Treatment of Peripheral Blood Mononuclear Cells with 8-Methoxypsoralen plus Ultraviolet A Radiation Induces a Shift in Cytokine Expression from a Th1 to a Th2 Response

Gabriele Klosner, Franz Trautinger, Robert Knobler, and Peter Neuner
Department of Dermatology, Division of Special and Environmental Dermatology, University of Vienna, Austria

Treatment with 8-methoxypsoralen plus ultraviolet A radiation and extracorporeal photochemotherapy (photopheresis) are widely used for the treatment of psoriasis and other inflammatory skin diseases, graft-*versus*-host disease, and mycosis fungoides. As the ratio of Th1 and Th2 cells appears to be critical for pathogenesis and progression of these disorders the effect of psoralen plus ultraviolet A on Th1 and Th2 cytokine production by CD4+ lymphocytes was investigated. Human peripheral blood lymphocytes were incubated in the presence of anti-CD3, rh-IL2, and rh-IL4 for 48 h. After subsequent stimulation with rh-IL2 and rh-IL4 for 72 h cells were treated with 8-methoxypsoralen (100, 500, 1000 ng per ml) plus ultraviolet A (2 J per cm^2) and incubated for a further period of 5 h in the presence of ionomycine, phorbol-12-myristate acetate and monensin. Fluorescence-activated cell sorter analysis revealed a significant reduction of interleukin-2- and interferon-γ-producing CD4+ cells upon psoralen plus ultraviolet A treatment depending on the concentration of 8-methoxypsoralen. In contrast, interleukin-4-producing CD4+ cells were increased, indicating a shift from Th1 to a Th2 cell cytokine profile upon psoralen plus ultraviolet A treatment. These results indicate that 8-methoxypsoralen photochemotherapy of lymphocytes is able to modulate their Th1/Th2 distribution. Inhibition of Th1 cytokine expression by psoralen plus ultraviolet A might help to explain its beneficial effects in the treatment of Th1 dominated skin diseases. ***Key words: flow cytometry/interferon-γ/interleukin-2/interleukin-4. J Invest Dermatol 116:459–462, 2001***

The initial event in photochemotherapy is the activation of a photosensitizer, usually 8-methoxypsoralen (8-MOP), by ultraviolet A radiation (UVA). Intracellularly the photoactivated psoralen molecules interact with nucleic acids, proteins, and lipids. Through these interactions not yet defined signaling events are induced that mediate the antiproliferative and immunomodulatory effects that are associated with the desired therapeutic response. For the treatment of psoriasis, mycosis fungoides, and a variety of other skin diseases psoralens are applied orally or topically with subsequent UVA exposure of the sensitized skin (PUVA). In photopheresis (extracorporeal photochemotherapy) photochemotherapy is applied extracorporeally to peripheral blood leukocytes, which are subsequently reinfused. Photopheresis has become standard treatment for Sezary syndrome and has shown efficacy in the treatment of systemic sclerosis, autoimmune diseases, organ rejection, and graft-*versus*-host disease (Knobler and Trautinger, 1997).

Although a major therapeutic mechanism of 8-MOP/UVA might be its antiproliferative effect based on DNA crosslinking, immunomodulatory effects have been observed as well. The type of immune response that predominates in a certain state of disease is influenced by functionally different subsets of CD4+ lymphocytes designated Th1 and Th2 (Murray, 1998). Th1 cells secrete interleukin 2 (IL-2), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α), and support macrophage activation, expansion of cytotoxic T lymphocytes, and class-switching to complement activating IgG isotypes. Th2 cells secrete mainly IL-4 and IL-5 and provide efficient help for B cells to produce antibody and for class-switching to neutralizing IgG and IgE.

There is evidence that photochemotherapy modulates the cytokine patterns in the target tissues and by this can influence the balance of Th1/Th2 subsets. Most of these data are obtained from clinical studies, however, and conflicting results have been reported, depending on the treatment modality and the disease under investigation. In mycosis fungoides, which in most cases is a malignancy of skin homing CD4+ lymphocytes with a Th2 phenotype, a reversal to a Th1 predominance has been described during photopheresis (Di Renzo *et al*, 1997). This result has recently been confirmed by an *in vitro* study using lymphocytes from a patient with Sezary syndrome as well as from healthy volunteers (Tokura *et al*, 1999). These studies were done either on malignant Th2-type lymphocytes or on normal-blood-derived lymphocytes from healthy donors, however. In contrast to the Th2-mediated diseases Sezary syndrome and mycosis fungoides, other diseases that respond to photochemotherapy are characterized by a Th1 predominance (e.g., acute graft-*versus*-host disease) and therapy should aim at restoring the protective function of Th2 cells

Manuscript received January 12, 2000; revised December 8, 2000; accepted for publication December 18, 2000.

Reprint requests to: Dr. Franz Trautinger, Department of Dermatology, Division of Special and Environmental Dermatology, University of Vienna, Währinger Gürtel 18–20, A-1090 Vienna, Austria. Email: franz.trautinger@akh-wien.ac.at

Abbreviations: PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; PI, propidium iodide.

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(Liblau *et al*, 1995; Ferrara *et al*, 1996). The seeming contradiction in the response of both Th1- and Th2-mediated diseases to photochemotherapy might be explained by different immunologic responses to photochemotherapy depending on the initial state of activation. As *in vitro* studies on the effect of photochemotherapy on activated lymphocytes have not been done, we established a model system where mononuclear cells from healthy donors were activated with anti-CD3, IL-2, and IL-4 prior to photochemotherapy *in vitro*. Th1/Th2 distribution was determined by fluorescence-activated cell sorter (FACS) analysis of intracellular cytokines. In contrast to the earlier studies analyzing cell extracts and culture supernatants this method for the first time allowed for the direct measurement of Th1/Th2 counts within a mixed population and their variation under the influence of photochemotherapy.

MATERIALS AND METHODS

Cells Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy human volunteers by Ficoll density gradient centrifugation (Pharmacia, Uppsala, Sweden). Monocytes were removed by adherence onto plastic Petri dishes under normal culture conditions (a humidified 5% CO_2 atmosphere, 37°C) for 2 h.

Cell culture and prestimulation Isolated cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U per ml streptomycin and penicillin, and 2 mM glutamine (all Bio Whittaker, Vervier, Belgium) at a density of 2×10^6 cells per ml. They were activated with 25 μg per ml anti-CD3 antibody (Ancell, Bayport), 10 ng per ml rh-IL-2, and 10 ng per ml rh-IL-4 (R&D Systems, Abingdon, U.K.) for 2 d. Subsequently cells were resuspended in fresh medium supplemented with rh-IL-2 and rh-IL-4 as above for another 3 d prior to photochemotherapy.

8-MOP/UVA treatment In order to avoid adherence of the cells onto plastic surfaces after UVA irradiation, Petri dishes and 24-well plates were coated with a layer of 0.8% agarose (Bio Rad, Hercules, CA) in phosphate-buffered saline (PBS) prior to 8-MOP/UVA treatment. Cells were washed and aliquoted at 2×10^7 in 20 ml of PBS (Bio Whittaker) and transferred into large size, coated Petri dishes. 8-MOP (Gerot, Vienna, Austria) was added to the cultures at concentrations of 100, 500, and 1000 ng per ml and they were incubated at room temperature in the dark for 60 min. A metal halide lamp (Mutzhas Supersun 5000, Munich, Germany) filtered for the emission of UVA (340–390 nm) was used for irradiation. Fluence rate was monitored using an IL700 radiometer (International Light, Newburyport). The applied dose was 2 J per cm^2. Temperature during irradiation was kept constant at 25°C; irradiation time was 50 s.

Sham-treated cultures (without 8-MOP and UVA treatment), 8-MOP-treated cultures (without UVA treatment), and UVA-treated cultures (without 8-MOP treatment) served as controls.

Stimulation Cells (treated and nontreated, as described above) were further divided into agarose coated 24-well tissue culture plates (Falcon, Becton Dickinson, San Jose, CA) at a concentration of 2×10^6 per ml and either stimulatd with 10 ng per ml phorbol-12-myristate acetate (PMA) and 1 μM ionomycin (both Sigma, St. Louis, MO) or left unstimulated. Monensin (Golgi-Stop, Pharmingen, San Diego, CA) was added to all cultures for the inhibition of cytokine secretion according to the manufacturer's instructions. Cells were harvested 5 h after restimulation, previously found to be the optimal time point for the analysis of this combination of cytokines. This also takes into consideration that prolonged incubation in the presence of the transport inhibitor monensin (used for the cytoplasmic retention of otherwise secreted cytokines) causes a major loss of CD4 surface expression, thus inhibiting the discrimination of subpopulations in multiparameter analysis.

Cell fixation and permeabilization Before harvest, cells were incubated for 5 min at 37°C with 20 μg per ml DNAse (Worthington Biochemical, NJ) to allow cell disaggregation. Cell viability was checked flow cytometrically by propidium iodide (PI) exclusion stain. Briefly, PI (Sigma) was added to aliquots of the cultures in PBS at a concentration of 1 μg per ml for 5 min at room temperature. Single-color (FL2) analysis was performed within the next 15 min and recorded as logarithmic amplified data. Statistical analysis revealed viabilities greater than 94% for all experiments.

Cells were then transferred from the culture plates into 15 ml Falcon tubes (Becton Dickinson) and washed twice in PBS. Cells were resuspended in a small amount of PBS to avoid aggregation and freshly prepared, ice-cold, 4% paraformaldehyde (Sigma) was added under constant agitation and left on cells for 10 min. After two further washes, cells were permeabilized in PBS containing 0.1% saponin (Fluka, Buchs, Switzerland), 1% bovine serum albumin (Sigma), and 0.1% HEPES (Sigma) for 30 min at room temperature. In order to keep cell membranes permeable (saponin acts in a reversible manner), all steps of the following staining procedure were performed in saponin-containing buffer.

Antibodes Commercially available antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP), or unlabeled, were used as recommended by the manufacturer: mouse antihuman IL-4, rat antihuman IL-2R, mouse antihuman IFN-γ (all Pharmingen); mouse antihuman CD4, mouse-Ig fluorescence controls (Becton Dickinson).

Multicolor staining for cell surface antigens and intracellular cytokines Cells from the different incubations were divided into FACS staining vials (Falcon, Becton Dickinson) at 1×10^6 in 100 μl of permeabilization buffer. Directly labeled antibodies for surface and intracellular staining were added simultaneously at the appropriate concentration and incubated at room temperature for 30 min. After two washing steps in permeabilization buffer, cells were resuspended in 400 μl of PBS for FACS analysis.

FACS analysis Samples were analyzed on a FACS-Calibur (Becton Dickinson) equipped with a 15 mW, 488 nm argon ion laser. FITC, PE, and PerCP fluorescence was measured through 530/30 and 585/42 bandpass and 650 longpass filters, respectively. Fluorescence measurements were made using a logarithmic amplifier.

Samples were gated on forward and side scatter to exclude debris and aggregates. In the case of triple-color labeling, CD4-positive cells were subsequently gated on the FL1 (FITC) positive fluorescent signal and correlated with intracellular cytokine expression on an FL2 (PE)/FL3 (PerCP) two-parameter display. For two-color analysis, CD4-positive cells were correlated with intracellular cytokine expression patterns directly on an FL3/FL2 dot blot.

Cell viability was determined through uptake of PI as described elsewhere (Schindl *et al*, 1998).

Ten thousand cells were collected for each sample, and data analysis was performed using Cell Quest software (Becton Dickinson).

Statistics The paired *t* test was used to calculate statistical significance for the comparison of 8-MOP/UVA to control in PBMC from multiple donors.

RESULTS AND DISCUSSION

Viability of PBMC after 8-MOP/UVA is independent of psoralen concentrations Viability of prestimulated PBMC was determined by dye exclusion and FACS analysis between 0 h and 48 h after exposure to 8-MOP/UVA (**Fig 1**). Neither 8-MOP alone nor UVA alone had an effect on cell survival. In accordance with what has been described earlier for lymphocytes after photopheresis, a time-dependent increase in the percentage of dye-uptaking cells was observed after 8-MOP/UVA treatment (Edelson *et al*, 1987). This effect was independent of 8-MOP concentration at a range between 100 ng per ml and 1000 ng per ml, indicating that, at least in our system, photochemotherapy-induced cell death and immunomodulation (see below) are not necessarily linked and that it is possible to increase 8-MOP levels without a concomitant increase in cytotoxicity.

At 5 h after UVA exposure, the time point of cytokine determination, cytotoxicity was the same as in the control samples, namely 4%–5%.

8-MOP/UVA leads to an increase in IL-4-producing Th2 cells and a concomitant decrease of IL-2- and IFN-γ-producing Th1 cells PBMC were pretreated as described in *Materials and Methods* and exposed to increasing doses of 8-MOP and UVA (2 J per cm^2). After blocking of cytokine secretion with monensin the percentage of IL-4-producing $CD4^+$ T cells was determined by FACS. **Figure 2(*a*)** shows the results of two independent experiments performed with PBMC from different donors. UVA alone had no effect whereas 8-MOP/UVA induced an S-shaped increase in the number of $IL\text{-}4^+/CD4^+$ cells that was

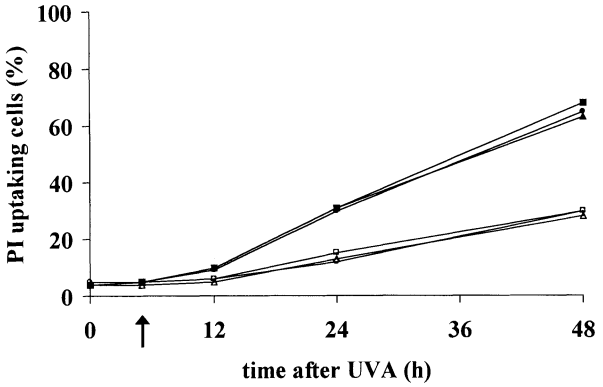


Figure 1. Viability of PBMC after 8-MOP/UVA is independent of psoralen concentrations. PBMC were treated as described in *Materials and Methods*. Viability was determined by FACS analysis of PI uptake. Open symbols denote controls (*triangles*, untreated; *squares*, UVA 2 J per cm^2; *circles*, 8-MOP, 1000 ng per ml); filled symbols denote cells treated with 8-MOP/UVA at various psoralen concentrations (*triangles*, 100 ng per ml; *squares*, 500 ng per ml; *circles*, 1000 ng per ml). At the time of cytokine measurements (5 h after UVA exposure, indicated by an arrow) viability is at the control level.

dependent on the 8-MOP concentration. At 1000 ng per ml a maximal induction of 338% and 240% of control levels was observed.

The percentage of IL-2^+/$CD4^+$ and IFN-γ^+/$CD4^+$ cells was determined after stimulation of the 8-MOP/UVA-treated cells with PMA and ionomycin in the presence of monensin. In two independent experiments we observed a decrease in the number of IL-2^+ as well as IFN-γ^+ cells that was inversely related to the 8-MOP concentration (**Fig 2*b*, *c***). At 1000 ng per ml 8-MOP the number of IL-2-producing Th cells was reduced to 5% and 15% of control levels. IFN-γ-producing cells were reduced to 19% and 62% of controls.

8-MOP alone (see below, **Fig 3**) had no effect on the production of IL-4, IL-2 and IFN-γ by $CD4^+$ T-cells.

8-MOP/UVA-induced Th1/Th2 skewing occurs at therapeutically relevant 8-MOP concentrations As described above, the effect of 8-MOP/UVA on the distribution of Th1/Th2-cytokine-producing $CD4^+$ T cells is more pronounced at 8-MOP levels that are usually not achieved in the clinical setting. Serum levels in photochemotherapy are usually in the range of 50 ng per ml to 200 ng per ml. To investigate the influence of therapeutically relevant 8-MOP concentrations on Th1/Th2 distribution, PBMC from six healthy volunteers were exposed to UVA (2 J per cm^2) and 8-MOP (100 ng per ml). Each individual sample served as its own control. We could demonstrate a small but significant increase of IL-4^+/$CD4^+$ cells from 4.4% ± 2.61% to 5.9% ± 3.0% (mean ± SD, **Fig 3*a***). Neither 8-MOP nor UVA alone had a significant effect. For IL-2^+/$CD4^+$ cells we observed a significant reduction from 11.0% ± 4.8% to 9.02% ± 4.2% and to 9.2% ± 4.4% (mean ± SD) for UVA alone and 8-MOP/UVA, respectively (**Fig 3*b***). 8-MOP alone had no effect. Although with our relatively small sample of donors the downregulation of IL-2 by UVA alone might be a chance finding a specific effect of UVA on IL-2-producing $CD4^+$ T cells cannot be ruled out, particularly as effects of low doses of UVA on lymphocytes have been described earlier (Yoo *et al*, 1996).

In parallel with IL-2 also IFN-γ^+/$CD4^+$ T cells were significantly reduced from 7.2% ± 3.7% to 5.3% ± 3.0% (**Fig 3*c***). UVA and 8-MOP alone had no effect on IFN-γ production.

The observed shift towards a Th2 expression pattern is weak at 100 ng per ml of 8-MOP, the currently recommended therapeutic serum level in PUVA and photopheresis. Thus, at least in clinical conditions where the enhancement of Th2 responses might be therapeutically desirable, higher 8-MOP levels might improve the

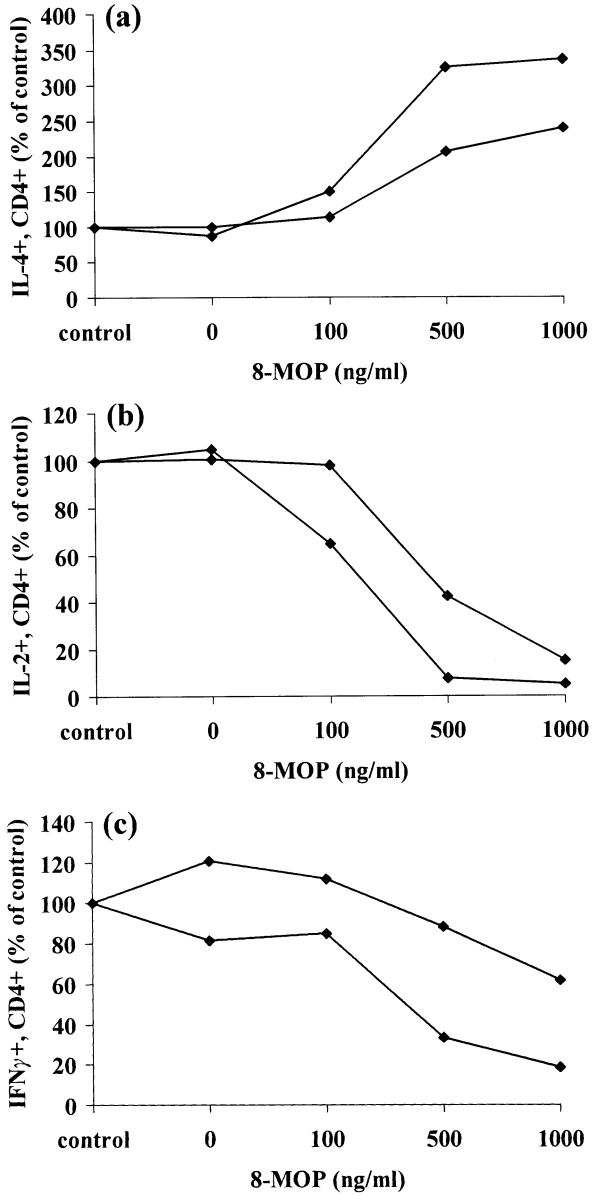


Figure 2. 8-MOP/UVA leads to an increase in IL-4-producing Th2 cells and a concomitant decrease in IL-2- and IFN-γ-producing Th1 cells. PBMC of two donors were treated as indicated in *Materials and Methods*. Cytokines were determined 5 h after exposure to increasing doses of 8-MOP and 2 J per cm^2 UVA in $CD4^+$ cells by double staining immunofluorescence and FACS analysis. Each line represents data from one single donor. (*a*) IL-4; (*b*) IL-2; (*c*) IFN-γ.

efficacy of photochemotherapy. This might be particularly true for photopheresis where the *ex vivo* exposure conditions are similar to our *in vitro* model. In photopheresis 8-MOP can be applied extracorporeally to the treatment bag without dose-limiting side-effects, making this treatment variant particularly suitable for dose escalation studies (Knobler *et al*, 1993).

Inhibition of IL-2 by photochemotherapy has been described earlier in murine spleen cells and in lymphocytes from psoriatic patients (Okamoto *et al*, 1985, 1987; Vonderheid *et al*, 1990). In contrast, the production of IL-2 and IFN-γ was transiently increased after *in vitro* PUVA of PBMC from healthy donors (Tokura *et al*, 1999). In this study a concomitant decrease of IL-4 and IL-10 was observed. This study provides only indirect evidence for the effect of photochemotherapy on Th1/Th2 distribution, however, as the methods used do not allow for the phenotypical characterization of the cytokine-secreting cells. Similarly, a reversal of the Th2 to a Th1 cytokine expression pattern was observed in

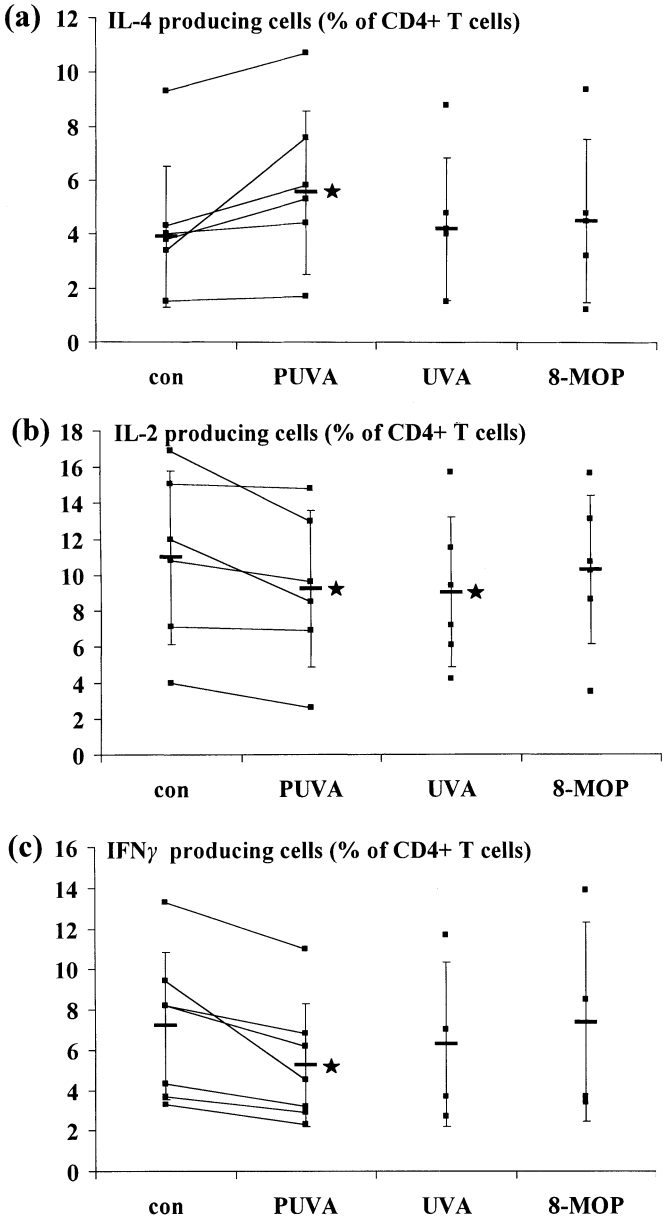


Figure 3. 8-MOP/UVA-induced Th1/Th2 skewing occurs at therapeutically relevant 8-MOP concentrations. PBMC from six healthy donors were treated as indicated in *Materials and Methods* prior to exposure to 8-MOP (100 ng per ml) and UVA (2 J per cm^2). Cytokine-expressing CD4+ cells were determined by FACS analysis 5 h after 8-MOP/UVA. Bars represent mean ± SD; asterisks indicate statistical significance compared with untreated controls at a significance level of $p < 0.05$ (paired *t* test). (*a*) IL-4; (*b*) IL-2; (*c*) IFN-γ.

patients with cutaneous T cell lymphomas under photopheresis (Di Renzo *et al*, 1997). It is unclear whether this change in cytokine secretion is a direct effect of photochemotherapy, however, or simply reflects the disappearance of the malignant Th2 clone from the circulation. Another study showed by competitive polymerase chain reaction that in a T cell line (HUT-87) derived from a patient with Sezary syndrome photochemotherapy can increase IFN-γ mRNA (Saed and Fivenson, 1994). Clearly this type of model is useful for the investigation of the molecular mechanisms of PUVA-induced gene regulation but cannot be applied to the clinical setting. Thus, to generalize that 8-MOP/UVA induces a shift towards a Th1 response in diseases other than mycosis fungoides and Sezary syndrome is not possible. Our results provide evidence that under different experimental conditions 8-MOP/UVA can have an opposite effect, namely the suppression of Th1 and induction of Th2 cytokines from $CD4^+$ lymphocytes. The clinical efficacy of photopheresis and PUVA in the treatment of graft-*versus*-host disease is within the context of these observations (Greinix *et al*, 1998). In particular the acute form of the disease is characterized by a Th1 response of graft-T cells against host tissues (Ferrara and Krenger, 1998) and according to our data photochemotherapy might help restore the protective function of Th2 cells. Another disease where a Th1 predominance has been recently described in lesional skin as well as in circulating T cells is psoriasis (Austin *et al*, 1999). Here the effect of PUVA might be at least in part mediated through exposure of skin-residing lymphocytes and their reversal from Th1 to Th2. Modification of the activity of other cytokine-producing cells in the skin and the peripheral blood such as keratinocytes, dendritic cells, other lymphocytes (B cells, $CD8^+$ T cells), and macrophages might further influence the immunomodulatory effect of photochemotherapy and may thus help explain the varied clinical effects ascribed to photochemotherapy.

Future experimental studies should identify the conditions that determine the effect resulting from 8-MOP photochemotherapy and elucidate the molecular basis of these differing cellular responses. Clinical research should integrate the results obtained in order to design the optimal conditions for photochemotherapy-induced T cell modulation.

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