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Production of hydrogen peroxide by cutaneous T-cell lymphoma following photopheresis with psoralens and ultraviolet light*

Diane E. Heck[1], Emil Bisaccia[2], Steven Armus[2], and Jeffrey D. Laskin[1]

[1] Department of Environmental and Community Medicine, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA
[2] Morristown Memorial Hospital, Morristown, NJ 07962, USA

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Summary. Treatment of peripheral blood mononuclear cells with 8-methoxypsoralen (8-MOP) and ultraviolet light, a procedure known as PUVA, has been found to be useful in the management of systemically disseminated cutaneous T-cell lymphoma (CTCL). In the present study we used a highly sensitive flow cytometric assay in conjunction with the hydroperoxide-sensitive dye 2′,7′-dichlorofluorescein diacetate to measure intracellular hydrogen peroxide in normal lymphocytes and CTCL following PUVA treatment. Based on their laser light-scattering properties, lymphocytes were separated into three major subpopulations. We found that ultraviolet light alone caused an increase in the hydrogen peroxide content of each of the subpopulations, a response that was augmented when the cells were pretreated with 8-MOP (50 ng/ml). Cells from CTCL patients were more sensitive to the effects of 8-MOP than were normal lymphocytes. In both cell types, the production of hydrogen peroxide was found to be inhibitable by catalase. We noted an increase in hydrogen peroxide production following photopheresis; however, this was observed only 24 h after treatment. In addition, a further increase in hydrogen peroxide production was observed in lymphocytes isolated from peripheral blood that had been obtained from patients at 15 min after a second photopheresis treatment. Hydrogen peroxide is known to modulate the action of cytokines as well as the immunological responses of leukocytes. Our data suggest that the production of hydrogen peroxide by lymphocytes may be important in the action of PUVA in CTCL.

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Abbreviations: HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; CTCL, cutaneous T-cell lymphoma; 8-MOP, 8-methoxypsoralen; UVA, ultraviolet light of wavelengths ranging from 320–400 nm; PUVA, 8-MOP and ultraviolet light; DCFH, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; BSA, bovine serum albumin

Offprint requests to: J. D. Laskin, Dept. of Environmental and Community Medicine, UMDNJ Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, N. J. 08854, USA

Introduction

Systemically disseminated cutaneous T-cell lymphoma (CTCL) consists of a group of T-lymphocyte disorders usually involving a mature T-helper/inducer phenotype [6, 17, 29]. As this series of hyperproliferative diseases, which include mycosis fungoides and Sézary syndrome, tend to be resistant to radiotherapy and chemotherapy, the prognosis for patients with CTCL is often poor [17]. Recently, the combination of 8-methoxypsoralen (8-MOP) and ultraviolet light of wavelengths ranging from 320 to 400 nm (UVA light), a procedure referred to as PUVA, has proved to be beneficial in the management of CTCL. 8-MOP is given orally to patients and then photoactivated either by treating the patients topically with UVA light or by using a process known as photopheresis, in which drug-treated lymphocytes are exposed to UVA light extracorporeally [17, 30]. The mechanism underlying the regression of CTCL following PUVA treatment is not well understood. It has been suggested that the clinical responsiveness is not due simply to cytotoxicity, but rather is attributable to an immune reaction elicited in the treated lymphocytes; on the reintroduction of the latter into the patients, they induce the suppression of actively growing tumor cells [29].

Many mammalian cell types are known to be sensitive to damage caused by reactive oxygen intermediates [13, 25, 35]. Various forms of active oxygen, including hydrogen peroxide, superoxide anion, and hydroxyl radicals, damage nucleic acids as well as protein and lipids [13, 18]. Damage to nucleic acids can inhibit DNA replication and lead to mutations [13, 18, 35]. Reactive oxygen intermediates also disrupt membranes through peroxidation of cellular phospholipids [13, 27, 34, 35]. T-cells are known to be particularly sensitive to damage caused by reactive oxygen intermediates, which modulate mitogen-stimulated T-cell proliferation [22] and activation [3, 26].

A number of reports have indicated that the ability of UV light to damage tissues and cells is mediated by reactive oxygen intermediates [9, 14]. Since UV light is used in the treatment of CTCL and reactive oxygen intermediates can alter lymphocyte function, the present investigations

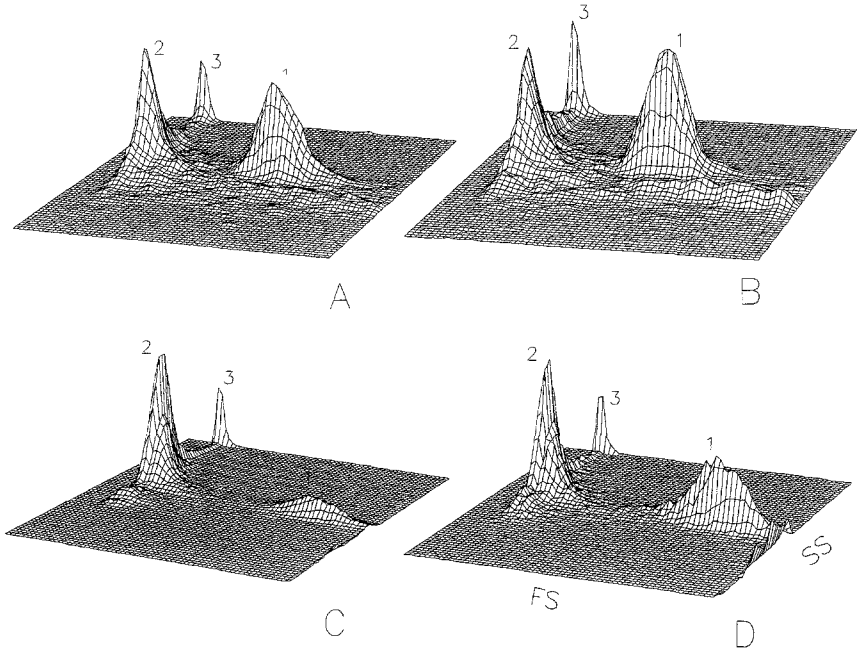

Fig. 1 A – D. Lymphocyte subpopulations in normal blood and in CTCL. Lymphocytes were separated from peripheral blood and analyzed for forward (*FS*) and right-angle (*SS*) light scatter by flow cytometry as described in Materials and methods. **A** Normal lymphocytes. **B** Normal lymphocytes following treatment with 8-MOP (50 ng/ml) and UVA light (0.69 J/cm^2) in vitro. **C** Lymphocytes obtained from CTCL patient A. G. prior to photopheresis. **D** Lymphocytes obtained from patient A. G. at 15 min after photopheresis. The origin is in the lower left hand corner of each figure

were focused on determining whether reactive oxygen intermediates are produced by CTCL following PUVA treatment. Using a highly sensitive flow cytometric assay, we found that UVA light alone was a potent stimulus for hydrogen peroxide production by CTCL as well as by normal T-lymphocytes. This effect was potentiated when cells had been pretreated with 8-MOP. We hypothesize that the generation of reactive oxygen intermediates by T-lymphocytes may at least in part contribute to the biological responses observed following PUVA treatment.

Materials and methods

Chemicals. 2-Mercaptoethanol, L-glutamine, bovine liver catalase, bovine serum albumin (BSA), sodium azide, and penicillin/streptomycin were obtained from Sigma Chemical Co. (St. Louis, Mo.). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, Ore.). 8-MOP was supplied by Elder Pharmaceuticals (Bryant, Ohio).

Cell isolation and immunophenotyping. Peripheral blood lymphocytes were isolated from the blood of healthy volunteers or CTCL patients after separation on a Ficoll gradient (Ficoll-Plaque Pharmacia LKB, Uppsala, Sweden). The cells were then washed two times with phosphate-buffered saline (PBS) and resuspended in culture medium consisting of RPMI 1640 (Gibco, Grand Island, N. Y.) supplemented with 15% heat-inactivated fetal calf serum (Biocell Laboratories, Carlson, Calif.), 2 mM L-glutamine (final concentration, 4 mM), 100 units penicillin/ml, 100 µg streptomycin/ml, and 5×10^{-5} M 2-mercaptoethanol. Blood from CTCL patients was obtained just prior to and at 15 min after photopheresis, which was performed as previously described [7, 17, 29, 30]. For assays for CD4 and CD8 surface antigens, isolated lymphocytes were suspended in PBS supplemented with 0.1% BSA and 0.05% sodium azide and were incubated for 30 min with T4-RD 1/T8-fluorescein isothiocyanate (FITC) or MsIgG1-RD/MsIgG1-FITC (isotypic control) monoclonal antibodies (Coulter Corp., Hialeah, Fla.). The lymphocytes were then washed and resuspended in PBS, and cell-associated fluorescence was analyzed using a Coulter Epics Profile flow cytometer equipped with a 25-mW argon laser. Fluorescence intensity was plotted on a 4-cycle log scale that was divided into 256 channels. For each analysis, 10,000 events were collected.

Hydrogen peroxide production. Hydrogen peroxide production was monitored by flow cytometry using DCFH-DA as previously described [5, 41, 48]. Briefly, approximately 2×10^5 cells/ml were incubated for 30 min at 37° C with 5 µM DCFH-DA. After being washed, cells (2×10^6) were inoculated into 35-mm plastic culture plates and pulsed with UVA light. For PUVA experiments, cells were pretreated for 5 min with 8-MOP (50 ng/ml). UVA light was emitted from a bank of four BLB fluorescent light tubes (F40BL, Sylvania) placed approximately 10 cm above the uncovered culture plates. Incident light on the culture dishes amounted to 2.8 mW/cm^2 as measured using an International Light UV-radiometer (model IL 442A) fitted with an IL-SE 115 probe and a 363 UVA pass filter (International Light, Newburyport, Mass.). In some experiments, 12,500 IU catalase/ml was added to the culture medium at 2 h prior to treatment. After PUVA treatment, the cells were placed on ice and immediately assayed for hydrogen peroxide production using flow cytometry as described above.

Results

Characterization of lymphocyte cell subpopulations by flow cytometry

Figure 1 (panels A and C) shows typical flow cytometic histograms of cells from the blood of a normal individual and a CTCL patient, respectively. Based on the light-scattering properties, we could identify three distinct subpopulations of cells that differed in size and density. Population 1 was relatively large in size and exhibited intermediate density, population 2 was intermediate in both size and density, and population 3 was smaller and highly dense.

CTCL was associated with a change in the distribution of lymphocytes within these three subpopulations. In the CTCL patient there were fewer lymphocytes in population 1 and greater numbers in populations 2 and 3. In general, a similar distribution of lymphocytes was obtained from other normal individuals. However, the lymphocyte distribution in two additional CTCL patients more closely resembled that in normal individuals. Using immuno-

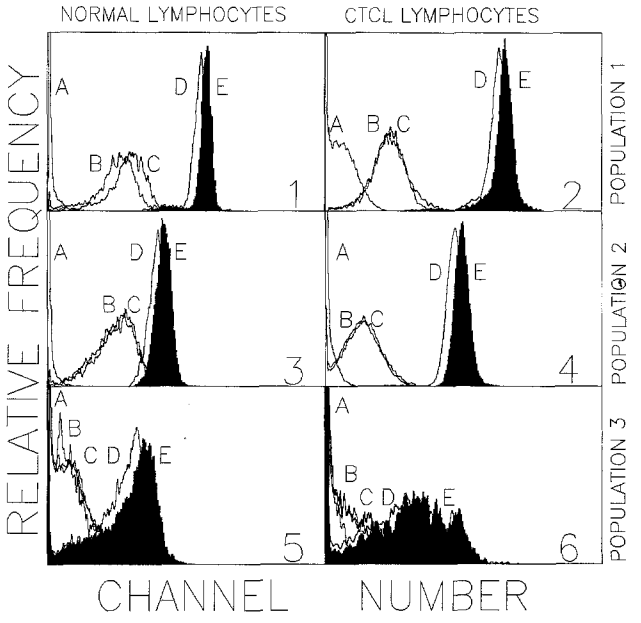

Fig. 2. Comparison of hydrogen peroxide production by lymphocytes from normal donors and from patients with CTCL following PUVA treatment in vitro. Electronic gates were drawn around the three subpopulations of cells shown in Fig. 1, which were analyzed for hydrogen peroxide production using DCFH-DA and flow cytometry as described in Materials and methods. *A*, Autofluorescence; *B*, cells labeled with DCFH-DA only (control); *C*, cells labeled with DCFH-DA and treated with 8-MOP (50 ng/ml); *D*, cells labeled with DCFH-DA and treated with UVA light (0.69 J/cm^2); *E*, cells labeled with DCFH-DA and treated with 8-MOP and UVA light. The channel number, which is proportional to the hydrogen peroxide levels in the cells, is presented on a 4-decade log scale. Areas are shaded to depict hydrogen peroxide production under conditions that would be observed following photopheresis

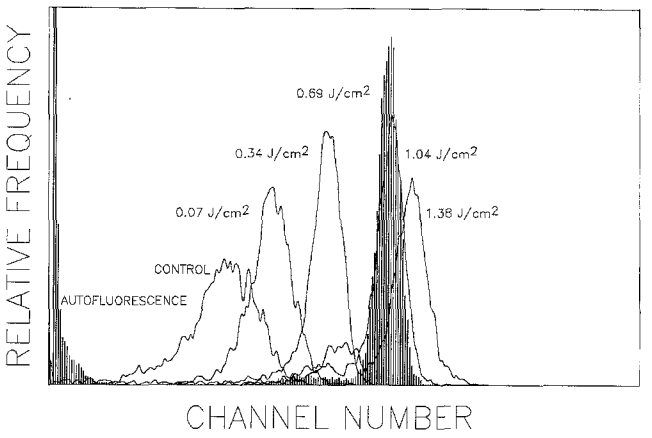

Fig. 3. Effect of increasing doses of UVA light on the production of hydrogen peroxide by human lymphocytes. Cells that had been prelabeled with DCFH-DA were treated with 8-MOP (50 ng/ml) and increasing doses of UVA light. Levels of hydrogen peroxide in population 1 were then determined by flow cytometry as described in Materials and methods. The channel number is presented on a 4-decade log scale. Areas are shaded to depict hydrogen peroxide production under conditions that would be observed following photopheresis

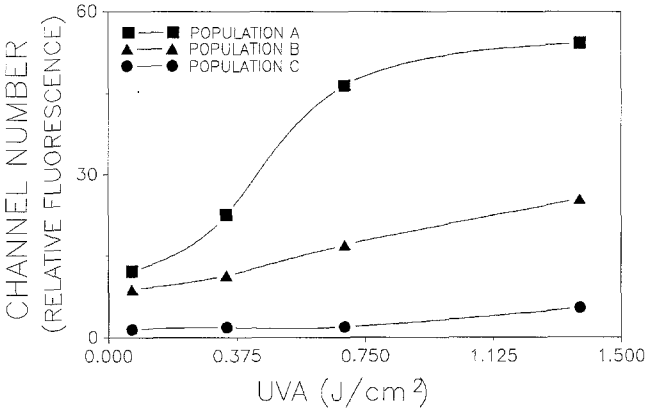

Fig. 4. Comparison of the effects of increasing doses of UVA light on the production of hydrogen peroxide in subpopulations of human lymphocytes. The three subpopulations of lymphocytes described in Fig. 1 were labeled with DCFH-DA and then treated with 8-MOP (50 ng/ml) and increasing doses of UVA light as described in Materials and methods. For each subpopulation, the amount of hydrogen peroxide (mean fluorescence channel number) produced by the cells was plotted against the dose of UVA light

fluorescence, we found that each of the subpopulations contained 81%–94% CD4+ cells and 35%–47% CD8+ cells; the ratio of CD4/CD8 cells ranged from 1.8 to 2.4. Similar results were found for lymphocytes from other normal individuals and CTCL patients (data not shown).

Effects of PUVA on hydrogen peroxide production by lymphocyte subpopulations

In our initial experiments, we compared hydrogen peroxide production by lymphocyte subpopulations from CTCL patients with that by equivalent subpopulations from normal individuals (Fig. 2). In these studies, cells were loaded with DCFH-DA and then analyzed for green fluorescence by flow cytometry both before and after treatment with 8-MOP and UVA light in vitro. We found that for both normal and CTCL lymphocytes, each of the subpopulations produced hydrogen peroxide in the absence of stimulation (Fig. 2). In general, populations 1 and 2 produced significantly more hydrogen peroxide than did cells from population 3.

Treatment of the cells with either UVA light or the combination of 8-MOP and UVA light was found to cause a dramatic increase in hydrogen peroxide production by the cells (Fig. 2). The greatest increase was observed in population 1, followed by populations 2 and 3. We also noted that populations 1 and 2 were homogeneous with respect to their responses to treatment, whereas population 3 exhibited an increased but heterogeneous response. In each case, more hydrogen peroxide was observed in cells treated with the combination of 8-MOP and UVA light than in those treated with UVA light alone. As compared with cells from normal individuals, lymphocytes from other CTCL patients consistently showed a stronger response to PUVA as well as to UVA light alone (Fig. 2). Figures 3 and 4 show that the increase in hydrogen peroxide production by the cells following UVA irradiation was dose-dependent within the range of 0.07 to 1.38 J/cm^2.

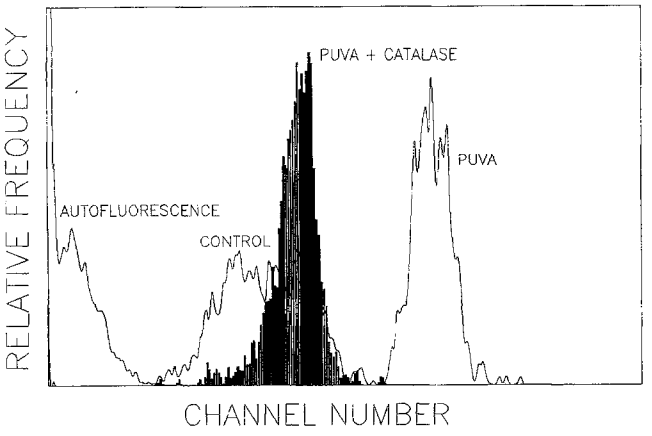

Fig. 5. Effect of catalase on hydrogen peroxide production by human lymphocytes. Lymphocytes from normal donors were preincubated with catalase and DCFH-DA and then treated with 8-MOP (50 ng/ml) and UVA light (0.69 J/cm^2) as described in Materials and methods. Cells were then assayed for the production of hydrogen peroxide by flow cytometry. The channel number is presented on a 4-decade log scale

Treatment with catalase

To determine the amount of DCFH oxidation that was specifically related to the formation of hydrogen peroxide, we pretreated normal lymphocytes with catalase prior to their exposure to 8-MOP and UVA light. Figure 5 shows that in population 1, fluorescence intensity was markedly reduced by catalase, indicating that hydrogen peroxide was the primary agent involved in DCFH oxidation. Similar results were found in populations 2 and 3 as well as in each of the three populations of CTCL cells (data not shown).

Effect of PUVA on hydrogen peroxide production after photopheresis

We next measured the levels of hydrogen peroxide produced by lymphocytes from CTCL patients following photopheresis. In these experiments, the hydrogen peroxide production by lymphocytes isolated from a CTCL patient just prior to photopheresis was compared with levels produced by cells recovered from patients at 15 min after the completion of therapy. Thus, in the post-photopheresis experiments, the blood sample contained both treated and untreated cells. We found that photopheresis had no immediate effect on the production of hydrogen peroxide by any of the lymphocyte subpopulations (Fig. 6; data not shown). In contrast, at 24 h following photopheresis, we observed a significant increase in hydrogen peroxide production by each of the lymphocyte subpopulations (Fig. 6; data not shown). Moreover, after a second photopheresis treatment, we noted an additional increase in hydrogen peroxide production by the lymphocyte subpopulations. This increase was most evident in subpopulation 3 (Fig. 6; data not shown).

Interestingly, we also found that treatment of the lymphocytes from CTCL patients by photopheresis caused a significant increase in the fraction of cells in population 1 (from 16.9% to 26.4% of the total lymphocyte population) along with a decline in the number of cells in population 2 (from 71.1% to 43.2% of the total lymphocyte population; Fig. 1, panel D). Only a small increase in the number of cells in population 1 was observed in normal lymphocytes and in those from CTCL patients that had been treated with UVA light alone or with PUVA in vitro (Fig. 1, panels A, B; data not shown). Changes in the distribution of lymphocytes in different subpopulations did not alter the ratio of CD4$^+$/CD8$^+$ cells (data not shown).

Discussion

In the present study, we demonstrated that both normal lymphocytes and lymphocytes from patients with CTCL produce significant quantities of hydrogen peroxide following treatment with psoralens and UVA light. Based on the light-scattering properties of the cells, these lymphocytes could be separated into three major subpopulations, each of which responded to the treatments. The identification of multiple lymphocyte subpopulations has previously been described, although the contribution of each to normal immune functioning is not well understood [43, 54].

Each of the subpopulations contained generally similar ratios of CD4$^+$/CD8$^+$ cells both before and after PUVA treatment. Although we observed changes in the distribution of the number of cells within the subpopulations fol-

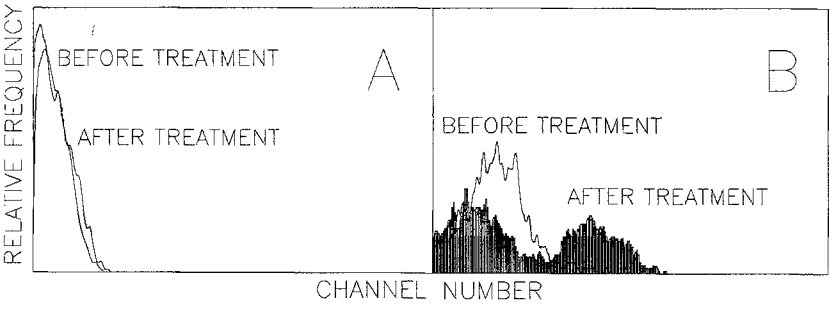

Fig. 6. Production of hydrogen peroxide by CTCL before and after photopheresis. Patient A. G. was treated with 8-MOP and then underwent photopheresis. Peripheral blood was withdrawn just prior to and immediately following UVA light treatment. **A** Production of hydrogen peroxide by cells from subpopulation 3 before and after photopheresis. **B** Hydrogen peroxide production by cells from subpopulation 3 from the CTCL patient just prior to and immediately following a second photopheresis treatment. *Unshaded area*, Prior to photopheresis; *shaded area*, 15 min after photopheresis. The channel number is presented on a 4-decade log scale

lowing photopheresis, there was no significant alteration in the ratio of $CD4^+/CD8^+$ cells. The significance of the changes in the distribution of cells in the lymphocyte subpopulations are not known. One could speculate that the treatments cause changes in the surface membranes of the lymphocytes, thus altering their light-scattering properties. In this regard, both the psoralens and UV light are known to modify the structure and function of cellular membranes [8, 24, 39, 42, 43, 46, 52, 55, 59].

The oxidation of dichlorofluorescein has been used as an indirect assay for the measurement of hydrogen peroxide production by cells using both fluorometry and flow cytometry [5, 45, 48]. The present observation that less fluorescence was emitted by the cells following treatment with catalase confirms that the fluorescence of the dye was for the most part attributable to hydrogen peroxide. However, it should be noted that low-molecular-weight peroxides other than hydrogen peroxide oxidize dichlorofluorescein, and we cannot presently rule out the possibility that these were also produced by the lymphocytes following treatment with UVA light and PUVA

The source of the hydrogen peroxide that was generated in the cells by the treatments is also not clear at the present time. Inflammatory leukocytes are known to release reactive oxygen intermediates as part of their normal phagocytic and bactericidal activity [13, 53, 57]. Reactive oxygen intermediates, including hydrogen peroxide, are also produced during mitochondrial respiration and via other reactions such as redox cycling in cells [13, 35]; although UVA light and PUVA may increase the intracellular levels by stimulating these reactions [9, 14], hydrogen peroxide may also be produced in cells by nonenzymatic mechanisms.

It is noteworthy that the different lymphocyte subpopulations from CTCL patients and normal individuals produced different levels of hydrogen peroxide following UVA light and PUVA treatment. Intracellular levels of reactive oxygen intermediates are known to be regulated by a variety of antioxidants and detoxifying enzymes, the concentrations of which may vary in the lymphocyte subpopulations, thus accounting for the different levels of hydrogen peroxide [13, 16, 44]. The present observation of higher levels of hydrogen peroxide in lymphocytes from CTCL patients as compared with normal lymphocytes following UVA light and PUVA treatment may have been due to an increase in the production of hydrogen peroxide by the former cells following stimulation or to a decrease in the intracellular content of antioxidants and/or detoxifying enzymes. Alterations in the profile of antioxidants, catalase, and superoxide dismutase activity have been reported during the tumor promotion process and in epidermal papillomas and carcinomas [12, 15, 19, 21, 23, 36, 37, 47, 50, 51, 56].

Our finding that UVA light alone caused an increase in hydrogen peroxide levels in the cells suggests that it may be active in photopheresis. Alternatively, since more hydrogen peroxide was produced by cells that had been pretreated with 8-MOP, critical levels might be required for the induction of biological effects. It is also possible that the amount of hydrogen peroxide induced in cells by PUVA alone is not sufficient to initiate a clinical response and that other biochemical events are required. In this regard, PUVA is known to alter a number of other biochemical pathways, including those involved in growth-factor-induced signal transduction [4, 40–42, 45, 59].

We also investigated whether increased levels of hydrogen peroxide could be detected in lymphocytes from the blood of CTCL patients following photopheresis. One limitation of this experiment is that treated lymphocytes are diluted with untreated lymphocytes in the blood when the former are reinfused into the patients. Although no changes in hydrogen peroxide levels were observed in lymphocytes from CTCL patients at 15 min after photopheresis, levels of hydrogen peroxide were elevated at 24 h after the initial treatment. Interestingly, after a second treatment with photopheresis, a relatively large increase in the hydrogen peroxide content of subpopulation 3 was observed. At the present time, the mechanisms by which levels of hydrogen peroxide change in the lymphocytes following photopheresis are unknown. It may be that these peripheral blood cells become "activated" and systemically respond to PUVA treatment.

The question arises as to whether hydrogen peroxide formed in lymphocytes following PUVA treatment plays a role in modifying the progression of CTCL. Reactive oxygen intermediates are known to be produced in human lymphocytes following mitogen stimulation, presumably as a result of increased oxidative metabolism [1, 3]. Hydrogen peroxide has been reported to act directly as a modulator of a number of immune functions. For example, treatment of T-lymphocytes with hydrogen peroxide inhibits proliferation induced by phytohemagglutin and concanavalin A but enhances that in response to pokeweed mitogen [22]. The mechanism for this enhancement is not known; however, hydrogen peroxide has been reported to modulate mitogen-induced signal transduction [22].

Hydrogen peroxide also blocks the generation of cytotoxic T-cells in mixed lymphocyte cultures [3], impairs the blastic transformation of human lymphocytes [49], and attenuates the suppressor activity of macrophages on T-lymphocytes [42]. Furthermore, it has been reported to induce a significant increase in the biological activity of both lymphocyte and macrophage-derived soluble immune suppressor factors [1, 2, 3, 10, 11, 58]. These factors are known to suppress antibody production by B-cells and mitogen-induced T- and B-cell proliferation [1, 11]; moreover, they have been reported to inhibit the growth of a number of different human leukemia cell lines [49]. Thus, it is possible that PUVA activates these suppressor factors during photopheresis and that their subsequent release into the blood could result in the inhibition of tumor-cell proliferation in patients with CTCL.

Both hydrogen peroxide and lipid peroxidation products are known to modulate the biosynthesis of prostaglandins [33], which are potent regulators of the growth and functional activity of leukocytes [10, 20, 25, 28, 32, 33, 38]. It is also possible that the hydrogen peroxide produced in CTCL leads directly to cell injury. Hydrogen peroxide damages cellular macromolecules and alters cellular metabolism, causing decreases in critical metabolites such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NAD^+) [13, 32, 35]. Dam-

aged cells may effectively decrease the tumor burden in CTCL patients [17, 29, 31] and/or stimulate an immune response against CTCL. Further studies are required to determine the potential role of hydrogen peroxide induced by PUVA in regulating the growth of tumor cells in CTCL.

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