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Cross-Reactivity in Murine Fluoroquinolone Photoallergy: Exclusive Usage of TCR Vβ13 by Immune T Cells That Recognize Fluoroquinolone-Photomodified Cells[1]

Yoshiki Tokura,[2] Naohiro Seo, Hiroaki Yagi, Fukumi Furukawa, and Masahiro Takigawa

Fluoroquinolone antibacterial agents are well known to elicit photosensitivity as an adverse effect, and their cross-reactivity has been clinically documented. The photoallergenicity of fluoroquinolones is mainly derived from their photohaptenic moiety, and photomodification of skin epidermal cells with fluoroquinolones is thought to be an initial step for this photoallergy. Here we have explored, both in vivo and in vitro, T cell responses to fluoroquinolone-photomodified cells, focusing on their photoantigenic cross-reactivity. Cells were derivatized with fluoroquinolones under exposure to UV-A, and fluoroquinolone photoadducts were detected in photomodified cells by immunostaining, flow cytometry, and cell ELISA using fluoroquinolone-specific mAb. T cell-mediated hypersensitivity induced and elicited by s.c. injection of fluoroquinolone-photomodified epidermal cells was cross-reactive among six fluoroquinolones. In addition, lymph node cells from mice sensitized with fluoroquinolone-photomodified cells proliferated well in vitro not only to Langerhans cell-enriched epidermal cells photoderivatized with corresponding fluoroquinolone, but also to those photomodified with any of five other fluoroquinolones, supporting their cross-reactivity. In three fluoroquinolones tested, Th1 populations that expanded after in vitro photoantigenic stimulation of immune lymph node cells expressed the same Vβ13 of TCR. The sensitivity could be transferred by the i.v. administration of this Vβ13$^+$ T cell line into naive recipients, in which a high percentage of Vβ13$^+$ cells infiltrated at the challenge site. These findings suggest that these fluoroquinolones carry the same photoantigenic epitope, which is recognized by Vβ13$^+$ T cells, leading to fluoroquinolone photosensitivity and cross-reactivity. ***The Journal of Immunology,*** **1998, 160: 3719–3728.**

Drugs are one of the important causative agents of photosensitivity that clinically manifests as cutaneous responses upon exposure to sunlight (1). Fluoroquinolones (FQs),[3] the representative quinolone derivatives, with fluorine at the 6 position of the structural backbone, exert their bacteriocidal effect by inhibiting DNA gyrase with a broad spectrum of great therapeutic activity that includes Gram-positive and Gram-negative aerobic bacteria, anaerobes, and even mycobacteria (2). Because of their oral administration, high tissue and plasma levels, and high rates of clearance, FQs are widely used, as 32 million prescriptions of ciprofloxacin (CPFX) had been recorded until 1993 in the U.S. (3). One of the major side effects that these drugs evoke is photosensitivity dermatitis (4), as reported for enoxacin (ENX) (5–7), CPFX (8), ofloxacin (OFLX) (9), lomefloxacin (LFLX) (10–12), norfloxacin (NFLX) (13), and sparfloxacin (SPFX) (14). In Japan, from 1980 to 1996 there have been reported 373 cases of drug-induced photosensitivity, and 142 (38%) patients are caused by FQs (15). The incidence of occurrence of photosensitivity in patients administered FQs varies from FQ to FQ; 0.07% of NFLX-treated patients developed photosensitivity (3), while we experienced five photosensitive individuals of 60 patients who took SPFX (14). UV-A light (UVA) is the main action spectrum (5, 10, 11), and UVB radiation occasionally augments the effect of UVA (14). Both phototoxicity (2, 4, 14, 16–20) and photoallergy (5, 10–12, 19, 21) play a role in the pathogenesis of FQ photosensitivity. In photoallergic sensitivity to FQs, cross-reactions between LFLX and ENX (11) and between LFLX and CPFX (12) have been clinically documented. However, it is not clear which FQs are photoantigenically cross-reactable and whether individuals photosensitive to one FQ should avoid medication with other FQs.

The current understanding of drug hypersensitivity is based on the hapten hypothesis: drugs bind covalently to proteins, and the resulting conjugates can be recognized as immunogenic determinants, as well studied for penicillin derivatives (22, 23). Likewise, a photosensitive drug, afloqualone (AQ) is proved to be a photohapten by immunochemical and immunobiologic studies (24), implying that some photoallergic drugs belong to photohaptens. A photohaptenic moiety is one of the salient properties of allergically photosensitizing chemicals, as exemplified by 3,3′,4′,5-tetrachlorosalicylanilide (TCSA). Upon exposure to UVA, protein is covalently coupled (25), and cells are easily conjugated (26) with a photohapten. Photohapten conjugation of cutaneous epidermal cells (EC) initiates T cell-mediated immunologic consequences for sensitization and elicitation (26–28). Thus, photohaptens are different from ordinary haptens in their requirement for UVA irradiation for covalent binding to protein. Our recent study has demonstrated that FQs also have photohaptenic properties. FQs are covalently coupled to BSA by irradiation with UVA, and EC are

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[3] Abbreviations used in this paper: FQ, fluoroquinolone; CPFX, ciprofloxacin; ENX, enoxacin; OFLX, ofloxacin; LFLX, lomefloxacin; NFLX, norfloxacin; SPFX, sparfloxacin; UVA, ultraviolet A; UVB, ultraviolet B; AQ, afloqualone; TCSA, 3,3′4′,5-tetrachlorosalicylanilide; EC, epidermal cell; LC, Langerhans cell; LC-EC, Langerhans cell-enriched EC; LNC, lymph node cells; TCL, T cell line; SI, stimulation index.

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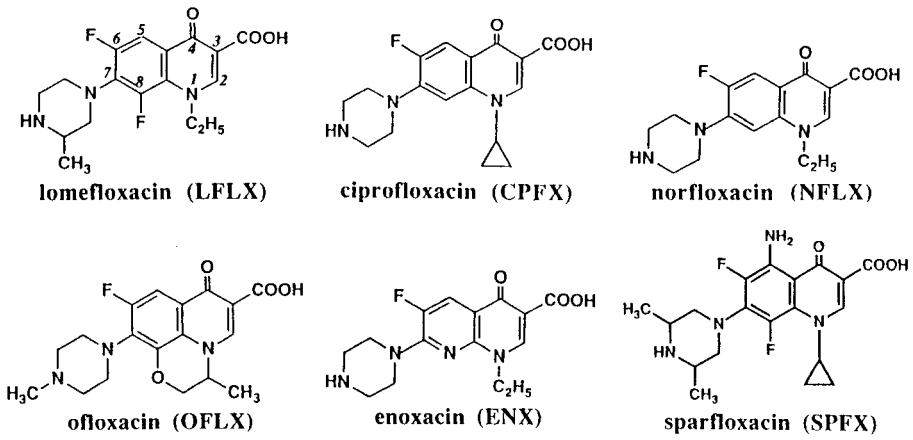

FIGURE 1. Chemical structures of FQs.

successfully photoderivatized with FQs by exposure to UVA (21). The photohaptenation of APC is necessary, by exposure to FQ in the presence of UVA irradiation, for induction of FQ photoallergy. Subcutaneous inoculation of FQ-photomodified cells induces a T cell-mediated reaction upon challenge with FQ-photomodified EC in mice (21), indicating the immunogenicity of FQ-protein photoadducts formed in the photomodified cells.

In this study, we explored the mechanism of cross-reactivity in FQ photoallergy by examining T cell responses to FQ photoantigen(s) with the use of structurally modified FQ derivatives in mice. Broad cross-reactivity was found among six FQs in both in vivo T cell-mediated cutaneous sensitivity induced and elicited by FQ-photomodified EC and in vitro primed T cell responses to Langerhans cell (LC)-enriched EC (LC-EC) photomodified with FQ. The cross-reactivity is explained in part by the exclusive usage of TCR Vβ13 in an FQ-reactive Th1 population(s). These findings suggest that various FQs have a common photoantigenic epitope and provide deeper insight at the T cell level for the clinically observed phenomenon of cross-reactivity.

Materials and Methods

Chemicals

The following FQs were provided: LFLX (Shionogi Pharmaceutical Co., Osaka, Japan), CPFX (Bayer Pharmaceutics, Osaka, Japan), NFLX (Kyorin Pharmaceutical Co., Tokyo, Japan), OFLX (Daiichi Pharmaceutical Co., Tokyo, Japan), ENX (Dai-Nippon Pharmaceutical Co., Osaka, Japan), and SPFX (Dai-Nippon Pharmaceutical Co.). The chemical structures are shown in Figure 1. These FQs have absorption peaks in UVB (280–320 nm) and UVA (320–400 nm) wavelength ranges as reported previously (21). Photodegradation, as assessed by alterations in the absorbance spectra after irradiation of aqueous solutions with UVA, was observed strongly in LFLX; moderately in CPFX, NFLX, OFLX, and ENX; and minimally in SPFX (21). AQ (Tanabe Seiyaku Co., Osaka, Japan) was used as a control photohapten.

FQ and AQ solutions saturated in PBS (pH 7.4) were prepared by vigorous stirring of excess amounts of these chemicals in PBS for 30 min. After removing unsolubilized materials by centrifugation at 3000 $\times$ *g* for 30 min, the supernatant was passed through a filter (0.20 μm; Toyo Roshi Kaisha, Tokyo, Japan). The saturated concentrations of FQs and AQ in PBS are as follows: LFLX, 19 mM; CPFX, 0.45 mM; NFLX, 3.0 mM; OFLX, >40 mM; ENX, 1.8 mM; SPFX, 0.62 mM; and AQ, 0.32 mM (21, 24). In the following experiments, solutions of these chemicals used for photocoupling of cells were prepared by diluting the saturated PBS solution.

Animals

Male BALB/c mice, 8 to 9 wk old, obtained from Japan SLC Co. (Hamamatsu, Japan), were maintained in our conventional animal facility. In in vivo experiments, each experimental group consisted of four to seven mice.

Light source

A black light (FL20SBLB) emitting UVA ranging from 320 to 400 nm with a peak emission at 365 nm was purchased from Toshiba Electric Co. (Tokyo, Japan). With a UV radiometer (Eisai Co., Tokyo, Japan), the energy output of three 20-watt tubes of black light at a distance of 20 cm was 2.7 milliwatt/cm^2 at 365 nm and 0.17 milliwatt/cm^2 at 305 nm.

Culture medium

RPMI 1640 (Life Technologies, Grand Island, NY) was supplemented with 10% heat-inactivated FCS, 5 $\times$ 10^{-5} M 2-ME, 2 mM L-glutamine, 25 mM HEPES, 1 mM nonessential amino acids, 1 mM sodium pyruvate, and 100 μg/ml streptomycin.

Preparation of FQ- and AQ-photomodified cells

EC suspensions were prepared as previously described (26) and contained 0.5 to 2% I-A$^+$ cells, representing LC, as determined by flow cytometry using an FITC-conjugated anti-I-A^d mAb (PharMingen, Sorrent Valley, CA). For enrichment of LC, EC suspensions were cultured in plastic dishes for 24 h, and nonadherent cells were centrifuged over a Histopaque density gradient (specific gravity, 1.083; Sigma Chemical Co.) (29). The cells at interface (LC-EC) were washed in PBS and contained 10 to 15% I-A$^+$ cells. For photomodification, freshly isolated EC, LC-EC, or erythrocyte-lysed spleen cells were suspended in an FQ or AQ solution at 0.2 mM dissolved in PBS (5 $\times$ 10^6 cells/ml), placed in a plastic dish, and irradiated with UVA (8 min, 1.2 J/cm^2 at 365 nm, measured through a plastic dish) (21, 24). The cells were used after washing them three times in PBS. The viability of photomodified EC, assessed by the trypan blue dye exclusion test, was as follows: LFLX, 74%; CPFX, 85%; NFLX, 71%; OFLX, 71%; ENX, 63%; SPFX, 53%; and AQ, 75% (21).

Generation of murine mAb, designated ST-Q-9, specific for FQs

BALB/c mice were immunized by i.p. injections of 3 $\times$ 10^6 erythrocyte-lysed, syngeneic spleen cells photomodified with CPFX under UVA (1.2 J/cm^2 at 365 nm). The injections were performed four times at a 2-wk interval, and spleen cells were obtained on day 4 after the final immunization. The spleen cells were fused with X63/AG8.653 cells using polyethylene glycol 1500 (Boehringer Mannheim GmbH, Mannheim, Germany) and selected with hypoxanthine, aminopterin, and thymidine, as described previously (30). Hybridoma cells were seeded into 288 wells of 96-well plates, and their culture supernatants were screened using cell ELISA. CPFX-photomodified spleen cells were immobilized in poly-L-lysine-coated 96-well plates by centrifugation (30). Hybridoma culture supernatants were added to each well of the ELISA plates and incubated for 90 min at room temperature. After washing, the wells were incubated with a goat anti-mouse Ig Ab conjugated with horseradish peroxidase (1/1000; Caltag Laboratories, San Francisco, CA). The presence of reactive mAb was detected by coloration after 100 μl of 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) peroxidase substrate and 100 μl of 0.02% hydrogen peroxide solution had been added to each well. Only 1 of 288 wells was found to contain Abs that reacted with CPFX-photomodified cells by cell ELISA. Recloning of hybridoma cells was conducted by limiting dilution 2 wk later. The mAb obtained, designated ST-Q-9, had an IgM κ

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isotype as determined using a mouse mAb isotyping kit (Isostrip, Boehringer Mannheim Corp., Indianapolis, IN). As assessed by cell ELISA, ST-Q-9 reacted with nine FQs, including the six FQs used in this study and levofloxacin, tosufloxacin, and fleroxacin, but not with AQ-photomodified spleen cells. Therefore, this mAb recognizes the common part of the structure of FQs.

Detection and semiquantification of FQ photoadducts on FQ-photomodified cells by immunostaining, flow cytometry, and cell ELISA using ST-Q-9

Freshly isolated EC and spleen cell suspensions were incubated with FQ under UVA exposure (FQ-photomodified cells), with UVA (1.2 J/cm^2 at 365 nm)-preirradiated FQ, or simply with FQ. Untreated EC and AQ-photomodified EC were used as controls. For indirect immunohistochemical staining, these treated EC were cytocentrifuged on glass slides coated with poly-L-lysine. The cytospun cells were incubated with normal goat serum (1/50) to reduce background staining and subsequently immersed in ST-Q-9 supernatant, or murine IgM specific for trinitrophenyl hapten (PharMingen; 10 μg/ml) as a control, for 60 min at room temperature. After washing in PBS, cells were soaked with goat F(ab′)$_2$ anti-mouse IgM conjugated with horseradish peroxidase (1/1000; Caltag Laboratories) for 60 min. As a chromagen, 3-amino-9-ethyl-carbazole was used with hydrogen peroxide. The slides were mounted in 50% glycerol in PBS and observed by microscopy.

For flow cytometry, EC and spleen cells variously treated as described above, along with untreated cells, were incubated with ST-Q-9 supernatant for 60 min at 4°C. After washing in HBSS containing 0.1% NaN_3 and 1% FCS, they were incubated with phycoerythrin-labeled goat F(ab′)$_2$ anti-mouse IgM (1/1000; Caltag Laboratories) for 30 min at 4°C. After three washes, 10^4 labeled cells were analyzed in a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

To semiquantify FQs photobound to cells, ST-Q-9 (50 μl of culture supernatant) was added to cell ELISA wells coated with spleen cells photomodified or simply incubated with each of the FQs. AQ-photomodified spleen cells were used as a control. The wells were washed with PBS, incubated with a peroxidase-conjugated, goat anti-mouse IgM (1/1000; Caltag Laboratories), and stained with 2,2′-azino-bis-(3-ethylbenz-thiazoline-6-sulfonic acid) and hydrogen peroxide. The intensity of coloration was measured by reading at A_{410}.

Sensitization and elicitation of photoallergy to FQ with FQ-photomodified EC (21, 24)

Freshly prepared FQ-photomodified EC (10^7/0.2 ml of PBS/mouse) were injected s.c. into the left lower dorsal flank. On day 5 after immunization, 4×10^6 photocoupled EC in 0.02 ml were injected intradermally into the left hind pad. Twenty-four hours after challenge, the thicknesses of left and right footpads were measured with a dial thickness gauge (Peacock Ltd., Tokyo, Japan), and swelling was expressed as the difference in thickness between the two. For all FQs used in this study, significant footpad swelling responses were inducible, but the response to SPFX (typically 8×10^{-3} cm) was significantly lower than those to the other five FQs (typically 13–23 $\times 10^{-3}$ cm) (21).

Epicutaneous sensitization and elicitation of photoallery to FQ with systemic administration of FQ plus UVA irradiation (21)

Mice were sensitized with an i.p. injection of FQ (2 mg/0.2 ml of PBS, equal to 100 mg/kg of weight) and irradiation of shaved abdominal skin with UVA (80 min, 12 J/cm^2 at 365 nm) by placing mice in a 2-cm height cage. On day 5 after sensitization, the animals were challenged with i.p. administration of the same dose of FQ and UVA irradiation (130 min, 20 J/cm^2 at 365 nm) of earlobes. Ear thickness was measured 24 h after irradiation with a dial thickness gauge and was expressed as the mean increment in thickness above the basal control value.

In vitro proliferation of immune lymph node cells (LNC) to photomodified cells

Single cell suspensions were prepared from inguinal and axillary lymph nodes on day 6 after s.c. immunization of mice with FQ-photomodified EC in the bilateral dorsal flank. Triplicate cultures of immune LNC (3×10^5 cells/well) or T cells purified by nylon-wool column passage (2×10^5 cells/well) (29) were incubated with various stimulants in a final volume of 150 μl in 96-well microtiter plates (Corning Glass Works, Corning, NY) for 72 h at 37°C in 5% CO_2 in air. FQ-photomodified LC-EC (10^5 cells/well) and nonirradiated and UVA-irradiated (1.2 J/cm^2 at 365 nm) FQ (final concentrations, 2 and 20 μM) were used as stimulants. Indomethacin (Sigma Chemical Co.) was added to the culture with photomodified LC-EC at a final concentration of 1 μg/ml. [Methyl-^{3}H]TdR (Amersham Corp., Arlington, IL) was added (1 μCi/well) 14 h before harvest. The cells were collected on glass-fiber filters using a cell harvester (Cambridge Technologies, Watertown, MA), and radioisotope uptake was measured in a scintillation counter. LNC taken from mice on day 6 after s.c. injection of AQ-photomodified EC served as control responders.

For blocking studies, the following mAb at the indicated final concentrations were added at the beginning of cultures as described previously (31): anti-I-A^d (MKD6, Becton Dickinson; 2 μg/ml), anti-CD80 (1G10, PharMingen; 1 μg/ml), anti-CD86 (GL1, PharMingen; 1 μg/ml), anti-CD54 (3E2, PharMingen; 1 μg/ml), anti-CD4 (Becton Dickinson; 1 μg/ml), and ST-Q-9 (2 μg/ml). Anti-I-a^k (II-5.2, Becton Dickinson; 2 μg/ml), murine IgM specific for trinitrophenyl hapten (PharMingen; 2 μg/ml), and purified rat myeloma IgG2a (Zymed, South San Francisco, CA) served as controls. The percent suppression of T cell proliferation was calculated with the following formula: (1 − cpm with mAb/cpm without mAb) $\times$ 100.

In vitro propagation of T cells reactive with FQ-photomodified cells and generation of T cell line (TCL)

Single cell suspensions of immune LNC were prepared on day 6 after sensitization of mice with FQ-photomodified EC and cultured (3×10^6/1.5 ml/well) in 24-well plates (Corning Glass Works) in the presence of FQ-photomodified syngeneic spleen cells (2×10^6/well). After 3-day cultivation, viable cells were obtained by Histopaque centrifugation, resuspended in medium, and further cultured for 10 to 14 days, with twice weekly medium changes, in the presence of murine rIL-2 (Genzyme Co., Boston, MA) at 5 U/ml. After separation with Histopaque, viable cells contained approximately 100% CD4$^+$ and approximately 90% TCR Vβ 13$^+$ cells, as mentioned in detail below, and were used as short term cultured TCL.

Flow cytometric analysis of T cells

HBSS containing 0.1% NaN_3 and 1% FCS was used as the staining buffer. Lymphocytes (5×10^5) were incubated with FITC-labeled mAb (1/40) for 30 min at 4°C. After three washes, 10^4 labeled cells were analyzed in a FACScan. FITC-labeled anti-CD4, anti-CD8, and anti-TCR Vβ6, Vβ7, Vβ8.1/8.2, Vβ9, Vβ10, Vβ13, and Vβ14 mAb were obtained from PharMingen.

RT-PCR

Total RNA was prepared from TCL originated from LFLX-, CPFX-, or NFLX-immune LNC, as reported previously (32). First-strand cDNA was reverse transcribed using each RNA sample and was amplified by PCR with an RNA PCR kit (GeneAmp RNA PCR Kit, Takara Biomedicals, Osaka, Japan) according to the manufacturer's directions. All pairs of primers for β-actin, IL-2, IL-4, IL-10, and IFN-γ and the procedure for PCR were described previously (33). The PCR products and DNA m.w. marker VI (Boehringer Mannheim GmbH, Mannheim, Germany) were separated in 2% agarose gels. The gel was stained with 1 μg/ml ethidium bromide, and amplified DNA bands were visualized with a UV transilluminator. The origin of amplified DNA bands was confirmed by a nonisotopic Southern blot hybridization as reported previously (33).

Keratinocyte-derived T cell-activating cytokine activity and IL-1α ELISA assay

Freshly isolated BALB/c EC were suspended in DMEM (Life Technologies) containing 10% heat-inactivated FCS, 5×10^{-5} M 2-ME, and 100 μg/ml streptomycin. Cells (7×10^6/well) were then cultured (1.2 ml/well) for 2 days in the presence or the absence of CFLX, OFLX, or SPFX at 0.2 μM in 24-well plates (Corning) at 37°C in 5% CO_2. Culture supernatants were harvested and subjected to PHA-stimulated thymocyte proliferation assay (29). Serially diluted murine rIL-1α (Genzyme) was used as a positive control. In a parallel experiment, the amount of IL-1α present in culture supernatants was measured with the use of an ELISA kit (Genzyme).

Cell transfer

Freshly isolated FQ-immune LNC or TCL generated from immune LNC were injected through a tail vein into recipient mice. Within 1 h, recipients were challenged with FQ-photomodified EC into a footpad or with i.p. administration of FQ plus UVA irradiation in an earlobe, and swelling

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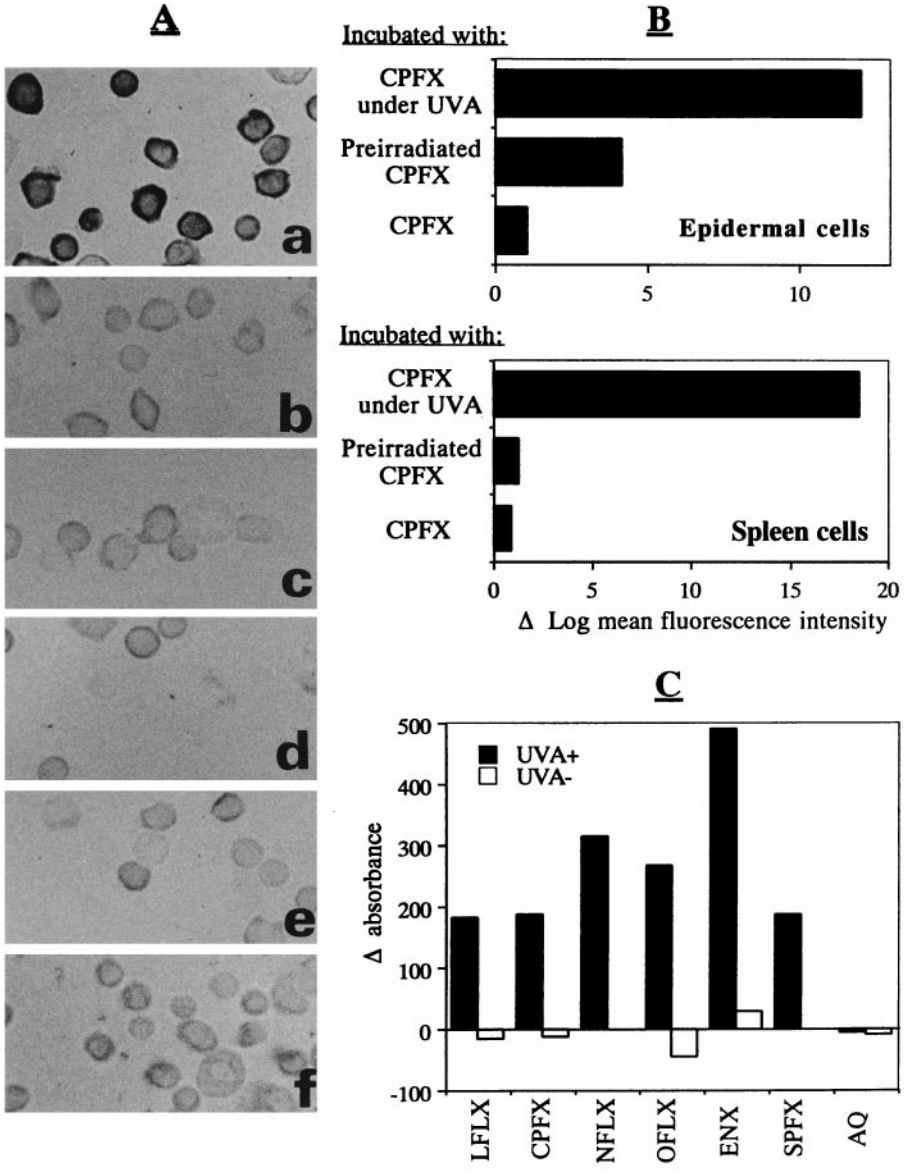

FIGURE 2. Presence of FQ photoadducts in FQ-photomodified cells. *A*, Immunostaining with ST-Q-9 of CPFX-photomodified EC (*a*), EC incubated with UVA-preirradiated CPFX (*b*), EC incubated with CPFX alone (*c*), and untreated EC (*d*). As a control, CPFX-photomodified EC were immunostained with control IgM specific for trinitrophenyl hapten (*e*), and AQ-photomodified EC were stained with ST-Q-9 (*f*). *B*, EC and spleen cells, untreated or treated as described above, were incubated with ST-Q-9 and subsequently with phycoerythrin-conjugated anti-mouse IgM Ab and subjected to flow cytometric analysis. Data are expressed as the Δ log mean fluorescence intensity, which represents the mean intensity of each treated cell group − the mean intensity of the untreated cell group. *C*, ST-Q-9 was added to cell ELISA plates covered with each of six FQ-photomodified or untreated spleen cells. The intensity of coloration was measured by reading at A_{410}. Data are the means of duplicate determinations. Δ absorbance represents the absorbance of treated spleen cells − the absorbance of untreated spleen cells.

responses were measured 24 h later as described above. In some experiments, immune LNC were treated with anti-TCR Vβ13 mAb plus complement (Low-Tox-M Rabbit Complement, Cedarlane Laboratories, Hornby, Ontario, Canada) to delete cells with this particular TCR or with complement alone as a control (33).

Immunofluorescence staining of challenged skin

Three mice were treated i.v. with Vβ13+ TCL derived from NFLX-immune LNC and challenged with NFLX-photomodified EC into footpads. Skin specimens were excised 48 h after challenge from footpads, dissected at the level of the subcutis, and mounted in OCT compound (Miles, Inc., Elkhart, IN). After being snap-frozen in liquid nitrogen, 6-μm cryostat sections on poly-L-lysine-coated glass slides were reacted with FITC-labeled anti-CD4, anti-CD8, and anti-TCR Vβ6, Vβ7, Vβ8.1/8.2, Vβ9, Vβ10, Vβ13, and Vβ14 mAbs at a 1/20 dilution; washed in PBS; mounted in 50% glycerol in PBS; and observed by Olympus fluorescent microscopy (BH-2, Olympus, Tokyo, Japan). Positive cells were enumerated by counting the ratio of the number of positive cells to the number of nuclei stained with hematoxylin. Three high power fields in the dermis were employed for enumeration. For comparison, spleen cells from naive BALB/c mice were examined in the expression of TCR Vβ by flow cytometry. Data represent the mean ± SD for three mice.

Statistical analyses

Student's *t* test was used to determine statistical differences between the means; $p < 0.05$ was considered significant.

Results

Requirement of UVA irradiation for derivatization of cells with FQs and semiquantification of FQ photoadducts formed in photomodified cells

FQ-photomodified EC, along with alternatively treated control EC, were immunohistochemically stained with ST-Q-9, which is an mAb specific to the common structure of FQs. As exemplified by CPFX-photomodified EC, positively stained substances were present in cell membrane and cytoplasm of photomodified cells (Fig. 2*A*, *a*). On the other hand, only weak background staining was seen in EC treated with UVA-preirradiated CPFX (*b*), EC incubated with CPFX alone (*c*), and untreated EC (*d*). Moreover, CPFX-photomodified EC were negatively stained with control IgM (*e*), and ST-Q-9 did not yield positive staining in control photomodified cells or AQ-photomodified EC (*f*). In a flow cytometric analysis using ST-Q-9, EC and spleen cells photomodified

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Table I. *Relationship between photoallergic responses induced by different photosensitization and photoelicitation methods*[a]

Sensitization	Challenge	Earlobe or Footpad Swelling ± SD ($\times 10^{-3}$ cm)
LFLX i.p. + UVA	LFLX i.p. + UVA (earlobes)	3.0 ± 0.80*
LFLX-photomodified EC	LFLX i.p. + UVA (earlobes)	4.1 ± 0.32*
	LFLX i.p. + UVA (earlobes)	1.3 ± 0.56
LFLX i.p. + UVA	LFLX-photomodified EC (footpad)	12.2 ± 2.64**
LFLX-photomodified EC	LFLX-photomodified EC (footpad)	14.2 ± 1.33**
	LFLX-photomodified EC (footpad)	3.3 ± 1.48

[a] Mice were sensitized with i.p. injection of LFLX (100 mg/kg of wt) plus UVA irradiation of shaved abdomen or with s.c. injection of LFLX-photomodified EC. On day 5 after sensitization, mice were challenged with exposure of earlobes to UVA or with s.c. injection of LFLX-photomodified EC into footpads. Increment of earlobes or footpad was measured 24 h after challenge.

*$p < 0.01$, **$p < 0.001$, compared with no sensitization group.

with CPFX had higher mean fluorescence intensity than cells incubated with UVA-preirradiated or untreated CPFX (Fig. 2*B*). These data confirmed that FQs are photohaptens that bind to cells under exposure to UVA (21).

To quantify the amount of FQ in FQ-photomodified cells, cell ELISA was performed with microtiter wells covered with spleen cells that were UVA photoderivatized or simply incubated with each of six FQs. ST-Q-9 was reactive with photoadducts formed on cells photoderivatized with any of six FQs (Fig. 2*C*), whereas cells incubated with FQ without exposure to UVA did not bear FQ photoadducts. No reactivity was found in AQ-photomodified cells. Notably, there were considerable differences among six FQs in the amount of photoconjugates; ENX had the greatest potential to photobind to cells.

Inoculation of FQ-photomodified EC induces and elicits sensitivity when mice are challenged and sensitized with systemic FQ administration plus UVA irradiation, respectively

We have established murine models of photoallergy to FQs in which sensitization and challenge are achieved by systemic (i.p.) administration of FQ plus UVA irradiation of skin or by s.c. inoculation of FQ-photomodified EC (21). The former method is more closely related to human photoallergy than the latter in terms of the manner of administration of photoallergen and of irradiation of skin with UVA. However, responses induced by the former method were weaker than those induced by the latter. To verify the use of FQ-photomodified cells for immunization and elicitation of mice, we compared photoallergic responses induced and elicited by either method. As shown in Table I, both sensitization procedures induced comparable swelling responses when challenged with FQ administration plus UVA irradiation in earlobes or with FQ-photomodified EC in footpads. Therefore, we assume that the immune response to FQ-photomodified EC is relevant to clinical photoallergy that is evoked by systemic administration of FQ plus UVA exposure.

In vitro stimulation of immune LNC with FQ-photomodified LC-EC

When individuals treated with FQ are exposed to UV, epidermal LC are one of the candidates that serve as APC in the initiation of FQ photoallergy. We therefore used LC-EC as accessory cells to stimulate primed T cells. LNC taken from mice sensitized s.c. with FQ-photomodified EC were cultured in the presence of LC-EC photoderivatized with corresponding FQ. In a preliminary experiment, we titrated the stimulatory activity of LC-EC treated with various concentrations of NFLX and a fixed dose of UVA. NFLX-immune LNC responded to LC-EC photomodified with varying concentrations of NFLX at the following stimulation indexes (SI): UVA (1.2 J/cm^2 at 365 nm) alone, 1.3; 0.02 μM, 2.5; 0.2 μM, 7.5; and 2 μM, 6.2. Thus, we used 0.2 μM FQ for photomodification of LC-EC.

Figure 3 shows representative data of six FQs in three independent experiments. Significant proliferative responses of immune LNC specific to photomodified LC-EC were found in all FQs tested. In any of six FQs, immune LNC did not respond significantly to corresponding FQ at 0.2 or 2 μM that was untreated or irradiated with UVA (1.2 J/cm^2 at 365 nm; data not shown). LC needed to be cultured and enriched for activation of LNC, because freshly isolated, unfractionated EC that were photomodified with FQ did not cause significant proliferation of immune LNC (data not shown). LFLX, CPFX, and NFLX had higher levels of SI (9.0, 10.5, and 8.9, respectively) than OFLX and ENX (3.0 and 2.8, respectively), and SPFX (2.0) was the lowest. High proliferative responses were also observed when NFLX-immunized, nylon wool-purified T cells were stimulated with NFLX-photomodified LC-EC (T cells, 496 ± 146 cpm; photomodified LC-EC, 525 ± 98 cpm; T cells plus photomodified LC-EC, 7828 ± 991 cpm), indicating that T cells were responsible for this proliferation.

Blocking studies with mAb were performed in NFLX-immune T cell proliferation to NFLX-photomodified LC-EC. The mean percentages of suppression in two independent experiments were: anti-I-A^d, 84 (control anti-Ia^k, 25); anti-CD80, 30; anti-CD86, 64; anti-CD54, 31 (control IgG2a for these three mAb, 5); anti-CD4, 90; and ST-Q-9, 71 (control IgM specific for trinitrophenyl hapten, 15). Thus, immune T cells recognize FQ photoadducts in the context of MHC class II with costimulatory molecules, mainly CD86.

Since it has been reported that CPFX has the potential to enhance the production of T cell-activating cytokines by PBMC (34), it was necessary to exclude the possibility that FQs stimulate keratinocytes residing in the LC-EC fraction to produce T cell-activating cytokines, because such a cytokine(s) might exaggerate FQ-specific responses of T cells. Freshly isolated murine EC were cultured with or without CPFX, OFLX, or SPFX at 0.2 μM for 2 days, and the culture supernatants were tested for their ability to enhance PHA-induced thymocyte proliferation (29, 35). Compared with the FQ-nontreated control, CPFX-, NFLX-, and SPFX-treated EC supernatants (final dilution, 1/16) augmented thymocyte [^{3}H]TdR incorporation by 5% (not significant), −6% (not significant), and −38% ($p < 0.01$), respectively. Thus, CPFX and NFLX did not affect, and SPFX suppressed the release of keratinocyte-derived T cell-activating cytokines. In addition, the amounts of IL-1α in the supernatants, a major T cell stimulatory cytokine from keratinocytes, measured by ELISA, were as follows: nontreated control, 80.1 pg/ml; CPFX, 86.1; OFLX, 74.2;

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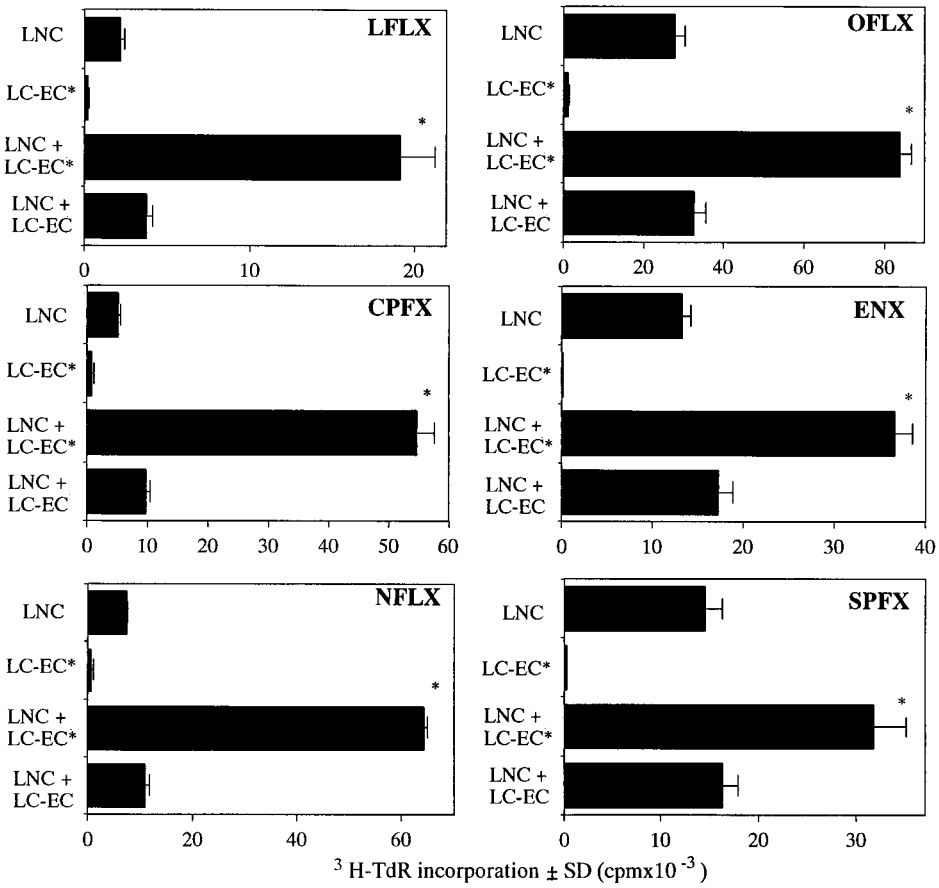

FIGURE 3. Proliferative responses of immune LNC to FQ-photomodified LC-EC. LNC from three mice immunized with FQ-photomodified EC were cultured in triplicate with LC-EC that were photomodified with the same FQ (LC-EC*) or with nontreated LC-EC (LC-EC). FQ photomodification reduced the [^{3}H]TdR incorporation of LC-EC by about 50%. * Indicates $p < 0.01$ compared with the corresponding LNC plus LC-EC group. Error bars represent the SD.

and SPFX, 40.2, supporting the idea of no augmentation by FQ. Therefore, these results negate the above-mentioned possibility.

Photoantigenic cross-reactivity among FQs in T cell recognition

Several clinical reports have documented cross-reactivity in FQs (11, 12, 36, 37). As shown in Figure 4, when mice sensitized with LFLX- or OFLX-photomodified EC were challenged with LFLX-, CPFX-, NFLX-, OFLX-, ENX-, or SPFX-photomodified EC, they exhibited T cell-mediated responses at comparable levels to the corresponding FQ-photocoupled cells. SPFX-photomodified EC induced lesser responses, and an irrelevant photohaptenic drug AQ did not elicit a significant response. This suggested that FQs were cross-reactive in the in vivo system.

The broad photoantigenic cross-reactivity was further confirmed in an in vitro study in which LNC from mice sensitized with FQ-photomodified EC were cultured in the presence of various FQ-photomodified LC-EC. As shown in Table II, LFLX-, CPFX-, and NFLX-immune LNC proliferated well in response not only to corresponding FQ-photomodified LC-EC but also to five other FQ-photoderivatized cells. When LNC immune to OFLX, ENX, and SPFX that showed low responsiveness to the corresponding FQ were used as responders, the pattern of reactivity was less definitive. AQ-immune LNC were not stimulated in response to any of FQs. Thus, the photoantigenic cross-reactivity in vivo parallels that in vitro when sensitization is successfully achieved.

Of note is the finding that, as typically seen in LFLX-immune LNC, the primed LNC proliferated more vigorously in response to ENX-, OFLX-, and NFLX-photomodified LC-EC than those photocoupled with the original immunogen, indicating heteroclitic cross-reactions. When the in vitro stimulatory activity (SI) of cells photomodified with each of six FQs for LFLX- or NFLX-immune LNC (see Table I) was plotted against the amount of FQ photobound to cells (Δ absorbance, see Fig. 2*C*), there were significant correlations between these two values (Spearman's rank test; correlation coefficiency = 0.83; $p < 0.05$). This suggests that in vitro stimulatory activity of FQ-photomodified cells is ascribed at least partly to the amount of FQ photobound to cells, which may account for the heterocliticity.

The same TCR Vβ13 usage in Th1 populations reactive with LFLX, CPFX, and NFLX

Immune LNC from LFLX-, CPFX-, or NFLX-sensitized mice were incubated with the corresponding FQ-photomodified spleen cells for 3 days, and viable cells were further cultured in the presence of rIL-2. The propagating cells were phenotyped by flow cytometry. As shown in Figure 5*A*, >90% of 7-day-cultured cells bore CD4. Moreover, 65 to 77% of the cultured LFLX-, CPFX-, and NFLX-treated LNC expressed Vβ13, and cells bearing six other Vβ segments made up <20% of the culture. The percentages of Vβ13$^+$ cells in LNC freshly isolated from naive mice and FQ-immunized mice were 4 and 8%, respectively, suggesting the dominant growth of Vβ13$^+$ cells. Prolongation of the culture period resulted in exclusive expansion of CD4$^+$Vβ13$^+$ T cells, as 81 to 89% of 11-day-cultured cells were positive for Vβ13 (Fig. 5*B*). Thus, T cell populations preferentially propagating in response to LFLX, CPFX, and NFLX commonly bore TCR Vβ13, supporting the antigenic cross-reactivity, at least among these three FQs. Furthermore, Vβ 13$^+$ cells also selectively expanded when LFLX-immune LNC were cultured in the presence of NFLX- or ENX-photomodified cells and when NFLX-immune LNC were cultured with LFLX- or ENX-photomodified cells (data not shown). In AQ-immune LNC, as a comparison, no selective expansion of T cells

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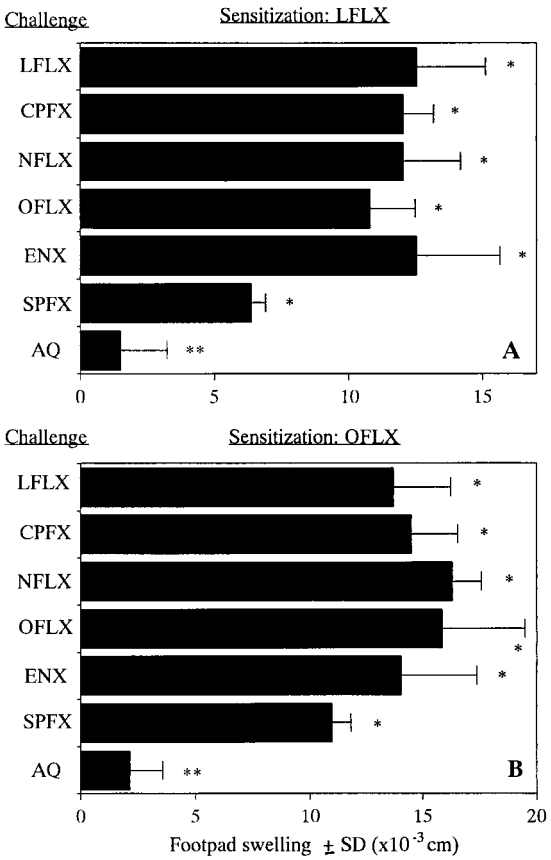

FIGURE 4. T cell-mediated responses elicited with various FQ-photomodified EC in mice sensitized with LFLX- or OFLX-photomodified EC. Mice sensitized with LFLX-photomodified (*A*) or OFLX-photomodified (*B*) EC were challenged with the indicated FQ-photomodified EC or with AQ-photomodified EC as a control. Each group consisted of four to seven mice. The footpad swelling in mice challenged without sensitization ranged from 1.5 to 3×10^{-3} cm. * Indicates $p < 0.05$, ** indicates statistically not significant (compared with corresponding negative control). Error bars represent the SD.

was found, as the 11-day-cultivated cells with AQ-photomodified cells expressed 3 to 20% of any of seven Vβ elements. In the following experiments, 13- to 14-day-cultivated NFLX-immune LNC containing >90% $CD4^+V\beta13^+$ T cells were used as $V\beta13^+$ TCL. By RT-PCR, IL-2 and IFN-γ mRNA were clearly found in $V\beta13^+$ TCL, whereas mRNA for neither IL-4 nor IL-10 was detected (Fig. 5*C*). The same pattern of cytokine messages was found in cultured LFLX- and CPFX-immune LNC (data not shown). These findings indicated that the $CD4^+V\beta13^+$ T cells selectively expanding in response to these FQs belonged to Th1 (38).

Table II. *Cross-reactivity of immune LNC proliferation among 6 FQs*[a]

LNC Immunized with:	SI of LC-EC Photomodified with:					
	LFLX	CPFX	NFLX	OFLX	ENX	SPFX
LFLX	9.07	13.7	18.7	22.7	22.5	13.2
CPFX	12.1	10.5	11.5	11.4	14.2	7.30
NFLX	5.01	5.46	8.85	8.62	8.54	5.32
OFLX	1.02	1.30	1.02	3.00	3.98	1.61
ENX	0.83	0.73	1.34	1.73	2.76	1.48
SPFX	1.07	1.38	1.65	1.45	4.09	2.21
AQ	1.00	1.20	1.22	1.12	1.30	1.31

[a] LNC immunized with EC photomodified with the indicated FQ were cultured with LC-EC photomodified with various FQs. Data shown in each row were obtained from experiments performed simultaneously using the same LNC. The mean cpms of each of immune LNC stimulated with corresponding FQ-photomodified LC were as follows: LFLX, 23,174; CPFX, 27,537; NFLX, 57,197; OFLX, 13,180; ENX, 29,333; and SPFX, 5,619. The mean cpm of nonstimulated AQ-immune LNC was 3,124, and those of FQ-photomodified LC-EC were 174 to 672. The SI of LNC stimulated with non-modified LC-EC was 1.1 to 1.8. The underlined values indicate more than 5.

Adoptive transfer of sensitivity with TCR $V\beta13^+$ T cells

LNC obtained from mice 6 days after sensitization with NFLX-photomodified EC were injected i.v. into syngeneic naive mice. Within 1 h of the transfer, recipients were challenged with NFLX-photomodified EC into footpads. A significant degree of sensitization was transferred to recipients with 3×10^7 LNC (Fig. 6*A*). Transfer of 6×10^6 LNC also induced significant but lesser degrees of footpad swelling responses (data not shown). Mice receiving 3×10^7 NFLX-immune LNC also exhibited a significant ear swelling response upon challenge with i.p. administration of FQ plus UVA irradiation of earlobes (Fig. 6*B*). Treatment of immune LNC with anti-Vβ13 mAb plus complement before transfer abrogated this reaction (Fig. 6*C*). When $CD4^+V\beta13^+$ TCL generated from NFLX-immune LNC were administered to mice, the sensitivity was also transferred (Fig. 6*D*). The data indicate that immune $V\beta13^+$ T cells were responsible for transfer of the sensitivity. As shown in Figure 6*E*, mice that received $V\beta13^+$ TCL produced from LFLX-immune LNC showed significantly greater ear swelling responses upon challenge with ENX administration and subsequent UVA irradiation than those from LNC treated with LFLX plus UVA. Thus, heterocliticity with ENX was also found in in vivo $V\beta13^+$ T cell-induced cutaneous photoallergy as well as in vitro primed T cell responses.

Infiltration of $V\beta13^+$ cells at challenge sites

Mice that received i.v. injection of $V\beta13^+$ TCL were examined by immunofluorescence staining in the Vβ usage of dermal infiltrating cells at the footpads where challenge with NFLX-photomodified EC was performed. Cells infiltrating perivascularly and in conjunction with the injected EC cluster consisted of 90% $CD4^+$ and 8% $CD8^+$ cells. The mean percentages of T cells bearing each Vβ in three mice were as follows: Vβ6, 10.5%; Vβ7, 11.0%; Vβ8.1/8.2, 17.8%; Vβ9, 10.5%; Vβ10, 3.2%; Vβ13, 22.0%; and Vβ14, 9.5%. Thus, T cell populations bearing particular Vβ did not exclusively infiltrate. However, the $V\beta13^+$ cell population was the largest, followed by $V\beta8^+$ cells, in number. Since in BALB/c mice, $V\beta13^+$ and $V\beta8.1/8.2^+$ cells are the minor (3.5% of spleen cells) and major (16% of spleen cells) T cell populations, respectively, these data suggested selective infiltration of $V\beta13^+$ T cells in the challenge sites.

Discussion

We have previously demonstrated the presence of FQs in FQ-photomodified cells by detecting their fluorescence under a fluorescence microscopy (19). This study more clearly proved FQ photocoupling to cells with the use of ST-Q-9. The current observation in mice that FQ-photomodified cells have in vivo and in vitro antigenic potency bears clinical importance in elucidating the mechanism of FQ photoallergy. Like ordinary haptens (39), it should be clarified whether FQs need to photobind directly to APC such as LC, or FQ-epidermal protein conjugates are processed by APC for T cell recognition. In contrast to TCSA-photomodified cells, which show low viability (<1%) due to its strong phototoxic property (27), FQ-photomodified EC remain highly viable (21), and FQ-photomodified LC-EC successfully presented photoallergen. This circumstantial evidence supports the concept that FQ-photomodified LC are capable of directly inducing hypersensitivity. In vitro Ag-presenting functions of LC-EC were inhibited

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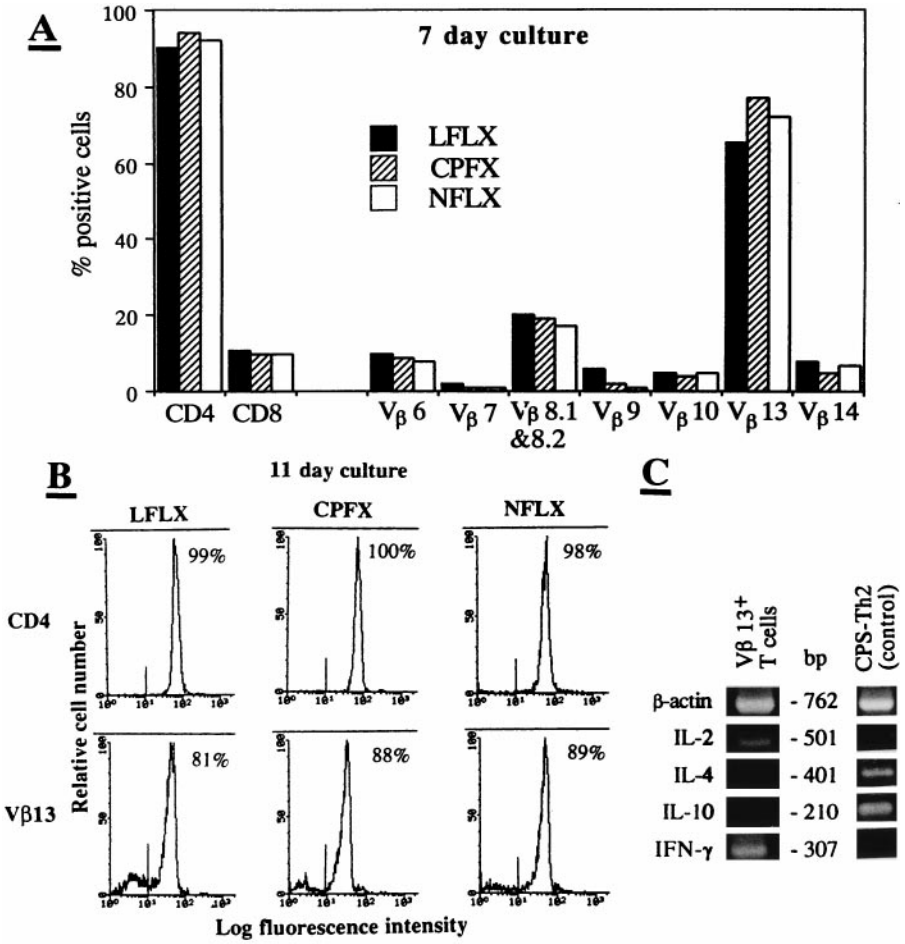

FIGURE 5. Immunophenotypes and cytokine profile of LFLX-, CPFX-, and NFLX-immune LNC propagating in response to FQ-photomodified cells. LNC from mice sensitized with LFLX-, CPFX-, or NFLX-photomodified EC were cultured in the presence of corresponding FQ-photomodified spleen cells. Expression of cell surface antigens was assessed by flow cytometry. *A*, The percentage of 7-day-cultured immune LNC positive for the indicated surface molecules. *B*, Flow cytometric graphs of 11-day-cultured LNC stained with anti-CD4 and anti-Vβ13 mAbs. *C*, RT-PCR for detection of cytokine mRNA in Vβ13$^+$ TCL; Vβ13$^+$ TCL and TCSA-photospecific TCL, as a Th2 control (33), were subjected to RT-PCR using primers specific for IL-2, IL-4, IL-10, and IFN-γ.

substantially by anti-MHC class II, ST-Q-9, and anti-CD86, but only partially by anti-CD54 and anti-CD80 mAb. Such predominant usage of CD86 costimulatory molecules in the interaction between LC and T cells has also been found in ordinary hapten (31, 40), allogeneic mixed EC lymphocyte reactions (41), and in vivo hapten-induced contact photosensitivity (42).

In both in vivo footpad swelling responses and in vitro proliferation of immune LNC, broad photoantigenic cross-reactivity existed among FQs. An enhanced expansion of CD4$^+$ TCR Vβ13$^+$ Th1 cells, but not of any other Vβ-bearing T cells, was observed after in vitro stimulation of these immune LNC with FQ-photomodified cells. This preferential propagation of T cells bearing identical TCR Vβ elements further supports the cross-reactive moiety in FQ-photomodified cells and the presence of a common epitope that is recognized by FQ-specific T cells. Such selectivity in an expanding T cell population was also reported in the response to several penicillin derivatives (22). The relevance of these Vβ13$^+$ T cells to in vivo photoallergy was evidenced by successful transfer of the sensitivity with Vβ13$^+$ TCL and selective accumulation of Vβ13$^+$ cells in the challenge sites. There have been several clinical reports concerning crossing photosensitivity (11, 12) and other cutaneous adverse reactions (36, 37) to FQs. Our study proves these clinical observations and further broadens the possible occurrence of cross-reactivity among various FQs.

Although all FQs tested in this study were photoantigenically active, there were considerable differences in the degree of photoallergenicity. In addition, the in vivo immunogenic activity did not parallel the in vitro stimulatory activity. As assessed by the proliferative response of immune LNC, the photosensitizing potencies of six FQs decreased in the order: LFLX, CPFX, NFLX > OFLX, ENX > SPFX. On the contrary, when evaluated by the in vitro restimulatory ability of photomodified LC-EC, ENX, OFLX, and NFLX activated primed T cells heteroclitically and more vigorously than the other FQs. Such discrepancy between sensitizing and eliciting activities was also reported in encephalitogenic myelin basic protein peptides (43). Like FQs, heteroclitic T cell responses have been found in various Ags: haptens (44, 45), protein/peptides (45–47), and glycosylated peptides (48). The mechanism(s) of heterocliticity in peptides has been explained by increased binding affinity to MHC class II (43, 48) and by a difference between antigenic sites for in vivo T cell priming and the residues contributing to the heteroclitic response of primed T cells (46, 47). On a cell ELISA analysis using ST-Q-9, the in vitro stimulatory ability of FQs was associated with the amount of photoadducts, as ENX, exhibiting a high value in the cell ELISA, strongly stimulated any immune LNC. Therefore, the heterocliticity in FQs may be caused at least partly by their photobinding affinity to APC. Mice transferred with Vβ13$^+$ TCL originating from LFLX-immune LNC exhibited stronger cutaneous photoallergy upon photochallenge with ENX than with LFLX, suggesting that heteroclitic responses may occur clinically as well as in in vitro T cell responses.

In addition to the photobinding affinity to cells, the phototoxic capacity is another important factor that influences the photoimmunogenicity of FQs. For example, SPFX is extremely phototoxic in DNA strand-breaking activity and mouse cutaneous reactions (14). Clinically, in the vast majority of SPFX-photosensitive patients, photodermatitis is evoked by a phototoxic, but not by a

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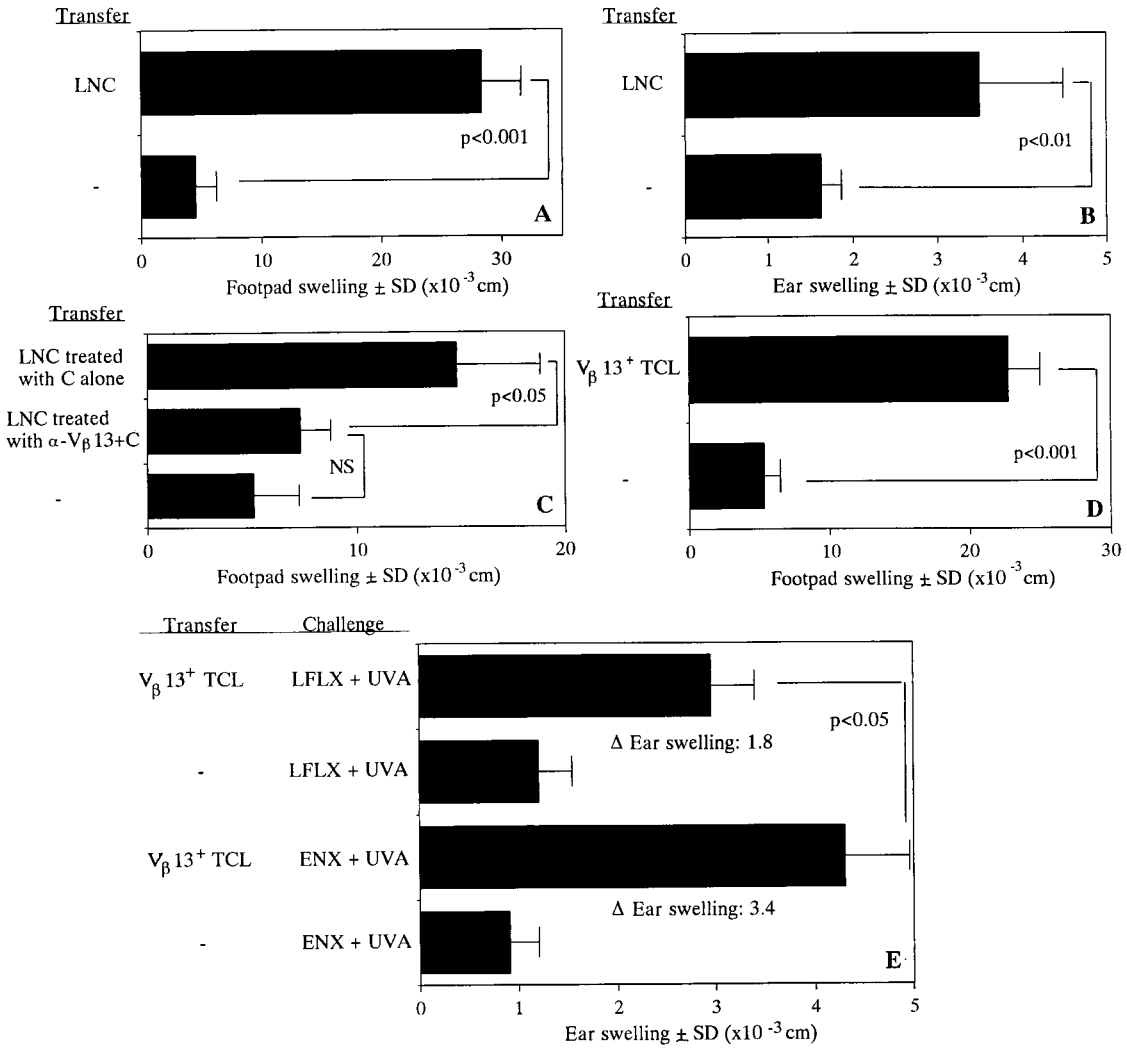

FIGURE 6. Cell transfer study of FQ-immune LNC and Vβ13$^+$ TCL. Recipient mice were treated i.v. with NFLX-immune LNC untreated (*A* and *B*), anti-Vβ13 mAb plus complement or complement alone (3×10^7 cells/mouse) (*C*), or TCL (4×10^6/mouse) produced from NFLX-immune (*D*) or LFLX-immune LNC (*E*). Recipients were challenged with NFLX-photomodified EC into the footpad (*A*, *C*, and *D*) or with i.p. NFLX (*B*), LFLX (*E*), or ENX (*E*) plus UVA irradiation of earlobes. In *E*, Δ ear swelling responses represent the ear swelling of transferred and challenged mice − the ear swelling of nontransferred and challenged mice. Each group consisted of five mice (*A–D*) or four or five mice (*E*). Error bars represent the SD.

photoallergenic, mechanism (14). In contrast, the weak photoallergenicity of SPFX has been documented by the low footpad response induced by SPFX-photomodified EC (21) and the inadequacy of priming LNC as shown in this study. The phototoxic and photoallergic activities are also inversely correlated for CPFX and NFLX (21). FQs with strong phototoxicity appear to exert adverse effects on immunocompetent cells, leading to a reduction in the Ag-presenting ability of photomodified cells.

There are several apparent relationships between the chemical structure of quinolones and photosensitivity (2, 21). The phototoxic potency is thought to be determined at least partly by the nature of substituents at the 8 position (49). FQs with fluorine at C_8, such as SPFX and LFLX, have strong phototoxic activity (2). However, this fluorine is not involved in the photoallergenicity, because CPFX and NFLX, possessing no fluorine at C_8, showed stronger photoallergenicity than SPFX. The recognition of various FQs by TCR Vβ13$^+$ T cells suggests that the photoantigenic epitope in FQs is the common nuclear structure. Recent photochemical studies have shown that the piperazinyl (or methylpiperazinyl) group, the major side chain of FQs linked at C_7, is altered by UVA irradiation (50, 51). One can assume that this piperazinyl photodegradation occurs commonly in FQs to various degrees depending on individual photostability (21). This raises the possibility that protein is covalently bound to the piperazinyl ring during its photodegradation, resulting in the formation of allergenic FQ-protein complex. This preferential piperazinyl photo-decomposition may account for the observed broad cross-reactivity.

On the basis of this study, FQ-photocoupled EC formed in UVA-exposed skin of patients medicated with FQs may induce immunologic reactions mediated by T cells that recognize the shared photohaptenic structure of FQs. However, since there are differences between FQs in the ability to photobind to cells and the phototoxicity toward immunocompetent cells, their photoantigenic abilities are substantially different. There have been 142 reported patients with photosensitivity to FQs in Japan from 1980 to 1996 (15). The majority of patients developed photosensitive dermatitis to ENX (16%), LFLX (15%), SPFX (42%), or fleroxacin (25%). The highest incidence of SPFX can be explained by its outstanding phototoxicity. Photosensitivity to ENX and fleroxacin seems to be mainly photoallergic based on clinical studies (5–7, 21), while LFLX is thought to have both potencies (10–12, 21). Considering that fleroxacin was newly marketed, and its usage and publications

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have been being increased recently, ENX as well as fleroxacin appear to be the major photoallergic FQs on a clinical basis. The current finding that ENX-photomodified cells strongly stimulate T cells primed with any of the FQs suggests that once sensitized with an FQ(s), individuals may develop photosensitive dermatitis easily upon administration of ENX. It is possible that photoimmunizing and photoeliciting FQs are different in some patients with FQ photosensitivity because of their cross-reactive and heteroclitic moiety.

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