

T-Cell Clonotypes Specific for *Dermatophagoides pteronyssinus* in the Skin Lesions of Patients With Atopic Dermatitis

Hideto Takahama, Kayo Masuko-hongo, Atsushi Tanaka,
Yoko Kawa, Nobuo Ohta, Kazuhiko Yamamoto,
Masako Mizoguchi, Kusuki Nishioka, and Tomohiro Kato

ABSTRACT: T-cell mediated immune response, toward the house dust mite (HDM) antigens in particular, has been reported to be involved in the pathogenesis of atopic dermatitis (AD). On the other hand, studies on the infiltrating lymphocytes in the skin lesion of AD revealed oligoclonal T-cell accumulation. However, it is not clear exactly what antigen(s) the accumulating T cells are exactly recognize *in situ*. Therefore, this study attempted to determine whether or not the clonally expanded T-cell clones in the diseased skin recognize HDM. Specifically, peripheral blood mononuclear cells (PBMC) obtained from six patients with AD, who revealed high titers of anti-HDM IgE, were stimulated with HDM antigens purified from *Dermatophagoides pteronyssinus* (Dp). T-cell clonotypes expanded by the stimulation were then identified by the analysis of their T-cell receptor (TCR) B-gene sequences using a combination of the reverse transcription–polymerase chain reaction and subsequent single strand conforma-

tion polymorphism separation. The Dp-responding T-cell clonotypes were compared with those that accumulated in the AD skin lesion *in vivo*. Nucleotide sequences of the TCR were also determined. As a result, the Dp stimulation induced oligoclonal T-cell expansion from the originally heterogeneous peripheral T-cell population of AD patients. However, only a small part of the Dp-reacting T-cell clonotypes detected in PBMC was identical to those accumulated in the AD skin lesion *in vivo*, and vice versa. This indicates that the frequency of the clonal expansion of Dp-specific T-cell clonotypes in the skin lesion of AD would be rather limited. *Human Immunology* 63, 558–566 (2002). © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: T-cell receptor; atopic dermatitis; subsequent single strand conformation polymorphism; house dust mite; human

ABBREVIATIONS

AD	atopic dermatitis
Dp	<i>Dermatophagoides pteronyssinus</i>
HDM	house dust mite
SSCP	subsequent single strand conformation polymorphism

PPD	purified protein derivative
PBMC	peripheral blood mononuclear cell
RT-PCR	reverse transcription–polymerase chain reaction
TCR	T-cell receptor

INTRODUCTION

Atopic dermatitis (AD), one of the central manifestations of atopic syndrome, is a relapsing inflammatory skin

disease [1]. Patients with AD are immunologically hypersensitive to various environmental antigens such as food or inhaled allergens. In fact, they display hypersensitivity to intracutaneously injected allergens (skin test) and high serum levels of allergen-specific IgE [2]. Among such candidate antigens, house dust mites (HDM) including *Dermatophagoides Pteronyssinus*, have been highlighted as a major immune response-driving factor in AD [2–6]. Supporting this view, HDM-specific T-cell clones were established from peripheral blood mononuclear cells (PBMC) and also from skin lesions of patients with AD [3, 4, 7, 8].

Previous studies revealed that the skin lesions of AD

From the Departments of Rheumatology (H.T., K.M.-H., A.T., K.Y., K.N., T.K.), Immunology and Genetics Program, Institute of Medical Science, and Dermatology (H.T., Y.K., M.M.), St. Marianna University School of Medicine, Kanagawa; and the Department of Zoology (N.O.), Nagoya City University, Nagoya; and the Division of Allergy and Rheumatology (K.Y.), University of Tokyo, Graduate School of Medicine, Tokyo, Japan.

Address reprint requests to: Dr. Hideto Takahama, Department of Dermatology and Rheumatology, Immunology and Genetics Program, Institute of Medical Science, St. Marianna University, 2-16-1 Sugao, Miyamae-ku, Kawasaki-shi, Kanagawa, 216-8512, Japan; Tel: +81 (44) 977-8111, ext. 3237; Fax: +81 (44) 977-3540; E-mail: bifu@marianna-u.ac.jp.

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were infiltrated mainly by activated T cells and monocytes/macrophages [9]. The infiltrating T cells were reported to secrete an array of cytokines that were different from those secreted from T cells of healthy donors [3, 4]. Thus, the skin-infiltrating T cells are suggested to be actively involved in the pathologic immune responses occurring in the skin. In this regard, we reported the presence of oligoclonal accumulation of T cells in the skin lesions of patients with AD [10], which was in good contrast with the high heterogeneity of T-cell population in the periphery. Because T cells are known to proliferate clonally after antigenic stimulation, the T-cell clonality detected in the AD skin is indirect evidence for the occurrence of an antigen-driven immune response in the skin lesion. Nevertheless, it has still not been directly evidenced which antigen(s) actually causes the T-cell clonal accumulation in the AD skin.

Because there is no established method to determine target antigens of each T-cell clonotype with unknown specificity, we focused on HDM and sought to determine whether the HDM accounts for the clonal accumulation of T cells in AD skin, even if in part. To explore this issue, we detected HDM-responding T-cell clonotypes from PBMC of patients with AD, and compared their TCR BV genes to those of the clones expanded in the *in vivo* skin lesions of the same patients. Our results revealed that only a small part of the Dp-reacting T-cell clonotypes detected in PBMC was identical to those accumulated in the AD skin lesion *in vivo*, and *vice versa*.

MATERIALS AND METHODS

Patients and Samples

Six patients, diagnosed to have AD using the criteria of Hanifin and Rajka [1], were enrolled in this study. Clinical features of these patients are summarized in Table 1. Severity of AD was assessed with the SCORAD index [11]. None of the patients had been treated by corticosteroids or other drugs either locally or systemically before the diagnosis. Skin samples (5-mm long) containing dermis and epidermis were biopsied from inflamed lesions of each AD patient under local anesthesia. Each sample was stored frozen in liquid nitrogen until use. All samples were obtained with informed consent. The protocol of the study was approved by our institutional ethical committee.

Antigens and Cell Culture

The crude HDM antigen was purified from *Dermatophagoides pteronyssinus* (Dp) as described previously [12–14]. This crude HDM antigen has been reported to be effective for establishing T-cell line [12–14]. This purified protein derivative (PPD) was purchased from Nihon BCG Seizo Co. (Tokyo, Japan). For stimulation of PBMC

TABLE 1 Clinical features of AD patients

Patient number	Age/sex	Family history of AD	Past history	Total IgE (k U/L)	Specific anti-dust mite IgE (UA/ml)		Other specific anti-allergen IgE (UA/ml)	Sampling site of skin	Condition of sampling site	SCORAD index
					<i>D. pteronyssinus</i>	<i>D. farina</i>				
1	23/M	sibling	asthma	1800	>100	83.44	HD1:87.19, HD2>100, CP:27.36	back	erythema	84
2	23/M	none	asthma	22,000	>100	90.89	HD1:98.6, HD2>100, CP:45.87	back	erythema	89
3	25/M	none	—	14,000	>100	>100	HD1>100, HD2>100, CP:26.27 dog epidermis:0.81, wheat:1.76	forearm	erythema	89
4	26/M	none	asthma	2000	>100	>100	HD1:87.19, HD2>100, CP:27.36 rice:2.01, wheat:2.04, soy beans:2.04	forearm	erythema	91
5	23/M	none	asthma	2200	>100	>100	HD1>100, HD2>100	jaw	lichenification	40
6	18/F	none	asthma	5800	>100	>100	HD>100, HD2>100, CP:25.38 milk:0.72	nape	lichenification	38

Abbreviations: AD = atopic dermatitis; CP = cedar pollen; HD = house dust; SCORAD = severity scoring of atopic dermatitis.

with the antigens, PBMC were separated from heparinized peripheral blood of the patients with AD by the standard Ficoll gradient centrifugation. The cells were then suspended at a concentration of 1×10^7 cells/ml in RPMI-1640 medium supplemented with 10% (v/v) pooled, heat-inactivated human serum, 100-mg/ml streptomycin, 100-U/ml penicillin, and 20-mM L-glutamine. The cells were incubated with or without 10 μ g/ml of the HDM protein or 25 μ g/ml of PPD in a 24-well culture plate (Becton Dickinson, Lincoln Park, NJ, USA) for 7 days. Then irradiated autologous PBMC (at 3000 rad) and 20-U/ml human recombinant IL-2 (Genzyme, Cambridge, MA, USA) were added to the culture together with the respective antigens (5 μ g/ml of HDM or PPD). The culture medium was changed on day 10. On day 14, the cultured cells were harvested and immediately immersed in denaturing guanidium solution for RNA isolation.

Analysis of Serum Total IgE Level and Serum Anti-Antigen Specific IgE Level

The serum total IgE level and anti-antigen specific IgE levels from each AD patient were measured using a capsulated hydrophobic carrier polymer system IgE fluorescent allergosorbent test [15].

RNA Isolation, cDNA Synthesis, and Analysis of TCR Clonotypes by SSCP

The samples, immersed in denaturing solution-D (4.0-M guanidine thiocyanate, 25-mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1-M 2-mercaptoethanol), were homogenized with a ultra-dispenser. Total RNA were isolated by the guanidium-phenol-chloroform method as described previously [16].

The clonality of the TCR B-chain messages was examined using the reverse transcription-polymerase chain reaction (RT-PCR) and subsequent single strand conformation polymorphism (SSCP) method as described previously [10, 17–21]. Briefly, the isolated RNA was converted to cDNA using RT (SuperscriptII; GibcoBRL, Gaithersburg, MD, USA) and random hexamer oligonucleotides (Gibco) at 42°C for 60 minutes. In the next step, 0.1- μ g cDNA was mixed with each primer set, *i.e.*, one of the BV1 ~ BV20 primers and a common BC primer of TCR B chain [22], and with 200-nM dNTP and 0.5-U Taq DNA polymerase (Promega Co., Madison, WI, USA). PCR was then performed for 35 cycles of 94°C for 1.5 minutes, 55°C for 2 minutes, 72°C for 23 minutes, and followed by extension at 72°C for 5 minutes. The amplified DNA was detected by 1.3% agarose gel electrophoresis.

For detection of accumulated TCR BV genes, the PCR products were denatured by incubation at 90°C for 2 minutes in a solution of 95% formamide, 10-mM

EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The samples were then electrophoresed on a non-denaturing 4% polyacrylamide gel containing 10% glycerol. The separated DNA was then transferred onto GeneScreen membranes (Millipore Intertech, Bedford, MA, USA) and hybridized overnight with a biotinylated internal BC probe as described previously [18]. The transferred DNA was finally visualized by a subsequent reaction with streptavidin, biotinylated alkaline phosphatase, and a chemiluminescent substrate (Phototope Detection Kit; New England Biolabs, Beverly, MA, USA). The numbers of detected band(s) on x-ray films were counted independently by at least three persons.

DNA Sequencing of the CDR3 Region of TCR

To determine the nucleotide sequence of the accumulated TCR BV gene transcripts, small pieces of the SSCP gel, which corresponded to the distinct TCR bands, were cut out and DNA was extracted into TE.1 (10-mM Tris-HCl/0.1-mM) by repeated boiling and freezing. TCR genes were amplified again by PCR using the BV primer with an EcoRI recognizing sequence (New England BioLabs, Beverly, MA, USA) and a common BC primer with a HindIII recognizing sequence (New England BioLabs, Beverly, MA, USA). The re-amplified TCR BV gene transcripts were subcloned into pBluescript II (TOYOBO, Osaka, Japan) and subjected to di-deoxy DNA sequencing (373A DNA Sequencing System; Perkin Elmer/Applied Biosystems, Foster, CA, USA). In a separate experiment, the first PCR products were subcloned into the plasmid without the SSCP separation and subjected to di-deoxy DNA sequencing, to directly determine the frequency of expanding T-cell clones in a BV family.

RESULTS

Stimulation of PBMC With the Dp Antigen Induced Distinct T-Cell Clonal Expansions

We previously analyzed the TCR BV gene segment usage of peripheral T cells in AD patients using the RT-PCR SSCP method, and demonstrated that the T cells were extremely diverse, as were those in PBMC of healthy donors [10]. This finding indicated that the frequency of T cells with TCR specific for AD-related antigens would be very low even if they exist. We first confirmed the phenomenon in PBMC of the six AD patients enrolled in this study. Specifically, TCR B genes were amplified by RT-PCR, and then separated by their SSCP on non-denatured gels. As illustrated in representative results from patients 1, 2, and 3 in Figure 1, the TCR BV genes were detected as smearlike broad bands in almost all of the tested BV families, indicating an extremely heterogeneous population (Figure 1, PBMC).

We then tried to expand Dp-specific T-cell clones by

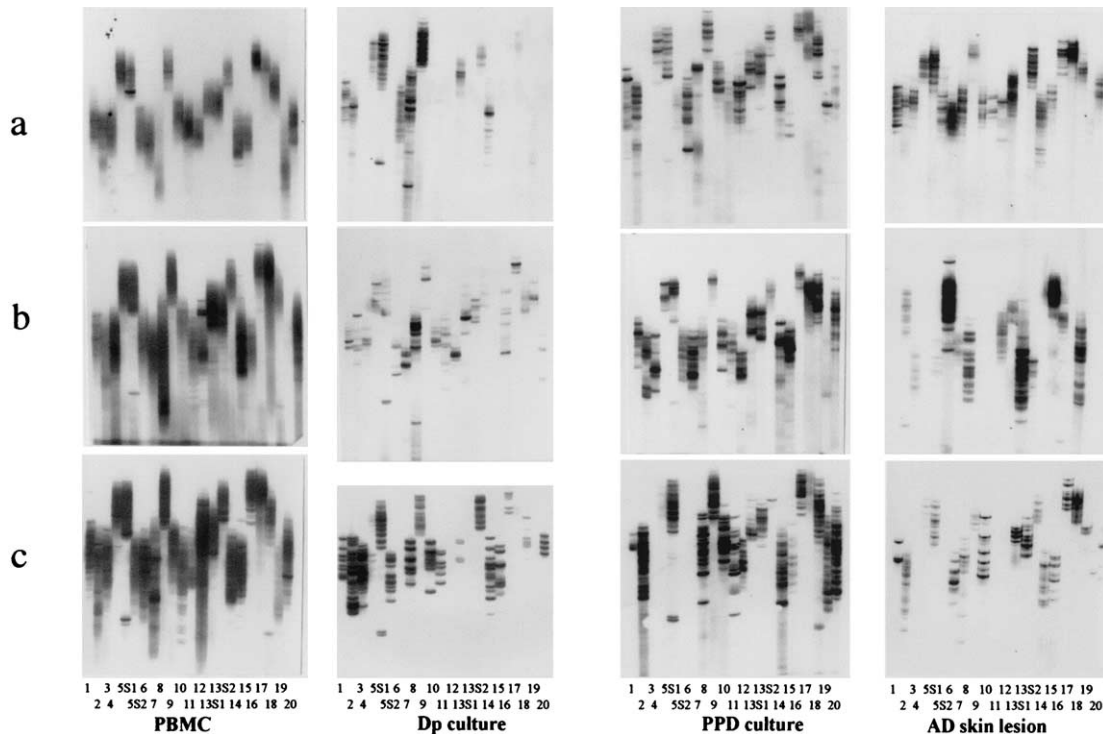


FIGURE 1 Clonotypic analysis of peripheral T cells, cultured T cells with *Dermatophagoides pteronyssinus* (Dp) or purified protein derivative (PPD), and the skin-infiltrating T cells from three patients with atopic dermatitis (AD). Peripheral blood mononuclear cells (PBMC) were analyzed for the T-cell clonality by reverse transcription–polymerase chain reaction (RT-PCR) subsequent single strand conformation polymorphism (SSCP); representative results illustrate PBMC. PBMC was cultured with the DP antigen or PPD and skin-infiltrating T cells also analyzed for the T-cell clonality by RT-PCR SSCP (representative results depict Dp culture, PPD culture, and AD skin lesion). Representative results of TCR BV1 to BV20 family as follows: (a) patient 1; (b) patient 2; (c) patient 3. RT-PCR SSCP analysis characterized smear pattern for most VB genes from T cells of PBMC from AD patients. RT-PCR SSCP analysis demonstrated several distinct bands for most VB genes from T cells of PBMC from three AD patients.

incubating the heterogeneous T cells with the Dp-antigen *in vitro*. PPD was used for a control. On day 14, T-cell proliferation against Dp or PPD was confirmed by incorporation of ^3H -thymidine (data not shown).

On the other hand, no or few cells remained alive microscopically in the culture without the antigens. In RT-PCR analysis, it was confirmed that the Dp-stimulated or PPD-stimulated samples, but not the cells incubated without antigens, produced PCR products for the TCR B genes (representative results of BV1, 2, and 3 are illustrated in Figure 2). This indicated the TCR BV gene of T cells that respond to Dp or PPD was amplified.

The amplified TCR B genes from the Dp- and PPD-stimulated samples were then separated by their SSCP. In contrast with the result from fresh PBMC (Figure 1, PBMC), results from Dp- and PPD-stimulated PBMC samples exhibited dozens of distinct TCR gene bands on the SSCP films, indicating oligoclonal expansions of T cells from the originally heterogeneous populations (Figure 1, Dp culture and PPD culture). Both Dp- and PPD-stimulation produced similar levels of oligoclonal

expansion of T cells (Figure 1, Dp culture and PPD culture). These newly accumulating T-cell clones were observed in almost all of the BV families studied, indicating that the clonal response was not restricted to the T cells with a particular TCR BV gene segment repertoire. Importantly, the expanding TCR clonotypes were completely different from each other between Dp and PPD stimulation (Figure 3). This also indicates that these clonal expansions of T cells are specific for the respective stimulating antigens.

Taken together, we found that Dp-responding T cells existed in PBMC, however, their frequency would be lower than that can be detected as expanded clonotypes by RT-PCR SSCP if not stimulated *in vitro*. The method used here was of use to detect such antigen-responding T-cell clonotypes *in vitro*.

Comparison of the Dp-Reactive and the Skin-Infiltrating T-Cell Clones

Next we questioned whether the T-cell clonotypes that proliferated in response to the Dp antigen were identical

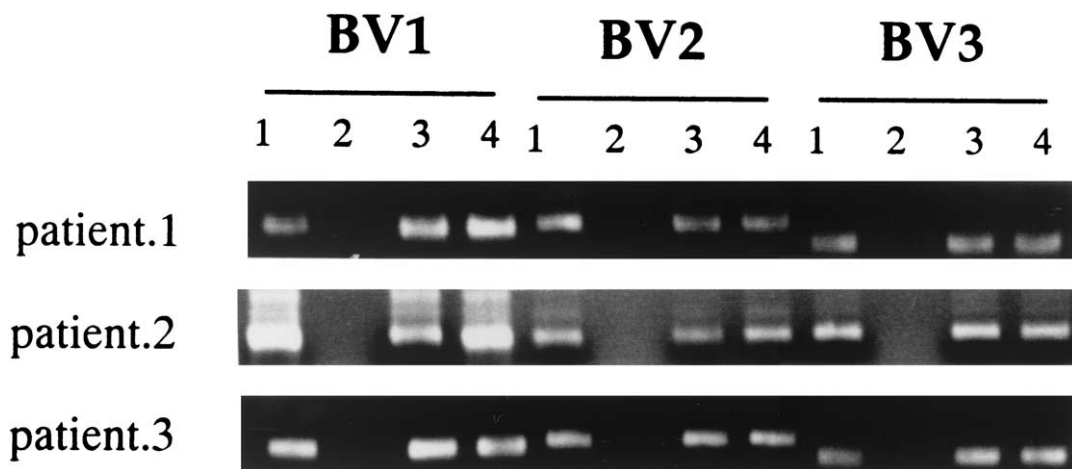
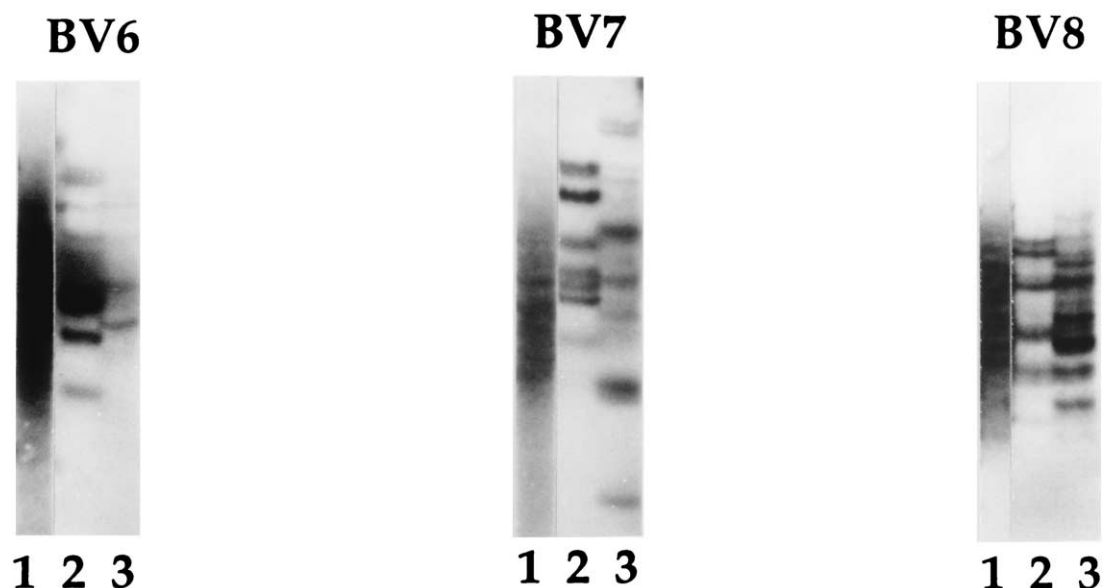


FIGURE 2 Agarose gel electrophoresis analysis of the polymerase chain reaction (PCR) amplified T-cell receptor (TCR) B genes of peripheral blood mononuclear cells (PBMC) with or without antigen stimulation in atopic dermatitis (AD) patients. PBMC were cultured with the *Dermatophagoides pteronyssinus* (Dp) antigen or purified protein derivative (PPD) for 14 days. Then cDNA was prepared, from which TCR B genes were amplified by PCR. Finally, the PCR products were separated by 1.3% agarose gel electrophoresis using with ethidium bromide staining. Representative results of TCR BV1 to BV3 family (patients 1, 2, and 3) are illustrated as follows: (1) preculture PBMC; (2) PBMC stimulated with rIL-2 only; (3) PBMC stimulated with Dp antigen and rIL-2; (4) PBMC stimulated with PPD and rIL-2). PCR products except PBMC stimulated with rIL-2 alone revealed distinct bands.

to the *in vivo* skin-infiltrating clonotypes in the same patients with AD. To this end, we amplified TCR B genes of the T cells in the AD skin by RT-PCR and analyzed them by the SSCP separation. As a result, being

consistent with our previous observation, all six patients tested characterized oligoclonal T-cell expansion in their skin lesions (data not shown). Thus we compared the T-cell clones expanding in the skin to the Dp-responding

FIGURE 3 Clonotypic analysis of preculture T cells and T cells cultured T cells with *Dermatophagoides pteronyssinus* (Dp) or purified protein derivative (PPD) from an atopic dermatitis (AD) patient. Peripheral blood mononuclear cells (PBMC) were cultured with the DP antigen or PPD and analyzed for the T-cell clonality by reverse transcription–polymerase chain reaction (RT-PCR) subsequent single strand conformation polymorphism (SSCP). Representative results of T-cell receptor (TCR) BV6 to BV8 family (patient 2) are illustrated as follows: (1) preculture PBMC; (2) PBMC stimulated with Dp antigen; (3) PBMC stimulated with PPD. PBMC cultured with the DP antigen or PPD revealed several distinct bands, whereas the precultured T cells revealed smear patterns.



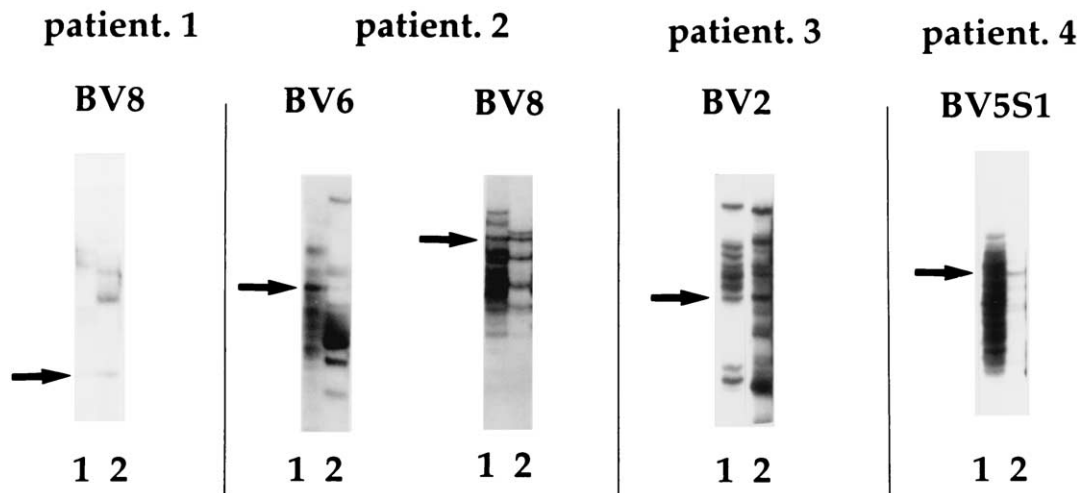


FIGURE 4 Comparison of *Dermatophagoides pteronyssinus* (Dp) induced and skin-infiltrating T-cell clones by reverse transcription-polymerase chain reaction (RT-PCR) subsequent single strand conformation polymorphism (SSCP). The skin biopsy samples were analyzed by RT-PCR SSCP and a specimen taken from each atopic dermatitis (AD) patient was compared with the Dp-responding T-cell clones induced by *in vitro* culture of their PBMC with the antigen. Lanes 1 and 2 characterize skin biopsy and Dp-stimulated PBMC samples, respectively. Arrows indicate identical T-cell clones found commonly between the samples (*i.e.*, between lane 1 and 2).

clonotypes detected from PBMC of the same patients by the *in vitro* stimulation. Specifically, the PCR products both from *in vivo* samples (*i.e.*, from skin) and the *in vitro* stimulated samples (*i.e.*, the T cells stimulated with Dp or PPD *in vitro*) were electrophoresed on one SSCP gel, because our previous experiments demonstrated that the TCR genes migrating to an identical position of the same SSCP gel were basically identical [19, 20].

As a result, it was revealed that a small part of the T-cell clones induced by the Dp-stimulation from PBMC were identical to those infiltrating the skin lesion (Figure 4, bands indicated by arrows). Although the skin samples from all of the six AD patients studies revealed clonal accumulation of T cells (Figure 1, AD skin lesion), we

could detect such identical clones only in four of six patients who exhibited severe disease activities (SCORAD index: 88 ± 3) (*i.e.*, in patients 1 to 4) [11]. Two patient who did not manifest any identical clone, exhibited mild or moderate disease activities (SCORAD index: 39 ± 1) and the samples were taken from chronic lichenified lesions [11]. To confirm the identity of two bands detected by SSCP, nucleotide sequences of the two TCR B gene clonotypes were determined by DNA sequencing in patient 2. Patient 2 revealed two pairs of identical clones. As shown in Table 2, identical sequences were obtained for each of the two pairs of clones indicated by arrows in Figure 4 (patient 2). Thus, the two expanded T-cell clonotypes were found to be identical to those which

TABLE 2 Nucleotide and amino acid sequences of the identical clones

Sample	TCR BV	nDN region	TCR BJ
skin	<u>GCC AGC AGC TTA</u> A S S L	<u>GCT GGA CAG CCT</u> A G Q P	<u>TAC AAT GAG CAG</u> Y N E Q
cultured PBMC	<u>GCC AGC AGC TTA</u> A S S L	<u>GCT GGA CAG CCT</u> A G Q P	<u>TAC AAT GAG CAG</u> Y N E Q
skin	<u>TGT GCC AGC AGT</u> C A S S	<u>CCC CAC CTA TTA GCG GGA GGG TTT</u> P H L L A G G F	<u>ATT CAG TAC TTC</u> I Q Y F
cultured PBMC	<u>TGT GCC AGC AGT</u> C A S S	<u>CCC CAC CTA TTA GCG GGA GGG TTT</u> P H L L A G G F	<u>ATT CAG TAC TTC</u> I Q Y F

Underline represents the sequence in patient 2.

Abbreviations: PBMC = peripheral blood mononuclear cells; TCR = T-cell receptor.

proliferated against the Dp stimulation *in vitro* at the TCR nucleotide sequence level in addition to SSCP separation. Further, these two pairs had a LAG sequence in CDR3 region in common, even though they belonged to different BV families.

We counted the number of visible band(s) on the SSCP x-ray films as individual T-cell clone(s), and calculated the ratio of T-cell clone(s) that were induced by the Dp-stimulation from PBMC and identical with the AD skin lesion to the skin-infiltrating T-cell clone(s) in AD *in vivo*. Patient 1 presented 0.28%, patient 2 0.83%, patient 3 0.28%, and patient 4 0.88%. As illustrated in Figure 1, the stimulation by the Dp antigen produced 124 to 212 clones from PBMC of AD patients. Thus, approximately 0.47% to 1.2% of the potential Dp-responding T cells were supposed to be identical to those in the skin. On the other hand, only 0.28% to 0.88% of the clones accumulating in the skin were found to be identical to the clones that were expanded from the peripheral population by the Dp stimulation *in vitro*. Because the number of accumulated T-cell clonotypes *in vivo* was 124.5 ± 64.8 , the evidenced Dp-responding clones accounted for only 0.5% to 1.2%.

DISCUSSION

Our findings here were as follows: Dp-reacting T cells were detected in PBMC of the patients with AD in most of the TCR BV families tested. In spite of this, only a small part of the accumulated T cells in the AD skin lesions was identical to the Dp-recognizing T cells detected in PBMC as above, and *vice versa*. On the first point, the peripheral T-cell populations in patients with AD without the Dp stimulation are highly heterogeneous, similar to those of healthy donors. However, once stimulated with Dp antigens, oligoclonal expansion of T cells was observed, the degree of which was similar to that observed by the PPD stimulation. This indicates that the patients would be deeply sensitized by the Dp antigens. Because the clonal accumulation of T cells was detected in almost all of the tested BV families in the Dp-stimulated PBMC samples, T cells in various BV subfamilies would be involved in the immune reaction of AD. This is different from multiple sclerosis and the experimental models of allergic encephalitis, in which pathogenic T cells were reported to be restricted to a couple of BV families [22–24].

On the second point, oligoclonal expansion of T cells was observed in almost all the tested BV families in the AD skin lesion, however, only four bands (<1% on average) were found to be identical to those of the Dp-reacting T cells detected in PBMC. Based on this observation, the frequency of the clonally expanded T-cell clones specific for the Dp antigen was roughly esti-

mated to be from 0.5% to 1.2% of the total number of clonally expanded T-cell clonotypes detected in the AD skin lesions. This ratio is compatible with the reported frequency of Ag-specific T cells (0.1%–2.2%) in affected sites of rheumatoid arthritis, tuberculous leprosy, leishmaniasis, and multiple sclerosis [25–29]. In the case of AD, Werfel *et al.* [8] reported a similar proportion of Dp-specific T cells in AD skin lesions, 0.72% to 0.02%. Combining their result and the present clonotype analysis, clonal expansion of only one or two Dp-specific T-cell clonotypes would be sufficient to account for the low percentage. Such a small number T-cell clonotypes may be enough to maintain inflammation of the skin lesion. In this meaning, HMD antigens are considered to play a causative role in AD. If so, these T-cell clonotypes would be a good therapeutic target for AD. Further, Dp-recognizing T-cell clonotypes in the skin lesion have an amino-acid motif in the CDR3 region. They may recognize the same epitope of the Dp.

Stimulation of PBMC with the Dp antigen produced clonal expansion of dozens of T cell clonotypes. However, only a small part of them was detected in the skin lesions as clonally expanded T cells. This indicates that some of the potentially Dp-reacting T cells were selected to expand clonally in the skin lesion by thus far unknown reasons. Migrating potential can vary between individual T-cell clonotypes. Alternatively, processing and presentation of the Dp antigens in the skin lesion can be different from that in *in vitro* stimulation. The T-cell clonotypes selected to expand in the skin lesion from the potential Dp-reacting T cells may be involved in the actual inflammation of the skin lesion. Although the skin samples from all of the six AD patients studies revealed clonal accumulation of T cells (Figure 1, AD skin lesion), we could detect such identical clones only in four of six patients who exhibited severe disease activities and were sampled from acute erythematous lesions (*i.e.*, patients 1, 2, 3, and 4 [Table 1]). Two patients who did not manifest any identical clones, exhibited mild or moderate disease activities and their sampled sites characterized chronic lichenified lesions (*i.e.*, patients 4 and 5 [Table 1]). Therefore, it seems that the oligoclonal proliferation of T cells by antigen stimulation makes AD lesions more severe, such as the acute erythematous lesion illustrated in Table 1. The more dense T-cell infiltrations were found in those acute erythematous AD lesions [10]. Such acute, erythematous AD skin lesions may be exposed to antigens, such as HDM before sampling.

It remains to be clarified what the majority of the skin accumulating T cells recognize. As a possible explanation, perhaps the T cells that recognize the Dp antigens and accumulate in the skin lesion trigger the inflammation of the skin lesion, and then peripheral T cells that

are already sensitized by other antigens infiltrate into the skin lesion. Alternatively, after the early inflammation caused by the Dp antigens, other exogenous antigens may sensitize T cells *in situ*. Such exogenous antigens might easily infiltrate into the skin with impaired barrier function due to in epidermal lipid abnormalities [30, 31]. This scenario may be supported by the fact that the impaired skin barrier is one of the major exacerbatory factors of AD.

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