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Formation of Antigenic Quinolone Photoadducts on Langerhans Cells Initiates Photoallergy to Systemically Administered Quinolone in Mice

Akihiro Ohshima, Naohiro Seo, Masahiro Takigawa, and Yoshiki Tokura

Department of Dermatology, Hamamatsu University School of Medicine, Hamamatsu, Japan

Quinolone antibacterial agents are well known to cause photoallergy as a side-effect. Murine photoallergy to fluoroquinolones is a T cell-mediated immune response, evoked either by systemic fluoroquinolone and subsequent exposure of skin to ultraviolet A light or by subcutaneous injection of fluoroquinolone-photomodified epidermal cells. In this photosensitivity, epidermal Langerhans cells may be photomodified initially with the drug and thus present photohaptenic moieties to sensitize and restimulate T cells. Although we have shown that Langerhans cells photocoupled *in vitro* with fluoroquinolones are capable of stimulating sensitized T cells, it remains unclear whether systemically given fluoroquinolone photomodifies Langerhans cells upon ultraviolet A irradiation of the skin and the Langerhans cells become photohapten-bearing, T cell-stimulatory cells. In a murine model of fleroxacin photoallergy induced by intraperitoneal injection of the drugs plus ultraviolet A irradiation of skin, we found that Langerhans cells as well as keratinocytes are photoderivatized with fleroxacin as demonstrated with a fluoroquinolone-specific monoclonal antibody. Langerhans-cell-enriched epidermal cells prepared from mice treated with fleroxacin and ultraviolet A induced proliferation of sensitized T cells, indicating that photomodified Langerhans cells are functional. There was an optimal range of ultraviolet A dose to quantitatively and qualitatively form fleroxacin-photomodified Langerhans cells, as excess ultraviolet A rather reduced the photoantigen-presenting capacity of Langerhans cells presumably because of drug phototoxicity. Our study suggests that Langerhans cells serve as photoantigen-presenting cells in drug photoallergy. ***Key words: fleroxacin/fluoroquinolone. J Invest Dermatol 114:569–575, 2000***

Fluoroquinolones (FQs) are a widely used, new class of quinolone antibacterials with a broad spectrum of activity towards Gram-negative and Gram-positive aerobic bacteria, anaerobes, and even mycobacteria (Domagala, 1994). One of the important and common side-effects of these drugs is photosensitive dermatitis (Ferguson, 1995). Although its incidence varies depending on the type of FQ, the vast majority of cases with FQ photosensitivity in Japan are caused by enoxacin, lomefloxacin, fleroxacin (FLRX), and sparfloxacin (Tokura, 1998). Ultraviolet A (UVA) light is the main action spectrum to elicit photosensitive skin reactions in patients medicated with FQ (Kawabe *et al*, 1989; Kurumaji and Shono, 1992; Yoshizawa *et al*, 1992). As with other photosensitive drugs, both phototoxic and photoallergic mechanisms have been proposed in the pathogenesis of FQ-induced photosensitivity (Ferguson, 1995; Tokura *et al*, 1996a, b; Tokura, 1998), although involvement of these potencies in photosensitivity is different with each FQ (Wagai and Tawara, 1991; Iwamoto *et al*, 1992; Horio *et al*, 1994; Tokura *et al*, 1996b). Based on clinical and experimental studies, enoxacin (Kawabe *et al*, 1989; Izu *et al*, 1992; Kang *et al*, 1993) and FLRX (Tokura *et al*, 1996b) seem to be mainly photoallergic, whereas sparfloxacin is highly phototoxic but less photoallergic than other FQs (Tokura *et al*, 1996a, 1998). There are some relationships between the chemical structure of FQs and photosensitivity (Domagala, 1994). The phototoxicity is partly dependent on the fluorine at C8 (Matsumoto *et al*, 1992), but this fluorine is not involved in the photoallergenicity.

The photoallergenicity of FQs is mainly derived from their photohaptenic moiety. FQs are covalently coupled to protein by irradiation with UVA (Tokura *et al*, 1996b). Recent photochemical studies have shown that the piperazinyl (or methylpiperazinyl) group, the major side chain of FQs linked at C7, is altered by UVA irradiation (Yoshida and Moroi, 1993; Tiefenbacher *et al*, 1994), suggesting the possibility that protein is covalently bound to the piperazinyl ring during its photodegradation to form an allergic FQ-protein complex (Tokura, 1998). Photoconjugation of epidermal cells with FQ initiates T cell-mediated immunologic consequences for sensitization and elicitation. In mice subcutaneous inoculation of FQ-photomodified epidermal cells induces and elicits photosensitivity to FQ (Tokura *et al*, 1996b), indicating that the photohaptenation of epidermal antigen-presenting cells is necessary for induction of FQ photoallergy. This FQ photoallergy is mediated at least by Th1 cells bearing T cell receptor Vβ13, and there exists broad cross-reactivity among FQs (Tokura *et al*, 1998), suggesting that FQs carry the same photoantigenic epitope recognized by $V\beta13^{+}$ T cells.

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Reprint requests to: Dr. Akihiro Ohshima, Department of Dermatology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431–3192, Japan. Email: a41114@hama-med.ac.jp

Abbreviations: FLRX, fleroxacin; FQ, fluoroquinolone; i.p., intraperitoneal; LC-epidermal cells, Langerhans-cell-enriched epidermal cells; LN, lymph node.

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Although Langerhans cells that are photomodified *in vitro* with FQ stimulate sensitized T cells (Tokura *et al*, 1998), it is unclear whether, upon systemic administration of FQ and subsequent exposure of skin to UVA, Langerhans cells are photomodified with FQ to become functional as antigen-bearing, T cell-stimulatory antigen presenting cells. Here, we investigated this issue in FQ systemic photoallergy, in which mice were injected intraperitoneally with drug and subsequently irradiated with UVA. We used FLRX in this study because it is a highly photoallergic FQ as seen in Japanese patients (Tokura, 1998). Our data demonstrate that FLRX diffuses to the epidermis after intraperitoneal (i.p.) injection and Langerhans cells are photomodified *in vivo* with FLRX and stimulate sensitized T cells.

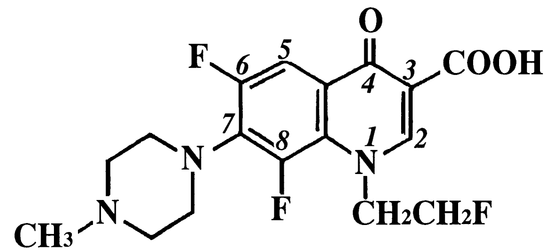

Figure 1. Chemical structure of FLRX.

MATERIALS AND METHODS

Mice Male BALB/C mice, 7–8 wk old, were obtained from Japan SLC (Hamamatsu, Japan) and were maintained in our conventional animal facility.

Reagents and monoclonal antibodies (MoAbs) The chemical structure of FLRX is shown in **Fig 1**. FLRX was kindly provided by Kyorin Pharmaceutical (Tokyo, Japan). As described previously, FLRX has absorption peaks at 280 and 327 nm, and the former peak is shifted to 274 nm after UVA irradiation (Tokura *et al*, 1996b).

ST-Q-9 MoAb (IgM, κ), specific to the common part of the structure of FQs on FQ-photomodified cells, has been described previously (Tokura *et al*, 1998). Briefly, ST-Q-9 reacts with spleen cells photomodified with any of the nine FQs tested. Given that the piperazinyl ring at C7 affords the protein-binding site upon photodegradation with UVA, ST-Q-9 may recognize all or a part of C2 to C6 and their residues, which is the common part of the structures. Isotype-matched control MoAb (G155–228, specific to trinitrophenyl hapten) was obtained from PharMingen (San Diego, CA). Fluorescein isothiocyanate (FITC)-labeled monoclonal rat antimouse IgM (μ chain-specific) was purchased from Zymed Laboratories, San Francisco, CA. Phycoerythrin (PE)-conjugated anti-I-A^d MoAb (AMS-32.1) and rat antimouse CD16/CD32 (Fcγ III/II receptor) MoAb (2.4G2) were from PharMingen.

RPMI-1640 (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum, 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 2 mM L-glutamine, 10^{-3} M sodium pyruvate, 10^{-4} M nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, 100 units penicillin per ml, and 100 μg streptomycin per ml was used as a culture medium (all from Gibco BRL Life Technologies).

Light source Black light (FL20SBLB) emitting 300–420 nm with a peak emission at 365 nm was purchased from Toshiba Electric, Tokyo, Japan. With a UV radiometer (UVR-305/365; Eisai, Tokyo, Japan), the energy output of three 20 W tubes of black light at a distance of 20 cm was 2.4 mW per cm^2 at 365 nm and 0.17 mW per cm^2 at 305 nm. Three tubes of black light were used as a UVA source, and irradiation was performed through a pane of 3 mm thick glass.

Preparation of epidermal cell suspension and Langerhans- cell-enriched epidermal cell suspension Ears were excised at the base and split along the plane of the cartilage, which was then removed together with the subcutaneous tissue. The specimens were incubated for 1 h at 37°C in 0.2% trypsin (Gibco BRL Life Technologies) in phosphate-buffered saline (PBS) (pH 7.4). Epidermal cells were dispersed in PBS supplemented with 10% heat-inactivated fetal bovine serum, filtered through a cotton column, and washed three times in PBS (Ohshima *et al*, 1998). The epidermal cell suspension thus prepared contained 0.5%–2% I-A^+ cells, representing Langerhans cells, as determined by flow cytometry using an FITC-conjugated anti-I-A^d MoAb (PharMingen). For enrichment of Langerhans cells, freshly isolated single cell suspensions of epidermal cells (5×10^6 cells per ml) were centrifuged over a Ficoll-Hypaque gradient (specific gravity 1.083; Sigma, St Louis, MO), and interface cells were collected and washed in PBS. Viability was greater than 90% as assessed by the trypan blue (Sigma) exclusion test. Langerhans-cell-enriched epidermal cells (LC-epidermal cells) contained typically 15% of I-A^+ cells (Tokura *et al*, 1994).

Preparation of FLRX-photomodified epidermal cells In order to maintain epidermal cells viable in FLRX solution in this study, excess amounts of FLRX were vigorously stirred for 30 min in PBS without use of any additional solvent. After centrifugation at $3000 \times g$ for 30 min to remove unsolubilized FLRX and sterilization through a millipore filter (0.20 μm; Toyo Roshi Kaisha, Tokyo, Japan), the saturated solution contained 2.1 mM FLRX (Tokura *et al*, 1996b). For photocoupling of cells, freshly isolated epidermal cells were suspended at 5×10^6 cells per ml in the FLRX solution diluted with PBS at a final concentration of 0.2 mM, and irradiated with UVA (1.5 J per cm^2 at 365 nm) in a plastic dish. The cells were used after washing three times in PBS. The viability of cells was 71%, as assessed by the trypan blue exclusion test.

Induction and elicitation of FLRX photoallergy For sensitization, mice were given i.p. injection of FLRX (0.2 mg per 0.2 ml PBS, equal to 10 mg per kg weight, unless otherwise mentioned) and their shaved abdominal skin was irradiated 24 h later with three tubes of black light at a distance of 20 cm (6 J per cm^2 at 365 nm). Alternatively, freshly prepared FLRX-photomodified epidermal cells (10^7 cells per 0.2 ml PBS per mouse) were injected subcutaneously into the bilateral lower dorsal flanks of mice (Tokura *et al*, 1996b, 1998). On day 5, the animals were challenged with i.p. injection of FLRX and UVA irradiation of earlobes at the same drug and UVA doses as for sensitization, according to the reported method (Giudici and Maguire, 1985). Ear thickness was measured before and 24 h after irradiation with a dial thickness gauge (Peacock, Tokyo, Japan) and was expressed as the mean increment in thickness above the basal control value. In some experiments for depletion of Langerhans cells, epidermal cells were incubated with 10 ml of monoclonal anti-I-A^d MoAb (IgG2a; Meiji Institute of Health Science, Tokyo, Japan) at a dilution of 1:500 in RPMI-1640 for 45 min at room temperature. The cells were sedimented by centrifugation, resuspended in 10 ml of a 1:10 dilution of complement (C′; Low-Tox-M rabbit C, Cederlane Laboratories, Ontario, Canada) in RPMI-1640 for 45 min at 37°C, and washed twice before FLRX photomodification (Tokura *et al*, 1991). Five days after sensitization, mice were challenged with i.p. injection of FLRX and UVA irradiation of earlobes (4 J per cm^2 at 365 nm).

Flow cytometric analysis Epidermal cells were taken from FLRX/UVA-treated mice immediately after UVA exposure and suspended in Hanks' balanced salt solution containing 0.1% NaN_3 and 1% fetal bovine serum. Cells (10^6 cells) were incubated with immunoaffinity-purified ST-Q-9 MoAb at a final concentration of 2 μg per ml for 40 min and subsequently with FITC-conjugated monoclonal rat antimouse IgM (1 μg per ml) for 30 min at 4°C. Purified mouse MoAb (IgM, κ isotype, 2 μg per ml) specific to trinitrophenyl hapten was used as a control antibody of ST-Q-9. For Langerhans cell staining, mice were treated with i.p. FLRX injection and earlobes were irradiated 24 h later with UVA; epidermal cell suspensions were prepared immediately or 48 h after UVA exposure. Cells were preincubated with anti-Fcγ III/II receptor MoAb (2 μg per ml) for 5 min to prevent nonspecific binding of the subsequent reagents to Fc receptors and were double-stained with ST-Q-9 with a subsequent second antibody and PE-conjugated anti-I-A^d MoAb. After three washes, 10^4 labeled cells were analyzed in a FACScan or a FACSCalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA). Dead cells were identified by propidium iodide uptake, and viable cells were subjected to flow cytometric analysis. Percentage augmentation of fluorescence intensity was calculated using the following formula: [(mean fluorescence intensity at each time point – mean fluorescence intensity at time 0)/mean fluorescence intensity at time 0] × 100.

Fluorescent microscopy analysis Mice were treated with i.p. injection of FLRX and the earlobes were irradiated 24 h later with UVA (4 J per cm^2). Epidermal cell suspensions obtained as above were incubated with PE-conjugated anti-I-A^d MoAbs, washed three times, mounted in 50% glycerol in PBS, and observed in an Olympus fluorescent microscope (BH-2; Tokyo, Japan).

T cell proliferative responses to photohaptenated Langerhans cells Mice were sensitized with subcutaneous injection of FLRX-photomodified epidermal cells into the bilateral lower dorsal flanks. On day 5, lymph node (LN) cells were collected from the inguinal and axillary regions and suspended in PBS. To obtain purified $CD4^+$ T cells, LN cells were incubated for 60 min at 4°C with anti-CD4 MoAb-conjugated magnetic beads (DYNABEADS mouse CD4, Dynal, Oslo, Norway) and the bound cells were detached from the beads with DETACHaBEAD mouse CD4 (Dynal) according to the manufacturer's directions. LC-epidermal cell suspensions were prepared from mice treated with i.p. FLRX plus UVA irradiation 24 h after FLRX injection or from control mice. LC-epidermal cells were preincubated with mitomycin C (Sigma) at 50 μg per ml for 30 min at 37°C and washed three times. $CD4^+$ T cells (2×10^5 cells per well) were cultured in a total volume of 150 μl of the culture medium with mitomycin-C-pretreated LC-epidermal cells (10^5 cells per well). In parallel experiments for depletion of Langerhans cells, LC-epidermal cells were incubated with anti-I-A^d MoAb and subsequently with C′, as described above, and cells were washed twice before incubation with mitomycin C (Tokura *et al*, 1991). Indomethacin (Sigma) was added to the culture at a final concentration of 1 μg per ml. Triplicate cultures were maintained in 96 well flat-bottom culture plates (Nunclon, Nunc, Denmark) at 37°C under 5% CO_2 in air for 72 h. One microcurie per well methyl tritiated thymidine ([^{3}H]TdR; Amersham International, Arlington, IL) was added 16 h before harvest. The cells were collected on glass fiber filters using an automated cell harvester (Cambridge Technologies, Watertown, MA) and [^{3}H]TdR uptake was measured in a scintillation counter.

Interleukin-1α (IL-1α) production and proliferation of epidermal cells Epidermal cell suspensions were prepared from photosensitized and untreated mice, and cultured at 2×10^6 cells per 1.5 ml of the culture medium for 72 h in 24 well flat-bottom plates (Nunclon). The amount of IL-1α in the culture supernatants was measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits (Genzyme, Boston, MA). In parallel experiments, epidermal cells (5×10^5 cells per ml) were cultured in 96 well flat-bottom culture plates for 24 h and [^{3}H]TdR (1 μCi per well) was added 6 h before termination of culture.

Statistical analysis Student's *t* test was used to determine statistical differences between the means, and $p < 0.05$ was considered a significant difference.

RESULTS

I.p. administration of FLRX plus UVA irradiation of skin induces and elicits photoallergy to FLRX In confirmation of our previous study with lomefloxacin (Tokura *et al*, 1998), i.p. administration of FQ plus UVA irradiation of skin and subcutaneous inoculation of FQ-photomodified epidermal cells induced significant degrees of photoallergic responses, as indicated by the increased ear thickness 24 h after challenge with i.p. FLRX plus UVA irradiation of earlobes (**Fig 2*A***, groups A and B). Neither mice treated with i.p. FLRX alone (group C) nor those untreated (group D) exhibited significant swelling of earlobes upon FQ plus UVA challenge. In addition, UVA irradiation or i.p. FLRX alone did not cause ear swelling in photosensitized mice (groups E and F). These data suggested that i.p. administration of FLRX plus UVA irradiation was feasible to study drug photomodification of epidermal cells relevant to FLRX photoallergy.

To examine the optimal irradiation dose of UVA, mice sensitized with subcutaneous injection of FLRX-photomodified epidermal cells were challenged with i.p. FLRX plus irradiation with varying doses of UVA. As shown in **Fig 2(*B*)**, mice challenged with systemic FLRX plus 2–12 J per cm^2 of UVA showed a significant degree of ear swelling responses (groups B to E) with the UVA range between 2 and 8 J per cm^2 being the most efficient.

We also examined the requirement of Langerhans cells for sensitization to FLRX photoallergy. Mice were sensitized with subcutaneous injection of FLRX-photomodified epidermal cells untreated or treated with anti-I-A^d MoAb + C′ or C′ alone, and challenged with i.p. FLRX plus UVA irradiation of earlobes. As shown in **Fig 2(*C*)** Langerhans-cell-depleted epidermal cells with anti-I-A^d + C′ yielded significantly suppressed ear swelling responses (group C), whereas no significant suppression was obtained with C′ treatment (group B). These results suggested that

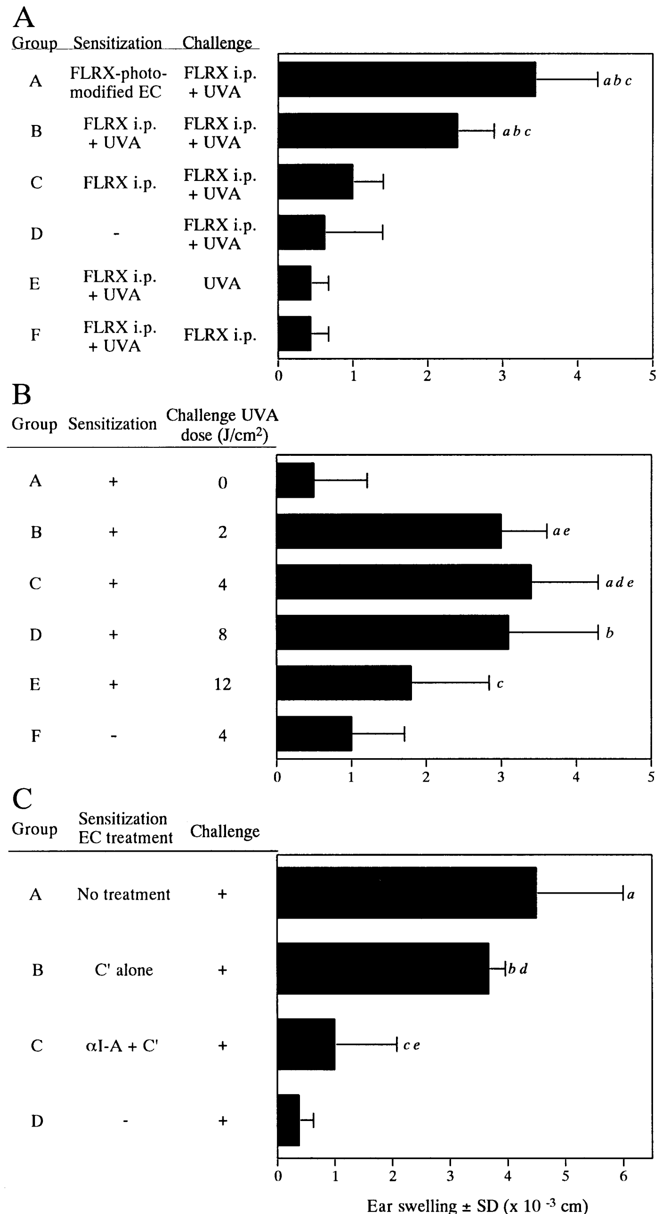

Figure 2. Induction and elicitation of photoallergic responses to FLRX. (*A*) Mice were sensitized with i.p. administration of FLRX (10 mg per kg weight) plus UVA irradiation (6 J per cm^2 at 365 nm) to clipped abdomen or subcutaneous inoculation of FLRX-photomodified epidermal cells (10^7 cells per mouse). Five days later, they were challenged with i.p. FLRX and/or subsequent UVA irradiation of earlobes. Ear swelling was measured 24 h after irradiation. Each group consisted of four to six mice. $^a p < 0.005$, compared with group C. $^b p < 0.01$, compared with group D. $^c p < 0.005$, compared with group E. (*B*) Mice were sensitized with subcutaneous inoculation of FLRX-photomodified epidermal cells. On day 5, they were subjected to i.p. administration of FLRX (10 mg per kg weight) and/or subsequent varying doses of UVA irradiation of earlobes. Ear swelling was measured 24 h after irradiation. Each group consisted of four to six mice. $^a p < 0.001$, $^b p < 0.005$, $^c p < 0.05$, compared with group A. $^d p < 0.005$, compared with group F. $^e p < 0.05$, compared with group E. Error bars represent SD. (*C*) Mice were sensitized with subcutaneous inoculation of FLRX-photomodified epidermal cells untreated or preincubated with anti-I-A^d MoAb + C′ or C′ alone. On day 5, they were challenged with i.p. FLRX (10 mg per kg weight) plus 4 J per cm^2 of UVA irradiation of earlobes. Ear swelling was measured 24 h after irradiation. Each group consisted of four mice. $^a p < 0.05$, $^b p < 0.001$, ^{c}statistically not significant, compared with group D. ^{d}Statistically not significant, $^e p < 0.05$, compared with group A. Error bars represent SD.

Langerhans cells were responsible for induction of FLRX photoallergy.

Presence of FLRX photoadducts on keratinocytes and Langerhans cells from mice treated with i.p. FLRX administration plus UVA irradiation The presence of FLRX photoadducts on epidermal cells from mice treated with i.p. FLRX and UVA irradiation was analyzed by flow cytometry using ST-Q-9. Mice were injected intraperitoneally with FLRX and their earlobes were irradiated with UVA (6 J per cm^2 at 365 nm) 24 h later. As shown in **Fig 3(*A*)**, in FLRX/UVA-treated mice, the fluorescence intensity of ST-Q-9-stained epidermal cells was significantly higher than that of control IgM-stained epidermal cells. Notably, in mice treated with systemic FLRX alone, the intensity was modestly higher with ST-Q-9 than control IgM. Thus, it seemed that epidermal cells were noncovalently coupled with FLRX without UVA irradiation and this binding was enhanced and became covalent after exposure to UVA. There was no significant difference in the fluorescence intensity between ST-Q-9 and control IgM staining in epidermal cells from mice treated with UVA alone or untreated.

Earlobes of mice were exposed to UVA (6 J per cm^2) at varying time points ranging from 0 to 72 h after i.p. injection of FLRX. **Figure 3(*B*)** shows that ST-Q-9 reactivity of epidermal cells reached the maximal level in mice irradiated with UVA 24 h after FLRX injection, and decreased gradually to the level of a UVA-nonirradiated group at 72 h. Epidermal cells did not react with ST-Q-9 in the absence of UVA exposure. These data indicated that FLRX photoadducts are produced most efficiently in the epidermis of FLRX/UVA-treated mice at 24 h. Therefore, mice were exposed to UVA 24 h after i.p. FLRX in the following experiments. To examine the optimal photomodification dose of UVA, mice given i.p. FLRX were irradiated with varying doses of UVA (0, 3, 6, 12 J per cm^2). Exposure of mice to UVA at 3 or 6 J per cm^2 significantly augmented the ST-Q-9 reactivity of epidermal cells compared with the nonirradiated group (**Fig 3*C***). The fluorescence intensity was not augmented at 12 J per cm^2, however, presumably because of high phototoxicity of FLRX photoadducts against epidermal cells. Thus, we chose 3 J per cm^2 UVA to irradiate mice in the following experiments unless otherwise mentioned.

We have suggested that epidermal Langerhans cells are one of the candidates that serve as antigen-presenting cells in murine FQ photoallergy (Tokura *et al*, 1996b, 1998). Flow cytometric analysis also showed that the fluorescence intensity of Langerhans cells, gated as I-A^+ cells, was significantly higher when stained with ST-Q-9 than control IgM in FLRX/UVA-treated mice (**Fig 4*A***). I-A^+ cells from FLRX-treated mice also exhibited a modestly higher level of ST-Q-9 reactivity than controls, although the intensity of I-A^+ cells was significantly higher in FLRX/UVA-treated mice than FLRX-treated mice. This indicated that Langerhans cells also modestly bind to FLRX even without UVA irradiation.

The percentage and ST-Q-9 immunoreactivity of Langerhans cells were monitored immediately and 48 h after UVA exposure in mice administered intraperitoneally with FLRX. Both the number of I-A^+ cells among ST-Q-9 reactive epidermal cells (**Fig 4*B***) and the ST-Q-9 fluorescence intensity of I-A^+ cells (**Fig 4*C***) were significantly decreased 48 h after UVA irradiation, whereas the ST-Q-9 intensity of I-A^- cells, representing keratinocytes, was unchanged. Thus, these data suggested that FLRX-photomodified Langerhans cells emigrated from the epidermis after UVA irradiation.

FQs have a fluorescence property and their presence in FQ-photomodified cells is visualized under fluorescent microscopy (Tokura *et al*, 1996b). **Figure 5(*A*)** is a phase contrast picture of

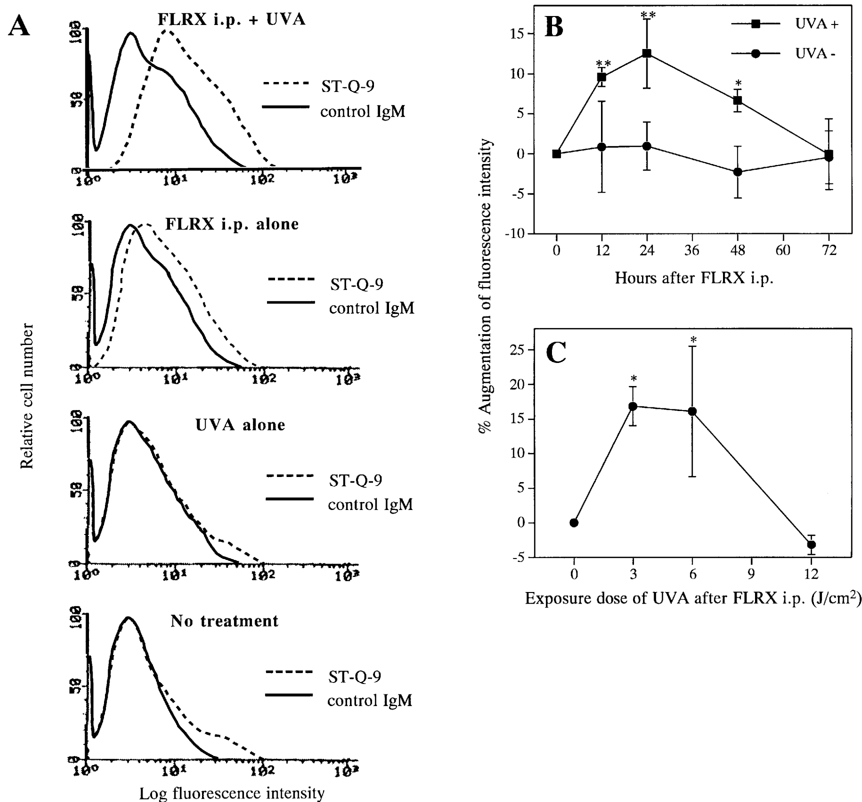

Figure 3. Presence of FLRX on epidermal cells from mice treated with different irradiation timing and dose of UVA after i.p. injection of FLRX (10 mg per kg weight). (*A*) Epidermal cells were prepared from mice 24 h after treatment with i.p. FLRX plus UVA irradiation (6 J per cm^2), i.p. FLRX alone, or UVA irradiation alone, or from untreated mice. Flow cytometric graph of epidermal cells from treated or untreated mice stained with ST-Q-9 or control IgM and subsequently with FITC-conjugated antimouse IgM antibody. (*B*) Epidermal cells were prepared from mice irradiated with UVA (6 J per cm^2 at 365 nm) (■, UVA+) or nonirradiated (●, UVA-) at the indicated time points after i.p. FLRX. Cells were stained with ST-Q-9 and subsequently with FITC-conjugated antimouse IgM antibody. Dead cells were identified by propidium iodide uptake, and viable cells were analyzed by flow cytometry. Percentage augmentation of fluorescence intensity was calculated using the following formula: [(mean fluorescence intensity at each time point – mean fluorescence intensity at time 0)/mean fluorescence intensity at time 0] × 100. Data are expressed as the mean of triplicates or quadruplicates. Error bars represent SD. $*p < 0.001$, $**p < 0.05$, compared with the corresponding UVA- group. (*C*) Epidermal cells prepared from mice irradiated with varying doses of UVA 24 h after i.p. FLRX were stained and analyzed as in (*A*). Percentage augmentation of fluorescence intensity was calculated using the following formula: [(mean fluorescence intensity at each dose of UVA group - mean fluorescence intensity of UVA-nonirradiated group)/mean fluorescence intensity of UVA-nonirradiated group] × 100. Data are expressed as the mean of triplicates. Error bars represent SD. $*p < 0.05$ compared with UVA-nonirradiated group.

FLRX-photomodified epidermal cells. Photocoupling of FLRX to epidermal cells was ascertained by the presence of membrane and cytoplasmic fluorescence, sparing nuclear fluorescence (**Fig 5*B***). Some of the fluorescent cells were positive for I-A^d (**Fig 5*C***), confirming that Langerhans cells were photomodified with FLRX.

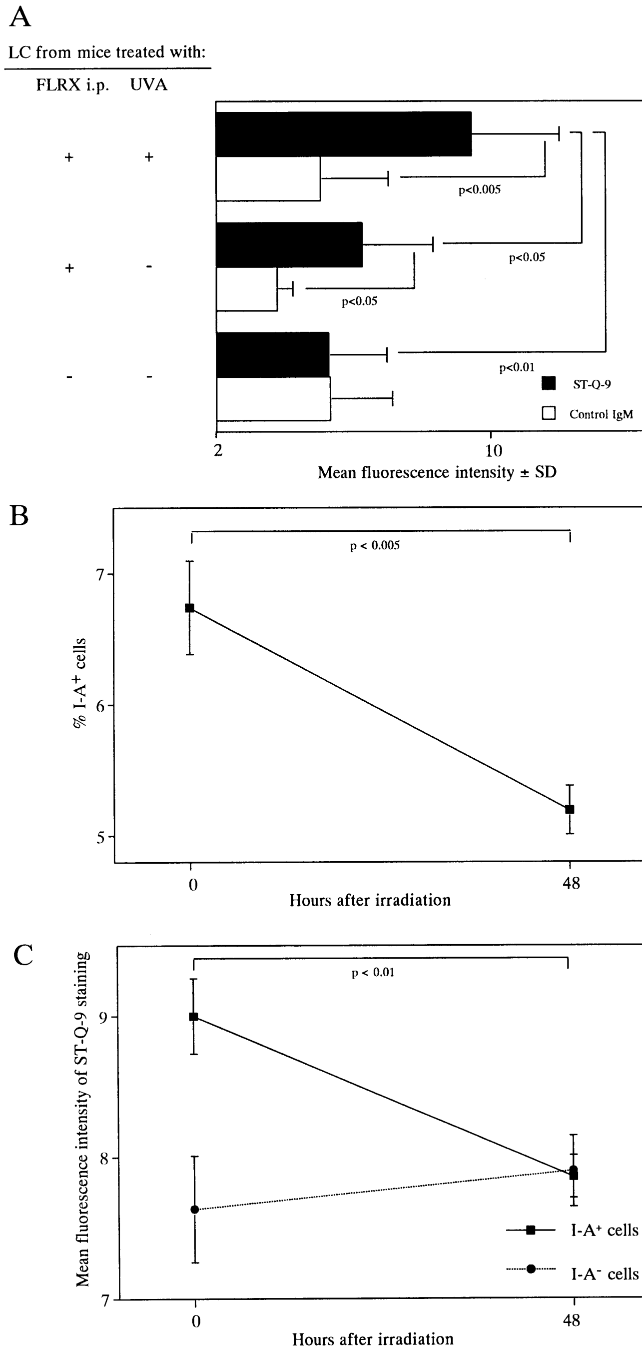

Figure 4. Presence of FLRX on Langerhans cells. (*A*) Epidermal cells were prepared from mice irradiated with UVA or nonirradiated 24 h after i.p. FLRX. The cells were taken immediately from treated mice and stained with ST-Q-9, followed by FITC-conjugated antimouse IgM antibody and PE-labeled anti-I-A^d after Fc blocking. Dead cells were identified by propidium iodide uptake, and viable cells were analyzed by flow cytometry. Gates were set to include MHC class II-bearing and size-gated epidermal cells without propidium iodide uptake in the analysis. (*B*, *C*) Epidermal cells were prepared from mice immediately and 48 h after UVA irradiation, and analyzed as in (*A*). The percentage of I-A^+ cells in ST-Q-9-reactive epidermal cells is shown in (*B*). The ST-Q-9 fluorescence intensities of I-A^+ cells (■) and I-A^- cells (●), representing Langerhans cells and keratinocytes, respectively, are indicated in (*C*). Data are expressed as the mean of quadruplicates. Error bars represent SD.

Langerhans cells from mice treated with i.p. FLRX administration plus UVA irradiation induce the proliferation of sensitized T cells $CD4^+$ T cells were purified from LN cells of mice immunized subcutaneously with FLRX-photomodified epidermal cells. They were cocultured with LC-epidermal cells from mice treated under various conditions. T cell responses to LC-epidermal cells from mice treated with FLRX alone (**Fig 6*A***, group B) was not significantly higher than the control group (group A). LC-epidermal cells from FLRX-injected mice that were subsequently exposed to 1.3 (group C), 4 (D) and 8 (E) J per cm^2 UVA induced significant proliferative responses of immune T cells. Radiation of 4 J per cm^2 UVA gave the most vigorous T cell responses, followed by 1.3 and 8 J per cm^2. Thus, Langerhans cells photomodified with optimal doses of UVA were functional in the proliferation of immune T cells.

LC-epidermal cells from mice receiving i.p. FLRX and UVA irradiation were untreated or treated with anti-I-A^d MoAb + C′ or C′ alone, and cultured with $CD4^+$ immune LN cells. As shown in **Fig 6(*B*)**, depletion of Langerhans cells by anti-I-A^d + C′ resulted in 72% suppression of T cell proliferation (group C), whereas only 27% suppression was obtained with C′ treatment (group B). This confirmed that Langerhans cells but not keratinocytes were responsible for the stimulation of immune T cells.

As LC-epidermal cells contained both Langerhans cells and keratinocytes, it was possible that the treatment with FLRX/UVA altered keratinocyte secretion of the cytokines that affect T cell proliferation. The amount of IL-1α in the culture supernatants from epidermal cells of FLRX/UVA-treated mice was reduced as the injected dose of FLRX was increased (**Fig 7**). In addition, FLRX administration and UVA irradiation did not change the proliferation capacity of keratinocytes (data not shown). Thus, we negated the possibility that the proliferation of immune $CD4^+$ cells was caused by enhanced production of T cell stimulatory cytokine(s) by keratinocytes.

DISCUSSION

In this study, both subcutaneous inoculation of FLRX-photomodified epidermal cells and systemic FLRX plus UVA irradiation of skin induced significant cutaneous responses when challenged with systemic FLRX plus UVA exposure. Together with our

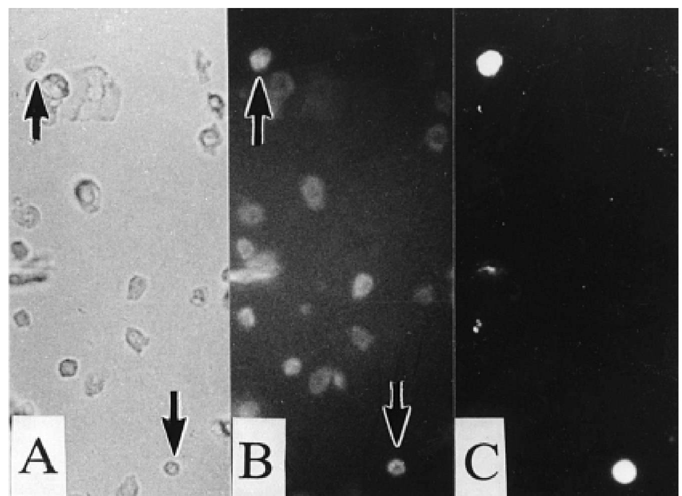

Figure 5. FLRX photomodification of Langerhans cells. Epidermal cells were prepared from mice irradiated with UVA 24 h after i.p. administration of FLRX. They were stained with PE-labeled anti-I-A^d and observed in a fluorescent microscope. (*A*) Phase contrast microscopy, (*B*) fluorescence of FLRX excited by blue light, and (*C*) I-A^+ cells, representing Langerhans cells.

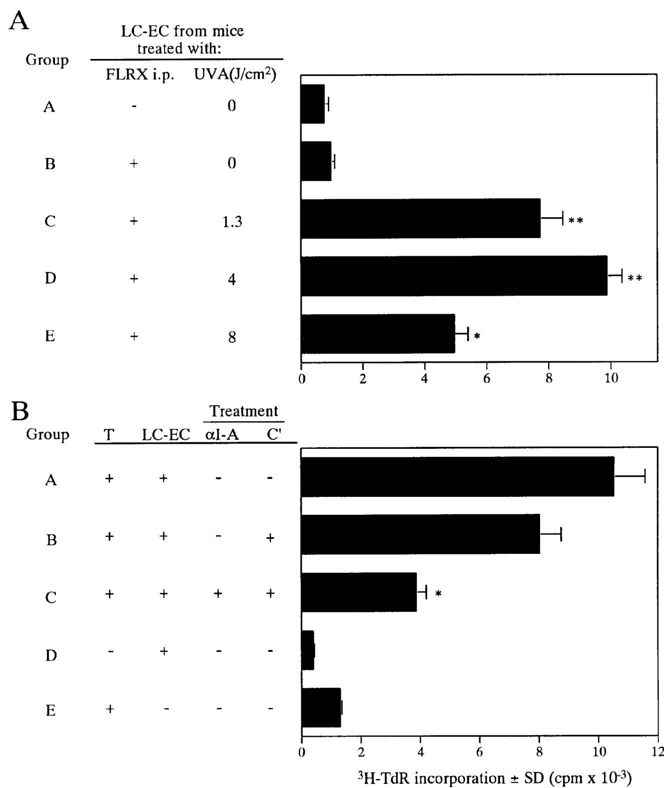

Figure 6. LC-epidermal cells from mice receiving i.p. FLRX and UVA irradiation are capable of inducing the proliferation of sensitized CD4$^+$ T cells. (*A*) CD4$^+$ LN T cells from mice immunized with FLRX-photomodified epidermal cells were cultured in triplicates in the presence of LC-epidermal cells either from mice treated with i.p. FLRX plus varying doses of UVA or from control mice. After 72 h cultivation, the [^{3}H]TdR incorporation was measured. The mean ± SD of CD4$^+$ T cells alone is 327 ± 67. Error bars represent SD. *p < 0.005, **p < 0.05, compared with groups A and B. (*B*) For depletion of Langerhans cells, LC-epidermal cells from mice receiving FLRX i.p. and UVA irradiation were untreated or treated with anti-I-A^d MoAb + C′ or C′ alone, and cultured with CD4$^+$ immune LN cells. Error bars represent SD. *p < 0.005, compared with group A.

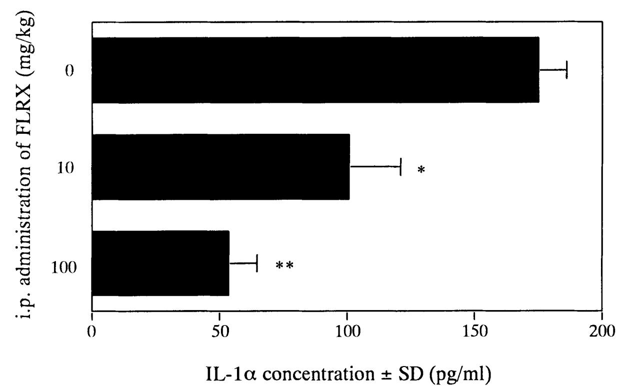

Figure 7. I.p. FLRX administration and UVA irradiation reduce keratinocyte IL-1α production. Epidermal cells were prepared from mice treated without or with varying doses of i.p. FLRX and subsequent UVA irradiation (6 J per cm^2) and were cultured for 72 h in triplicates. The supernatants were subjected to ELISA for IL-1α. Error bars represent SD. *p < 0.01, **p < 0.001, compared with FLRX-nonadministered, UVA-irradiated group.

previous study (Tokura *et al*, 1996b), this suggests that the initial event in FQ photoallergy is photoconjugation of epidermal antigen-presenting cells with FQ under UVA exposure, and the resultant formation of FQ photoadducts initiates a T cell-mediated allergic reaction. The current flow cytometric analysis using ST-Q-9 showed that systemically administered FLRX photobinds to viable Langerhans cells as well as keratinocytes upon UVA exposure maximally 24 h after i.p. FLRX administration. These *in vivo* FLRX-photomodified Langerhans cells were capable of stimulating sensitized T cells, indicating the clinical relevance of this photomodification. The decreased number of Langerhans cells photomodified with FLRX 48 h after UVA irradiation suggests that photoantigen-bearing Langerhans cells migrate to LNs as does an ordinary hapten (Okamoto and Kripke, 1987). We tried to trace ST-Q-9 reactive cells in LNs of mice treated with FLRX plus UVA by flow cytometry and immunofluorescence staining without success, despite the presence of enough ST-Q-9 bound to Langerhans cells as revealed in epidermal cell suspensions. This may be because the frequency of Langerhans cell migration to LN was very low as suggested by Casares *et al* (1997).

As determined by ST-Q-9 reactivity, FLRX modestly bound to Langerhans cells even without UVA irradiation and this binding was enhanced by UVA exposure. As LC-epidermal cells from FLRX-administered, UVA-nonirradiated mice could not induce a proliferation of sensitized T cells, it seems that this UVA-independent binding is noncovalent and does not yield the antigenic determinant. Maximal FLRX photobinding was observed in epidermal cells of mice irradiated with 3–6 J per cm^2 of UVA, and immune T cells proliferated most effectively in response to LC-epidermal cells of mice exposed to 2–8 J per cm^2 UVA. Thus, optimal doses of UVA for the formation of FLRX photoadducts and immunologically active FLRX-photomodified Langerhans cells were in the same range. Excess doses of UVA rather abrogated quantitative and qualitative photoderivatization. Consistent with the *in vitro* finding, 4 J per cm^2 UVA evoked the highest level of ear swelling response in FLRX-administered mice. Therefore, these data suggest that involvement of Langerhans cells in the development of cutaneous photoallergic response to FLRX occurs only under appropriate conditions, even in the clinical setting.

Each FQ has different levels of phototoxicity as well as photoallergenicity. As the phototoxicity is determined partly by the fluorine at C8 (Matsumoto *et al*, 1992), FLRX, like sparfloxacin and lomefloxacin, is estimated to have a high phototoxic activity. In fact, the phototoxicity of FLRX has been reported to be stronger than that of ofloxacin, norfloxacin, and ciprofloxacin (Ferguson and Johnson, 1992). It is thought that FLRX exerts phototoxicity when irradiated with a high dose of UVA, thereby damaging the antigen-presenting function of photomodified Langerhans cells. The existence of an optimal dose of UVA for FLRX photoallergy may stem from the positive (photomodification) and negative (phototoxicity) effects of UVA in the context of FLRX. It is considered that highly photoderivatized epidermal cells may be dead and excluded from flow cytometric counting. In addition to Langerhans cells, such a phototoxic effect of FLRX was also found in keratinocytes, whose IL-1α production was reduced by UVA in a dose-dependent manner.

On the basis of this study, FLRX-photocoupled Langerhans cells in UVA-exposed skin of patients medicated with FLRX may interact with T cells to induce immunologic reactions. In our previous study, *in vitro* antigen-presenting function of norfloxacin-photomodified Langerhans cells for immune T cells was inhibited by anti-major histocompatibility complex (MHC) class II, ST-Q-9, and anti-CD86, but only partially by anti-CD54 and anti-CD80 MoAb (Tokura *et al*, 1998). Thus, these molecules on Langerhans cells are involved in T cell recognition of photohaptenic FQ, as observed in ordinary hapten (Katayama *et al*, 1997; Nishijima *et al*, 1997). This study further suggests participation of Langerhans cells in the pathogenesis of photosensitive dermatitis evoked by systemically administered FQ.

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