be between that of SJS K+ and SJS K− with respect to keratinization-related molecules.

We also found that hyperproliferation-related keratins (CK6/16/17)[15-17] as well as the cell-proliferation marker Ki67 were significantly increased in the pannus epithelia of SJS and OCP patients. These results suggest that the pannus epithelium was in a hyperproliferating state, consistent with the results that we previously reported regarding the conjunctival epithelium of Sjögren syndrome patients.[18,19] Possibly, the sustained epithelial damage resulting from intercurrent severe dry eye led to a wound-healing process in their epithelia.

In summary, our clinical observations based on our recently proposed grading system agreed with the immunohistological status with respect to keratinization, cell proliferation, and corneal/conjunctival cell typing. We believe that our findings will provide useful information toward further understanding of the pathogenesis of OCP and SJS. In addition, we hope that our current results will contribute to the development of future treatment strategies as well as to the prediction of the postoperative prognosis of ocular surface reconstruction in patients with OCP and SJS.

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CORRESPONDENCE

Systemic and Local Management at the Onset of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis with Ocular Complications

EDITOR:

STEVENS-JOHNSON SYNDROME (SJS) AND TOXIC EPIDERMAL necrolysis (TEN) are recognized as two of the most devastating ocular surface disorders and are extremely difficult to treat both at the acute and later stages. Massive inflammation on the ocular surface at the acute stage often is uncontrollable. Even after the acute-stage impairments subside, ocular complications such as serious visual impairment with dry eye and keratinization remain at the later stage. Recently, we reported in our retrospective analysis that visual outcomes at the later stage were significantly better in the group receiving topical steroids at the acute stage compared with the no-treatment group ($P < .00001$).[1] Furthermore, using a prospective study, we confirmed the therapeutic importance of steroid therapy at disease onset for reducing the degree of ocular complications.[2] Therefore, we read with great interest the editorial entitled "Acute Management of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis to Minimize Ocular Sequelae" by Scheffer C. G. Tseng,[3] in which the author states that "systemic high-dose of glucocorticoids may heighten the risk of inciting (existing) infections in the wake of managing patients with life-threatening diseases." In this editorial, the author suggests that amniotic membrane transplantation (AMT) may be a better strategy for reducing immunoreactions on the ocular surface. However, in some aspects, we are not in agreement with the author's comments.

First, the author pointed out the difficulties of distinguishing SJS and TEN from erythema multiforme, which is known to be a high underlying cause of herpes simplex virus infection.[4] However, a biopsy of the skin lesion can reveal the histologically characteristic appearance of SJS and TEN such as apoptosis, necrosis, and epidermal detachment.[5] Moreover, bilateral eye symptoms before or concurrently with the onset of skin eruptions and oral and nail involvement help the clinician to recognize SJS and TEN with ocular involvement.[1] Thus, we can distinguish most cases of SJS and TEN with ocular involvement from erythema multiforme at disease onset. As we mentioned in our report, the possibility of common viral and microbial infections can be excluded by polymerase chain reaction and microbial cultures of the tissue and the blood. The delay of the diagnosis itself increases the risk of secondary infection at erosive sites.

Second, our recent study proved that the patients with SJS and TEN are severely affected systemically and on the ocular surface because of a cytokine storm that occurs at the acute phase (unpublished data). Thus, a systemic therapeutic method such as steroid pulse treatment is essential for subsiding this cytokine storm, controlling the ocular surface inflammation in an indirect manner. Although we understand that AMT may be effective in diminishing ocular surface inflammation and immunoreactions, AMT alone cannot manage systemic inflammation. Furthermore, details of the general management of SJS and TEN by AMT are uncertain in most published reports.[6] In addition, in the patients with poor general conditions, surgical treatment is very difficult and general anesthesia is impossible.

In conclusion, steroid pulse therapy and topical betamethasone should be considered to prevent corneal epithelial stem cell loss in the limbal region and cicatricial changes, and AMT may prove to be a beneficial addition to this therapy.

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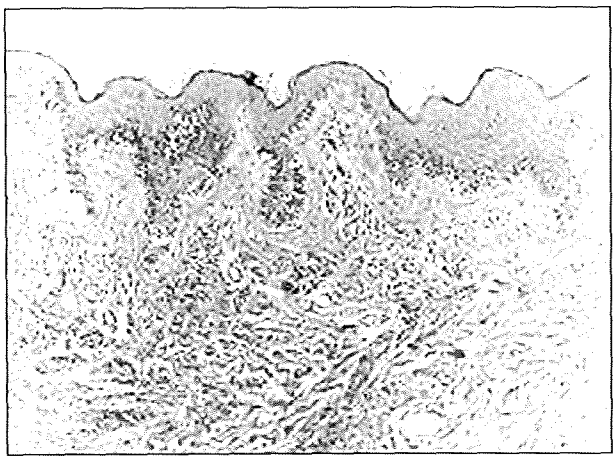

Figure 9. Cockade nevus demonstrating a central compound nevus with a peripheral, nonpigmented junctional pattern (hematoxylin-eosin, original magnification ×10).

plastic than are nevi elsewhere on the body.[6] Moreover, scalp nevi may have clinically worrisome features, but their unique histologic characteristics often do not meet the criteria for melanoma, a dysplastic nevus, or a Clark nevus.[7]

In our experience, nevi that fit the clinical description of a cockade or eclipse nevus do not warrant surgical excision. Consequently, children with these nevi do not need surgery. However, they would benefit from parental instruction on how to conduct home mole checks, and if they have numerous melanocytic nevi, multiple atypical nevi, and/or a history of melanoma in a first-degree family member, they should undergo regular skin examinations by a dermatologist.

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Additional Contributions: Jean Bolognia, MD, Earl Glusac, MD, and Ayca Yazici, MD, provided photographic contributions.

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COMMENTS AND OPINIONS

The Management of Severe Ocular Complications of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are recognized as 2 of the most devastating ocular surface diseases, and they are extremely difficult to treat both at the acute and later stages. Massive inflammation on the ocular surface at the acute stage is often uncontrollable. Even after the acute-stage impairments subside, ocular complications such as serious visual impairment with dry eye and keratinization remain. With these facts in mind, we read with great interest the article titled "Risk Factors for the Development of Ocular Complications of Stevens-Johnson Syndrome and Toxic Epidermal Necrolysis," by Gueudry et al,[1] in which the authors assert that the only significant risk factor of late ocular involvement of SJS and TEN is the severity of initial eye involvement. In their report, they suggest that the use of systemic and local corticosteroids might not be able to prevent late ocular complications. However, we believe that the authors should not draw such a conclusion because topical corticosteroid treatment was used in only 1 case in their study.

Contrary to the conclusions drawn by Gueudry et al,[1] our research group[2] found, in a retrospective analysis, that vision outcomes at the later stages of SJS and TEN were significantly better in patients treated with topical steroids during the acute-stage than in patients who received no treatment ($P<.001$). Furthermore, in a prospective study,[3] our group confirmed the therapeutic importance of steroid therapy at disease onset for reducing the degree of ocular complications.

We believe that the patients described by Gueudry et al[1] had less severe disease than did the patients in our studies.[2,4] None of the patients described by Gueudry et al developed ankyloblepharon, and only 4 patients mani-

fested visual loss. However, 44 of 138 eyes in our studies showed ankyloblepharon (32%), and visual impairment was serious.

In patients with SJS and severe ocular complications, the total loss of the palisades of Vogt is the key ocular surface manifestation, which implies a complete loss of corneal epithelial stem cells.[4,5] The loss of these cells can occur during the acute stage due to severe ocular surface inflammation, thus resulting in conjunctivalization and vascularization on the cornea that leads to severe visual impairment. Therefore, to rescue corneal epithelial cells, steroid pulse therapy and topical steroid application are therapeutic techniques that must be considered. We are in general agreement with Gueudry et al[1] that all patients with SJS or TEN should undergo initial ophthalmologic screening and ophthalmologic follow-up during the acute phase of the disease, but it is of vital importance also to examine the ocular surface of those patients and pay special attention to epithelial defects and ocular surface inflammation at the acute stage.

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Additional Contributions: John Bush assisted with the preparation of the manuscript.

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In reply

We appreciate the interest and comments of Sotozono et al regarding our article on the severity and management of ocular complications of SJS and TEN.[1] Both conditions are recognized as 2 of the most devastating ocular surface diseases, and there is no standardized ophthalmologic treatment for the prevention of ocular complications.

Because accumulated clinical and experimental data suggest that the mechanisms of SJS and TEN depend on a medication-specific immune response, corticosteroids and immunosuppressive drugs are used by many physicians. However, these drugs are of unproven benefit at the acute stage, and their use remains highly controversial.[2,3] Furthermore, results of retrospective analyses performed in several burn departments in the United States suggest that the prolonged use of high doses of systemic corticosteroids is associated with increased mortality.[4] That is why we noted in our discussion[1] that current management strategies based on the use of systemic and local corticosteroids seem unable to prevent late ocular complications, even though our study did not assess corticosteroid efficacy.

More recently, 2 studies were published concerning corticosteroid use with the aim of preventing ocular complications associated with SJS and TEN.[5,6] The results of these studies are promising, and the authors are to be congratulated. However, the studies have several limitations. Concerning the article about the use of corticosteroid eyedrops,[6] we think that the control group is not quite relevant. Thus, the authors compared vision outcomes at the later stage in a group of patients who had received topical corticosteroids with the vision outcomes of a group of patients who had not received any treatment for their eyes during the acute stage. We cannot agree with the conclusion that corticosteroids improve the vision prognosis. In fact, ophthalmologic supportive care might have a similar effect. Furthermore, the care associated with topical corticosteroid administration might have had some effect in the patients who received treatment.

Concerning the study that examined the use of systemic corticosteroids,[5] the number of patients was too small to allow discrimination between the efficacy of corticosteroids and the natural evolution of SJS and TEN. Therefore, further clinical studies are essential to settle the controversy regarding the appropriate treatment of SJS and TEN. We suggest that controlled prospective studies may be useful, even though such studies would be difficult to organize because of the low incidence of these conditions.

The different rates of severe ocular complications associated with SJS and TEN found by Araki et al[5] and Sotozono et al[6] compared with the rates we found[1] may reflect selection bias. In their studies, patients were referred for ocular complications management, while our study analyzed all patients with SJS or TEN who were hospitalized in a dermatology department during the acute stage of the disease.

We completely agree that the ocular surfaces of these patients must be examined and special attention paid at the acute stage. In addition, more aggressive ophthalmologic treatment protocols might be offered to patients with severe ocular complications to prevent the occurrence of sequelae.

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Linking Publication About Efalizumab Effectiveness With Safety Concerns

The Cutting Edge article by Muñtos-Santos et al,[1] "Response of Keratosis Lichenoides Chronica to Efalizumab Therapy," published in the August 2009 issue of the *Archives*, was accepted on August 12, 2008, which is prior to the recognition of the safety concerns of efalizumab. As noted by Korman et al[2] in the commentary in the same issue, reports of progressive multifocal leukoencephalopathy (PML) in patients treated with efalizumab in the absence of other immunosuppressive agents led the manufacturers to voluntarily withdraw the drug from the market.

At the time the drug was withdrawn from the market, the *Archives* ceased considering manuscripts discussing the dermatologic indications of this drug. The final manuscript in our inventory of manuscripts accepted prior to withdrawal of the drug was published in the same issue as the commentary by Korman et al[2] to alert our readers to concerns about using efalizumab for dermatologic indications. On February 19, 2009, both the European Medicines Agency and the US Food and Drug Administration issued safety concerns regarding the risk of PML with the use of efalizumab for the treatment of psoriasis.[3] This letter to the editor ensures that the National Library of Medicine links the article by Muñoz-Santos et al[1] to articles discussing the reasons for withdrawal of the drug.[2,3]

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Additional Information: Dr Robinson is Editor of the *Archives of Dermatology*.

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VIGNETTES

Leukonychia Related to Vorinostat

We herein describe a new clinical finding of leukonychia occurring in 3 patients with mycosis fungoides (MF) being treated with vorinostat (Zolinza; Merck & Co Inc, Whitehouse Station, New Jersey), a zinc-dependent histone deacetylase (HDAC) inhibitor approved for the treatment of cutaneous T-cell lymphoma.[1]

Report of Cases. *Case 1*. Patient 1 was a 70-year-old white woman with stage IVA MF treated previously with methotrexate, interferon alfa, bexarotene, topical nitrogen mustard, and psoralen–UV-A therapy (PUVA). She had been taking vorinostat, 300 to 400 mg/d, for 6 months when diffuse leukonychia of the fingernails was noted (**Figure 1**). Vorinostat treatment was discontinued after 10 months owing to persistent disease, and by 10 months later, her nails had returned to normal.

Case 2. Patient 2 was a 70-year-old white man with stage IIB MF previously treated with interferon alfa, bexarotene, local and total-body electron beam irradiation, and PUVA. The patient had taken vorinostat, 400 mg/d, for 40 weeks when leukonychia totalis of all fingernails was noted (**Figure 2**). Four months after discontinuing vorinostat treatment, the leukonychia had completely resolved.

Case 3. Patient 3 was a 44-year-old African American man with stage IIB MF previously treated with interferon alfa, denileukin diftitox, methotrexate, bexarotene, local and total-body electron beam irradiation, etoposide, topical nitrogen mustard, topical carmustine, and PUVA. Six months after treatment with vorinostat, 400

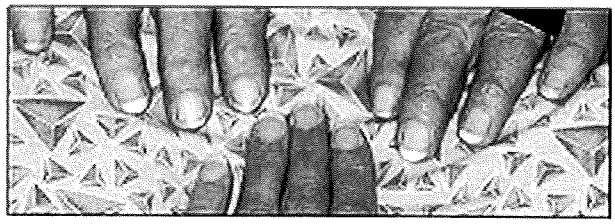

Figure 1. Clinical comparison of the fingernails of patient 1 after 6 months of vorinostat treatment (top, 2 hands) with normal fingernails (bottom, 1 hand).

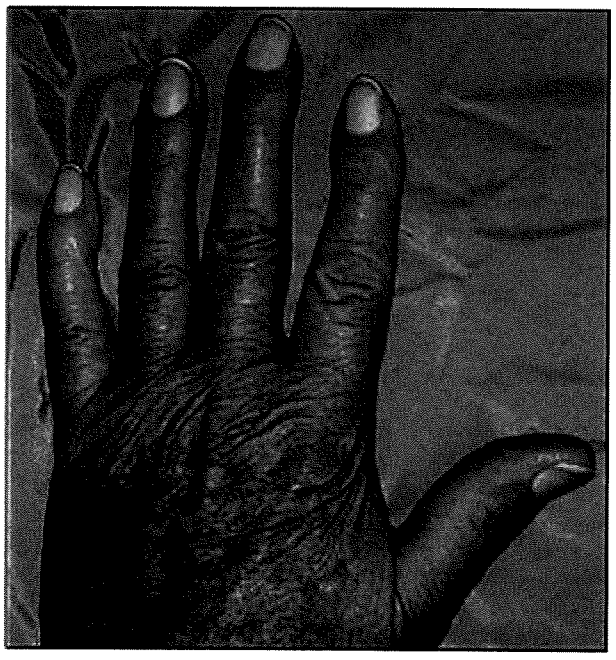

Figure 2. Clinical appearance of the fingernails of patient 2 after 10 months of vorinostat treatment.

Identification of a novel HLA-B allele, HLA-B*5904

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Key words: B*5904; human leukocyte antigen-B; new allele

The new human leukocyte antigen (HLA) class I allele, HLA-B*5904 was identified in Japanese individual. HLA-B*5904 differs from HLA-B*5901 by two non-synonymous nucleotide exchanges at codon 163 (ACG to CTG).

The human leukocyte antigen (HLA)-B locus is one of the most polymorphic regions of the human genome. Its members increase continuously with the development of genotyping technology. Thousand one hundred and nine alleles were found at HLA-B locus up to now. HLA-B*59 is

a small family among HLA-B genes. There are only three members altogether from HLA-B*5901 to B*5903 (1). Here, we report on a novel allele, HLA-B*5904.

The HLA-B*5904 was found in a Japanese patient suffered from Stevens–Johnson syndrome with ocular complications. The sample was determined by the low-to-intermediate resolution kit (HLA-B, WAKFlow HLA typing kit; Wakunaga, Hiroshima, Japan). Typing result was inconclusive with one missing probe, resulting with a clear software interpretation of no match found. Closest possible match was indicated as B*5101 and B*5901. To further analysis of the sample, exons 2 and 3 were sequenced in forward and reverse directions. The sequencing result showed a new HLA-B*59 allele in the sample.

To confirm the presence of a novel allele in the sample, DNA fragment including exons 2 and 3 was cloned into the pT7Blue-2 vector (Merck, Darmstadt, Germany). DNA inserts from the cloned alleles were sequenced using M13F (−20) and T7 primers in both directions. The sequence analysis showed that the new allele differed from B*5901 at positions 559 and 560, codon 163 (ACG to CTG), that change the amino acid from threonine to leucine (Figure 1). The sequence 'CTG' at codon 163 is seen in many other HLA-B groups (B*15, B *35, B*44, B*45, B*46, B*49, B*51, B*52, B*53, B*56, B*57, B*58, and B*78). The HLA-B*5904 might have been arisen by gene-conversion-like event of a short fragment in B*5901 with some allele in these groups as donor. Residue 163 is located at the alpha helix of alpha 2 domain, which is the part of the binding pocket of the HLA class I molecule contributing to the binding properties and specificity of the HLA class I molecule (2). Thus, the new HLA class I allele, HLA-B*5904, may substantially differ in its peptide-binding repertoire from the most common B*5901 allele. The sample was typed as A*0201, 0206; B*5101, B*5904; Cw*0102, 1502; DRB1*0405, 1202; DQB1*0301, 0401.

The nucleotide sequence can be viewed in the DDBJ database under the accession number AB467317. The name HLA-B*5904 has been officially assigned by the World Health Organization (WHO) Nomenclature Committee in November 2008. This follows the agreed policy that, subject to the conditions stated in the most recent Nomenclature report (3), names will be assigned to the new sequences as they are identified. Lists of such new names will be published in the following WHO Nomenclature report.

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                      -20                 -15                 -10                 -5
B5901    ATG CGG GTC ACG GCA CCC CGA ACC CTC CTC CTG CTG CTC TGG GGG GCC CTG GCC CTG ACC GAG ACC TGG GCC G|GC
B5904    *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *|--

                    5                   10                  15                  20                  25
B5901    TCC CAC TCC ATG AGG TAT TTC TAC ACC GCC ATG TCC CGG CCC GGC CGC GGG GAG CCC CGC TTC ATC GCA GTG GGC
B5904    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                   30                  35                  40                  45                  50
B5901    TAC GTG GAC GAC ACG CAG TTC GTG AGG TTC GAC AGC GAC GCC GCG AGT CCG AGA GAG GAG CCG CGG GCG CCG TGG
B5904    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                   55                  60                  65                  70                  75
B5901    ATA GAG CAG GAG GGG CCG GAG TAT TGG GAC CGG AAC ACA CAG ATC TTC AAG ACC AAC ACA CAG ACT TAC CGA GAG
B5904    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                   80                  85                  90                  95                  100
B5901    AAC CTG CGG ATC GCG CTC CGC TAC TAC AAC CAG AGC GAG GCC G|GG TCT CAC ACT TGG CAG ACG ATG TAT GGC TGC
B5904    --- --- --- --- --- --- --- --- --- --- --- --- --- --- -|-- --- --- --- --- --- --- --- --- --- ---

                  105                 110                 115                 120                 125
B5901    GAC CTG GGG CCG GAC GGG CGC CTC CTC CGC GGG CAT AAC CAG TTA GCC TAC GAC GGC AAG GAT TAC ATC GCC CTG
B5904    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                  130                 135                 140                 145                 150
B5901    AAC GAG GAC CTG AGC TCC TGG ACC GCG GCG GAC ACC GCG GCT CAG ATC ACC CAG CGC AAG TGG GAG GCG GCC CGT
B5904    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                  155                 160                 165                 170                 175
B5901    GTG GCG GAG CAG CTG AGA GCC TAC CTG GAG GGC ACG TGC GTG GAG TGG CTC CGC AGA TAC CTG GAG AAC GGG AAG
B5904    --- --- --- --- --- --- --- --- --- --- --- CT- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                          ACG --> CTG
                                          Thr --> Leu
                                              163

                  180                 185                 190                 195                 200
B5901    GAG ACG CTG CAG CGC GCG G|AC CCC CCA AAG ACA CAC GTG ACC CAC CAC CCC ATC TCT GAC CAT GAG GCC ACC CTG
B5904    --- --- --- --- --- --- -|** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** *** ***
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Figure 1 Alignment of the nucleotide sequence of exon2 and 3 of the new allele HLA-B*5904 with HLA-B*5901. Dashes indicate identity with the B*5901 sequence. Numbers above correspond with the amino acid position in the mature protein. HLA, human leukocyte antigen.

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Examination of *Staphylococcus aureus* on the Ocular Surface of Patients With Catarrhal Ulcers

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Purpose: The purpose of this study was to investigate the role of *Staphylococcus aureus* in the onset of ocular catarrhal ulcers.

Methods: We examined the colonization by *S. aureus* of the ocular surface (conjunctival sac, upper and lower lid margins) of 3 patients with catarrhal ulcers and analyzed the *S. aureus* isolates by pulsed-field gel electrophoresis.

Results: *S. aureus* organisms were found on the lid margin of all eyes affected by catarrhal ulcers. The contralateral eye without ulcers harbored *S. aureus* exhibiting a pulsed-field gel electrophoresis pattern identical to that of the affected eye.

Conclusions: Although *S. aureus* on the lid margin plays an important role in the onset of catarrhal ulcers, its presence is one among several risk factors.

Key Words: *Staphylococcus aureus*, catarrhal ulcer, pulsed-field gel electrophoresis (PFGE)

(*Cornea* 2009;28:780–782)

INTRODUCTION

Catarrhal ulcers are usually a complication of long-standing staphylococcal blepharitis, conjunctivitis, or meibomitis,[1,2] which might sometimes be subclinical, and cultures from the lid margins of affected patients usually yield colonies of *Staphylococcus aureus*,[2] although lid margins of normal eyes also sometimes, but not usually, have *S. aureus*.[3,4] Because corneal cultures are usually negative for the organisms, it has been suggested that catarrhal ulcers are not the result of direct infection of the cornea, but rather derive from an antigen–antibody reaction with complementary activation and neutrophil infiltration in patients sensitized to staphylococcal antigens.[1,5,6]

Catarrhal infiltrates and ulcers are frequently seen by ophthalmologists and because they readily respond to adequate treatment, they do not attract much attention in the literature. To the best of our knowledge, this is the first pulsed-field gel electrophoresis (PFGE) analysis of the relationship between catarrhal ulcers and the presence of *S. aureus*.

MATERIALS AND METHODS

The diagnosis of catarrhal ulcer in our 3 patients was based on ocular surface manifestations. The patients were 15- (Case 1, Fig. 1A), 81- (Case 2, Fig. 1B), and 55-year-old (Case 3, Fig. 1C) females. In all patients, the right eye was involved. Clinical examinations revealed oval infiltrates and ulcers separated from the limbus by a distinct lucid border and adjacent conjunctival inflammation. We examined 3 ocular sites (the conjunctival sac and the upper and lower lid margins) for the presence of bacteria; in cases 1 and 2, we examined both eyes and in case 3 only the affected eye. Using PFGE analysis, we analyzed and compared the *S. aureus* organisms isolated from 2 or more sites in each patient.

The isolates obtained were stored (–20°C) in ANAport BIKEN culture medium (BIKEN, Osaka, Japan) at the Department of Ophthalmology of Kyoto Prefectural University of Medicine; they were sent to The Research Foundation for Microbial Diseases of Osaka University the next day. The isolates were cultured in both methods, direct culture and enrichment culture, as previously reported.[3,7]

We used the GenePath system (Bio-Rad Laboratories, Hercules, CA) to perform PFGE according to the manufacturer's instructions (GenePath Group I Reagent Kit; Bio-Rad) and visually compared the DNA banding patterns as described by Tenover et al.[8]

RESULTS

The colonization by *S. aureus* is shown schematically in Figure 2A. In case 1, *S. aureus* was detected in the lower lid margin of the affected and the conjunctiva of the unaffected eye. The PFGE patterns of the organisms from both eyes were identical (Fig. 2B-1), suggesting that they derived from the same clone. In case 2, *S. aureus* was detected in the upper lid margin and conjunctiva of the affected and in the lower lid

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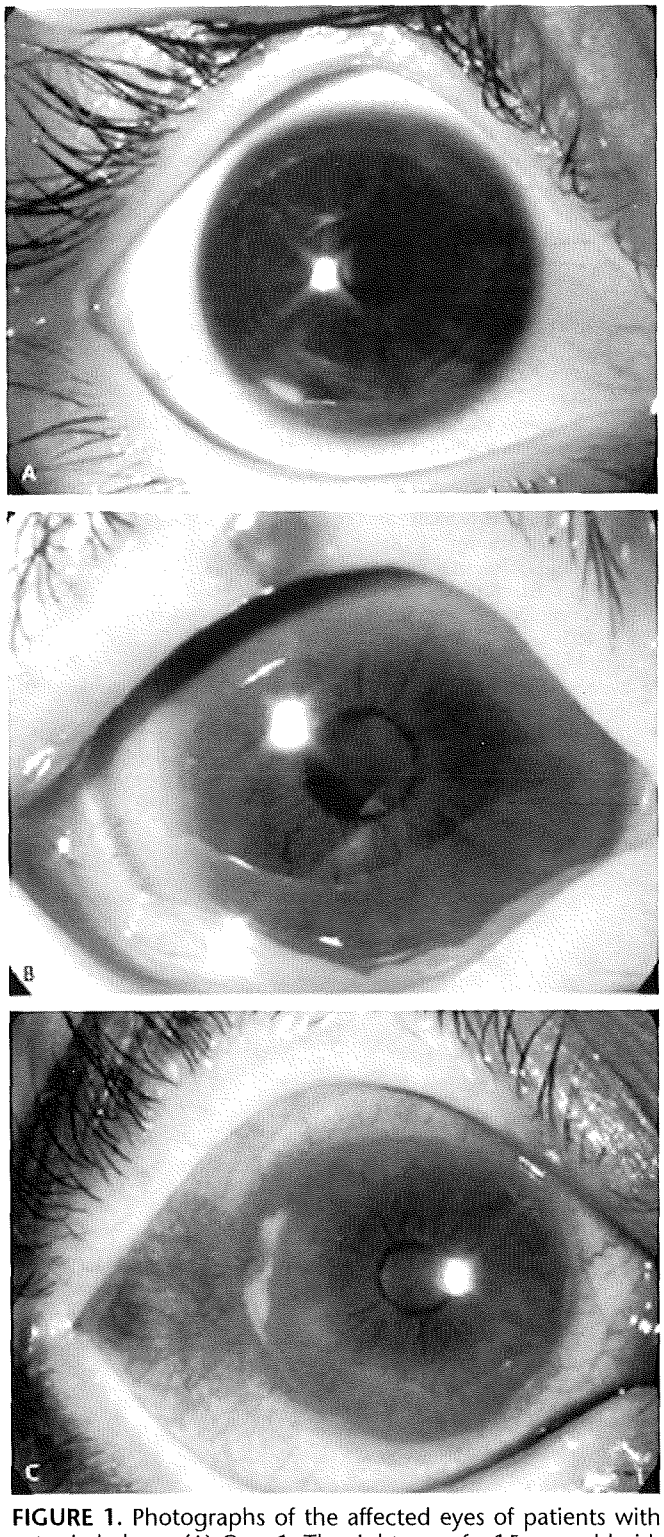

FIGURE 1. Photographs of the affected eyes of patients with catarrhal ulcers. (A) Case 1: The right eye of a 15-year-old girl. (B) Case 2: The right eye of an 81-year-old woman. (C) Case 3: The right eye of a 55-year-old woman.

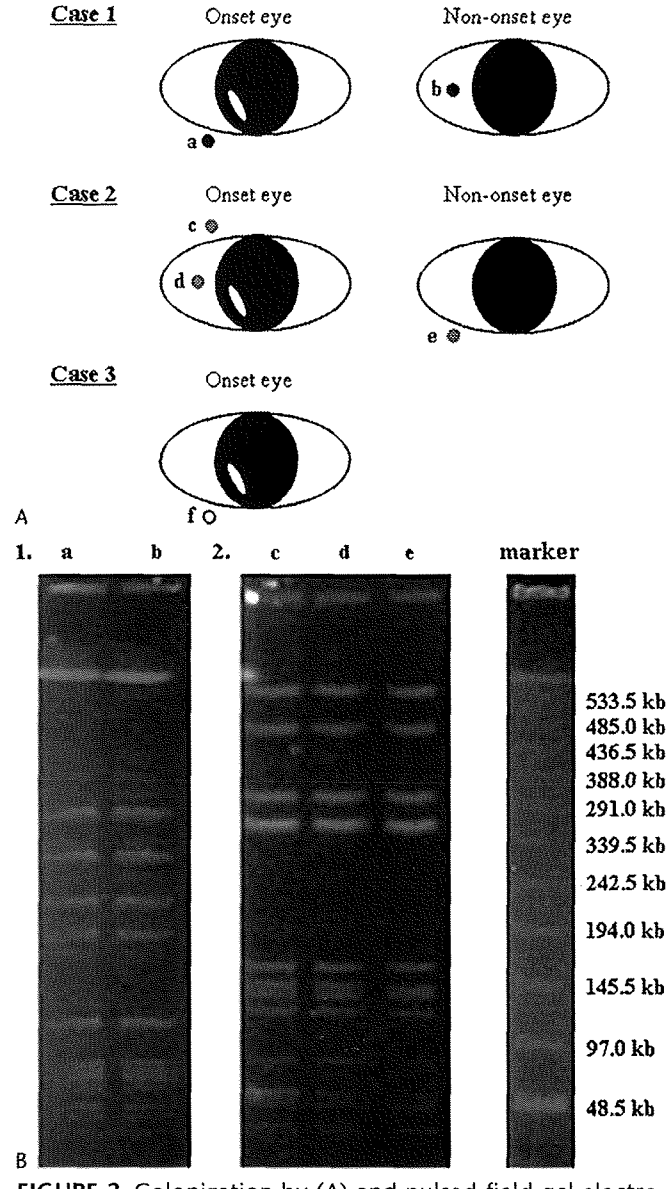

FIGURE 2. Colonization by (A) and pulsed-field gel electrophoresis (PFGE) analysis of (B) *Staphylococcus aureus*. Case 1: *S. aureus* was detected in the lower lid margin (A) of the affected and the conjunctiva (B) of the unaffected eye. The organisms from the 2 sites showed identical PFGE patterns (Fig. 2B-1). Case 2: *S. aureus* was detected in the upper lid margin (C) and the conjunctiva (D) of the affected eye and in the lower lid margin (E) of the unaffected eye. These organisms from the 3 sites exhibited identical PFGE patterns (Fig. 2B-2). Case 3: *S. aureus* was detected in the lower lid margin of the affected eye (F). We used the GenePath System for PFGE. Bacterial chromosomal DNA was cut with SmaI. The PFGE patterns were obtained by running digested DNA on 1% agarose gels in a CHEF-DR Mapper. A lambda ladder was used as the molecular size marker.

margin of the unaffected eye. They also manifested identical PFGE patterns (Fig. 2B-2), suggesting that they originated from the same clone. In case 3, *S. aureus* were detected in the lower lid margin of the affected eye; the unaffected eye could not be examined because the patient gave consent to examine only the affected eye.

DISCUSSION

Although our study included only a small number of patients, we found *S. aureus* to be present in the lid margin of the eyes affected by catarrhal ulcers. This might suggest that their presence in the lid margin, rather than the conjunctival sac, is important for the development of catarrhal ulcers. Because we were able to detect all *S. aureus* organisms in enrichment cultures, it appears that the development of catarrhal ulcers does not require the presence of large amounts of the bacterium.

Interestingly, in case 2, we also found *S. aureus* in the lid margin of the unaffected eye. It means that if the patient, who was sensitized to staphylococcal antigens, has *S. aureus* on both eyes, the catarrhal ulcer may occur on only one eye but not the fellow eye. Moreover, our PFGE analysis showed that *S. aureus*, which was detected in both eyes, might be derived from the same clone, suggesting that the kind of clone of *S. aureus* is not necessarily important for the initiation of the catarrhal ulcers.

Although our study included only a small number of patients, our findings might suggest that other factors may be necessary for the initiation of the catarrhal ulcers in addition to the existence of *S. aureus* on the lid margin and the patients' sensitivity to staphylococcal antigens. One possible factor may be an immune abnormality of the ocular surface of the affected eye. A second possible factor may be the condition of contact between the cornea and the lid margin such as a subtle difference of pressure and/or angle of the lid margin, although it is not apparent in clinical findings. A third possible factor may be a difference in the amount of bacterium between the affected eye and unaffected eye, although the development of catarrhal ulcers does not require the presence of large amounts of the bacterium. Investigations are underway to shed light on the pathogenesis of catarrhal ulcers.

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The influence of hepatic damage on serum soluble Fas ligand levels of patients with drug rashes

To the Editor:

We read with interest the report of Murata et al[1] stating that increases in soluble Fas ligand (sFasL) levels were observed in patients with Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) in the very early phases but not in maculopapular (MP) types of drug rash. However, we reported recently that increases in serum sFasL levels were observed not only in patients with SJS and TEN but also in those with drug-induced hypersensitivity syndrome (DIHS) and MP-type drug eruptions.[2] Examinations were performed in groups of 4 patients each, and the changes in sFasL levels showed a similar pattern among all 3 types of drug rash. The sFasL levels peaked within a week after onset of the rash and then decreased rapidly thereafter. These observations suggested that increases in sFasL levels in patients with drug rashes are not a specific indicator of SJS/TEN. To further confirm this suggestion, we focused on serum sFasL levels within 5 days after onset of drug rash and examined a larger number of cases.

Serum samples were collected from patients with DIHS, MP-type drug rash, erythema multiforme (EM)–type drug rash, SJS, and TEN and subjected to clinical laboratory testing within 5 days of onset. Nineteen serum samples from 13 patients with DIHS, 37 serum samples from 31 patients with MP/EM-type drug rashes, and 13 serum samples from 11 patients with SJS/TEN, which excluded 12 cases reported previously from this examination, were examined. The highest sFasL level was chosen in each patient. sFasL levels were measured with an ELISA kit (R&D Systems, Minneapolis, Minn), and the detection limit was 16 pg/mL. The average sFasL level of patients with SJS/TEN was 96 ± 29 pg/mL, which was similar to that of 16 healthy control subjects (90 ± 59 pg/mL). However, the sFasL levels of patients with DIHS and MP/EM-type drug rashes were significantly higher (patients with DIHS: 214 ± 127 pg/mL, $P = 0.0082$; patients with MP/EM-type drug rashes: 160 ± 81 pg/mL, $P = .0012$) than those of healthy control subjects and patients with SJS/TEN. This result was in agreement with the findings of our previous study.[2]

The Fas–Fas ligand (FasL) system mediates hepatocyte apoptosis in various liver diseases.[3] Upregulated Fas on hepatocytes is engaged with FasL expressed on cytotoxic T cells, inducing apoptosis of hepatocytes.[4] As FasL is shed and released into the serum,[5] serum sFasL levels are increased in patients with acute and fulminant hepatitis.[6,7] Because all patients with DIHS and many patients with MP/EM-type drug rashes were associated with liver dysfunction in the present study, we speculated that the increases in sFasL levels in these patients reflected liver damage. Increases in alanine aminotransferase (ALT) levels within 5 days of onset were observed in 31 of 55 patients. Interestingly, 14 cases in which serum ALT levels were increased by more than 5-fold compared with the normal limit showed significantly increased sFasL levels (Fig 1). In addition, all patients with sFasL levels of greater than 300 pg/mL were associated with increased ALT levels of greater than 5-fold compared with the normal limit.

Taken together, these observations indicate that increase of serum sFasL levels is not specific for SJS and TEN. Rather, higher sFasL levels might indicate hepatic damage. Therefore these values should not be used as an indicator of the development of SJS or TEN.

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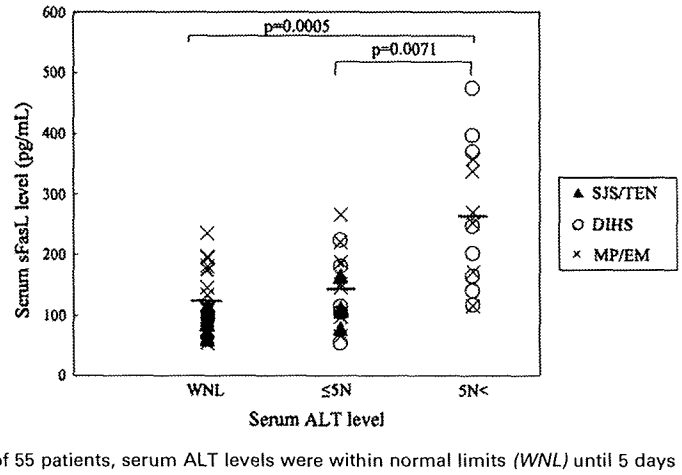

FIG 1. In 24 of 55 patients, serum ALT levels were within normal limits *(WNL)* until 5 days after onset. Increases in ALT levels of 5-fold or less compared with the normal limit *(≤5N)* and of greater than 5-fold compared with the normal limit *(5N<)* were observed in 17 and 14 patients, respectively. The Student *t* test was used for comparison of paired conditions.

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Disclosure of potential conflict of interest: The authors have declared that they have no conflict of interest.

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Reply

To the Editor:

We thank Dr Tohyama et al[1] for their interest in our study.[2] They reported that the difference of serum soluble Fas ligand (sFasL) levels between patients with toxic epidermal necrolysis (TEN)/Stevens-Johnson syndrome (SJS) and maculopapular types of drug rash was not observed.[1,3] This observation is in contrast to our study, in which we detected the highest concentrations of sFasL in 71.4% of patients with TEN/SJS before disease onset (approximately day −4 to −2). Increased sFasL levels decreased rapidly within 5 days of disease onset. In all of 32 patients with ordinary types of drug-induced skin reactions (ODSR), no increase in sFasL level was detected.[2,4]

Several molecules have been reported as important mediators in the pathogenesis of TEN/SJS. A very recent article reported that granulysin is a key molecule responsible for the development of TEN/SJS. In this article it was also shown that sFasL is confirmed as a highly expressed molecule in patients with TEN/SJS.[5]

Several points are warranted in the interpretation of the results of Tohyama et al.[1] In particular, we reported that sFasL levels were decreased after day 3.[2] Detailed information on sample collection was not described in the correspondence by Tohyama et al.[1] If the serum samples were collected after day 3, sFasL levels should have returned to within the normal range. We have to emphasize that it is very difficult to distinguish clinical presentations of TEN/SJS at the early stage from ODSR. Therefore it is crucial to collect and analyze the samples of TEN/SJS at an early stage.

Additionally, in the correspondence by Tohyama et al,[1] serum levels of sFasL seemed to be higher than those seen in our study. In our study sFasL levels of healthy control subjects were 42.8 ± 8.2 pg/mL,[2] whereas they were 90 ± 59 pg/mL in Tohyama et al.[1]

Furthermore, Tohyama et al[1] used a different definition of disease onset of TEN/SJS compared with ours. A major previous report defined disease onset as when erosion/ulceration of mucocutaneous or ocular lesions are first developed,[6] and we followed that precedent. In contrast, in Tohyama et al's correspondence,[1] onset is defined as the day when the rash appears. It is well known that the disease course of TEN/SJS is variable; some patients have erosion/ulceration without erythema, and other show only erythema for several days before erosion/ulceration appears. Because erosion/ulceration or ocular lesions are essential manifestations of TEN/SJS, the presence of markers such as sFasL or granulysin to distinguish the early stage of TEN/SJS from ODSR is crucial.

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Activation of the blood coagulation cascade is involved in patients with chronic urticaria

To the Editor:

Chronic urticaria (CU) is a continuously recurrent whealing of the skin with pruritus and usually defines its course as 6 weeks and more. As reported in the JACI, Asero et al[1] found that the tissue factor pathway of blood coagulation might be active in patients with CU.

Thirty adult patients (men/women, 11/19; median age, 40.88 years; range, 18-66 years) with CU and 30 normal subjects (men/women, 11/19; median age, 39.93 years; range, 18-58 years) were enrolled in the study. Disease activity was estimated according to the number of wheals presented. Prothrombin time (PT) and partial thromboplastin time (APTT) were measured by the coagulation method (Dade Behring Marburg GmbH, Marburg, Germany). The level of D-dimer was tested with a turbidimetric immunoassay kit (Dade Behring Marburg GmbH). Levels of plasma activated Factor VII (FVIIa) and thrombin-antithrombin complex (TAT) were measured by ELISA kit (FVIIa: American Diagnostic Inc, Stanford, Conn; TAT: AssayPro, St Charles, Mo). Means were compared by *t* test. Differences in the levels of FVIIa, TAT, and D-dimer were assessed by the Wilcoxon-Mann-Whitney nonparametric test. Correlations between the various parameters were assessed by the Spearman test.

Disease activity of patients was graded as slight, 4; moderate, 11; severe, 7; and very severe, 8. The results in Table I show that PT and APTT were in the normal range, and the levels of FVIIa,

cell to detach from their substratum, thus allowing metastatization; on the other hand it is now clear that TnC produced from stromal cells has an opposite function. A favourable function of stromal TnC would be that it helps to create a dense desmoplastic stroma which may act as a boundary to contain tumor cells, thus hindering cancer invasion. Our results may suggest that in the analysed SLNs, negative for the presence of tumoral cells as demonstrated by histology and immunohistochemistry, TnC is mainly produced by stromal cells, probably playing a biological defensive role. We believe that this preliminary study, as well as other performed by our group at molecular level on the SLNs may be useful to outline molecular prognostic factors in melanoma patients [7–10]. Furthermore, it would be crucial to evaluate if the positive expression of TnC in SLNs is due to the stroma or to tumoral cells eventually present in the SLN. Secondary, it should be appropriate to investigate the expression of TnC by IHC assay in an adequate number of SLN in order to verify if the significance of the statistical analysis is maintained using a technique more easily applied to prognostic purpose, even if less sensitive than RT-PCR. Finally it is our future aim to investigate the role of different spliced isoforms of TnC in SLNs to identify which isoform may better represent a molecular marker for melanoma patients.

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Letter to the Editor

Effects of non-steroidal anti-inflammatory drugs (NSAIDs) on serum allergen levels after wheat ingestion

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To the Editor

Absorption of food allergens from the intestinal tract is thought to be important in the development of food allergies. A recent report showed that intestinal permeability to macromolecules was increased in patients with adverse reactions to food [1]. Passage of intact dietary allergens, such as β-lactoglobulin and ovalbumin in meal, into the blood has been documented [2]. In our previous study, we demonstrated that the circulating levels of gliadin, which is recognized as an allergen for a variety of wheat allergies, were correlated with the clinical symptoms in patients with wheat-dependent exercise-induced anaphylaxis (WDEIA) [3,4]. Interestingly, increased serum gliadin levels induced by exercise and aspirin were also observed in healthy subjects. In addition, a recent case report described a patient with WDEIA induced by low-dose aspirin therapy for secondary prevention of cardiovascular events [5]. Intake of aspirin enhances intestinal permeability, which is associated with gastrointestinal disorders [6]. Thus, it is hypothesized that aspirin facilitates the absorption of intact gliadin due to an increase in gastrointestinal permeability. However, the dose effects of aspirin and administration of other

non-steroidal anti-inflammatory drugs (NSAIDs) on the absorption of wheat gliadin remain unclear. The purpose of the present study was to evaluate the effects of administration of low-dose aspirin and conventional-dose of diclofenac, loxoprofen and meloxicam on serum gliadin levels after wheat ingestion in healthy subjects.

Seven healthy volunteers (5 males, 2 females; mean age: 39.1 years; range: 25–48 years) abstained from food for at least 6 h before eating udon, a type of Japanese noodle, made from 120 g of wheat flour. Since common bread wheat includes approximately 6% gliadin, the amount of gliadin ingestion in this study was estimated to be 7.2 g. Each dose of aspirin was administered 30 min before wheat ingestion. Blood samples were taken serially and the serum concentrations of gliadin were determined as described previously [3].

The serum concentrations of gliadin increased very little after wheat ingestion without administration of aspirin. However, various amounts of serum gliadin (17.7–297 pg/mL) were detected in all subjects after administration of 1000 mg of aspirin (Fig. 1). Slight increases in the serum gliadin level (16.9–39.0 pg/mL) were observed in 5 of 7 subjects (subjects 1, 2, 3, 5 and 6) by administration of low-dose aspirin (100 mg). The maximum concentrations of serum gliadin increased in an aspirin dose-dependent manner.

Next, we studied the effects of loxoprofen sodium, diclofenac and meloxicam on the serum gliadin levels after wheat ingestion in 5 subjects (subjects 1–5). Increases in the serum gliadin level were observed in 3 subjects (subjects 1, 2 and 3) after administration of 120 mg of loxoprofen and in 4 subjects (subjects 1, 2, 3 and 5) after administration of 50 mg of diclofenac (Fig. 2). The maximum concentrations of serum gliadin were lower than those when 1000 mg of aspirin was administered. On the other hand, no or only marginal elevation of serum gliadin was induced by administration of 15 mg of meloxicam. In subject 4, the three tested NSAIDs did not increase the serum gliadin levels after wheat ingestion.

To assess the allergenicity of the gliadin detected in sera, the histamine release activities of serum samples containing the maximum concentrations of gliadin from subjects 1, 6 and 7 before and after wheat challenge with 1000 mg of aspirin were examined. Peripheral blood leukocytes containing about 1% basophils were obtained from a WDEIA patient with specific IgE antibodies against recombinant ω5-gliadin (1.57 kUA/L) as described previously [7,8]. When the sera collected from the three subjects after wheat and aspirin challenge were incubated with the basophils, the histamine releases were 7.3, 14 and 9.6%, respectively. In contrast, the sera collected before wheat challenge did not induce histamine release. This result indicates that the gliadin with allergenic activity is absorbed from the intestine.

NSAIDs have the common property of inhibiting cyclooxygenase (COX) activity, which is essential for prostaglandin production. COX exists in two forms, COX-1 and COX-2, and NSAIDs are targeted against COX-2 to reduce inflammation and lower fever. Traditional NSAIDs generally inhibit both isoforms of COX, and inhibition of COX-1 in the gastrointestinal mucosa causes small intestinal damage related to alterations in intestinal permeability. It has been reported that NSAIDs such as aspirin, diclofenac and meloxicam increase intestinal permeability [6,9]. Thus, passage of gliadin into the blood was suggested to be enhanced under the condition of increased intestinal permeability by NSAIDs.

Increased serum gliadin levels induced by 1000 mg of aspirin, 50 mg of diclofenac or 120 mg of loxoprofen, which represent the clinical doses, were observed in almost all the tested subjects. Low-dose aspirin (100 mg) also slightly increased the serum gliadin level. In contrast, an increased serum gliadin level was only observed in 1 of 5 subjects administered 15 mg of meloxicam. Meloxicam, a preferential COX-2 inhibitor, produce fewer gastrointestinal side effects than older non-selective NSAIDs [10]. It has been suggested that meloxicam has less effect on intestinal gliadin permeability. Loxoprofen is a preferential COX-1 inhibitor, similar to aspirin, but did not increase the serum gliadin levels in 2 of 5 subjects. This may be attributed to the weak gastrointestinal ulcerogenicity of loxoprofen due to its prodrug

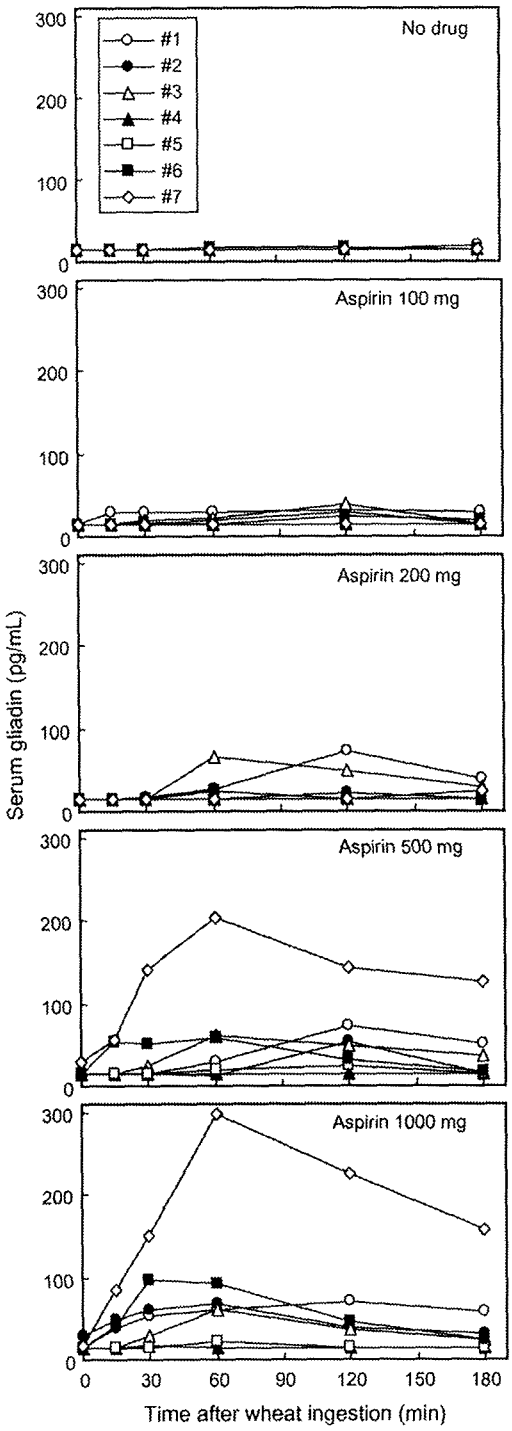

Fig. 1. Individual serum gliadin concentration versus time plots after ingestion of a wheat product and aspirin. Aspirin was administered 30 min before ingesting 120 g of the wheat product. Gliadin levels were measured by ELISA and values under the detection limit are depicted as 15 pg/mL.

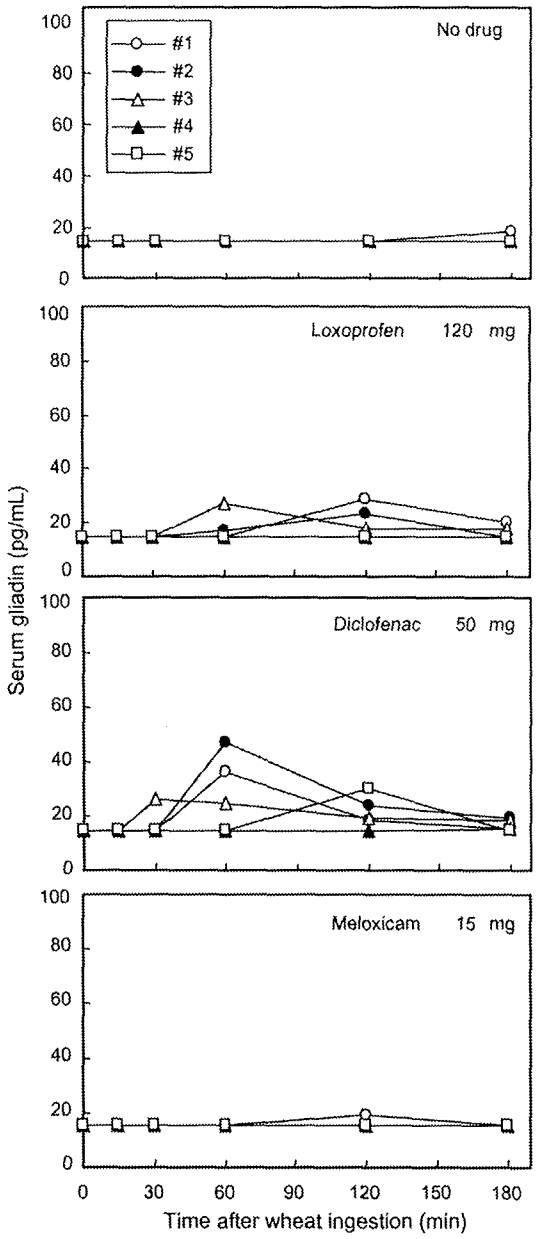

Fig. 2. Individual serum gliadin concentration versus time plots after ingestion of a wheat product and individual NSAIDs. Each NSAID (loxoprofen at 120 mg; diclofenac at 50 mg; meloxicam at 15 mg) was administered 30 min before ingesting 120 g of the wheat product. The gliadin levels were measured by ELISA and values under the detection limit are depicted as 15 pg/mL.

form. Our present results, together with those of previously published studies regarding intestinal alterations, suggest that NSAIDs that cause mucosal injury in the gastrointestinal tract as a side effect facilitate gliadin absorption from the gastrointestinal tract in humans.

In conclusion, the present study clearly demonstrates that aspirin, diclofenac and loxoprofen facilitate the absorption of wheat gliadin with allergenic activity from the gastrointestinal tract into the circulation. Our results suggest that, even for low-dose aspirin, NSAIDs that can cause gastrointestinal hyperpermeability may accelerate symptom development in gliadin-sensitized patients with wheat allergy.

Acknowledgements

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Prostaglandin E_2–EP_3 signaling suppresses skin inflammation in murine contact hypersensitivity

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Background: Prostaglandin (PG) E_2 exerts a variety of actions through 4 G protein–coupled receptors designated as EP_1, EP_2, EP_3, and EP_4. We have reported that PGE_2 acts on EP_3 in airway epithelial cells and exerts anti-inflammatory actions in ovalbumin-induced murine allergic asthma. Although EP_3 is also expressed in skin and PGE_2 is produced abundantly during skin allergic inflammation, the role of PGE_2-EP_3 signaling in skin allergic inflammation remains unknown.
Objective: We sought to investigate whether PGE_2-EP_3 signaling exerts anti-inflammatory actions in skin allergic inflammation.
Methods: We used a murine contact hypersensitivity (CHS) model and examined the role of EP_3 by using an EP_3-selective agonist, ONO-AE-248 (AE248), and EP_3-deficient mice. The inflammation was evaluated by the thickness and histology of the hapten-challenged ear. Inflammation-associated changes in gene expression and effects of AE248 were examined by means of microarray analysis of the skin. Localization of EP_3 was examined by staining for β-galactosidase knocked in at the EP_3 locus in EP_3-deficient mice. EP_3 action was also examined in cultured keratinocytes.
Results: Administration of AE248 during the elicitation phase significantly suppressed CHS compared with that seen in vehicle-treated mice. Microarray analysis revealed that administration of AE248 inhibited the gene expression of neutrophil-recruiting chemokines, including CXCL1, at the elicitation site. X-gal staining in EP_3-deficient mice revealed EP_3 expression in keratinocytes, which was further confirmed by anti-EP_3 antibody in wild-type mice. In cultured keratinocytes AE248 suppressed CXCL1 production induced by TNF-α.
Conclusion: PGE_2-EP_3 signaling inhibits keratinocytes activation and exerts anti-inflammatory actions in murine CHS. (J Allergy Clin Immunol 2009;124:809-18.)

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Murine contact hypersensitivity (CHS) is widely used as a model for contact dermatitis, a common allergic skin disorder of human subjects. The CHS model is composed of 2 phases: the sensitization phase, in which skin dendritic cells take up antigens, migrate to regional lymph nodes, and stimulate T-cell activation and differentiation, and the elicitation phase, in which effector T cells evoke immune inflammation on exposure to antigens.[1] Although the elicitation reaction is known to be mediated by IFN-γ–producing T_H1 cells and T cytotoxic type 1 cells, it is suggested that initial neutrophil infiltration is required for subsequent recruitment of T cells and development of inflammation.[2,3] On exposure to antigens in the elicitation phase, keratinocytes produce neutrophil-recruiting chemokines, such as CXCL1 and CXCL2, as well as T cell–recruiting chemokines, such as CCL17 or CCL27, which contribute to neutrophil recruitment within 12 hours after elicitation and after T-cell infiltration, respectively.[3-5] At an inflammatory site, other than chemokines or cytokines, lipid mediators, such as prostanoids, are produced abundantly, which might regulate CHS responses.[6,7]

Prostanoids, including prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxane A_2, are oxygenated metabolites of arachidonic acid produced by sequential catalysis of COX and respective synthases. They are produced in large amounts during inflammation in response to various stimuli and exert a variety of actions, including inflammatory swelling, pain sensation, and fever generation. Prostanoids exert these actions by acting on a family of G protein–coupled receptors, which include PGD receptor, 4 subtypes of PGE receptor (EP_1, EP_2, EP_3, and EP_4), PGF receptor, PGI receptor, and thromboxane A receptor.[8] In addition, another receptor belonging to the chemokine receptor family, CRTH2, also responds to PGD_2. PGE_2 and PGD_2 are abundantly produced in the skin during the elicitation phase of CHS.[6,9] It has been shown that PGD_2 promotes neutrophil infiltration through CRTH2 and contributes to progression of inflammation during elicitation.[9] However, the role of PGE_2 in the elicitation phase has not been fully investigated. Furthermore, if the above action of the PGD_2-CRTH2 signaling is the only PG-mediated action involved in elicitation of a CHS response, nonsteroidal anti-inflammatory drugs (NSAIDs) that inhibit COX and suppress PG production would suppress or lessen allergic inflammation in the skin. However, NSAIDs are usually without significant effects on the inflammation of CHS, suggesting the presence of other PG receptor–mediated processes that suppress inflammation.

On the basis of this hypothesis, we have examined the action of PGE_2 in allergic skin inflammation. Among EPs, we focused on

Abbreviations used
AE248: ONO-AE-248
CHS: Contact hypersensitivity
DNFB: 2,4-Dinitrofluorobenzene
HE: Hematoxylin and eosin
LT: Leukotriene
NSAID: Nonsteroidal anti-inflammatory drug
PG: Prostaglandin
PMN: Polymorphonuclear leukocyte
WT: Wild-type

EP_3 because EP_3 is expressed abundantly in the skin[10,11] and mediates suppression of allergic inflammation in the murine allergic asthma model.[12] Although EP_3 has been reported to have both proinflammatory and anti-inflammatory roles in patients with acute skin inflammation,[13,14] the role of EP_3 signaling in allergic skin inflammation has not been investigated. Here we used an EP_3-selective agonist and EP_3-deficient *(Ptger3*$^{-/-}$*)* mice and examined whether PGE_2-EP_3 signaling has anti-inflammatory action during the elicitation phase of CHS.

METHODS

Materials

Female 8- to 12-week-old C57BL/6 mice (Japan SLC, Shizuoka, Japan) and mice lacking EP_3 that were backcrossed to a C57BL/6 background for more than 10 generations[15] were used. Mice were bred at the Institute of Laboratory Animals of Kyoto University on a 12-hour light/dark cycle under specific pathogen-free conditions. All experimental procedures were approved by the Committee on Animal Research of Kyoto University Faculty of Medicine. The EP agonists ONO-DI-004 (EP_1 agonist), ONO-AE1-259 (EP_2 agonist), ONO-AE-248 (AE248; EP_3 agonist), and ONO-AE1-329 (EP_4 agonist) were kindly provided by Ono Pharmaceutical Co (Osaka, Japan). The structures, ligand-binding affinities and selectivities, and pharmacokinetic properties of each EP agonist were described.[8] 2,4-Dinitrofluorobenzene (DNFB) was purchased from Nacalai Tesque (Kyoto, Japan). Indomethacin was purchased from Sigma (St Louis, Mo).

CHS experiment

CHS was induced as previously described.[16] Briefly, mice were shaved and painted on the abdomen with 25 μL of 0.5% DNFB in acetone/olive oil (4:1). Five days later, the mice were challenged by painting with 10 μL of 0.3% DNFB on both sides of the ear. Ear thickness was measured with a thickness gage (Teclok, Nagano, Japan) before and 24 hours after the challenge, and the difference was used as a parameter of ear swelling. AE248 was diluted with saline or acetone and administered either subcutaneously in the dorsal skin or topically applied to the ear 3 times a day (30 minutes before and 3 and 8 hours after the DNFB challenge, respectively) at indicated doses. For repeated DNFB application, mice were sensitized first by means of topical application of 20 μL of 0.15% DNFB to both ears and challenged with 20 μL of 0.15% DNFB on both ears once a week for 4 weeks. Vehicle (acetone) or indomethacin (0.2 mg/mL in acetone, 20 μL per ear) was applied 30 minutes before each challenge.

Bone marrow transplantation

Bone marrow cells were taken from femurs from wild-type (WT) or *Ptger3*$^{-/-}$ donor mice and transplanted to recipient WT green fluorescent protein transgenic mice (2×10^6 cells for each mouse from the tail vein) irradiated with 8 Gy. Four weeks after transplantation, more than 97% of whole blood cells were reconstituted with donor-derived cells, which was confirmed by analyzing the expression of green fluorescent protein–positive cells among blood cells with flow cytometry, and we used those mice for experiments.

Histology

Ears were isolated 24 hours after elicitation, fixed in 10% formalin, and embedded in paraffin. Sections of 7 μm in thickness were prepared and stained with hematoxylin and eosin (HE). The number of neutrophils per a ×40 field was determined in 4 randomly chosen fields, and the average counts were determined. For EP_3 localization, X-gal staining was performed as previously described.[12] The sections were then counterstained with HE or anti-keratin 5 antibody (R&D Systems, Minneapolis, Minn). For staining of EP_3, the rabbit polyclonal antibody reactive with murine EP_3 (Cayman, Ann Arbor, Mich) was used as previously described.[17]

Real-time RT-PCR

Total RNA was obtained from keratinocytes of murine ear skin by using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Complementary DNA was synthesized with Superscript III (Invitrogen, Carlsbad, Calif). The amount of mRNA for CXCL1 and glyceraldehyde-3-phosphate dehydrogenase was quantified by means of real-time RT-PCR with the LightCycler 2.0 (Roche Diagnostic, Foster City, Calif). The primer sequences of glyceraldehyde-3-phosphate dehydrogenase were previously described.[18] Primers used for CXCL1 were 5′-GCC TAT CGC CAA TGA GC-3′ (forward) and 5′-TGG ACA ATT TTC TGA ACC AAG-3′ (reverse). Data were analyzed by using LightCycler Software Version 4.0.

Keratinocyte culture and ELISA

Normal human epidermal keratinocytes were obtained from Kurabo (Okayama, Japan) and cultured in Humedia KG2 medium (Kurabo). Cells in the third passage were seeded in triplicate at 5×10^4 cells/well onto 24-well plates in 0.5 mL of Humedia KB2 and cultured for 24 hours. The cells were washed, incubated with 10 μmol/L AE248 for 15 minutes, and then incubated with 10 ng/mL TNF-α in the continued presence of AE248 in Humedia KB2 containing 1 μmol/L indomethacin for 6 hours. The supernatant was collected, and the amount of CXCL1 was determined by means of ELISA (R&D Systems).

DNA microarray analysis

Total RNA was prepared from DNFB-challenged ears by using TRIzol reagent (Invitrogen) and purified by using the RNeasy Mini Kit (Qiagen), and 3.5 μg of purified RNA was used for microarray analysis with a Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara, Calif), according to the manufacturer's protocol. Data were analyzed by using Statistical Algorithm with the Affymetrix GeneChip Expression Analysis software (Microarray Suite 5.0). All microarray data are deposited in Gene Expression Omnibus (GEO).

Statistics

Data were expressed as means ± SEMs, and statistical analyses were performed by means of ANOVA or the Student *t* test, as appropriate. A *P* value of less than .05 was considered statistically significant.

RESULTS

Effect of EP_3 agonist on the elicitation phase of CHS

We first examined whether stimulation of EP_3 had an anti-inflammatory effect on CHS. To investigate this, we administered an EP_3 agonist CAE248, 100 μg/kg subcutaneously 3 times a day during the elicitation phase. This dose of AE248 exerts a significant effect *in vivo*.[12] The DNFB challenge caused ear swelling in both vehicle-treated and AE248-treated mice. However, the mice treated with AE248 showed significant reductions in swelling

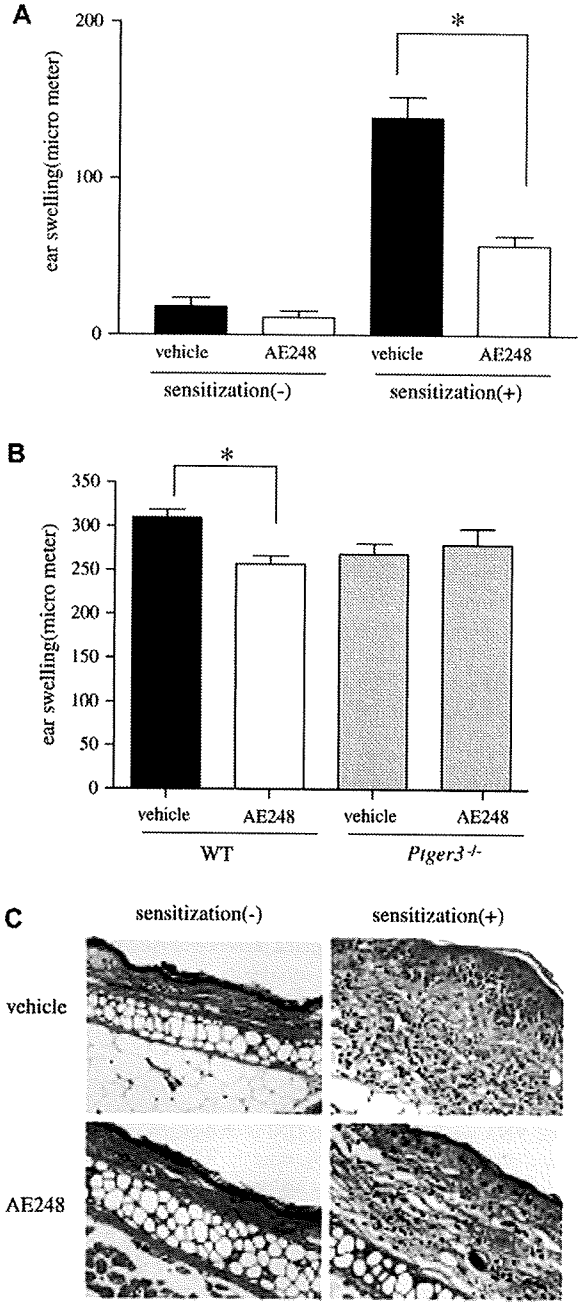

FIG 1. Suppressive effects of EP_3 agonist on the ear-swelling response in mice with CHS. **A** and **B**, Results are expressed as means ± SEMs (n = 5 in both groups). Data are representative of 3 experiments. **C**, HE staining of control- and DNFB-challenged ears treated with either vehicle or EP_3 agonist. Representative samples of each group are shown.

compared with that seen in the vehicle-treated mice 24 hours after elicitation (Fig 1, *A*). This suppressive effect of AE248 was completely absent in *Ptger3*$^{-/-}$ mice (Fig 1, *B*), suggesting that the effect was elicited through the EP_3 receptor. Histology of the ear from sensitized mice showed edema and marked inflammatory cell infiltration in the dermis 24 hours after elicitation (Fig 1, *C*). Consistently, the extent of the edema and inflammatory cell infiltration was markedly reduced in the AE248-treated mice compared with that seen in the vehicle-treated mice. These findings together demonstrate that EP_3 stimulation in the elicitation phase elicits suppressive effect on CHS.

We next examined the localization of EP_3 in the normal murine ear. X-gal staining was performed in *Ptger3*$^{-/-}$ mice, in which the β-galactosidase gene was knocked in at the EP_3 gene locus. Positive signals were detected mostly in the basal layer of epidermis in the skin of control mice (Fig 2, *A*). Similar signals were also observed in the ears of mice after elicitation, whereas little signals were detected in the cells infiltrating the dermis (data not shown). These findings suggest that the main cell species expressing EP_3 in the skin is keratinocytes and that they express it constitutively. To examine the EP_3 expression in keratinocytes of the basal layer, we costained for keratin 5, a specific marker of basal keratinocytes, and found that signals for keratin 5 colocalized with those of the X-gal staining (Fig 2, *A*). EP_3 expression in keratinocytes was confirmed by means of immunohistochemical analysis with anti-EP_3 antibody in WT mice (Fig 2, *B*). These results, together with our finding that little X-gal staining was detected in lymph nodes (data not shown), suggest a possibility that AE248 acts on keratinocytes and not on immune cells to exert its anti-inflammatory actions. Therefore we next examined the effects of topical application of AE248 to the ear in CHS. AE248 was dissolved in acetone and topically applied to the ear 3 times in the elicitation phase. This topical application of AE248 showed significant dose-dependent suppression of ear swelling in CHS 24 hours after elicitation (Fig 2, *C*), whereas that of agonists specific to other EP subtypes was without effect (Fig 2, *D*). Administration of AE248 showed a suppressive effect 24, 48, and 72 hours after elicitation, suggesting that the effect of AE248 did not induce just the delay in the development of inflammation (Fig 2, *E*). To confirm that the effect of AE248 was not caused by immune cells, we made bone marrow chimera in which stromal cells, such as keratinocytes, express EP_3, whereas bone marrow–derived cells do not express EP_3, as described in the Methods section. A suppressive effect of AE248 on ear swelling in CHS of the bone marrow chimera was detected (Fig 2, *F*), which supports our hypothesis that AE248 acts on EP_3 in keratinocytes to exert an anti-inflammatory effect.

Reduced expression of genes related to inflammatory cell infiltration caused by topical treatment with AE248

Various inflammation-related genes, including those for chemokines, are upregulated during the elicitation phase of CHS.[5] We therefore compared gene expression between vehicle-treated control mice and mice treated with AE248 to examine the role of EP_3 in this process. We first examined the time course of gene expression during the elicitation phase in our model. Ears challenged with DNFB were isolated at 1, 3, 6, 12, and 24 hours after elicitation for microarray analysis by using an Affymetrix Mouse Genome 430 2.0 GeneChip that contains 45,101 genes. We screened for gene expression, which exhibited a more than 2-fold increase at any given time during the elicitation phase over basal expression at 0 hours (Table I). Among the genes with increased expression, we focused on chemokine genes. At 1 hour after challenge, 130 genes were detected as genes showing a more than 2-fold increase in expression, and none of chemokine genes was among those genes. At 3 hours, 263 genes were upregulated, and 4 kinds of chemokines were included in this group. The analysis similarly picked up 408 genes with 5 kinds of chemokine genes at 6 hours, 655 genes with 8 kinds of chemokine genes at 12 hours, and 902 genes with 14 kinds of chemokine

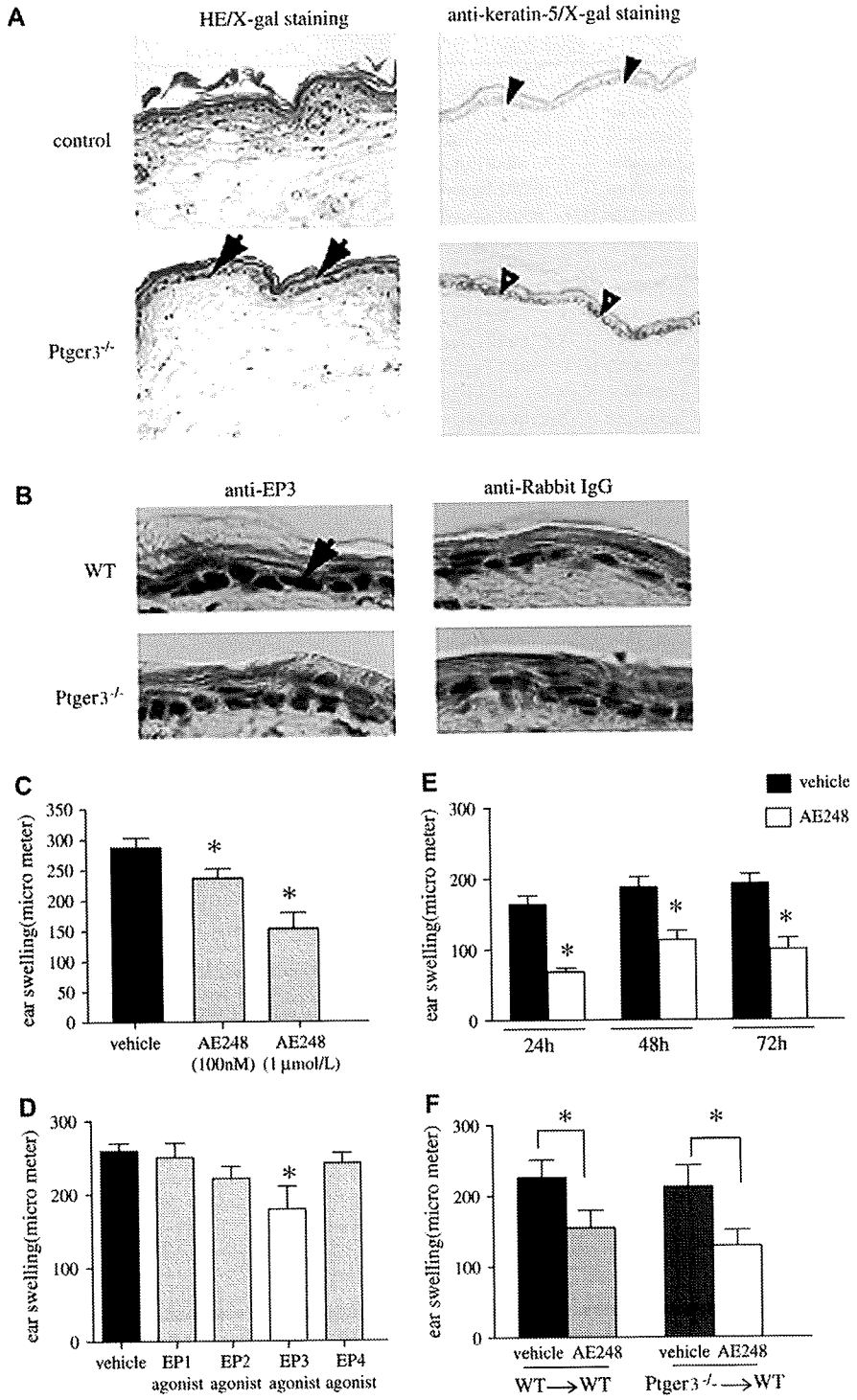

FIG 2. Localization of EP_3 receptors and effect of topical application of EP_3 agonist on mice with CHS. **A**, Histochemical staining for EP_3 (X-gal) counterstained with HE or anti–keratin 5 antibody. *Arrows*, Positive signaling (blue); *black arrowheads*, positive staining of keratin 5; *white arrowheads*, colocalization of positive signals in X-gal and anti–keratin 5 staining. **B**, Immunohistologic analysis for EP_3. The *arrow* indicates positive signals. **C-E**, Suppressive effects of topical administration of EP_3 agonist and effects of various EP agonists (1 μmol/L) on murine CHS (n = 5 per group [Fig 2, *C*] and n = 4 per group [Fig 2, *D* and *E*]). Data are representative of 2 experiments. **F**, Effect of AE248 on murine CHS of bone marrow chimera (n = 13-15 per group). Results are a combination of 3 independent experiments.