Treatment of T Lymphocytes with 8-Methoxypsoralen Plus Ultraviolet A Induces Transient but Biologically Active Th1-Skewing Cytokine Production

Yoshiki Tokura, Naohiro Seo, Hiroaki Yagi, Hisashi Wakita, Shin-ichi Moriwaki, Fukumi Furukawa, and Masahiro Takigawa

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

8-Methoxypsoralen plus ultraviolet A light is suggested to shift T lymphocytes from Th2 to Th1 cells. To clarify this issue, we examined the effects of 8-methoxypsoralen/ultraviolet A on the expression/production of cytokines in peripheral blood mononuclear cells from normal subjects and a Sézary syndrome patient. 8-Methoxypsoralen/ultraviolet A augmented the expression of mRNAs for interferon-γ and interleukin-2 and reduced those for interleukin-4 and interleukin-10. It seems that this enhancement of Th1 cytokines is caused by increment of cytokine production by Th1 cells but not by conversion of Th2 cells to produce Th1 cytokines. The number of interferon-γ-secreting lymphocytes was markedly increased in 8-methoxypsoralen/ultraviolet A-treated peripheral blood mononuclear cells 20 h after treatment, whereas that of Th2 cytokine-producing cells was decreased. Accordingly, the amount of interferon-γ was elevated in culture supernatants from 8-methoxypsoralen-phototreated peripheral blood mononuclear cells, whereas interleukin-4 was significantly reduced. This enhanced production of interferon-γ, however, was found only until 3 d after 8-methoxypsoralen phototreatment and was declined by 5 d after treatment. Finally, 8-methoxypsoralen/ultraviolet A treatment of T cells regulated their ability to induce keratinocyte CD54 expression. Our results show that 8-methoxypsoralen/ultraviolet A has a transient but biologically active Th1-skewing action in human T cells, suggesting that 8-methoxypsoralen/ultraviolet A exerts a beneficial therapeutic effect on Th2-mediated or Th2-malignant diseases. ***Key words: interferon-γ/photochemotherapy/psoralen/T cells/ultraviolet light. J Invest Dermatol 113:202–208, 1999***

Clinical efficacy of 8-methoxypsoralen (8-MOP) in conjunction with ultraviolet A light (UVA), referred to as PUVA, has been well documented in the treatment of a number of skin disorders such as psoriasis (Pathak and Fitzpatrick, 1992). In the more recently developed method, the extracorporeal photochemotherapy (ECP) or photopheresis, peripheral blood lymphocytes of the 8-MOP-administered patients are irradiated extracorporeally with UVA and then returned to the patients (Edelson *et al*, 1987). ECP was originally approved for the treatment of cutaneous T cell lymphoma (CTCL), a malignancy derived from $CD4^+$ T helper (Th) cells (Heald and Edelson, 1991). Various mechanisms have been proposed to explain the mode of action by which PUVA and ECP exert their biologic actions. As these psoralen photochemotherapies are used for immunologic disorders (Rook *et al*, 1990, 1992) or immunomodulatory purposes (Edelson *et al*, 1987; Besnier *et al*, 1997), it is important to investigate functional changes in immunocompetent cells phototreated with 8-MOP. Among the immunologic consequences, alterations in the cytokine production (Tokura *et al*, 1991a; Tokura, 1999) as well as expression of surface molecules (Kozenitzky *et al*, 1992; Urano *et al*, 1995; Cheng *et al*, 1996) are critical events in 8-MOP-phototreated cells. The vast majority of studies have shown reduced production of cytokines in 8-MOP/UVA-treated keratinocytes, lymphocytes, monocytes, and endothelial cells (Tokura, 1999). For example, treatment of peripheral blood mononuclear cells (PBMC) with 8-MOP/UVA reduces the expression of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α (Boehncke *et al*, 1994; Neuner *et al*, 1994). This phototreatment at certain doses, however, can stimulate T lymphocytes to produce certain cytokines (Saed and Fiverson, 1994), suggesting that some activational events may occur in 8-MOP-phototreated T cells.

$CD4^+$ Th cells can be divided into two distinct subsets with mutually counteract properties: Th1 cells, producing interferon-γ (IFN-γ) and IL-2, and Th2 cells, secreting IL-4, IL-5, and IL-10 (Mosmann and Coffman, 1989). Recently, 8-MOP/UVA has been reported to skew T cell cytokine production from Th2 to Th1 type. By competitive reverse transcription–polymerase chain reaction (reverse transcription–PCR), Saed and Fiverson (1994) have shown that 8-MOP/UVA augments IFN-γ mRNA expression in HUT-78 cells, a malignant T cell line established from a patient with Sézary syndrome. A clinically oriented study by Di Renzo *et al* (1997) demonstrated that PBMC of patients with the early stage of CTCL produced higher levels of IL-4 and lower levels of IFN-γ and IL-12. This showed that cytokine production was normalized

Manuscript received May 6, 1998; revised April 6, 1999; accepted for publication April 7, 1999.

Reprint requests to: Dr. Yoshiki Tokura, Department of Dermatology, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-3192, Japan.

Abbreviations: ECP, extracorporeal photochemotherapy; ELISPOT, enzyme-linked immunospot; PBMC, peripheral blood mononuclear cells.

after ECP treatment of 1 y. Considering that malignant T cells in the majority of CTCL patients are of a Th2 type (Vowels *et al*, 1992; Saed *et al*, 1994; Rook *et al*, 1995; Yagi *et al*, 1996a), this Th1 skewing effect of 8-MOP/UVA may at least partly be responsible for the therapeutic effect of ECP.

This study was conducted to dissect whether the Th1-skewing effect of 8-MOP/UVA effectively operates and is clinically relevant. Our results show that the Th1-preponderance of 8-MOP-photo-treated cells is seen in mRNA expression, the number of cytokine-secreting cells, and the cytokines released into the supernatant. Although the enhanced production of Th1 cytokines by the treated T cells is transient, this change is biologically relevant when examined by induction of CD54 expression in keratinocytes.

MATERIALS AND METHODS

Culture medium RPMI-1640 (Gibco BRL Life Technology, Grand Island, NY) was supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM HEPES, 1% nonessential amino acids, 100 units per ml penicillin, and 100 μg per ml streptomycin (all from Gibco).

PBMC and T cell clones PBMC were isolated from normal subjects and a 59 y old patient with Sézary syndrome by the standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) method. The patient's history and cytologic features of his malignant T cells were described previously in detail (Seo *et al*, 1998). Briefly, he had a marked peripheral blood lymphocytosis ($300–400 \times 10^9$ per liter) with a high CD4/CD8 ratio fluctuating from 9:1 to 370:1. Seventy to 90% of PBMC were $CD3^+$ $CD4^+$ $CD7^-$ $CD45RO^+$ $CD45RA^-$ cells, typical of Sézary cells (Wood *et al*, 1990). $CD4^+CD7^-$ Sézary cells exhibited a clonally rearranged band for a T cell receptor β-chain (Cβ1) by Southern blot analysis and expressed mRNA for IL-4, IL-5, and IL-10 but not for IL-2 or IFN-γ, indicating that the tumor cells are of Th2 type (Seo *et al*, 1998). The patient had not received any treatment except for topical application of corticosteroid ointments for at least 2 wk before the study.

Keyhole limpet hemocyanin + $I\text{-}A^k$-specific murine T cell clones, 28-4 and 24-2 (Nakayama *et al*, 1988, 1989), were kindly provided by Dr. Yoshihiro Asano, Department of Bacteriology, Ehime University; 28-4 and 24-2 cells belong to Th1 and Th2 cells, respectively, in their cytokine profile (Nakayama *et al*, 1988, 1989). These cell clones were used 3 wk after feeding with keyhole limpet hemocyanin (Sigma, St. Louis, MO; final concentration, 10 μg per ml), feeder cells, and concanavalin A (Con A)-stimulated rat spleen cell supernatants.

Human $CD4^+$ Th1 clones were generated from a normal subject by culturing PBMC with mitomycin C-treated, autologous feeder cells and recombinant IL-2 (5 units per ml; Genzyme, Boston, MA) after limiting dilution (0.5 cells/well). Cells were restimulated with feeder cells and IL-2 and expressed CD3 and CD4 but not CD8 by flow cytometric analysis. On reverse transcription–PCR analysis, the clones expressed mRNA for IFN-γ and IL-2, but not IL-4 or IL-10.

Light source Black light (FL20SBLB) emitting UVA ranging from 320 to 400 nm with a peak emission at 365 nm were purchased from Toshiba Electric (Tokyo, Japan). With a UV radiometer (Eisai, Tokyo, Japan), the energy output of three 20 W tubes of black light at a distance of 20 cm was 2.7 mW per cm^2 at 365 nm and 0.17 mW per cm^2 at 305 nm.

Treatment of cells with 8-MOP/UVA Stock solutions were prepared by dissolving crystalline 8-MOP (Sigma) in absolute ethanol (100 μg per ml) and diluted with phosphate-buffered saline (PBS; pH 7.4) just before use. The procedure to treat cells with 8-MOP/UVA was described previously (Tokura *et al*, 1991a, 1993). Briefly, PBMC and human $CD4^+$ T cell clones were incubated with 8-MOP for 30 min in the dark and transferred to plastic dishes (Sumilon Dish, Sumitomo Bakelite, Tokyo, Japan; 60 mm in diameter). The dishes were irradiated with UVA, whose irradiance was measured through the plastic dishes. After irradiation, cells were washed twice with PBS. The proportionate 8-MOP/DNA photoadduct formation has been observed over an 8-MOP concentration range of 1–200 ng per ml and a UVA dose range of 0.2–24 J per cm^2 at 365 nm (Edelson *et al*, 1987; Tokura *et al*, 1991b). Furthermore, the plasma concentration of 8-MOP in humans receiving oral PUVA therapy falls in the range of 10–250 ng per ml (Gasparro *et al*, 1988). Thus, we chose 100 ng per ml of 8-MOP concentration plus 1 J per cm^2 of UVA dose to treat cells in this study unless otherwise mentioned. Treatment of keratinocytes with 8-MOP/UVA at these doses reduced their IL-1α production by 55%–60% (Tokura *et al*, 1991a).

Reverse transcription–PCR for cytokines PBMC and 28-4 and 24-2 cells were untreated or treated with 8-MOP and/or UVA. PBMC were cultured with 5 μg Con A (Sigma) per ml and 28-4 and 24-2 cells cultured with Con-A-stimulated rat spleen cell supernatants for 2 h at 37°C in 5% CO_2 in air. A preliminary study showed that steady-state levels of cytokine mRNA were too low to evaluate the modulatory effect of 8-MOP/UVA and that incubation of PBMC with Con A augments mRNA expression for all cytokines examined as compared with untreated cells. Therefore, cells were exposed to Con A before extraction of RNA. In some experiments, 8-MOP/UVA-treated PBMC were stimulated with solid-phase anti-CD3 monoclonal antibody (MoAb) and soluble anti-CD28 MoAb (Thompson *et al*, 1989). Twenty-four well plates were precoated with anti-mouse immunoglobulin G (1:500; goat affinity purified antibody; Cappel, Aurora, OH) at 4°C overnight and coated with anti-human CD3 MoAb (2 μg per ml; OKT3; Ortho Diagnostic, Raritan, NJ). Cells were cultured for 18 h in the plate in the presence of anti-CD28 MoAb (1 μg per ml; PharMingen, San Diego, CA).

Total RNA was prepared from these cells, as reported previously (Chomczynski and Sacchi, 1987). First-strand cDNA was reverse transcribed using each RNA sample and was amplified by PCR with a RNA PCR kit (GeneAmp RNA PCR Kit; Takara Biomedicals, Osaka, Japan) according to the manufacturer's directions. All pairs of primers for β-actin, IFN-γ, IL-2, IL-4, and IL-10 in humans (Yagi *et al*, 1996a,b) and mice (Yagi *et al*, 1996c), and the procedure for PCR were described previously (Yagi *et al*, 1996a–c). The PCR products and DNA molecular weight marker VI (Boehringer Mannheim GmbH, Mannheim, Germany) were separated by electrophoresis in 2% agarose gels. The gel was stained with 1 μg per ml ethidium bromide and amplified DNA bands were visualized with an UV transilluminator. The density of the scanned band was quantitated by image-analyzing software (NIH Image). The intensity of cytokine mRNA expression was estimated by calculating the relative density of cytokine PCR bands against β-actin in the same sample.

Reverse enzyme-linked immunospot (ELISPOT) assay The ELISPOT assay was performed as described (Czerkinsky *et al*, 1988; Kabilan *et al*, 1990). Normal PBMC were untreated or treated with 8-MOP/UVA and cultured for 20 h with or without Con A at 5 μg per ml. Nitrocellulose-bottom MultiScreen-GV 96 well Filtration Plates (Millipore, Bedford, MA) were coated with 100 μl of the first monoclonal antibody against IFN-γ, IL-2, IL-4, IL-5, or IL-10 (PharMingen, San Diego, CA) in PBS at a concentration of 10 μg per ml and incubated overnight at 4°C. Unbound antibodies were removed by washing with sterile PBS and the outer surface of the nitrocellulose membrane of the plate was treated with PBS containing 5% fetal bovine serum to block nonspecific binding of cytokines. The wells were filled in duplicate with 2×10^5 cultured PBMC suspended in 100 μl of culture medium. After incubation for 20 h at 37°C in 5% CO_2 in air, the plates were rinsed four times with PBS by vigorous shaking for 5 min. Then, 100 μl of PBS containing 5% fetal bovine serum and the second anti-IFN-γ, anti-IL-2, anti-IL-4, anti-IL-5, or anti-IL-10 MoAb conjugated with biotin (PharMingen, 10 μg per ml) was added to each well and incubated for 2 h at room temperature. After washing with PBS, wells were exposed for 45 min to 100 μl of PBS containing avidin conjugated with horseradish peroxidase (Gibco BRL Life Technology) at a final concentration of 1 μg per ml. The enzyme substrate solution (3,3′-diaminobenzidine tetrahydrochloride-H_2O_2-chromagen substrate) was added, and spots detectable as a brown precipitate were counted under low magnification (×40) with a stereomicroscope. The results were expressed as the number of cytokine-forming spots per 10^6 cells.

Preparation of culture supernatants and assay for cytokines Normal and Sézary syndrome PBMC, and $CD4^+$ Th1 clones were treated with 8-MOP and/or UVA, and cultured in 24 well plates (Corning Glass Works, Corning, NY; 3×10^6/1.5 ml of culture medium for PBMC and 3×10^5/1.5 ml for T cell clones) in the presence of Con A (1 μg per ml unless otherwise mentioned) at 37°C in 5% CO_2 in air. The culture supernatants were harvested after 3 d cultivation. In some experiments, supernatants (95%) were harvested and replaced by fresh medium containing Con A (1 μg per ml) on days 1, 3, and 5. As alternative stimulation, PBMC were cultured in anti-CD3 MoAb-coated plates in the presence of anti-CD28 MoAb as described above. The amounts of IFN-γ and IL-4 present in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) (Genzyme, or Special Reference Laboratories, Tokyo, Japan).

PBMC proliferation assay and enumeration of cultured PBMC PBMC treated with 8-MOP and/or UVA (2×10^5 cells per well) were

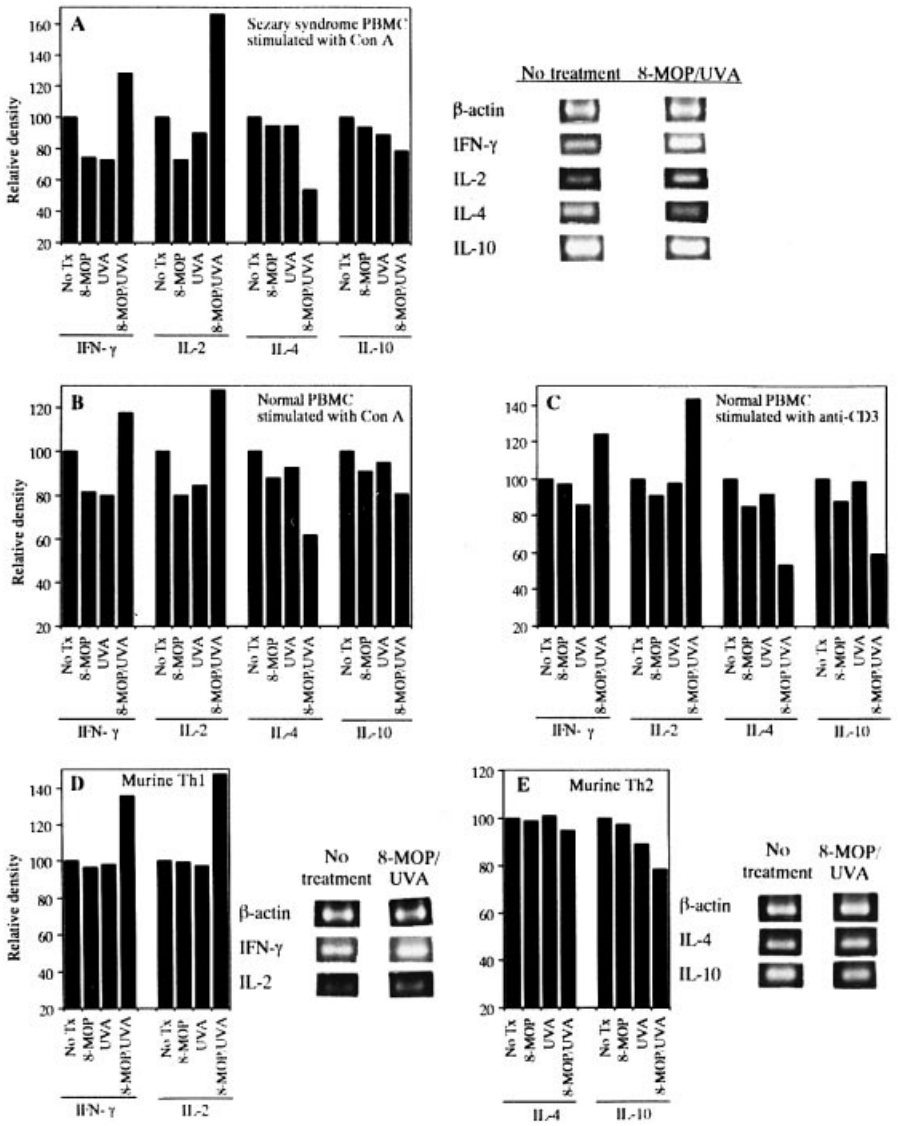

Figure 1. Elevated and depressed mRNA expression for Th1 and Th2 cytokines, respectively, in 8-MOP/UVA-treated PBMC and murine Th1 and Th2 clones on reverse transcription–PCR analysis. PBMC from the Sézary syndrome patient (*A*) and normal subjects (*B*, *C*) and murine Th1 (28-4; *D*) and Th2 (24-2; *E*) clones were treated with 8-MOP (100 ng per ml) and/or UVA (1 J per cm^2). They were stimulated for 2 h with Con A (*A*, *B*) or Con-A-stimulated rat spleen cell supernatants (*D*, *E*), or with anti-CD3 and anti-CD28 MoAb for 18 h (*C*), and subjected to reverse transcription–PCR. Photographed DNA bands show PCR products, whose size (base pairs, bp) was 661 bp for β-actin, 501 bp for IFN-γ, 458 bp for IL-2, 456 bp for IL-4, and 352 bp for IL-10 in humans, and 762 bp for β-actin, 307 bp for IFN-γ, 501 bp for IL-2, 401 bp for IL-4, and 210 bp for IL-10 in mice. Graphed bars represent the relative density of cytokine PCR bands against β-actin, analyzed with NIH software. The data are a representative of two series of independent experiments with similar results.

cultured in triplicate in a final volume of 150 μl of culture medium in 96 well, flat-bottomed microtiter plates (Corning Glass Works) for 3 d and pulsed with methyl ^{3}H-thymidine (Amersham, Arlington, IL; 1 μCi per well) 16 h before harvest. Con A (1 μg per ml) was added at the beginning of the culture. The cells were collected on glass fiber filters using a cell harvester (Cambridge Technologies, Watertown, MA) and their radio-uptake was measured in a scintillation counter.

PBMC (2×10^6 per ml) untreated or treated with 100 ng 8-MOP per ml and/or 1 J UVA per cm^2 were cultured in the presence of Con A (1 μg per ml) in duplicate in a final volume of 1 ml of culture medium in 24 well plates. On days 0–8, the number of viable cells, assessed by Trypan Blue dye exclusion, was counted after pipetting the culture. As the cultured cells were aggregated and made colonies, it was difficult to count the absolute number of cells. Therefore, we used the relative number of cells to untreated control, which represents (the number of PBMC treated with 8-MOP and/or UVA)/(the number of untreated PBMC) × 100.

CD54 (intercellular adhesion molecule-1) expression on keratinocytes (Wakita *et al*, 1995) Normal human keratinocytes (Epipack, Clonetics, CA) were grown in serum-free keratinocyte growth medium (Gibco BRL, Gaithersburg, MD), supplemented with 10 ng per ml of epidermal growth factor and 60 ng per ml of whole bovine pituitary extract in 24-well plates. Keratinocytes at 90% confluency were used for CD54 expression experiments by refeeding with 1 ml of medium without epidermal growth factor or whole bovine pituitary extract. Supernatants obtained from untreated or 8-MOP/UVA-treated PBMC after 3 d-cultivation with or without Con A (1 μg per ml) were added to keratinocyte cultures at a final dilution of 1/10. After 2 d cultivation, single cell suspensions of keratinocytes prepared with trypsin/EDTA were incubated with fluorescein isothiocyanate-conjugated anti-CD54 MoAb (Immunotech, Marseilles, France) and analyzed in a FACScan (Becton Dickinson, Mountain View, CA). Dead cells were identified by propidium iodide uptake. Mean fluorescence intensity on the abscissa was expressed as a logarithm.

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

RESULTS

Th1-preponderant augmentation of cytokine mRNA expression by 8-MOP/UVA The modulatory effect of 8-MOP/UVA on mRNA expression for T cell cytokines was examined by reverse transcription–PCR. PBMC from the Sézary syndrome patient were treated with 8-MOP and/or UVA and stimulated with Con A. Treatment with 8-MOP or UVA alone reduced the expression of all cytokines to various degrees (**Fig 1*A***). The combination of 8-MOP and UVA, however, augmented the levels of mRNA for IFN-γ and IL-2, whereas this treatment further reduced transcription of IL-4 and IL-10. Similar results were obtained from experiments in which normal subject's PBMC were stimulated with Con A (**Fig 1*B***) or anti-CD3 plus anti-CD28 MoAb (**Fig 1*C***). PBMC from the Sézary syndrome patient, however, exhibited more pronounced changes in Th1 and Th2 cytokine production than those of normal subjects, presumably because of the presence of a high percentage (≈80%) of malignant Th2 cells. This notion was

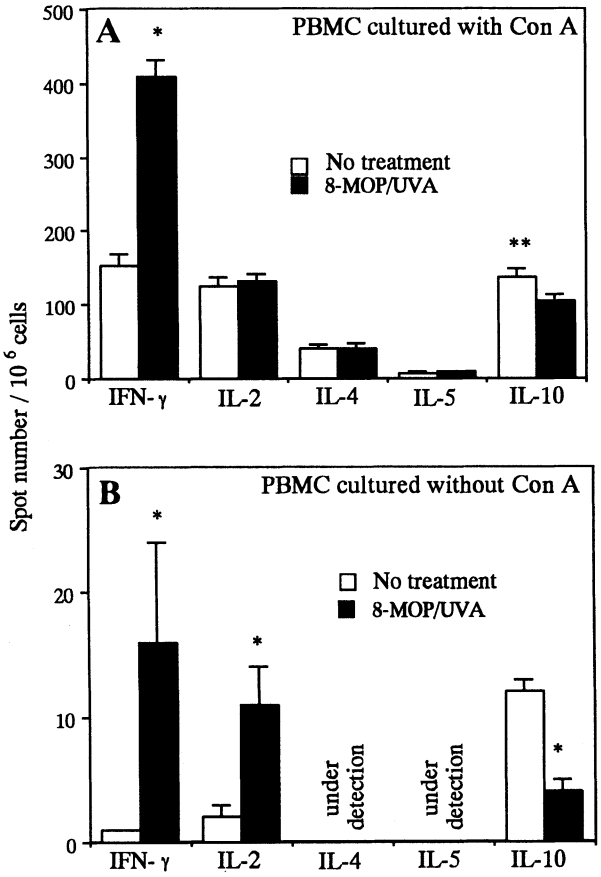

Figure 2. Increased Th1-producing and decreased Th2-producing cell numbers in 8-MOP/UVA-treated PBMC in ELISPOT assay analysis. Normal subject's PBMC were treated with 8-MOP (100 ng per ml) and/or UVA (1 J per cm^2 at 365 nm), cultured with Con A (*A*) or without Con A (*B*) for 20 h, and subjected to ELISPOT assay in triplicate. The data are expressed as brown chromagen spot number per 10^6 cells. The values of PBMC treated with 8-MOP or UVA alone were not significantly changed compared with those of untreated PBMC. The data are representative of three normal subjects with similar results. Vertical bars represent SD. *$p < 0.01$, **$p < 0.05$, compared with the corresponding no treatment group.

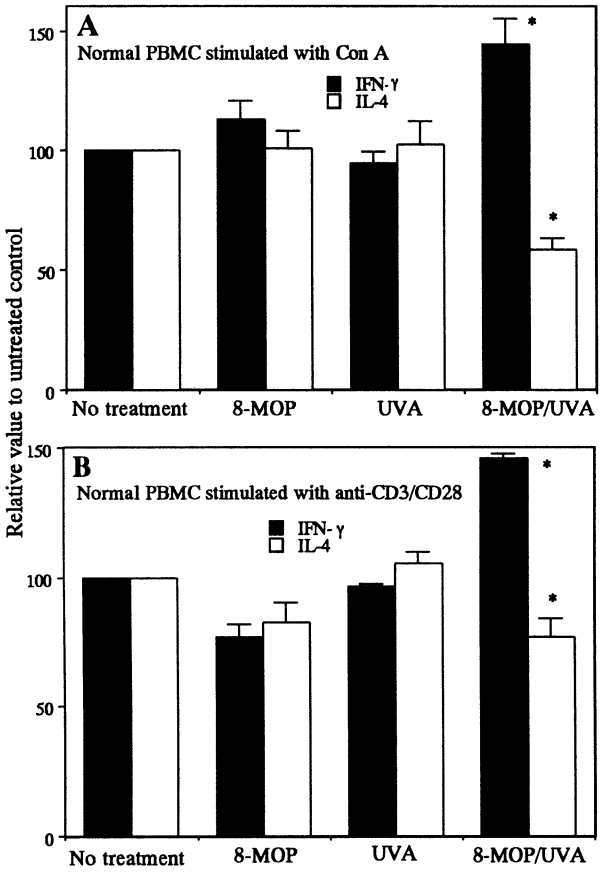

Figure 3. Increased IFN-γ and decreased IL-4 amounts in the culture supernatants from 8-MOP/UVA-treated PBMC. Normal PBMC were treated with 8-MOP (100 ng per ml) and/or UVA (1 J per cm^2) and cultured for 3 d with Con A at 1 μg per ml (*A*) or with anti-CD3 and anti-CD28 MoAb (*B*). The supernatants were assayed for IFN-γ and IL-4 by ELISA. The relative value to untreated control represents (concentration of experimental group)/(concentration of no treatment group) × 100, so that the values of no treatment groups are always 100. Data are expressed as mean ± SD of the relative values in two independent experiments. The mean concentrations of no treatment groups in the two experiments are as follows: IFN-γ of Con A-stimulated PBMC, 109 units per ml; IL-4 of Con A-stimulated PBMC, 31 units per ml; IFN-γ of anti-CD3/CD28-stimulated PBMC, 158 units per ml; and IL-4 of anti-CD3/CD28-stimulated PBMC, 5.6 units per ml. *$p < 0.01$, compared with corresponding 8-MOP or UVA alone group.

supported by the observation that PBMC from another patient with Sézary syndrome, which contained ≈20% Th2 Sézary cells, showed a less marked increase in IFN-γ expression upon phototreatment than those from the patient shown in **Fig 1(*A*)** (data not shown).

When keyhole limpet hemocyanin-specific murine Th1 and Th2 clones were tested, 8-MOP/UVA upmodulated the mRNA expression of IFN-γ and IL-2 in Th1 cells (**Fig 1*D***), but not IL-4 or IL-10 in Th2 cells (**Fig 1*E***). Thus, Th1-skewing modulation by 8-MOP/UVA was found at the mRNA level.

Increased numbers of Th1 cytokine-secreting cells in 8-MOP/UVA-treated PBMC The number of cytokine-secreting T cells in normal PBMC untreated or treated with 8-MOP/UVA was enumerated by ELISPOT assay after 20 h-cultivation with Con A. As shown in **Fig 2(*A*)**, lymphocytes secreting IFN-γ were increased in number in 8-MOP/UVA-treated PBMC compared with untreated cells. The number of IL-10-producing cells was marginally decreased by this treatment, and IL-2-, IL-4-, or IL-5-secreting cells were not significantly changed in number. When examined in PBMC without Con A preincubation, the absolute numbers of cytokine-producing cells were low, but 8-MOP/UVA augmentation of IFN-γ and inhibition of IL-10 were more clearly observed (**Fig 2*B***). Furthermore, the number of IL-2-secreting cells was remarkably increased with 8-MOP/UVA. Thus, 8-MOP/UVA augmented the number of lymphocytes producing Th1 cytokines.

Increased IFN-γ and decreased IL-4 levels in culture supernatants from 8-MOP/UVA-treated lymphocytes Normal PBMC were treated with 8-MOP and/or UVA and cultured for 3 d in the presence of Con A at 1 μg per ml or in anti-CD3 MoAb-coated plates in the presence of anti-CD28 MoAb. IFN-γ was augmented in supernatants by 8-MOP/UVA treatment, whereas IL-4 was reduced in both Con A-stimulated (**Fig 3*A***) and anti-CD3 and anti-CD28 MoAb-stimulated PBMC (**Fig 3*B***). In $CD4^+$ T cell clones from a normal subject and PBMC from a Sézary syndrome patient, that were stimulated with Con A, IFN-γ production was marginally but significantly enhanced (38% and 25%, respectively) by 8-MOP/UVA. Thus, the Th1-skewing effect of 8-MOP/UVA was also observed in the culture supernatants but to a lesser degree than in the ELISPOT asssay.

8-MOP/UVA-treated PBMC rapidly lose the ability to produce increased IFN-γ Treatment with 8-MOP/UVA leads to inhibition of cell proliferation (Nielsen and Linnane, 1983; Kaidbey, 1985). When normal and Sézary syndrome PBMC untreated or

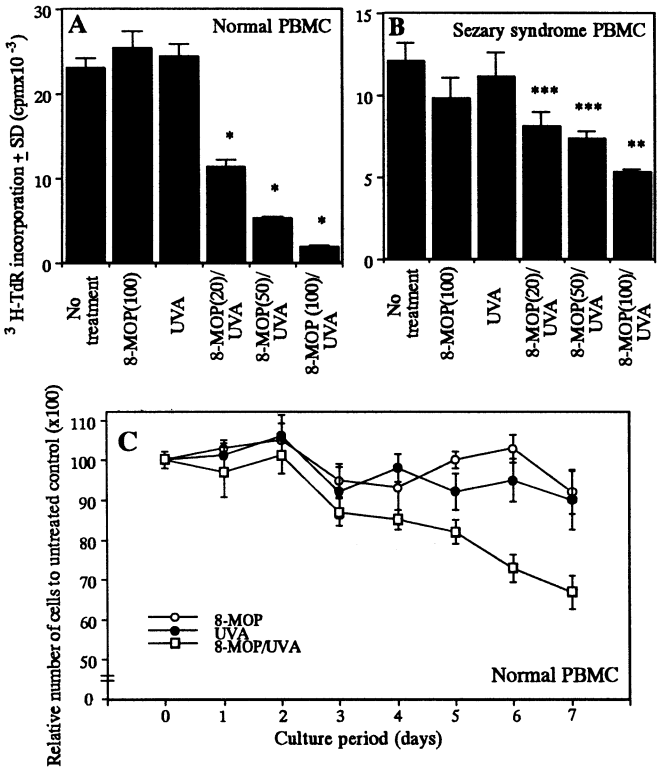

Figure 4. Decreased proliferative responses of 8-MOP/UVA-treated PBMC to Con A and subsequent reduction in their number. (*A*, *B*) Normal subject's and Sézary syndrome PBMC were treated with 8-MOP (20, 50, or 100 ng per ml) and/or UVA (1 J per cm^2) and cultured for 3 d in the presence of Con A (1 μg per ml). DNA synthesis was measured by the incorporation of methyl ^{3}H-thymidine pulsed for the last 16 h. (*C*) Normal PBMC were untreated or treated with 8-MOP (100 ng per ml) and/or UVA (1 J per cm^2) and cultured for 7 d in the presence of Con A (1 μg per ml). On each day, the viable cells numbers were counted and expressed as the relative number of cells to untreated control. *$p < 0.001$, **$p < 0.01$, ***$p < 0.05$, compared with the corresponding no treatment group. Vertical bars represent SD.

treated with 8-MOP and/or UVA were cultured with Con A for 3 d and DNA synthesis was measured, the proliferative responses were inhibited by 8-MOP/UVA in an 8-MOP concentration-dependent manner (**Fig 4*A*, *B***). Following this inhibition of DNA synthesis, 8-MOP/UVA-treated PBMC were decreased in number compared with PBMC treated with 8-MOP or UVA alone (**Fig 4*C***). As the decrease in cell number began to be significant on day 5, there was a time lag between the decline in DNA synthesis and that in cell number.

We examined the time course of IFN-γ production by untreated and treated PBMC. PBMC cultures were started on day 0 in the presence of Con A at 1 μg per ml, and collection of supernatants and replacement by the same volume of Con A (1 μg per ml)-containing medium were performed on days 1, 3, and 5. As shown in **Fig 5**, PBMC did not release substantial amounts of IFN-γ until day 1. During days 1–3, the production of IFN-γ was higher in 8-MOP/UVA-treated cells than in untreated, 8-MOP-treated or UVA-treated cells. On day 3, there was no difference in the number of cells between the variously treated groups (see **Fig 4*C***), suggesting that IFN-γ production was also enhanced by 8-MOP/UVA treatment when related to the number of viable cells. During days 3–5, IFN-γ release was reduced in 8-MOP/UVA-treated cells, compared with the other groups. Thus, the enhanced ability of 8-MOP-phototreated PBMC to produce IFN-γ was transient and declined rapidly in association with a decreased proliferative response.

8-MOP/UVA treatment of T cells augments their capacity to induce keratinocyte CD54 expression To evaluate the

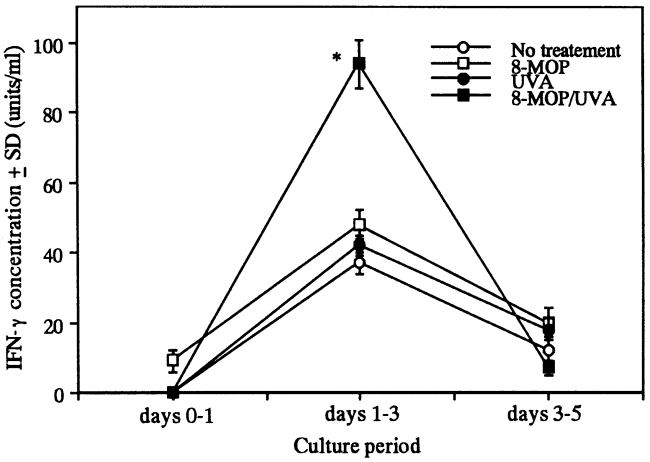

Figure 5. Rapid decline in IFN-γ production by 8-MOP/UVA-treated PBMC. Normal subject's PBMC were untreated or treated with 8-MOP (100 ng per ml) and/or UVA (1 J per cm^2). Cultivation of these cells was started on day 0 with Con A (1 μg per ml) in triplicate. Ninety-five per cent of supernatants were harvested and replaced by the same volume of Con A (1 μg per ml)-containing medium on days 1, 3, and 5. The supernatants were assayed for IFN-γ. *$p < 0.001$, compared with the other groups. Vertical bars represent SD.

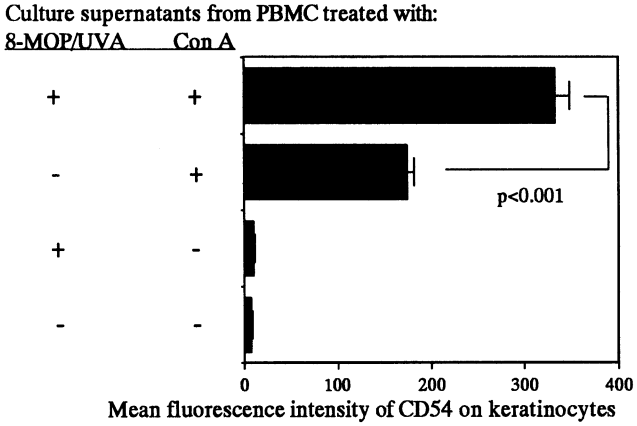

Figure 6. Augmentative effect of 8-MOP/UVA on PBMC culture supernatants to induce keratinocyte CD54 expression. Human keratinocytes were incubated for 2 d with 3 d culture supernatants from normal PBMC that were untreated or treated with 8-MOP (100 ng per ml) and UVA (1 J per cm^2) and incubated with or without Con A. The levels of CD54 expression on keratinocytes were measured by flow cytometry using anti-CD54 MoAb. Error bars represent SD.

Th1-enhancing and Th2-inhibitory effects of 8-MOP/UVA and the overall biologic relevance, 3 d culture supernatants from normal PBMC treated or not with 8-MOP/UVA were added to semiconfluent cultures of normal human keratinocytes. After 2 d cultivation with the supernatants, keratinocytes were tested for their expression of CD54 by flow cytometry. IFN-γ is known to enhance the expression of CD54 on keratinocytes (Griffiths *et al*, 1989), whereas IL-4 modestly reduces the IFN-γ-induced expression of CD54 when added simultaneously with IFN-γ and cultured for 2 d (Viac *et al*, 1994). As shown in **Fig 6**, supernatants from PBMC that were treated with 8-MOP/UVA and stimulated with Con A promoted CD54 expression more markedly than those from non-phototreated, Con-A-stimulated cells. No substantial augmentation of CD54 expression was found with supernatants from either PBMC nonstimulated with Con A. Thus, Th1-skewing cytokine modulation by 8-MOP/UVA was also observed in this assay system.

DISCUSSION

Our study provides a deeper insight into 8-MOP/UVA effects on T cell cytokine production. In mRNA expression, a marked enhancement of Th1 cytokines and a reduction in Th2 cytokines were found in 8-MOP/UVA-treated PBMC. This was more definitely observed in PBMC from a Sézary syndrome patient than those from normal subjects, presumably because Th2-type Sézary cells and Th1-type normal T cells clearly coexisted in the former PBMC. Similarly, when murine Th1 and Th2 clones specific for the same antigen were examined, the levels of mRNA for IFN-γ and IL-2 were elevated in Th1 cells after 8-MOP/UVA, whereas the treatment did not affect or it modestly decreased the expression of IL-4 or IL-10 in Th2 cells. These results are in accordance with the findings of Saed and Fiverson (1994), which show that treatment of HUT-78 cells with 8-MOP/UVA increases IFN-γ mRNA levels.

HUT-78 cells have steady-state levels of mRNA for IFN-γ as well as Th2 cytokines including IL-4, IL-5, and IL-10 (Saed and Fiverson, 1994). Thus, 8-MOP/UVA is considered to enhance the constitutional ability of HUT-78 cells to transcribe IFN-γ. On the other hand, Sézary Th2 cells used in this study expressed mRNA for IL-4, IL-5, IL-10, but not IFN-γ or IL-2, when examined after purification by CD4-positive and CD7-negative selections (Seo *et al*, 1998). Furthermore, the expression of IFN-γ mRNA was not detected by reverse transcription–PCR even after mitogenic stimulation of these purified Sézary cells. Therefore, it is most likely that the 8-MOP/UVA-induced elevation of IFN–γ in Sézary syndrome PBMC is due to increased IFN-γ expression by normal Th1 cells but not to conversion of malignant Th2 cells to produce IFN-γ. In addition, 8-MOP/UVA is thought to downmodulate mRNA expression for Th2 cytokines, IL-4 and IL-10, in Sézary cells. The reduced production of Th2 cytokines may contribute to further augmentation of cytokine production by normal Th1 cells as a result of abrogation of antagonistic effects of Th2 cytokines. Such an antagonistic interaction between Th1 and Th2 cells may exaggerate Th1-skewing cytokine production in Th1/Th2-mixed populations.

Increased Th1 and decreased Th2 cytokine production was also clearly observed at the protein level in 8-MOP/UVA-treated PBMC by ELISPOT assay, which exhibited an increase in the number of IFN-γ-secreting or IL-2-secreting lymphocytes at 20 h after phototreatment. Accordingly, when IFN-γ was quantitated in the culture supernatants, its level was elevated in 8-MOP-phototreated cells, whereas IL-4 was significantly reduced. The treated cells, however, rapidly lost the IFN-γ-producing ability during 3–5 d after phototreatment. The proliferation of 8-MOP-phototreated PBMC was dramatically decreased by day 3 after treatment, and 8-MOP/UVA has been reported to retard cell cycle progression, leading to cell cycle arrest and apoptotic cell death (Johnson *et al*, 1996). In association with this inhibition of proliferation, the enhanced ability of phototreated T cells to synthesize Th1 cytokines may be lowered, accounting for the only transient increase in released IFN-γ. Although transient, 8-MOP/UVA exerted a substantial biologic effect on T cells when released cytokines were bioassayed in keratinocyte CD54 expression, which is promoted by IFN-γ (Griffiths *et al*, 1989) and reduced by IL-4 (Viac *et al*, 1994). Therefore, repeated infusions of 8-MOP/UVA-treated lymphocytes in ECP potentially led to continuous restoration of Th1/Th2 balance in Th2-dominant individuals. In fact, the impaired ability of PBMC to produce Th1 cytokines was normalized in CTCL patients after 1 y of ECP (Di Renzo *et al*, 1997). This notion may also be supported by an older observation that IFN-like activity in sera of patients with psoriasis was increased following PUVA treatment (Diezel *et al*, 1983).

The Th1-skewing action of 8-MOP/UVA is thought to participate in the therapeutic mechanisms of 8-MOP photochemotherapy. Tumor-specific $CD8^+$ cytotoxic cells are proved to exist in PBMC of Sézary syndrome (Berger *et al*, 1996; Seo *et al*, 1998). On one hand, 8-MOP/UVA promotes the expression of major histocompatibility complex class I molecules and cell-adhesion molecules on the surface of tumor cells (Schmitt *et al*, 1995; Gasparro *et al*, 1997), which potentially enhances the action of cytotoxic T cells. On the other hand, the tumoricidal function of $CD8^+$ cells is profoundly activated by Th1 cytokines, IL-2 and IFN-γ (Seo *et al*, 1998) and inhibited by Th2 cytokines (Salvucci *et al*, 1996). Thus, Th1-preponderant modulation by 8-MOP/UVA may lead to activation of cytotoxic T cells. 8-MOP/UVA is considered to exert beneficial effects on CTCL as a cytokine modifier, in addition to an antigen enhancer. The relative Th2 cytokine-inhibitory action also may be involved in the therapeutic mechanism of ECP for atopic dermatitis (Richter *et al*, 1998). It is suggested that ECP at least partly shares the therapeutic action with IFN-γ administration, which is used in the treatment of Th2-type CTCL (Yagi *et al*, 1996a) and Th2-mediated cutaneous disorders (Fushimi *et al*, 1996; Tokura *et al*, 1997; Stevens *et al*, 1998).

Our study indicates that this photoactivatable agent is not merely cytotoxic but can induce some activational events. The molecular mechanisms of this activation remain to be elucidated, but several possibilities can be proposed. Like ionizing radiation (Valerie *et al*, 1995), transcription factors such as nuclear factor-κB might be activated by psoralen photodamage, leading to an upregulation of cytokine genes (Gasparro, 1996). Mitogen-activated protein kinase(s) is involved in the signaling pathway for the production of IFN-γ (Rincon *et al*, 1998). Prolonged activation of mitogen-activated protein kinase by low doses of radiation (Carter *et al*, 1998) raises the possibility that 8-MOP/UVA promotes IFN-γ-producing signaling pathway by activating mitogen-activated protein kinase(s). More specifically to 8-MOP phototreatment, in cells transfected with chloramphenicol acetyltransferase gene under the control of the human immunodeficiency virus-1 promoter, 8-MOP/UVA was proved to activate the promoter of this virus (Zmudzka *et al*, 1993). Finally, p53 assists in the recognition of DNA damage and causes cell cycle arrest at the G_1 phase until the damage has been repaired (Chen *et al*, 1998). There is a difference in DNA content between cells treated with 8-MOP/UVA and those with UVA alone (Hornicek *et al*, 1989). 8-MOP/UVA may thus induce G_1 arrest and apoptosis (Johnson *et al*, 1996). As the late G_1 phase is the most cytokine-productive period in Th1 cells (Dröge, 1986), cytokines may be produced at high levels when p53 is induced by 8-MOP/UVA (Gasparro, 1996). Signal transduction pathways of Th1 and Th2 cells are different from each other in terms of participation of protein tyrosine kinases (Tamura *et al*, 1995) and mitogen-activated protein kinases (Rincon *et al*, 1998). Future studies are required for clarifying the mechanisms underlying Th1-preponderant activational events induced by 8-MOP and UVA.

We thank Ms. Keiko Sugaya for technical assistance. This work was supported in part by a grant from the Ministry of Education, Science, Sports and Culture of Japan.

REFERENCES

Berger CL, Wang N, Christensen I, Longley J, Heald P, Edelson RL: The immune response to class I-associated tumor-specific cutaneous T-cell lymphoma antigens. *J Invest Dermatol* 107:392–397, 1996

Besnier DP, Chabannes D, Mahe B, *et al*: Treatment of graft-versus-host disease by extracorporeal photochemotherapy: a pilot study. *Transplantation* 64:49–54, 1997

Boehncke WH, Konig K, Kaufmann R, Schefford W, Prummer O, Sterry W: Photodynamic therapy in psoriasis: suppression of cytokine production *in vitro* and recording of fluorescence modification during treatment *in vivo*. *Arch Dermatol Res* 286:300–303, 1994

Carter S, Auer KL, Reardon DB, *et al*: Inhibition of the mitogen activated protein (MAP) kinase cascade potentiates cell killing by low dose ionizing radiation in A431 human squamous carcinoma cells. *Oncogene* 16:2787–2796, 1998

Chen QM, Bartholomew JC, Campisi J, Acosta M, Reagan JD, Ames BN: Molecular analysis of H_2O_2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. *Biochem J* 332:43–50, 1998

Cheng T-Y, Shen F-W, Lin R-H: The immunological effect of 8-methoxypsoralen and UVA treatment on murine T-cell leukemia. *Photochem Photobiol* 64:594–600, 1996

Chomcznski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987

Czerkinsky C, Andersson G, Ekre H-P, Nilsson L-Å, Klareskog L, Ouchterlony Ö: Reverse ELISPOT assay for clonal analysis of cytokine production. I. Enumeration of gamma interferon-secreting cells. *J Immunol Methods* 110:29–36, 1988

Di Renzo M, Rubegni P, de Aloe G, *et al*: Extracorporeal photochemotherapy restores Th1/Th2 imbalance in patients with early stage cutaneous T-cell lymphoma. *Immunology* 92:99–103, 1997

Diezel W, Waschke SR, Sonnichsen N: Detection of interferon in the sera of patients with psoriasis, and its enhancement by PUVA treatment. *Br J Dermatol* 109:549–552, 1983

Dröge W: Protein kinase C in T-cell regulation. *Immunol Today* 7:340–343, 1986

Edelson R, Berger C, Gasparro F, *et al*: Treatment of cutaneous T-cell lymphoma with extracorporeal photochemotherapy. *N Engl J Med* 316:297–303, 1987

Fushimi M, Tokura Y, Sachi Y, *et al*: Eosinophilic pustular folliculitis effectively treated with recombinant interferon-γ: suppression of mRNA expression of interleukin 5 in peripheral blood mononuclear cells. *Br J Dermatol* 134:766–772, 1996

Gasparro FP: Psoralen photobiology: recent advances. *Photochem Photobiol* 63:553–557, 1996

Gasparro FP, Battista J, Song J, Edelson RL: Rapid and sensitive analysis of 8-methoxypsoralen in plasma. *J Invest Dermatol* 90:234–236, 1988

Gasparro FP, Felli A, Schmitt IM: Psoralen photobiology: the relationship between DNA damage, chromatin structure, transcription, and immunogenic effects. *Recent Results Cancer Res* 143:101–127, 1997

Griffiths CEM, Voorhees JJ, Nockoloff BJ: Gamma interferon induces different keratinocyte expression of HLA-DR, DQ, and intercellular adhesion molecule-1 (ICAM-1) antigens. *Br J Dermatol* 120:1–8, 1989

Heald P, Edelson R: The immunobiology of cutaneous T-cell lymphoma. *J Natl Cancer Inst* 83:400–404, 1991

Hornicek FJ, Malinin TI, Gratzner H, Malinin GI: Cytometric analysis of the proliferative capacity of HUT 102 lymphoblasts exposed to long-wave UV light and psoralen. *J Invest Dermatol* 93:96–99, 1989

Johnson R, Staiano-Coico L, Austin L, Cardinale I, Nabeya-Tsukifuji R, Krueger JG: PUVA treatment selectively induces a cell cycle block and subsequent apoptosis in human T-lymphocytes. *Photochem Photobiol* 63:566–571, 1996

Kabilan L, Andersson G, Lolli F, Ekre H-P, Olsson T, Troye-Blomberg M: Detection of intracellular expression and secretion of IFN-γ at the single cell level after activation of human T cells with tetanus toxoid *in vitro*. *Eur J Immunol* 20:1085–1089, 1990

Kaidbey KH: An action spectrum for 8-methoxypsoralen-sensitized inhibition of DNA synthesis *in vivo*. *J Invest Dermatol* 85:98–101, 1985

Kozenitzky L, David M, Sredai B, Albeck M, Shohat B: Immunomodulatory effects of AS101 on interleukin-2 production and T-lymphocyte function of lymphocytes treated with psoralens and ultraviolet A. *Photodermatol Photoimmunol Photomed* 9:24–28, 1992

Mosmann TR, Coffman RL: TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 7:145–173, 1989

Nakayama T, Kubo RT, Kubo M, Fujisawa I, Kishimoto H, Asano, Y, Tada T: Epitopes associated with the MHC restriction site of T cells. IV. I-J epitopes on MHC-restricted cloned T cells. *Eur J Immunol* 18:761–765, 1988

Nakayama T, Kubo RT, Kishimoto H, Asano Y, Tada T: Biochemical identification of I-J as a novel dimeric surface molecule on mouse helper and suppressor T cell clones. *Int. Immunol* 1:50–58, 1989

Neuner P, Charvat B, Knobler R, Kirnbauer R, Schwarz A, Luger TA, Schwarz T: Cytokine release by peripheral blood mononuclear cells is affected by 8-methoxypsoralen plus UVA. *Photochem Photobiol* 59:182–188, 1994

Nielsen PE, Linnane WP: Differentiated inhibition of DNA, RNA and protein synthesis in L1210 cells by 8-methoxypsoralen. *Biochem Biophys Res Commun* 112:965–971, 1983

Pathak MA, Fitzpatrick TB: The evolution of photochemotherapy with psoralens and UVA (PUVA): 200 BC to 1992 AD. *J Photochem Photobiol B Biol* 14:3–22, 1992

Richter HI, Billmann-Eberwein C, Grewe M, Stege H, Berneburg M, Ruzicka T, Krutmann J: Successful monotherapy of severe and intractable atopic dermatitis by photopheresis. *J Am Acad Dermatol* 38:585–588, 1998

Rincon M, Enslen H, Raingeaud J, *et al*: Interferon-γ expression by Th1 effector T cells mediated by the p38 MAP kinase signaling pathway. *EMBO J* 17:2817–2829, 1998

Rook AH, Jegasothy BV, Heald P, *et al*: Extracorporeal photochemotherapy for drug-resistant pemphigus vulgaris. *Ann Intern Med* 112:303–305, 1990

Rook AH, Freundlich B, Jegasothy BV, *et al*: Treatment of systemic sclerosis with extracorporeal photochemotherapy: results of a multicenter trial. *Arch Dermatol* 128:337–346, 1992

Rook AH, Kubin M, Cassin M, *et al*: IL-12 reverses cytokine and immune abnormalities in Sézary syndrome. *J Immunol* 154:1491–1498, 1995

Saed GM, Fiverson DP: Quantification of interferon gamma mRNA levels in psoralen/UVA-treated HUT-78 cells by competitive PCR. *Biochem Biophys Res Commun* 203:935–942, 1994

Saed G, Fivenson DP, Naidu Y, Nickoloff BJ: Mycosis fungoides exhibits a Th1-type cell-mediated cytokine profile whereas Sézary syndrome expresses a Th2-type profile. *J Invest Dermatol* 103:29–33, 1994

Salvucci O, Chouaib FM, Moreau JL, Theze J, Chehimi J, Chouaib S: Differential regulation of interleukin-12 and interleukin-15-induced natural killer cell activation by interleukin-4. *Eur J Immunol* 26:2736–2741, 1996

Schmitt IM, Moor AC, Patrignelli R, *et al*: Increased surface expression of class I MHC molecules on immunogenic cells derived from the xenogenization of P815 mastocytoma cells with 8-methoxypsoralen and long-wavelength ultraviolet radiation. *Tissue Antigens* 46:45–49, 1995

Seo N, Tokura Y, Matsumoto K, Furukawa F, Takigawa M: Tumor-specific cytotoxic T-lymphocyte activity in Th2 type–Sézary syndrome: its enhancement by interferon-γ and interleukin-12 and fluctuations in association with the disease activity. *Clin Exp Immunol* 112:403–409, 1998

Stevens SR, Hanifin JM, Hamilton T, Tofte SJ, Cooper KD: Long-term effectiveness and safety of recombinant human interferon-γ therapy for atopic dermatitis despite unchanged serum IgE levels. *Arch Dermatol* 134:799–804, 1998

Tamura T, Nakano H, Nagase H, *et al*: Early activation signal transduction pathways of Th1 and Th2 cell clones stimulated with anti-CD3. Roles of protein tyrosine kinases in the signal for IL-2 and IL-4 production. *J Immunol* 155:4692–4701, 1995

Thompson CB, Lindsten T, Ledbetter JA, *et al*: CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc Natl Acad Sci USA* 86:1333–1337, 1989

Tokura Y: Modulation of cytokine production by 8-methoxypsoralen and UVA. *J Dermatol Sci* 19:114–122, 1999

Tokura Y, Yagi J, Edelson RL, Gasparro FP: Inhibitory effect of 8-methoxypsoralen plus ultraviolet-A on interleukin-1 production by murine keratinocytes. *Photochem Photobiol* 53:517–523, 1991a

Tokura Y, Edelson RL, Gasparro FP: Formation and removal of 8-MOP-DNA photoadducts in keratinocytes: effects of calcium concentration and retinoids. *J Invest Dermatol* 96:642–649, 1991b

Tokura Y, Edelson RL, Gasparro FP: Modulation of 8-methoxypsoralen-DNA photoadduct formation by cell differentiation, mitogenic stimulation and phorbol ester exposure in murine T lymphocytes. *Photochem Photobiol* 58:822–826, 1993

Tokura Y, Yagi H, Hanaoka K, *et al*: Subacute and chronic prurigo effectively treated with recombinant interferon-γ: implications for participation of Th2 cells in the pathogenesis of prurigo. *Acta Derm Venereol (Stockh)* 77:231–234, 1997

Urano K, Matsuyama T, Urano R, Matsuo I, Habu S: PUVA suppresses the expression of cell adhesion molecules of lymphocytes. *Exp Dermatol* 4:36–41, 1995

Valerie K, Laster WS, Kirkham JC, Kuemmerle NB: Ionizing radiation activates nuclear factor kappa B but fails to produce an increase in human immunodeficiency virus gene expression in stably transfected human cells. *Biochemistry* 34:15768–15776, 1995

Viac J, Gueniche A, Gatto H, Lizard G, Schmitt D: Interleukin-4 and interferon-γ interactions in the induction of intercellular adhesion molecule-1 and major histocompatibility complex class II antigen expression of normal human keratinocytes. *Exp Dermatol* 3:72–77, 1994

Vowels BR, Cassin M, Vonderheid EC, Rook AH: Aberrant cytokine production by Sézary syndrome patients: cytokine secretion pattern resembles murine Th2 cells. *J Invest Dermatol* 99:90–94, 1992

Wakita H, Tokura Y, Furukawa F, Takigawa M: Staphylococcal enterotoxin B upregulates expression of ICAM-1 molecules on IFN-γ-treated keratinocytes and keratinocyte cell lines. *J Invest Dermatol* 105:536–542, 1995

Wood GS, Hong SR, Sasaki DT, Abel EA, Hoppe RT, Warnke RA, Norhenn VB: Leu-8/CD7 antigen expression by $CD3^+$ T cells: comparative analysis of skin and blood in mycosis fungoides/Sézary syndrome relative to normal values. *J Am Acad Dermatol* 22:602–607, 1990

Yagi H, Tokura Y, Furukawa F, Takigawa M: Th2 cytokine mRNA expression in primary cutaneous CD30-positive lymphoproliferative disorders: successful treatment with recombinant interferon-γ. *J Invest Dermatol* 107:827–832, 1996a

Yagi H, Tokura Y, Furukawa F, Takigawa M: CD7–positive Sézary syndrome with a Th1 cytokine profile. *J Am Acad Dermatol* 34:368–374, 1996b

Yagi H, Tokura Y, Wakita H, Furukawa F, Takigawa M: TCRVβ 7^+ Th2 cells mediate UVB-induced suppression of murine contact photosensitivity by releasing IL-10. *J Immunol* 156:1824–1831, 1996c

Zmudzka BZ, Strickland AG, Miller SA, Valerie K, Dall'Acqua F, Beer JZ: Activation of the human immunodeficiency virus promoter by UVA radiation in combination with psoralens or angelicins. *Photochem Photobiol* 58:226–232, 1993