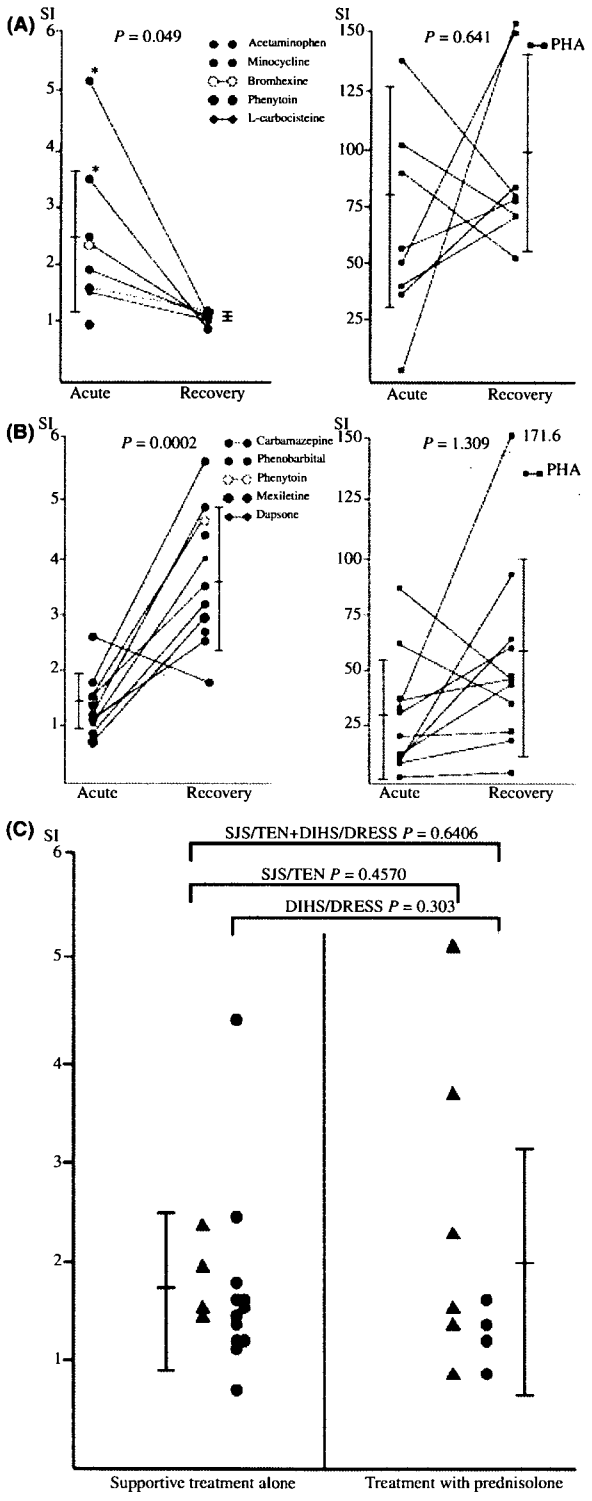

Figure 1. Alterations of SI in LTT and PHA-induced lymphocyte proliferative responses at the acute stage and recovery stage in SJS/TEN (A) and in DIHS/DRESS (B). Comparison of SI in LTT between patients treated with prednisolone and those with supportive therapy alone (C); closed triangles indicate SJS/TEN; closed circles indicate DIHS/DRESS; the mean of SI in SJS/TEN is 2.488 ± 1.561 (patients with supportive therapy alone) *vs* 1.855 ± 0.374 (those treated with prednisolone), $P = 0.457$; that in DIHS/DRESS is 1.228 ± 0.244 *vs* 1.758 ± 0.962, $P = 0.303$, respectively. SJS/TEN, Stevens–Johnson syndrome/toxic epidermal necrolysis; *TEN; DIHS/DRESS, drug-induced hypersensitivity syndrome/drug rash and eosinophilia with systemic symptoms; SI, stimulation index; LTT, lymphocyte transformation test; PHA, phytohemagglutinin.

average, treatment with systemic prednisolone was begun 5 days after the onset of symptoms, with a range of 2–14 days; and the initial dose of prednisolone used was 0.8–1 mg/kg daily. Prednisolone was then tapered gradually after full control was achieved. In an attempt to assess the impact of systemic prednisolone on the LTT, patients whose samples were initially obtained within 4 weeks after onset were retrospectively divided into two groups depending on the use of systemic prednisolone at the time of sample collection. We initially analyzed patients with SJS/TEN and DIHS/DRESS together. As shown in Fig.1C, when comparing the SI values in those patients who received systemic prednisolone and those who did not, no statistically significant differences were noted (SI: 1.782 ± 0.842 *vs* 1.984 ± 1.341, $P = 0.6406$). Similar results were obtained when patients with SJS/TEN and DIHS/DRESS were individually analyzed.

Discussion

Our results conclusively demonstrate the importance of the timing of the LTT performed during the course of the disease, depending on the type of drug reactions. In patients with DIHS/DRESS, false negative LTT reactions were constantly observed when examined at the acute stage, usually within 2–3 weeks after onset, regardless of whether the patients are on therapy with systemic prednisolone. Positive LTT reactions can be observed when tests were performed after remission, usually 5–8 weeks after onset. In contrast, totally opposite results were observed in patients with SJS/TEN. Our results clearly indicate that a dramatic increase in LTT reactions dependent on a function of the time-point in its course is specific to patients with DIHS/DRESS. If in patients with DIHS/DRESS the LTT is performed at the right timing as demonstrated here, the diagnostic sensitivity and specificity of this test would dramatically improve.

There are two possible explanations for why expansion of drug-specific T cells is undetectable at the acute stage but detectable at the recovery stage in patients with DIHS/DRESS. One possibility is that during the acute stage, drug-specific T cells would be strongly activated *in vivo*;

therefore, these drug-specific T cells obtained from the peripheral blood could have no longer reacted to the drug given *in vitro* (1). However, this possibility is unlikely, because similar activation of drug-specific T cells would also occur in SJS/TEN. Another possibility is that T cells that play a suppressive role in the proliferation of T cells might expand excessively in patients with DIHS/DRESS. In this regard, we have recently examined the frequencies of CD4+ CD25+ Foxp3+ regulatory T cells between the two stages in patients with MP, SJS/TEN, and DIHS/DRESS (R. Takahashi and T. Shiohara, manuscript in preparation). Our preliminary result demonstrated that the regulatory T cells dramatically increased in frequency in the acute stage and have returned to normal upon recovery in patients with DIHS/DRESS: this change was specifically observed in patients with DIHS/DRESS but not in those with other types of drug reactions, indicating that expansions of regulatory T cells may contribute to the negative LTT reactions at the acute stage.

One of the most important questions raised by the present findings is why positive LTT reactions in patients with DIHS/DRESS could persist for months to a year while, in patients with other types of drug reactions, they became negative 5–8 weeks after the onset. In view of the notion that expansions of drug-specific T cells are unlikely to be maintained at such a high rate for long times without stimulation by specific drug antigen, the present results are difficult to explain by the widely accepted view that most of adverse drug reactions including DIHS/DRESS is primarily mediated by activation of drug-specific T cells (21, 22). How do the present findings described here fit with a scenario of T cell-mediated drug reactions coupled to the *in vivo* expansion and long-term maintenance of drug-specific T cells in the absence of apparent stimulation by the causative drug. Some clues may come from studies of graft *vs* host disease (GVHD), in which virus-reactive T cells that cross-react with alloantigens have been suggested to be involved (23, 24). Indeed, previous studies demonstrated that T cells with dual specificity for viral and alloantigenic determinants contribute to alloreactive T cell expansions *in vitro* during the acute phase of viral infections (25, 26); and that the incidence and severity of GVHD could be influenced by alloreactive T cell expansions induced by past viral infections (27). Among various viruses, the herpesvirus family is the most likely candidate responsible for the development of GVHD (28), because the herpesviruses induce and maintain a potent specific memory T cell response due to their common properties of ubiquitous prevalence in human populations. In this regard, we have recently demonstrated interesting findings that several herpesviruses including human herpesvirus 6 and cytomegalovirus are specifically reactivated during the course of DIHS/DRESS in the same sequential order as in GVHD (29–31). These findings, together with clinical similarities between DIHS/DRESS and GVHD, led us to consider the hypothesis that these herpesviruses sequentially reactivated would serve to maintain drug/virus-cross-reactive T cell expansions for long times after recovery. However, in another study, the persistence of positive LTT reactions over years has been also reported to occur even in patients with MP (12). These conflicting results suggest that not only cross-reactivity of the T cells but also the strength of the original reaction observed at the acute stage would determine the persistence of positive LTT reactions. If so, then one important question arises why positive LTT reactions cannot be detected at later time points in patients with SJS/TEN, in which most potent drug-specific T cell responses would occur. At present, we have no satisfying explanation; one possible explanation could be that T cell responses to drugs in patients with SJS/TEN are potent enough but not cross-reactive in nature with herpesviruses that can be abundantly detected in humans.

Although systemic prednisolone is widely used to treat patients with severe drug eruptions, little is known about the effect on the SI levels of LTT. Systemic prednisolone has been long thought to have an inhibitory effect on the SI value. Indeed, Pichler and Tilch recommended that the LTT be performed with blood from patients receiving ≤ 0.2 mg/kg prednisolone (1). However, our results have revised this opinion and found that there was no significant effect of systemic prednisolone therapy on the SI value of LTT, although the retrospective nature of our study and small series of patients preclude any valid assessment of the impact of systemic prednisolone. In view of our finding that systemic prednisolone appeared to affect the background proliferation rather than the SI value, drug-driven T cell proliferation may be relatively refractory to the suppressive effect of prednisolone. Thus, our results indicate that the LTT provides a very useful method of monitoring drug-specific T cell responses even in the setting of therapy with systemic prednisolone.

In conclusion, our observations demonstrate that LTT is a promising method to define the causative agent in drug eruptions; however, it is crucial to perform LTT at the right timing. To determine the causative agent, LTT should be performed within 1 week after the onset of skin rashes in patients with MP and SJS/TEN: 5–8 weeks after that in patients with DIHS/DRESS.

Acknowledgments

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CXCL16 is a novel mediator of the innate immunity of epidermal keratinocytes

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Keywords: anti-microbial activity, chemokine, CXCL16, innate immunity, keratinocyte

Abstract

The epidermis is constantly exposed to a variety of microbial pathogens and plays a vital role in resisting them. Soluble CXC chemokine ligand (CXCL) 16, which is one of the ELR$^-$ CXC chemokines, acts as a mediator of innate immunity by attracting CXC chemokine receptor (CXCR) 6-expressing cells, such as activated T cells and NKT cells. However, the production of CXCL16 by non-immune cells remains unclear. We found that cultured keratinocytes produced a significant amount of CXCL16 (2–3 ng per 10^6 cells per 24 h). Stimulation with tumor necrosis factor α, IL-1α, IFN-γ, peptidoglycan and polyinosinic-polycytidylic acid [poly(I:C)] enhanced CXCL16 production. The forms of CXCL16 in the culture supernatants had molecular weights of 14, 28 and 50 kDa. Immunohistochemical analysis revealed that the normal human epidermis expressed CXCL16. As several chemokines have anti-microbial activities, we studied the anti-microbial activity of CXCL16. The chemokine domain of CXCL16 at concentrations >5 μg ml^{-1} had significant anti-microbial activity against *Staphylococcus aureus* and *Escherichia coli*. Killing activity was retained at the physiological salt concentration in the presence of carbonate. In conclusion, CXCL16 is a novel mediator of the innate immune reactivities of epidermal keratinocytes.

Introduction

The CXC chemokine ligand (CXCL) 16 was discovered as a ligand for CXC chemokine receptor (CXCR) 6. CXCL16 is a membrane-bound chemokine that consists of four distinct domains: the chemokine domain, mucin-like domain, transmembrane domain and ad cytoplasmic (1, 2). This structure is similar to that of fractalkine/CX3CL1, which is another membrane-bound chemokine. After cleavage, soluble CXCL16 acts as a chemoattractant for activated CD8 T cells, NKT cells and T_h1-polarized T cells that express CXCR6 (1, 2). Cleavage is considered to be mediated by a disintegrin and metalloproteinase (ADAM) family protease, ADAM 10 (3, 4).

Shimaoka *et al.* (5) have reported a novel protein, designated SR-PSOX (scavenger receptor that binds phosphatidylserine and oxidized lipoprotein), which acts as a receptor for oxidized low-density lipoprotein (OxLDL). They have demonstrated that SR-PSOX is expressed by human macrophages and dendritic cells and that it specifically binds OxLDL, leading to its internalization and degradation. In atherosclerotic lesions, macrophages express SR-PSOX. This finding suggests that SR-PSOX may play important roles in the formation of atherosclerotic lesions. Recently, this SR-PSOX has been found to be identical to CXCL16 (6).

In addition to these functions, Shimaoka *et al.* (6) have demonstrated that CXCL16 on macrophages and dendritic cells mediates the adhesion and phagocytosis of bacteria, such as *Escherichia coli* and *Staphylococcus aureus*, and bacterial recognition is mediated by the chemokine domain of CXCL16. These findings indicate that CXCL16 is not only a chemokine, but is also a multifunctional protein, which suggests that CXCL16 has some novel function.

CXCL16 expression has been studied mainly in macrophages and dendritic cells. CXCL16 production by non-immune cells remains controversial. Hofnagel *et al.* (7) reported the detection by reverse transcription (RT)–PCR of CXCL16 mRNA in cultured aortic smooth muscle cells and umbilical endothelial cells. However, another group did not detect CXCL16 mRNA by northern blot analysis (8). There are no reports describing the production of CXCL16 by keratinocytes.

The epidermis is the primary barrier between the body and the outside environment. In addition to this physical

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barrier, the epidermis functions as an innate immune barrier that resists microbial pathogens. Epidermal keratinocytes produce anti-microbial peptides, such as human β-defensins and hCAP18/LL-37, following differentiation or wounding (9–11). Furthermore, epidermal keratinocytes recognize bacteria and virus-associated, double-stranded RNA via Toll-like receptor (TLR)2 and TLR3, respectively, to produce anti-microbial peptides, cytokines and chemokines (12–15). Since CXCL16 is an important chemokine for host defense, it seems reasonable to assume that keratinocytes also produce CXCL16.

In this study, we report for the first time that epidermal keratinocytes constitutively produce CXCL16. Furthermore, we show that the chemokine domain of CXCL16 has anti-microbial activity. Thus, CXCL16 is a novel mediator of the innate immune reactivities of keratinocytes in the human epidermis.

Methods

Reagents and antibodies

Tumor necrosis factor α (TNF-α) and IL-1α were generous gifts from Dainippon Pharmaceutical Co., Ltd (Osaka, Japan). IFN-γ was a generous gift from Otsuka Pharmaceutical Co., Ltd (Tokyo, Japan). Recombinant CXCL16 [chemokine domain and extracellular domain (ECD)] and recombinant IFN-γ-inducible protein of 10 kDa (IP-10/CXCL10) were purchased from R&D Systems (Minneapolis, MN, USA). Peptidoglycan purified from *S. aureus* was purchased from Fluka (Buchs, Switzerland). Polyinosinic-polycytidylic acid [poly(I:C)] was purchased from Amersham (Piscataway, NJ, USA). The monoclonal antibodies against CD1a were purchased from Dako Japan (Kyoto, Japan). The antibodies against the chemokine domain (aa 51–68) and cytoplasmic domain (aa 248–260) of human CXCL16 (Fig. 4) were generated by immunization of rabbits with the synthetic peptides. The respective reactivities of the antibodies were confirmed by ELISA. IgG was affinity purified using the synthetic peptides.

Skin samples

Normal human skin was obtained from plastic surgery under a protocol approved by the Institutional Review Board of Ehime University School of Medicine.

Cell preparation and culture

Normal human keratinocytes, dermal fibroblasts and dermal microvascular endothelial cells (DMECs) were cultured as described previously (16–18). Keratinocytes were cultured in MCDB153 medium that was supplemented with insulin (5 μg ml^{-1}), hydrocortisone (0.5 μM), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM) and bovine hypothalamic extract (50 μg ml^{-1}). Cells that were passaged four times were used in the experiments. $CD14^+$ monocytes were isolated from the buffy coats of healthy donors using the MACS cell isolation kit (Milteny Biotec, Bergisch Gladbach, Germany).

RNA preparation, RT–PCR, and real-time PCR

Total RNA was prepared from cells and epidermis using Isogen (Nippon Gene, Tokyo, Japan). The epidermis was separated from the dermis by treatment at 60°C for 1 min followed by immediate cooling in ice-cold PBS. RT–PCR was performed using RT–PCR High Plus (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Twenty-five PCR cycles were used to amplify the CXCL16 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequences. The following primers were used: for CXCL16, 5′-GGCCCCTCA-TTTAAAAACGG-3′ and 5′-GCCTGGTCAACATGGTGAAAC-3′ and for GAPDH 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′. The PCR products were visualized on 2% agarose gels that contained ethidium bromide and confirmed by size and direct DNA sequencing.

The primer and probes used in the real-time PCR of CXCL16 mRNA were selected using the Primer Express software (Applied Biosystems, Norwalk, CT, USA) as follows: forward, 5′-AAGCCATTGAGACACCAGCTG-3′; reverse, 5′-AC-CTCGCTCTGACTCCCAGA-3′ and 6FAM-ACGTCACGCGC-CGGAGCAT-TAMRA. cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems) following the manufacturer's suggested protocols. Real-time quantitative RT–PCR was performed with reagents recommended by the manufacturer (Applied Biosystems) and using the ABI PRISM 7700 Sequence Detection System. The levels of mRNA expression were normalized to that of GAPDH.

Protein preparation and western blot analysis

The supernatants of keratinocyte cultures were concentrated from 35 to 0.5 ml using Centriprep-3 (Amicon, Beverly, MA). Twenty-microliter samples were separated on 12% SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, Tokyo, Japan). After blocking with 1% non-fat dry milk, the membrane was treated with rabbit anti-chemokine domain antibodies of CXCL16. The antibody was detected with the Vistra ECF kit (Amersham). Fluorescence was observed on a FluorolImager (Molecular Dynamics, Sunnyvale, CA, USA).

Immunofluorescence microscopy

Frozen skin sections (5 μm) were fixed in cold acetone for 5 min and then reacted with the rabbit anti-CXCL16 antibody for 1 h at room temperature. After washing with PBS, the sections were incubated with fluorescence-labeled goat anti-rabbit IgG for 30 min at room temperature. For mouse anti-CD1a antibodies, anti-mouse rat antibodies labeled with Alexa Fluor 594 (Molecular Probes) was used as secondary antibodies. The fluorescence was observed under a fluorescence microscope.

ELISA

The ELISA development kit for CXCL16 was purchased from R&D Systems. The concentrations were measured according to the manufacturer's instruction. Optical density was measured with the Immuno Mini NJ-2300 microplate reader (Nalge Nunc International K.K, Tokyo, Japan).

Anti-bacterial assays

Staphylococcus aureus (209P) was grown in trypticase soy broth at 37°C. *Escherichia coli* strain HB101 was grown aerobically in Luria–Bertani broth. Overnight cultures of

S. aureus and *E. coli* were harvested, washed with PBS and suspended in 10 mM sodium phosphate buffer (NaPi; pH 6.8). The bacterial suspension was diluted to 10^7 cells per ml with NaPi (pH 6.8), and 10 μl of the bacterial suspension (10^5 cells) was inoculated into 200 μl of NaPi with or without various concentrations of chemokines and incubated for 2 h at 37°C. An appropriate dilution of the reaction mixture (100 μl volume) was plated on trypticase soy agar and incubated at 37°C overnight. The colony-forming units were assessed, and the anti-bacterial effect was calculated as the ratio of surviving cells to total cells. To demonstrate the effects of NaCl and $NaHCO_3$, NaCl (150 mM) or $NaHCO_3$ (50 mM) was added together with 10 mM NaPi (pH 6.8) in the anti-bacterial assay described above.

Results

CXCL16 production by cultured normal human keratinocytes

We analyzed, by RT-PCR, the levels of CXCL16 mRNA expression in cultured keratinocytes, normal human epidermis and $CD14^+$ monocytes. Cultured keratinocytes and normal human epidermis, as well as $CD14^+$ monocytes, expressed CXCL16 mRNA (Fig. 1A). We also evaluated, using real-time PCR, the levels of CXCL16 mRNA expression in keratinocytes, dermal fibroblasts, DMEC and $CD14^+$ monocytes (Fig. 1B). The level of CXCL16 mRNA expressed by keratinocytes was one-sixth that of monocytes but was 5-fold higher than the levels expressed by dermal fibroblasts and DMEC.

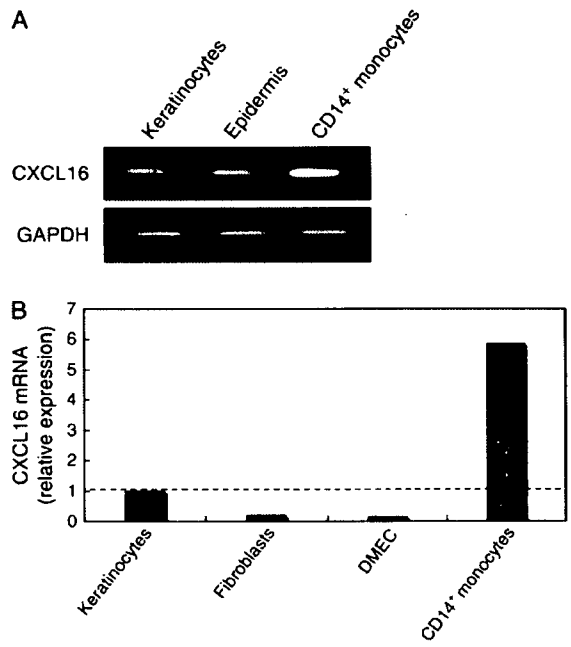

Fig. 1. CXCL16 mRNA expression. (A) CXCL16 mRNA expression by cultured keratinocytes, normal human epidermis and monocytes was analyzed by RT-PCR. GAPDH was used as an internal standard. (B) CXCL16 mRNA expression by monocytes, keratinocytes, dermal fibroblasts and DMEC was analyzed by real-time PCR. The expression levels were normalized to that of GAPDH. The level of mRNA expression by keratinocytes is expressed as one unit.

We also examined, by ELISA, the production of CXCL16 by cultured keratinocytes. CXCL16 was detected at 2–3 ng per 10^6 cells per 24 h in the culture supernatant of unstimulated keratinocytes. Although cultured keratinocytes produce several chemokines, such as IL-8, MIP-3α and CTACK, in the absence of stimulation (19–21), the levels are significantly lower than that of CXCL16 (data not shown). CXCL16 mRNA expression (Fig. 2A) and production (Fig. 2B) increased following stimulation with inflammatory cytokines, which included TNF-α, IL-1α and IFN-γ, either alone or in combination.

CXCL16 production increased by ligands for TLR2 and TLR3

We studied whether TLR stimulation increased CXCL16 production. Peptidoglycan and poly(I:C), which are ligands for TLR2 and TLR3, respectively, increased CXCL16 mRNA expression and production in cultured keratinocytes, as shown by real-time PCR and ELISA, respectively (Fig. 3).

Generation of antibodies to the chemokine and cytoplasmic domains of CXCL16

We raised rabbit antibodies against the chemokine domain (Fig. 4A) and cytoplasmic domain (Fig. 4B) of CXCL16 to evaluate CXCL16 shedding. The anti-chemokine domain and anti-cytoplasmic domain antibodies were used for western blot (Fig. 5) and immunohistological (Fig. 6) analyses. The anti-cytoplasmic domain antibody was used for immunohistological analysis (Fig. 6).

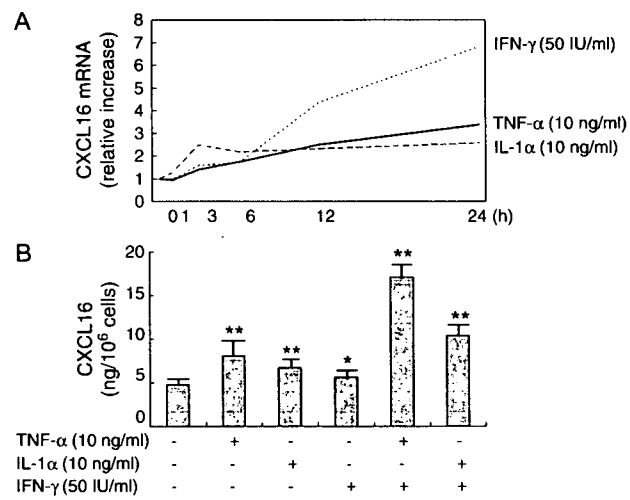

Fig. 2. Increased production of CXCL16 induced by pro-inflammatory cytokines. (A) Keratinocytes were treated with IFN-γ (50 U ml^{-1}), TNF-α (10 ng ml^{-1}) and IL-1α (10 ng ml^{-1}). The levels of CXCL16 mRNA expression were analyzed at the indicated time point by real-time PCR. The expression levels were normalized to that of GAPDH as one unit. (B) Soluble CXCL16 in the culture supernatant was measured by ELISA. Culture supernatants were harvested after 48 h of treatment with IFN-γ (50 U ml^{-1}), TNF-α (10 ng ml^{-1}) and IL-1α (10 ng ml^{-1}), either alone or in combination. Data are presented as means ± SDs (n = 3). Statistical analysis was performed using the Student's t-test with comparisons to the control. $^*P < 0.05$ and $^{**}P < 0.005$.

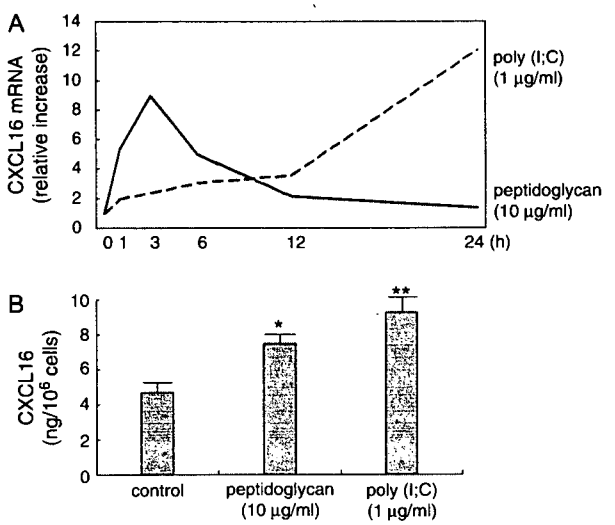

Fig. 3. Increased production of CXCL16 induced by ligands for TLR2 and TLR3. (A) Keratinocytes were treated with 10 μg ml $^{-1}$ peptidoglycan or 1 μg ml $^{-1}$ poly(I:C). CXCL16 mRNA expression was examined by real-time PCR at various time points. The expression levels were normalized to that of GAPDH as one unit. (B) Soluble CXCL16 in the culture supernatant was measured by ELISA. Culture supernatants were harvested after 48 h of treatment with 10 μg ml^{-1} peptidoglycan or 1 μg ml^{-1} poly(I:C). Data are presented as means ± SDs (n = 3). Statistical analysis was performed using the Student's t-test with comparisons to the control. *P < 0.05 and **P < 0.005.

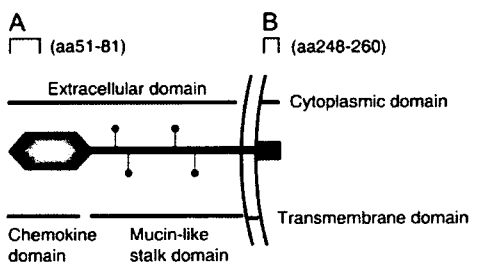

Fig. 4. Structure of CXCL16. CXCL16 consists of a chemokine domain, mucin stalk-like domain, transmembrane domain and cytoplasmic domain. Rabbit anti-CXCL16 antibodies were raised against the chemokine domain (A; aa 51–68) and cytoplasmic domain (B; aa 248–260).

Shedding of CXCL16 by keratinocytes

The culture supernatants were concentrated and subjected to western blotting. The 55-kDa recombinant ECD and 10-kDa recombinant chemokine domain were used as positive controls (Fig. 5A and B). Proteins of 14, 28 and ~50 kDa were detected in the culture supernatant (Fig 5A and B; arrow head). Antibodies treated with corresponding peptide of chemokine domain could not react with these proteins (data not shown). These results indicate that these proteins contain the chemokine domain.

CXCL16 expression in normal human epidermis

Next, we studied the expression of CXCL16 in normal human epidermis by immunohistological analyses using the anti-

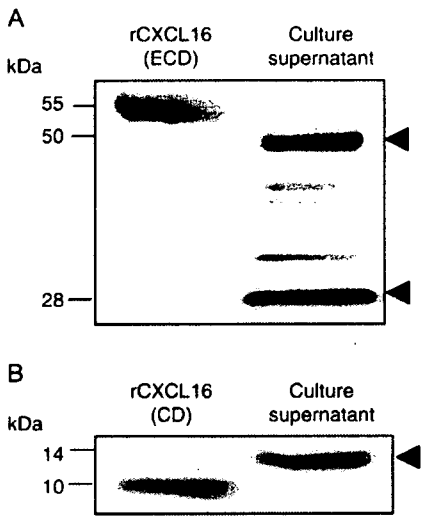

Fig. 5. Molecular size of CXCL16 produced from keratinocytes. The molecular size of CXCL16 was determined by western blot analysis using the anti-chemokine domain antibody. The culture supernatants were concentrated and subjected to western blot analysis with 10% (A) and 20% (B) SDS-PAGE. The CXCL16 recombinant ECD in (A) and chemokine domain (CD) in (B) are positive controls.

bodies against the chemokine domain and cytoplasmic domain of CXCL16. Normal human epidermis strongly expressed both the chemokine domain and cytoplasmic domain of CXCL16 (Fig. 6A and C), although there were no positive cells in the dermis, which indicates that the major source of CXCL16 is epidermal keratinocytes. This result is consistent with those shown in Fig. 1B.

Previous reports described that monocytes and dendritic cells produce much CXCL16 (1, 2). To examine a production of CXCL16 by Langerhans cells, immature dendritic cells existing in the epidermis, we performed double staining of CXCL16 and CD1a which is a major marker of Langerhans cells in normal human skin. As shown in Fig. 7, the expression levels of CXCL16 chemokine domain and cytoplasmic domain were weaker than that of surrounding keratinocytes. This result suggests that the production level of CXCL16 is not so much as compared with keratinocytes. Tabata *et al.* (22) reported that immature dendritic cells expressed a lower level of CXCL16 than monocytes and macrophages. We think that the ability of keratinocytes to produce CXCL16 is lower than that of monocytes, but is higher than Langerhans cells.

Anti-microbial activity of CXCL16

Recently, non-ELR CXC chemokines, such as CXCL9, CXCL10 and CXCL11, have been shown to have β-defensin-like anti-bacterial activities (23). Since CXCL16 is a non-ELR CXC chemokine, we hypothesized that CXCL16 also had anti-bacterial activity.

We analyzed the anti-bacterial activities of CXCL10 and the chemokine domain and ECD of CXCL16 against *E. coli* and *S. aureus* (Fig. 8). The chemokine domain of CXCL16 showed anti-bacterial activity against *E. coli* (with about 60% cell survival) at concentrations >5 μg ml^{-1}. The killing

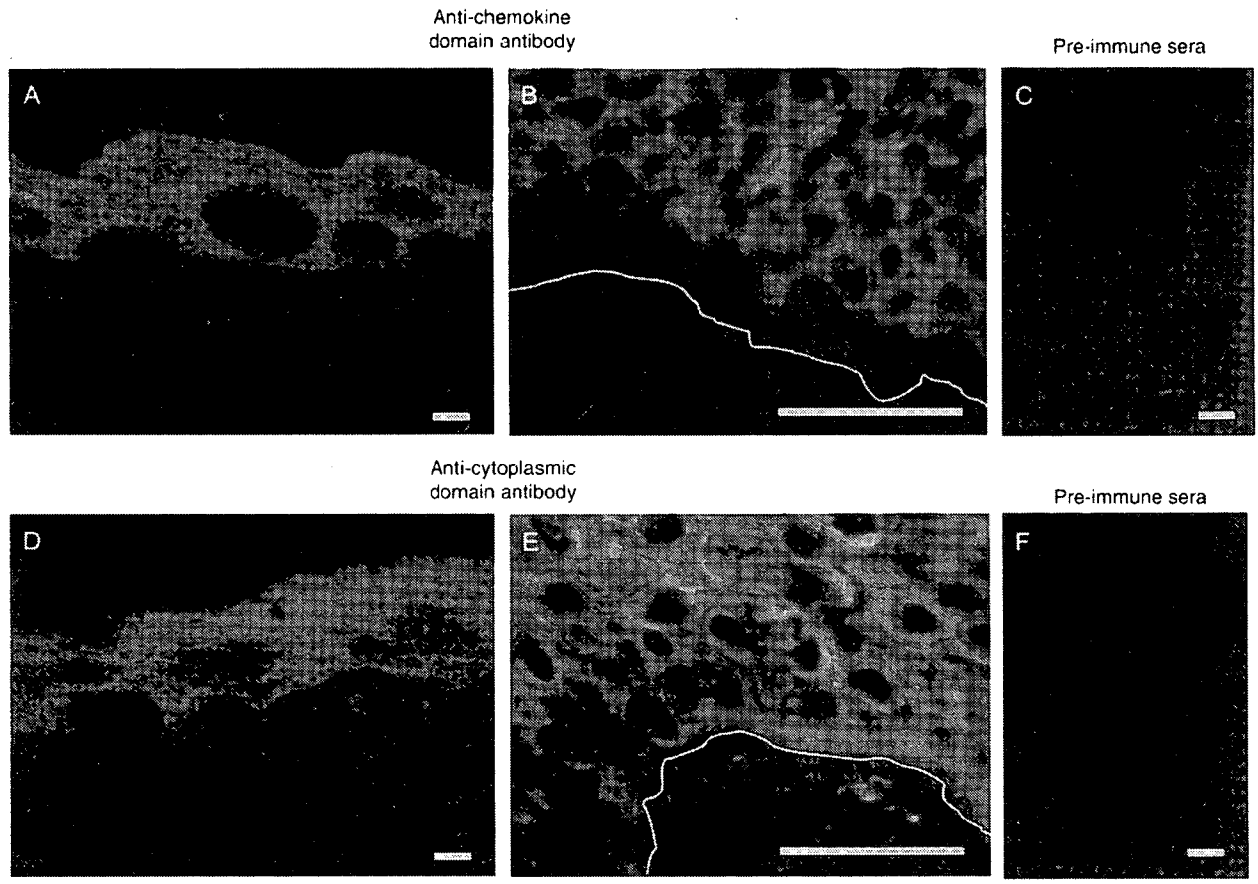

Fig. 6. Expression of CXCL16 in normal human epidermis. Frozen sections of normal human skin were stained with antibodies against the chemokine domain (A and B) and cytoplasmic domain (D and E) of CXCL16. (B) and (E) are higher magnifications of (A) and (B), respectively. Pre-immune sera of anti-chemokine domain (C) and anti-cytoplasmic domain (F) could not stain normal human skin. Immunofluorescence was observed under the fluorescence microscope. The dashed lines indicate the dermo-epidermal junctions. Scale bar: 50 μm.

effect on *S. aureus* was more impressive. The chemokine domain of CXCL16 killed *S. aureus* at concentrations >1 μg ml^{-1}, with only 30% of the bacteria surviving at 10 μg/ml of the peptide.

Although the anti-microbial activities of peptides are often lower at physiological salt concentrations (24), Dorschner *et al.* (25) found that carbonate, which is naturally present in the bodily fluids of mammalian tissues, preserves the activities of anti-microbial peptides at physiological salt concentrations. As expected, CXCL16 lost the ability to kill *S. aureus* following the addition of 150 mM NaCl and, indeed, the bacteria grew better under this condition (Fig. 9). However, the addition of 50 mM $NaHCO_3$ clearly restored the killing activity of CXCL16. The anti-microbial activity of CXCL16 at 10 μg ml^{-1} was almost equal to that of human β-defensin 2 at 1 μg ml^{-1}.

Discussion

In the present study, we demonstrate for the first time that epidermal keratinocytes produce CXCL16. In addition, we show that the chemokine domain of CXCL16 has anti-microbial activity. Since the epidermis is part of the innate immune defense system, CXC16 represents a novel mediator of epidermal innate immunity.

CXCL16 is one of the most important chemokines that recruit NKT cells (26). Microbial pathogens activate NKT cells via CD1d on activated dendritic cells during bacterial infection (reviewed in 27). These cells become active very early in the immune response against microbial pathogens (reviewed in 28). In the present study, we show that CXCL16 production by keratinocytes increases following stimulation with peptidoglycan. Therefore, keratinocytes may play an important role in the recruitment of NKT cells during bacterial infection, although it remains unclear whether NKT cells contribute to the immune response to gram-positive bacteria. NKT cells are also important in the defense against viral infections (28). Herpes simplex virus type 1 infection leads to the development of larger skin lesions in $CD1d^{-/-}$ mice than in control mice, which suggests that the immune response of CD1d-restricted NKT cells is impaired (29). In the present study, we show that poly(I:C) enhances the production of CXCL16 by keratinocytes, which suggests that epidermal keratinocytes are able to recruit NKT cells during

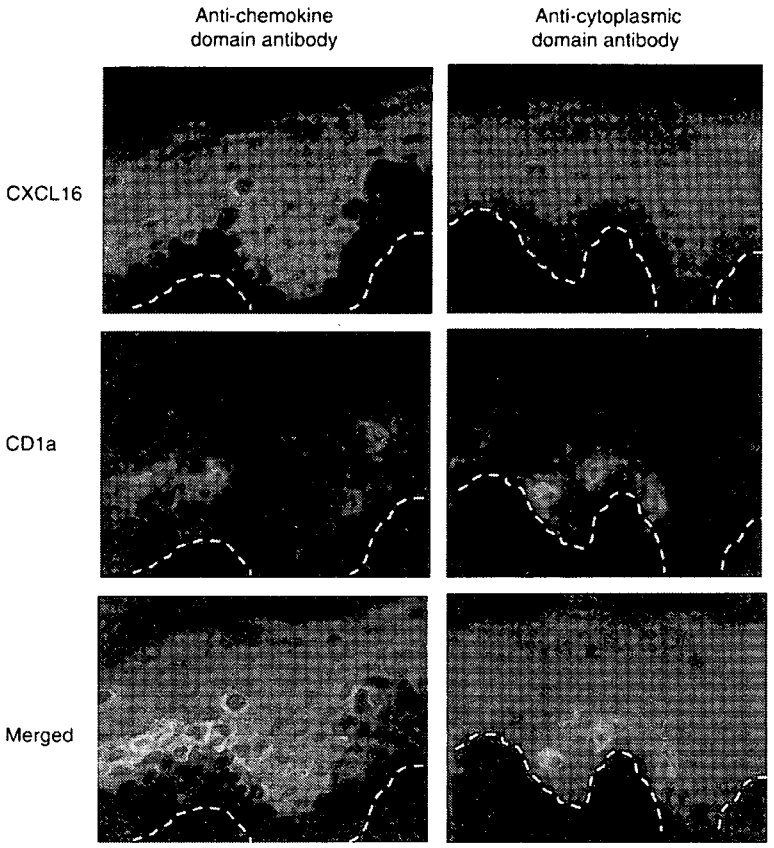

Fig. 7. Expression of CXCL16 on Langerhans cells. Frozen sections of normal human skin were double stained with antibodies against the CXCL16 chemokine domain (green) and CD1a (red) or CXCL16 cytoplasmic domain (green) and CD1a (red).

viral infection. Therefore, CXCL16 production by keratinocytes is an important step in the initiation of host defenses against bacterial and viral infections.

Moreover, the constitutive expression of CXCL16 by keratinocytes may participate in the recruitment of resident T cells to the skin, so-called skin-homing T cells, which exist under the resting condition and can initiate immune reactions in the absence of T-cell recruitment from the circulation (30). These cells are characterized by the expression of cutaneous lymphocyte-associated antigen (CLA). A recent report has demonstrated that 50% of CLA^+ skin-resident T cells express CXCR6 (31). In contrast, only 2% of circulating CLA^+ T cells express CXCR6. Constitutive production of CXCL16 by keratinocytes may contribute to the residence status of T cells and may play a role in cutaneous immune surveillance.

Another important innate immune system exists in the skin. Human epidermal keratinocytes produce anti-microbial peptides, such as β-defensins and CAP18/LL-37 (9–12). Recently, several chemokines have been shown to have similar anti-microbial activities (23, 32, 33). CXC chemokines that lack the ELR (Glu-Leu-Arg) motif, which include CXCL9, CXCL10 and CXCL11, have anti-microbial peptides due to a highly positive charge at the C-terminus (23). CXCL16 belongs to the ELR^- CXC chemokine family, and it also contains positively charged amino acids. In the present study, we show that the chemokine domain of CXCL16 exerts potent anti-microbial activities against *E. coli* and *S. aureus*. Therefore, CXCL16 can act as an anti-microbial peptide in the epidermis. Although keratinocytes produce CXCL9, CXCL10 and CXCL11 (34, 35), production is limited to those keratinocytes that are stimulated by cytokines, such as IFN-γ. In contrast, keratinocytes constitutively produce CXCL16, which indicates that CXCL16 is one of the first lines of innate immune defense before the start of inflammation.

The anti-microbial activities of chemokines and anti-microbial peptides are salt sensitive (24, 36, 37). Therefore, there is a question as to whether the anti-microbial activities of these chemokines function *in vivo* at the physiological salt concentration. Dorschner *et al.* (25) have clearly shown that carbonate enhances anti-microbial activity at the physiological salt concentration. The present study also shows that CXCL16 retains anti-microbial activity at the physiological salt concentration in the presence of carbonate. This result suggests that CXCL16 is active *in vivo* as an anti-microbial peptide.

ADAM 10 causes the shedding of CXCL16 from the membranes of macrophages (3, 4). Since keratinocytes express ADAM 10 (38), it is conceivable that ADAM 10 also mediates the shedding of CXCL16 from keratinocytes. In a previous report, human CXCL16-transfected COS-7 cells were shown

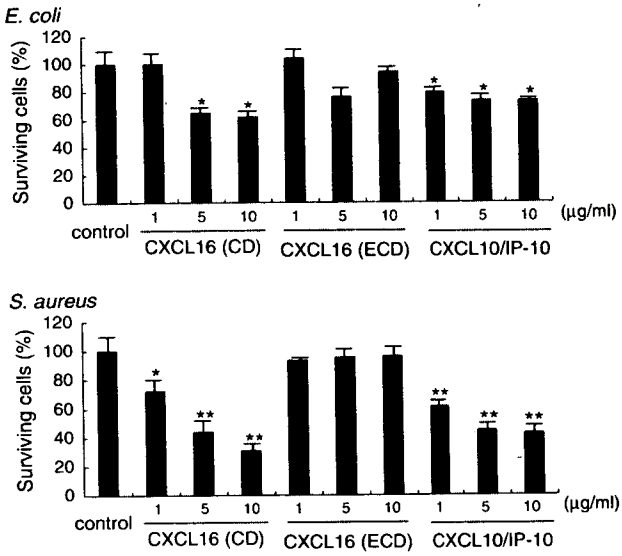

Fig. 8. Anti-microbial activities of CXCL16. The anti-microbial activities of CXCL10 and the chemokine domain (CD) and ECD of CXCL16 were studied. The anti-bacterial effect was calculated as the ratio of surviving cells to total cells. The activities for *Escherichia coli* (A) and *Staphylococcus aureus* (B) were examined in 10 mM NaPi. Data are presented as means ± SDs (n = 3). Statistical analysis was performed using the Student's t-test with comparisons to the control. $*P < 0.05$ and $**P < 0.005$.

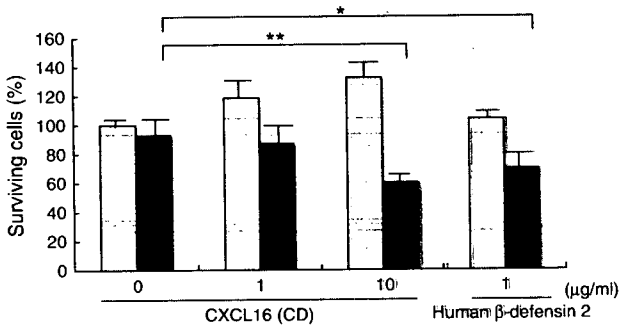

Fig. 9. Recovery of the anti-microbial activity of CXCL16 in high-salt medium by carbonate. The anti-microbial activities of the chemokine domain of CXCL16 and human β-defensin 2 against *Staphylococcus aureus* were examined in media that contained 150 mM NaCl without $NaHCO_3$ (gray bars) or with 50 mM $NaHCO_3$ (black bars). Data are presented as means ± SDs (n = 3). Statistical analysis was performed using the Student's t-test. $*P < 0.05$ and $**P < 0.005$.

to secrete a 32-kDa protein into the culture supernatant, the level of which was decreased by the addition of an ADAM 10 inhibitor (4). However, the CXCL16 produced from keratinocytes in the present study appeared as 14, 28 and 50 kDa proteins. The mechanism for proteolytic cleavage of CXCL16 in keratinocytes may differ from that in macrophages.

In conclusion, CXCL16 is a mediator of the innate immune reactivities of epidermal keratinocytes.

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Abbreviations

ADAM	disintegrin and metalloproteinase
CLA	cutaneous lymphocyte-associated antigen
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DMEC	dermal microvascular endothelial cell
ECD	extracellular domain
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
OxLDL	oxidized low-density lipoprotein
poly(I:C)	polyinosinic-polycytidylic acid
RT	reverse transcription
TLR	Toll-like receptor

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trying dietary manipulation (30%) is similar to the 40% using CAM to treat proven allergic contact dermatitis.[1] However, the majority of our patients reported that their skin failed to improve as a result of dietary manipulation. Of ongoing concern is the significant number of patients who attempt dietary manipulation without seeking appropriate expert advice.

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Key words: allergic contact dermatitis, complementary and alternative medicine, diet

Conflicts of interest: none declared.

Chromosomal integration of human herpesvirus 6 DNA in anticonvulsant hypersensitivity syndrome

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Sir, Anticonvulsant hypersensitivity syndrome (AHS), which is also referred to as drug rash with eosinophilia and systemic symptoms (DRESS) or drug-induced hypersensitivity syndrome (DIHS), is a multiorgan systemic reaction characterized by rashes, fever, lymphadenopathy, leucocytosis with eosinophilia and atypical lymphocytes, liver dysfunction and a close relationship to the reactivation of herpesvirus, especially human herpesvirus 6 (HHV-6), in patients on long-term anticonvulsant therapy.[1–3] AHS tends to show relatively later onset (2–8 weeks or more after commencing administration of the causative drug) than the other types of drug eruption, and HHV-6 DNA is detected in the serum at around 3–5 weeks after the onset, followed by a dramatic rise in anti-HHV-6 IgG titres.[3] We report a patient with AHS caused by carbamazepine, in whom serum HHV-6 DNA levels had persistently been extremely high and there had been chromosomal integration of the HHV-6 genome.

A 47-year-old Japanese man was admitted to our hospital on April 7th 2005, with a 12-day history of generalized erythematous rash and a fever. He had a 20-year history of schizophrenia. He had been treated with chlorpromazine hydrochloride, levomepromazine, biperiden, nitrazepam, bromazepam and clonazepam for 2 years. On January 7th 2005, oral carbamazepine had been added to his treatment. The erythematous lesions began on the 78th day of administration of carbamazepine, becoming generalized over the next 5 days. Physical examination on admission revealed a diffuse erythema with scaling over the whole body (Fig. 1). He had a high fever (38.8 °C) and bilateral cervical and inguinal lymphadenopathy. He was negative for Nikolsky's sign. Laboratory investigations revealed white blood cell count 23.5×10^9 L^{-1}, with 35% eosinophils and 6% atypical lymphocytes; aspartate aminotransferase 70 IU L^{-1}; alanine aminotransferase 73 IU L^{-1}; γ-glutamyltransferase 129 IU L^{-1}; lactate dehydrogenase 686 U L^{-1}; hypogammaglobulinaemia (IgG 609 mg dL^{-1}, IgA 34 mg dL^{-1}, IgM 27 mg dL^{-1}) and C-reactive protein 2.6 mg dL^{-1}. Analysis of peripheral blood lymphocyte surface markers showed 49.3% CD4+ T cells and 39.0% CD8+ T cells. Hepatitis B surface antigen, hepatitis C virus antibody, human immunodeficiency virus-1 antibody and adult T-cell leukaemia-associated antigen were all negative. Skin biopsy from the abdomen revealed hydropic and vacuolar degeneration of epidermal basal cells, lymphocytic infiltration in the epidermis and a dense upper dermal infiltrate consisting mainly of mononuclear cells. Histological examination of inguinal lymph nodes and ultrasonography of the cervical and inguinal lymph nodes bilaterally showed a benign lymphoid hyperplasia. The diagnosis of an AHS due to carbamazepine was made and carbamazepine therapy was discontinued. Because previous reports have demonstrated that cross-reactivity to multiple drugs with different structures including those used after onset of the rash can be detected in patients with DRESS/DIHS,[4] we mainly use systemic corticosteroid for treatment of these cases. Oral prednisolone 20 mg daily was given for 3 days but this was ineffective, so the dose was increased to 60 mg daily with good clinical effect. The dose was tapered over the next 21 days in line with improvement of clinical symptoms. We also gave intravenous immunoglobulin (5 g daily for 3 days, from the first day of the higher steroid dose), as recommended by other groups.[5,6] It took 6 months for complete resolution of all symptoms including eosinophilia and lymphadenopathy.

After the rash had resolved completely, we conducted patch testing with all drugs the patient had been taking before admission. A positive reaction (++) according to the International Contact Dermatitis Research Group scoring system was observed only for carbamazepine. We also performed a drug-induced lymphocyte stimulation test (DLST) to identify the causative drug. The DLST stimulation index for carbamazepine was 391% (cut-off for DLST is 180%).

Six weeks after onset, HHV-6 IgG titres increased 128-fold compared with those at admission but anti-HHV-6 IgM titres

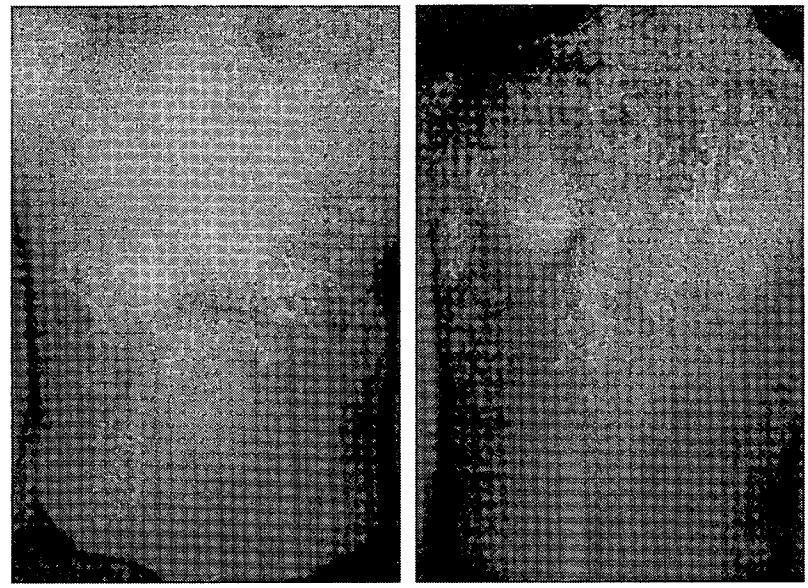

Fig 1. Clinical features on admission. Diffuse erythema with scaling on the trunk, consistent with erythroderma.

did not change during the whole course, indicating that HHV-6 reactivation had occurred in this patient. We also examined our patient's HHV-6 DNA in serum samples. In DRESS/DIHS, HHV-6 DNA has previously been detected in the serum at around 3–5 weeks after onset (and only at this point),[3] whereas in our patient HHV-6 DNA was persistently extremely high (> 10 000 copies mL^{-1} serum). Our patient also showed persistently very high HHV-6 DNA levels in the whole blood.

Because the HHV-6 genome has been detected in several human lymphoproliferative disorders,[7,8] and found to be integrated into chromosomes in some cases, we examined whether our patient carried chromosomally integrated HHV-6 DNA. Fluorescent *in situ* hybridization (FISH) with the HHV-6-specific ph6Z-101 probe was performed on metaphase chromosomes from the peripheral blood as previously described.[7,8] The result of FISH analysis in this patient showed the symmetrical doublet hybridization signals seen on chromosome 1q44 (Fig. 2).

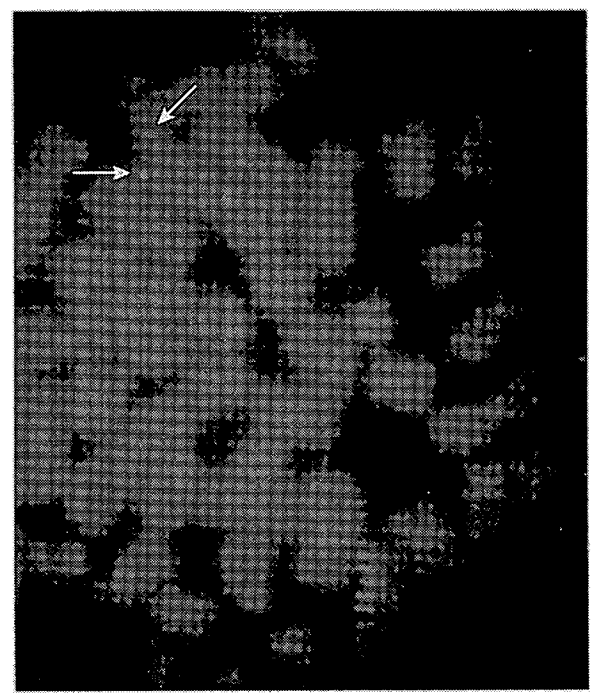

Fig 2. Fluorescent *in situ* hybridization on metaphase chromosomes from the patient. Hybridization with a human herpesvirus 6 (HHV-6)-specific probe showed HHV-6 integration with symmetrical doublet signals at homologous sites on both chromatids. Arrows indicate the hybridization signals on chromosome locus 1q44. Chromosomes were counterstained with propidium iodide. Ethical approval was sought from the Ethics Committee of Showa University School of Medicine, and the patient and his parents provided written, informed consent.

HHV-6 latency in adults is usually characterized by a very low copy number of HHV-6 genome in peripheral blood mononuclear cells.[9,10] In this respect, chromosomally integrated HHV-6 with a high copy number of HHV-6 genome should be distinct from latency after primary infection.[9] Recently, Ward *et al.* demonstrated that immunocompetent individuals who show persistently very high HHV-6 DNA levels in whole blood and serum have viral integration that has been inherited chromosomally from either of their parents.[9] Although we could not get agreement from the patient's family to look for the HHV-6 DNA in their peripheral blood, this patient's viral genome was most likely inherited chromosomally from either of his parents as described above.

This is the first report in which chromosomally integrated HHV-6 DNA is identified in an allergic disease such as AHS. Further investigation of these phenomena will improve the understanding of adverse drug reactions by providing further insight into the mechanisms underlying immune responses to viruses.

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Key words: *anticonvulsant hypersensitivity syndrome, chromosomal integration, drug eruption, drug-induced hypersensitivity syndrome, human herpesvirus 6*

Conflicts of interest: none declared.

Successful use of etanercept in type I pityriasis rubra pilaris

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SIR, Pityriasis rubra pilaris (PRP) is a rare, idiopathic, papulosquamous disease. It has many histological and clinical features in common with psoriasis and differentation of these two diseases is sometimes difficult. Systemic retinoids are the mainstay of treatment; however, PRP is often a therapeutic challenge. We describe a patient with type I PRP responding to etanercept. To the best of our knowledge, this is the first report of etanercept used successfully in PRP.

A 37-year-old man presented with a skin eruption of approximately 2 months' duration which was initially diagnosed as psoriasis. Narrowband ultraviolet (UV) B was commenced; however, after the first session, an increase in erythema developed which progressed into erythroderma. The patient underwent an urgent appendectomy after the second session of phototherapy. Dermatological examination revealed erythroderma with islands of sparing on the upper back, and marked palmoplantar keratoderma in association with thickened nails (Fig. 1a). Histopathological examination showed alternating orthokeratosis and parakeratosis in both vertical and horizontal directions as well as dilated hair follicle orifices and keratotic and parakeratotic plug formations which were consistent with PRP. Phototherapy was discontinued after three sessions. Routine biochemistry revealed mild elevations in liver enzymes [aspartate aminotransferase (AST) 56 IU L^{-1} (normal 10–37), alanine aminotransferase (ALT) 85 IU L^{-1} (normal 10–40)]. A 20-day trial of ciclosporin 3·5 mg kg^{-1} daily was unhelpful. As liver enzymes had returned to normal during this time, acitretin was started at an initial dose of 0·3 mg kg^{-1} daily which was later increased to 0·5 mg kg^{-1} daily. Although 1 month of acitretin therapy led to only a slight improvement, it also resulted in progressive elevation of liver enzymes (AST 57 IU L^{-1}, ALT 125 IU L^{-1}) which persisted despite dose reduction. Hepatitis serology and autoimmune hepatitis markers were all negative, whereas hepatomegaly was detected in ultrasonographic examination. Owing to its hepatotoxic effects, acitretin was finally stopped 2 months after initiation. For the following 3 months, the patient was treated only with emollients and oral antihistamines. His erythrodermic condition remained unchanged during this period and was a great concern for the patient, but liver enzymes were normalized.

At this stage, etanercept was started at a dose of 50 mg twice a week. During the first 2 months of treatment, there was a slow regression in erythroderma and palmoplantar keratoderma which showed considerable improvement especially after the third month (Fig. 1b–d). This was followed by reducing the dose to 25 mg twice a week. The patient has been on etanercept for 6 months now and is almost clear. Treatment was well tolerated and no adverse effects were observed other than slight increases in liver enzymes without any need for treatment modification.

PRP usually has no preceding event. However, infection and UV radiation can be provocative.[1] Our patient underwent an urgent appendectomy concurrent with phototherapy. These two factors together may have contributed to the rapid progression of the disease.

PRP has been divided into five categories by Griffiths according to the age at onset, clinical presentation and natural

Paraben allergic contact dermatitis in a patient with livedo reticularis

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Key words: contact dermatitis; excited skin syndrome; paraben; patch test.

Despite extensive use of parabens, incidence of allergy to them is lower than other common preservatives. However, there is a well-known phenomenon called 'paraben paradox': although it is usually safe to use paraben-containing cosmetics, sensitization to the paraben in topical therapeutic manufactures is not rare. We report a case of paraben allergic contact dermatitis in a patient with livedo reticularis.

Case Report

A 48-year-old man was examined in February 1996 with cutaneous lesions in the lower thighs. Past medical history included anaphylactoid purpura and acute pyelonephritis at 14 years of age. Leg lesions developed in 1986. Although topical application of corticosteroid ointment produced clinical resolution, new lesions developed several times over 10 years. Physical examination showed red-blue mottling in the lower thighs. Several ulcers with yellowish or blackish crust and dark reddish swelling were seen on both lower thighs and dorsal feet. An erythematous lesion showed perivascular lymphocytic infiltration and dermal vessels with a thickened wall. Livedo reticularis was diagnosed on the above findings.

Course and Patch Testing

He was hospitalized 3 times (1996, 1997, and 2005), when ulcers developed on the legs. During those hospitalizations, erythematous lesions developed around the ulcers several times (Fig. 1). Suspecting contact dermatitis to topical medicaments, we performed patch testing on each admission. Medicaments were applied on the back for 2 days, with vinyl plaster (Patch tester TORII®; Torii Pharmaceutical Co. Ltd, Tokyo, Japan). The results were read using the International Contact Dermatitis Research Group scoring system, 2 and 3 days after application (1). In the first testing, only Reflap® ointment showed a positive reaction (Table 1). In addition to that ointment, positive reactions to other 3 medicaments were seen (Acuatim® cream, Geben® cream, and Olcenon® ointment) in the second testing (Table 1). The third testing with Reflap® ointment and 15% paraben [the standard series of the Japanese Society for Contact Dermatitis (2)] were patch test positive (Table 1).

Discussion

Parabens, including propyl, methyl, ethyl, butyl, and benzyl parabens are commonly used as topical medicament preservatives (3). Among topical medicaments patch tested in our patient, 4 contained parabens (Table 2). Based on these and patch test results, this case was diagnosed as contact dermatitis from methyl and propyl parabens in Reflap® ointment (Tables 1 and 2). Despite their extensive use of parabens, incidence of allergy to them is lower than other common preservatives (3). However, there is a well-known phenomenon called 'paraben paradox': although it is usually safe to use paraben-containing cosmetics, sensitization to the paraben in topical therapeutic manufacturers is not rare (4). The answer seemed to be derived from differences between normal and damaged skin (3). In particular, in ulcerative lesions, topical medicaments are common causes of allergic contact dermatitis (5). Therefore, we consider

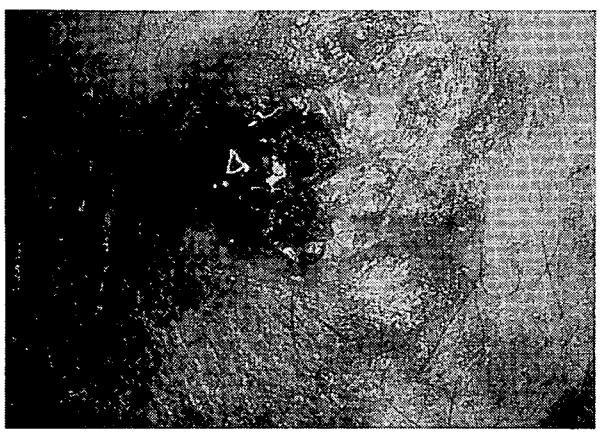

Fig. 1. Dark reddish, erythematous lesions around an ulcer.

Table 1. Results of patch testing[a,b]

Trade name	Ingredients	1996	1997	2005
Gentacin® ointment	0.1% Gentamicin sulfate	IR	–	–
Baramycin® ointment	Bacitracin + fradiomycin sulfate	–	–	ND
Acuatim® cream	0.1% Nadifloxacin cream	ND	+	–
Dalacin® T gel	0.1% Clindamycin phosphate	ND	ND	–
Geben® cream	1% Sulfadiazine silver	ND	+–	–
Olcenon® ointment	0.25% Tretinoin tocoferil	ND	++	ND
Prostandin® ointment	0.003% Alprostadil alfadex	ND	ND	–
U-pasta® ointment	Povidoneiodine + sucrose	ND	ND	–
Actosin® ointment	3% Bucladesine sodium	ND	ND	–
Bromelain® ointment	50 000 Bromelain unit/g	ND	ND	–
Reflap® ointment	Lysozyme chloride	+	++	++
15% Paraben		ND	ND	++

ND, not determined; IR, irritant reaction.
[a]Readings were performed 3 days after application.
[b]Individual parabens were not tested.

Table 2. Parabens in topical medicaments

Trade name	Results of patch testing	Methylparaben	Propylparaben	Butylparaben	Ethylparaben
Gentacin® ointment	–	*	*		
Geben® cream	+–,–	*		*	
Olcenon® ointment	+–	*	*		
Reflap® ointment	+–,+	*	*		*

*, Contained.

contact dermatitis seeing long-lasting exacerbation of cutaneous ulcers.

In our case, there were dissociations of patch testing results to 2 medicaments (Acuatim® cream and Geben® cream) between the second and third testing. We speculated the following 4 possibilities: (i) the dissociation was by immunological tolerance, (ii) positive reactions in the second patch testing were irritant reactions, (iii) conditions for patch testing were different between the second and third testing, and (iv) positive reactions in the second were caused by 'excited skin syndrome' (6, 7). We speculated that (iv) is the most likely because positive reactions in the second testing were strong (+). In addition, Acuatim® cream does not contain paraben (Table 2). In Geben® cream, however, (iii) could not be ruled out because it contains methylparaben and butylparaben (Table 2). In patch testing with paraben, false-negative reactions are seen because the paraben concentration is too low to give a positive reaction on normal skin. In this respect, Gentacin® ointment is also unavailable for further therapy in our patient.

This case emphasizes the value of patch testing with ingredients as well as 'as is' for adequate selection of topical medicaments for further treatment.

Acknowledgements

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Patient Report

Toxic epidermal necrolysis in a child successfully treated with cyclosporin A and methylprednisolone

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Key words cyclosporin A, G-CSF, leukopenia, methylprednisolone, toxic epidermal necrolysis.

Toxic epidermal necrolysis (TEN) is an acute and life-threatening disease that is characterized by severe necrosis of the skin. It also involves visceral organs and manifests systemic symptoms.[1,2] Some medicines and infections are known to be major precipitating factors of the disease.[3] TEN and Stevens–Johnson syndrome (SJS) are now categorized into the same type of disorder.[4] The mortality rate of TEN is 30–35% and prompt management including withdrawal of causative drugs with long half-lives is essential for a favorable outcome.[5] Many drugs such as corticosteroids, i.v. immunoglobulin,[3] and thalidomide[6] are used as treatment for TEN, but these drugs are unsatisfactory in efficacy. In the current report we present a pediatric case of TEN associated with leukopenia successfully treated with i.v. cyclosporin A (CsA), methylprednisolone and granulocyte-colony stimulating factor (G-CSF).

Case report

A 10-year-old girl, previously in good health, had a high fever of 39°C and took acetaminophen and commercially available cold medicine (Paburon; Taisho Pharmaceutical, Tokyo, Japan) on 19 January 2001. The next day she developed erythematous eruption on the face, trunk and extremities and went to a nearby hospital. She was diagnosed with acute tonsillitis. The bullous eruptions progressed within 2 days to include the conjunctival, oropharyngeal mucosae with eye and oral pain. On suspicion of SJS she was admitted to hospital. She was treated with oral prednisolone (PSL, 40 mg daily), and i.v. ulinastatin without any improvements. Therefore, she was transferred to Department of Pediatrics, Yokohama City Medical Center on 26 January 2001 (Fig. 1). On admission she had skin eruptions with bullae and erythema on her face and upper extremities (>30% of total body surface area), bilateral conjunctivitis, pseudomembrane formation, spotty erosions at bilateral conjunctivas, and erosions and ulcers of the entire buccal mucosa, and tongue and lips with erosions and bloody crustae (Fig. 2). Nikolsky's sign was easily demonstrated, and her body temperature was 37.2°C. Her aphthous stomatitis was so severe that she could not ingest anything. Also, she presented with erosions in the genital region. There was neither lymphadenopathy nor visceral organomegaly.

Laboratory data on admission were as follows: white blood cells count 2300/μL, hemoglobin 12.6 g/dL, platelet count 28.6×10^4/μL, total protein 6.4 g/dL (normal, N: 6.9–8.3), albumin 3.5 g/dL (N: 4.3–5.4), creatine phosphokinase 91 IU/L (N: 39–163), aspartate aminotransferase 291U/L (N: 14–32), alanine aminotransferase 25 IU/L (N: 9–25), lactic dehydrogenase 2051U/L (N: 116–199), blood urea nitrogen 7 mg/dL (N: 8–20), and creatinine 0.37 mg/dL (N: 0.48–0.82). C-reactive protein was 1.5 mg/dL. Antinuclear antibody was negative, and C3, C4 and CH50 were 109 mg/dL, 29 mg/dL, and 59 U/mL, respectively. The serum IgG, IgA, IgM and IgE were 1130 mg/dL, 246 mg/dL, 156 mg/dL and 781U/mL, respectively. Fibrin/fibrinogen degradation products-E was 108 ng/mL (N: <60). Soluble IL-2 receptor was 993 U/mL (N: 220–530).

The patient was diagnosed with TEN based on her clinical conditions and laboratory data. Because of the lack of efficacy of oral PSL therapy we administered CsA (1 mg/kg per day i.v.) and high-dose methylprednisolone (30 mg/kg per day, 3 successive days). In addition, the patient initially had leukopenia with a neutrophil count of 1426/μL and was commenced on G-CSF (75 μg) s.c. once at 24 h after starting CsA. Within 24 h, her neutrophil count rose to a normal range. In view of her neutropenia and pyrexia, empirical panipenem/betamipron (1 g/day) was started. Careful topical treatments for eyes and skin lesions were started. She also required parenteral nutrition and a urinary catheter. Within 24 h of starting CsA, the rapid

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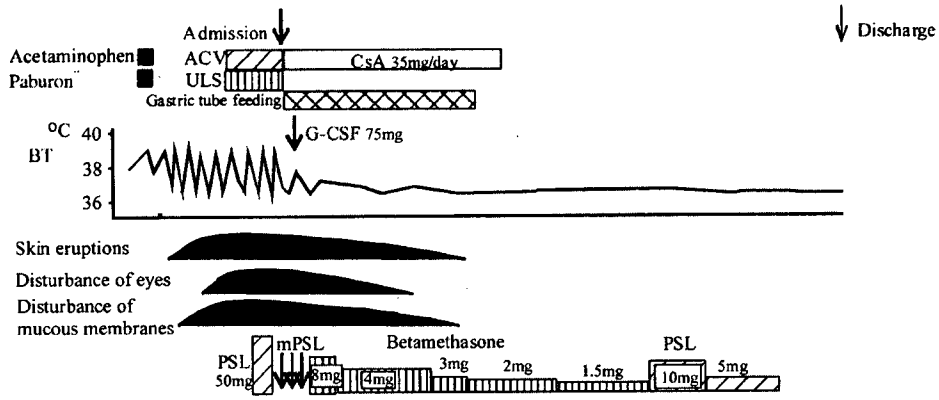

Date	Jan.19	22	26	28	Feb.1	8	13	20	27	Mar.5
Days	1	4	8	10	14	21	26	33	40	46
WBC	(/μL)		2,300	32,010	9,650	10,910	9,860	8,010	8,860	6,120
Neu	(%)		67.0	ND	74.0	76.5	54.5	57.5	ND	54.9
TP	(g/dL)		6.4	5.9	5.9	6.4	6.3	6.8	7.0	7.0
ALB	(g/dL)		3.5	3.3	3.5	4.0	4.1	4.6	4.8	4.7
LDH	(IU/L)		205	ND	183	190	211	210	220	232
FDP-E	(μg/mL)		108	231	103	ND	124	124	ND	76

Fig. 1 Clinical course and patient laboratory data. Day 1, day the suspected causative drugs were taken. ALB, albumin; ACV, aciclovir; BT, body temperature; CsA, cyclosporin A; FDP-E, fibrin/fibrinogen degradation products-E; LDH, lactic dehydrogenase; Neu, neutrophil; PSL, prednisolone; TP, total protein; ULS, ulinastatin; WBC, white blood cells.

progression of the skin lesions had stopped, and by 3 days there was a visible improvement in the erythema. CsA was stopped within 14 days, by which time re-epithelialization was almost complete. She was successfully treated and oral corticosteroid was gradually tapered until 2 March and she was discharged on 5 March without any sequelae (Fig. 2).

To determine the causative agents and to elucidate the mechanism of the disease we performed serological tests for antibodies against pathogens including HSV, VZV, HHV6, HHV7, EBV, adenovirus, and *Mycoplasma pneumoniae*, and these were negative (data not shown). In addition, drug-induced lymphocyte stimulation tests (DLST) for acetaminophen and Paburon (Taisho Pharmaceutical) were negative on the control (151% and 125%) on 13 March 2001. We also measured the serum levels of seven cytokines using Cytometric Bead Array (BD PharMingen, San Diego, CA, USA), and Human IL-6 ELISA Ready-SET (eBioscience, San Diego, CA, USA). The serum levels of interleukin (IL)-6, interferon-γ (IFN-γ),

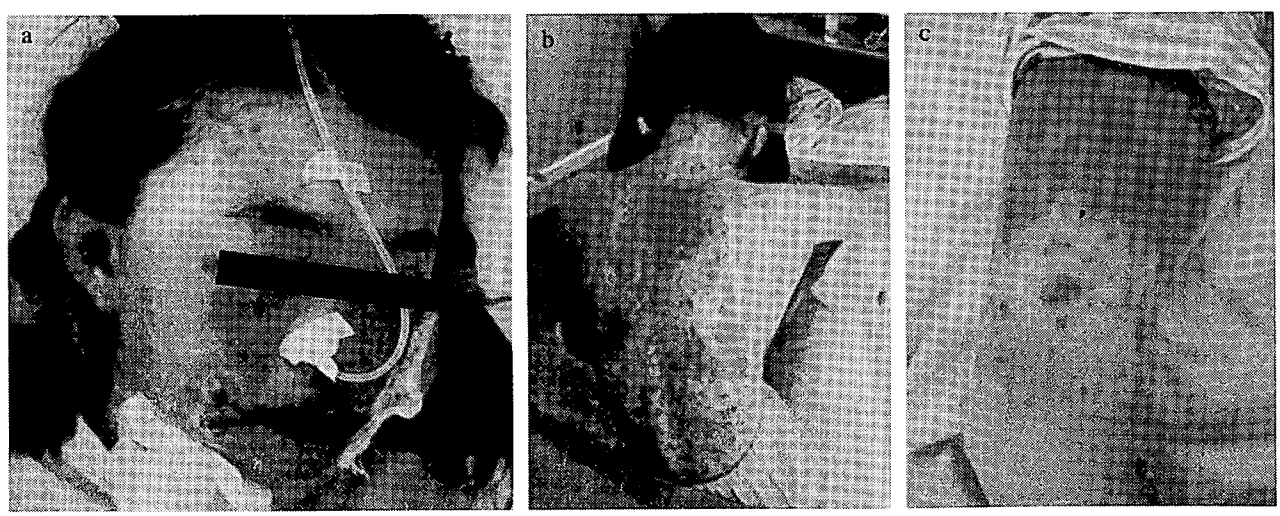

Fig. 2 Patient on admission. (a) Face; (b) back; (c) right upper limb.

IL-10 but not tumor necrosis factor-α (TNF-α) were elevated on admission and decreased gradually (Table 1).

Discussion

Toxic epidermal necrolysis and SJS are acute and life-threatening disorders. Current classification scheme defines SJS and TEN according to the amount of epidermal loss and whether pre-existing focal lesions or a diffuse redness are present.[2] Clinical features of TEN are characterized by the acute onset of erythema and tenderness of mucosal and cutaneous surfaces followed by extensive epidermal exfoliation. The initial symptoms of the diseases include high fever, as well as conjunctivitis, pharyngitis and pruritus. The most common cause of TEN is adverse reaction to drugs, such as sulfonamides, antibiotics, barbiturates, hydantoins and non-steroidal anti-inflammatory agents.[7] Infections, malignant disorders and graft-versus-host reactions might be other precipitating factors.[8]

In the present patient, based on clinical features we determined that she had extended TEN from SJS. She took acetaminophen and Paburon (Taisho Pharmaceutical) prior to the onset of the disease. Because DLST and serological analyses were negative, we could not determine the offending drug or the causative virus. However, she did seem to have drug-induced TEN based on her clinical course and the timing of the exposure of the drugs under preceding infection with an undetermined pathogen.

There have been several reports of TEN but the pathophysiology remains unknown. It has been speculated that cytotoxic T cells expressing the skin homing receptor cause epidermal necrolysis with subepidermal bulla formation in TEN. The epidermis splits at the basal layer and the inflammatory reaction is rich in CD8+ T cells. Leyva *et al.* have reported that an overexpression of TNF-α, IFN-γ and IL-2 was observed in peripheral blood cells of patients with TEN.[9] These cytokines might induce the aberrant expression of human leukocyte antigen-DR and Fas on the keratinocyte, leading to keratinocyte death, and it was also reported that increased production of TNF-α leads to necrosis of the epidermis.[9] In the present case we detected slightly, but not significantly, increased levels of inflammatory cytokines in the serum on admission. However, the steroid therapy administered at the nearby hospital might explain this discrepancy.

Table 1 Cytokines in peripheral blood of the patient

Date	IFN-γ	TNF-α	IL-10	IL-5	IL-4	IL-2	IL-6
Day 8	12.4	0	5.5	1.1	2.2	1.6	6.2
Day 14	0	0	3.4	0	4.3	1.2	2.8
Day 33	0	0	1.5	0	0	0	ND
Day 54	2.6	0	3.7	1.6	1.9	ND	ND

Day 1, day the suspected causative drugs were taken. Normal ranges of all seven cytokines in serum are <0.1 pg/mL. IFN, interferon; IL, interleukin; ND, not determined; TNF, tumor necrosis factor.

Therapy for TEN has progressed and several kinds of drugs including systemic corticosteroids, cyclophosphamide, immunoglobulin,[3] ulinastatin[10] and thalidomide[6] have been used for the treatment of TEN with unsatisfactory outcomes. Recent reports of TEN adult patients successfully treated with CsA have been published.[11–14] CsA has many inhibitory effects on the main cell populations involved in TEN (T cells, macrophages and keratinocytes) including anti-apoptotic properties, and it also promotes re-epithelialization. For this reason and because oral PSL was not effective, we decided to treat the present patient with i.v. CsA and methylprednisolone. G-CSF was also effective and her neutropenia improved promptly.

Many other organs are also affected in TEN. Notably, skin involvement can result in moderate to severe sequelae. Therefore, topical treatments for eyes, mouth and genital and urinary regions are the most important. Monitoring of fluid and electrolyte balance, nutritional support, and prevention of infection are indispensable.

Although oral corticosteroids did not stop the disease progression in the present patient, high-dose methylprednisolone together with CsA controlled severe epidermal necrolysis. TEN is a rare disease especially in children and this is the first report describing a pediatric patient successfully treated with CsA. Further studies of the treatment and etiology of TEN are necessary.

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