body (Fig. 1). The entire body surface was erythematous and the extremities were oedematous. Circumscribed alopecia was more pronounced on the right side of the scalp. The skin eruptions cleared within several weeks, although slightly scaly skin remained over her whole body. Skin biopsy of a hyperkeratotic lesion on the trunk taken during the neonatal period revealed orthohyperkeratosis. A marked calcification in the stratum corneum was seen by van Kossa staining. From these clinical and histological features, a diagnosis of CHH was made in this case.

All four coding exons 2–5 of EBP were amplified using previously described polymerase chain reaction (PCR) primers.[4] Direct sequencing of the PCR products from the patient and her parents revealed that the patient was a heterozygote for a missense mutation p.Arg147His [G to A substitution at nucleotide position 440: arginine 147 (CGC) to histidine (CAC)], which was not found in her parents. This mutation was not found in 100 normal unrelated alleles (50 normal unrelated Japanese individuals) by direct sequencing analysis. Direct sequencing of all the coding exons and exon/intron borders of EBP failed to detect any other pathogenic mutations in the patient's DNA. The p.Arg147His is a known mutation reported in an aborted fetus affected with CHH.[3]

We performed ultrastructural observation of the patient's epidermal keratinocytes using ruthenium tetroxide postfixation. Lamellar granules with abnormal contents, lacking the normal lamellar structure, were seen in the granular layer keratinocytes in the patient's epidermis (Fig. 2). The lamellar granule contents were secreted into the intercellular space in the stratum corneum. Secreted lipid material trapped in the cytoplasm of corneocytes, corresponding to the membranous remnants reported by Emami *et al.*,[5] was distributed sparsely throughout the stratum corneum. In addition, irregularly dilated intercellular spaces were often observed between the keratinized cells.

We performed CD1a staining on the skin biopsy specimen in order to evaluate Langerhans cell density in the epidermis. Langerhans cell density was 8·8 cells/high power field (HPF) in the patient's epidermis and 4·2 cells/HPF and 16·0 cells/HPF in two age/gender-matched normal controls. Thus, no significant reduction of Langerhans cell density was confirmed in the patient's epidermis.

EBP mutations were reported to underlie CHH. EBP has a dual function: on the one hand it serves as a binding protein for the Ca^{2+} antagonist emopamil and thus is a high-affinity acceptor protein for several anti-ischaemic drugs,[6] and on the other hand it acts as a delta8–delta7 sterol isomerase.[7] It has been suggested that the skeletal manifestations in CHH may be caused by an accumulation of toxic sterol intermediates which interfere with the function of cholesterol-modified hedgehog proteins.[8] Furthermore, the molecular pathology of the ichthyotic phenotype in CHH can also be explained by EBP function in sterol biosynthesis pathways. EBP is a key enzyme involved in cholesterol biosynthesis[7] and dysfunction of EBP results in cholesterol deficiency and accumulation of cholesterol pathway products such as 8-dehydrocholesterol.[2,3] In a review of the

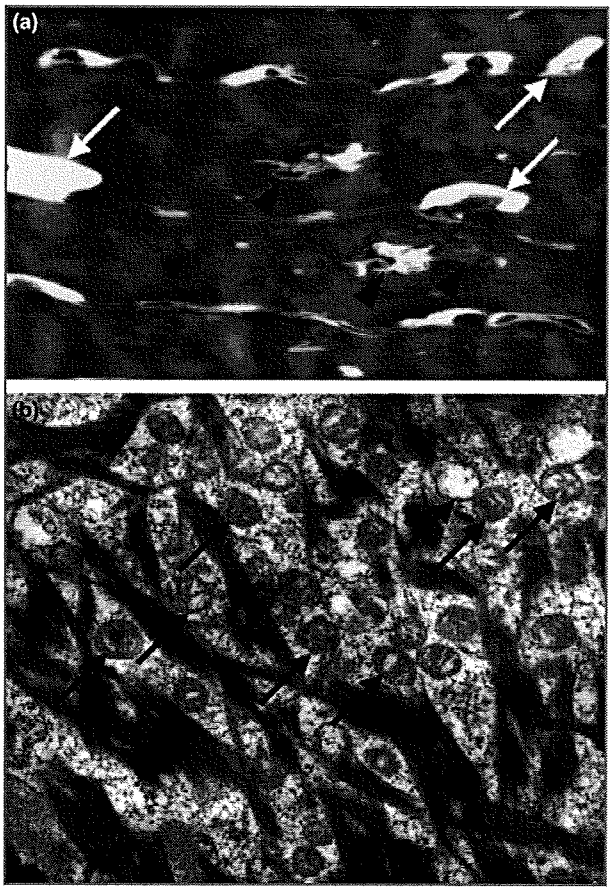

Fig 2. (a) Electron microscopy revealed irregularly dilated intercellular spaces (white arrows) between the keratinized cells and a small number of membranous remnants (black arrows) within the cytoplasm of keratinized cells. (b) In a granular layer cell, lamellar granules lacking lamellar structure, vacant or containing irregular sized vesicles (black arrows) were observed. Original magnification: (a) × 12 000, (b) × 60 000.

pathophysiology of ichthyosis disease, Elias *et al.*[9] hypothesized that a deficiency of bulk cholesterol accumulation in keratinocyte membrane function may be a major factor contributing to the ichthyosis phenotype seen in CHH.

In 1984, Kolde and Happle[10] reported morphological changes in the lesional skin of patients with CHH. In their report, numerous small to medium-sized vacuoles measuring between 0·4 and 1·5 μm in diameter were observed in the granular layer keratinocytes, as seen in the present study. In 1994, vacuolated lamellar granules and a lack of intercellular lamellar structures were shown in an infant with CHH,[5] although the causative molecule or gene was not elucidated in those studies. In this report, for the first time, in a patient with CHH with a confirmed EBP mutation, we demonstrated abnormal lamellar granule contents in the granular layer cells in the lesional epidermis. The present ultrastructural findings suggest that EBP mutations and consequent cholesterol deficiency lead to defective lamellar granule contents, resulting in malformed intercellular lipid layers and the ichthyotic skin phenotype characteristic of patients with CHH.

Kolde and Happle[11] reported Langerhans cell degeneration and reduced density of Langerhans cells in the patients' epidermis and suggested that the ichthyotic phenotype of CHH is caused by Langerhans cell depletion. However, in the present study, no significant reduction of Langerhans cell density was observed in the patient's epidermis.

Kolde and Happle[10] reported that hair follicles showed signs of atrophy which was in an early stage in a 4-week-old baby, and was fully developed in a 14-year-old girl, although the other morphological abnormalities were similarly observed in both the baby and the 14-year-old girl. In the present study, no apparent atrophy of hair follicles was seen, probably because the skin biopsy sample was taken in the neonatal period.

Acknowledgments

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Key words: cholesterol, EBP, ichthyosis, lipid

Conflicts of interest: none declared.

Acquired idiopathic generalized anhidrosis: possible pathogenic role of mast cells

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SIR, A 37-year-old man experienced anhidrosis of almost his entire body and cholinergic urticaria accompanied by severe heat intolerance for 5 months, which caused him to leave his job as an electrical engineer. A physical examination revealed no abnormalities except for slightly dry skin on his trunk and extremities. A neurological examination yielded no abnormal findings for his sensory system and sympathetic function. Laboratory tests, including blood counts, antinuclear antibody, anti-SS-A/Ro, anti-SS-B/La, total IgE and other biochemical profiles were normal except for a slightly elevated total bilirubin (1·2 mg dL^{-1}; normal 0·0–1·0 mg dL^{-1}).

Intradermal injection of 0·05 mL acetylcholine (100 μg mL^{-1}) produced no local sweating (Fig. 1). A thermoregulatory sweating test using the iodine–starch method showed almost generalized anhidrosis except for the axillary zones (Fig. 1). After 15 min of exercise on a treadmill, only 0·06 mL of sweat was collected from both forearms; pinpoint-sized weals characteristic of cholinergic urticaria were observed. In order to check his responsiveness to autologous sweat, autologous sterilized sweat (diluted 1 : 100) was injected intradermally, resulting in a negative response.[1] A skin biopsy specimen was taken from his right forearm where sweating did not occur. The eccrine glands and ducts were surrounded by infiltrates of CD3-positive lymphocytes and a considerable number of mast cells (0·88 mast cells per gland) that were metachromatically stained with toluidine blue (Fig. 2). Serial sections of the skin biopsy revealed focal hyperkeratosis at the acrosyringium and normal eccrine glands (Fig. 2).

The patient was diagnosed with acquired idiopathic generalized anhidrosis (AIGA) accompanied by cholinergic urticaria. Firstly, loratadine 10 mg daily was administered but this treatment was not effective for the cholinergic urticaria or anhidrosis. Next, methylprednisolone 1000 mg daily was

eradication treatment will probably give a huge advantage in terms of social health, especially in high-risk areas.

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Potential Conflicts of Interest: None disclosed.

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CLINICAL OBSERVATIONS

Granulysin as a Marker for Early Diagnosis of the Stevens–Johnson Syndrome

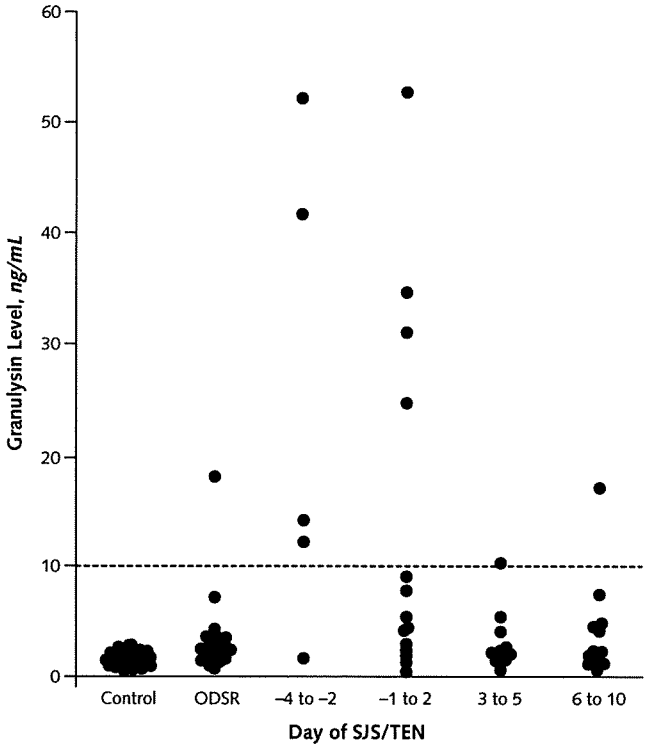
Background: The Stevens–Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening adverse drug reactions characterized by massive epidermal necrosis. In the early stage, clinical presentations of SJS/TEN are very similar to those of ordinary drug-induced skin reactions (ODSRs); therefore, SJS/TEN is difficult to diagnose and the start of treatment is often delayed, resulting in high mortality rates. Other investigators (1) reported that granulysin is highly expressed in blisters of SJS/TEN and causes disseminated keratinocyte death. Because SJS/TEN progresses and spreads rapidly, the granulysin level should be increased in the serum of patients with active SJS/TEN if it is a key mediator of these diseases.

Objective: To determine whether serum granulysin levels are higher in patients with SJS/TEN than in healthy control participants or those with ODSRs.

Methods: We measured granulysin in the sera of 31 healthy control participants, 24 patients with ODSR, 13 patients with SJS, and 7 patients with TEN by using enzyme-linked immunosorbent assay (2). Disease onset in patients with SJS/TEN was defined as the day (day 1) on which the mucocutaneous or ocular lesion first eroded or ulcerated (3), and we collected sera from these patients from 4 days before to 10 days after ulceration. We used the Tukey–Kramer test to conduct multiple comparisons between groups.

Results: None of the 31 healthy control participants had a granulysin level greater than the upper limit of normal, which was 10 ng/mL (0% elevated; mean, 1.6 ng/mL [SD, 0.6]), and among 24 patients with ODSRs, only 1 patient had an elevated granulysin level (4.2% elevated; mean, 3.5 ng/mL [SD, 3.4]) (**Figure**). We obtained samples from 5 patients with SJS/TEN on day −4 to day −2, and we detected the highest granulysin concentrations (elevated in 80% of patients); mean, 24.8 ng/mL [SD, 21.2]). Granulysin levels were lower in the 14 samples collected on day −1 to day 2 (28.6% elevated; mean, 13.7 ng/mL [SD, 16.0]), and were even lower in the 10 samples collected from day 3 to day 5 (10.0% elevated; mean, 4.2 ng/mL [SD, 3.0]) and in the 13 samples collected from day 6 to day 10 (7.7% elevated; mean, 4.5 ng/mL [SD, 4.5]). When we compared granulysin levels from day −4 to day −2 among patients with SJS/TEN, patients with ODSRs, and healthy control participants, the differences were statistically significant ($P < 0.010$).

***Figure.* Granulysin levels of healthy control participants, patients with ODSRs, and patients with SJS/TEN.**



ODSR = ordinary drug-induced skin reaction; SJS/TEN = Stevens–Johnson syndrome/toxic epidermal necrolysis.

Discussion: Granulysin is cytotoxic for tumor cells, transplant cells, bacteria, fungi, and parasites, in which it damages negatively charged cell membranes because of its positive charge (4). It plays an important role in the host defense against pathogens, and it induces apoptosis of target cells by using a mechanism involving caspases and other pathways (4). Its potency makes it a credible mediator of skin damage in patients with SJS/TEN. Adding to this credibility is a report (1) that granulysin is the most highly expressed cytotoxic molecule in the blisters of patients with SJS/TEN. We show that serum granulysin levels in 4 of 5 patients with SJS/TEN were elevated before skin detachment or mucosal lesions develop. Soluble Fas ligand (sFasL) shares some properties with granulysin: It contributes to keratinocyte death in SJS/TEN (3, 5), and levels are elevated in the sera of patients with SJS/TEN (3). Serum granulysin levels, however, are approximately 100 times higher than those of sFasL on day

−4 to day −2 (23.1 ng/mL [SD, 16.6] vs. 147.76 pg/mL [SD, 104.4]). Therefore, we believe it would be easier to develop bedside granulysin serum measurement, for example, by using immunochromatography, than it would be to develop a similar sFasL measurement. Monitoring serum granulysin might enable early diagnosis of SJS/TEN in patients with cutaneous adverse drug reactions that otherwise could not be distinguished from ODSRs.

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Potential Conflicts of Interest: None disclosed.

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Localized Amyloidosis at the Site of Enfuvirtide Injection

Background: Enfuvirtide is the first of a new class of antiretroviral agents that block fusion of the viral particle with the host target cell. Its safety and antiviral activity have been demonstrated (1, 2). In clinical trials, injection site reactions occurred in 80% to 100% of patients (3). The most common signs and symptoms reported were induration in 94%, erythema in 91%, and subcutaneous nodules and cysts in 70% (4).

Objective: To describe a case of amyloidosis at the injection site of enfuvirtide.

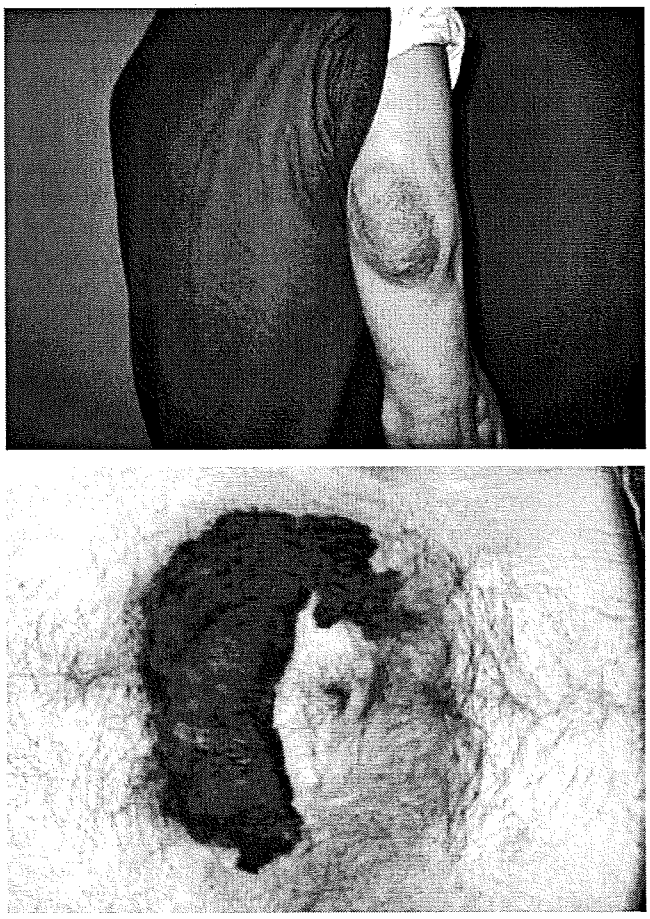
Case Report: The patient was a man aged 47 years who had a history of sexual intercourse with men and extensive treatment for HIV with a triple-class viral resistance profile. He also had long-standing leg pain thought to be secondary to HIV neuropathy and no history of intravenous drug use. There was no history of opportunistic or chronic infections.

Because of a persistently elevated viral load, enfuvirtide by subcutaneous injection was added to his highly active antiretroviral treatment regimen for 41 months; enfuvirtide therapy was then stopped in February 2007 because of intolerable injection site reactions. While he was receiving enfuvirtide, his viral loads were completely suppressed. Eighteen months after enfuvirtide therapy was stopped, large, tender, indurated reactions with fragile epithelial surfaces persisted at all injection sites (**Figure**, *top*). These reactions bled extensively into the subcutaneous tissue with minor trauma (**Figure**, *bottom*). A lesion on the triceps was excised surgically, and the wound healed without complications. Pathologic examination showed extensive deposits of proteinaceous material with intense Congo red staining that was consistent with amyloid. A lesion on the opposite arm was resected and showed similar findings. The patient had a normal leukocyte count and normal hemoglobin, blood urea nitrogen, and creatinine levels and had no evidence of plasma cell dyscrasia and no history of organ dysfunction to suggest systemic amyloidosis.

Discussion: In 7 patients receiving enfuvirtide, biopsy of injection site reactions revealed an inflammatory response consistent with a localized hypersensitivity reaction (5), and other studies (3) have reported similar findings. Other reports (6) have described 3 histologic patterns: an acute urticaria- or vasculitis-like pattern with inflammation of the fat tissue, a subacute pattern with an initial dermal sclerosis, and a long-term scleroderma-like pattern.

In our patient, surgical excision of enfuvirtide injection site reactions revealed subcutaneous nodular amyloidosis. Localized

Figure. **Lesion in right triceps area (*top*) and periumbilical site with spontaneous intradermal and subcutaneous hemorrhage (*bottom*).**



A randomized double-blind trial of intravenous immunoglobulin for pemphigus

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Background: Pemphigus is a rare life-threatening intractable autoimmune blistering disease caused by IgG autoantibodies to desmogleins. It has been difficult to conduct a double-blind clinical study for pemphigus partly because, in a placebo group, appropriate treatment often must be provided when the disease flares.

Objective: A multicenter, randomized, placebo-controlled, double-blind trial was conducted to investigate the therapeutic effect of a single cycle of high-dose intravenous immunoglobulin (400, 200, or 0 mg/kg/d) administered over 5 consecutive days in patients relatively resistant to systemic steroids.

Methods: We evaluated efficacy with time to escape from the protocol as a novel primary end point, and pemphigus activity score, antidesmoglein enzyme-linked immunosorbent assay scores, and safety as secondary end points.

Results: We enrolled 61 patients with pemphigus vulgaris or pemphigus foliaceus who did not respond to prednisolone (≥20 mg/d). Time to escape from the protocol was significantly prolonged in the 400-mg group compared with the placebo group ($P < .001$), and a dose-response relationship among the 3 treatment groups was observed ($P < .001$). Disease activity and enzyme-linked immunosorbent assay scores were significantly lower in the 400-mg group than in the other groups ($P < .05$ on day 43, $P < .01$ on day 85). There was no significant difference in the safety end point among the 3 treatment groups.

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Other investigators in the Pemphigus Study Group are listed in the Appendix.

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Disclosure: Drs Amagai, Ikeda, Kitajima, Nishikawa, and Hashimoto report receiving consulting and lecture fees from Nihon Pharmaceutical Co Ltd. Drs Shimizu, Iizuka, Hanada, Aiba, Kaneko, Izaki, Tamaki, Ikezawa, Takigawa, Seishima, Tanaka, Miyachi, Katayama, Horiguchi, Miyagawa, Furukawa, Iwatsuki, Hide, Tokura, Furue, Ihn, Fujiwara, Ogawa, and Hashimoto have no conflicts of interest to declare.

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Limitation: Prednisolone at 20 mg/d or more may not be high enough to define steroid resistance.

Conclusion: Intravenous immunoglobulin (400 mg/kg/d for 5 d) in a single cycle is an effective and safe treatment for patients with pemphigus who are relatively resistant to systemic steroids. Time to escape from the protocol is a useful indicator for evaluation in randomized, placebo-controlled, double-blind studies of rare and serious diseases. (J Am Acad Dermatol 2009;60:595-603.)

Pemphigus is a life-threatening, rare intractable autoimmune blistering disease caused by IgG autoantibodies to desmoglein (Dsg) (epidermal adhesion factor). It is characterized by the development of blisters and erosions of the skin and mucosa.[1] Currently, oral steroids are the drugs of first choice for pemphigus, and may be used in combination with immunosuppressants or plasma exchange. However, many patients with pemphigus experience cycles of remission and recurrence, and accordingly become unresponsive to conventional therapy. On the other hand, patients with complications such as diabetes mellitus, gastrointestinal disease, osteoporosis, infection, or immunodeficiency are relatively contraindicated for use of high-dose (HD) steroids. For such patients, an alternative effective treatment strategy is required.

Although several reports suggesting the effectiveness of HD intravenous immunoglobulin (IVIG) in the treatment of pemphigus have been published since its introduction as monotherapy in 1989, most are case reports with a low evidence level or involved clinical research with a limited number of patients using multiple treatment cycles.[2] No well-controlled, double-blind clinical study to demonstrate the efficacy of HD-IVIG has been conducted.[3-13] This is because: (1) pemphigus is a rare intractable disease; (2) appropriate treatment must be provided in a timely manner if symptoms are aggravated or unchanged for a certain period of time; (3) inclusion of a placebo group compromises compliance with the study protocol; and (4) it is not ethical to treat patients with pemphigus using placebo because mortality is high.

We developed a novel evaluation end point to solve these problems and verified the usefulness of HD-IVIG in a single treatment cycle for this rare intractable disease.

Abbreviations used:

ADRs:	adverse drug reactions
Dsg:	desmoglein
HD:	high dose
IVIG:	intravenous immunoglobulin
PAS:	pemphigus activity score
PF:	pemphigus foliaceus
PV:	pemphigus vulgaris
TEP:	time to escape from the protocol

METHODS

Patients

This study was conducted in 27 medical institutions in Japan with affiliated dermatologists specialized in autoimmune blistering disease. Patients were given the diagnosis of pemphigus vulgaris (PV) or pemphigus foliaceus (PF) as confirmed based on our national diagnostic criteria as follows: pemphigus was diagnosed when at least one item from every 3 findings, or two items from clinical findings and one item from immunologic findings were satisfied.

1. Clinical findings
   - Multiple, easily rupturing, flaccid blisters of the skin
   - Subsequent progressive, refractory erosions or crust after blisters
   - Noninfectious blisters or erosions of visible mucosa including oral mucosa
   - Nikolsky sign
2. Histologic findings
   - Intraepidermal blisters caused by loss of adhesion between epidermal cells (acantholysis)
3. Immunologic findings
   - IgG (or complement) deposition in the intercellular spaces of the lesional or normal-appearing skin and mucosa as detected by direct fluorescent antibody assay
   - Antiepidermal intercellular IgG autoantibody (anti-Dsg IgG autoantibody) identified by indirect fluorescent antibody assay or enzyme-linked immunosorbent assay

The study patients had to meet all the following inclusion criteria and none of the exclusion criteria.

1. Inclusion criteria: patients aged 20 years or older who provided written informed consent to participate in the study and met all of the following criteria.

Table I. Criteria for pemphigus activity score

Variable score	Skin lesion area*	No. of new blisters/d	Oral mucosal lesions†
3	≥15%	≥5	≥30%
2	≥5% and <15%	1 to 4	≥5% and <30%
1	<5%	Occasionally‡	<5%
0	None	None	None

*Percentage of entire surface area.
†Score is doubled for patients who have only oral mucosal lesions at time of study enrollment.
‡Blisters sometimes newly develop within 1 week but not every day.

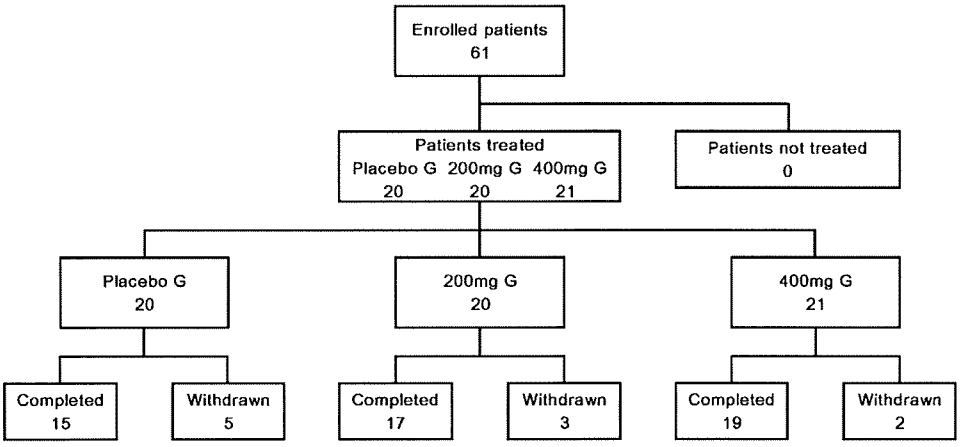

Fig 1. Disposition of patients. *G*, Group.

- Treatment with any steroid at greater than or equal to 20 mg/d (prednisolone equivalent)
- Symptoms (total pemphigus activity score [PAS] [Table I]) did not respond to steroid therapy

2. Exclusion criteria: patients who met any of the following criteria were excluded from the study because efficacy evaluation of the test drug might be affected and to assure the safety of patients.
   - Patients treated with plasma exchange therapy, steroid pulse therapy, or HD-IVIG within 30, 14, or 42 days, respectively, before informed consent and the start of study treatment
   - Patients with a history of shock or hypersensitivity to the test drug
   - Patients with IgA deficiency, hepatic disorder, renal disorder, or hemolytic or blood loss anemia
   - Patients with any previous or existing cerebrovascular or cardiovascular disorder

Study design

This was a multicenter, randomized, placebo-controlled, double-blind, parallel-group study. The study protocol and written informed consent form approved by the institutional review board at each study institution were used in the study. Observation of the first patient was started on November 4, 2004, and that of the last patient was completed on September 25, 2006.

Treatment groups

The IVIG group received IV drip infusion at 200 or 400 mg/kg/d administered in divided dose over 5 consecutive days. The placebo group received IV drip infusion of physiologic saline for 5 consecutive days.

Investigational drugs manufactured by Nihon Pharmaceutical Co Ltd (Higashikanda, Tokyo, Japan) were used in the study.

Methods of allocation

Patients were randomized by a central enrollment system to the treatment groups according to a dynamic allocation scheme to ensure that there were no between-group differences in the dose of prior steroid, total PAS, or disease type.

Blinding

Because the investigational drugs were distinguishable in terms of appearance and viscosity after

Table II. Demographic and other baseline characteristics

Characteristic	Category	Dose: Placebo n = 20	Dose: 200 mg n = 20	Dose: 400 mg n = 21	Between-group comparison
Sex	Male	9	10	8	NS* (P = .766)
	Female	11	10	13	
Age, y	Mean ± SD	53.1 ± 10.9	57.0 ± 14.6	50.1 ± 11.7	NS† (P = .225)
Body weight, kg	Mean ± SD	57.8 ± 11.6	58.0 ± 10.4	57.7 ± 9.1	NS* (P = .686)
Disease type	PV	13	14	13	NS* (P = .942)
	PF	7	6	8	
Disease duration, mo	Mean ± SD	16.1 ± 13.6	28.6 ± 32.3	28.5 ± 46.9	NS† (P = .414)
Baseline PAS	Mean ± SD	3.3 ± 1.4	3.6 ± 1.8	3.7 ± 1.1	NS† (P = .660)
Steroid dose, mg	Mean ± SD	27.6 ± 9.7	23.9 ± 11.1	27.4 ± 11.1	NS† (P = .461)
Immunosuppressants	No. of patients (%)	2 (10.0)	7 (35.0)	5 (23.8)	NS* (P = .179)

NS, Not significant difference; *PAS*, pemphigus activity score; *PF*, pemphigus foliaceus; *PV*, pemphigus vulgaris.
Two-sided test for both analyses.
*Fisher exact test.
†One-way analysis of variance.

reconstitution, independent staff at each study institution separately prepared and administered the dosing solution, and evaluated efficacy and safety in each patient to maintain blinding. The bottles of the investigational drugs were covered with a masking cover and provided to the independent staff member in charge of administration. Each independent staff member involved signed a blinding confirmation form at the end of the study to assure that blinding was maintained.

End points

Time to escape from the protocol (TEP) was used as the primary efficacy end point. TEP was defined as the length of the period until a patient stayed on the protocol without any additional treatment. When symptoms were unchanged for 2 weeks or aggravated, the treatment given was considered to be ineffective and additional treatment was required such as increase in steroid dose, change in steroid type, use of additional immunosuppressive agents, or plasma exchange; these patients were considered escaped from the protocol. This methods allow doctors in charge to have flexibility to rescue patients with other treatment when needed.

The secondary end points used in the study included: (1) PAS over time (scores [0-3 point] for skin lesion area, number of new blisters/d, and oral mucosal lesions, and their total scores [Table I]); and (2) the titers of pemphigus autoantibodies over time (anti-Dsg1 autoantibody titer and anti-Dsg3 autoantibody titer). Titers of pemphigus autoantibodies were determined by enzyme-linked immunosorbent assay.[14,15] As a safety end point, the occurrence of adverse events by 85 days after the start of the study treatment (day 85) was investigated. Adverse events were recorded up to day 43 if patients escaped from the protocol by day 43 or up to TEP if patients escaped from the protocol after day 44.

Statistical analysis

The cumulative rate of TEP, which was estimated by evaluation of the dose-response relationship of TEP and by analysis using the Kaplan-Meier method, was compared among the treatment groups by log rank test. Scores for skin lesion area, number of new blisters/d, and oral mucosal lesions, and total score, the secondary end point, up to day 85 were compared with baseline data by the paired t test for each treatment group. The data after TEP were imputed from the data at the TEP (last observation carried forward). Adverse events occurring up to day 85 for which the causal relationship with HD-IVIG or placebo was judged to be other than "not related" were handled as adverse drug reactions (ADRs). A two-sided significance level of .05 was used for analyses.

RESULTS

Disposition of patients

The disposition of patients enrolled in the study is shown in Fig 1. A total of 61 patients were treated with the investigational drug (placebo, 20; 200 mg, 20; and 400 mg, 21). All the enrolled patients including 10 patients (placebo, 5; 200 mg, 3; and 400 mg, 2) who were withdrawn from the study according to the requirements in the protocol were included in the analyses. The main reasons for study withdrawal were the evaluator's decision to withdraw the patient and the occurrence of adverse events. The demographic and other baseline

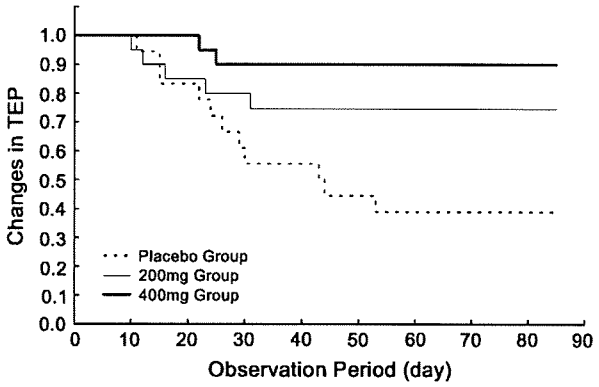

Fig 2. Changes in time to escape from protocol (*TEP*). TEP was significantly prolonged in 400-mg group compared with placebo group with dose-dependent fashion. Cumulative TEP on day 85 was 10.0% in 400-mg group, 25.0% in 200-mg group, and 61.0% in placebo group (log rank test). Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group ($P < .001$, log rank test). In contrast, difference between 200-mg and placebo groups was not significant ($P = .052$). In addition, dose-response relationship was observed in TEP ($P < .001$). Data are stated using TEP ratio.

characteristics are presented in Table II. There were no significant between-group differences in the distribution of baseline characteristics. The average disease durations of 200- and 400-mg groups are longer than in the placebo group, but this is because the former group happened to contain patients with extremely long duration (116 months in 200 mg; 142 and 169 months in 400 mg) and the difference was not statistically significant.

Efficacy (primary end point): TEP

TEP was evaluated as the primary end point (Fig 2). In the 400-mg group, 19 of 21 patients stayed on the protocol during the observation period. Two patients escaped from the protocol with TEPs of 22 and 25 days. In the 200-mg group, 15 of 20 patients stayed on the protocol and the shortest TEP was as early as 10 days among the 5 escaped patients. In the placebo group, only 9 patients stayed on the protocol, and the shortest TEP was as early as 11 days. TEP was within 30 days for 8 patients.

TEP in the active treatment groups was compared with that in the placebo group (log rank test). The TEP in the 400-mg group was significantly longer than that in the placebo group ($P < .001$), whereas the difference between the 200-mg and placebo groups was not significant ($P = .052$). Log rank test of TEP for the 61 patients indicated a dose-response relationship for this parameter ($P < .001$).

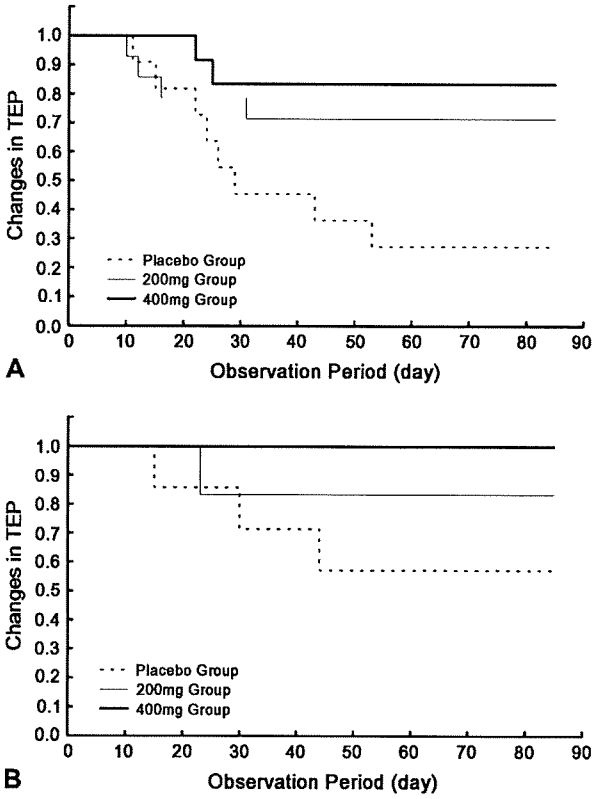

Fig 3. Cumulative time to escape from protocol (*TEP*) shown by pemphigus subtype. Cumulative TEP estimated by Kaplan-Meier method was divided in disease subtype of pemphigus vulgaris (PV) (**A**, n = 13 in 400-mg group, n = 14 in 200-mg group, n = 13 in placebo group) and pemphigus foliaceus (PF) (**B**, n = 8 in 400-mg group, n = 6 in 200-mg group, n = 7 in placebo group). Cumulative TEP in patients with PV on day 85 was 15.0% in 400-mg group, 29.0% in 200-mg group, and 73.0% in placebo group, whereas that of patients with PF was 0.0% in 400-mg group, 17.0% in 200-mg group, and 43.0% in placebo group. Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group (PV, $P = .007$; PF, $P = .044$; log rank test). In contrast, difference between 200-mg and placebo groups was not significant (PV, $P = .055$; PF, $P = .416$). In addition, dose-response relationship was observed in TEP (PV, $P = .007$; PF, $P = .043$).

Analyses stratified by baseline characteristics (disease type and PAS) also demonstrated dose-response relationships and significant differences between the 400-mg and placebo groups, as in the overall analyses (Figs 3 and 4).

Efficacy (secondary end point)

Pemphigus activity score. Efficacy was also evaluated based on the changes in clinical symptoms, ie, changes in PAS determined based on skin lesion area, number of new blisters/d, and oral mucosal lesions. In the 400-mg group, total PAS

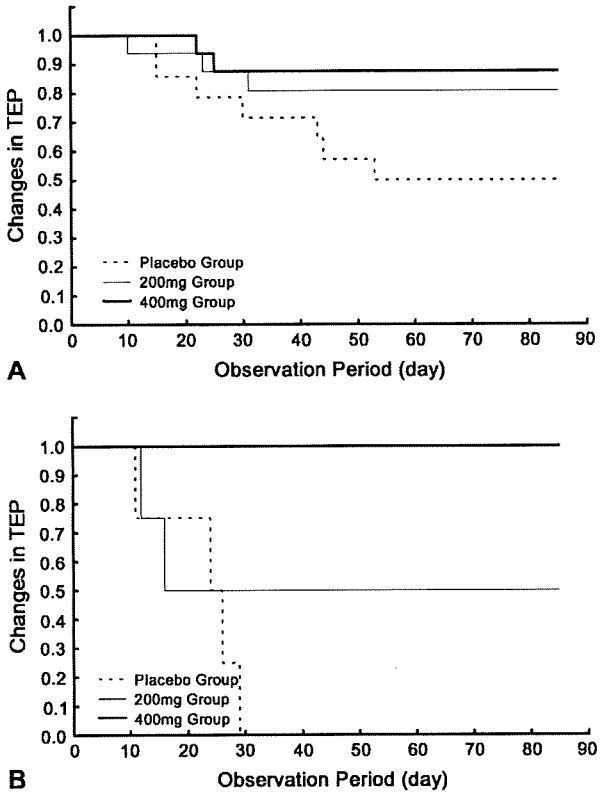

Fig 4. Cumulative time to escape from protocol (*TEP*) shown in different pemphigus activity score (PAS). Data were divided by PAS into two groups: total PAS of 0 to 4 (**A**, n = 17 in 400-mg group, n = 16 in 200-mg group, n = 16 in placebo group) and total PAS of 5 to 9 (**B**, n = 4 in 400-mg group, n = 4 in 200-mg group, n = 4 in placebo group). Cumulative TEP in patients with total PAS of 0 to 4 on day 85 was 12.0% in 400-mg group, 19.0% in 200-mg group, and 50.0% in placebo group, whereas those of patients with total PAS of 5 to 9 was 0.0% in 400-mg group, 50.0% in 200-mg group, and 100.0% in placebo group. Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group (total score 0-4, $P = .028$; total score 5-9, $P = .006$). In contrast, difference between 200-mg and placebo groups was not significant (total score 0-4, $P = .109$; total score 5-9, $P = .345$). In addition, dose-response relationship was observed in TEP (total score 0-4, $P = .024$; total score 5-9, $P = .012$).

was significantly decreased from the baseline score at all points of observation (day 8, $P = .05$; after day 15, $P < .01$). It was decreased from 3.7 on day 1 to 2.0 on day 85 (by 46.8%) (Fig 5). In the 200-mg group, total PAS was significantly decreased from the baseline score at all points of observation after day 15 (day 15-43, $P < .05$; day 57-85, $P < .01$). It was decreased from 3.7 on day 1 to 2.3 on day 85 (by 36.6%). On the other hand, in the placebo group, no significant decrease from baseline score was observed at any of the points of observation. Each PAS

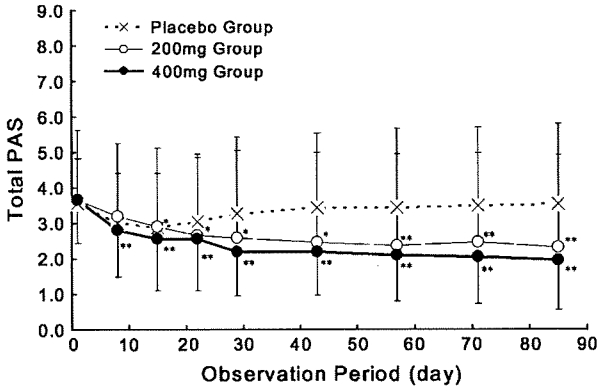

Fig 5. Changes of pemphigus activity score (*PAS*) over time. Total PAS was significantly lower in 400- and 200-mg groups than in placebo group. Significant difference from day 1 at hazard ratio of *0.05 and **0.01.

(skin lesion area, number of new blisters/d, and oral mucosal lesions) also exhibited a significant change from baseline in the 400-mg group ($P < .01$) but not in the placebo group (data not shown).

Titers of anti-Dsg IgG autoantibodies

It has been reported that levels of IgG autoantibodies to Dsg1 and Dsg3 in patients with pemphigus correlate with disease activity.[14,15] Accordingly, efficacy was also evaluated based on the changes in anti-Dsg1 IgG autoantibody titer for patients with PF and PV or in anti-Dsg3 IgG autoantibody titer for patients with PV (Fig 6). In the 400-mg group, anti-Dsg1 and −Dsg3 IgG antibody titers were significantly decreased from baseline on days 43 and 85 (day 43 and 85, $P < .01$). In the 200-mg group, anti-Dsg1 and −Dsg3 IgG antibody titers also exhibited significant decreases on day 85 but not day 43 (day 43, $P < .05$; day 85, $P < .01$). On the other hand, in the placebo group, no significant decrease from baseline was observed in either anti-Dsg1 or −Dsg3 IgG antibody titer.

Safety

The incidence of ADRs was 28.6% (n = 6/21) in the 400-mg group, 35.0% (n = 7/20) in the 200-mg group, and 25.0% (n = 5/20) in the placebo group. No significant difference was observed between the placebo and 200- or 400-mg groups. ADRs reported in the study included: headache in two patients, aggravated chronic hepatitis C, decreased lymphocytes, palpitations, abdominal discomfort, constipation, nausea, pain at the injection site, increased creatinine, increased blood pressure, and decreased platelet count in one patient each in the 400-mg group; and increased alanine aminotransferase in 3 patients; increased γ-glutamyltranspeptitase, hepatic dysfunction, and increased bilirubin in two patients each; and common cold, muscle pain, increased

aspartate aminotransferase, increased blood pressure, decreased lymphocytes, increased neutrophils, decreased white blood cell count, bleeding tendency, anorexia, hypoalbuminemia, hepatic encephalopathy, gastrointestinal bleeding, malaise, fever, increased ammonium, increased C-reactive protein, decreased hematocrit, decreased hemoglobin, decreased platelet count, decreased red blood cell count, and decreased urine volume in one patient each in the 200-mg group. All these ADRs were consistent with the information displayed on the Food and Drug Administration Web site (http://www.fda.gov/cber/gdlns/igivimmuno.htm).

One patient in the 200-mg group died of hepatic failure as a result of aggravation of hepatitis C, which was an underlying complication reported before the start of the study.

This event was judged as probably related to the investigational drug in the evaluator's opinion.

DISCUSSION

Most clinical research involving a rare disease is based on case reports or data from limited samples obtained in open-label studies. In particular, in life-threatening, serious, and intractable diseases, such as pemphigus, appropriate treatment must be provided in a timely fashion if symptoms are aggravated or unchanged for days. This makes performance of a placebo-controlled, double-blind comparison study infeasible. On the other hand, the efficacy of new drugs for malignant tumors or for patients requiring pain relief is evaluated based on the time to recurrence of tumor or the number of patients requiring rescue analgesia.[16-21] Based on these considerations, we developed a novel efficacy indicator (ie, TEP) with reference to the end points used for efficacy evaluation of drugs for malignant tumors or for patients requiring pain relief, to conduct a placebo-controlled, double-blind comparison study in patients with pemphigus who were relatively resistant to systemic steroids. This new efficacy end point provides flexibility for physicians to rescue patients when required and proved to be useful to evaluate the efficacy of a single cycle of HD-IVIG in a double-blind comparison design. However, some concerns remain regarding the rigidity: a period of 3 to 7 days before the start of study treatment was required to confirm the unresponsiveness of patients to steroids, and switching to other treatments was prohibited during the first 5-day treatment period.

The mode of action of HD-IVIG is complex. It is found to exert its effect through modulation of expression and function of Fc receptors, interference with complement activation and the cytokine

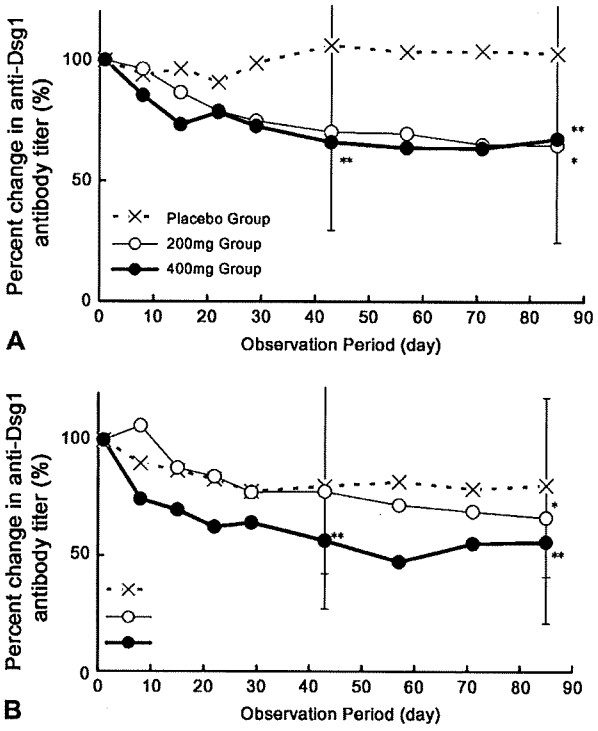

Fig 6. Changes of anti-desmoglein (*Dsg*) IgG titers. Anti-Dsg IgG titers were significantly lower in 400-mg intravenous immunoglobulin group than in placebo group over time. Changes of titers in anti-Dsg1 IgG autoantibodies (**A**) in patients with pemphigus vulgaris (PV) and pemphigus foliaceus and in anti-Dsg3 IgG autoantibodies (**B**) in patients with PV were shown (mean ± SD). Significant difference from day 1 at hazard ratio of *0.05 and **0.01.

network, provision of anti-idiotypic antibodies, modulation of dendritic cell, T- and B-cell activation, differentiation, and their effector functions.[22,23] Thus, HD-IVIG has multiple modes of action and is thought to act synergistically. HD-IVIG exerts immunomodulatory effects in autoimmune and inflammatory disorders without suppressing the immune system, which provides a distinctive advantage over conventional treatment.

Most of the previous studies suggesting efficacy of HD-IVIG for treatment of pemphigus involved multiple treatment cycles. However, our study demonstrated that a single cycle with HD-IVIG for 5 days has a therapeutic benefit to suppress the disease activity of pemphigus. Like rituximab, for which efficacy was recently reported in a single cycle,[24] IVIG is expensive and should be considered for patients who show difficulty with or resistance to conventional treatments.

In conclusion, our study suggests that TEP is a useful indicator for evaluation for rare intractable diseases such as pemphigus, and that a single cycle of HD-IVIG appears to be an effective treatment for

patients with pemphigus who are relatively resistant to systemic steroids.

We thank members of the Pemphigus Study Group described below for cooperation with registration and precise observation of patients with pemphigus. We also acknowledge data management and analysis support from Bellsystem24 Inc and EPS Co Ltd, Tokyo, Japan.

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APPENDIX

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SPECIAL NOTICE REGARDING CASE REPORTS

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Expression of toll-like receptor 2, NOD2 and dectin-1 and stimulatory effects of their ligands and histamine in normal human keratinocytes

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dectin-1, histamine, keratinocyte, NOD2, toll-like receptor 2

Conflicts of interest
None declared.

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Summary

Background Epidermal keratinocytes are involved in the skin innate immunity and express toll-like receptors (TLRs) and other innate immune proteins. The epidermis is continuously exposed to pathogenic Gram-positive bacteria or fungi. However, few studies have examined the function and expression of innate immune proteins in keratinocytes. Histamine, which is well known for itch and allergy, is closely associated with innate immunity, but its influence on epidermal innate immunity is still unclear.

Objectives To clarify the expression of innate immune proteins in keratinocytes stimulated by ligand pathogen-associated molecules, and the function of histamine in this process.

Methods We investigated the effects of lipopeptide (MALP-2, 1–100 ng mL^{-1}; ligand for TLR2), peptidoglycan (PGN, 0·02–2 μg mL^{-1}; ligand for NOD2) and β-glucan (1–100 μg mL^{-1}; ligand for dectin-1) in the presence or absence of histamine on mRNA expression of TLR2, NOD2 and dectin-1 as well as human β-defensin 2 by quantitative real-time polymerase chain reaction in cultured normal human epidermal keratinocytes. TLR2 expression was also examined at the cell surface and intracellularly, as determined by flow cytometry and confocal microscopy. The quantities of interleukin (IL)-1α and IL-8 produced by keratinocytes were measured using enzyme-linked immunosorbent assay.

Results At the mRNA level, TLR2 was enhanced by PGN but not by its ligand MALP-2 or by β-glucan; NOD2 was easily induced by all three ligands; and dectin-1 was enhanced by its ligand β-glucan. These enhanced expressions were further augmented by histamine at 1 μg mL^{-1}. While the surface expression of TLR2 was barely detectable by flow cytometry even after stimulation, the intracellular expression of TLR2 was apparently elevated by PGN and further promoted by histamine. A confocal microscopic analysis also revealed the enhanced expression of TLR2 in the cytoplasm. The expression of TLR2, NOD2 and dectin-1 was functional, as these pathogen-associated molecules induced the production of IL-1α, IL-8 and defensin, and again, histamine greatly enhanced this production.

Conclusions Our study demonstrated that the expression of functional innate immune receptors is augmented by the pathogen-associated molecules in a ligand-feed forward or nonrelated manner in keratinocytes, and histamine promotes their expression and the resultant production of cytokines and defensins.

Toll-like receptors (TLRs) are involved in the innate immune system and recognize various pathogen-associated molecular patterns of microorganisms, such as lipopeptide, lipopolysaccharide, RNA and unmethylated CpG DNA.[1] It has been shown by many studies that epithelial cells from several defensive organs play a pivotal role in the primitive defence system against microorganisms such as bacteria, fungi and viruses, and accordingly, they express TLRs.[2–4] TLR2 is a key

receptor for epithelial cells, because the epithelium is continuously exposed to pathogenic Gram-positive bacteria whose products stimulate TLR2. The expression of TLRs is induced by cytokines such as tumour necrosis factor (TNF)-α and interferon-γ,[4,5] and by TLR agonists *per se*.[6] In addition, it has been strongly suggested that mast cells are deeply involved in innate immunity[7] and in the epithelial defence system.[8,9] A recent finding that histamine, a key product from mast cells, induces TLR expression on endothelial cells,[10] implicates the ability of histamine to increase TLR expression in epithelial cells as well.

In the skin, keratinocytes are the first responders to external invaders and serve as initiators in innate immunity by producing cytokines/chemokines and antimicrobial peptides.[11–13] Several studies have documented that keratinocytes express TLRs such as TLR1, TLR2, TLR3, TLR4, TLR5, TLR6 and TLR10.[14] However, the induction and detection of these molecules remain unclear or even controversial among the previous reports. As assessed by flow cytometry, human keratinocytes were shown to express TLR4 as well as CD14,[15] whereas another group of investigators reported that only TLR2 was detected on the surface of cultured normal human keratinocytes.[16] The HaCaT keratinocyte cell line was reported to express both TLR2 and TLR4 by flow cytometry.[17] On the other hand, an immunohistochemical study of human skin demonstrated that TLR1, TLR2 and TLR5 are constitutively expressed in the cytoplasm of normal keratinocytes and that TLR2 expression is increased in psoriasis.[18]

Upon stimulation of keratinocytes via TLRs, they produce cytokines/chemokines and antimicrobial peptides as an outcome of operation of the innate immunity. These substances include interleukin (IL)-1α, TNF-α, granulocyte/macrophage colony-stimulating factor (GM-CSF), IL-8, macrophage inflammatory protein-1α,[13,16,19–23] and major antimicrobial peptides, the β-defensins.[24–26] The production of these molecules is one of the hallmarks of expression of functional TLRs by keratinocytes. As a consequence, IL-1α, TNF-α and GM-CSF produced by keratinocytes subsequently activate the cutaneous acquired immunity by enhancing the antigen-presenting ability of dendritic cells.[13]

In this study, we aimed firstly to clarify the agents that induce the expression of TLR2 and its cooperating receptors in keratinocytes, focusing on lipopeptide, peptidoglycan (PGN), the fungal element β-glucan, and histamine. Secondly, we explored whether TLR2 expression can be assessed by flow cytometry at the surface or intracellular level. We chose these agents because lipopeptide is a TLR2 ligand[27] and β-D-glucan binds to dectin-1.[28] PGN, which is recognized by NOD2,[29,30] also augments expression of TLR2.[27,31] In addition to these external stimulants for epidermal keratinocytes, we also examined histamine, which is a physiological stimulator for TLR2 expression,[10] and whose receptors, H_1 and H_2,[32] are expressed on keratinocytes. Results suggest that PGN and histamine strongly elevate the intracellularly detectable TLR2 and augment the production/expression of cytokines/chemokines and β-defensin in normal human keratinocytes. It is notable that there is a synergism between the pathogen-associated molecules and histamine in the expression of TLR2 and the production of inflammatory and antimicrobial molecules.

Materials and methods

Culture and stimulation of keratinocytes

Normal human epidermal keratinocytes (NHEK) isolated from neonatal foreskin were obtained from Cambrex Bio Science Walkersville (Walkersville, MD, U.S.A.) and grown in the serum-free keratinocyte growth medium KGM-2 (Clonetics, San Diego, CA, U.S.A.) or Dulbecco's modified Eagle's medium (Gibco BRL Life Technology Inc., Gaithersburg, MD, U.S.A.) at 37 °C in a 5% CO_2 incubator. NHEK were subcultured using trypsin–ethylenediamine tetraacetic acid (Clonetics), and semiconfluent cells at third passage were used in all experiments. Unless otherwise mentioned, semiconfluent keratinocytes in six-well plates (Corning Glass Works, Corning, NY, U.S.A.) with 2 mL of medium were stimulated with the following substances: lipopeptide (MALP-2; Axora, San Diego, CA, U.S.A.), PGN (InvivoGen, San Diego, CA, U.S.A.), β-glucan (MP Biomedicals, Aurora, OH, U.S.A.) and histamine (MP Biomedicals).

Quantitative real-time polymerase chain reaction

Total RNA from NHEK was isolated using the SV Total RNA Isolation System (Promega, Madison, WI, U.S.A.) according to the manufacturer's instructions with inclusion of the DNase step. Purified RNA was reverse transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, U.S.A.) with oligo d(T)16 primers. TaqMan experiments were carried out in an ABI PRISM 7000 Sequence Detector System using TaqMan Gene Expression Assays for TLR1, TLR2, TLR6, NOD2, human β-defensin 2 (hBD2) and dectin-1 (Applied Biosystems). Endogenous β-actin was used to normalize the gene expression between different samples.

Flow cytometry of toll-like receptor 2 expression

NHEK were incubated for 48 h with the stimulants. For the surface expression of TLR2, cells were stained with phycoerythrin (PE)-conjugated anti-TLR2 (TL2.1) monoclonal antibody (mAb) or isotype control mouse IgG2a (all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). For the intracellular expression of TLR2, cells were first stained with nonconjugated anti-TLR2 mAb, fixed with Cytofix/Cytoperm (BD Pharmingen, San Jose, CA, U.S.A.) for 40 min at 4 °C, and stained with the PE-conjugated anti-TLR2 mAb as above. Hanks' balanced salt solution containing 0·1% NaN_3 and 1% fetal calf serum was used as the staining buffer. After incubation for 60 min at room temperature, cells were washed twice and analysed: fluorescent profiles were generated using a FACSCanto (Becton Dickinson, San Jose, CA, U.S.A.).

Quantification of cytokines and chemokines in keratinocyte culture supernatants

Three-day culture supernatants from NHEK were collected, stored at −80 °C, and assayed for IL-1α and IL-8 using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's directions. Optical density was measured with a microplate reader (model 3550; Bio-Rad, Hercules, CA, U.S.A.).

Construct and transfection

The pCMV-SPORT6-TLR2 (IMAGE clone 5213439) including full-length TLR2 cDNA (GenBank BC033756) and pCMV-SPORT6 (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) were purchased from Open Biosystems (Huntsville, AL, U.S.A.) and used for the transfection study. NHEK (5×10^5 cells) were cultured without serum and antibiotics in a 60 × 15 mm TC dish (Nalge Nunc International, Naperville, IL, U.S.A.) and used at 40–50% confluency. Transfection was performed with 4 μg of plasmid DNA and 8 μL of Hily Max (Dojindo Laboratories, Kumamoto, Japan) in Opti-MEM I (Gibco). Four hours after transfection, the cells were washed and soaked in the fresh medium.

Toll-like receptor (TLR) 2 blocking with anti-TLR2 antibody in interleukin-8 production

Semiconfluent NHEK were incubated with 10 μg mL^{-1} of Function Grade (FG) antihuman TLR2, clone TL2.1, or FG mouse IgG2a isotype control (eBioscience, San Diego, CA, U.S.A.) at room temperature for 1 h. PGN (0·02 μg mL^{-1}) or MALP-2 (1 ng mL^{-1}) was then added to the culture and incubated for 72 h. The concentration of IL-8 in the supernatants was measured by ELISA.

Immunocytoplasmic staining

NHEK were cultured in chamber slides (Nalge Nunc International). Slides were fixed and stained as for flow cytometric analysis. After staining, cells were analysed by confocal microscopy (LSM5 Pascal; Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Statistical differences were determined by Student's t-test or Welch's t-test; $P < 0{\cdot}05$ was considered to be significant.

Results

Expression of mRNA for toll-like receptor (TLR) 2, its cooperating receptors TLR1 and TLR6, dectin-1, NOD2 and human β-defensin 2 in normal human epidermal keratinocytes

We first examined whether TLR2, its cooperating receptors (TLR1 and TLR6), dectin-1, NOD2 and hBD2 are expressed in unstimulated or stimulated NHEK. TLR2 is a receptor for lipopeptide (MALP-2), and dectin-1 in combination with TLR2 is a receptor for zymosan, and they collaborate with each other in recognition of microbes and induction of inflammation.[33–35] NOD2 is an intracytoplasmic molecule that recognizes PGN.[29,30] hBD2 is an antimicrobial peptide known to be produced following TLR ligation.[36,37]

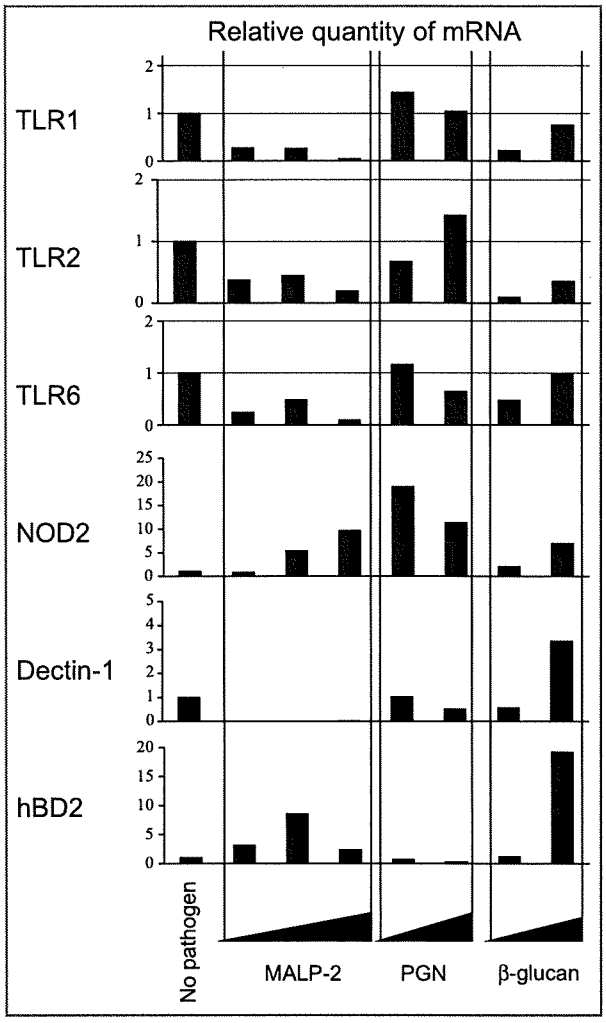

Fig 1. Expression of toll-like receptor (TLR) 2, its cooperating receptors TLR1 and TLR6, NOD2, dectin-1 and human β-defensin 2 (hBD2) in normal human epidermal keratinocytes (NHEK). NHEK were cultured for 2 h with MALP-2 (1, 10, 100 ng mL^{-1}), peptidoglycan (PGN) (0·2, 2 μg mL^{-1}) or β-glucan (1, 10 μg mL^{-1}) and subjected to real-time polymerase chain reaction analysis.

The levels of mRNA for TLR2, TLR1 and TLR6 were augmented by PGN to some extent at certain concentrations, but not by MALP-2 or β-glucan (Fig. 1). NOD2 expression was remarkably enhanced by all the stimulants, with variations. The expression of dectin-1 was elevated by β-glucan but not by MALP-2 or PGN. hBD2 expression was increased by MALP-2 and β-glucan. It is thus suggested that (i) NOD2 is easily inducible by various pathogenic stimulants, (ii) TLR2 is enhanced by PGN but not by its ligand lipoprotein or by

β-glucan; and (iii) dectin-1 is enhanced by β-glucan. These findings partly support the concept that TLR expression is often augmented by pathogen-associated molecules other than the corresponding specific ligand.[5,6]

Detection of augmented expression of intracellular toll-like receptor 2 by flow cytometry

We tested whether TLR2 is detectable at the protein level in NHEK, either unstimulated or following stimulation with PGN and β-glucan at relatively high doses. A flow cytometric analysis showed that the surface expression of TLR2 was very low, and that it was not enhanced by PGN (Fig. 2a) or β-glucan stimulation at either low or high Ca concentration, which induces basal and cornified keratinocytes, respectively. However, when these cells were subjected to intracellular staining, we found that PGN upregulated the expression of TLR2 (Fig. 2b). The addition of β-glucan also elevated TLR2 expression in the cytoplasm but not at the surface. Thus, an increased level of TLR2 was detected by flow cytometry in the cytoplasm but not at the surface of keratinocytes.

Functional expression of toll-like receptor 2, NOD2 and dectin-1 assessed by cytokine/chemokine production

To confirm the functional expression of TLR2, NOD2 and dectin-1, NHEK were cultured with MALP-2, PGN or β-glucan and the amounts of IL-1α and IL-8 secreted in the supernatants were measured. As shown in Figure 3, MALP-2 stimulated NHEK to produce these cytokines/chemokines. The NOD2 ligand PGN also augmented the production at an optimal concentration as low as 0·2 μg mL^{-1}. Similarly, β-glucan, a ligand for TLR2 and dectin-1, promoted the production of these cytokines/chemokines at an optimal concentration of 10 μg mL^{-1}. Thus, TLR2 and NOD2 seemed to be functionally expressed in NHEK.

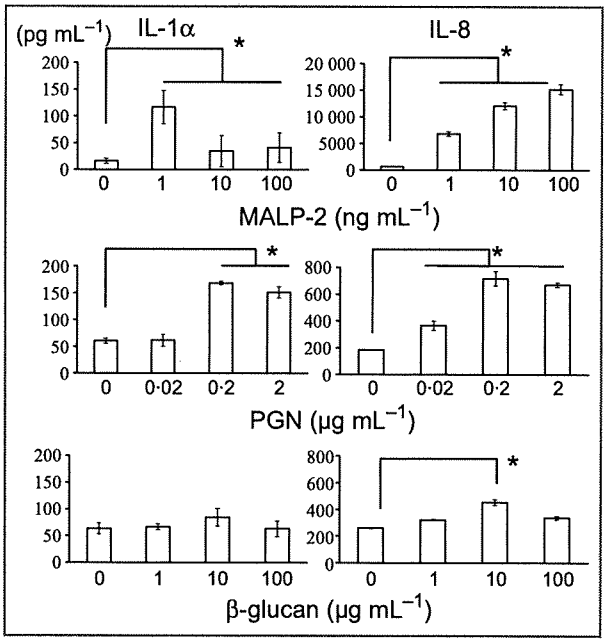

Fig 3. Augmentative effect of MALP-2, peptidoglycan (PGN) and β-glucan on cytokine/chemokine production by normal human epidermal keratinocytes (NHEK). NHEK were cultured for 72 h in the presence or absence of pathogen-associated molecules at the indicated concentration. The amounts of interleukin (IL)-1α and IL-8 in the supernatants were measured by enzyme-linked immunosorbent assay. The values are means of quadruplicate determinations with SD shown by vertical bars. *P < 0·05, compared with the nonaddition group.

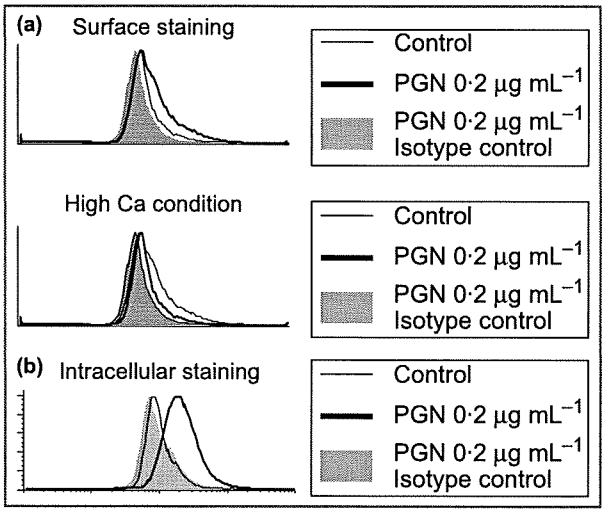

Fig 2. Flow cytometric detection of toll-like receptor (TLR) 2 expression in the cytoplasm but not on the surface of normal human epidermal keratinocytes (NHEK). NHEK were cultured in the presence or absence of peptidoglycan (PGN) for 48 h. Cells were stained with anti-TLR2 monoclonal antibody (mAb) or isotype-matched control antibody (a). The levels of isotype-matched control were evaluated in the stimulated NHEK. Simultaneously, NHEK cultured with the stimulant were intracellularly stained with anti-TLR2 mAb (b).

It is known that PGN is not a ligand for TLR2 but is an activator.[31,38,39] To confirm this notion, we performed two studies. In one study, TLR2-transfected keratinocytes were stimulated with PGN or MALP-2, and the concentration of IL-8 was measured in the culture supernatants. Compared with the control (mean ± SD 1970·45 ± 15·14 pg mL^{-1}), addition of MALP-2 at 1 ng mL^{-1} (2518·01 ± 26·34 pg mL^{-1}) but not PGN at 0·02 μg mL^{-1} (1957·83 ± 28·44 pg mL^{-1}) gave a significantly higher level of IL-8 secretion in the supernatants. In the other study, NHEK were cultured with PGN (0·02 μg mL^{-1}) or MALP-2 (1 ng mL^{-1}) in the presence of blocking antibody to TLR2 or IgG2a as isotype-matched control. Following stimulation with PGN, neither anti-TLR2 antibody-treated nor isotype-matched control showed decreased production of IL-8. Following stimulation with MALP-2, however, treatment with anti-TLR2 antibody, but not control IgG2a, significantly decreased IL-8 production by 20%. Taken together, these findings suggest that PGN is not a specific ligand for TLR2.

Synergistic effects between pathogen-associated molecules and histamine on toll-like receptor 2 expression and cytokine production

NHEK were cultured with PGN or β-glucan in the presence or absence of histamine, and the levels of mRNA expression for TLR1, TLR2 and TLR6, NOD2, dectin-1 and hBD2 were measured by quantitative real-time polymerase chain reaction (PCR). Histamine at 1 μg mL^{-1} clearly amplified the expression of all the receptors and hBD2 that were induced by MALP-2 at 10 ng mL^{-1}, PGN at 2 μg mL^{-1} or β-glucan at 10 μg mL^{-1} (Fig. 4), demonstrating their synergistic effects.

Further to confirm the synergism by intracellular flow cytometry, we tested the combined effect of PGN at 0·2 μg mL^{-1} and histamine at 1 μg mL^{-1} on the expression of TLR2. The synergism between PGN and histamine was clearly observed, as the addition of both further elevated the TLR2 expression induced by individual stimulants (Fig. 5a), but again the surface expression was undetectable (data not shown). A confocal image analysis of keratinocytes showed that augmentation of the cytoplasmic level of TLR2 induced by synergism between PGN and histamine was greater than that of the cell surface level (Fig. 5b).

Finally, the synergism with histamine in cytokine/chemokine production was examined. NHEK were cultured with MALP-2, PGN or β-glucan in the presence or absence of histamine, and the amounts of IL-1α and IL-8 in the supernatants were measured. As shown in Figure 6, histamine at 1 μg mL^{-1} alone did not increase the production of IL-1α or IL-8 (solid bars of ligand 0). However, histamine upmodulated IL-1α and IL-8 production in the presence of MALP-2, PGN or β-glucan.

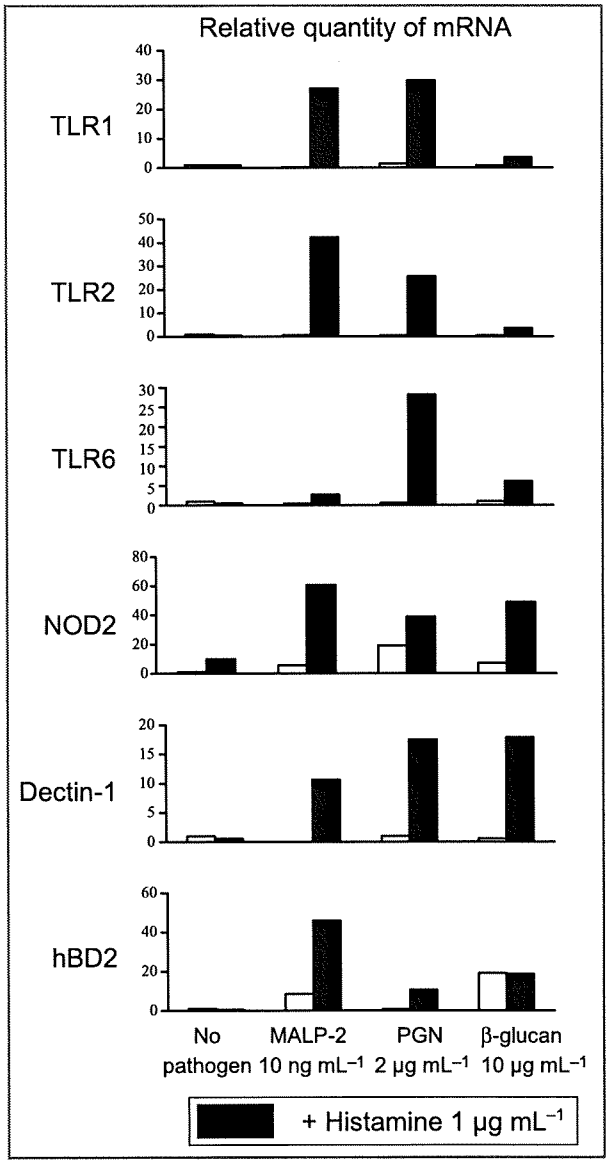

Fig 4. Synergism of MALP-2, peptidoglycan (PGN) or β-glucan, with or without histamine, in mRNA expression for toll-like receptor (TLR) 1, TLR2, TLR6, NOD2, dectin-1 and human β-defensin 2 (hBD2) by real-time polymerase chain reaction (PCR) analysis. Normal human epidermal keratinocytes were cultured for 2 h with each of the pathogen-associated molecules and/or histamine and subjected to real-time PCR analysis.

Discussion

Our study demonstrated that NHEK express both TLRs and nontoll-like innate immune proteins, including the intracellular Nod-like protein NOD2 and the surface C-type lectin dectin-1. As MALP-2, PGN and β-glucan stimulated keratinocytes to produce cytokines and antimicrobial peptides, it is considered that TLR2, NOD2 and dectin-1 are functionally expressed and efficiently mount the innate immunity in keratinocytes. More interestingly, the innate immune receptors were augmented by pathogen-associated molecules relevant or irrelevant to the corresponding receptors, as has been suggested in other types of cells.[5,6] The feed-forward stimulation was observed between PGN and NOD2 and between β-glucan and dectin-1, while TLR2 was induced by PGN but not by its ligand MALP-2. Therefore, the pathogen-driven acceleration of receptor expression appears to be complicated. However, NOD2 might be a key molecule, because all the pathogen-associated molecules tested enhanced NOD2 expression, and its ligand PGN was most stimulatory for the cytokine/hBD2 production among the pathogen-associated molecules.

TLR2 was induced by PGN and was detected at both protein and mRNA levels. We successfully evaluated the intracellular, but not surface, expression of TLR2 by flow cytometry. The expression of each TLR in keratinocytes has been a matter of debate.[40] In the present study, the surface expression of TLR2 was very low compared with the isotype-matched control, and was not enhanced by any of the stimuli at either low or high Ca concentration. Nevertheless, we found that the intracellular expression of TLR2 was significantly elevated upon stimulation. As the TLR2 ligand MALP-2 promoted the production/expression of cytokines and of hBD2, it is considered that TLR2 is expressed on keratinocytes as a functional surface molecule. Although the change of surface expression was

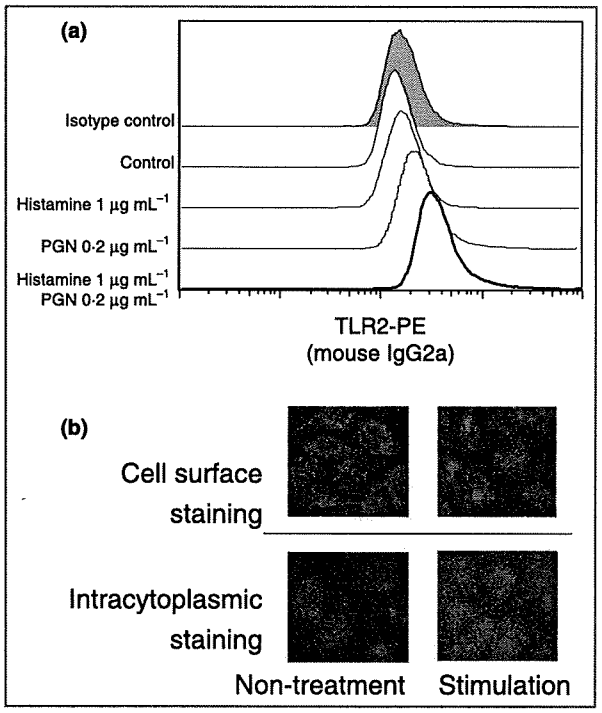

Fig 5. Synergism between peptidoglycan (PGN) and histamine in intracellular toll-like receptor (TLR) 2 expression and confocal microscopic analysis for detection of TLR2 in normal human epidermal keratinocytes (NHEK). (a) NHEK were cultured in the presence of histamine (1 μg mL^{-1}) and/or PGN (0·2 μg mL^{-1}), and were subjected to intracellular staining for TLR2. The level in the isotype-matched control was evaluated in NHEK stimulated with both histamine and PGN. (b) NHEK were cultured in a chamber slide in the presence or absence of PGN at 0·2 μg mL^{-1} and histamine at 1 μg mL^{-1} for 48 h. After incubation with nonconjugated isotype control mouse IgG2a, intracellular or cell surface staining of keratinocytes was performed with phycoerythrin (PE)-conjugated anti-TLR2 monoclonal antibody and visualized by confocal microscopy.

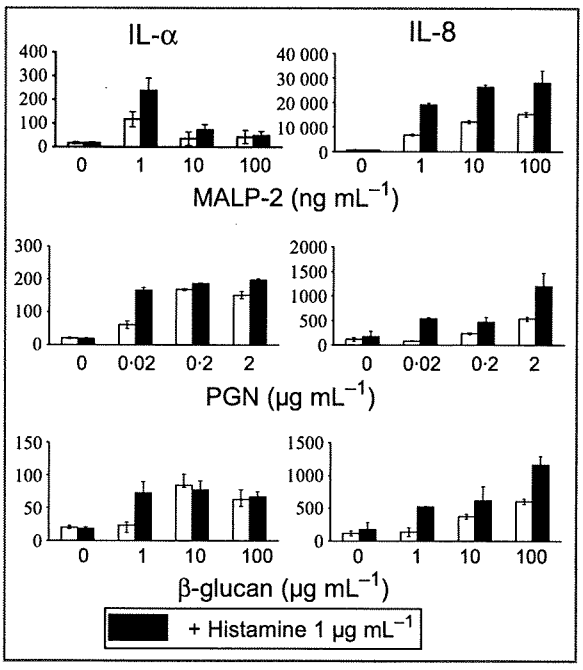

Fig 6. Synergism between pathogen-associated molecules and histamine in cytokine/chemokine production by normal human epidermal keratinocytes (NHEK). NHEK were cultured for 72 h with each of the pathogen-associated molecules and/or histamine. The concentrations of interleukin (IL)-α and IL-8 in the supernatants were measured by enzyme-linked immunosorbent assay. The values are means of quadruplicate determinations with SD shown by vertical bars. PGN, peptidoglycan.

under the detection level, the intensity of its intracellular expression may predict an alteration of the surface expression. A similar finding has been reported in dendritic cells[41] and has recently been reported in keratinocytes.[42] On the other hand, a recent finding has suggested that the intracellular TLR2 functions as a receptor for the infecting pathogen.[43] Considering that skin is constantly exposed to microorganisms, the low level of surface expression of TLR2 might be reasonable. If the quantity of TLR of the surface of keratinocytes were easily increased, this would always give rise to an irritable response to pathogens and to the occurrence of inflammation. In this respect, keratinocytes should be different from monocytes/macrophages, and intracellular TLR2 appears to recognize the pathogens when they invade the cell. There might exist a certain protein that regulates the surface expression of TLR2 like that of TLR4,[44] and it may act on the homeostasis of the epithelial tissue.

It is already known that histamine plays an important role for innate immunity.[45] We stimulated keratinocytes with the pathogen-associated molecules in combination with histamine. Histamine amplified the expression of TLRs and proinflammatory cytokines synergistically with the pathogen-associated molecules. The augmentation of TLR2 expression by histamine was also observed in endothelial cells.[10] We found that there was synergism between histamine and MALP-2, PGN or β-glucan in TLR2 expression, as assessed by both intracellular staining and real-time PCR. Such synergistic effects were also found in the expression of hBD2 and dectin-1 and the production of IL-1α and IL-8. In the skin, keratinocytes are potentially exposed to histamine that is released from dermal mast cells in certain pathological conditions. As mast cells also express TLRs and can produce histamine by TLR ligation,[46] pathogens may stimulate keratinocytes directly or indirectly via mast cells with their produced histamine. This scenario suggests the potential involvement of histamine in the natural defence system and may result in an exaggerated response to pathogens.

Our findings are of clinical significance. As the skin is constantly exposed to Gram-positive bacteria and fungi as exemplified by *Staphylococcus aureus* colonization[47] and superficial fungal infection,[48] the upmodulation of TLR2, NOD2 and dectin-1 in keratinocytes may be beneficial for the defence system. The expression of these molecules is enhanced by pathogens, resulting in the augmented production of

proinflammatory cytokines and chemokines for neutrophils and lymphocytes. These cytokines/chemokines eventually protect the host from bacteria and fungi by inducing inflammation and immune reactions. In addition, the upregulated production of the defensins effectively eliminates microorganisms. Histamine may support these events as an internal stimulus for innate immune protein expression. It is suggested that the activation of keratinocytes via innate immune proteins leads to pluripotential responses in the cutaneous innate immunity and subsequent acquired immunity.[13]

Acknowledgments

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