sequential halving of cellular fluorescent intensity with each successive generation. Although CFSE dilution is usually a useful technique for the measurement of cell proliferation, cells that have proliferated are sometimes indistinguishable from a non-specific peak of dead cells, especially when the cell proliferation is small in magnitude, which it often is in a DLST reaction. 5-bromo-2′-deoxyuridine (BrdU) is a non-radioactive thymidine analogue that becomes incorporated into DNA during the S-phase of the cell cycle (Fig. 1A). Here, drug-specific proliferating cells were identified by a flow cytometric DLST (FCM-DLST) protocol that combines CFSE dilution and BrdU incorporation and utilizes them as a substitute for ^{3}H-thymidine incorporation. The combination of the CFSE and BrdU assays allows for the clear identification of the very small proliferating cell population as $CFSE^{low}$ $BrdU^{high}$ cells. A FCM-DLST protocol that uses the combination of CFSE and BrdU assays can reveal the proliferating drug-specific cell population responsible for the proliferation found by conventional DLST. We took advantage of this feature and analyzed the drug-specific T cells of anti-convulsant hypersensitivity patients during the acute and recovery stages of the disease. Interestingly, drug-specific $CD8^{+}$ T cells were detected only in the acute stage of severe drug hypersensitivity, whereas drug-specific $CD4^{+}$ T cells were found to be dominant in the recovery stage. Moreover, the percentage of drug-specific $CD4^{+}$ T cells that were $Foxp3^{+}$ regulatory T cells (Tregs) was increased during the recovery stage in one of the DIHS cases, suggesting that different subsets of drug-specific T cells are induced during different disease stages of a cADR.

2. Materials and methods

2.1. Patients

Sixteen patients clinically diagnosed with anticonvulsant-induced cADR were enrolled in this study from July 2008 to July 2011. Conventional DLST was performed in all 16 cases, while FCM-DLST was performed in six of the cases. Our institutional review board approved this study, and informed consent for all diagnostic procedures and research was obtained from all patients and healthy controls.

2.2. Cell preparation and culture

Cell preparation and culture for DLST were performed in accordance with standard DLST protocols [6,10,11]. Briefly, PBMCs were isolated with Ficoll-Hypaque solution (Sigma–Aldrich), labeled with 6 mM CFSE (Invitrogen), and cultured at 2×10^{5} cells/well in two 96-well flat-bottomed plates for 7 days. After addition of the identified culprit drug, one plate was used for conventional DLST and one for FCM-DLST (Fig. 1B).

2.3. Preparation of culprit drugs

Culprit drugs were dissolved in phosphate-buffered saline (PBS), or PBS with 0.025% dimethyl sulfoxide (Wako) if the drug was PBS-insoluble, and added to the PBMC culture medium at the beginning of incubation (day 0). Sodium valproate was PBS-soluble, whereas phenytoin, zonisamide, and carbamazepine were PBS-insoluble. The final drug concentrations were 100, 10, and 1 μg/ml. PBMCs were also incubated without a drug (negative control) and with 10 μg/ml phytohemagglutinin (PHA, Sigma–Aldrich; positive control).

2.4. ^{3}H-thymidine incorporation assay for DLST

Conventional DLST that used ^{3}H-thymidine was performed as previously described [10,11]. The results are presented as the stimulation index (SI), which was the ratio of the highest count per minute of the samples cultured with diluted drug to that of the control cultured without a drug. A SI value >2.0 was interpreted as a positive result.

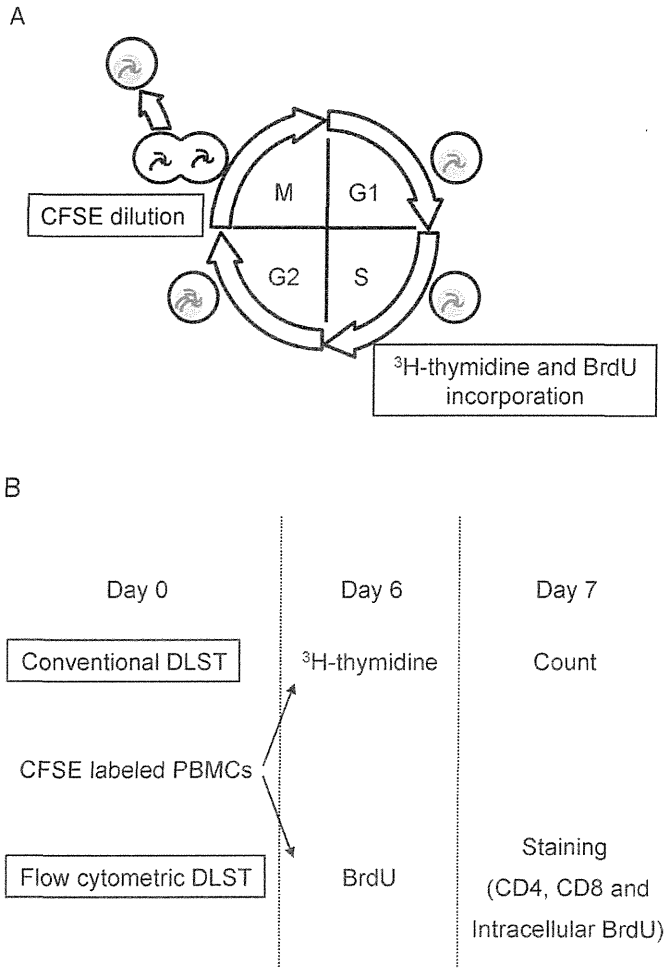

Fig. 1. (A) Cell cycle scheme. ^{3}H-thymidine and BrdU are incorporated into S-phase cells, while the CFSE intensity of a labeled cell is reduced by half every cell division. G1: gap 1 phase; S: synthesis phase; G2: gap 2 phase; M: mitotic phase; BrdU: 5-bromo-2′-deoxyuridine; CFSE: carboxyfluorescein diacetate succinimidyl ester. (B) Comparison of the conventional and flow cytometric drug-induced lymphocyte stimulation tests (DLST). A patient's peripheral blood mononuclear cells (PBMCs) were isolated and CFSE-labeled. They were cultured for 7 days in two culture plates with a culprit drug for conventional and flow cytometric DLST (one plate for conventional, one for flow cytometric). Six days after incubation, ^{3}H-thymidine or BrdU were added. After an additional 20–24 h of incubation, the cells were harvested, and ^{3}H-thymidine incorporation was measured for conventional DLST. For flow cytometric DLST, BrdU-pulsed cells were stained with anti-CD4 and anti-CD8 antibodies, fixed, permeabilized, and then intracellularly stained for BrdU.

2.5. Flow cytometric DLST

The other plate of CFSE-labeled PBMCs was incubated with or without culprit drug in the same manner (Fig. 1B). Six days after the start of incubation, 10 μm BrdU (Sigma–Aldrich) was pulsed into the wells instead of ^{3}H-thymidine. After 20–24 h of further incubation, the cells were collected and stained with the following antibodies and reagents: Peridinin chlorophyll protein (PerCP)-conjugated CD4, allophycocyanin (APC)-conjugated CD8, streptavidin–phycoerythrin (PE) (all BD Biosciences), and biotin-conjugated BrdU (Abcam). For intracellular BrdU staining, cells were labeled with CD4 and CD8, fixed and permeabilized with BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences), and treated with 0.3 mg/ml Deoxyribonuclease I

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(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	Glioblastoma	MP	16	1.6	
		Carbamazepine			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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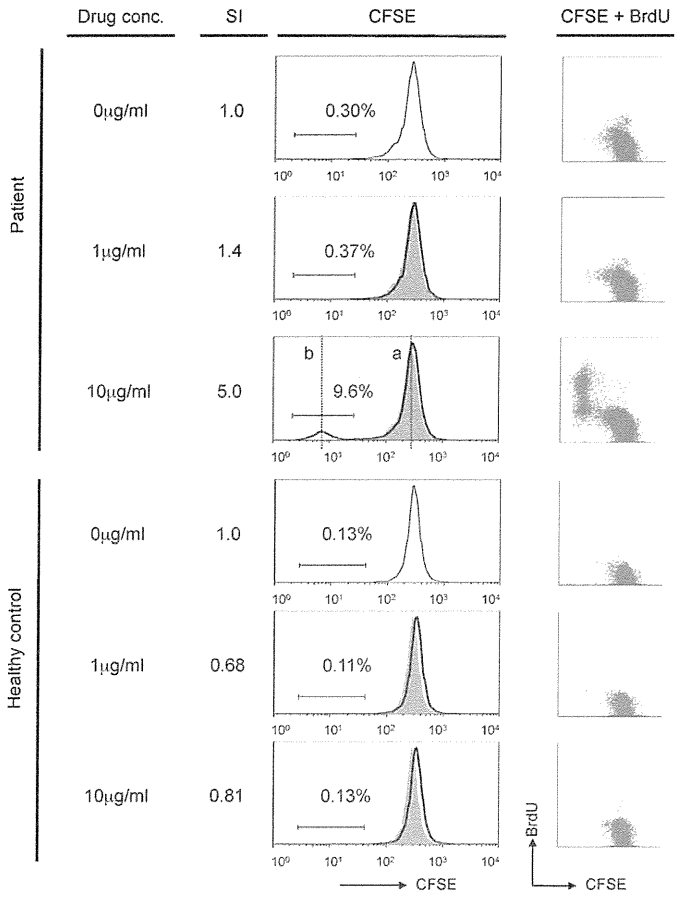

Fig. 2. Representative data of conventional DLST, the CFSE dilution assay alone, and the CFSE dilution assay combined with the BrdU incorporation assay (Case 1 and a healthy control). Drug conc: concentrations of culprit drugs, SI: stimulation index. Corresponding with a positive SI value, 9.6% of the 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 ($\log_2 a/b$).

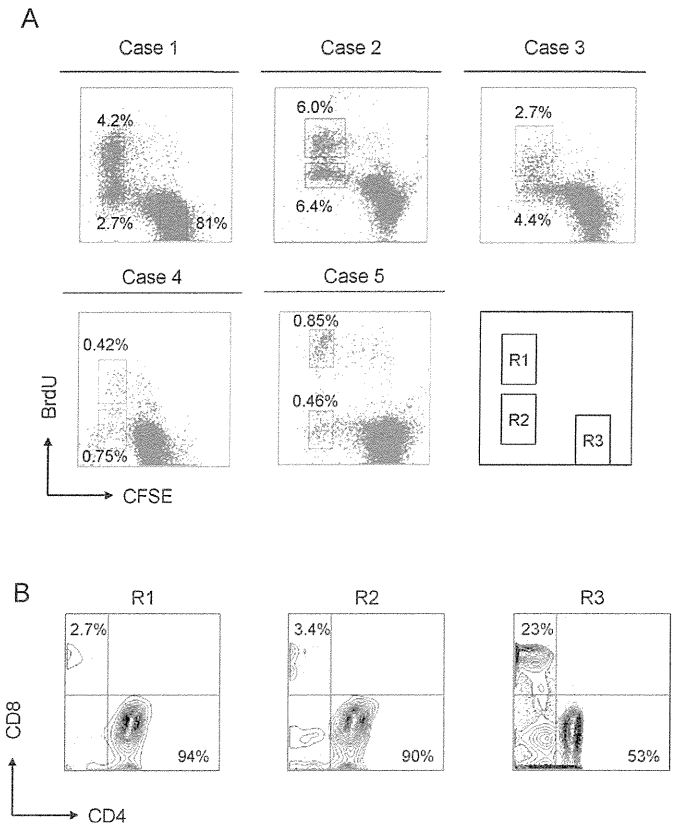

Fig. 3. (A) The results of flow cytometric DLST in five cases and the T-cell subsets found in the flow cytometric DLST of Case 1. Seven-day-cultured PBMCs were categorized into three subpopulations: R1, the $CFSE^{low}$ $BrdU^{high}$ drug-specific proliferating cells that had incorporated BrdU within 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.

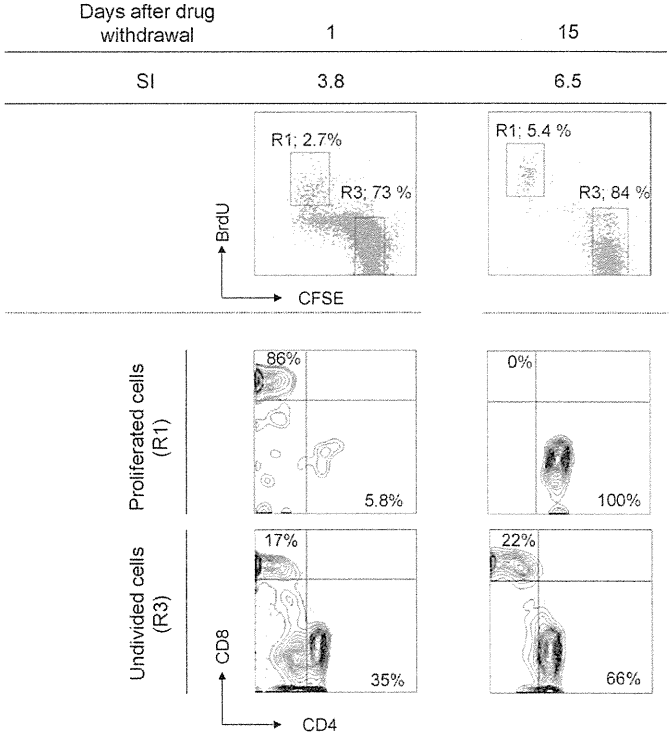

Fig. 4. The predominant drug-specific proliferating cell population changed from $CD8^+$ CTLs to $CD4^+$ T lymphocytes during the clinical course of DIHS (Case 3). When conventional and flow cytometric DLST were examined on day 0 after the withdrawal of phenytoin, the major drug-specific proliferating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

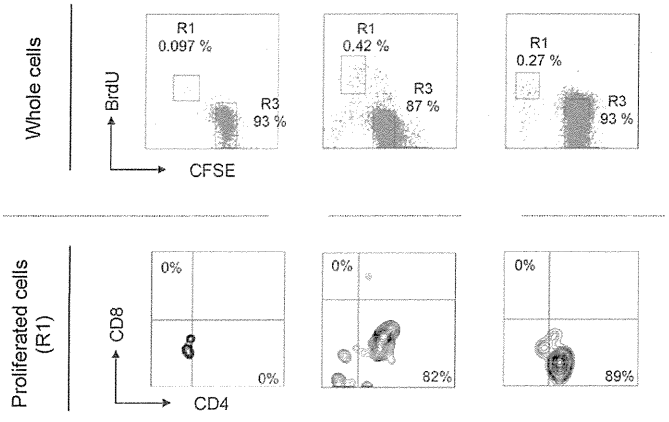

Fig. 5. The predominant drug-specific proliferating cell population remained CD4$^+$ T lymphocytes during the recovery course of DIHS (Case 4). From day 15 to day 35 after the withdrawal of zonisamide, the major drug-specific proliferating cell population remained the CD4$^+$ T lymphocyte population.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

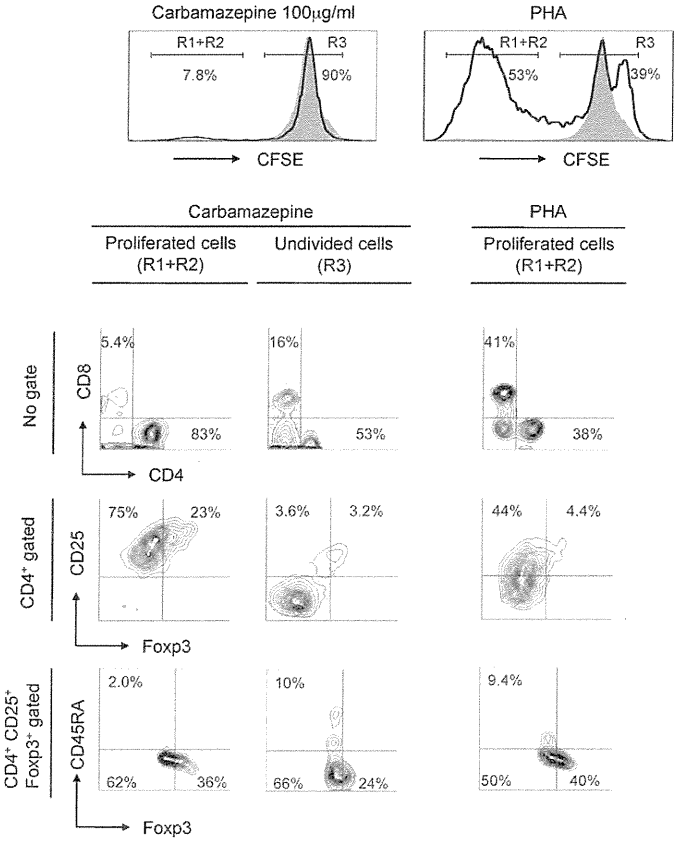

Fig. 6. Drug-specific Tregs were increased in DLST at the recovery stage. A case of a patient with a carbamazepine-induced DIHS was examined by CFSE dilution assay combined with Foxp3 staining. On day 52 after drug withdrawal, the major CFSE^{low} drug-specific proliferating cell population was CD4$^+$ 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.

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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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特集II　重症薬疹の診断と治療

重症薬疹の発症機序*

橋爪秀夫**

Key Words：drug-induced hypersensitivity syndrome (DIHS), immunology, mechanism, Stevens-Johnson syndrome (SJS), toxic epidermal necrolysis (TEN)

はじめに

薬疹の多くは，薬剤の中止によって速やかに回復する．しかし，なかには薬剤中止後も病勢が進行し，炎症の鎮静が必要な，致命率および後遺症の合併率も高い重症薬疹が存在する．スティーブンス・ジョンソン症候群(Stevens-Johnson syndrome；SJS)，中毒性表皮壊死症(toxic epidermal necrolysis；TEN)および薬剤性過敏症症候群(drug-induced hypersensitivity syndrome；DIHS)の3疾患は，この重症薬疹に分類されている．SJSとTENは類似した臨床像および皮膚病理組織像を呈し，病因的に同じスペクトラムの疾患ととらえられており，SJS/TENとして一括して論じられることが多い．この組織学的特徴は，表皮細胞の壊死であり，熱傷のように体表から多くの水分を奪い，循環不全を生じさせる．さらに，皮膚からの細菌の侵入によって重症感染症をひき起こす．また，眼球結膜や口腔粘膜に生じた病変は，重篤な後遺症をもたらす．一方，DIHSは，欧米ではdrug rash with eosinophilia and systemic symptoms (DRESS)と呼ばれる疾患と同一であると考えられている．1998年にShioharaら[1]とHashimotoら[2]のグループから同時にヒトヘルペスウイルス(human herpesvirus；HHV)-6再活性化との関連が見出され，その独立性が検証されて，DIHSという疾患概念が確立された．原因薬剤がかなり限定されていること，皮疹以外に肝障害，腎障害をはじめ種々の臓器障害を合併することとともに，薬疹が治癒した後に種々の自己免疫疾患を合併するという奇妙な特徴を持つ．また，臓器障害はときに重篤で死亡率はおよそ10%とされている．これらの重症薬疹に関する最近の知見をはさみながら，その謎について考えてみる．

薬剤は抗原としてどのように認識されているか

ゲノムワイド関連解析によって，ある種の薬剤では，特有のHLA保有者に高頻度に薬疹が起こることが明らかになった．たとえば，カルバマゼピン薬疹は，漢民族ではHLA-B15:02[3]，わが国ではHLA-B31:01保有者[4]に高率に起こる．また，アロプリノール薬疹は，洋の東西を問わずHLA-B58:01保有者に高率に起こることが明らかになった[5]．HIV治療薬のアバカビルでは，HLA-B57:01保有者が高率に起こるだけでなく，このハプロタイプを持った患者に同薬剤を投与しないことで，同薬剤の薬疹の発症を有意に抑えることが可能であることが検証された[6]．わが国でも，カルバマゼピン投与に際し，HLA-B31:01

* Pathomechanisms of severe adverse drug reactions.

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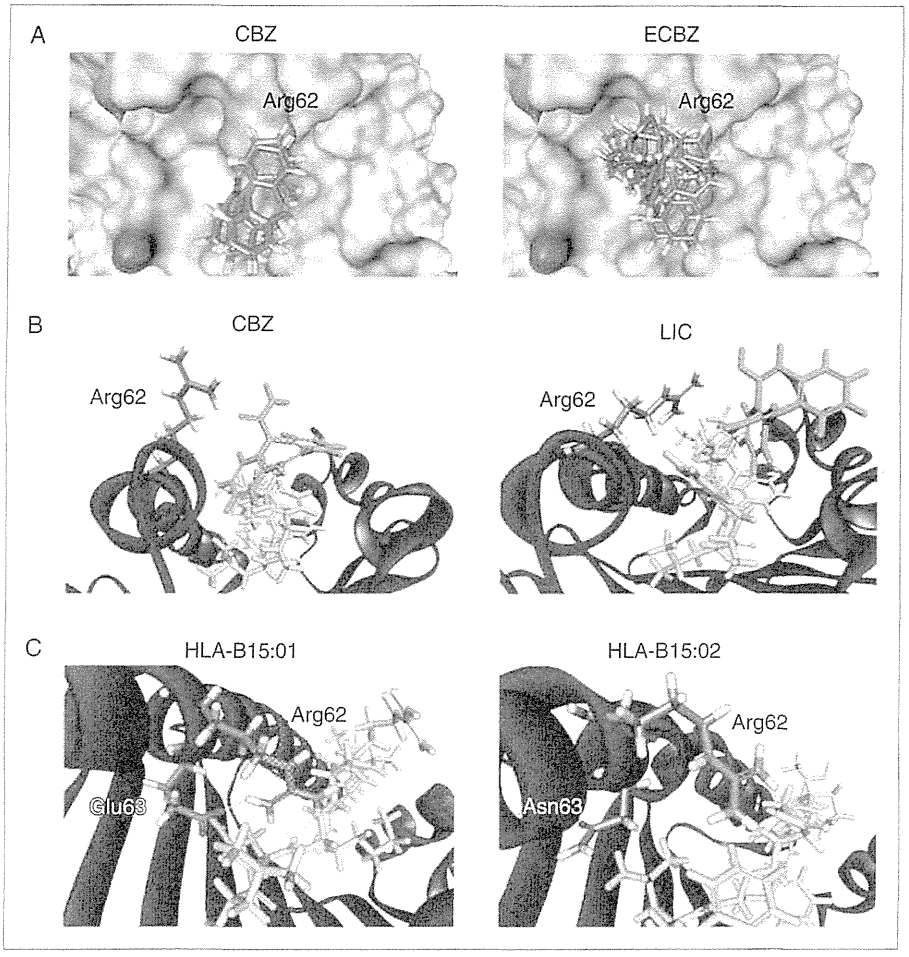

図1　HLA-B15:02分子とカルバマゼピン(CBZ)またはその類薬(ECBZとLIC)との相互作用のコンピュータ解析
CBZおよびECBZはArg62に接するが(A)，三環部分が置換したLICでは構造的にArg62に接することはないので，反応は起きない．HLA-B15:01ではArg62とGlu63とペプチドに安定した結合が生じるため，薬剤がArg62に結合することができない(C)．　(文献7より引用)

保有者はこれを使用しないということで，薬疹発症が抑えられるかを検討する臨床治験が開始されはじめた．薬疹発症を回避するテーラーメイド薬物療法の幕開けである．一方，in silico解析の進歩によって分子間構造から，どのような部位に薬剤が結合するかという研究が進んでいる．特にカルバマゼピンに関してはこの薬剤が共有結合などの強固な結合ではなく，弱い分子間の電子的結合による可能性が確認されている(図1)[7]．また，近年ではアバカビルとHLA-B57:01との結合における分子構造解析が行われ，薬剤がHLAと結合することによって，自己ペプチドが新しい抗原決定基を提示して免疫反応を誘導する可能性も示された(図2)[8]．すなわち，薬剤がHLAに結合することによって，多種類の自己抗原が非自己の抗原と認識されてしまうのである．アバカビル薬疹が重症であることの一つの根拠なのかもしれない．今後も薬疹発症に関する薬剤とHLAとの結合の詳細が明らかになることが期待される．

一部の報告とは異なるが，日本においてはカルバマゼピン薬疹の重症度とHLAとの関連性は明らかになっていない．すなわち，HLAは薬疹の発症しやすさを規定するだけで，その重症度

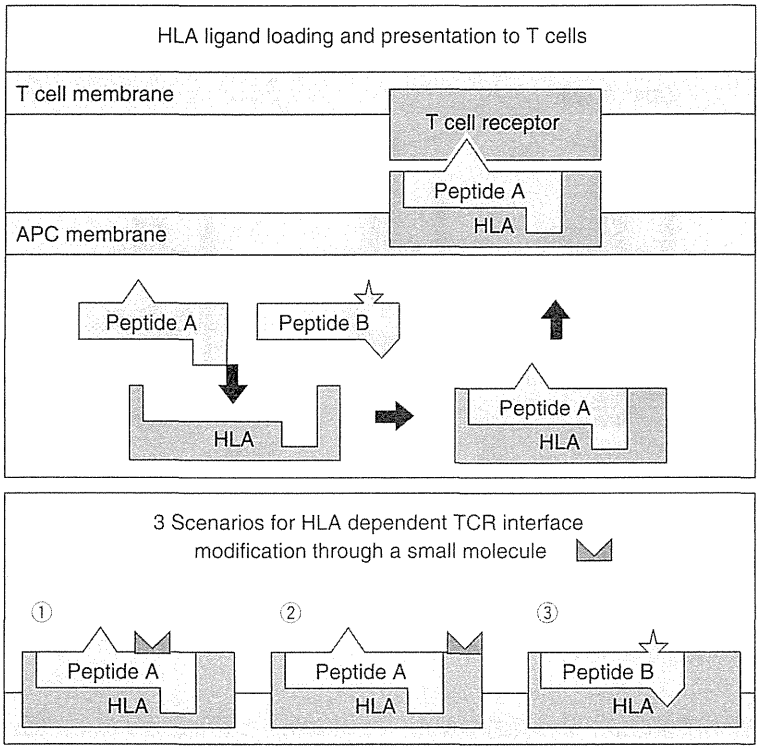

図2　薬剤抗原が提示される仕組み
薬剤が自己ペプチドを修飾して提示される場合(①)，MHCが薬剤によって修飾され抗原として提示される場合(②)があるが，アバカビルの場合は，MHCに薬剤が結合することによって，自己ペプチドが新しい抗原決定基を提示してしまう場合(③)があることが判明した．

とは関連がなかったのである．この事実は，HLA以外の他の因子が，薬疹の重症度を規定することを示唆している．薬疹の多くはT細胞を介したアレルギー反応と理解され，皮疹の重症度を決定するのは，活性化するT細胞の量と質と考えられている．T細胞は産生するサイトカインやフェノタイプによって機能的に分類されている．一般的にCD8$^+$細胞は，細胞傷害性であり，表皮細胞障害が強い程，末梢血および組織に浸潤する本細胞は多い傾向にある．また，膿疱を臨床的特徴とする急性汎発性発疹性膿疱症では，好中球遊走因子であるCXC-8を産生するTh17細胞の浸潤を認める．皮疹に浸潤するT細胞の特性は，発症する皮疹の形成に密接に関連しているはずである．

それでは，活性化するT細胞の質(種類)や量を規定するものは何なのだろうか．薬剤代謝の遅い患者では，SJS/TENなどの重症薬疹が起こりやすいことは，古くから知られている[9]．代謝されない薬剤の血中濃度の上昇に比例してT細胞の活性化が増強することに起因すると考えられる．また，抗原提示細胞の活性化状況によって，T細胞の活性化の強弱は修飾される．すなわち，免疫応答における強弱は，抗原の量や免疫担当細胞の状態によって規定されていると考えられる．しかし，活性化するT細胞の質は何が規定するのかはよくわかっていない．その答えの一つのヒントとして，Pichlarが提唱するpharmacological-interaction concept(*p-i* concept)がある(図3)[10]．そして，最近実際にこの機構の存在が分子レベルで確認された．これは，T細胞と薬剤，抗原提示細胞との反応の間には，通常のハプテン抗原のような共有結合を介した強固な結合は必ずしも存在せず，電気的または分子間の弱い結合によっても成立するという考えである．この説の重要な点は，この反応系が存在す

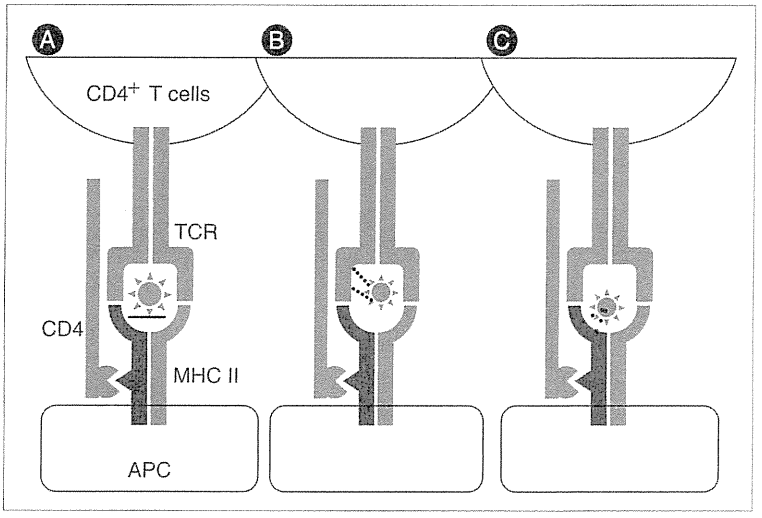

図3　*p-i* concept
CD4⁺細胞が薬剤抗原を認識する場合，薬剤が自己ペプチドやMHCに共有結合してハプテン抗原となる場合(A)以外にも，薬剤が電気的にT細胞受容体(B)またはMHC(C)と緩く結合した場合にも反応が起こることがあり，Pichlerらはこれを*p-i* conceptと名づけた．

る場合，既存の感作T細胞が，ある条件で薬剤や抗原提示細胞と接触する機会を持つならば，薬剤感作なしに薬剤によって刺激されることである．ヒトは種々の病原体に接し，その抗原がT細胞を感作させて，記憶T細胞のプールができる．したがって，個々で異なる既往感染やワクチン接種による感作がすんでいる記憶T細胞の質の違いが，薬剤刺激によって活性化するT細胞の質を決定づけ，皮疹にバリエーションを与えている可能性がある．

SJS/TENの謎

SJS/TENの末梢血中および皮膚または水疱内容液には，多数の活性化CD8⁺細胞およびNK細胞を認める．しかし，特に皮膚組織において，その重症度と浸潤細胞数は必ずしも相関しないことは，表皮細胞の壊死が，必ずしもCD8⁺細胞やNK細胞の直接攻撃によってもたらされたものではない可能性を示している．かかる疑問に答えるべく，数十年前から本疾患における液性表皮攻撃因子の探索が行われてきた．SJS/TENにおける表皮細胞壊死の原因分子はCD8⁺細胞が産生する液性細胞傷害性分子が想定され，パーフォリン[11]，Fasリガンド[12]，グラニュライシン[13,14]など，その主役の座は次々と移り変わっている(図4)．そして，最近話題のグラニュライシンでさえ，DIHSなどの表皮壊死を伴わない薬疹でも高値を示すことが明らかになり[15]，その意義は薄れてしまった．これまでCD8⁺細胞が産生する液性因子のみ注目されてきたが，本症の重症度とCD11c⁺CD16⁺樹状細胞の皮膚病変浸潤との相関が明らかとなったことから[16]，樹状細胞が産生する分子に関する探索も必要であろう．SJS/TENの発症にかかわる新規の液性細胞性障害性因子の探索は，ただちに治療に結びつく可能性が期待されるため，今後も精力的な研究が望まれる．

DIHSの謎

DIHSに関する奇妙な現象は多い．DIHSの診断基準は，この奇妙な現象を言い表しており，本疾患の独立性を明確にしている(表1)．すなわち，①薬剤投与後の発症は，他の薬疹に比べて明らかに長い(3週間以上)，②薬剤中止後も進行する，③経過中にHHV-6, HHV-7, EBV, サイトメガロウイルスなどのヘルペスウイルス再活性化がみられる[17]，④限定した薬剤によって発症するなどである．本症は，ダイナミックな症状変化が特徴的である．典型的には，発熱と播種状

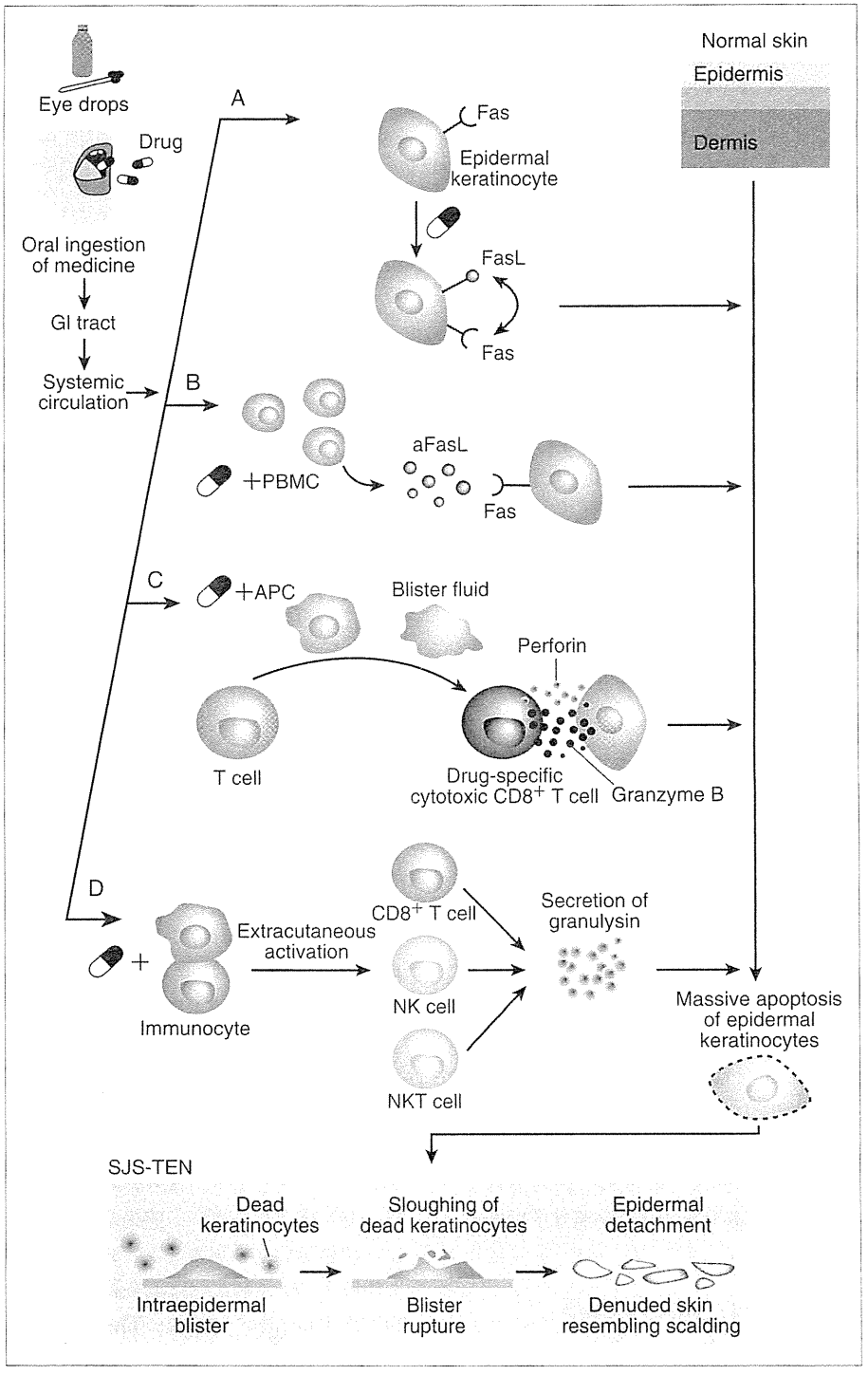

図4　SJS/TENにおける表皮細胞障害のメカニズム
薬剤は表皮細胞(A)や単核細胞(B)にFasLを産生させ，Fasが高発現する表皮細胞をアポトーシスに陥らせたり，$CD8^+$細胞傷害性細胞を活性化させ，パーフォリンやグランザイムを分泌させて表皮細胞を壊死においやる(C)．また，$CD8^+$細胞，NK細胞またはNKT細胞は，グラニュライシンを産生して表皮細胞を殺す(D)．（文献[26]より引用）

表1　DIHSの診断基準

1. Maculopapular rash developing > 3 weeks after starting with a limited number of drugs
2. Prolonged clinical symptoms 2 weeks after discontinuation of the causative drug
3. Fever(>38℃)
4. Liver abnormalities(alanine aminotransferase>100 U/l)*
5. Leucocyte abnormalities(at least one present)
   a. Leucocytosis(>11×10^9/l)
   b. Atypical lymphocytosis(> 5 %)
   c. Eosinophilia(>1.5×10^9/l)
6. Lymphademopathy
7. Human herpesvirus 6 reactivation

The diagnosis is confirmed by the presence of the seven criteria above(typical DIHS)or of the five(1～5)(atypical DIHS). *This can be replaced by other organ involvement, such as renal involvement.　（文献[27]より引用）

の淡い紅斑，顔面浮腫，リンパ節腫脹が出現した後，皮疹はゆっくりと紅皮症化し色素沈着を伴いながら消退していく．そのころより異型リンパ球の出現を伴う白血球増多，肝機能障害を含む種々の臓器障害を生じる．本症の制御性T細胞(Treg)に焦点を当てた報告[18]によると，初期に機能亢進し，後期に機能不全に陥っていることが明らかとなっており，これがダイナミックに変化する臨床症状と関連する可能性がある．ヘルペスウイルスの再活性化の一つの可能性として，Tregの機能亢進以外にも，薬剤が直接潜伏感染している細胞を刺激し，ウイルスの増殖を起こすことがin vitroの実験で示されている[19]．われわれは最近，経過中に一過性に出現するmono/myeloid precursorにHHV-6が潜伏感染していることを見出し，これが皮膚に移行して浸潤しているCD4$^+$細胞にHHV-6を感染させることによって，ウイルス増殖を起こしている可能性を示した(図5)[20][21]．なぜmono/myeloid precursorが骨髄より末梢へ動員するのかは明らかではないが，われわれは，少なくとも最近注目されているalarmins, HMGB-1が一つの候補分子であると考えている[21]．

SJS/TENとDIHSの関係

SJS/TENの病態に最も重要なのは，CD8$^+$細胞，NK細胞などの細胞傷害性分子を含む細胞群であることに異論はないが，皮膚浸潤細胞には，この種の細胞以外にも無視できない数のCD4$^+$細胞が存在する．一方，DIHSの皮疹にも，CD8$^+$細胞とCD4$^+$細胞の両者が浸潤している．CD8$^+$細胞の主たる機能は，もっぱら標的細胞に対する直接または間接的な細胞障害である．一方，CD4$^+$細胞は，機能的にいくつかのサブタイプに分類されており，この違いがCD8$^+$細胞による炎症にディバーシティーを与えている可能性がある．

私たちは，SJS/TENおよびDIHSの皮疹浸潤細胞を採取し，CD4$^+$細胞に焦点を当てて，その産生サイトカインを調べてみた．すると，SJS/TENにおいてCD4$^+$細胞はCCR6を発現し，IL-17を産生するTh17が多く，DIHSにおいてはCD25$^+$CD127$^-$で，Foxp3を発現するTregが多いことが明らかとなった(図6：未発表データ)．今までわれわれは，SJS/TENやDIHSの患者末梢血から薬剤反応性T細胞クローンを樹立してきたが，このデータは，これらの細胞でもそれぞれIL-17およびIL-10産生細胞が多かったという結果と一致する[21]．Th17とTregは同じprecursorからTGF-β下で誘導され，それはIL-6の有無によって運命づけられている．すなわち，IL-6が豊富な環境ではTh17へ分化しやすくなり，IL-6が欠如した環境下ではTregが生成されやすい．感染症や急性炎症性疾患など急性炎症サイトカインであるIL-6が高値である環境下で抗生剤または消炎鎮痛剤によってSJS/TENが出現しやすいのは，Th17によってCD8$^+$細胞を中心とした炎症反応が促進されるからであると理解できる．SJS/TENにおいてTh17が関与する可能性は，乾癬治療の手法が応用できる可能性を持つ．台湾のグループは，抗TNF-α抗体療法を用いるとSJS/TENが急速に治癒に向かうことを経

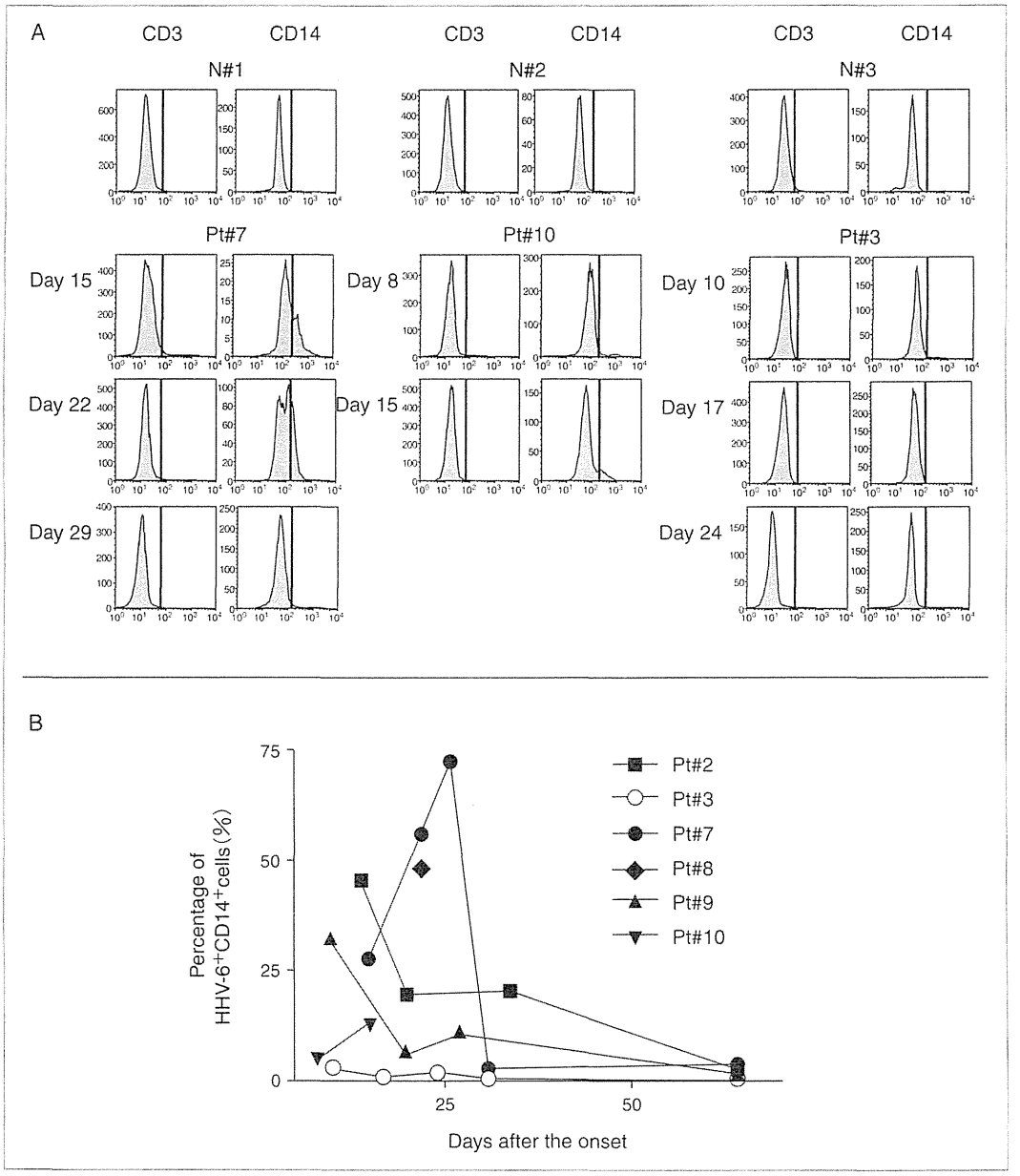

図5　DIHS発症経過中の患者(Pt)および健常人(N)の末梢$CD3^+$細胞および$CD14^+$細胞におけるHHV-6抗原の発現(A)と$CD14^+$細胞中のHHV-6抗原陽性細胞の割合の経時的変化(B)
Pt#3はHHV-6に未感染の患者.

験しているという(Cung WH, et al. personal communication). 一方, 精神神経疾患で抗けいれん剤によって突然発症するDIHSでは, 薬剤反応性の$CD8^+$細胞による炎症はなんらかの誘因によってTregの割合が多くなり, これによって抑制されているために, 皮疹発現までの期間が長いと想像できる.

最近, SJS/TENで発症し, 後にDIHSと診断された症例の報告がある. このような症例ではTh17とTregとのバランスの時間的変化によって説明可能なのかもしれない.

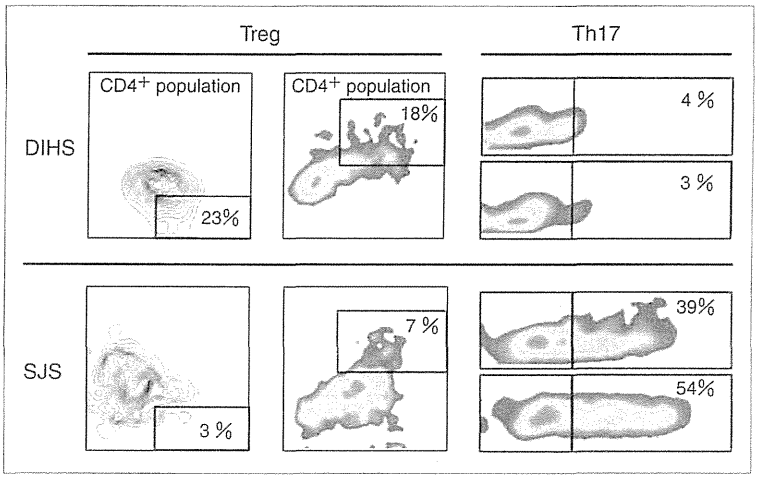

図6　DIHS(上段)およびSJS(下段)における皮疹部浸潤細胞のTregとTh17
皮疹部浸潤細胞を抗CD3/CD28抗体ビーズとIL-2で増幅してから，常法に従い発現分子(左)とIL-17産生(右)をフローサイトメーター解析した．Tregに関する発現分子はY軸，左：CD127，右：Foxp3でX軸は両者ともCD25．それぞれ一名の患者について示す．IL-17産生はPMA刺激後24時間後にintracytoplasmic cytokine stainingを行った．図はそれぞれ患者2名のデータを示す．

自然免疫系と薬疹

定常状態では細胞代謝にかかわるが，いったん細胞が障害されたときに細胞外へ放出されると，樹状細胞や単球に作用し，炎症を惹起し，組織修復反応を促進する分子があることが判明し，これをalarminsと呼んでいる．Alarminsはこれらの細胞膜表面に発現する自然免疫に重要なToll-like receptorやpattern recognition moleculesに結合し，単球や樹状細胞の活性化や遊走を促進して炎症反応を促進し，組織修復に関与している[22]．これらは膠原病や熱傷など感染が関与しない炎症反応に深くかかわっていることが明らかになっている．

最近，SJS/TENなどの薬疹の末梢血においてalarminsの分子群が高発現することが，DNAアレイを用いた網羅的解析によって明らかになった[23]．さらに，本疾患の血中HMGB-1濃度が高いことも確認されている[24]．われわれは，最近DIHSの皮疹や末梢血中に特にHMGB-1が高発現していることを見出し，これが骨髄からHHV-6を潜伏感染するmono/myeloid precursorsの末梢への放出を促している可能性を想定している．また，SJS/TENにおいて直接表皮細胞を障害する分子として注目されてきたグラニュライシンのうち15kDaアイソフォームは，TLR4に結合して，樹状細胞の活性化と遊走をもたらすalarminsであることが確認された[25]．

DIHS患者の多くは，すでに脳疾患や高尿酸血症，膠原病などを合併している患者が多く，障害細胞から放出されるalarmins値は健常人より高い可能性がある．すなわち，このような疾病を持つ患者は，骨髄からの樹状細胞や単球系前駆細胞が末梢へと動員されやすい状況がある．HHV-6を含む種々のヘルペスウイルスは，骨髄内の単球や樹状細胞をreservoirとしていることから，これらの患者の血中内には，HHV-6潜伏感染細胞が循環している可能性がある．HHV-6の爆発的な増殖には，$CD4^+$細胞への感染が必須であるから，この状況下ではHHV-6の再活性化は起こらない．しかしながら，薬疹などによって皮膚に対するT細胞の攻撃が始まると，皮膚由来のalarminsによってHHV-6感染細胞が皮膚内へ動員され，皮膚浸潤$CD4^+$細胞にウイルスを伝播させてしまうのだろうとわれわれは推測している．皮膚は再活性化をもたらすためのHHV-6増殖の場と考えられる．

将来への展望

病気を治療するための薬剤副作用によって，後遺症を起こしたり，命を脅かすことはあってはならない．商業化と結びつきやすい新規薬剤の開発において多額の研究費が投じられるのに対し，残念ながら薬剤の副作用に関する研究に対する助成は，なかなか得られにくいのが現状である．しかし，目の前には多くの薬疹患者が存在する．この現実を私たち医療者は直視し，薬疹を減らす努力をしなければならない．ゲノムワイド関連解析の進歩は，薬疹発症危険因子を明らかにし，薬疹を起こさないためのテーラーメイド薬物療法が現実となる日も近い．今後，薬疹の治療や予防，さらには薬疹を起こさない創薬に繋げるためにも，薬疹の発症機序解明の研究は進めなければならない現代医療の課題だともいえる．

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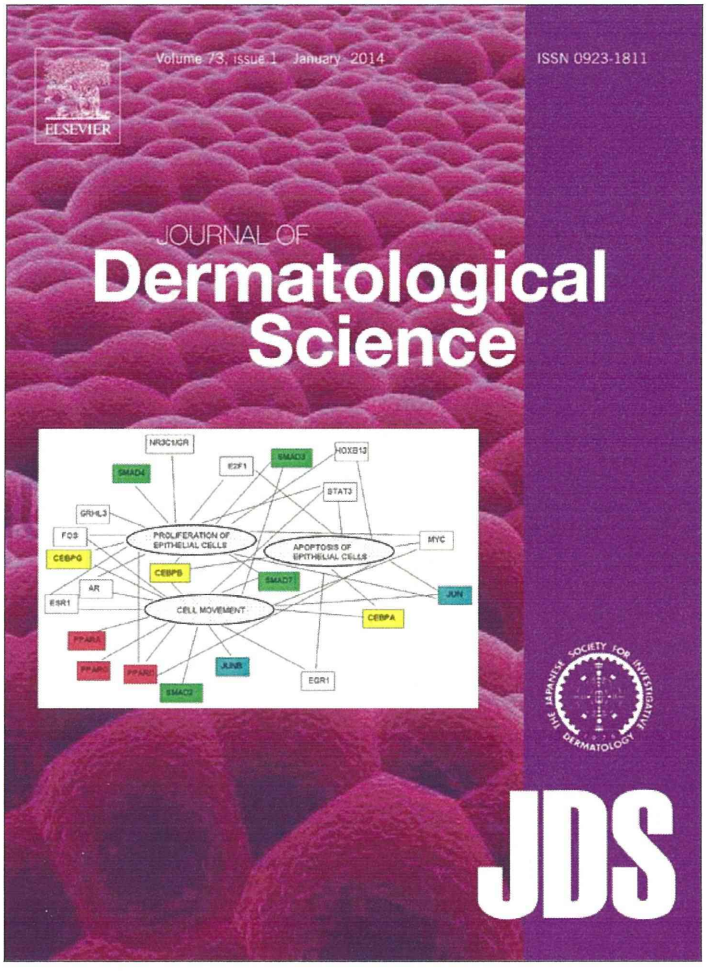

Table 1
Result of immunohistochemical analysis for immune competent cells infiltration. Data represented as mean number of cells and SD. Statistical analysis for comparison was performed by using unpaired *t*-test.

	MelanA	CD8	Foxp3	CD4	CD4+ IL17A+	Epi. CD1a	CD11c	AHR	HLA-DR	CD123	Der. CD1a+ CD207−	CD56
LS ($n=6$)	1.7±3.5**	39.5±22.7*	10.2±3.4	16.5±3.8	15.6±6.3*	33.5±10.0*	3.7±3.2	15.8±11.5	31.1±21.1	11.6±5.5	14.4*	4.5
LE ($n=6$)	8.5±3.6**	19.9±15.4*	9.16±3.4	14.5±4.2	10.3±2.8	22.6±7.1*	17.3±8.3*	12.0±9.2	26.5±15.1	13.2±4.0	10.2	4.5
NL ($n=6$)	19.2±7.8	14.3±10.5	9.1±3.3	10.7±3.3	5.2±1.4	19.2±3.9	5.8±3.7	7.8±2.3	18.1±8.6	10.3±3.3	7.6	1.75
Psoriasis ($n=1$)	41.3	37.7*	18.3	32.3*	28.3**	46.7**	24.7**	13	28	11.3	ND	2
Normal ($n=3$)	17.8±2.2	7.8±2.4	7.9±3.2	12.3±2.1	8.5±1.0	5.9±0.9	5.88±1.1	7.8±3.3	16.3±9.2	7.9±2.5	0	0

ND represent not done.
* *p*-Value < 0.01.
** *p*-Value < 0.05.

TNF-α and IL-6 [8]. Since LCs are commonly activated under IL-1α and TNF-α condition in case of contact hypersensitivity [9], it is conceivable that activated LCs may be an important factor on the occurrence of vitiligo as the interface of melanocyte-specific adoptive immunity cooperating with cytotoxic T cells and may also induce innate immunity in participation with Th17 cells. Following increased infiltration of CD11c+ myeloid dendritic cells and dermal CD1a+ dendritic cells in vitiligo skin can act as antigen trafficking to draining lymph nodes and can produce proinflammatory cytokines such as IL-6 and TNF-α leading to determine helper T cells polarization [10]. Taken together with the effect of Th17 cell-related cytokines on surrounding keratinocyte and fibroblast [6], this positive feedback linage of local cytokines is possibly important for transient appearance of indeterminate dendritic cells and subsequent mature melanocyte disappearance. Given this idea on the underlying immunogenic mechanism, early therapeutic intervention of molecular targeting biologics is considerable for the treatment with progressive nonsegmental vitiligo.

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Letter to the Editor

Increased frequencies of Th17 cells in drug eruptions

T cell-mediated hypersensitivity is the most frequent type of drug eruptions [1] and includes maculopapular eruption (MPE), erythema multiforme (EM), Stevens–Johnson syndrome (SJS), toxic epidermal necrolysis (TEN), and drug-induced hypersensitivity syndrome (DIHS). SJS and TEN, and some of EM are characterized by cytotoxic T (Tc) cell attack toward epidermal keratinocytes with granulysin and Fas–Fas ligand [2], and DIHS is associated with

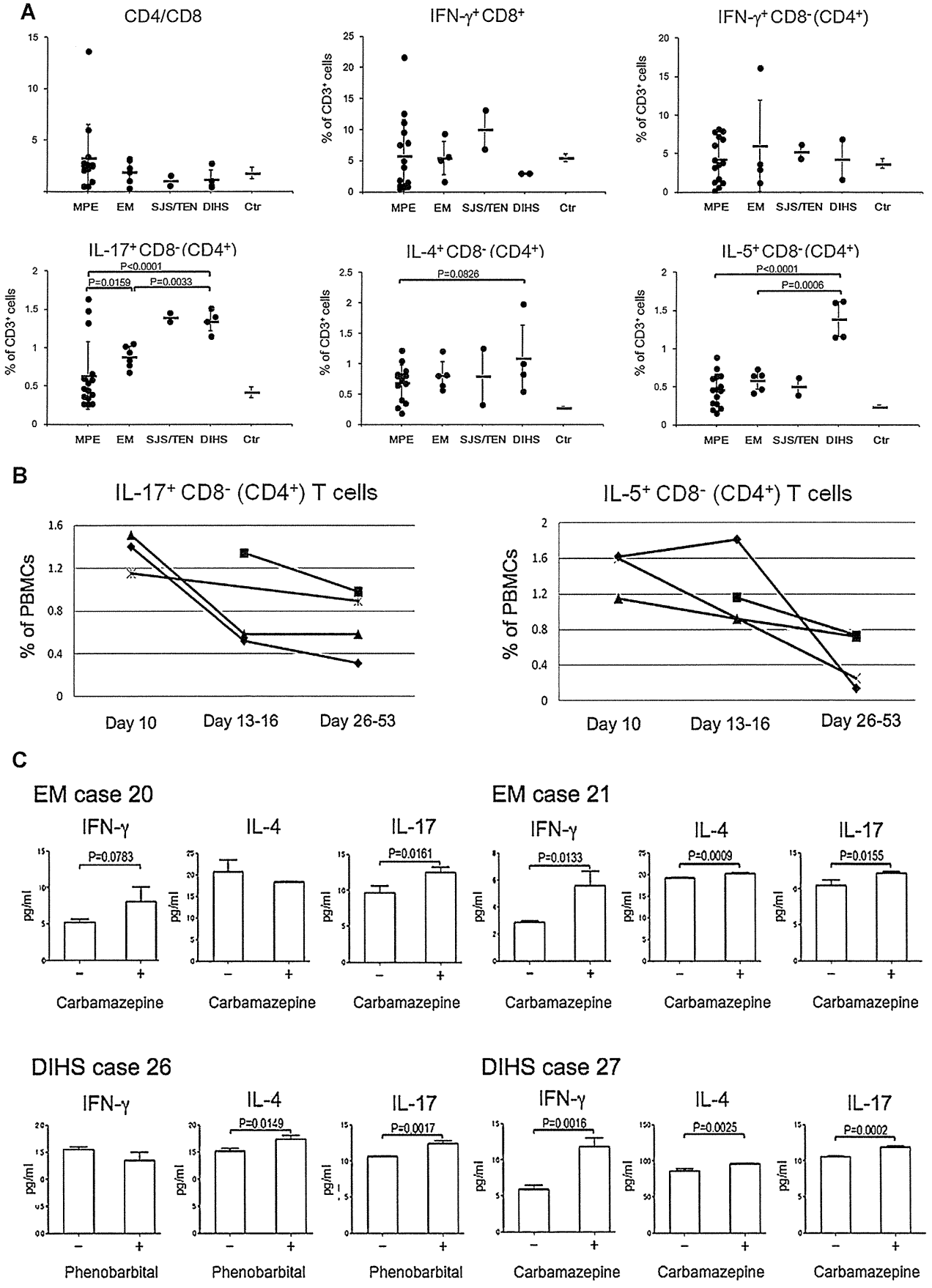

Fig. 1. (A) Percentages of IL-17, IFN-γ, IL-4, or IL-5-producing T cells in the peripheral blood. Blood samples were collected on 2–6 days after onset in EM and SJS/TEN and on 10 days after onset in DIHS and analyzed on a FACSCanto (BD Bioscience, San Diego, CA). In MPE, EM and SJS/TEN, the mean percentages of $CD3^+$, $CD4^+$, and $CD8^+$ cells were within normal ranges ($CD3^+$ cells, 58–84%; $CD4^+$ cells, 25–56%; $CD8^+$ cells, 17–44%). Intracellular cytokines of PBMCs were stained according to the protocol of Cytostain with a few

Table 1
Cytokine conncentrations in the culture supernatants from skin-infiltrating T cells. Cytokine concentration (pg/ml).

Case	Type	Biopsy day	IL-2	IL-4	IL-6	IL-10	TNF-α	IFN-γ	IL-17A	CD4/CD8
28	EM	2	892	14,191	12	32	1495	1565	25	0.10
29	EM	2	4330	3640	9	878	4300	1145	32	0.60
30	EM	7	8	7332	25	519	395	10,115	3497	0.63
31	EM	1	242	5177	92	2988	1587	18,525	41	0.21
32	SJS	7	23	4959	1011	2	1264	5840	15	0.50
33	DIHS	21	20	8175	782	333	465	16,060	3700	6.1
34	DIHS	12	432	3452	264	545	3944	22,770	1659	0.77
35	DIHS	2	8	10,245	436	2684	637	4725	27	0.73
36	DIHS	7	63	7380	338	37	467	317	35	30
37	DIHS	12	19	11,965	89	1035	1423	4210	176	8.7
38	DIHS	24	21	11,130	636	1119	2976	3053	24	15
39	DIHS	6	19,650	19,395	118	3279	12,300	20,115	25	1.4

Biopsy day: the day of biopsy after skin eruption onset. For expansion of T cells, 4-mm skin samples were immersed in complete RPMI supplemented with 20–50 U/ml human recombinant IL-2 and anti-CD3/CD28 mAb-conjugated microbeads (T-cell Expander; Dynal, Copenhagen, Denmark) as previously reported [8]. We obtained $>10^7$ cells/specimen by this method. After phenotyped by flow cytometry, the cells (2×10^5/well) were cultured in an immobilized anti-CD3 mAb-coated 96-well plate for 48 h. The culture supernatants were then harvested to measure the levels of IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α with CBA.

reactivation of human herpesvirus 6 (HHV6) [3]. Upon occurrence of these eruptions, the populations of circulating T cells may be polarized [4]. Interleukin (IL)-17A-producing Th17 cell is a $CD4^+$ T helper subset, and dysregulated Th17 responses mediate psoriasis, allergic diseases, and others [5]. Drug eruption merits Th17 investigation to see the biased relationship between Th17 cells and the other T cell subsets [6] and the host-defensive role of Th17 cells in DIHS. We investigated circulating and skin-infiltrating Th17 cells in drug eruptions.

This study consisted of two types of experiments using different samples: peripheral blood mononuclear cells (PBMCs) and biopsied skin specimens. Totally 39 cases of drug eruption were enrolled in this study (Supplemental Table 1). Cases 1–27 (MPE, 15; EM, 6; SJS, 1; TEN, 1; and DIHS, 4) were seen in University of Occupational and Environmental Health for the PBMC study, and cases 28–39 (EM, 4; SJS, 1; and DIHS, 7) were in Hamamatsu University School of Medicine for the skin-infiltrating T cell study. In 14 patients, the lymphocyte transformation test (LTT) was assessed by ^{3}H-thymidine uptake after 3-day culture with drugs [1], and 9 patients showed positive results. The diagnoses of SJS, TEN [7], and DIHS (seroconversion of anti-HHV6 IgG) [3] were reported. All patients were informed and agreed to participate. The skin-infiltrating T cell analysis was approved by the ethical committee of Hamamatsu University.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2013.08.008.

PBMCs were taken from the patients with MPE, EM, SJS or TEN 2–6 days after the onset of eruption and from those with DIHS2–3 times (on day 10, 13–16, and 26–53), and analyzed by flow cytometry. There was no significant difference in CD4/CD8 ratio between the eruption types (Fig. 1a). However, $CD8^+$ cell percentage in DIHS (43%, day 13–16) was significantly higher ($P = 0.0054$) than that in MPE (18%).

Intracellular cytokines were stained after stimulation of freshly isolated PBMCs with phorbol 12-myristate 13-acetate (PMA) and ionomycin [5]. Since CD4 expression on T cells is downregulated with the stimulants, Th17 cells were expressed as $IL\text{-}17A^+CD3^+$ and $IL\text{-}17A^+CD8^-$ T cells, and the major source of IL-17A, IL-4, and IL-5 was $CD8^-(CD4^+)$ cells [5]. The percentages of $IL\text{-}17A^+CD8^-(CD4^+)$ were dramatically increased in DIHS (10 days after onset) and tended to be high in SJS/TEN (2–6 days after onset) as compared to normal subjects (NS, $n = 9$) and MPE patients (2–6 days after onset) (Fig. 1a). In Tc1 ($IFN\text{-}\gamma^+CD8^+$) and Th1 ($IFN\text{-}\gamma^+CD8^-$) cells, no significant differences were found among the groups. $IL\text{-}5^+$ cells were significantly higher and $IL\text{-}4^+$ cells tended to be higher in DIHS. During the clinical course of DIHS, $IL\text{-}17A^+CD8^-$ cells (normal, 0.4%) and $IL\text{-}5^+CD8^-$ cells (normal, 0.3%) were declined in percentage (Fig. 1b).

To investigate the *in vitro* cytokine production, LTT-positive PBMCs from 2 patients with EM (cases 20 and 21) and 2 patients with DIHS (cases 26 and 27) were cultured with the causative drugs. The drug concentration was determined by the highest LTT proliferation, and the culture supernatants were measured for cytokines. In all four cases, the causative drug significantly stimulated the PBMCs taken 26–53 days after onset to produce IL-17A (Fig. 1c). The produced IL-17A amounts were not high, however, considering the relatively low percentages (0.8–1.3% of PBMCs) of Th17 cells and the inability of IL-17A to stimulate bystander T cells, the stimulation degrees seem to be significant. The productions of IFN-γand IL-4 were also significantly increased in 2 EM and 1 DIHS cases, and 2 EM and 1 DIHS cases, respectively.

The biopsied skin specimens from 4 EM (cases 28–31), 1 SJS (case 32), and 7 DIHS patients (cases 33–39) were recruited for the production of cytokines by skin-infiltrating T cells. T cells were expanded with IL-2 and anti-CD3/CD28 mAb-conjugated microbeads [8] and further cultured in an immobilized anti-CD3 mAb-coated 96-well plate (2×10^5/well) for 48 h. IL-17A variously produced in these cases (Table 1). In the 7 patients with DIHS, however, the IL-17A levels appeared to fluctuate in parallel with the clinical courses. The timing of skin biopsy after eruption onset and the IL-17 values are as follows: day 2, 27 pg/ml (case 35); day

modifications. Briefly, freshly isolated PBMCs (2×10^6 cells/ml) were incubated in complete RPMI in a 24-well plate with 10 ng/ml of PMA (Sigma Chemical Co.), 10^{-6} M of ionomycin (Wako, Osaka, Japan) and 0.7 μl of Golgistop (BD Biosciences) for 8 h [5]. The cells were washed and directly stained with PerCP-conjugated anti-CD8 mAb and subsequently with APC-conjugated anti-CD3 mAb. After washing, 100 μl of Cytofix/Cytoperm buffer was added to each well and incubated for 20 min at room temperature, and washed with Perm/Wash solution as manufacture's protocol. They were stained with PE-labeled anti-IL-17, IL-4, or IL-5 and FITC-labeled anti-IFN-γ mAb. Fluorescence profiles were analyzed by flow cytometry in FACSCanto. The $IL\text{-}17^+$, $IL\text{-}4^+$, and $IL\text{-}5^+$cell population was found exclusively in $CD3^+CD8^-$ T cells. Vertical bars represent the mean ± SD.Ctr: healthy control. (B) Changes in the percentages of IL-17-or IL-5-producting cells in patients with DIHS. PBMCs were taken from the patients at the indicated time points, stimulated with PMA and ionomycin, and intracytoplasmically stained with antibodies to IL-17 and IL-5. (C) Cytokine production by PBMCs from patients with EM or DIHS in response to causative drugs. PBMCs were taken from patients 26–53 days after onset. Patients' PBMCs were cultured in triplicate with a causative drug in complete RPMI in 24-well plates (2×10^6 cells/1 ml/well). The final concentration of drugs was determined on the basis of known C_{max} of each drug. In LTT, the drugs were added to the culture at 1/10, 1, and 10 times of C_{max}. For cytokine production, PBMCs were cultured with the causative drug at the concentration that yielded the highest proliferation, and the culture supernatants were subjected to cytokine measurement. Three-day culture supernatants were measured for IFN-γ, IL-4, and IL-17 with the Human Th1/Th2/Th17 Cytokine Beads Array kit (CBA, BD). Vertical bars represent the mean ± SD. Wilcoxon test was employed to evaluate statistical significance between the means. $P < 0.05$ was considered significant.