprits

Kenichi Kato[a,1], Asami Kawase[c,1], Hiroaki Azukizawa[a,c,*], Takaaki Hanafusa[a,d], Yukinobu Nakagawa[a], Hiroyuki Murota[a], Shimon Sakaguchi[b,e], Hideo Asada[c], Ichiro Katayama[a]

[a] Department of Dermatology, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[b] Department of Experimental Immunology, Immunology Frontier Research Center, Osaka University, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan
[c] Department of Dermatology, Nara Medical University, 840, Shijo-cho, Kashihara, Nara 634-8521, Japan
[d] Department of Dermatology, Tokyo Medical and Dental University, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8519, Japan
[e] Department of Experimental Pathology, Institute for Frontier Medical Sciences, Kyoto University, 53 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

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ABSTRACT

Background: The drug-induced lymphocyte stimulation test (DLST), also referred to as lymphocyte transformation test (LTT), is used to identify the culprit drug in cases of cutaneous adverse drug reactions (cADR). Although DLST is a widely used in vitro test, its sensitivity and specificity are unsatisfactory. Recent reports suggest that the detection of drug-induced interferon (IFN)-γ production using enzyme-linked immunoSpot (ELISpot) assay (conventional IFN-γ ELISpot) is useful for identifying culprit drugs in cADR cases.

Objective: The aim of this study was to establish a novel method for identifying culprit drugs in patients with cADR by efficiently detecting drug-specific IFN-γ production using activated cells.

Methods: Sixteen patients with cADR, including drug-induced hypersensitivity syndrome, erythema multiforme-like eruption, maculopapular exanthema, Stevens-Johnson syndrome, and toxic epidermal necrolysis, caused by clinically convincing culprit drugs were enrolled in this study. In some cases, the blood samples were obtained at two or three different time points. Peripheral blood mononuclear cells (PBMCs) from total 20 samples were analyzed using both the DLST and drug-induced conventional IFN-γ ELISpot. In addition, drug-induced IFN-γ ELISpot was performed using PBMCs, which were stimulated with anti-cluster of differentiation (CD)-3/CD28 antibody-coated microbeads and interleukin (IL)-2 for 7 days before exposure to the culprit drugs (modified IFN-γ ELISpot).

Results: Among the culprit drugs tested in each patient, the modified IFN-γ ELISpot was positive in 17 samples (13 patients) while DLST and conventional IFN-γ ELISpot were positive in eight and four samples (six and three patients), respectively.

Conclusion: The modified IFN-γ ELISpot using activated PBMCs was more sensitive than the conventional IFN-γ ELISpot was for detecting drug-induced IFN-γ production, which could be a useful in vitro tool for identifying culprit drugs in cADR cases.

Abbreviations: DLST, drug-induced lymphocyte stimulation test; LTT, lymphocyte transformation test; cADR, cutaneous adverse drug reactions; PBMCs, peripheral blood mononuclear cells; ELISpot, enzyme-linked immunospot; OVA, ovalbumin; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis; DIHS, drug-induced hypersensitivity syndrome; DRESS, drug rash with eosinophilia and systemic symptoms; EM, erythema multiforme; MPE, maculopapular exanthema; PHA, phytohemagglutinin; SMX/TMP, sulfamethoxazole/trimethoprim.

* Corresponding author. Present address: 840 Shijo-cho, Kashihara, Nara 634-8521, Japan.
E-mail addresses: bykenbykenbyken@yahoo.co.jp (K. Kato), 41600401a@naramed-u.ac.jp (A. Kawase), azukizaw@naramed-u.ac.jp (H. Azukizawa), takafussa0710@gmail.com (T. Hanafusa), nakagawa@derma.med.osaka-u.ac.jp (Y. Nakagawa), h-murota@derma.med.osaka-u.ac.jp (H. Murota), shimon@ifrec.osaka-u.ac.jp (S. Sakaguchi), asadah@naramed-u.ac.jp (H. Asada), katayama@derma.med.osaka-u.ac.jp (I. Katayama).

[1] These authors equally contributed to this work as first authors.

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1. Introduction

T cell-mediated delayed hypersensitivity is responsible for the pathogenesis of severe cutaneous adverse drug reactions (cADRs), including Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome (DIHS), also called drug rash with eosinophilia and systemic symptoms (DRESS) syndrome [1–4]. In severe cADR, the drug-induced lymphocyte stimulation test (DLST), also referred to as the lymphocyte transformation test (LTT), is used to identify culprit drugs because oral provocation test using culprit drugs cannot be recommended for ethical safety reasons. Although patch testing is safer than the oral provocation test and is useful when contrast media or anti-epileptics are suspected to be the causative drugs, its sensitivity is not always satisfactory [5]. In the DLST, peripheral blood mononuclear cells (PBMCs) are incubated with the culprit drug for approximately 7 days and then the cell proliferation is measured using ^{3}H-thymidine incorporation for the last 24 h. The DLST is commonly performed in Japan because this test is covered by health insurance as a diagnostic method for cADR. While DLST is a widely used in vitro test, its sensitivity and specificity are unsatisfactory. Previous reports indicate that the sensitivities of DLST are 47% in highly probable patients and 74% in definite patients of cADR [6].

The determination of antigen-specific IFN-γ production using ELISpot assay is a well-established in vitro diagnostic method for tuberculosis infections. T-SPOT.TB (Oxford Immunotec, Oxford, UK) is a commercially available IFN-γ ELISpot for detecting tuberculosis infection, which is highly sensitivity in high-risk populations (84.1–94.1%) and shows a high specificity in low-risk populations (98.7–99.1%) [7–12]. Hashizume et al. [13] reported the detection of drug-induced IFN-γ production using conventional IFN-γ ELISpot in PBMCs that were freshly isolated from phenobarbital-induced cADRs. Recent reports suggest that drug-induced conventional IFN-γ ELISpot is more sensitive than DLST is, and this method is suitable for identifying the culprit drug in cADR cases [14–18].

Previously, we reported a mouse model of TEN established using T cells from OT-I transgenic mice [19]. Most of the CD8^{+} T cells in OT-I mice express a class I-restricted ovalbumin (OVA)-specific T cell receptor. These antigen-specific T cells activated by OVA peptide show cell division and IFN-γ production, leading to cytotoxicity. A previous study using T cells from OT-I mice demonstrated that the determination of IFN-γ production requires the activation of T cells by a 7-day stimulation with anti-cluster of differentiation 3 (CD)-3/CD28 antibody-coated microbeads and interleukin (IL)-2 before exposure to the OVA peptide [20]. Therefore, we speculated that IFN-γ production by drug reactive T cells might be efficiently detected following activation of patients' PBMCs with anti-CD3/CD28 microbeads and IL-2. We performed a drug-induced ELISpot assay using similarly activated human PBMCs. In this study, we demonstrated that drug-induced IFN-γ ELISpot using activated PBMCs was more sensitive than DLST and conventional IFN-γ ELISpot were.

2. Materials and methods

2.1. Patients and drugs

Sixteen patients with cADR caused by the clinically convincing culprit drugs sulfamethoxazole/trimethoprim (SMX/TMP), celecoxib, allopurinol, lamotrigine, and phenytoin and three healthy volunteers were enrolled in this study. The culprit drugs that caused the cADR in each case were determined based on the clinical course and drug history. In the assessment of all cADR cases using the Naranjo algorithm, which is one of the most widely used causality assessment tools, enrolled cases were categorized as 14 probable cases and two definite cases, but none of them were unlikely cases or possible cases.

In cases 2, 5, and 10, the blood samples were obtained at two or three different time points. PBMCs freshly prepared using Ficoll gradient separation were used for the three different assays: DLST, as well as conventional and modified IFN-γ ELISpot. The samples used in the three methods were collected during the same blood draw and were assayed within 24 h after their collection. Irrelevant drugs were selected as those administered safely in each patient without the recurrence of rashes after the onset of cADR. The culprit drugs were dissolved in phosphate-buffered saline (PBS) or PBS with 0.025% dimethyl sulfoxide (Wako) if the drug was PBS-insoluble. This study was approved by our Institutional Review Board (Osaka University, No. 08088 and Nara Medical University, No. 1257) and conducted according to the Helsinki declaration. Informed consent for all diagnostic procedures and research was obtained from all the patients and healthy volunteers.

3. DLST/LTT

PBMCs were distributed in duplicate in 96-well flat-bottom microwell plates (2×10^5 cells/well) in 1640 Roswell Park Memorial Institute (RPMI) medium supplemented with 10% AB serum, in the absence or presence of the culprit drugs (100, 10, and 1 μg/mL) or irrelevant drugs (100 μg/mL). The positive controls were phytohemagglutinin (PHA)-stimulated cultures (10 μg/mL). The cultures were incubated for 7 days at 37 °C in 5% CO_2, and ^{3}H thymidine was added to each well for the last 24 h. The drug-specific proliferation was determined based on ^{3}H thymidine incorporation. The results are expressed as the stimulation index (SI), which is the ratio of the highest count per minute of the samples cultured with diluted drug to that of the control cultured without a drug. An SI value > 2.0 was interpreted as a positive result.

3.1. Cell activation and IFN-γ ELISpot

For the modified IFN-γ ELISpot assay, the PBMCs were stimulated with Dynabeads Human T-activator CD3/CD28 according to manufacturer's protocol except for the timing of IL-2 addition. In addition, PBMCs were used without T cell purification. Briefly, the PBMCs were cultured in 10% AB serum in RPMI-1640 (1×10^6 cells/mL) containing 25 μL/mL anti-CD3/CD28 antibody-coated microbeads (Gibco, Life Technologies) and 30 IU/mL human recombinant IL-2 (Miltenyi Biotec) for 7 days at 37 °C exposed to an atmosphere of 5% CO_2. IL-2 was added only at the beginning of cell activation. The activated cells were harvested, and the bound microbeads were detached using a magnetic device. Both the conventional and modified IFN-γ ELISpot were performed using a human IFN-γ ELISpot kit (Mabtech). Cells were distributed in duplicate in 96-well ELISpot plates (2×10^5 cells/well) in 1640 RPMI medium supplemented with 10% AB serum, and incubated for 24 h in the absence or presence of the culprit drugs (100 and 10 μg/mL) or irrelevant drugs (100 μg/mL). The positive controls were PHA-stimulated cultures (10 μg/mL). The number of IFN-γ spots in each well was counted using an ELISpot plate reader (CTL), and the results are shown as the largest number of IFN-γ spots/2×10^5 PBMCs. All the experiments were read using the ELISpot reader, and were also checked by visual reading. The results were interpreted by subtracting the spot count in the negative control (no drug) from the spot count in the drug-treated wells. Based on the criteria for T-SPOT.TB, a difference in the value of the IFN-γ spot > 6 was defined as a positive result in this study because differences in values of 5, 6, and 7 are interpreted as borderline in the T-SPOT.TB.

3.2. Flow cytometric analysis

Flow cytometric analysis of the PBMCs was performed using a FACS LSRFortessa instrument (BD Biosciences) before and after cell activation by CD3/CD28 microbeads and IL-2. The following antibodies and reagents were used for immunofluorescence: AF700-conjugated CD4 (eBioscience), V500-conjugated CD8 (BD Biosciences), PE-conjugated CD20 (eBioscience), APC-Cy7-conjugated Fixable Viability Dye (Invitrogen), and Percp-Cy5.5-conjugated CD3 (eBioscience). Subsequent analyses were performed using the FlowJo software (TreeStar).

4. Results

4.1. Modified IFN-γ ELISpot was more sensitive than conventional IFN-γ ELISpot and DLST

To improve the sensitivity of the drug-induced IFN-γ ELISpot assay, IFN-γ ELISpot using stimulated PBMC was conducted after CD3/CD28 bead expansion for 7 days (modified IFN-γ ELISpot). We tested the three different methods (DLST and conventional and modified IFN-γ ELISpot) using freshly isolated PBMCs sampled on the same day. The clinical data of 20 samples from 16 patients are summarized in Table 1. In case 1, the conventional IFN-γ ELISpot was positive for the culprit drug SMX/TMP but was negative for vancomycin, which was irrelevant for cADR in this case (Fig. 1A). The drug, which was continuously administered without recurrence of rash after the onset of cADR, was used as the irrelevant drug.

Compared with the conventional IFN-γ ELISpot assay, a larger number of IFN-γ spots were detected in the presence of SMX/TMP in the modified assay. Although <10 IFN-γ spots were detected in the negative control (no drug) and irrelevant drug (vancomycin) wells, 74 spots were detected in the presence of the culprit drug. Similar results were obtained in cases 6 and 10-1, although the conventional IFN-γ ELISpot assay was negative (Fig. 1B and C). The number of IFN-γ spots in the modified IFN-γ ELISpot assay of 16 patients is shown in Table 2. The results were interpreted by subtracting the spot count of the negative control (no drug) from that of the drug-containing wells (Table 3).

The DLST was performed in all cases using each clinically convincing culprit drug, and positive results were obtained from 8 of the 20 samples (6 out of 16 patients). On the contrary, the conventional IFN-γ ELISpot assay was positive for 4 out of 20 samples (3 out of 16 patients), indicating that the conventional IFN-γ ELISpot was less sensitive than the DLST was in this study. Strikingly, the modified IFN-γ ELISpot assay was positive for 17 out of 20 samples (13 out of 16 patients) as shown in Table 2. These three assays were also conducted for the three healthy donors, and none of the culprit drugs used in this study showed positive results, including in the modified IFN-γ ELISpot assay (Table 4). To exclude the possibility of non-specific IFN-γ production after stimulation by the anti-CD3/28 antibody and IL-2, the modified IFN-γ ELISpot assay was performed using the clinically irrelevant drugs in all cases (Table 3). None of the irrelevant drugs showed a positive result in the modified and conventional IFN-γ ELISpot assays, although the DLST performed using the irrelevant drug was positive in case 10-1. Although the number of cADR cases in this study was limited, these results indicate that the modified IFN-γ ELISpot assay demonstrated the highest sensitivity (85.0%) and specificity (100%) among the three methods.

It has been reported that high dose administration or high blood concentrations of culprit drugs are related to the pathogenesis of severe cADRs [21–23]. We hypothesized that drug-specific IFN-γ production in the modified IFN-γ ELISpot assay was induced in a dose-dependent manner. Therefore, we compared the frequencies of drug-induced IFN-γ producing cells at two different drug concentrations (100 and 10 μg/mL) in the modified IFN-γ ELISpot assay (Fig. 2). The results revealed that 15 of the 17 positive samples showed a dose-dependent increase in the number of IFN-γ-producing cells. This result indicates that a drug concentration of 100 μg/mL was better for inducing drug-specific IFN-γ production in the modified IFN-γ ELISpot assay than a drug concentration of 10 μg/mL was among the culprit drugs in this study.

To verify the effect of the anti-CD3/28 antibody and IL-2, the $CD3^+$ proportion of the cells was compared before and after stimulation. Flow cytometric analysis was performed using PBMCs from healthy donors. Approximately 90% of the cells were $CD3^+$ after stimulation and the cells consisted of 43–51% and 31–42% of

Table 1
Clinical data of 16 cutaneous adverse drug reactions (cADR) cases.

Case	Type	Age–sex	Culprit drug	Underlying disease	Days after onset	Days after drug withdrawal
1	DIHS	61M	Sulfamethoxazole/trimethoprim	Leg ulcer	362	359
2-1	EM-like	52F	Lamotrigine	Anxiety neurosis, psoriasis vulgaris	11	11
2-2					79	79
3	DIHS	66M	Allopurinol/oxpurinol	Gout, alcoholism	527	520
4	DIHS	64F	Lamotrigine	Bipolar disorder	1376	1364
5-1	EM-like		Celecoxib	Dental caries, emphysema	3	0
5-2					98	95
6	EM-like	63F	Celecoxib	Rheumatoid arthritis	66	64
7	EM-like	86M	Celecoxib	Femoral neck fracture, osteoporosis	49	47
8	TEN	78F	Celecoxib	Chondrocalcinosis	10	0
9	EM-like	33M	Sulfamethoxazole/trimethoprim	Dilated cardiomyopathy	343	337
10-1	EM-like	71F	Lamotrigine	Epilepsy, schizophrenia	1	0
10-2					15	14
10-3					25	24
11	DIHS	45F	Lamotrigine	Bipolar disorder	114	115
12	DIHS	70F	Lamotrigine	Meningioma, symptomatic epilepsy	1500	1416
13	MPE	20M	Phenytoin	Cerebral contusion, symptomatic epilepspy	2223	2221
14	EM-like	73F	Sulfamethoxazole/trimethoprim	ANCA-associated vasculitis	413	411
15	SJS	49M	Allopurinol/oxpurinol	Gout	601	597
16	TEN	43M	Lamotrigine	Bipolar disorder	764	764

Type of cADR, culprit drug, underlying disease, the number of days after onset of cADR, and the number of days after withdrawal of the culprit drugs are presented. M, male; F, female; DIHS, drug-induced hypersensitivity syndrome; EM-like, erythema multiforme-like eruption; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis.

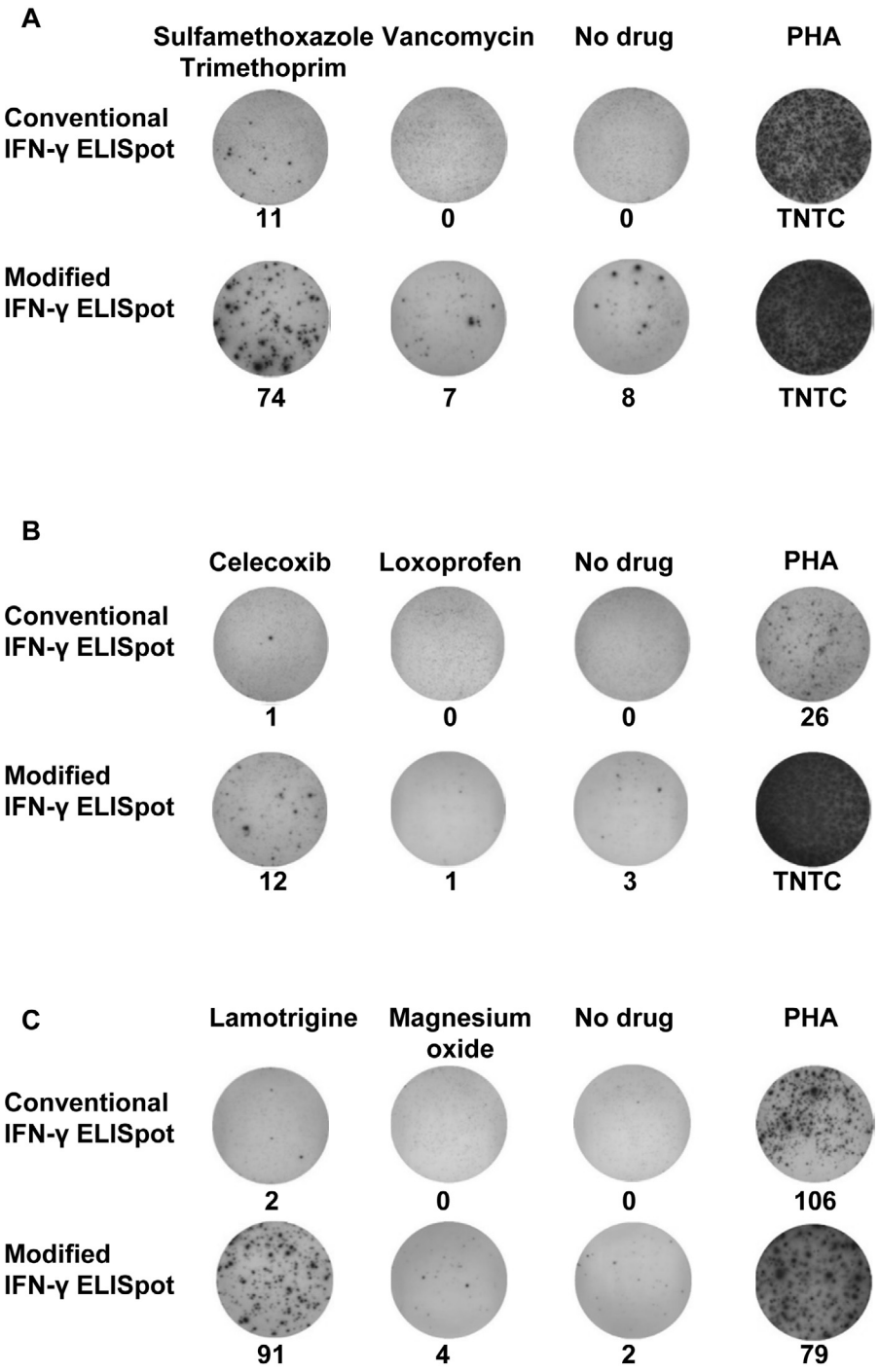

Fig. 1. Comparison of conventional and modified interferon (IFN)-γ enzyme-linked immunoSpot (ELISpot) assays. Representative images of IFN-γ spots in a 96-well plate. IFN-γ ELISpot was performed in the absence or presence of culprit drug, irrelevant drug, or PHA for 24 h. For modified IFN-γ ELISpot, PBMCs were stimulated with anti-CD3/CD28 antibody-coated microbeads and IL-2 for 7 days before exposure to drugs or PHA. (A) Case 1 was DIHS due to sulfamethoxazole/trimethoprim, and vancomycin was an irrelevant drug. (B) Case 6 was EM-like due to celecoxib, and loxoprofen was an irrelevant drug. (C) Case 10-1 was EM-like due to lamotrigine, and magnesium oxide was an irrelevant drug. PHA, phytohemagglutinin; PBMCs, peripheral blood mononuclear cells; IL, interleukin; DIHS, drug-induced hypersensitivity syndrome; EM-like, erythema multiforme-like eruption; TNTC, too numerous to count.

$CD4^+$ and $CD8^+$ cells, respectively, while the $CD20^+$ B cells were few (0.022–0.10%, Fig. 3).

5. Discussion

The determination of the culprit drug that causes cADR is an important challenge for physicians and patients because a severe cADR-inducing drug should be contraindicated for the lifetime of the patient. Drug-specific T cells are extremely rare in the total PBMC population with a frequency of approximately one in tens of thousands of PBMCs [14]. The DLST may produce false negative results because T cell viability in the DLST is not always adequate to detect cell division after a 7-day incubation, even if drug-specific T cells are present. Intracellular cytokine staining for flow cytometric analysis may prove difficult for detecting the cytokine production by T cells on an individual cell basis using samples from actual

Table 2
Summary of number of interferon (IFN)-γ spots in modified IFN-γ enzyme-linked immunoSpot (ELISpot) assay using culprit drugs.

Case	Type	Culprit drug	Modified IFN-γ-ELISpot		
			Culprit drug	Irrelevant drug	No drug
1	DIHS	Sulfamethoxazole/trimethoprim	74	7	8
2-1	EM-like	Lamotrigine	10	5	3
2-2			10	3	1
3	DIHS	Allopurinol/oxpurinol	3/12	5	3
4	DIHS	Lamotrigine	11	4	3
5-1	EM-like	Celecoxib	43	5	2
5-2			17	9	7
6	EM-like	Celecoxib	12	1	3
7	EM-like	Celecoxib	9	2	2
8	TEN	Celecoxib	8	0	1
9	EM-like	Sulfamethoxazole/trimethoprim	15	2	8
10-1	EM-like	Lamotrigine	91	4	2
10-2			17	0	0
10-3			56	33	28
11	DIHS	Lamotrigine	44	28	30
12	DIHS	Lamotrigine	14	9	7
13	MPE	Phenytoin	19	11	9
14	EM-like	Sulfamethoxazole/trimethoprim	0	0	0
15	SJS	Allopurinol/oxpurinol	0/1	0	0
16	TEN	Lamotrigine	8	9	9

IFN-γ spots/2×10^5 PBMCs in 96-well plates were counted using ELISpot plate readers (CTL). PBMCs, peripheral blood mononuclear cells; M, male; F, female; DIHS, drug-induced hypersensitivity syndrome; EM-like, erythema multiforme-like eruption; MPE, maculopapular exanthema; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis.

Table 3
Summary of drug-induced lymphocyte stimulation test (DLST) and conventional and modified interferon (IFN)-γ enzyme-linked immunoSpot (ELISpot) assays.

Case	Type	Culprit drug	Irrelevant drug	Culprit drug			Irrelevant drug		
				DLST	Conventional IFN-γ ELISpot	Modified IFN-γ ELISpot	DLST	Conventional IFN-γ-ELISpot	Modified IFN-γ ELISpot
1	DIHS	Sulfamethoxazole/trimethoprim	Vancomycin	8.7↑	11↑	66↑	1.6	0	-1
2-1	EM-like	Lamotrigine	Zolpidem	4.6↑	25↑	7↑	ND	0	2
2-2				3.2↑	0	9↑	1.9	0	2
3	DIHS	Allopurinol/oxpurinol	Flunitrazepam	1.1/5.1↑	0/0	0/9↑	1.4	0	2
4	DIHS	Lamotrigine	Lithium carbonate	3.6↑	1	8↑	1.1	0	1
5-1	EM-like	Celecoxib	Loxoprofen	1.4	0	41↑	ND	ND	3
5-2				1.5	1	10↑	ND	0	2
6	EM-like	Celecoxib	Loxoprofen	0.95	1	9↑	0.70	0	-2
7	EM-like	Celecoxib	Lansoprazole	1.5	0	7↑	0.81	0	0
8	TEN	Celecoxib	Loxoprofen	1.7	0	7↑	1.4	0	-1
9	EM-like	Sulfamethoxazole/trimethoprim	Rabeprazole	1.4	0	7↑	1.6	0	-6
10-1	EM-like	Lamotrigine	Magnesium oxide	3.1↑	2	89↑	3.3↑	0	2
10-2				1.4	19↑	17↑	1.5	0	0
10-3				3.5↑	17↑	28↑	1.4	0	5
11	DIHS	Lamotrigine	Flunitrazepam	1.3	0	14↑	1.3	0	-2
12	DIHS	Lamotrigine	Atrorvastatin	2.3↑	1	7↑	1.1	0	2
13	MPE	Phenytoin	Carbamazepine	1.4	1	10↑	1.1	0	2
14	EM-like	Sulfamethoxazole/trimethoprim	Bisoprolol	1.2	0	0	0.80	0	0
15	SJS	Allopurinol/oxpurinol	Famotidine	1.1/1.5	0/0	0/1	1.2	0	0
16	TEN	Lamotrigine	Loxoprofen	1.6	3	-1	1.7	0	0

For DLST, an SI value >2.0 was interpreted as a positive result (upper arrow). For IFN-γ ELISpot assay, a difference in value of IFN-γ spot > 6 was interpreted as a positive result by subtracting the spot count in the negative control (no drug) from the spot count in the wells with drugs (upper arrow). DIHS, drug-induced hypersensitivity syndrome; EM-like, erythema multiforme-like eruption; MPE, maculopapular exanthema; SJS, Stevens-Johnson syndrome; TEN, toxic epidermal necrolysis; ND, not done.

patients with cADRs. On the contrary, the IFN-γ ELISpot assay has the potential to recognize a single drug-specific T cell among tens of thousands of PBMCs.

As described above, a recent report suggested that a penicillin-induced conventional IFN-γ ELISpot assay was more sensitive than the DLST [16]. It is noteworthy that the conventional IFN-γ ELISpot

Table 4
Summary of drug-induced lymphocyte stimulation test (DLST) and conventional and modified interferon (IFN)-γ enzyme-linked immune Spot (ELISpot) assays in three healthy donors.

Drug	Donor	DLST	Conventional IFN-γ-ELISpot	Modified IFN-γ-ELISpot
Sulfamethoxazole/trimethoprim	A	1.0	0	0
	B	1.0	1	2
	C	1.3	0	1
Lamotrigine	A	0.87	0	1
	B	0.91	0	0
	C	0.79	0	−2
Allopurinol	A	0.89	0	1
	B	0.69	0	0
	C	0.62	0	−1
Oxpurinol	A	0.81	0	0
	B	0.99	0	1
	C	0.88	0	0
Celecoxib	A	0.83	0	1
	B	1.1	0	1
	C	0.88	0	−1
Phenytoin	A	0.91	0	2
	B	1.2	0	0
	C	0.89	0	1

In three healthy donors (designated as A, B, and C), none of the three assays showed positive results with the drugs tested (Table 2). Result was calculated by subtracting the spot count of the negative control (no drug) from that of the drug-containing wells. Difference in value of IFN-γ spot > 6 was defined as a positive result.

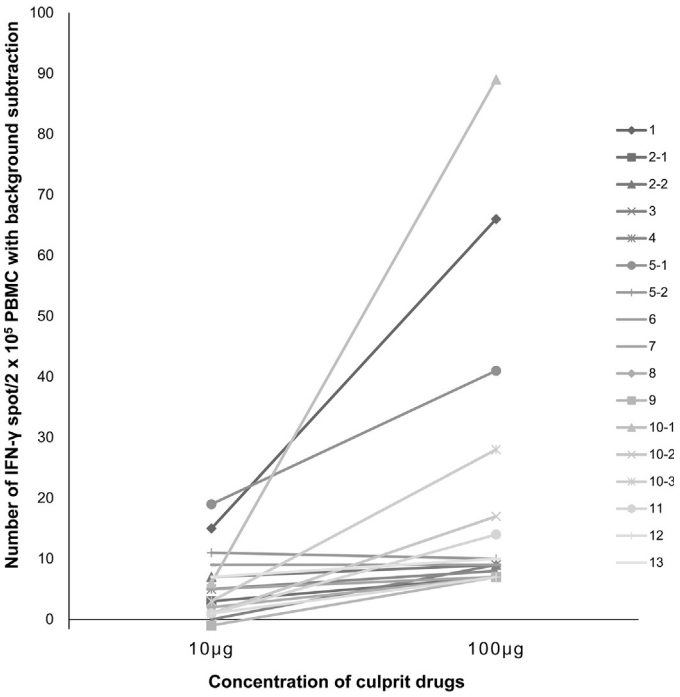

Fig. 2. Interferon (IFN)-γ production in modified enzyme-linked immunoSpot (ELISpot) was dose-dependent manner. Activated PBMCs from each patient were cultured with two different concentrations (100 and 10 μg/mL) of culprit drugs in modified IFN-γ ELISpot assay. Frequencies of drug-induced IFN-γ-producing cells were increased in a dose-dependent manner in 13 out of 17 samples. PBMCs, peripheral blood mononuclear cells.

assay was less sensitive than the DLST in this study. However, penicillin was not one of the tested drugs in this study. Furthermore, all the DLST-positive cases showed positive results in the modified IFN-γ ELISpot assay. Although the number of cases in this study was limited, our findings suggest that the modified IFN-γ ELISpot assay can detect drug-induced IFN-γ production in any case where the drug-induced T cell reactivity is strong enough to induce cell division in response to a culprit drug. In addition, seven DLST-negative cases, which were also negative in the conventional IFN-γ ELISpot assay, were positive in the modified assay. In most of the cases enrolled in this study, the blood samples were collected after a long-term remission (Table 1). Previous reports indicate that positive DLST reactions were obtained in the acute but not the recovery stage of maculopapular (MP) drug eruptions and SJS/TEN, while the exact opposite was observed in DIHS [24]. In this study, the DLST was negative for the clinically convincing culprit drugs in more than half of the cases. In contrast, we detected drug-specific T cells using the modified IFN-γ ELISpot assay in more than 80% of the cases, regardless of the cADR types.

A drug concentration of 100 μg/mL was better for inducing drug-specific IFN-γ production in the modified IFN-γ ELISpot assay compared with a drug concentration of 10 μg/mL among the culprit drugs in this study. Similar to the case of DLST, this concentration may not be applicable for all kinds of causative drugs. Appropriate drug concentration in the culture media of modified IFN-γ ELISpot assay should be determined for each culprit drug.

Concerns regarding the use of the modified IFN-γ ELISpot assay include the possibility that the stimulation of PBMCs with anti-CD3/CD28 antibody-coated microbeads and IL-2 may lead to non-specific IFN-γ production. However, less than 10 spots of generated IFN-γ were detected in the majority of cases in the wells with no drug, except in two cases (10-3 and 11, Table 2). According to the T-SPOT.TB criteria samples showing more than 10 spots in the negative control should be interpreted as invalid. In some cases, small IFN-γ dots, which were likely non-specific, were detected without exposure to the culprit drugs, and these dots were smaller than the detection threshold of the ELISpot reader. Most of the patients presented with prolonged rash, suggesting DIHS, and some of them met the diagnostic criteria for DIHS. Therefore, we speculated that non-specific spots might represent a continuous immunological abnormality associated with DIHS after long-term

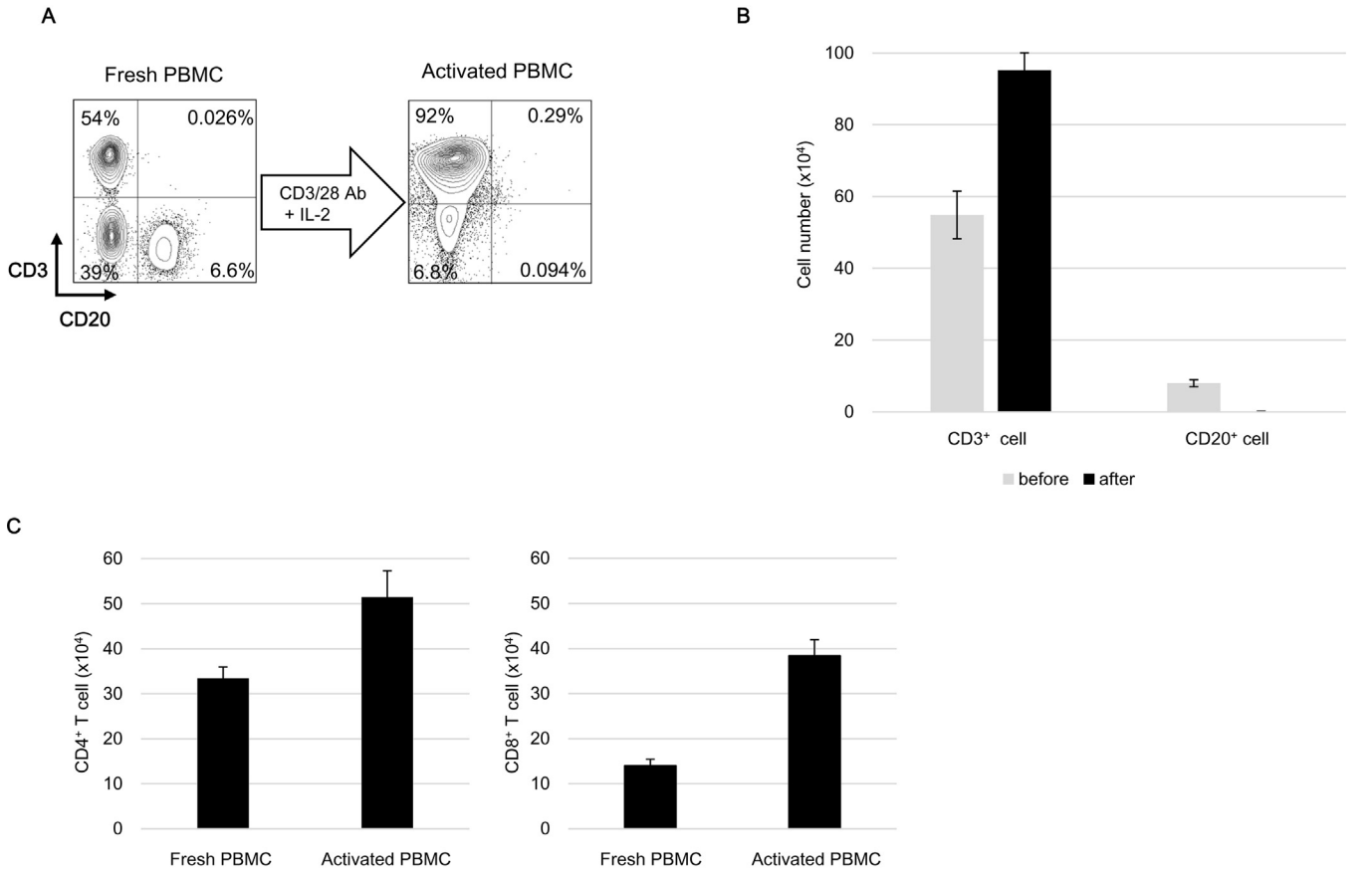

Fig. 3. Flow cytometric analysis of PBMC before and after cell activation. (A) The percentage of $CD3^+$ T cells and $CD20^+$ B cells in the lymphocyte gate of one representative healthy donor. (B) The average number of $CD3^+$ T cells and $CD20^+$ B cells in 10^6 PBMCs of three healthy donors before and after cell activation. (C) The average number of $CD4^+$ T cells and $CD8^+$ T cells in 10^6 PBMCs of three healthy donors before and after cell activation. Error bars indicate standard deviations. Fresh PBMC: PBMC before cell activation; activated PBMC: PBMC after cell activation.

remission of the rash. We have to be cautious in interpreting the results, especially with DIHS cases tested using the modified IFN-γ ELISpot assay. Thus, the modified IFN-γ ELISpot assay may not be reliable when more than 10 spots appear in the negative control.

In this study, we compared DLST, conventional IFN-γ ELISpot, and modified IFN-γ ELISpot assay, which were performed simultaneously using blood samples from patients with cADRs induced by clinically convincing culprit drugs. Among these three assays, the modified IFN-γ ELISpot assay was far more sensitive in detecting the culprit drug than the other conventional methods were. Our results suggest that the modified IFN-γ ELISpot assay could be a useful in vitro tool for identifying culprit drugs in cADR cases. It is worth noting that the present study was performed with selected patients with cADRs caused by well-known culprit cADR-inducing drugs. Although the identification of the culprit drug in cADR cases requires the careful assessment of the clinical course and drug history, our novel method provides a useful option for determining culprit drugs.

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Conflicts of interest

The authors have no conflict of interest to declare.

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