Apoptosis Induction by Ultraviolet Light A and Photochemotherapy in Cutaneous T-Cell Lymphoma: Relevance to Mechanism of Therapeutic Action

Elisa K. Yoo,* Alain H. Rook,* Rosalie Elenitsas,* Francis P. Gasparro,† and Benjamin R. Vowels*

*Department of Dermatology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, U.S.A.; and †Department of Surgery (Vascular), School of Medicine, Yale University, New Haven, Connecticut, U.S.A.

The anti-tumor action of many chemotherapeutic agents has recently been attributed to the induction of apoptosis in the malignant cell population. In this study, we investigated the ability of extracorporeal photopheresis (ExP) and *in vitro* PUVA (8-methoxypsoralen + ultraviolet A) therapy to induce apoptosis in peripheral blood mononuclear cells from Sezary syndrome patients and normal controls. Flow cytometric analysis of ExP- or PUVA-treated peripheral blood lymphocytes demonstrated two distinct cell populations within 24 h of treatment. One population was similar to untreated controls with the other exhibiting characteristics of apoptotic cell death, i.e., a loss of cell volume and an accompanying increase in cell density. This latter population was comprised of cells with DNA strand breaks as determined by the Tdt-mediated deoxyuridine triphosphate-biotin nick end labeling assay. Apoptosis was also confirmed morphologically by fluorescent and electron microscopy as well as by demonstration of characteristic DNA strand breaks (laddering) using gel electrophoresis. Apoptosis was not observed with 8-methoxypsoralen (≤300 ng per ml) alone; however, ultraviolet A alone at doses ≥ 2 J per cm^2 induced apoptosis in lymphocytes. Peripheral blood T-cell subpopulations of Sezary syndrome patients, including the malignant clone, were equally susceptible to apoptosis subsequent to either photopheresis or PUVA treatment. In contrast, monocytes (CD14+/CD45+) appear to be resistant to apoptosis induction by ExP or PUVA treatment. Moreover, ExP-treated and untreated monocytes phagocytized apoptotic, but not untreated, peripheral blood mononuclear cells. ExP and PUVA have been shown to be efficacious and well-tolerated therapies in the treatment of dermatologic diseases and transplant rejection. These data suggest that induction of apoptosis may be an important event for therapeutic efficacy. ***Key words: Sezary syndrome/psoralen/extracorporeal photopheresis. J Invest Dermatol 107:235–242, 1996***

Apoptosis is an active form of cell death characterized by morphologic and biochemical changes very different from necrotic cell death (Cohen, 1993; Kerr *et al*, 1994). Apoptosis has been demonstrated to play an important role in embryonic development (Goldman *et al*, 1983), in the generation and maintenance of the immune system (Cohen, 1991; Cohen and Duke, 1992), in T-cell lymphoma (CTL)-mediated immune reactions (Cohen and Duke, 1992), and in the normal turnover of differentiated cells (Martin and Johnson, 1991). A growing body of evidence also indicates that the action of various anti-tumor agents, including irradiation (Sellins and Cohen, 1987), hormonal therapy (Redding *et al*, 1992), and cytotoxic chemotherapy (Gorczyca *et al*, 1993a, 1993b) may in fact be efficacious by inducing apoptosis in the malignant cell population (Dive and Hickman, 1991; Hickman, 1992; Kerr *et al*, 1994).

A unique type of anti-tumor therapy is photochemotherapy (PCT). Two types of PCT, both of which employ the DNA intercalator, 8-methoxypsoralen (8-MOP), and long wavelength ultraviolet radiation (320–400 nm, UVA), are used in the treatment of cutaneous T-cell lymphoma (CTCL) and its leukemic variant, Sezary syndrome, which is characterized by erythroderma and circulating malignant atypical lymphocytes (Sezary cells) in the skin and peripheral blood (Edelson, 1980; Wieselthier and Koh, 1990).

Psoralen in combination with UVA (PUVA) is used to treat early stage CTCL (Gilchrest *et al*, 1976; Gilchrest, 1979; Roenigk, 1979; Honigsmann *et al*, 1984) whereas extracorporeal photopheresis (ExP) therapy is used in the treatment of patients with Sezary syndrome (Edelson *et al*, 1987; Rook *et al*, 1992). PCT has also been used successfully for the treatment of psoriasis (Parrish *et al*, 1974), systemic sclerosis (Rook *et al*, 1992), acute transplant rejection (Costanzo-Nordin *et al*, 1992), and other T-cell–mediated diseases (Rook *et al*, 1993). Although PCT has been used clinically for many

Manuscript received November 21, 1995; revised March 27, 1996; accepted for publication April 17, 1996.

Reprint requests to: Dr. Alain Rook, Department of Dermatology, 2 Rhoads, 3600 Spruce Street, University of Pennsylvania, Philadelphia, PA 19104-6065.

Abbreviations: dUTP, deoxyuridine triphosphate; ExP, extracorporeal photopheresis; FSC, forward light scatter; PCT, photochemotherapy; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; SSC, side light scatter.

0022-202X/96/$10.50

years, very little data currently exist regarding the mechanisms by which this therapy is effective.

Several different lines of evidence indicate that ExP results in apoptotic cell death. Previous studies have demonstrated that psoralens, including 8-MOP, intercalate into double-stranded nucleic acids and bind to pyrimidine bases (Song and Tapley, 1979). Upon exposure to UVA light, photo-addition occurs with the formation of monoadducts and covalent crosslinks of the DNA (Gasparro, 1994). Following 8-MOP + UVA treatment, lymphocytes are unable to proliferate as evidenced by reduced incorporation of [^{3}H]thymidine into nuclear DNA in unstimulated and mitogen-stimulated lymphocytes; however, assessment of cell membrane integrity, as determined by trypan blue dye exclusion, indicates that the membrane remains intact. These cells, however, eventually die over a period of about 72 h (Song and Tapley, 1979; Warwick *et al*, 1981; Gasparro *et al*, 1989). A previous study by Marks *et al* indicated that treatment of peripheral blood mononuclear cells (PBMC) with ExP resulted in an increase in DNA single strand breaks with a concomitant decrease in cellular ATP and NAD; this damage then leads to cell death (Marks *et al*, 1990). In subsequent studies, they demonstrate that peripheral blood lymphocytes (PBL) as well as a T-cell line (CEM) undergo apoptosis as a result of PUVA treatment; however, the doses of UVA utilized in these experiments were approximately five times those used in ExP (Marks and Fox, 1991).

In this study, we investigated the ability of ExP and *in vitro* PUVA therapy to induce apoptosis in CTCL and normal patient PBMC. PCT-treated and untreated cells were examined by several methods, including flow cytometry, fluorescence microscopy, electron microscopy, and DNA gel electrophoresis for the characteristic morphologic and biochemical features of apoptosis. We also examined the kinetics and effects of PCT on specific subpopulations of PBMC, including peripheral blood T-cell subpopulations.

MATERIALS AND METHODS

Reagents Complete media consisted of RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU penicillin per ml, and 100 μg streptomycin per ml (Life Technologies, Inc., Grand Island, NY). 8-MOP (Sigma Chemical Co., St. Louis, MO) stock solution in absolute ethanol (100 μg per ml) was stored at −20°C in the dark for no longer than 6 mo. All fluorochrome-labeled antibodies (CD4-PE, CD7-FITC, CD8-FITC, CD45-FITC/CD14-PE, and mouse IgG_1FITC/mouse IgG_{2a}PE isotype control) were obtained from DAKO (Santa Barbara, CA)

Patients Patients participating in this study were diagnosed with CTCL (Sezary syndrome, n = 9) as defined by clinical and laboratory findings (Sen and D'Incalci, 1992). Donations of blood by patients or normal volunteers in this study conformed to institutional review board-approved protocols, and informed consent was obtained.

Isolation of PBMC Venous blood was collected in heparin from normal volunteers and from CTCL patients immediately prior to ExP. Post-ExP blood was removed directly from the photopheresis unit upon conclusion of treatment immediately prior to infusion into the patient. The blood was diluted 1:2 with phosphate-buffered saline (PBS) and layered on a Ficoll-Hypaque gradient (Pharmacia Biotech, Pitscataway, NJ). The gradient was centrifuged for 15 min at 2200 rpm at room temperature, and the PBMC layer was collected and washed × 2 in PBS. The cells were resuspended in complete media.

***In Vitro* PUVA Treatment** PBMC in complete media (10^6 cells per ml) were incubated with various concentrations of 8-MOP (0–300 ng per ml) for at least 15 min and then exposed to different doses of UVA light (0–3 J per cm^2). Cell suspensions were irradiated in cell culture cluster dishes (Costar Corp., Cambridge, MA) with a UVA light box (Therakos, West Chester, PA) containing a series of nine UVA bulbs (emission spectra of 320–400 nm, peak at 352 nm) in the top and the bottom banks separated by two glass plates. An IL700A research radiometer (International Light, Newbury Port, MA) was used to calibrate the UVA light box, which emitted between 10.14 and 11.71 mW per cm^2 when corrected for passage through the polystyrene plate. The emission spectra, peak wavelength of emission, and total average joules per cell volume delivered were identical to those utilized in the photopheresis instrument. The treated and untreated control samples were subsequently incubated at 37°C in a humidified atmosphere of 5% CO_2 for different periods of time up to 48 h following UVA exposure and then assessed for apoptosis.

Extracorporeal Photochemotherapy Patients were treated using the ultraviolet A radiation (UVA) photopheresis system (Therakos) as previously described (Edelson *et al*, 1987; Rook *et al*, 1992). Briefly, ExP involves the ingestion of 8-MOP (0.6 mg per kg) in order to achieve therapeutic plasma levels (>50–100 ng per ml). Two hours after ingestion of the psoralen, the patient undergoes a discontinuous leukopheresis procedure with exposure of removed leukocytes to UVA. During the procedure, approximately 240 ml of leukocyte-enriched blood are mixed with 300 ml of the patient's plasma and 200 ml of sterile saline with heparin. This results in approximately 15% of the patient's circulating leukocytes being treated during the photopheresis process (Lee and Garro, 1989). The buffy coat is then passed as a 1-mm film through a sterile cassette surrounded by UVA-emitting bulbs (320–400 nm), permitting 180-min exposure of the cells to UVA, and yielding an average exposure per lymphocyte of 2 J per cm^2. The treated buffy coat is then returned to the patient.

Flow Cytometric Analysis To assess apoptosis by flow cytometry using light scatter measurements, pre-, and post-PCT-treated cell samples (10^6 cells per ml) at various incubation times were fixed in 2% paraformaldehyde, pH 7.4. Forward and side light scatter were analyzed at a cell flow rate of 500 cells per sec using a FACScan Flow Cytometer (Becton Dickinson, San Jose, CA). To identify the different cell types, surface antigens of human PBMC were stained with fluorochrome-labeled antibodies (CD4-PE/CD7-FITC, CD8-FITC or CD45-FITC/CD14-PE) by incubating 10^6 cells in 100 μl of PBS with 10 μl of antibody for 30 min in an ice bath. The cells were washed with PBS and resuspended in 0.5 ml of 2% paraformaldehyde, pH 7.4. Mouse IgG_1 FITC/mouse IgG_{2a}PE was used for determination of background staining. Flow cytometry was performed using the Becton Dickinson FACScan Flow Cytometer equipped with an argon laser for fluorescein isothiocyanate (FITC) (488 nm) and phycoerythrin (480–550 nm) excitation. Green and red fluorescence of individual cells using logarithmic amplification were measured. The data from 10^4 events per sample were collected and analyzed using LYSYS II software.

***In Situ* Terminal Deoxynucleotidyl Transferase (Tdt) Assay** Tdt-mediated dUTP-biotin nick end labeling (TUNEL) assay of DNA fragmentation as previously described by Sgonc *et al* (Sgonc *et al*, 1994) was adapted for use with flow cytometry. Briefly, 1–2 × 10^6 cells were fixed in 2% paraformaldehyde for 30 min at room temperature on a horizontal shaker, washed in PBS and then permeabilized with 0.1% Triton X100/0.1% sodium citrate. The cells were then incubated in a humidified chamber (1 h, 37°C) with 0.3 nmol of FITC-12-dUTP (Boehringer Mannheim Biochemicals, Indianapolis, IN), 3 nm of deoxyadenosine triphosphate, 25 U of Tdt (Promega Corp., Madison, WI), and Tdt buffer (1 mM $CoCl_2$, 100 mM cacodylate buffer, pH 6.8, 0.1 mM dithiothreitol) in a total reaction volume of 50 μl. The reaction was stopped by adding 2 μl of 0.5 M ethylenediamine tetraacetic acid (EDTA). After washing twice with PBS, the samples were analyzed within 6 h using FACScan with an argon laser (488 nm). The data from 10^4 events per sample were collected and stored using LYSYS II software.

DNA Extraction and Agarose Gel Electrophoresis About 5–10 × 10^6 cells in media were collected by centrifugation, washed in PBS, and then resuspended in 1 ml of freshly made lysis buffer containing 100 mM NaCl, 50 mM EDTA, pH 8.0, 0.1% sodium dodecyl sulfate, and 0.1 mg Proteinase K per ml (Fisher Scientific, Fair Lawn, NJ). Samples were incubated overnight at 37°C with gentle rocking. The DNA was then extracted with an equal volume of phenol:chloroform:isoamyl alcohol solution (25:24:1) and precipitated with 3 M sodium acetate, pH 5.2, and 100% ice-cold ethanol. The DNA precipitates were washed twice in 70% ethanol, dried by Speedvac evaporator, and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA), pH 8.0, overnight at room temperature. The DNA was quantified by UV absorbance at 260 and 280 nm. About 10–15 μg of DNA were added to each well, and horizontal electrophoresis of DNA was performed for 2 h at 75 V on 2% agarose gel. The running buffer contained 90 mM Tris(hydroxymethyl)-aminoethane, 90 mM boric acid, and 2 mM EDTA, pH 8.0. DNA was stained with ethidium bromide and visualized under UV light.

Quantification of Apoptotic Cells by Fluorescence Microscopy Assessment of apoptosis by fluorescent microscopy was done using previously described methods (Duke and Cohen, 1992). Briefly, a 4-μl aliquot of dye mixture consisting of 100 μg acridine orange per ml and 100 μg ethidium bromide per ml in PBS was added to 100 μl of cell suspension (10^6cells per ml) and mixed gently. A 25-μl aliquot of the stained cells was then placed on a slide, coverslipped, and viewed on a fluorescent microscope using epi-illumination and a filter combination suitable for observing

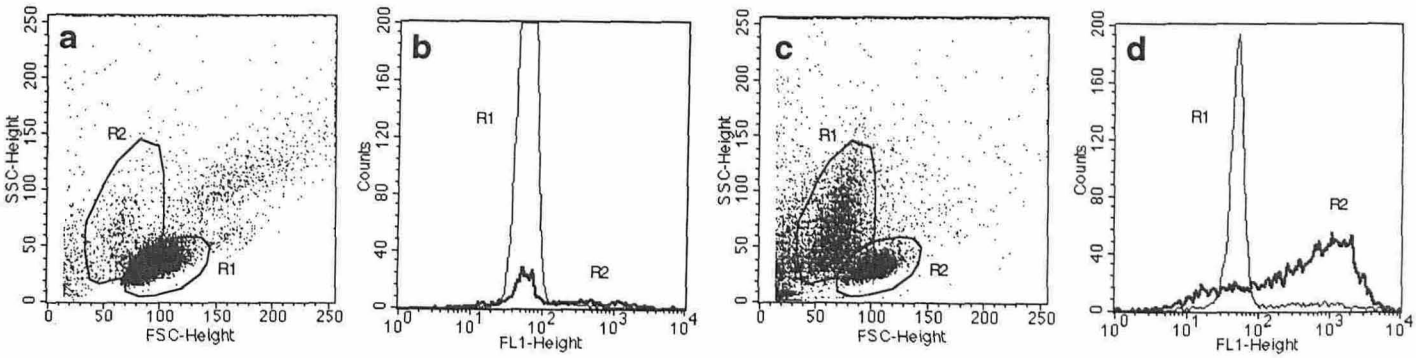

Figure 1. Flow cytometric analysis of apoptosis following PCT of Sezary Syndrome patient. *Panels a* and *c* show forward *versus* side light scatter prior to and 24 h after PCT, respectively. The shift to the R2 gate is indicative of nuclear condensation and decreased cell size. *b* and *d)* Binding of fluorescein-labeled dUTP to 3′-end of DNA (TUNEL assay) prior to and 24 h after PCT, respectively. The increase in binding observed at 24 h in the R2 gate indicates increased DNA fragmentation.

fluorescein. Apoptotic cells were determined based on cell size, nuclear condensation, and fluorescence. A minimum of 200 total cells were examined.

Electron Microscopy PBMC were fixed as pellets in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, overnight at 4°C and subsequently post-fixed with 1% osmium tetroxide in the same buffer for 1 h at room temperature. After washing, the cell pellets were dehydrated through graded alcohols to absolute ethanol and then propylene oxide. Embedding was done using Taab 812 epoxy resin (Marivac, Ltd., Halifax, Nova Scotia) in BEEM capsules (Bronx, NY). Thin sections were cut on an LKB Ultratome III (LKB Instruments, Rockville, MD) post-stained with alcoholic uranyl acetate and bismuth subnitrate, and viewed with a Hitachi 7000 electron microscope. Apoptotic cells were identified morphologically as determined by condensation of nuclear chromatin, decrease in cell size, and cell-surface blebbing. For quantification, a minimum of 200 cells were evaluated.

Phagocytosis Assay To obtain monocyte-derived macrophages, 1 ml of untreated or ExP and PUVA-treated PBMC cell suspension in complete media (10^6 cells per ml) was added to a 24-well polystyrene tissue culture plate (Corning, Corning, NY). After 60-min incubation at 37°C, the nonadherent cells were removed. The plate was washed three times with PBS, and 1 ml of fresh complete media was added. The cultures were then incubated at 37°C and 5% CO_2 for 4–5 days. One milliliter of untreated or ExP- and PUVA-treated PBMC at 0.5–1.0 × 10^6 cells per ml was then added to the monocyte cultures. Cultures were incubated for 1 h at 37°C, 5% CO_2. The wells were washed twice with warm PBS to remove nonadherent cells and then stained with Wright's stain.

RESULTS

Apoptosis of ExP and PUVA-Treated PBMC Is Demonstrated by Flow Cytometry Normal and patient (CTCL) PBMC were either treated with ExP or *in vitro* PUVA therapy using doses of 8-MOP and UVA similar to those routinely used clinically in ExP (100–200 ng 8-MOP per ml + 2 J UVA per cm^2). The treated and untreated cells were then examined by flow cytometry.

The characteristic changes in the morphology of cells undergoing apoptosis, including cell shrinkage and nuclear condensation, affect the cellular light scattering properties as detected by flow cytometry. Several investigators have observed that cells undergoing apoptosis have a decrease in forward light scatter (FSC) and increase in side light scatter (SSC), indicating a loss of cell volume with an accompanying increase in cell density (Swat *et al*, 1991; Darzynkiewicz *et al*, 1992; Dive *et al*, 1992; Zamai *et al*, 1993; Huschtscha *et al*, 1994). In order to assess apoptosis in normal human PBMC treated with ExP or PUVA, we examined changes in their light scatter measurements. By 24 h post-ExP or PUVA treatment, there were two distinct cell populations (**Fig 1*a*** and ***c***). One population was similar to untreated controls and the second, comprised of ≥ 50% of the total cells, had a decreased FSC and increased SSC, characteristic of light scatter changes in apoptotic cells. In order to assess whether the PCT-treated cells were undergoing the characteristic internucleosomal DNA fragmentation of apoptosis (Sgonc *et al*, 1994), an adaptation of the TUNEL assay for flow cytometry was used to detect DNA strand breaks. This assay demonstrated that the treated cells incorporated much more FITC-dUTP when compared to the untreated controls. Gating on the shifted population (R2) with low FSC/high SSC showed that these cells stained more brightly with FITC-dUTP than the nonshifted population (R1) (**Fig 1*b*** and ***d***) demonstrating that the R2 population are indeed apoptotic cells.

ExP AND PUVA Cause DNA Fragmentation Consistent with Apoptotic Cell Death Double-stranded DNA fragmentation at the linker regions between nucleosomes as part of the apoptotic process leads to the formation of DNA fragments that are multiples of 180–200 bp in size (Arends *et al*, 1990; Sellins and Cohen, 1987). Internucleosomal DNA fragmentation was verified by agarose gel electrophoresis of genomic DNA from cells treated with ExP and PUVA after 24 and 48 h of incubation but not from control (untreated) cells (data not shown)

DNA-Binding Fluorescent Dyes Demonstrate Apoptotic Cell Death Following ExP and PUVA Further confirmation of apoptosis as the mode of cell death in the PCT-treated PBMC was obtained using DNA-binding fluorescent dyes (acridine orange and ethidium bromide) along with fluorescent microscopy.

Fluorescence microscopy was performed 24 and 48 h after exposure to doses of UVA-8-MOP used in clinical ExP. In order to determine the percentage of cells undergoing apoptosis, a minimum of 200 cells were examined and classified into one of four categories described previously. The morphologic features of apoptosis including cell shrinkage, compaction of nuclear chromatin into sharply circumscribed dense masses around the periphery of the nuclei, and cytoplasmic condensation (Cohen, 1993; Kerr *et al*, 1994) were all observed in the PBMC treated with ExP or PUVA but not in the controls **(Table I)**.

Table I. Comparison of Methods Used to Assess Apoptosis[a]

Apoptosis Assessment Method	Untreated	6 h post-PUVA[b]	24 h post-PUVA	48 h post-PUVA
Flow cytometry	2.6 ± 2.1	3.9 ± 2.4	61.2 ± 19.9	84.8 ± 13.2
Fluorescent microscopy	3.9 ± 1.7	2.7 ± 1.15	37.7 ± 8.9	43.2 ± 9.6
Electron microscopy	1.2 ± 0.3	1.5 ± 0.4	24.3 ± 7.5	n.d.

[a] Values expressed as percent of total PBMC ± SD that were apoptotic. A minimum of four subjects were assessed for each group.
[b] PBMC were treated with 200 ng 8-MOP per ml and 2 J of UVA per cm^2.

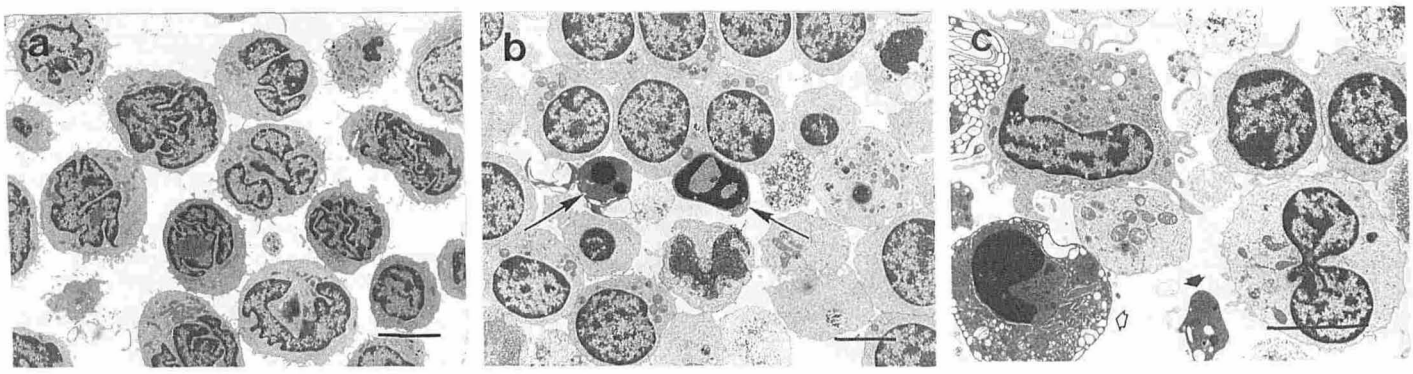

Figure 2. Visualization of apoptosis by electron microscopy. Venous blood was collected from CTCL patients and treated *in vitro* with PUVA. PBMC were isolated and visualized by EM. Photomicrographs shown are representative of three patients examined. *a)* PBMC prior to PUVA. No apoptotic cells are observed. Note cerebriform nuclei characteristic of Sezary cells (*scale bar*, 5 μm). *b)* PBMC 24 h after PUVA. Arrows indicate several cells at different stages of apoptosis (*scale bar*, 5 μm). *c)* PBMC 24 h after PUVA. ◊ indicates apoptotic cell; ◆ indicates apoptotic 'bleb' (*scale bar*, 5 μm).

Characteristic Morphological Changes of Apoptosis Occur Following ExP and PUVA PBMC isolated from three CTCL patients were treated *in vitro* with 200 ng 8-MOP per ml and 2 J UVA per cm^2 and examined by electron microscopy for changes characteristic of apoptosis at 6 and 24 h after PUVA treatment. Apoptotic cells totaled <2% in untreated PBMC or at 6 h post-PUVA treatment **(Table I)**; however, 24.2 ± 5.6% apoptotic cells were observed in PBMC obtained at 24 h after PUVA **(Fig 2)**. Characteristic nuclear condensation as well as blebbing of the cytoplasm were observed in all three samples examined.

Kinetics of Apoptosis Induction Are Similar for Both ExP and PUVA Treatment To determine the kinetics and time necessary to detect apoptotic cells following ExP, PBL obtained from the treatment device immediately prior to reinfusion into the patient were examined by flow cytometry at 6, 24, and 48 h following treatment using light scattering properties. Only the live and apoptotic lymphocytes were gated in order to eliminate all debris. No apoptotic cells were detected 6 h after treatment when compared to patient-matched untreated PBL; however, 24 h after treatment >50% of the cells were apoptotic. By 48 h after treatment, approximately 80–90% of the cells were apoptotic **(Fig 3*a*)**. Similar results were obtained for PBL treated *in vitro* with 100–200 ng 8-MOP per ml with subsequent exposure to 2 J UVA per cm^2, the same conditions used in ExP **(Fig 3*b*)**.

Psoralen Concentration and UVA Dose Correlate with the Induction of Apoptosis Preliminary studies indicated that variations in the concentration of 8-MOP or dose of UVA affected the percentage of cells that became apoptotic. To determine the minimum effective dose of UVA that was necessary to induce apoptosis in human PBL in the presence of various concentrations of 8-MOP, a series of experiments using a checkerboard titration of UVA (0–3.0 J per cm^2) *versus* 8-MOP (0–300 ng per ml) were performed.

At 6 h post-treatment, no apoptotic cells were evident at all UVA doses or 8-MOP concentrations **(Fig 4*a*)**; however, by 12 h, apoptotic cells were present in the PBL treated with 2.0 and 3.0 J UVA per cm^2 at all concentrations of 8-MOP, including no 8-MOP, suggesting that the UVA alone was inducing apoptosis **(Fig 4*b*)**.

After 24 h, the population of apoptotic cells present in PBL treated with 2.0 and 3.0 J UVA per cm^2 had doubled (from 25% to 50%) at concentrations of 0 and 25 ng 8-MOP per ml, whereas the number of apoptotic cells in the PBL treated with 50–300 ng 8-MOP per ml at the same dosage of UVA tripled (from 25% to 75%). PBL treated with 1.0 J UVA per cm^2 had an increased apoptotic population when 8-MOP concentration was ≥ 50 ng per ml. No apoptotic changes above baseline were observed in PBL treated with 0.5 J UVA per cm^2 at all concentration of 8-MOP **(Fig 4*c*)**.

At 48 h post-treatment, nearly all cells treated with 2.0 or 3.0 J UVA per cm^2 and ≥ 50 ng 8-MOP per ml were apoptotic. A significant number of cells (>%50) treated with 0 or 25 ng 8-MOP per ml and 2.0 or 3.0 J UVA per cm^2 were also apoptotic. Cells treated with 1.0 J UVA per cm^2 and ≥ 25 ng 8-MOP per ml also had an increased percentage of apoptotic cells **(Fig 4*d*)**.

To determine whether there was correlation between the percent of apoptotic cells and the dose of UVA × [8-MOP] used to treat the cells, linear regression analysis was performed on data collected at 48 h post-treatment. This analysis revealed a highly significant correlation ($r = 0.90$) for values of UVA × [8-MOP] of <150 (e.g. 2 J UVA per cm^2 × 50 ng 8-MOP per ml = 100). When the value of UVA × [8-MOP] was >150, the entire lymphocyte population was apoptotic.

ExP AND PUVA Induce Apoptosis in Lymphocyte Subpopulations but not in Monocytes Two-color flow cytometry was utilized in order to identify subtypes of lymphocytes from

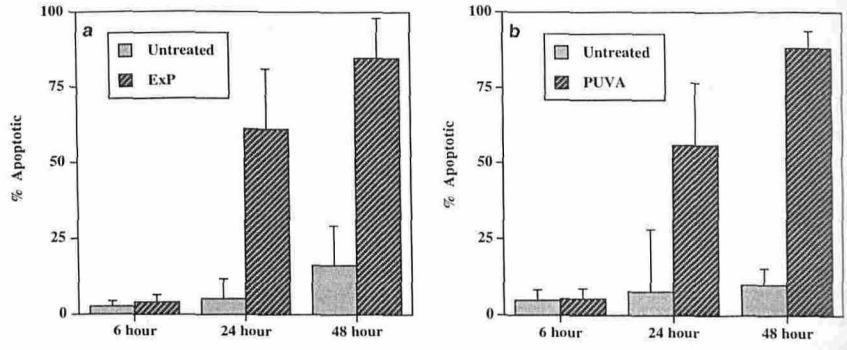

Figure 3. Kinetics of apoptotic cell appearance following PCT. PBL treated with ExP (*A*) or 8-MOP (200 ng per ml)/UVA (2 J per cm^2) (*B*) were examined for apoptotic cells by flow cytometry at 6, 24, and 48 h after treatment. Untreated cells were obtained prior to ExP and cultured under the identical conditions as the treated cells. Data presented are the mean ± SD of a minimum of six subjects with Sezary Syndrome.

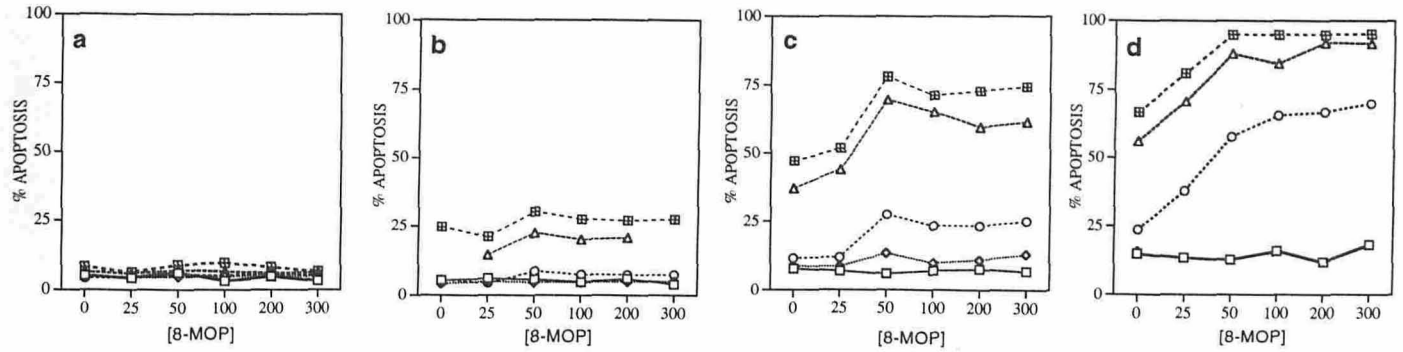

Figure 4. Effect of 8-MOP concentration and UVA dose on induction of apoptosis. A checkerboard titration of 8-MOP (0–300 ng per ml) and UVA (0.0–3.0 J per cm^2) was utilized to determine the minimum combination necessary to induce apoptosis. PBL were examined for apoptotic changes by flow cytometry at 6 h (*a*), 12 h (*b*), 24 h (*c*), and 48 h (*d*). UVA dosage: 0 (—□—), 0.5 (—◇—), 1.0 (—○—), 2.0 (—△—), 3.0 (—⊞—). Data presented are representative of that obtained from three subjects.

normal or patient (CTCL) PBMC and their susceptibility to apoptosis subsequent to either ExP or PUVA treatment. In order to ensure that the cells being examined were lymphocytes, simultaneous two-color staining with CD45-FITC/CD14-PE and light scatter measurements were used (Loken *et al*, 1990). In normal and CTCL lymphocytes, we observed the characteristic apoptotic shift (decrease FSC/increase SSC) in different peripheral blood T-cell subpopulations (CD4+/CD7+ and CD8+) including the putative malignant clone from the CTCL patients (CD4+/CD7-) (40, 41) subsequent to ExP treatment **(Fig 5*a-e*)**. Of particular note was the observation that the malignant clone from a Sezary syndrome patient, as identified by a monoclonal anti-Vβ20 antibody, was also susceptible to apoptosis induction by ExP **(Fig 5*d*)**. In contrast, no shift in the monocyte population (CD14+/CD45+) was observed, suggesting that these cells may be more resistant to induction of apoptosis by ExP or PUVA treatment **(Fig 5*f*)**.

Phagocytosis of ExP and PUVA-Treated Cells Is Not MHC-Restricted Previous investigations have shown that apoptotic cells undergo specific changes including alteration of surface carbohydrates (Morris *et al*, 1984), and expression of vitronectin (Savill *et al*. 1990) or phosphatidylserine ligands (Fadok *et al*, 1992a, 1992b), which allow them to be bound and phagocytized by macrophages in a non-MHC restricted manner (Duvall *et al*, 1985; Savill *et al*, 1993). To determine whether the PCT-induced apoptotic cells were phagocytized at an increased frequency as compared to untreated cells, 4–5 day cultures of monocyte-derived macrophages were incubated with ExP-treated or untreated nonadherent PBMC. When macrophages were incubated with ExP-treated PBMC, we observed phagocytosis of apoptotic cells by the majority of macrophages (two to three apoptotic cells per macrophage). When untreated PBMC were added to the macrophage cultures, however, phagocytized cells were not observed. This difference was not due to settling rates of apoptotic *versus* normal cells because normal cells settled within 20 min. Moreover, ExP-treated monocytes that were cultured for 5 days were also able to phagocytize apoptotic cells but not untreated cells (data not shown). This further suggests monocytic recalcitrance to PCT.

Electron micrographs of PBMC 24 h post-ExP treatment were examined for evidence of apoptotic phagocytosis. Several instances of phagocytosis of apoptotic cells and probable apoptotic 'blebs' were identified **(Fig 6)**, further supporting the observation of phagocytosis of PCT-treated cells.

DISCUSSION

In this study we have demonstrated that PCT is a potent inducer of apoptosis in PBL. Whereas this was accomplished by utilizing several methodologies, the most sensitive and objective means of determining apoptotic cells was flow cytometry. This approach offers important advantages for the study of apoptosis in that it can reliably and accurately quantify apoptosis and can also be combined with labeling of surface antigens in order to identify specific cell subpopulations in a heterogeneous population. Several investigators have observed this characteristic "apoptotic shift" of cells from

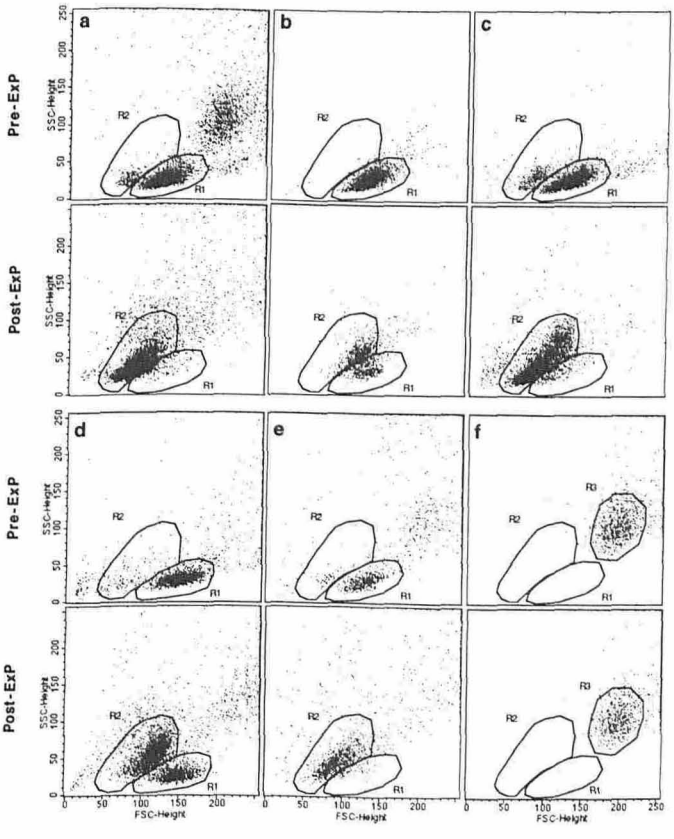

Figure 5. Flow cytometric analysis of apoptosis in lymphocyte subpopulations and monocytes. PBMC from Sezary Syndrome patients were stained with specific fluorochrome-labeled monoclonal antibodies and then gated for the positively staining population. The *top panel* of each group represents the pre-treatment cells; *bottom panel* represents the cells 24 h after PCT. R1 and R2 gates are identical for each group. In each group except f, a characteristic apoptotic shift is observed. *a)* CD4+/CD7+ cells. *b)* CD4+/CD7- cells. *c)* CD4−/CD7+ cells. *d)* Vβ20+ cells (clone from Sezary Syndrome patient) *e)* CD8+ cells. *f)* CD14+ cells; no shift in the population is observed (lymphocyte gates are included for comparison). Data presented are representative of that obtained from five subjects.

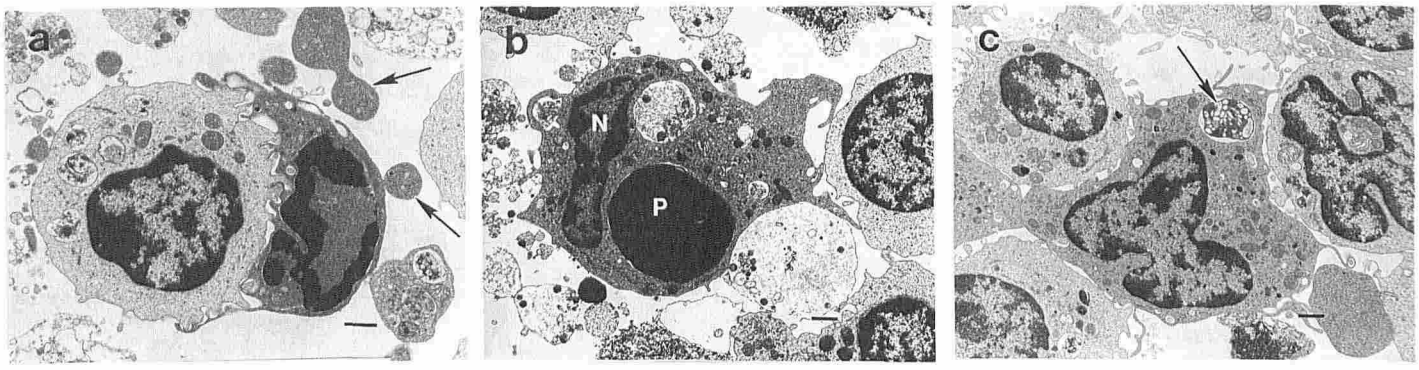

Figure 6. Electron microscopy of phagocytosis of PUVA-treated PBMC. Venous blood was collected from Sezary syndrome patients and treated with PUVA. PBMC were isolated and visualized by EM. *a)* Phagocytosis of an apoptotic lymphocyte by peripheral blood monocyte. Arrows indicate probable apoptotic 'blebs' (*scale bar*, 1 μm). *b)* Apoptotic lymphocyte completely engulfed by peripheral blood monocyte. N = monocyte nucleus, p = phagocytized apoptotic lymphocyte (*scale bar*, 1 μm). *c)* Phagocytosis of probable apoptotic 'bleb' (→; *scale bar*, 1 μm).

relatively high FSC/low SSC to low FSC/high SSC in apoptotic thymocytes and selected human-derived cell lines (Swat *et al*, 1991; Darzynkiewicz *et al*, 1992; Dive *et al*, 1992; Huschtscha *et al*, 1994).

To confirm that the "shifted" population of cells were truly apoptotic and not merely cell debris or necrotic cells, an adaptation of the TUNEL assay for flow cytometry was used. This method is based on fluorescent-labeling of DNA strand breaks that are associated with apoptosis. Using back-gating analysis, we confirmed that the population of cells with the highest fluorescence, i.e., more DNA strand breaks, was also the same population of cells that had "shifted" due to a low FSC/high SSC alteration. Because DNA fragmentation is often an early event in apoptosis, this assay appears to be a more sensitive early indicator of apoptosis as compared to morphologic changes (Gorczyca *et al*, 1994). This was evident in our comparisons of flow cytometry, electron microscopy, and fluorescent microscopy.

Whereas previous studies have indicated that DNA damage as a result of 8-MOP and UVA treatment leads to cell death (Gasparro *et al*, 1989; Marks and Fox, 1991), this study establishes that the minimum concentrations of 8-MOP and UVA necessary to induce apoptosis in PBL from patient and normal volunteers is well within the range delivered during ExP. Generation of a dose-response curve showed that the minimum combination of UVA and 8-MOP that was necessary to induce greater than background levels of apoptosis within 24 h of treatment was 1.0 J UVA per cm^2 and 50 ng 8-MOP per ml. We previously showed that cell division of T cells can almost be completely blocked by 1 J UVA per cm^2 in the presence of 50 ng 8-MOP per ml (Gasparro *et al*, 1989). Moreover, these data are consistent with clinical observations that patient plasma levels of 8-MOP must exceed 50 ng per ml during the period of UVA irradiation (1.0–2.0 J per cm^2) in order to exert a therapeutic effect (Wagner *et al*, 1979; Edelson *et al*, 1987).

One of the most striking observations was that UVA alone at doses $\geq$ 2 J per cm^2, but not 8-MOP alone (0–300 ng per ml), induced significant apoptosis in the PBL within 24–48 h. Whereas the percentage of apoptotic PBL induced by UVA alone was not as great as that of 8-MOP and UVA in combination, this observation may have major ramifications regarding the role of sunlight in the induction of apoptosis in cutaneously trafficking PBL. Sunlight is the most common source of UV exposure; however, UVC rays do not reach the earth and UVB represents only a minor component with UVA present at 100-fold levels over UVB. Although UVA is 1000 times less effective in inducing erythema, which can occur in prolonged exposure, the majority of sunscreens do not block it. The observation that exposure of lymphocytes to a single, low dose of UVA ($\geq$ 2.0 J per cm^2) results in apoptosis might suggest that sunlight, or other UVA exposure such as that incurred in tanning booths, could destroy cutaneous infiltrating lymphocytes. This is particularly relevant given that approximately 20% of UVA can penetrate to the level of the dermal vasculature (Everett *et al*, 1966), and that an individual receives approximately 16 J UVA per cm^2 with a 1-h summer sun exposure in Philadelphia. UVA-induction of apoptosis in lymphocytes may be of benefit in conditions such as psoriasis; however, it could be deleterious in other situations such as when the lymphocytes are trafficking in response to an infectious agent. Furthermore, if low doses of UVA can induce apoptosis in a population of lymphocytes via DNA damage and inasmuch as it has demonstrated ability to cause mutagenesis (Gasparro *et al*, 1993), it is likely that UVA can also have mutagenic effects on lymphocytes. This may represent a possible means of triggering certain types of leukemias or lymphomas. Although no direct evidence for this exists, several epidemiologic studies suggest that there is a link between the development of non-Hodgkin's lymphoma (NHL) and UV exposure (Fisher, 1994; Stewart, 1994; Stellar, 1995). Most recently, Adami and colleagues examined the possibility of a link between UV light and non-Hodgkin's lymphoma (NHL) (including chronic lymphocytic leukemia) by using skin cancer (melanoma or squamous cell carcinoma) as surrogate markers for UV exposure. In surveying more than 110,000 Danish and Swedish individuals with either NHL or skin cancer, a strong association between NHL and skin cancer, particularly squamous cell carcinoma, was observed (Fisher, 1994).

Of particular importance to the treatment of Sezary syndrome by ExP was whether the malignant clone was differentially affected, i.e., became apoptotic, as compared to other lymphocytic populations. Using immunophenotyping and flow cytometry, we found that there was no difference in the susceptibility of the different T-cell subpopulations (CD4+/CD7+ and CD8+) to apoptosis following PCT treatment. More importantly, these data show that the malignant clone found in the peripheral blood of Sezary syndrome patients undergoes apoptosis at a rate similar to the other subpopulations subsequent to PCT treatment. This may have important clinical implications. Sezary syndrome patients treated with ExP maintain their absolute number of normal T cells with a disproportionately greater decrease in their total body burden of malignant cells (Edelson *et al*, 1987). These data indicate that although the normal T cells undergo apoptosis as a result of ExP, they are quickly replaced by new T cells at a rate that is substantially greater than the rate at which malignant cells migrate to the peripheral blood. In this regard, we have previously shown that in some Sezary syndrome patients the malignant clone disappears completely from the peripheral blood in response to ExP therapy (Rook *et al*, 1991, 1993). This suggests that apoptosis may be an essential pathway by which ExP mediates its clinical efficacy. Because ExP treats only 2–5% of the total body lymphocyte population (Lee and Garro, 1989), this may explain why it is often necessary for patients to undergo many rounds of therapy to achieve a clinical response. Moreover, we have observed that in

order for ExP to be clinically effective in CTCL, a malignant clone must be present in the peripheral blood (Rook and Vowels, unpublished observations).

An important aspect of apoptosis is that cells undergoing this phenomenon are recognized by phagocytes and taken up before the release of intracellular contents, protecting the surrounding tissue from potential tissue damage (Cohen and Duke, 1992). In our investigations, monocytes appeared to be resistant to apoptosis, as defined by cellular light scattering properties by flow cytometry and fluorescent microscopy, subsequent to PCT treatment. Furthermore, ExP-treated, as well as untreated, monocytes that were cultured for 5 days were able to recognize and phagocytize ExP-induced apoptotic PBL, suggesting no alterations in monocyte function. This resistance to apoptosis may be due to the increased elaboration of GM-CSF by ExP-treated monocytes (Vowels and Rook, unpublished observations), because GM-CSF has been demonstrated to inhibit apoptosis of human monocytes (Strasser *et al*, 1994). One hypothesis on the mechanism for the clinical efficacy of ExP is that an antigen associated with the malignant clonal population, possibly the unique T-cell receptor, is altered as a result of ExP such that an immune response can be specifically generated to remove the malignant clone from the body. Such a scenario would require antigen uptake, processing, and presentation and could be enhanced as a result of macrophage uptake of apoptotic cells.

An alternative hypothesis to the generation of clone-specific immunity is that ExP may result in the cellular elaboration of apoptosis-inducing factor which would be capable of causing apoptosis in cells not treated by ExP. In this regard, tumor necrosis factor-alpha (TNF-α) has been demonstrated to induce apoptosis in certain cell types including tumor cell lines (Wong and Goeddel, 1994). Our previous studies have demonstrated that significant amounts of TNF-α are produced by monocytes following ExP, which also suggests that monocytes are resistant to apoptosis induction by PUVA (Vowels *et al*, 1991). The observation that TNF can induce apoptosis suggests a possible physiologic role for the elaboration of this cytokine in the therapeutic effectiveness of ExP.

As these various hypotheses are tested or as new hypotheses are formulated, it will be important to keep in mind that nearly all PBL treated with ExP will undergo apoptosis and as such cannot act in an effector fashion. Morphologic changes associated with apoptosis are not apparent until approximately 12 h post-treatment, but the expression of ligands necessary for recognition by phagocytes may occur earlier. As a result, PBL treated by ExP would be removed from the circulation soon after reinfusion. In this regard, attempts to identify apoptotic cells in the peripheral blood of patients at various timepoints (1, 6, and 24 h) after reinfusion of ExP-treated PBMC were uniformly unsuccessful (Vowels *et al*, unpublished observations).

Although we have described the induction of apoptosis by PCT, it remains unknown how PCT induces apoptosis. Studies by Marks and Fox have shown that PUVA increases Ca^{2+} uptake and poly (ADP-ribose) synthetase activity and that a protein synthesis inhibitor, cyclohexamide, can decrease the incidence of PUVA-induced apoptosis in T-cell lines (Marks *et al*, 1990; Marks and Fox, 1991). Our observation that induction of apoptosis by PUVA is titratable suggests that additional factors such as stage of cell cycle or expression of specific receptors, such as CD95, may be important determinants in whether a cell enters the apoptotic pathway. Apoptosis induction by UVC and UVB radiation is accompanied by a significant upregulation of the tumor suppressor gene, p53, a finding we have recently demonstrated in lymphocytes from patients treated with ExP (Gasparro and Vowels, manuscript in preparation). Whether p53, in either wild type or mutant form, plays a role in apoptosis induction by UVA or PUVA remains to be determined.

We dedicate this manuscript to the memory of our senior author, Dr. Benjamin R. Vowels, whose sudden and untimely death has left us with his legacy of enthusiasm for discovery, dedication to science, and loyalty to his colleagues. We also thank Dr. Jonni Moore, Dr. Floyd Fox, Hank Pletcher, Ray Douglas, and Donna Frazier for technical assistance and helpful discussions with flow cytometry.

This work was supported by National Institutes of Health Training Grant CA4 0008 (to E.K.Y.) and National Institutes of Health Grant RO1 CA58841 (to A.H.R.).

REFERENCES

Arends M, Morris R, Wyllie A: Apoptosis: the role of the endonuclease. *Am J Pathol* 136:593–608, 1990

Cohen JJ: Programmed cell death in the immune system. *Adv Immunol* 50:55–83, 1991

Cohen JJ: Overview: mechanisms of apoptosis. *Immunol Today* 14:126–130, 1993

Cohen JJ, Duke RC: Apoptosis and programmed cell death in immunity. *Ann Rev Immunol* 10:267–293, 1992

Costanzo-Nordin MR, Hubbell EA, O'Sullivan EJ, Johnson MR, Mullen GM, Heroux AL, Kao WG, McManus BM, Pifarre R, Robinson JA: Successful treatment of heart transplant rejection with photopheresis. *Transplant* 53:808–815, 1992

Darzynkiewicz Z, Bruno S, DelBino G, Gorczyca W, Hotz MA, Lassota P, Traganos F: Features of apoptotic cells measured by flow cytometry. *Cytometry* 13:795–808, 1992

Dive C, Gregory CD, Phipps D, Evan DL, Milner AE, Wyllie AH: Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim Biophys Acta* 1133:275–285, 1992

Dive C, Hickman JA: Drug-target interactions: only the first step in the commitment to a programmed cell death? *Br J Cancer* 64:192–196, 1991

Duke RC, Cohen JJ: Morphological and biochemical assays of apoptosis, In: Coligan JC, Kruisbeek AM, Margulies DH, Shevach EM, Strober W (eds) *Current Protocols in Immunology*. Vol. 1. John Wiley & Sons, Inc., New York, 1992, pp 3.17.11–13.17.13.

Duvall E, Wyllie AH, Morris RG: Macrophage recognition of cells undergoing programmed cell death (apoptosis). *Immunology* 56:351–358, 1985

Edelson R, Berger C, Gasparro F, Jegasothy B, Heald P, Wintroub B, Vonderheid E, Knobler R, Wolff K, Plewig G, McKierman G, Christiansen I, Oster M, Honigsmann H, Wilford H, Kokoschaka E, Rehle T, Perez M, Stingl G, LaRoche L: Treatment of cutaneous T cell lymphoma by extracorporeal photochemotherapy: Preliminary results. *N Engl J Med* 316:297–303, 1987

Edelson RL: Cutaneous T cell lymphoma: mycosis fungoides, Sezary syndrome, and other variants. *J Am Acad Dermatol* 2:89–106, 1980

Everett M, Yeargers E, Sayre R, Olson R: Penetration of epidermis by ultraviolet rays. *Photochem Photobiol* 5:533–542, 1966

Fadok VA, Campbell DR, Cohen JJ, Bratton DL, Henson PM: Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148:2207–2216, 1992a

Fadok VA, Savill JS, Haslett C, Bratton D, Doherty D, Campbell PA, Henson PM: Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol* 149:4029–4035, 1992b

Fisher DE: Apoptosis in cancer therapy: Crossing the threshold. *Cell* 78:539–542, 1994

Gasparro F, Dall'amico R, Goldminz D, Simmons E, Weingold D: Molecular aspects of extracorporeal photochemotherapy. *Yale J Biol Med.* 62:579–593, 1989

Gasparro FP: *Extracorporeal Photochemotherapy: Clinical Aspects and the Molecular Basis for Efficacy*. RG Landes, Georgetown TX, 1994

Gasparro FP, Malane MS, Maxwell VM, Tigelarr RE: The treatment of mastocytoma cells with 8-methoxypsoralen and long wavelength ultraviolet radiation enhances cellular immunogenicity: preliminary results. *Photochem Photobiol* 58:682–688, 1993

Gilchrest BA: Methoxsalen photochemotherapy for mycosis fungoides. *Cancer Treat Rep* 63:663–667, 1979

Gilchrest BA, Parrish JA, Tanenbaum L, Haynes HA, Fitzpatrick TB: Oral methoxsalen photochemotherapy for Mycosis Fungoides. *Cancer* 38:683–689, 1976

Goldman AS, Baker MK, Piddington R, Herold R: Inhibition of programmed cell death in mouse embryonic palate *in vitro* by cortisol and phenytoin: receptor involvement and requirement of protein synthesis. *Proc Soc Exp Biol Med* 174:239–243, 1983

Gorczyca W, Bigman K, Mittelman A, Ahmed T, Gong J, Melamed MR, Darzynkiewicz Z: Induction of DNA strand breaks associated with apoptosis during treatment of leukemias. *Leukemia* 7:659–670, 1993a

Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynkiewicz Z: The cell cycle related difference in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res* 53:3186–3192, 1993b

Gorczyca W, Tuziak T, Kram A, Melamed MR, Darzynkiewicz Z: Detection of apoptosis-associated DNA strand breaks in fine-needle aspiration biopsied by *in situ* end labeling of fragmented DNA. *Cytometry* 15:169–175, 1994

Hickman JA: Apoptosis induced by anticancer drugs. *Cancer Metast Rev* 11:121–139, 1992

Honigsmann H, Brenner W, Rauschmeier W, Konrad K, Wolff K: Photochemotherapy for cutaneous T cell lymphoma. A follow up study. *J Am Acad Dermatol* 10:238–245, 1984

Huschtscha LI, Jeitner TM, Andersson CE, Bartier WA, Tattersall MHN: Identifica-

tion of apoptotic and necrotic human leukemic cells by flow cytometry. *Exp Cell Res* 212:161–165, 1994

Kerr JFR, Winterford CM, Harmon BV: Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 73:2013–2016, 1994

Lee KH, Garro J: Engineering aspects of extracorporeal photochemotherapy. *Yale J Biol Med* 62:621–628, 1989

Loken M, Brosnan J, Bach B, Ault K: Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. *Cytometry* 11:433–439, 1990

Marks DI, Fox RM: Mechanisms of photochemotherapy-induced apoptotic cell death in lymphoid cells. *Biochem Cell Biol* 69:754–760, 1991

Marks DI, Rockman SP, Oziemski MA, Fox RM: Mechanisms of lymphocytotoxicity induced by extracorporeal photochemotherapy for cutaneous T cell lymphoma. *J Clin Invest* 86:2080–2085, 1990

Martin DP, Johnson FM: Programmed cell death in the peripheral nervous system. In: Tomei LD, Cope FO (eds) *Apoptosis: The Molecular Basis of Cell Death*. Vol. 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1991, pp 263–278

Morris RG, Hargreaves AD, Duvall E, Wyllie AH: Hormone-induced cell death. 2. Surface changes in thymocytes undergoing apoptosis. *Am J Pathol* 115:426–436, 1984

Parrish JA, Fitzpatrick TB, Tanenbaum L, Pathak MA: Photochemotherapy of psoriasis with oral methoxsalen and long wave ultraviolet light. *N Engl J Med* 291:1207–1211, 1974

Redding TW, Shally AV, Radulovic S, Milovanovic S, Szepshazi K, Isaacs JT: Sustained release formulations of leuteinizing hormone-releasing hormone antagonist SB-75 inhibit proliferation and enhance apoptotic cell death of human prostate carcinoma (PC-82) in male nude mice. *Cancer Res* 52:2538–2534, 1992

Roenigk HH: Photochemotherapy for mycosis fungoides: long-term follow-up study. *Cancer Treat Rep* 63:669–673, 1979

Rook AH, Cohen JH, Lessin SR, Vowels BR: Therapeutic applications of photopheresis. *Dermatol Clin* 11:339–347, 1993

Rook AH, Freundlich B, Jegasothy BV, Perez M, Barr W, Jimenez S, Rietschel R, Wintroub B, Kahaleh MB, Varga J, Heald P, Steen V, Massa M, Murphy GF, Perniciaro C, Istfan M, Ballas SK, Edelson RL: Treatment of systemic sclerosis with extracorporeal photochemotherapy: results of a multicenter trial. *Arch Dermatol* 128:337–346, 1992

Rook AH, Prystowsky MB, Cassin M, Boufal M, Lessin SR: Combined therapy of the Sezary syndrome with extracorporeal photochemotherapy and low dose interferon alpha: clinical, molecular, and immunologic observations. *Arch Dermatol* 127:1535–1540, 1991

Savill J, Dransfield I, Hogg N, Haslett C: Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature* 343:170–173, 1990

Savill J, Fadok V, Henson P, Haslett C: Phagocyte recognition of cells undergoing apoptosis. *Immunol Today* 14:131–136, 1993

Sellins KS, Cohen JJ Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J Immunol* 139:3199–3206, 1987

Sen S, D'Incalci M: Apoptosis: biochemical events and relevance to cancer chemotherapy. *FEBS Lett* 307:122–127, 1992

Sgonc R, Boeck G, Dietrich H, Gruber J, Recheis H, Wick G: Simultaneous determination of cell surface antigens and apoptosis. *Trends Genet* 10:41–42, 1994

Song PS, Tapley KJ: Photochemistry and photobiology of psoralens. *Photochem Photobiol* 29:1177–1197, 1979

Stellar H: Mechanisms and genes of cellular suicide. *Science* 267:1445–1449, 1995

Stewart BW: Mechanisms of apoptosis: integration of genetic, biochemical, and cellular indicators. *J Natl Cancer Inst* 86:1286–1295, 1994

Strasser A, Harris AW, Jacks T, Cory S: DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by bcl-2. *Cell* 79:329–339, 1994

Swat W, Ignatowicz L, Kisielow D: Detection of apoptosis of immature CD4+8+ thymocytes by flow cytometry. *J Immunol Methods* 137:79–87, 1991

Vowels B, Cassin M, Boufal M, Walsh L, Rook A: Extracorporeal photochemotherapy induces production of tumor necrosis factor and IL-6 by adherent peripheral blood mononuclear cells. *J Invest Dermatol* 96:585, 1991

Wagner G, Hofmann C, Busch U, Schmid J, Plewig G: 8-MOP plasma levels in PUVA problem cases with psoriasis. *Br J Dermatol* 101:285–292, 1979

Warwick LM, Parrish J, McAuliffe DJ, Bloch KJ: Sensitivity of mononuclear cells to PUVA: effect on subsequent stimulation with mitogens and on exclusion of trypan blue dye. *Clin Exp Dermatol* 6:273–277, 1981

Wieselthier JS, Koh HK: Sezary syndrome: diagnosis, prognosis, and critical review of treatment options. *J Am Acad Dermatol* 22:381–401, 1990

Wong GH, Goeddel DV: Fas antigen and p55 TNF receptor signal apoptosis through distinct pathways. *J Immunol* 152:1751–1755, 1994

Zamai L, Falcier E, Zauli G, Cataldi A, Vitale M: Optimal detection of apoptosis by flow cytometry depends on cell morphology. *Cytometry* 14:891–897, 1993