(Sigma–Aldrich) before BrdU staining [12]. In one case, cells were stained with the following antibodies and reagents: Pacific-Blue-conjugated CD4 (BioLegend), AmCyan-conjugated CD8, PE-conjugated CD45RA, APC-Cy7-conjugated CD25 (BD Biosciences), and APC-conjugated Foxp3 (eBioscience). For intracellular Foxp3 staining, cells were first incubated with anti-CD4, anti-CD8, anti-CD45RA, and anti-CD25 antibodies, then fixed, permeabilized with the Anti-Human Foxp3 Staining Set APC (eBioscience), and stained with anti-Foxp3 (eBioscience). All stained cells were analyzed with a FACS Calibur or FACS Canto II cytofluorometer (BD Biosciences). Subsequent analysis was performed with FlowJo software (TreeStar).

3. Results

3.1. Utility of conventional DLST in the clinical course of cADR

Positive results are not always obtained when conventional DLST is performed during a cADR case. However, in many cADR cases, conventional DLST is positive at certain times of the clinical course. Kano et al. previously reported that regardless of whether patients were treated with systemic prednisolone, positive DLST reactions were obtained in the acute, but not the recovery, stage of MP and SJS/TEN, while the exact opposite was observed in DIHS, where positive reactions were obtained in the recovery, but not the acute, stage [13]. Therefore, we analyzed 16 patients with anti-convulsant-induced delayed-type hypersensitivity to examine the correlation between the SI value of conventional DLST and the examination date after cADR onset. The clinical data of our 16 cADR patients are summarized in Table 1. The SI values dramatically changed in individual patients over the course of the disease, and some patients who were negative by conventional DLST during the acute stage were positive 30 days after disease onset. These results indicated that the drug-specific immune reactions detected by conventional DLST could vary during different clinical stages of the disease course. Therefore, we speculated that the variations in the SI value of conventional DLST might reflect alterations in the immune status and the magnitude of the drug-specific immunity. This led us to focus on the drug-reactive proliferating cells that lead to positivity on conventional DLST through the use of FCM.

3.2. Drug-specific proliferating T cells in conventional DLST are detected as $CFSE^{low}$ $BrdU^{high}$ cells in flow cytometric DLST

To visualize the proliferating cells that incorporate ^{3}H-thymidine in conventional DLST, samples were examined for both CFSE dilution and BrdU incorporation. To exclude the effects of CFSE labeling, CFSE-labeled PBMCs were divided into two samples before incubation, with one aliquot used for conventional DLST and the other for FCM-DLST. PBMCs used for FCM-DLST analysis were incubated in the same manner as for conventional DLST for 6 days and pulsed with BrdU for 24 h (Fig. 1B). Theoretically, CFSE dilution reflects the total number of divided and proliferated cells during the 7-day culture, whereas ^{3}H-thymidine and BrdU incorporation into cells during the synthesis phase of the cell cycle represent cells that proliferated during the last 24 h before cell harvesting.

PBMCs of a patient (Case 1) in the acute stage of phenytoin-induced maculopapular rash were used for FCM-DLST and conventional DLST (Fig. 2). In conventional DLST, PBMCs incorporated ^{3}H-thymidine (SI 5.0) after treatment with 10 μg/ml phenytoin, whereas PBMCs from healthy controls did not (SI 0.81).

Back-gating analysis revealed that the $CFSE^{low}$ population was distributed in the lymphocyte area, indicating that the cells that incorporated ^{3}H-thymidine in conventional DLST were lymphocytes,

Table 1
Summary of the 16 cADR cases examined by conventional DLST. M: male; F: female; cADR: cutaneous adverse drug reaction; d: days; MP: maculopapular rash; SJS: Stevens-Johnson syndrome; DIHS: drug-induced hypersensitivity syndrome; EM: erythema multiforme; TEN: toxic epidermal necrolysis; SI: stimulation index.

Conventional DLST patient number	Age·sex	Culprit drug	Underlying disease	Type of cADR	Days after cADR onset	SI	FCM-DLST patient number
1	38F	Phenytoin	Cerebral arteriovenous malformation	MP	25	5.0	Case 1
					38	24	
2	76M	Phenytoin	Post-operative subdural hematoma	SJS	3	6.4	Case 2
					14	7.5	
3	70M	Phenytoin	Brain metastasis of lung cancer	DIHS	13	3.8	Case 3
					27	6.5	
					68	3.3	
4	53M	Phenytoin Carbamazepine	Glioblastoma	MP	16	1.6	
					16	1.5	
5	65M	Phenytoin	Brain metastasis of lung cancer	EM	27	2.6	
6	71F	Phenytoin	Glioblastoma	EM	9	16	
					17	24	
7	71F	Phenytoin	Cerebral aneurysm	MP	55	1.7	
8	77M	Phenytoin	Epilepsy	TEN	15	3.2	
9	61F	Phenytoin	Epilepsy	MP	42	1.4	
10	20F	Zonisamide	Epilepsy	DIHS	6	2.4	Case 4
					20	8.9	
					40	2.7	
					97	1.9	
11	30F	Sodium valproate	Migraine	EM	5	4.6	Case 5
					47	2.2	
					84	1.3	
12	74F	Carbamazepine	Peritoneal cancer	DIHS	4	1.4	Case 6
					51	11	
13	67F	Carbamazepine	Mononeuropathy multiplex	MP	11	1.9	
14	51F	Carbamazepine	Pituitary tumor	SJS	16	0.9	
					71	22	
15	29M	Carbamazepine	Herpes encephalitis	SJS	5	1.7	
					33	2.6	
16	24F	Carbamazepine	Epilepsy	SJS	4	2.5	
					11	1.3	
					60	2.5	

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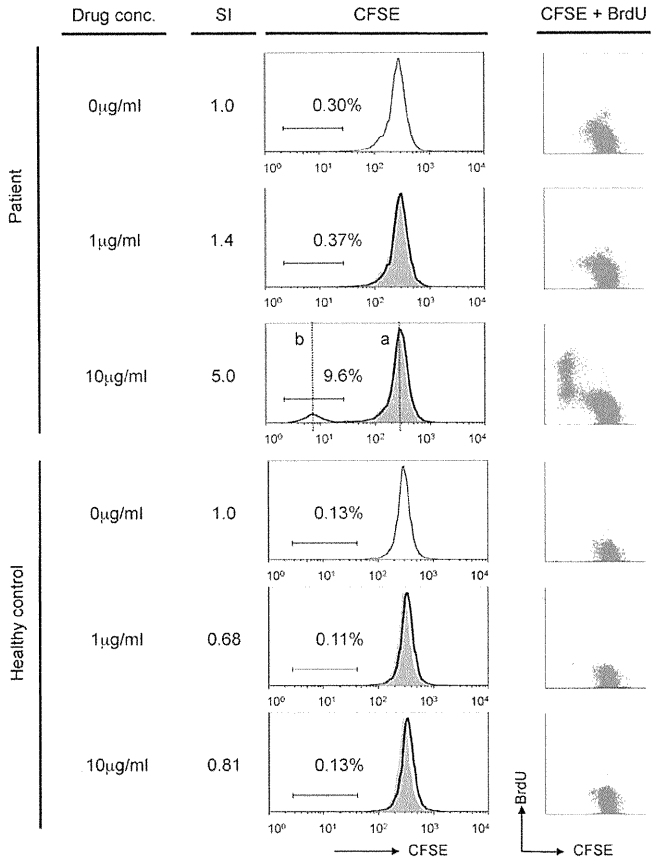

Fig. 2. Representative data of conventional DLST, the CFSE dilution assay alone, and the CFSE dilution assay combined with the BrdU incorporation assay (Case 1 and a healthy control). Drug conc: concentrations of culprit drugs, SI: stimulation index. Corresponding with a positive SI value, 9.6% of the cells were found to be drug-specific proliferating cells by the CFSE dilution assay in the culture treated with 10 μg/ml of a culprit drug. The $CFSE^{low}$ $BrdU^{high}$ population was detected by the dilution assay combined with the BrdU incorporation assay in PBMCs treated at the same concentration (10 μg/ml) of the culprit drug. On the other hand, no cell proliferation was detected at any concentration of culprit drug in healthy control PBMCs. The number of cell divisions was estimated as follows: *a* the value of the peak CFSE fluorescence intensity of the non-proliferating cell population, *b* the value of peak fluorescence intensity of the proliferating cell population. Since CFSE intensity is reduced by half per single cell division, the number of cell divisions can be roughly calculated by taking the binary logarithm of *a* by *b* times ($\log_2 a/b$).

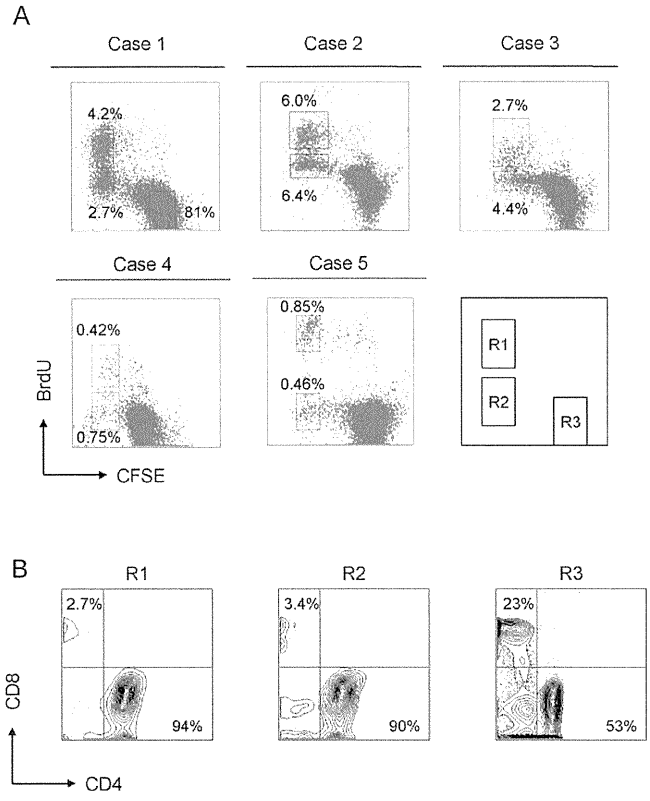

Fig. 3. (A) The results of flow cytometric DLST in five cases and the T-cell subsets found in the flow cytometric DLST of Case 1. Seven-day-cultured PBMCs were categorized into three subpopulations: R1, the $CFSE^{low}$ $BrdU^{high}$ drug-specific proliferating cells that had incorporated BrdU within the last 24 h of culture (days 6–7); R2, the $CFSE^{low}$ $BrdU^{low}$ drug-specific proliferating cell population that had divided until day 6 but did not incorporate BrdU within the last 24 h of culture; and R3, the $CFSE^{high}$ $BrdU^{low}$ non-proliferating cell population. The $CFSE^{low}$ $BrdU^{high}$ population, the population that corresponded to the cells that incorporated ^{3}H-thymidine, was composed entirely of $CD4^+$ and $CD8^+$ T lymphocytes even though the undivided cell population ($CFSE^{high}$ $BrdU^{low}$) contained $CD4^-$ $CD8^-$ non-T cells (R3).

which was consistent to what had been previously reported [13]. Although a few contaminating granulocytes in PBMCs showed greater BrdU incorporation (likely due to these cells being larger than lymphocytes), they never appeared as $CFSE^{low}$ cells, suggesting that granulocytes did not proliferate (Supplementary Fig. 1). Therefore, the lymphocyte gate was used for all further analysis. As shown in a histogram of CFSE fluorescence intensity (Fig. 2), the $CFSE^{low}$ proliferated population (9.6%), the cell population that led to the positive result by conventional DLST, appeared as a small peak when the cells were treated with 10 μg/ml phenytoin. However, cells cultured with 1 μg/ml phenytoin were found to be negative for proliferation by conventional and FCM-DLST. In FCM-DLST, the phenytoin-specific proliferating cells were detected as a $CFSE^{low}$ $BrdU^{high}$ population, which clearly correlated with the SI value determined by conventional DLST.

FCM-DLST allows for a detailed and precise analysis of the drug-specific proliferating cells that correspond to cells that incorporate ^{3}H-thymidine in conventional DLST. We examined CD4 and CD8 expression in the cells that proliferated in FCM-DLST. In a positive case of FCM-DLST, cultured PBMCs were categorized into three populations: R1, the $CFSE^{low}$ $BrdU^{high}$ drug-specific proliferating cells that incorporated BrdU within the last 24 h; R2, the $CFSE^{low}$ $BrdU^{low}$ drug-specific proliferating cell population that did not incorporate BrdU within the last 24 h; and R3, the $CFSE^{high}$ $BrdU^{low}$ non-proliferating cell population (Fig. 3A). The $CFSE^{low}$ $BrdU^{high}$ population, the population that incorporated ^{3}H-thymidine, was composed entirely of $CD4^+$ and $CD8^+$ T lymphocytes even though the undivided cell population ($CFSE^{high}$ $BrdU^{low}$) contained $CD4^-$ $CD8^-$ non-T cells (R3 in Fig. 3B). When four additional cases were analyzed, these three populations were identified in each case (Fig. 3A). Proliferating drug-specific T cells were better isolated in R1 than R2, suggesting that a FCM-DLST protocol that used both CFSE and BrdU was superior to DLST that used CFSE only (Supplementary Fig. 2). To date, we have not detected proliferation in any cells other than T cells.

Since CFSE intensity is reduced by half with every cell division, the number of cell divisions can be calculated (Fig. 2) [14]. The average number of divisions was 5.5 (range: 3.54–6.47; Table 2). Thus, the drug-specific proliferating lymphocytes divided approximately five or six times in the 7 days in DLST-culture medium.

3.3. $CD8^+$ T cells are the predominant proliferating population in a DLST culture of the PBMCs from a severe cADR patient

Drug-specific $CD4^+$ T cells produce cytokines, including interferon-gamma, and this production is related to the

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Table 2
Summary of the six cADR cases examined by conventional and flow cytometric DLST concurrently. M: male; F: female; cADR: cutaneous adverse drug reaction; PBMC: peripheral blood mononuclear cell; d: days; MP: maculopapular rash; SJS: Stevens-Johnson syndrome; DIHS: drug induced hypersensitivity syndrome; EM: erythema multiforme; N.D: no data.

Case	Age sex	Days after onset	Days after drug withdrawal	Type of cADR	Culprit drug	SI	Drug-specific T cells in FCM-DLST		Calculated the number of cell division times	CD4(%)/CD8(%) in PBMC
							CD4(%)/CD8(%) (CFSE BrdU)	CD4(%)/CD8(%) (CFSE only)		
1	38F	25d	23d	MP	Phenytoin	5.0	94/2.7	88/3.3	5.38	N.D.
		38d	36d			24	78/0.1	73/2.3	6.21	N.D.
2	76M	14d	12d	SJS	Phenytoin	7.5	41/57	47/47	5.58	N.D.
3	70M	13d	1d	DIHS	Phenytoin	3.8	5.8/86	11/78	4.52	N.D.
		27d	15d			6.5	100/0	85/2.6	6.45	N.D.
4	20F	6d	1d	DIHS	Zonisamide	2.4	N.D.	N.D.	N.D.	15/48
		20d	15d			8.9	82/0	49/4.9	3.54	25/26
		40d	35d			2.7	89/0	77/2.6	5.43	40/26
5	30F	47d	33d	EM	Sodium valproate	2.2	100/0	66/4.1	6.00	N.D.
6	74F	51d	52d	DIHS	Carbamazepine	11	N.D.	83/5.4	6.47	N.D.

pathogenesis of cADR [9]. However, recent reports suggested that $CD8^+$ CTLs are the major effector cells in SJS/TEN [15] and are involved in DIHS development [16]. Therefore, we evaluated the percentage of $CD4^+$ and $CD8^+$ drug-specific T cells in FCM-DLST. PBMCs from six cADR patients who were conventional DLST-positive were analyzed by conventional DLST and FCM-DLST concurrently (Table 2). As previously reported, the drug-specific proliferated cells were mainly $CD4^+$ T cells in four of the six cases. Interestingly, the $CFSE^{low}$ $BrdU^{high}$ population was predominantly $CD8^+$ CTLs in the PBMCs of an SJS patient (Case 2) and those from a patient in the acute stage of DIHS (Case 3). Drug-specific CTLs were preferentially detected in these cases soon after the withdrawal of the culprit drug, demonstrating that ^{3}H-thymidine incorporation in conventional DLST actually represents a complex immune reaction against a drug antigen that could be classified into at least two subgroups according to the type of drug-specific proliferating T cell.

3.4. The predominant drug-specific proliferating cell population in DLST dramatically changes from $CD8^+$ CTLs to $CD4^+$ T lymphocytes in the clinical course of DIHS

Conventional DLST is sometimes measured several times during the course of a cADR, and SI values in the acute stage differ considerably from those in the recovery stage. To study differences in DLST during the clinical course of DIHS, conventional and FCM-DLST were concurrently examined at different time points in two cases of DIHS (Cases 3 and 4). In Case 3, a case of phenytoin-induced DIHS, the human herpes virus-6 immunoglobulin G (HHV-6-IgG) titer increased from 40 × (day 0 after the withdrawal of the culprit drug) to 2560 × (day 14), indicating that HHV-6 was reactivated (Fig. 4). The percentage of the $CFSE^{low}$ $BrdU^{high}$ population (R1) increased from 2.7% (day 1) to 5.4% (day 15) in accordance with the SI. Surprisingly, the major drug-specific proliferating cell population dramatically changed from $CD8^+$ CTLs on day 0 (86%) to $CD4^+$ T lymphocytes on day 14 (100%), indicating that the drug-specific T-cell subsets may play different roles in the pathogenesis of DIHS at different clinical stages.

In Case 4, a case of zonisamide-induced DIHS, the HHV6-IgG titer increased from 20× to 1280×, confirming a reactivation of HHV-6. From days 15 to 35, $CD4^+$ T lymphocytes were the predominant population that exhibited drug-specific proliferation (Fig. 5). However, drug-specific T cells were not detected in the acute stage of this DIHS case, likely because this case was only weakly positive on conventional DLST.

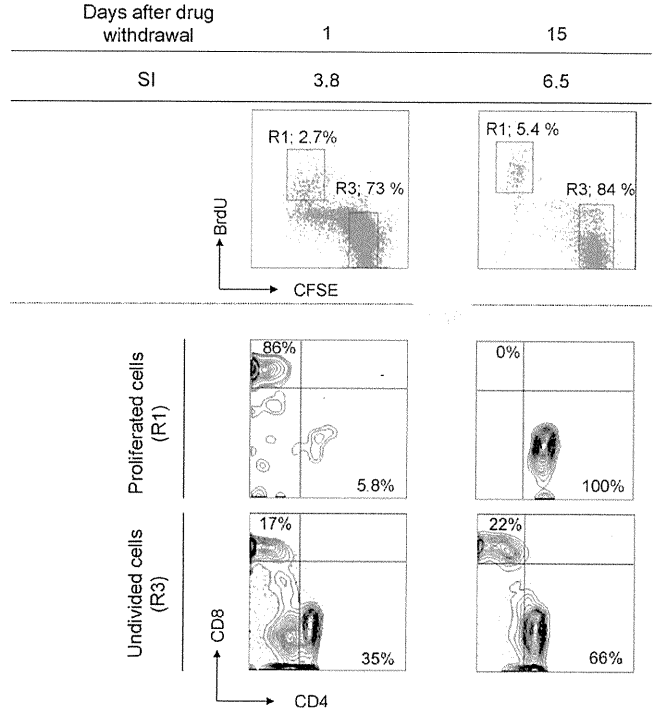

Fig. 4. The predominant drug-specific proliferating cell population changed from $CD8^+$ CTLs to $CD4^+$ T lymphocytes during the clinical course of DIHS (Case 3). When conventional and flow cytometric DLST were examined on day 0 after the withdrawal of phenytoin, the major drug-specific proliferating cell population was $CD8^+$ CTLs (86% of proliferating cells). However, on day 14, the proliferating population was composed entirely of $CD4^+$ T lymphocytes (100%). The undivided cell population remained predominantly $CD4^+$ T lymphocytes.

3.5. Drug-specific Tregs increase in DLST during the recovery stage

Next, we evaluated drug-specific Tregs by FCM-DLST in a case (Fig. 6) of carbamazepine-induced DIHS (Case 6). DLST was performed at the recovery stage (52 days). A BrdU incorporation assay was not performed in this case because intracellular Foxp3 staining is not compatible with the BrdU staining protocol. We compared the $CFSE^{low}$ population with the $CFSE^{high}$ population and used PHA-stimulated $CFSE^{low}$ proliferated $CD4^+$ T cells as a positive control (Fig. 6). The $CFSE^{low}$ drug-specific proliferating cells were mainly $CD4^+$ T cells. Almost all drug-specific $CD4^+$ T cells highly expressed CD25, and the ratio of $CD4^+$ $CD25^+$ $Foxp3^+$ drug-specific

Days after drug withdrawal	1	15	35
SI	2.4	8.9	2.7

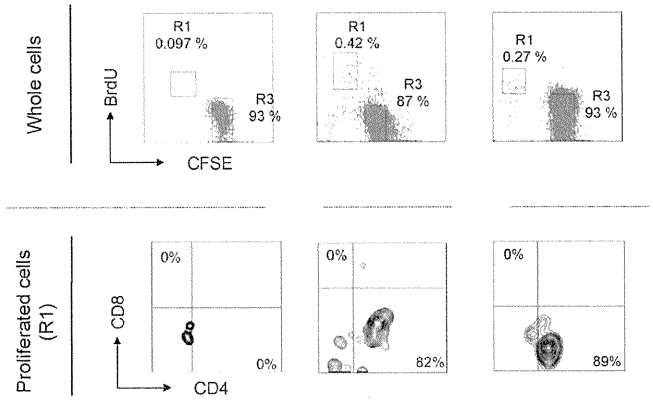

Fig. 5. The predominant drug-specific proliferating cell population remained $CD4^+$ T lymphocytes during the recovery course of DIHS (Case 4). From day 15 to day 35 after the withdrawal of zonisamide, the major drug-specific proliferating cell population remained the $CD4^+$ T lymphocyte population. Drug-specific T cells were not detected on day 1, perhaps because the SI was only weakly positive in this case (2.4).

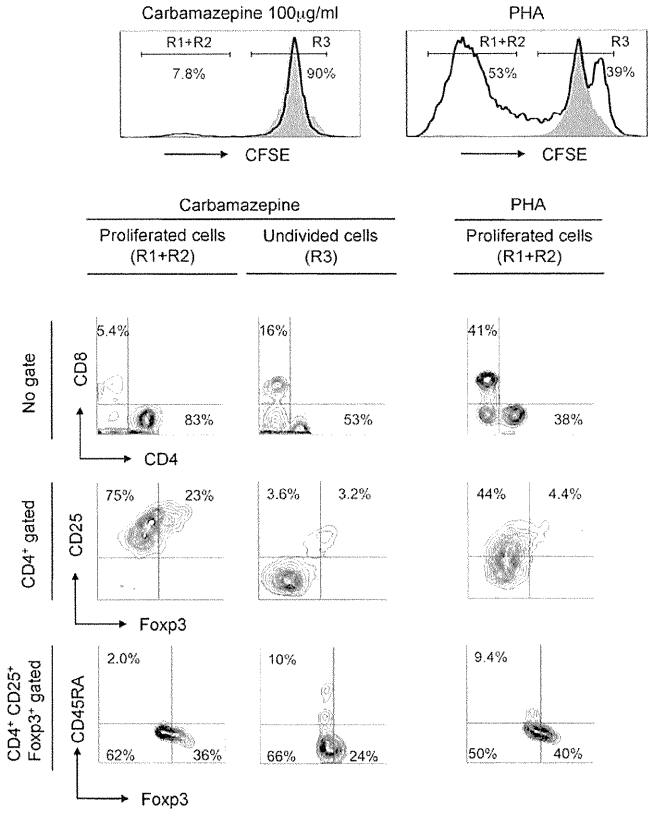

Fig. 6. Drug-specific Tregs were increased in DLST at the recovery stage. A case of a patient with a carbamazepine-induced DIHS was examined by CFSE dilution assay combined with Foxp3 staining. On day 52 after drug withdrawal, the major $CFSE^{low}$ drug-specific proliferating cell population was $CD4^+$ T lymphocytes (83%). Almost all of the drug-specific $CD4^+$ T cells highly expressed the activation marker CD25, the $CD4^+$ $CD25^+$ $Foxp3^+$ drug-specific Treg population was expanded in the proliferated population compared to the $CFSE^{high}$ non-proliferated population and a $CFSE^{low}$ PHA-stimulated population, and the $CD45RA^+$ $Foxp3^{low}$ resting Treg population almost disappeared. R1 + R2: the $CFSE^{low}$ drug-specific or PHA-stimulated proliferated cell population; R3: the $CFSE^{high}$ non-proliferated cell population.

Tregs was increased in this population compared to the $CFSE^{high}$ population and controls. In contrast, the $CD45RA^+$ $Foxp3^{low}$ resting Treg population almost disappeared in the $CFSE^{low}$ population. These results indicate that drug-specific Tregs expand during the recovery stage of drug hypersensitivity.

4. Discussion

DLST, a widely used in vitro diagnostic tool for drug hypersensitivity, is used irrespective of the effector mechanism and clinical phenotype of the hypersensitivity reaction [10]. However, the sensitivity and specificity of conventional DLST is sometimes problematic, particularly when the SI value is not that high [10]. In our study, the SI values of conventional DLST dramatically changed in individual patients over the course of the disease. FCM-DLST determined that the percentage of drug-specific proliferating cells was very small even when the SI value was much higher than the current standard cut-off value. These results indicated that conventional DLST might be useful for the screening of the causative drug in a cADR case and that FCM-DLST, due to its ability to provide more detailed information about the drug-specific T-cell population, could be a suitable method for the determination of the culprit drug.

In vitro detection of drug-specific cytokine production by PBMCs appears to be an adequate alternative for the detection of drug hypersensitivities [6,9,17–19]. In many reports, the total T-cell population in the cultures, including the non-proliferating T cells, was analyzed. However, a few reports focused on the drug-specific proliferating T cells. In our FCM-DLST, the proliferating $CD4^+$ lymphocytes and CTLs, the cells that take up ^{3}H-thymidine in conventional DLST, are clearly visualized. Hashizume et al. previously reported that when DLST was performed with CFSE alone, the $CFSE^{low}$ proliferated population appears even in the absence of the culprit drug. However, the $CFSE^{low}$ population in a CFSE dilution assay not coupled to a BrdU assay might include non-specific dead cells as described above. Our FCM-DLST has the advantage of differentiating the overlapping dead cells as well as any cells with non-specific CFSE dilution from the proliferated population through BrdU labeling.

In previous clinical reports, $CD4^+$ T cells were the predominant population that infiltrated into maculopapular rash skin lesions [20], and most drug-specific T cells were $CD4^+$ T cells. In contrast, recent reports suggested that SJS and TEN result from HLA class I-restricted drug hypersensitivity. CTLs were the predominant population that infiltrated into the epidermis of skin lesions of SJS and TEN patients, and HLA B1502 was found to be fully associated with carbamazepine-induced SJS in Han-Chinese [21–23]. In addition, we reported that epidermal antigen-specific CTLs in Treg-depleted mice induce severe epidermal damage that mimics human TEN, suggesting the effector cells of SJS and TEN are CTLs [24–26].

Interestingly, although the number of patients is limited in this study, FCM-DLST revealed that drug-specific CTLs predominantly proliferated during the acute stages of SJS and DIHS, indicating that this proliferation corresponded to the administration of the culprit drug. On the other hand, drug-specific $CD4^+$ T cells, which likely included suppressive $Foxp3^+$ Tregs, were detected during the recovery stage of a DIHS patient after the withdrawal of the culprit drug. Moreover, unlike the previous report [13], positive DLST reactions were clearly observed during the acute stage of DIHS. This is likely because drug administration had been continued for

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12 days after the onset of cADR, which could have led to the drug-specific CTLs becoming greatly expanded.

In conclusion, FCM-DLST demonstrated that the cell proliferation detected by conventional DLST is a heterogeneous proliferation of both $CD8^+$CTLs and $CD4^+$ T cells that likely includes Tregs. However, the conclusions that can be drawn from this study are limited due to the limited number of cases. As these T-cell populations recognize antigen on different MHC molecules, it will be interesting to test how a single drug antigen presented on MHC class I and class II independently primes and activates drug-specific T cells.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jdermsci.2011.12.002.

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CORRESPONDENCE

Lichen planus-type chronic graft-versus-host disease complicated by mucous membrane pemphigoid with positive anti-BP180/230 and scleroderma-related autoantibodies followed by reduced regulatory T cell frequency

Chronic graft-versus-host disease (cGVHD) remains one of the most frequent complications of allogeneic hematopoietic stem cell transplantation. cGVHD often presents with clinical manifestations that resemble those of autoimmune diseases such as scleroderma [1]. Autoimmune bullous diseases are also associated with cGVHD via the production of circulating basement membrane zone antibodies, such as collagen VII, BP230, collagen XVII/BP180 or p200/laminin γ1 [2]. We report a lichen planus-type cGVHD complicated by mucous membrane pemphigoid with positive anti-BP180/230 and scleroderma-related autoantibodies followed by reduced regulatory T cell (Treg) frequency.

A 17-year-old Japanese man presented with recurrent oral aphtha and scaly erythemas on the trunk and extremities in October 2010. Peripheral blood stem cell transplantation (PBSCT) had been performed from human leukocyte antigen-identical unrelated donors 18 months before for his mixed lineage leukemia. One month after PBSCT, acute GVHD (aGVHD)-induced diarrhea developed and was improved by prednisolone (PSL), tacrolimus, and methotrexate (MTX). In October 2009, he presented with systemic scaly erythemas, diarrhea, and liver dysfunction *(figure 1A)*. The histology of the erythema was compatible with lichen planus-like reaction *(figure 1B)*. Therefore, he was diagnosed as lichen planus-type cGVHD, which was temporarily improved by the same medication. However, the recurrent oral aphtha and scaly erythemas exacerbated, and he was admitted to our hospital in February 2011. On admission, he presented with multiple erythemas with thick scales on the trunk and extremities, as well as severely painful oral ulcers *(figure 1C)*. Laboratory findings were as follows (abnormal values are underlined): white blood cell count, $4.18\times10^3/\mu L$ (neutrophils: 56.0%; lymphocytes: 34.0%; monocytes: 10.0%; eosinophils: 0.0%, and basophils: 0.0%) C-reactive protein: 5.3 mg/L; creatinine: 0.49 mg/dL; aspartate aminotransferase: 32 U/L; alanine aminotransferase: 42 U/L; antinuclear antibody: 1:5120 (<1:40); anti-topoisomerase-1 (anti-Scl-70) antibody: 52.7 index (<16); anti-centromere antibody: 181.0 index (<10); anti-BP180 antibody: 59.5 index (<15); anti-BP230 antibody: 39.0 index (<9). The positive autoantibodies were all negative when cGVHD initially developed in October 2009. Indirect immunofluorescence assay with salt-split skin test demonstrated that serum IgG (×40) reacted to the epidermal side of the basement membrane zone of normal human skin. The patient serum also reacted with recombinant BP180-NC16A by immunoblot analysis. These results indicated that the intractable oral ulcers

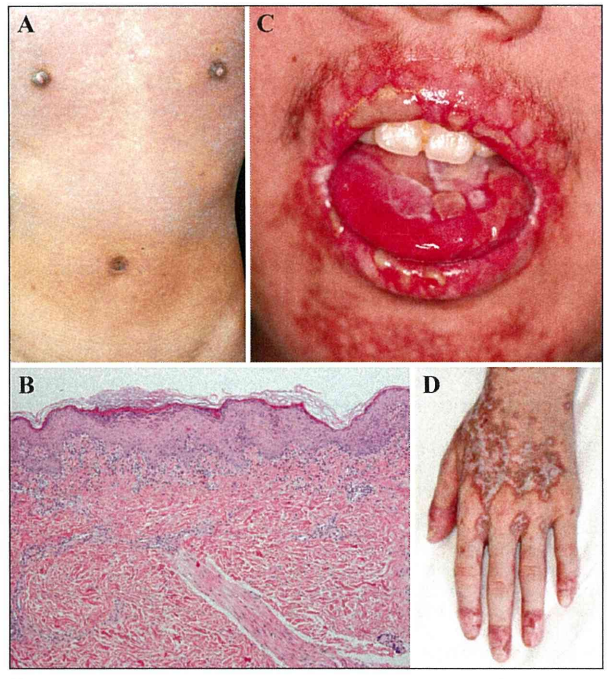

Figure 1.
A) Clinical appearance of scaly erythemas on the patient's trunk when cGVHD initially developed in October 2009. Scaly erythemas were fused on the chest.
B) Skin biopsy from the left abdomen revealed lichen planus-like reactions when cGVHD initially developed. Hematoxylin-eosin staining indicated irregular acanthosis, Civatte bodies, liquefaction degeneration and band-like dermal lymphocytic infiltration. (Original magnifications: ×40).
C) Clinical appearance of oral aphtha with lip and lingual erosion when cGVHD became exacerbated in October 2010.
D) Clinical appearance of scaly erythemas on the patient's left hand once cGVHD was in remission after treatment in April 2011. His hand exhibited pigmentation and depigmentation with mild sclerodactyly.

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were anti-BP180/BP230 autoantibody-induced mucous membrane pemphigoid. PSL and MTX rapidly improved the skin erythemas *(figure 1D)*. However, the oral ulcers remained severe. As previously reported for cGVHD [3], topical tacrolimus dramatically improved the oral lesions, and he has maintained complete remission. Once in remission, we evaluated the number of peripheral blood Tregs. Flow cytometric analysis demonstrated that the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, especially $Foxp3^{low}$ $CD45RA^+$ naïve Tregs [4], was lower than that of a healthy individual.

The pathogenesis of autoimmune disease development in cGVHD remains unclear, but there are two possible mechanisms. One is that Treg expansion is limited and its function is inhibited, attributable to the cytokine milieu of interleukin (IL)-6 elevation without IL-2 elevation in GVHD. Limited Treg expansion leads to the transition from acute to chronic GVHD, resulting in an increase of donor-derived $CD4^+$ T cells, which may explain why autoimmunity occurs in some cGVHD patients [5]. Alternatively, the peripheral T-cell receptor repertoire is skewed and *de novo* T-cell generation is impaired, attributable to defects in regular thymopoiesis in GVHD [6]. In our case, peripheral blood Tregs were decreased during the clinical course, most likely leading to the breakdown of tolerance and production of multiple autoantibodies.

In conclusion, production of multiple autoantibodies in cGVHD provides useful information for the understanding of cGVHD-associated autoimmune disease development, especially the involvement of Tregs. Long-term observation of the T-cell repertoire, especially Tregs, is essential in cGVHD patients. ■

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CORRESPONDENCE

Acute generalized exanthematous pustulosis induced by topical diphenhydramine

Acute generalized exanthematous pustulosis (AGEP) is characterized by the rapid onset of many sterile erythematous pustules, often accompanied by leukocytosis and fever. In almost all cases, systemic administration of drugs is causative, but AGEP can sometimes be induced by the application of topical medicines, such as bufexamac [1, 2]. To our knowledge, this is the first case report of AGEP probably induced by the application of diphenhydramine cream. A 67-year-old woman presented with itchy erythematous plaques at the site of insertion of a catheter on the left arm for intravenous hyperalimentation, 15 days after partial pancreatic resection for serous cystadenoma. A low-grade fever of approximately 37.0°C had persisted for several days, but no infectious symptoms were noted as she had a normal white blood cell (WBC) count and normal C-reactive protein levels. On the following day, the adhesive film for the catheter was removed since we hypothesized that the dressing might induce contact dermatitis. Treatment with topical diphenhydramine cream was subsequently begun for the erythema. The pruritic erythemas were exacerbated; 2 days later, she presented with developing erythematous patches and scattered pustules, extending to the site of the diphenhydramine cream application on both the trunk and extremities *(figure 1A, B)*. No mucosal involvement was noted. Physical examination revealed a high body temperature of 38.4°C. Laboratory findings showed leukocytosis (WBC count, 15,850/μL) with 89.0% neutrophilia, but neither were infectious symptoms observed nor were bacterial infections identified in a bacteriological culture of the contents of one of the pustules. A drug lymphocyte stimulation test (DLST), performed 2 days after the erythematous pustules appeared, was positive for diphenhydramine (stimulation index: 480%). A skin biopsy from a left femoral pustule revealed subcorneal neutrophilic pustules and perivascular infiltration of neutrophils, lymphocytes, and eosinophils *(figure 1C, D)*. The AGEP validation score (EuroSCAR group criteria) was 9 (8-12: definite) [1]. Therefore, we diagnosed the patient with AGEP mostly likely induced by diphenhydramine cream application. Replacement of diphenhydramine cream with betamethasone ointment dramatically improved her erythematous skin reactions within 3 days, with post-pustular desquamation. Her leukocytosis and high fever also improved. A patch test, performed 3 days after the erythematous pustules resolved, was negative for diphenhydramine cream (as is) (diphenhydramine 1%) at both the 48- and 72-hour time points.

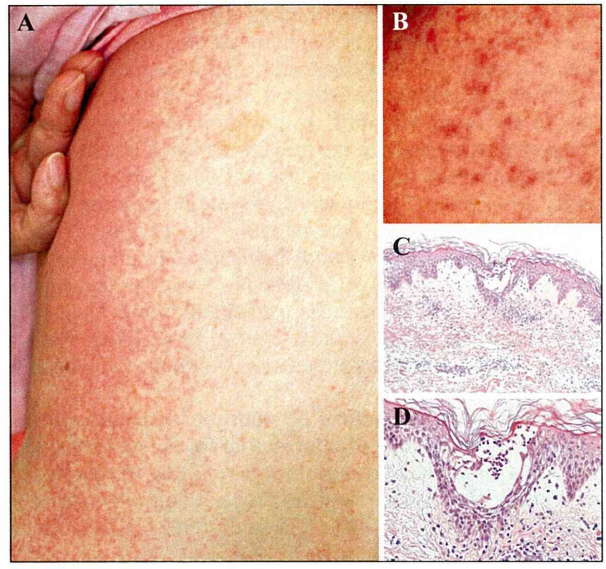

Figure 1. Clinical appearance and histological findings. **A)** Left back. Erythema appeared limited to the site of diphenhydramine cream application. The border between the erythematous regions and normal areas was comparatively clear.
B) Left anterior thigh. Many sterile erythematous pustules appeared on both thighs.
C, D) Skin biopsy from a left femoral pustule revealed subcorneal neutrophilic pustules and perivascular infiltration of neutrophils, lymphocytes, and eosinophils with marked papillary dermal edema. Spongiotic changes were rarely found in the epidermis [original magnifications: **(C)** ×40, **(D)** ×200].

Diphenhydramine, one of the most effective sedating antithistamines, is often used in topical medicines [3]. Nevertheless, it can induce contact sensitization and photodermatitis [4]. In this case, we concluded that the preceding low-grade fever was non-specific and temporary under post-operative conditions, but the concurrent high fever was closely related to diphenhydramine cream application, because she did not present any symptoms of infection and her body temperature rose after drug application and fell rapidly after drug stoppage, corresponding to the clinical course of the erythematous pustular reactions. Localized pustular contact dermatitis as a differential diagnosis could be ruled out, since it does not accompany either leukocytosis or high fever. Histologically, spongiotic changes were rarely found in the epidermis, as commonly seen in contact dermatitis. Recently, the definition of acute localized exanthematous pustulosis (ALEP) was introduced [5], which may be an appropriate diagnosis in our case, because the

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skin reaction was limited to the application site. On the other hand, a patch test after resolution of the pustules was negative in our case. One reason for our findings could be a false-negative result or inflammasome signaling of IL-1β from some preceding post-operative inflammation, which would convert diphenhydramine cream into a sensitizer, inducing neutrophilic and eosinophilic reactions through IL-8 and IL-5 [6]. ■

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INVITED ARTICLE

Animal models of toxic epidermal necrolysis

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ABSTRACT

Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are severe acute exfoliative skin diseases developing extensive epidermal detachment and mucosal damage. Although SJS and TEN are mostly caused by drugs, an animal model of TEN using drugs has not been established yet. We have established an autoimmune skin disease model mouse reproducing the devastating skin damage of TEN by a combination of transgenic mice expressing an epidermal model antigen and its specific $CD8^+$ T-cell receptor. In this model mouse, we found that the thymus-derived $CD4^+CD25^+$ regulatory T cell (Treg) is a critical regulator of cytotoxic T lymphocytes (CTL) causing TEN. Indeed, loss of Treg function was recently demonstrated by human studies of TEN patients. Although how drug-reactive CTL is activated *in vivo* is still unknown, this model elucidated the immunological pathomechanism of TEN after CTL obtained cytotoxicity against epidermal keratinocyte. In this review, roles of CTL, Treg, cytotoxic granules and antigen-presenting cells were discussed on pathogenesis of TEN.

Key words: cytotoxic T cell, dendritic cell, ovalbumin, transgenic.

INTRODUCTION

Cutaneous adverse drug reaction (cADR) is a frustrating problem for the patient and physician, because withdrawal of the causative drug is often necessary to end the symptom. Allergic drug reaction is usually idiosyncratic in both immediate reaction and non-immediate reaction, classified as type I allergy and type IV allergy by Coombs and Gell,[1] respectively. Physicians sometimes encounter mild cases of type IV delayed allergic reaction of cADR, such as maculopapular rash, in daily clinical practice. On the other hand, severe cADR, especially Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), is a rare and unanticipated event.[2] Although drugs are the major cause of SJS/TEN, herpes simplex virus or *Mycoplasma* infection is also occasionally associated with the acute phase.[3] In addition, certain causative drugs or infections cannot be identified in some SJS/TEN cases, suggesting that part of SJS/TEN cases is related to unknown factors.

As a defense system, the immune system protects our bodies against microbes, whereas it sometimes creates an unfavorable situation by being involved in the pathogenesis of various diseases. Innate immunity and adaptive immunity are the two major defense systems, in particular, T-cell immunity plays a key role on pathogenesis of many inflammatory diseases.[4] For example, donor-derived T cell, which is non-self, kills recipient cells expressing the host self-antigen in acute graft-versus-host disease (GVHD) after hematopoietic cell transplantation.[5] On the other hand, autoreactive T cells cause immune reaction to the self-antigen and lead to organ destruction in autoimmune diseases. Obviously, T-cell-mediated, delayed drug hypersensitivity reaction can be classified into neither GVHD nor autoimmune disease.[6] In drug hypersensitivity reaction, the drug is a non-self-antigen recognized by drug-reactive "native" T cells. Conversely, clinical symptom of acute GVHD are often difficult to distinguish from drug eruption, because maculopapular rash and TEN can be seen in both acute GVHD and delayed drug hypersensitivity

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reaction.[7] Unlike acute GVHD or autoimmune diseases, the target of T-cell immunity is not peptides derived from tissue, but low molecular weight chemical substances in the delayed drug hypersensitivity reaction, suggesting that the culprit drug may haptenize self-antigen like in contact hypersensitivity reaction. Moreover, drug-reactive T cells can be activated by drugs binding to major histocompatibility complex (MHC) molecules without self-peptides, and this phenomenon is so-called the pharmacological interaction (p-i) concept.[8,9] Nevertheless, T cells activated by a drug cannot remove a drug from the body, but induce skin eruption, and drug eruption unconsciously alarms patients and physicians against continuation of the causative drug.

ADOPTIVE TRANSFER MODELS OF TEN

In humans, TEN is a most severe clinical symptom in delayed drug hypersensitivity and acute GVHD. In mice, acute GVHD, identical to the human disease, can be induced by allo-specific T-cell transfer. Asagoe *et al.*[10] demonstrated that BALB/c nude mice that have been adoptively transferred T cells from C57BL/6 mouse spleen develop a lethal acute GVHD. This mouse develops bodyweight loss, diarrhea, erythematous skin changes and erosions with positive Nikolsky's sign. Because the target of the T cells in acute GVHD is MHC molecules, allo-specific T cells attack multiple organs distributed through the whole body. Thus, adoptive transfer is a simple and established method to induce cytotoxic immune response.

Animal model of TEN induced by drug antigen have not been established so far. The major target organs of this severe cADR is skin and mucosa including the cornea, although liver dysfunction and intestinal symptoms are sometimes observed. As previously reported, the effector cell of TEN was assumed to be $CD8^+$ T cells (cytotoxic T cells) akin to acute GVHD,[11] however, there was no direct evidence demonstrating that the epidermotropic cytotoxic T lymphocyte (CTL) is crucial for pathogenesis of TEN. By using an ovalbumin (OVA) model antigen system that never expresses in wild-type mice, we established an adoptive transfer model of TEN without using drugs. In K5-mOVA transgenic mice, OVA was expressed in the epidermal keratinocyte under the human keratin 5 promoter.[12] As an epidermal self-antigen-specific

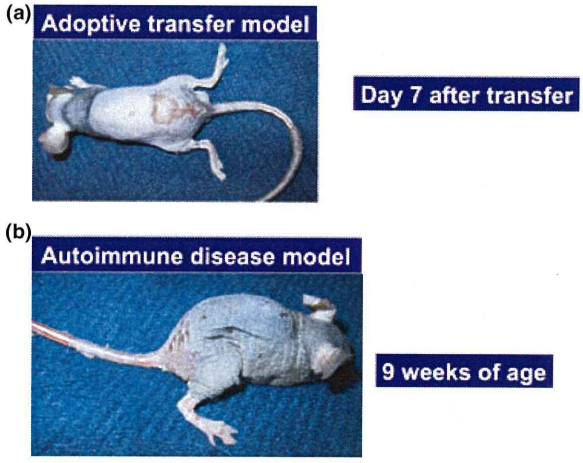

Figure 1. Adoptive transfer model and autoimmune disease model of toxic epidermal necrolysis. (a) Athymic K5-mOVA nude mouse transferred OT-I cells developed erosion with positive Nikolsky's sign in dorsal skin and tail skin at day 7 after transfer. (b) Athymic K5-mOVA.OT-I double transgenic nude mouse spontaneously developed epidermal detachment on dorsal skin and tail skin at 9 weeks of age.

T cell, OT-I transgenic mouse having MHC class I restricted OVA-specific T-cell receptor on CD8 T cells was used.[13]

OT-I cells proliferated in the skin-draining lymph node, infiltrated into the epidermis, and induced apoptosis of keratinocytes in the K5-mOVA mouse, however, the K5-mOVA mouse never developed TEN even when an extremely large number of OT-I cells were transferred. Interestingly, when OT-I cells were transferred into K5-mOVA on an athymic nude mouse background, a large sheet of epidermal detachment was induced (Fig. 1). Because athymic nude mice lack T cells that normally mature in the thymus, transferred T cells can proliferate rapidly by homeostatic expansion.

AUTOIMMUNE DISEASE MODEL OF TEN

Obviously, TEN is not an autoimmune disease. Nassif *et al.*[11] demonstrated that keratinocyte added to the culprit drug, but not keratinocyte alone, is the target of drug-specific CTL from blister fluid of TEN patients. This result confirms that drug-reactive T cells are not autoreactive against the keratinocyte itself. However, we could also establish an animal model of TEN as an autoimmune disease.[14] When K5-mOVA.OT-I

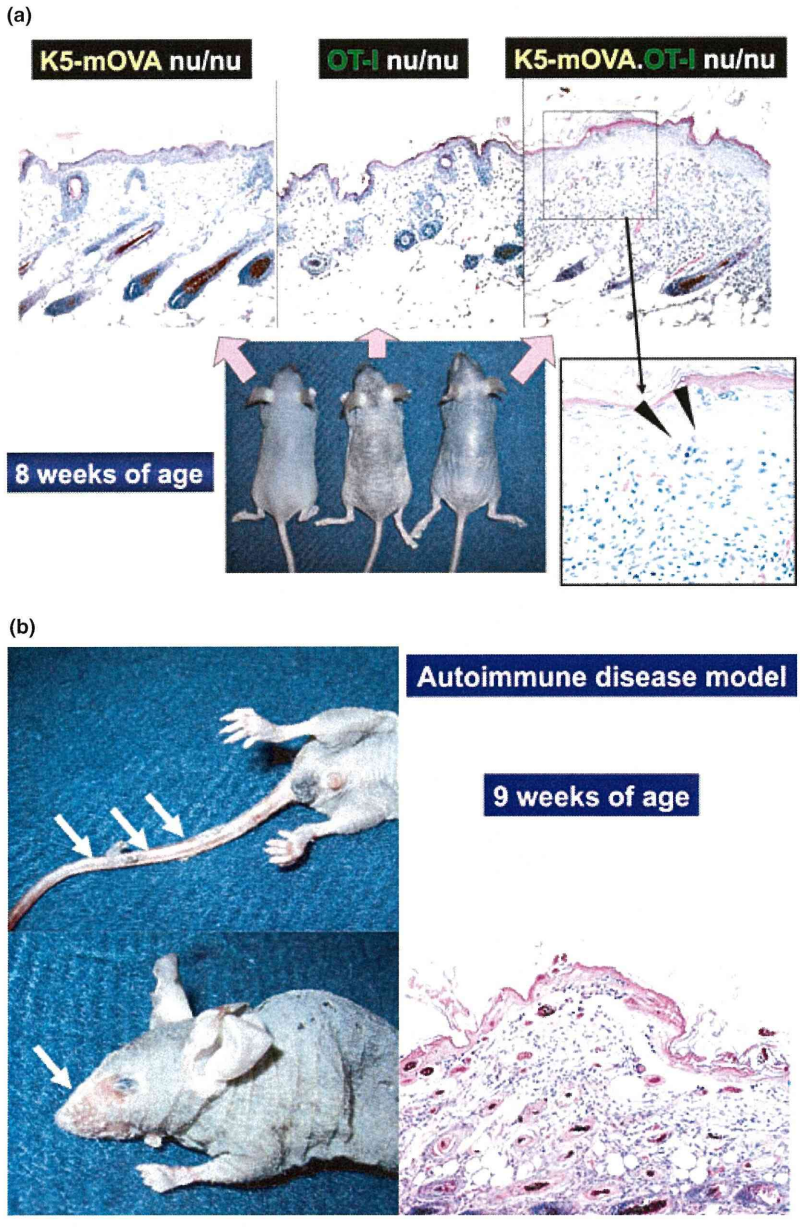

Figure 2. (a) Athymic K5-mOVA.OT-I double transgenic nude mice showed microscopic skin changes just before developing toxic epidermal necrolysis (TEN)-like devastating skin disease. Neither K5-mOVA or OT-I single transgenic athymic nude mouse developed any skin diseases. (b) Autoimmune disease model of TEN. Athymic K5-mOVA.OT-I nude mice developed large area of skin erosion at ~8–12 weeks of age. Histopathologically, full thickness of epidermal necrosis and blister formation were observed (hematoxylin–eosin, original magnification ×200).

double transgenic mice were generated in either euthymic mouse or athymic nude mice, only the athymic double transgenic mouse spontaneously developed a lethal exfoliative skin disease closely resembling human TEN at approximately 8–12 weeks of age (Figs 1,2). Interestingly, this athymic double transgenic mouse developed not only large sheets of detachment but also mucosal damage in the eyes, mouth and genital area. Because TEN was induced by a "native" transgenic T cell without adoptive

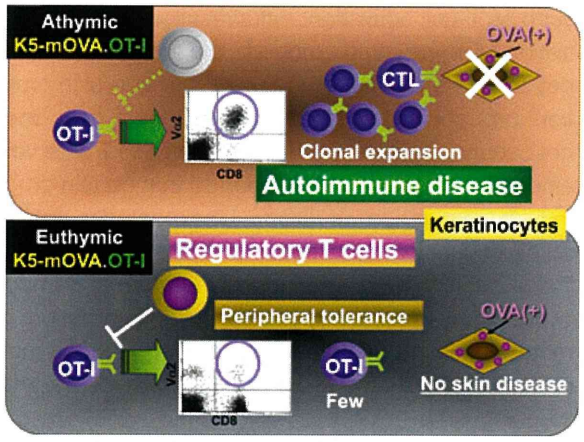

Figure 3. The differences between euthymic and athymic K5-mOVA.OT-I double transgenic mice. Thymus-derived regulatory T cells prevent expansion and activation of cytotoxic T lymphocytes causing toxic epidermal necrolysis.

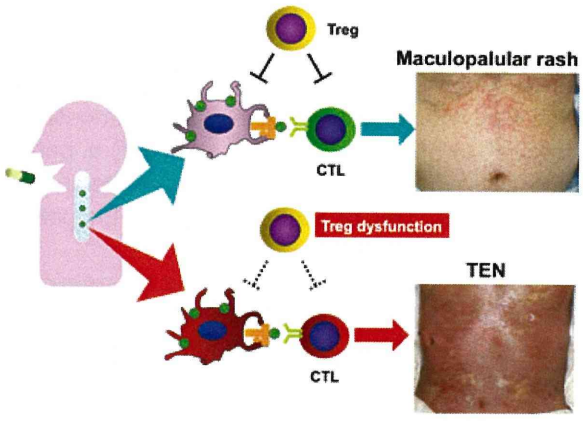

Figure 4. Regulatory T cell (Treg) function may be a "turning point" between mild and severe delayed drug hypersensitivity. CTL, cytotoxic T lymphocytes; TEN, toxic epidermal necrolysis.

transfer, an autoimmune disease model may be a more favorable model than an adoptive transfer model of TEN.

By analyzing the autoimmune model of TEN, we could find out a new pathomechanism of TEN that determines the severity of delayed drug hypersensitivity. Interestingly, a mouse having naturally-occurring regulatory T cell (Treg), named the euthymic K5-mOVA OT-I double transgenic mouse, did not develop erosion, whereas the mouse lacking $CD4^+CD25^+$Treg, named the athymic K5-mOVA OT-I double transgenic mouse, developed lethal large sheets of epidermal detachment clinically and histopathologically similar to TEN (Fig. 3). Surprisingly, adoptive transfer of OT-I cells separated from the euthymic K5-mOVA OT-I double transgenic mouse into the athymic K5-mOVA mouse lacking OT-I transgene induced TEN-like devastating skin damage, indicating that tolerance is induced on CTL by thymus-derived T cells. Furthermore, *in vivo* depletion of $CD4^+$ T cells, including Treg, induced TEN in the euthymic K5-mOVA OT-I double transgenic mouse, suggesting that thymus-derived Treg prevents a runaway reaction of CTL that develops robust apoptosis of keratinocytes.

Treg DYSFUNCTION IN HUMAN TEN

Clinically, lymphocytopenia is often seen in TEN patients. Roujeau *et al.*[15] reported that $CD4^+$ T-cell number is decreased in TEN patient, however, the number of $CD8^+$ T cells is relatively maintained. Recently, Takahashi *et al.*[16] nicely demonstrated a dysfunction in Treg of the patient peripheral blood of TEN. They showed that the percentage of Treg in TEN patients was within normal range at both the acute stage and resolution stage, although a drug-induced hypersensitivity syndrome patient showed an increased percentage of Treg. Very interestingly, the suppressive function of Treg is profoundly impaired in acute-stage TEN patients. These findings strongly support our findings in mouse TEN, and clearly demonstrated that loss of Treg function is a new exacerbating factor of cADR (Fig. 4).

CYTOLYTIC GRANULES IN TEN

Our understanding of the effector mechanism of keratinocyte apoptosis in TEN has been dramatically changed in the last decade. Although inflammatory cells infiltrating into the epidermis are relatively few, robust apoptosis of keratinocytes is observed in the epidermis. This contrasting finding leads us to assume that keratinocyte apoptosis in TEN might be mediated by cytolytic granules without cell–cell contact. Indeed, several candidates for the cytotoxic molecules of TEN have been reported. Viard *et al.*[17] showed that high concentration of soluble Fas-L is detected in the serum of TEN patients; epidermal keratinocytes of TEN patients expressed not only Fas, that is expressed in the normal keratinocytes,

but also Fas-ligand (FasL). In this report, they demonstrated that pooled human intravenous immunoglobulins contained antibodies that can directly block Fas–FasL interaction. Posadas *et al.*[18] demonstrated that mRNA of tumor necrosis factor (TNF)-α, perforin and granzyme B from blisters or peripheral blood mononuclear cells of acute-stage SJS/TEN patients were increased.

Recently, Chung *et al.*[19] found that granulysin produced by CTL and natural killer (NK) cells can be detected in vesicles of SJS/TEN patients, and that granulysin concentration is 100–10 000-fold higher than in Fas-L and granzyme B. Interestingly, when granulysin is injected into the skin of a nude mouse or shaved C3H mouse, epidermal necrosis similar to TEN was induced within several hours. This model is also a very important animal model of TEN, demonstrating a final effector molecule produced by drug-reactive CTL or NK cell.

ROLE OF ANTIGEN-PRESENTING CELL IN TEN

Delayed drug hypersensitivity reaction including TEN is induced by a drug-specific T cell.[20] T cells recognize peptides presented in the context of MHC molecules on antigen-presenting cells (APC). Although the drug must be presented by certain APC in delayed drug hypersensitivity reaction, the APC responsible for drug-antigen presentation have not been identified yet. In contact dermatitis, APC activating a hapten-specific immune response are well studied. Because hapten applied onto the skin is presented by migratory dendritic cells (DC) that migrate from the skin to draining lymph node, then hapten-reactive T cells infiltrate into the skin where hapten originally existed and induce epidermal damage. On the contrary, the sensitization site of the culprit drug in delayed drug hypersensitivity reaction is still unknown. In delayed drug hypersensitivity reaction, most causative drugs are administrated p.o. or i.v., and spread into organs and tissues of the whole body through the bloodstream. However, it is still unclear whether p.o. or i.v. administrated medicine really binds to epidermal self-antigen like contact dermatitis.[6]

Before drug-reactive T-cell immunity attacks epidermal keratinocytes, many immunological steps, such as T-cell activation, extravasation and skin infiltration, are required. Indeed, the vast majority of patients who receive drugs never develop skin eruption, probably because activation of the T cells does not occur. DC are APC which can activate T cells very efficiently, and it has been reported that certain drugs induce maturation of DC.[21] Unexpectedly, in our autoimmune disease model of TEN, a naive $CD8^+$ T cell became CTL and induced epidermal damage without any exogenous trigger of inflammation or maturation stimuli. In other words, drug-induced maturation of DC seems to be not always necessary for inducing TEN.

When T cells migrate to the target organ (e.g. skin, intestinal tract) by responding to the foreign antigen, recognizing the destination where the foreign antigen is originally engulfed by DC is important for T cells to reach the inflammation site. If drug hypersensitivity is induced by epidermal self-antigen haptenized by causative drugs, skin-migratory DC may play a major role in the sensitization phase and elicitation phase. Recent mouse studies showed that epidermal self-antigen is presented by $Langerin^+CD103^+$ dermal DC, but not by epidermal Langerhans cell, that migrate from skin to the draining lymph node, indicating that dermal DC might be important for activation of the effector T cell of drug eruption.[22,23]

CONCLUSION

Animal models of TEN effectively help us to understand the immunological background of this life-threatening cADR, that is difficult to study in humans because of rare incidence. Especially, the importance of Treg in TEN was recently confirmed in human studies by great efforts using Treg separated from TEN patients. According to recent clinical and genetic studies, drug, virus infection and human leukocyte antigen (HLA) are known as the three major factors of severe drug eruption. Our animal model and the clinical study of Shiohara's group strongly suggest that Treg function should be included in major factors of severe drug eruption. Although the animal model of TEN using drugs have not been established yet, further understanding of TEN for developing effective prophylaxis and treatment should be achieved by continuous efforts in this field through basic and clinical research.

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重症薬疹の発症機序update

Recent advances in pathogenesis of severe skin adverse reaction

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◎薬疹の発症機序に関してはいまだ数々の謎がある．しかし近年の研究の進歩によって，その重症化に関与する事象が明らかとなりつつある．薬剤抗原認識機序にはハプテン抗原認識以外に，蛋白に結合しないでT細胞を活性化する経路が発見されている．この経路は薬剤抗原による感作を要しないもので，この経路による爆発的なT細胞活性化が薬疹の重症化に関与する可能性がある．また，活性化するT細胞のフェノタイプは臨床像を反映することが検証されている．さらに，薬剤性過敏症症候群(DIHS)のように経過中内在性ウイルスの再活性化を生じ，重症化するものがある．この機序はいまだ不明であるが，著者らはひとつの仮説を提唱している．本稿では，これら薬疹の重症化に関するこれまでの知見をまとめて概説した．

Key word：薬疹，HLA，ハプテン，pharmacological interaction（*p-i*）concept，high mobility group box（HMGB）-1

薬疹の謎

薬疹にまつわる未解決の謎はまだ多い．なぜ，薬疹を起こすヒトがいるのか．なぜヒトによって重症度が異なるのか．しかし，この分野の研究は着実に進歩している．最近のエポックメーキングは，ゲノムワイド関連解析による薬疹関連遺伝子の発見であろう[1]．カルバマゼピン，アバカビル，アロプリノールによる薬疹は，それぞれ特有のHLAハプロタイプ保有者に限定して起こりやすいことが検証される．一方，たしかにHLA検索は薬疹の予見に有用であるが，重症度の予測には無用らしいことが判明しつつある．

薬剤性過敏症症候群(drug-induced hypersensitivity syndrome：DIHS)では，ヒトヘルペスウイルス再活性化が予後に関連することが明らかとなった[2]．さらに，スティーブンス・ジョンソン症候群(Stevens-Johnson syndrome：SJS)でも内在ウイルスの再活性化をきたす症例が報告されている．ウイルス感染と重症薬疹との強い関連はいったい何を意味するのであろうか．いくつもの謎のなかに薬疹の発症メカニズムの本質が隠れている．本稿では最近の薬疹に関する研究を総括し，これからの薬疹研究の展望について考察する．

ハプテンとしての薬剤とT細胞反応(図1-A)

薬疹の原因は投与された薬剤である．薬剤が低分子であり，それ自身に免疫原性がないことから，自己蛋白に共有結合することによって生成された抗原(ハプテン抗原)によって薬疹は惹起されることが示されている．薬剤反応性T細胞を樹立することで，その詳細が明らかにされた．ペニシリンやセフェム系薬剤における反応は，おもにアルブミンを構成するリジンに結合してペニシロール基を生成する[3]．また，サルファメキサゾール薬疹では肝のCyP450によって生じた中間代謝物が，自己蛋白のチオール基に結合する[4]．最近，HLA-B57:01保有者にアバカビルによる薬疹が高頻度に起こることが明らかとなり，HLAの特定領域にアバカビルが結合することが判明した[5]．ハプテン抗原は通常の抗原と同様な方法でT細胞受容体

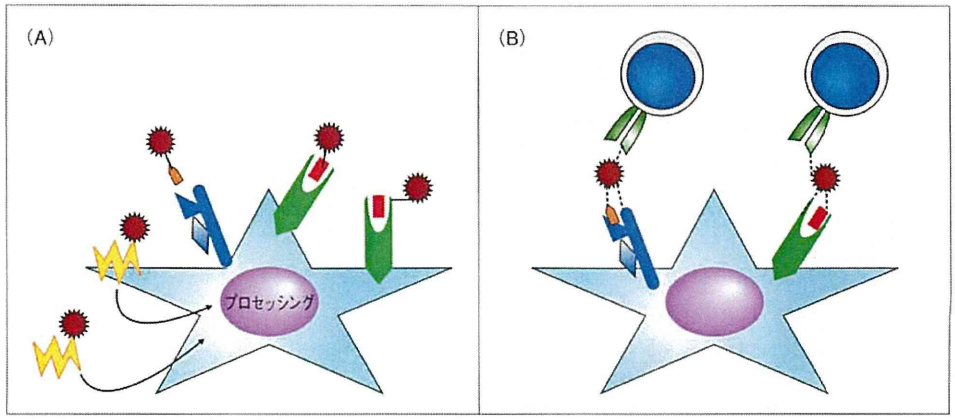

図 1　ハプテン抗原認識(A)と，ハプテンによらない薬剤の抗原認識モデル(B)[19]
薬剤が自己蛋白と結合し新しい抗原として認識される経路(A)と，薬剤の電気的または分子間結合力による親和性によってT細胞が活性化する経路(B)が想定されている．この場合にはT細胞は薬剤に対する感作の必要はない．

を介して認識され，免疫反応が惹起される．

ハプテンによらないT細胞活性化(図 1-B)

薬疹のハプテン抗原認識が検証される一方で，ハプテンによらない薬剤によるT細胞反応の存在が提唱され，注目されている．この反応はハプテン抗原の認識とは異なり，MHCやハプロタイプに非依存性を示す点でユニークである．Pichlerらはこのような反応をpharmacological interaction (p-i)conceptとよび，その重要性を強調している[6]．

通常，個々の薬疹ではハプテンによるもの，よらないもの両者の経路が確認されている．ハプテン抗原の場合は，さきに抗原感作が成立していることがT細胞反応の必要条件である．反応には数時間から100時間ぐらいの時間を要する．一方，非ハプテンとして働く場合についての詳細は明らかでない部分が多い．一般に共有結合，電気的結合，分子間力(ファンデルワース結合)の順で物質間の結合力は弱くなるが，ハプテンによらない抗原認識では，電気的または分子間力による緩い結合が関与していると考えられている．薬剤やその代謝物は，T細胞受容体または抗原提示細胞上のMHCと緩く結合し，両者が十分に接近することによって活性化のセカンドシグナルが入る．カルバマゼピンはHLA-B15:02ペプチド複合体に緩く結合することが判明している[7]．また，薬剤がMHCよりもT細胞受容体に緩く結合する場合もある．抗てんかん剤反応性T細胞ではとくにVβ5.1を発現するT細胞が多いことから[8,9]，抗てんかん剤にはこのT細胞受容体に高い親和性を示す共通の化学・物理特性が推測されている．これらのT細胞反応は感作を必要とせず，薬剤刺激後数秒から数分で起こる．したがって，メモリーT細胞が抗原提示細胞の周辺に存在し，T細胞受容体や抗原提示細胞上のMHCと薬剤との緩い結合が起これば，T細胞はすばやく活性化する．メモリーT細胞のプールサイズは個体の感染の既往やワクチンの接種歴によって決定し，このサイズが大きいほど，薬剤反応性が確率的に増加するはずであるから，メモリーT細胞数と抗原提示細胞との接触頻度が薬疹の重症度を決定する因子になるのかもしれない．ハプテンによるものはT細胞反応と抗体産生がともに起こるのに対し，ハプテンによらないものは抗体産生を伴わない．

活性化T細胞のフェノタイプ

薬疹において，薬剤刺激によって反応するT細胞はその皮疹の臨床像を反映する．すなわち，Th2型細胞はIL-5を産生して好酸球を誘導し[10]，Tc1細胞はgranulysinやperforinを高発現し，表皮障害性に機能する[11](図 2，3)．Acute generalized exanthematous pustulosis患者から得た薬剤反応性T細胞は，GM-CSFやCXCL-8を産生する[12]．

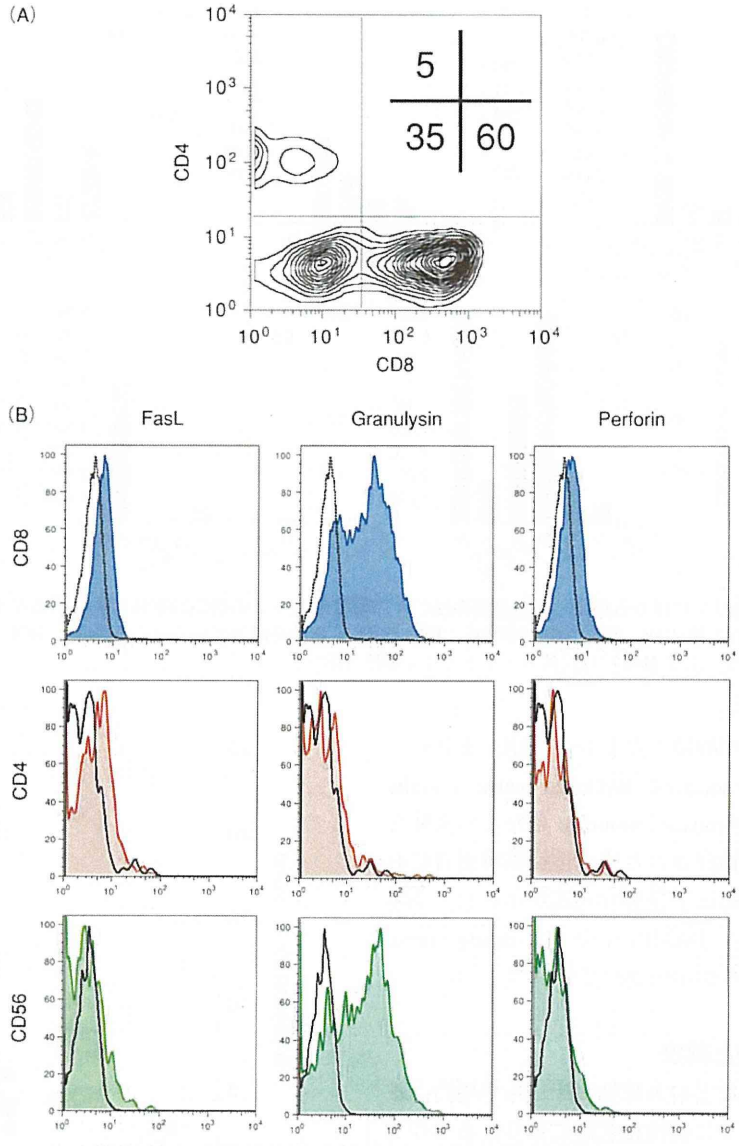

図 2　中毒性表皮壊死融解症の水疱内リンパ球の細胞傷害性分子(FasL, granulysin, perforin)の発現のフローサイトメータ解析
$CD8^+$細胞と $CD56^+$細胞には，細胞障害機能をもつ granulysin の高発現がみられる．

最近，中毒性表皮壊死融解症の水疱内のリンパ球から樹立したある薬剤反応性 T 細胞はきわめて高い IL-17 を産生したことから，著者らは本細胞の出現が重症度を規定するのではないかと考えている(未発表データ)．一方，薬疹皮膚病変に浸潤する制御性 T 細胞の存在の有無が薬疹の重症度を決定するかもしれないという興味深い仮説がある[13]．

Innate immunityと薬疹

これまで薬疹の研究においてはいかに免疫反応が起こるかが注目され，獲得免疫(acquired immunity)の成立機序に焦点がおかれてきた．しかし，薬疹の発症機序においても，innate immunity の発動が関与し，重要な役割を演じている可能性が示されてきている．アセトアミノフェンによって生じる肝障害の機序では，中間代謝物の過剰な蓄積

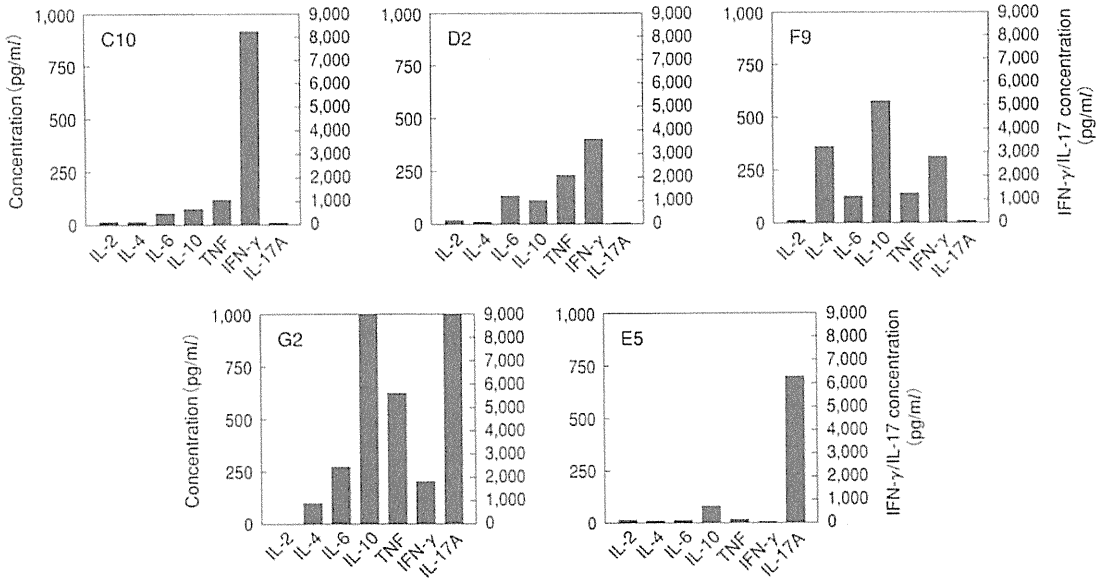

図 3　水疱内リンパ球から樹立した薬剤反応性CD4$^+$T細胞クローンの抗CD3抗体刺激によるサイトカイン産生
CD4$^+$細胞は Th1 型(C10，D2)，Th2 型(F9)，Th17 型(G2，E5)を混じている．右 Y 軸は，IFN-γ および IL-17 のサイトカイン濃度，左 Y 軸はそれ以外のサイトカイン濃度を示す．

による直接的肝細胞障害とともに，DNA を含む一連の damage-associated molecular pattern molecules(DAMPs)が innate immunity を介して炎症を増幅することが検証された[14]．重症薬疹患者においても DAMPs 遺伝子発現の亢進が示され，薬疹の重症化において DAMPs を介した innate immunity の発動の関与が示唆されている[15]．

ウイルス感染と薬疹

ウイルス感染などの炎症性疾患をもつ場合，抗原提示細胞はすでに活性化状態にあり，あらゆる抗原に対して感作が成立しやすい．加えて感染症に伴う発熱や菌体などによって抗原提示細胞に存在する代謝酵素が機能低下を起こし，薬剤代謝が停滞することによって自己蛋白と結合しやすい薬剤中間代謝物が生じて感作されやすくなるという[16]．

DIHS は薬疹の経過中に，遅れてヘルペスウイルス群の再活性化を生じる点で，先述した状況とは異なる．著者らは，DAMPs のひとつである血中 high mobility group box(HMGB)-1 がおそらく表皮細胞障害によって放出されることにより，DIHS や SJS 患者で上昇することを見出した(図4，発表準備中)．この分子は骨髄から単球や骨髄由来幹細胞を局所に動員し，炎症を促進させるとともに，組織修復に関与する．DIHS 患者において一過性に通常の単球分画とは異なる，HHV-6 を内在させた未熟な単球様細胞が循環するが[17]，これに HMGB-1 が関与しているのではないかと著

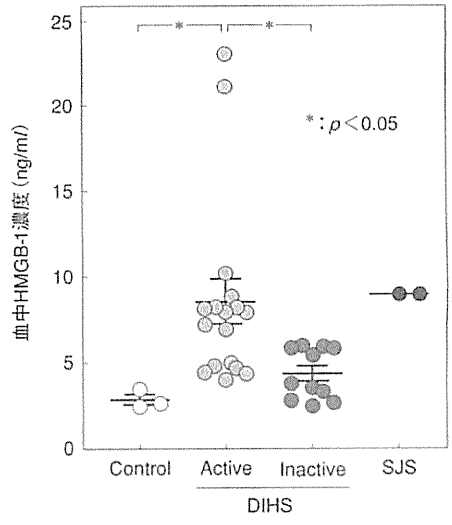

図 4　DIHSとSJS患者の血中HMGB-1濃度
活動期にある DIHS では，健常人や非活動期に比べて有意に高値を示す．

者らは考えている．DAMPsは自己免疫疾患の発症に深い関連があることから，DIHSとこの経過中に発症する自己免疫疾患との共通分子として今後の研究の展開が期待される．

薬疹研究の展望

薬疹の臨床像はおそらく，体質(HLA)によって決定される部分と，環境や経験からくる後天的要素の両面が影響していると考えられる．前者は進行中のゲノムワイド関連解析を用いた網羅的な探索によって，近い将来つぎつぎと謎が解き明かされるであろう．後天的要素に関する研究は，わが国は公的な情報データベースを有さないという点で立ち後れている．著者らは，最近，日本皮膚アレルギー学会・接触皮膚炎学会の共同研究として薬疹データベースの構築に着手し，有意義な情報源の確立をめざしている．

さらに，ヒトにおける薬疹の研究はまだ十分とはいえない．動物を用いた純粋な免疫応答では推し量れない複雑なシステムが，ヒト薬疹には介在する可能性がある．ニッケルのヒトToll-like receptor-4に対する特異的結合と活性化がみられることが見出され，ニッケルアレルギー発症機序として重要な現象であることが示された[18]．同様の現象が低分子薬剤でも起こりうる．ヒトに関する薬疹研究の充実は今後のもうひとつの課題となるであろう．

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