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METHODS

mRNA extraction of purified keratinocytes from murine ear skin

The hairs on murine ear skin were removed with depilatory cream. After removing hairs, the ears were split into dorsal and ventral halves, and the cartilage was removed. Then the skin was floated on 0.25% trypsin/EDTA for 30 minutes at 37°C and separated into epidermis and dermis. Single epidermal cell suspension (EC suspension) was done by means of vigorous trituration of the epidermal sheet. Because EC suspensions are mixtures of keratinocytes, Langerhans cells, and γδ T cells, we purified keratinocytes by removing Langerhans cells and γδ T cells from the EC suspension with the autoMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany) using magnetic microbeads coated with anti-mouse CD45 antibody. The purity of keratinocytes is greater than 99%. Then we extracted RNA from keratinocytes with the RNeasy mini kit (Qiagen).

Real time RT-PCR primer

The primer sequences of CXCL2 were 5′-GCC TAT CGC CAA TGA GC-3′ (forward) and 5′-TGG ACA ATT TTC TGA ACC AAG-3′ (reverse).

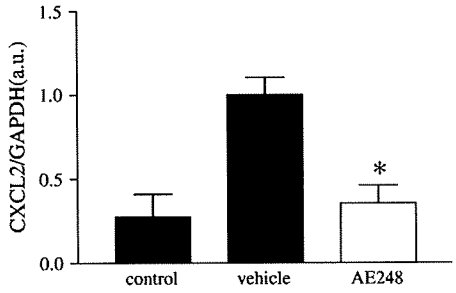

FIG E1. Real-time RT-PCR analysis on mRNA expression of CXCL2 in the purified keratinocytes in hapten-challenged ears of vehicle- or AE248-treated mice (n = 3 per group). Results are expressed as means ± SEMs. *$P < .05$ versus the vehicle-treated group. *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase.

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Induction of eosinophil-infiltrating drug photoallergy in mice

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ABSTRACT

Background: Drug photoallergy is one of the highly incident adverse effects. Several different histological patterns have been recognized.
Objective: To establish a murine model of the eosinophil-infiltrating type of drug photoallergy by using afloqualone (AQ), a representative photosensitive drug.
Methods: AKR/J mice were sensitized by intraperitoneal injection of afloqualone solution (2 mg/kg/mouse) and irradiation of shaved abdomen with ultraviolet A light (UVA) (12 J/cm^2). This sensitization procedure was repeated 2–12 times, and 3 days after the last immunization, mice were challenged by a subcutaneous injection of AQ solution and irradiation of the same site with UVA. The draining lymph node cells (LNCs) were used for transfer and cytokine production studies, and the challenged skin was analyzed for chemokine expression.
Results: More than 10 times of sensitization induced a massive infiltrate of eosinophils and lymphocytes at the challenged site. AKR/J mice were a high responder strain. The sensitivity was transferred with 5–8 $\times$ 10^7 immune lymph node and spleen cells into naïve mice. CD4$^+$ T cells were mainly responsible for this sensitivity, since 1 $\times$ 10^7 CD4$^+$ cells alone induced a high level of sensitivity, but CD8$^+$ T cells evoked the sensitivity to a lesser degree. Culture supernatants from AQ-photoimmuned lymph node cells contained a higher level of IL-4 and lower interferon-γ than those from mice immunized with dinitrofluorobenzene. Finally, the skin of AQ-photochallenged site exhibited high expression of CCL24/eotaxin-2, a chemokine for eosinophils.
Conclusion: It is suggested that eosinophilic drug photoallergy is mediated by sensitized Th2 cells and locally produced eosinophil-attracting chemokines.

1. Introduction

Drug photosensitivity is one of the adverse reactions and clinically recognized as photosensitivity dermatitis [1]. The action spectrum is usually ultraviolet A light (UVA) and UVB exceptionally may induce this sensitivity or augment the UVA-evoked sensitivity level [2]. Various drugs have been reported to induce photosensitivity, including quinolones [3], afloqualone (AQ) [4], piroxicum [5], non-steroidal anti-inflammatory drugs [6], and others. There are phototoxic [2] and photoallergic [3–7] mechanisms in drug sensitivity, and the incidence of photoallergy is higher than that of phototoxicity [3]. Two theories of photoantigen formation have been put forward to explain the photoallergic mechanisms, prohapten and photohapten [8]. Prohapten is converted to ordinary hapten by UVA exposure and can bind to protein. On the other hand, photohapten needs to coexist non-covalently with protein prior to UVA exposure, and UVA induces a covalent bond between them. The vast majority of photosensitizing drugs are photohapten rather than prohapten [3,4,9].

We have taken several different approaches to establish mouse models of drug photoallergy with the use of afloqualone [4], quinolones [10], and ketoprofen [6]. Drug photoallergy is successfully induced and elicited by systemic administration of a drug and subsequent UVA irradiation of the skin [4,6,10], which is mimicry of clinical drug photoallergy. In another system, photoallergy is induced by sensitization and elicitation with subcutaneous injections of epidermal cells that are *in vitro* photomodified with a drug under UVA exposure [4,9]. The involvement of T cells in drug photoallergy has been clearly demonstrated by mouse models of photoallergy to fluoroquinolone, afloqualone, and ketoprofen [6,10]. Drug photoallergy is mediated by CD4$^+$ T cells [10], and CD8$^+$ T cells may enhance CD4$^+$ T cell responses [6]. Epidermal Langerhans cells are photomodified with a given drug and capable of inducing the proliferation of primed CD4$^+$ T cells [10].

Clinically, drug photoallergy shows eythematous eruption [1] and lichenoid eruption [11,12], and rarely bullous eruption [13] and leukomelanoderma. The erythematous eruption is

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the common type of drug photoallergy and may have scaling on the surface. The lichenoid eruption is not uncommon and similar to lichen planus. This type is characterized clinically by erythematous but dark-colored papules and histologically by $CD8^+$ T cells infiltrating in the upper dermis and attacking keratinocytes [11]. Leukomelanoderma displays a unique clinical appearance of mixture of pigmentation and depigmentation and occurs in dark-colored individuals such as Japanese. Thus, drug photoallergy is heterogeneous as ordinary allergic drug eruptions, and different populations of T cells may induce different clinical and histological appearances. In some patients with the erythematous, lichenoid, and bullous eruptions, biopsied specimens exhibit infiltration of eosinophils as well as lymphocytes [12,3].

In this study, we attempted to establish a murine model of eosinophil-infiltrating photoallergy by administration of AQ in combination with UVA irradiation. Repeated sensitization with AQ + UVA successfully induced eosinophil infiltration upon challenge with subcutaneous AQ + UVA irradiation in AKR/J mice. Here we present the procedure for eosinophil-infiltrating photoallergy and characterize T cell subsets and cutaneous chemokines responsible for this sensitivity.

2. Materials and methods

2.1. Animals

Eight-week-old female AKR/J and BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained on a 12-h light/dark cycle under specific pathogen-free conditions. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Occupational and Environmental Health.

AQ was obtained from Tanabe Pharmaceutical Co. (Osaka, Japan) and enoxacin (ENX) from Shionogi Pharmaceutical Co. (Osaka, Japan). 2,4-Dinitro-1-fluorobenzene (DNFB) was purchased from Nacalai Tesque Co. (Tokyo, Japan).

2.2. Sensitization and challenge of drug photoallergy to AQ

An AQ solution (10 mg/ml) in phosphate-buffered saline (PBS; pH 7.4) was prepared as described previously [4]. For sensitization, AKR/J and BALB/c mice received an intraperitoneal (i.p.) injection of AQ solution (2 mg/kg/mouse), and 2 h after injection, they were irradiated with UVA (12 J/cm^2) on the shaved abdomen, as reported previously in quinolone photoallergy [9]. This sensitization procedure was repeated twice a week for 2–6 weeks. Three days after the last immunization, mice were challenged by a subcutaneous (s.c.) injection of AQ solution (2 mg/kg/mouse), or ENX solution (2 mg/kg/mouse) as control, into the shaved abdomen and subsequent irradiation of the same site with UVA (12 J/cm^2).

2.3. Adoptive lymphocytes transfer

AKR/J mice were sensitized with AQ + UVA 10 times. Single cell suspensions were prepared from axillary and inguinal lymph node cells (LNCs) and spleen cells of the sensitized mice. Cells were purified for $CD4^+$ cells (purity >95%) by the AutoMACS magnetic separation system with a kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's directions. Crude immune cells (5×10^7 cells/mouse) or purified $CD4^+$ T cells (1×10^7 cells/mouse) were transferred to naïve AKR/J mice by intravenous (i.v.) injection. Two days after injection, they were challenged by a subcutaneous injection of AQ + UVA irradiation of the shaved abdomen.

2.4. Histological assessment

Two days after the challenge with AQ + UVA, mice were sacrificed, and the abdominal skin was excised. Two to three micrometers-thick sections were cut and stained with hematoxylin and eosin.

2.5. Measurement of cytokines produced by LNCs

The culture medium was RPMI-1640 (Gibco BRL Life Technology Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% nonessential amino acids, and 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Gibco). Immune LNCs from mice sensitized with AQ + UVA 10 times were cultured in 24-well plates (Corning Inc., Corning, NY; 1×10^6/ml of culture medium) at 37 °C in 5% CO_2 in air in the presence of anti-CD3 mAb (soluble form stimulatory for T cells; BD Biosciences (San Diego, CA) at 5 μg/ml and anti-CD28 mAb (Immunotech, Marseille, France) at 5 μg/ml. After 72-h culture, the supernatants were collected and were stored at −80 °C until use. The concentrations of interferon-γ (IFN-γ), IL-4, and IL-5 in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA) with kits (R&D Systems, Minneapolis, MN) according to the manufacturer's directions.

2.6. Real-time quantitative PCR

Total mRNA was extracted from the mice ears with the SVTotal RNA Isolation system (Promega, Madison, WI) according to the manufacturer's protocol. Target gene expression was quantified in a two-step RT-PCR. cDNA was reverse transcribed from total RNA samples using the TaqMan RT reagents (Applied Biosystems, Foster City, CA). Murine CXCL12 (Assay ID: Mm00445552_m1) expression was quantified using TaqMan Gene Expression Assay (Applied Biosystems) in the ABI PRISM 7000 sequence detection system (Applied Biosystems). As an endogenous reference for these PCR quantification studies, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene expression was measured using the TaqMan rodent GAPDH control reagents (Applied Biosystems). The relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method [14]. The expression of the target gene normalized to an endogenous reference and relative to calibrator is given by the formula $2^{-\Delta\Delta C_T}$. Gene expression in control mice was used as a calibrator expression to calculate $\Delta\Delta C_T$. As control, earlobes painted with 25 μl of 0.3% DNFB in 4:1 (v/v) acetone/olive oil was used.

2.7. Statistical analysis

Data were analyzed using an unpaired two-tailed *t*-test. *P* value of less than 0.05 was considered to be significant.

3. Results

3.1. Induction of eosinophil-infiltrating photoallergy by AQ + UVA in AKR/J mice

We have previously induced photosensitivity to AQ with AQ-photomodified epidermal cells in BALB/c mice. AQ-photomodified epidermal cells were prepared *in vitro* by UVA irradiation of epidermal cell suspensions in AQ solution, and mice were sensitized and elicited by s.c. injections of those photomodified cells into lateral aspects of the back and footpads, respectively [4]. Here we attempted to sensitize mice by systemic administration of AQ and UVA irradiation of the skin. Since UVA irradiation is

performed *in vivo* in this procedure, melanin pigment may affect the degree of photosensitivity as seen in contact photosensitivity [15]. Therefore, we used albino mice, BALB/c and AKR/J mice in the present study.

The histological changes at the challenged skin were investigated in mice sensitized with AQ + UVA. BALB/c and AKR/J mice were sensitized by an i.p. injection of AQ and subsequent UVA irradiation of shaved abdomen. When this sensitization was performed 10 times and the challenge was done with the same procedure, we could not obtain substantial infiltration of inflammatory cells at the challenged site in either mouse strain (data not shown). Therefore, we immunized mice 10 times (twice a week for 5 weeks) with AQ + UVA, and 3 days after the last sensitization, they were challenged by s.c. injection of AQ on the shaved abdomen and subsequent irradiation of the same site with UVA. As shown in Fig. 1, while AKR/J mice sensitized with AQ + UVA 2 times had no induction of the sensitivity (Fig. 1A), 10-time sensitization exhibited a massive infiltrate of inflammatory cells at the challenged site (Fig. 1B). A high power view revealed that the infiltrate consisted of a high number of eosinophils with occasional lymphocytes (Fig. 1C). BALB/mice sensitized even 10 times did not show infiltration of eosinophils (data not shown), indicating strain dependency of this photoallergy. There were clinical differences in the skin between the 10-time sensitized and challenged mice and the non-sensitized and challenged mice. While the only challenged mice showed virtually no skin change, the 10-time sensitized and challenged mice exhibited scaly and crusty lesions with erythema (Fig. 2).

3.2. Number of sensitization with AQ + UVA to induce eosinophil infiltration and requirement of both AQ and UVA for induction

The frequency of sensitization that induces eosinophilic infiltration upon challenge was tested in AKR/J mice. Mice were sensitized 2–12 times (twice a week) and challenged with AQ + UVA. A significant increase in the number of eosinophils was observed in mice sensitized 8 times (Fig. 3A). Ten-time sensitization induced a higher eosinophil number (more than 30 cells/25 μm^2), which was comparable to 12-time sensitization. Thus, we sensitized AKR/J mice 10 times with AQ + UVA in the following study. We also counted the number of lymphocytes and the found that 5.0 + 1.0 lymphocytes/25 μm^2 infiltrated in the 2-time sensitized and challenged mice and 6.7 + 1.2 lymphocytes/25 μm^2 did in the 10-time sensitized and challenged mice. Therefore, the number of lymphocytes was smaller than that of eosinophils.

AKR/J mice sensitized with AQ + UVA were challenged with AQ or ENX, another representative photosensitizing drug [9,10], in combination with UVA. AQ or UVA alone did not elicit a substantial infiltrate of eosinophils, although AQ alone slightly increased eosinophil infiltration presumably because of serving as an ordinary antigen (Fig. 3B). ENX + UVA evoked no eosinophil infiltration, indicating its photoantigenic specificity.

3.3. Transfer of AQ photoallergy with immune lymphocytes

Immune LNCs and spleen cells were obtained from AKR/J mice sensitized with AQ + UVA 10 times and were injected i.v. into naïve syngeneic recipients. Recipient mice challenged by a s.c. injection of AQ and UVA irradiation of the shaved abdominal skin exhibited an intense skin inflammatory change with a massive infiltrate of eosinophils and lymphocytes (Fig. 4A). The same histological change was obtained by transfer of $CD4^+$ T cells purified from draining LNCs (Fig. 4B), while transfer of $CD8^+$ T cells evoked the response to a lesser degree (Fig. 4C). Mice receiving transfer of LNCs and spleen cells did not show the skin response upon challenge. Thus, $CD4^+$ T cells were mainly responsible for the sensitivity, but $CD8^+$ T cells at least partly participated in the response.

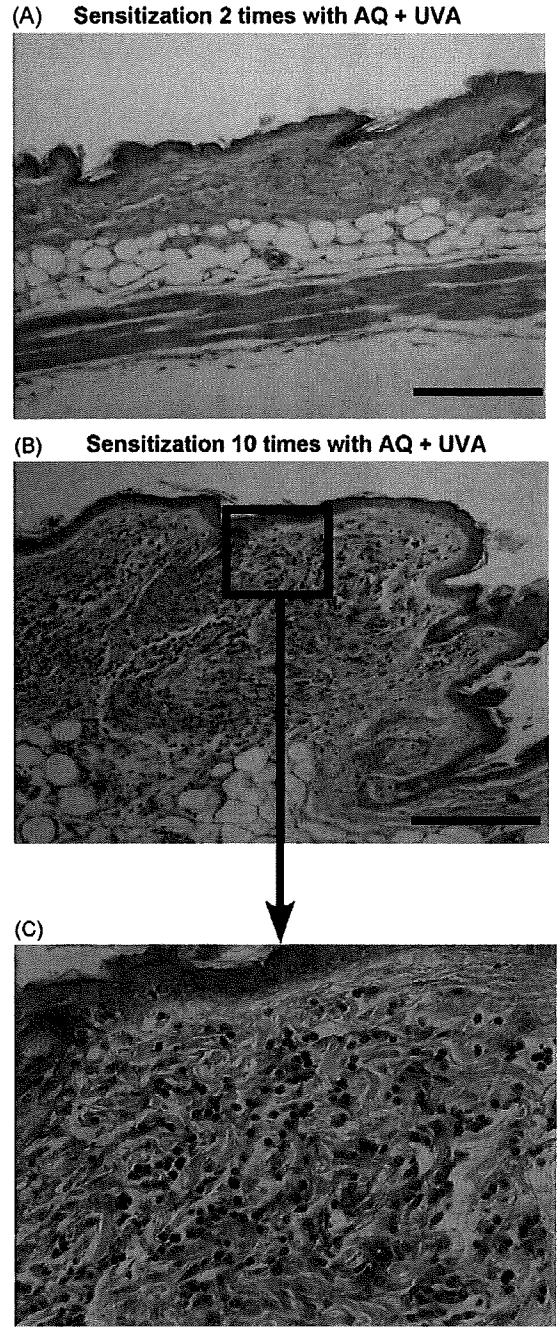

Fig. 1. Histological pictures of abdominal skin challenged with AQ + UVA in AKR/J mice sensitized 2 or 10 times with AQ + UVA. AKR/J mice were sensitized by i.p. injection of AQ (2 mg/kg/mouse) + irradiation with UVA (12 J/cm^2) 2 (A) or 10 times (B) and challenged by a s.c. injection of AQ (2 mg/kg/mous) + irradiation with UVA (12 J/cm^2). (C) High magnification of B, showing massive infiltration of eosinophils and lymphocytes. Scale bar, 100 μm.

3.4. Cytokine production pattern of draining LNCs

Draining LNCs from AKR/J mice sensitized with AQ + UVA 10 times were cultured in the presence of anti-CD3 and anti-CD28 antibodies, and 72-h culture supernatants were measured in the concentrations of IFN-γ, IL-4, and IL-5. As a control, draining LNCs from AKR/J mice sensitized by skin application of 0.5% DNFB 5 days

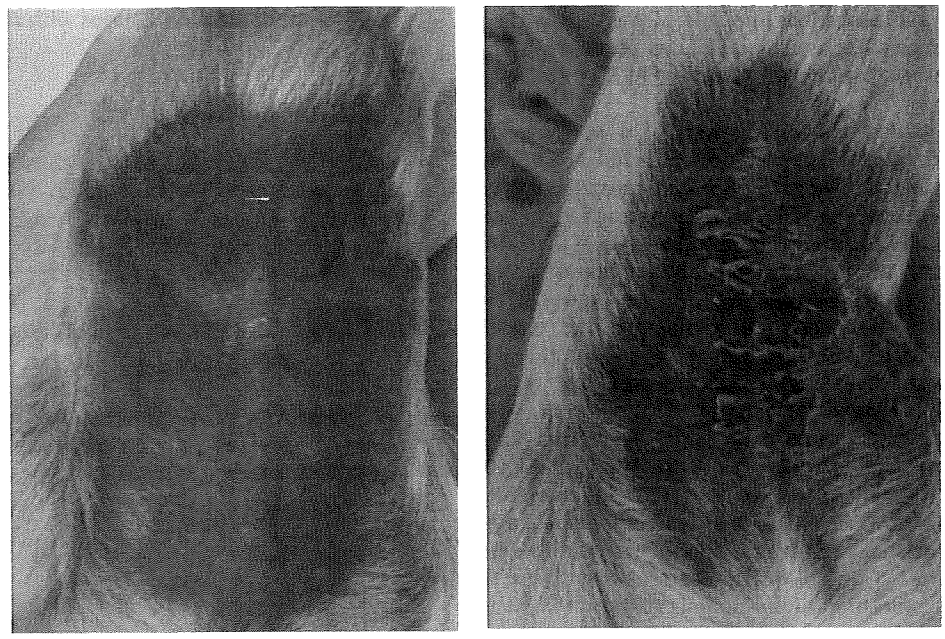

Fig. 2. Skin reaction evoked by AQ + UVA in 10-time sensitizes mice. AKR/J mice were sensitized 10 times and challenged with AQ + UVA (right), while the control mice were challenged with AQ + UVA without sensitization (left).

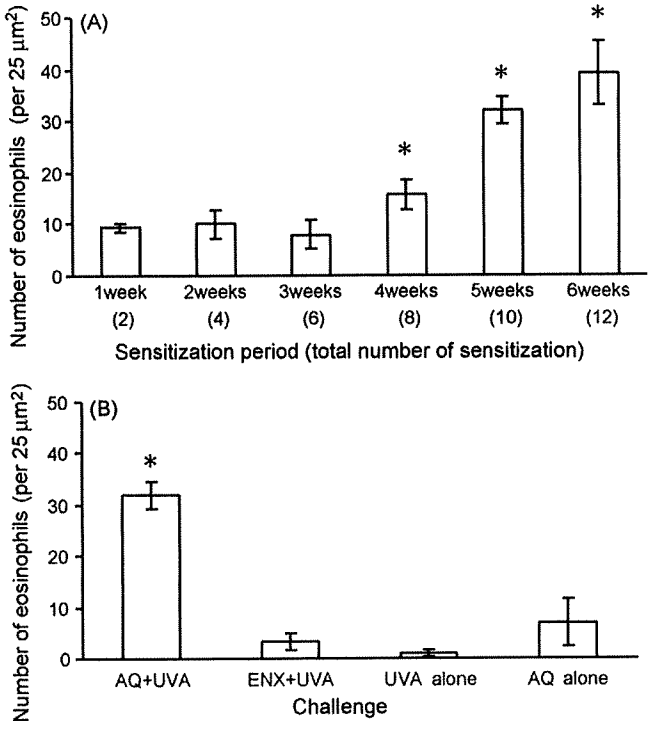

Fig. 3. Frequency of sensitization with AQ + UVA and elicitation procedure for eosinophil infiltration. (A) AKR/J mice were sensitized by i.p. injection of AQ + irradiation with UVA 2–12 times (twice a week) and challenged by a s.c. injection of AQ + irradiation with UVA. In the excised abdominal skin, eosinophils were enumerated as the number per 25 mm^2. Data are the mean + SD of four mice. $^*P < 0.05$, compared with the 2 time challenged group. B: AKR/J mice were sensitized by i.p. injection of AQ + irradiation with UVA 10 times (2 times a week) and challenged by intradermal injection of AQ (or ENX as control) and/or irradiation with UVA. The number of eosinophils was counted and expressed as the number per 25 mm^2. Data are the mean + SD of four mice. $^*P < 0.001$, compared with the other three groups.

before were used. LNCs from the AQ-photosensitized mice produced a higher amount of IL-4 and a lower amount of IFN-γ than those from the DNFB-sensitized mice (Fig. 5). The amounts of IL-5 was too low to discriminate the both. The results suggest that LNCs from the AQ-photosensitized mice contained a high frequency of Th2 cells.

3.5. Chemokine production in AQ-photochallenged skin

AKR/J mice were sensitized 10 times and challenged with AQ + UVA. The elicited skin was subjected to quantitative real-time PCR to measure mRNA levels for chemokines, including eosinophil-associated chemokines (CCL24/eotaxin-2 and CCL5/RANTES), Th1-associated chemokines (CXCL10/IP-10 and CXCL9/Mig), Th2-associated chemokines (CCL17/TARC and CCL22/MDC). As a control, AKR/J mice were painted with 0.2% DNFB on the abdomen, and the skin was excised 2 days later. In the level relative to β-actin, the expression of CCL24 was elevated (Fig. 6A), suggesting its involvement in eosinophil infiltration.

To further characterize the chemokine expression, we compared the AQ + UVA-challenged skin with normal abdominal skin of mice. However, since the normal skin had extremely low levels of chemokine expression, it was difficult to calculate the relative intensity of each chemokine mRNA in AQ + UVA-challenged skin. Then, we used hapten-applied skin for comparison. We compared the expression of chemokines between AQ + UVA-challenged abdominal skin and DNFB-challenged ear skin of DNFB-sensitized mice. Although we found that AQ + UVA-challenged skin was skewed to Th2 cells as observed with low CXCL10 and high CCL22 levels, a discrepancy existed with a relatively high level of CXCL9 (data not shown). It is possible that these results were influenced by the difference in the skin specimens used for AQ + UVA and DNFB, namely abdomen and ears. We therefore used the abdominal skin of DNFB single application. This was not a challenged skin, but the expression levels of chemokines were appropriate for comparison with AQ + UVA-challenged abdomen. When compared to the DNFB-painted abdominal skin of mice without sensitization, both CCL24 and CCL5 were elevated along

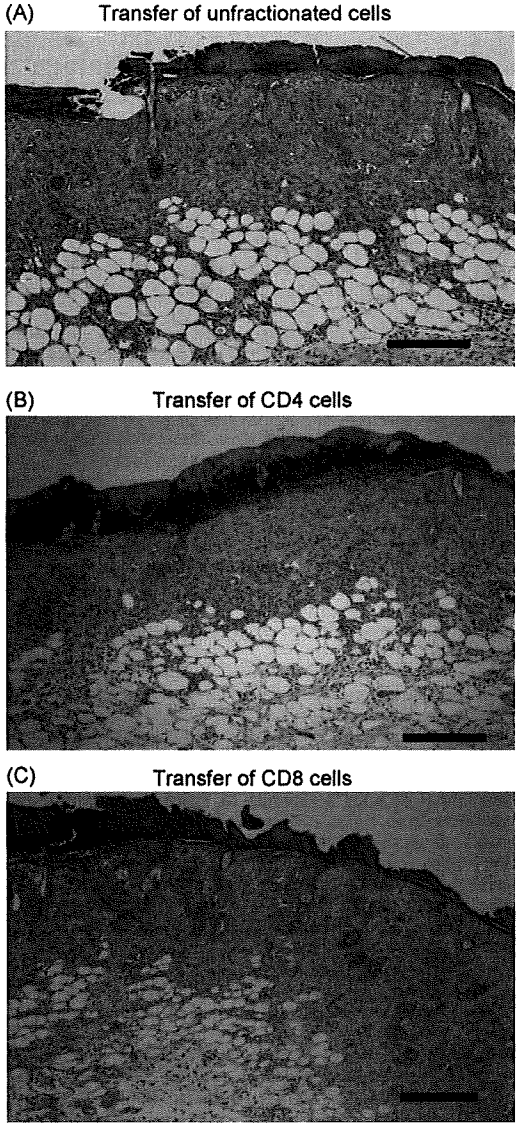

Fig. 4. Histological pictures of challenged abdominal skin of recipients transferred with AQ + UVA-immune LNCs. Donor AKR/J mice were sensitized with AQ + UVA 10 times. Draining LNCs were taken from the mice and non-purified or purified for $CD4^+$ or $CD8^+$ T cells. Recipient mice were administered i.v. with crude LNCs (A), $CD4^+$ T cells (B), or $CD8^+$ T cells (C) and challenged with AQ + UVA. Bar = 50 μm.

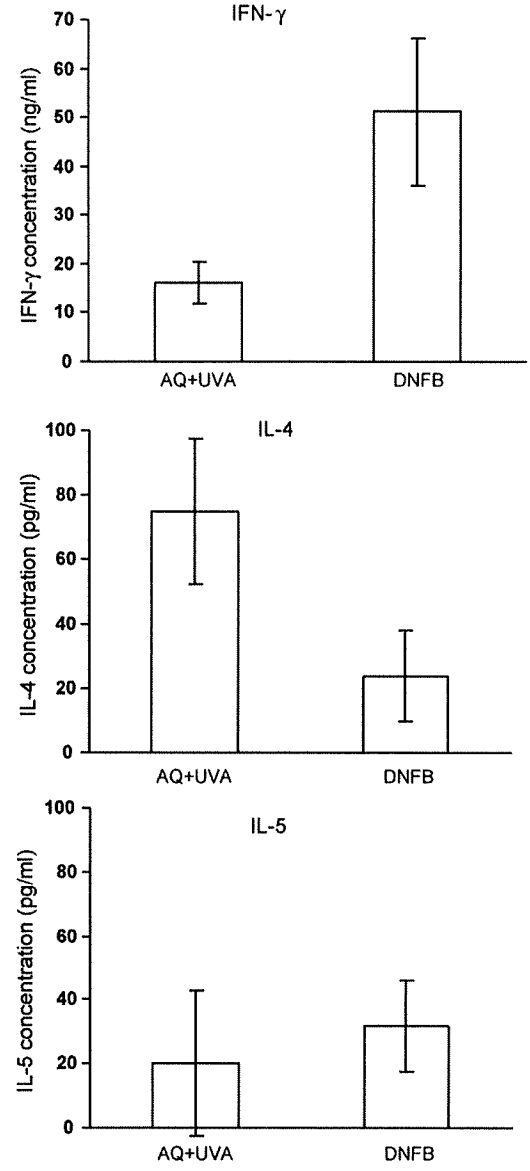

Fig. 5. Cytokine concentration in culture supernatants of immune lymph node and spleen cells. Immune lymph nodes and spleen cells were taken from mice sensitized with AQ + UVA 10 times or from mice sensitized with DNFB as control. They were cultured for 72 h in the presence of anti-CD3 mAb (soluble form stimulatory for T cells; BD Biosciences, San Diego, CA) at 5 μg/ml and anti-CD28 mAb (Immunotech, Marseille, France) at 5 μg/ml. Culture supernatants were subjected to ELISA for measurement of IFN-γ, IL-4, and IL-5. Data are the mean + SD ($n = 3$). *$P < 0.05$, compared with AQ + UVA group. These data represent one of three independent experiments.

with marked elevation of both Th2 chemokine CCL22 and CCL17 in the AQ + UVA-sensitized and challenged mice (Fig. 6B).

4. Discussion

In this study, we established a mouse model of photoallergy to AQ using AKR/J mice, which shows marked skin infiltration of eosinophils upon challenge with both AQ and UVA. This eosinophilic photoallergy is induced by repeated systemic administration of AQ and irradiation of UVA to the skin. The sensitivity was photoantigen-specific, and AKR/J strain was a high responder. The eosinophil-infiltrating histology was transferred to naïve recipients by i.v. injection of immune IL-4-producing $CD4^+$ T cells, indicating that Th2 cells mediate the tissue eosinophilia.

In this photoallergy to AQ, sensitization of more than 8 times was required for the induction of Th2 cells and the resultant eosinophilia. This is in accordance with the observation found in contact hypersensitivity, a repeated hapten application leads to Th2-mediated cutaneous sensitivity upon epicutaneous challenge [16]. However, since the infiltrate consists largely of neutrophils and eosinophils with occasional lymphocytes [17], the level of eosinophil infiltration is higher in our system. By using AQ, we have previously found that s.c. injection of AQ-photomodified epidermal cells sensitize and elicit a delayed-type hypersensitivity reaction [4]. In that study, the photocoupling of cells with AQ was efficiently but artificially performed *in vitro*, and thus the AQ-photomodified cells seem to have strong photoantigenicity, thereby immunizing rodents by only one s.c. sensitization procedure. In contrast, the combination of systemic administration of AQ and UVA exposure of the skin is a clinically mimic but weaker sensitization procedure, and therefore, repeating of immunization is required for sensitizing mice. The repeated treatments, however, may result in polarization of the induced immunological state to a

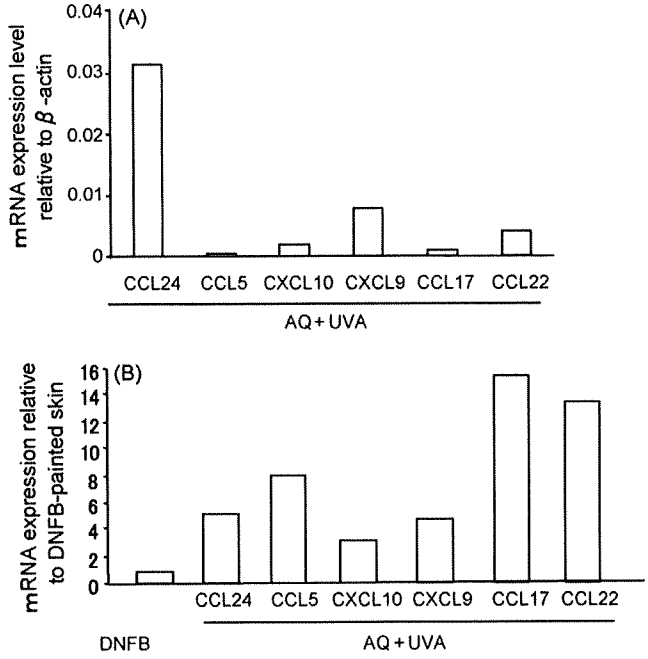

Fig. 6. Chemokine production pattern in AQ-photochallenged skin. AKR/J mice were sensitized 10 times and challenged with AQ + UVA. The elicited skin was subjected to real-time quantitative PCR to measure mRNA levels for chemokines, including CCL24, CCL5, CXCL10, CXCL9, CCL17, and CCL22. As control, AKR/L mice were painted with 0.2% DNFB on the abdomen, and the skin was excised 2 days later. Data are expressed as mRNA expression level relative to β-actin (A) or relative to each level of DNFB-painted skin (B). These data represent one of three independent experiments.

Th2-dominant condition [16]. The necessity of long duration of drug administration and UVA exposure necessary for the development of eosinophilic photoallergy seems to be in accordance with our observation.

In our mouse model of AQ photoallergy, Th2 induction, chemokine production, and eosinohpil infiltration are closely related, but the precise mechanism involving these events is still speculative. During sensitization with AQ + UVA, Th2 cell population(s) specific for AQ photoantigen is duly induced, and additionally, IgE specific for photodegradated AQ possibly develops. In the process of elicitation with AQ + UVA, the initial event that evokes the sensitivity is photomodification of antigen-presenting cells with AQ. Since AQ is injected s.c. for the challenge, the candidates for those modified cells include dermal dendritic cells, macrophages, and others. Then, cells bearing AQ photoantigen may restimulate specific Th2 cells, which directly release IL-5 and indirectly elaborate CCL24 and/or CCL5 by stimulating fibroblasts [18-20] or macrophages [21], leading to tissue eosinophilia [19,20].

There are differences between drug-specific T cell clones established from individual patients with drug eruption, as they can be Th1, Th2 or Tc1 cells [22]. In addition, some but not all patients exhibit blood and/or tissue eosinophilia [23]. In photoallergic drug eruption to AQ, the patients may show an eythematous eruption or lichenoid eruption. Whereas the latter type is mediated by Th1 and Tc1 cells [24,25], the former may be caused by Th2 cells and possibly associated with tissue eosinophilia. The present model may be a mimicry of the former type. Our study suggests that even photohapten can sensitize Th2 cells and induce tissue eosinophilia, when weak sensitization is prolonged.

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