

Apoptotic Depletion of CD4+ T Cells in Idiopathic CD4+ T Lymphocytopenia

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Abstract

Progressive loss of CD4+ T lymphocytes, accompanied by opportunistic infections characteristic of the acquired immune deficiency syndrome, has been reported in the absence of any known etiology. The pathogenesis of this syndrome, a subset of idiopathic CD4+ T lymphocytopenia (ICL), is uncertain. We report that CD4+ T cells from seven of eight ICL patients underwent accelerated programmed cell death, a process facilitated by T cell receptor cross-linking. Apoptosis was associated with enhanced expression of Fas and Fas ligand in unstimulated cell populations, and partially inhibited by soluble anti-Fas mAb. In addition, apoptosis was suppressed by aurintricarboxylic acid, an inhibitor of calcium-dependent endonucleases and proteases, in cells from four of seven patients. The *in vivo* significance of these findings was supported by three factors: the absence of accelerated apoptosis in persons with stable, physiologic CD4 lymphopenia without clinical immune deficiency; detection of serum antihistone H2B autoantibodies, one consequence of DNA fragmentation, in some patients; and its selectivity, with apoptosis limited to the CD4 population in some, and occurring among CD8+ T cells predominantly in those individuals with marked depletion of both CD4+ and CD8+ peripheral T lymphocyte subsets. These data suggest that patients with idiopathic loss of CD4+ T lymphocytes linked to clinical immune suppression have evidence for accelerated T cell apoptosis *in vitro* that may be pathophysiologic and amenable to therapy with apoptosis inhibitors. (*J. Clin. Invest.* 1996. 97:672–680.) Key words: aurintricarboxylic acid • cell death, programmed • immunologic deficiency syndromes • T lymphocyte

Introduction

Marked CD4+ T lymphopenia in the absence of HIV infection has been documented by our group (1) and others (2, 3). This syndrome is termed idiopathic CD4+ T lymphocytopenia (ICL)¹ by the U.S. Centers for Disease Control and Prevention

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1. Abbreviations used in this paper: ATA, aurintricarboxylic acid; ICL, idiopathic CD4+ T lymphocytopenia; PHA, phytohemagglutinin; PKA, protein kinase A; RT, reverse transcriptase; TMX, tamoxifen.

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(2), and severe unexplained HIV seronegative immune suppression (SUHIS) by the World Health Organization (4). It is defined by an absolute CD4 count $< 300/\text{mm}^3$ or $< 14\%$, either alone (ICL) or accompanied by clinical signs and symptoms of cellular immune deficiency (SUHIS). The etiology of this syndrome is unclear and, given the lack of risk factors for acquisition of HIV infection in most individuals, absence of a clear epidemiologic pattern, diversity of prodromes, and transient nature of many cases, almost certainly multifactorial.

We recently distinguished a subset of ICL patients mimicking HIV disease: severe, prolonged decrement in peripheral CD4+ T cells with progressive decline in such counts documented in some; CD4/CD8 T cell ratios ≤ 1 ; and a history of opportunistic infections (5, 6). This finding served as the impetus to investigate mechanisms associated with T cell loss in HIV infection and other disorders which might also be etiologic in ICL.

Apoptosis of T lymphocytes *in vitro*, linked to transient T cell loss *in vivo*, is a consequence of certain viral infections, including lymphocytic choriomeningitis (7) and vaccinia (8) in mice and EBV (9) in humans. It has also been reported in association with HIV-1 (10, 11) in humans, although whether or not direct HIV infection is required for this effect, and its specificity for CD4+ T cells, are subjects of debate (10–12). We sought to test the hypothesis that apoptosis plays a role in ICL associated with clinical immune deficiency and attempted to relate the accelerated *in vitro* death of CD4+ T cells from these individuals with expression of two molecules linked to programmed cell death, Fas and Fas ligand (13). We also evaluated the ability of anti-Fas mAb and two drugs, aurintricarboxylic acid (ATA) and tamoxifen (TMX), known inhibitors of two common pathways to apoptosis, Ca^{2+} -dependent endonuclease activation and cAMP signalling, respectively, to mitigate this cell death. *In vivo* correlates were sought by assays for circulating antihistone H2B autoantibodies, one consequence of DNA fragmentation (12), and examination of lymphocytes from patients with stable, “physiologic” CD4 lymphopenia. Our results suggest the discrimination of a subset of ICL patients with sustained T cell apoptosis that is clinically relevant and may be amenable to therapy with inhibitors of programmed cell death.

Methods

Patient selection. PBMC were prepared from heparinized venous blood of eight ICL patients and four controls by density gradient centrifugation (14). The clinical data and risk factors for retroviral infection, if any, are shown in Table I. Only one of the ICL patients, ICL-1, has been previously reported (1). All subjects were seronegative for HIV-1, -2, and human T cell lymphotropic virus types I, II by ELISA and, HIV-1, by immunoblotting. No patient had an active infection at the time of study. All were receiving trimethoprim-sulfamethaxazole prophylaxis against *Pneumocystis carinii* pneumonia. ICL-2, ICL-3, and ICL-8 were also taking fluconazole as prophylaxis against cryptococcosis. Ig isotype levels were normal in all individuals except for

Table I. Summary of Case Histories and DNA Histogram Analysis

Subject	Age/sex	Risk factors for retroviral infection	Clinical findings	Lymphocyte phenotype per mm ³			Cell cycle Analysis
				CD4+	CD8+	CD4/CD8 ratio	CD4+ apoptotic peak* peak (A ₀) percent
ICL-1	44/M	Needlestick; bisexual	<i>Herpes zoster</i> , intractable papillomatosis, Bowen's disease	124	125	0.99	43.8
ICL-2	35/F	Needlestick	Cryptococcal meningitis	100	1200	0.08	39.0
ICL-3	55/M	Worker, biohazardous waste disposal co.	Cryptococcal meningitis, B cell lymphoma	73	350	0.21	3.8
ICL-4	32/M	None	<i>Herpes simplex</i> , fever	110	244	0.45	33.8
ICL-5	43/F	None	<i>Pneumocystis carinii</i> pneumonia	120	133	0.90	39.9
ICL-6	27/M	None	Cryptococcal meningitis	22	51	0.43	19.4
ICL-7	35/F	Needlestick	Thrush, progressive, global dementia	157	365	0.43	19.2
ICL-8	34/F	None	Cryptococcosis	104	100	0.96	30.7

* Determined by DNA histogram analysis of CD4+ T lymphocytes in PBMC maintained in culture for 72 h in the absence of exogenous stimuli.

ICL-1, who had a monoclonal IgM spike on immunofixation. This had been noted for the past seven years, without evidence for malignancy. No evidence for active EBV or cytomegalovirus infection by culture or serology was noted in any patient at the time of study. PBMC cocultures with assay for HIV-1,2 p24 Gag antigen, and particulate reverse transcriptase in culture supernatants, and amplification of PBMC DNA for HIV-1,-2 by PCR using *gag* primers (SK145 and SK431, Perkin-Elmer, Foster City, CA), performed as previously described (1, 14), were negative.

T cell subset analysis and CD4/CD8 ratios are recorded in Table I. All patients were followed for at least 12 mo (range 12–52 mo), over which time they had sustained decrements in CD4 count or progressive CD4+ T cell loss. Controls, two females and two males age 32–44, had CD4+ T cell counts > 1,000/mm³ (normal limits, 830±288, range 410–1,540 [15]). An additional control, a 36 year old homosexual male, was originally reported (“Case 5”) as an instance of ICL based upon two CD4+ T cell counts < 300/mm³ and severe *Mycobacterium tuberculosis* pneumonia (1). However, his CD4/CD8 ratio remained > 1 and, 6 mo subsequent to successful antitubercule therapy, the CD4 count returned to the low normal range. This transient decrease in CD4 count is characteristic of a small segment of otherwise normal individuals with pulmonary or miliary tuberculosis (5).

Apoptosis assays. PBMC were washed in PBS, pH 7.2, assessed for viability by trypan blue dye exclusion, and plated at 0.5 × 10⁶ viable cells/ml, in culture medium alone (RPMI 1640 plus 10% FBS) or with various reagents, for 12–120 h. Cells were harvested, washed, and labeled with anti-CD4 mAb Leu-3a, followed by exposure to FITC-conjugated goat anti-mouse Ig. They were then fixed in 70% cold ethanol, incubated for 20 min at 4°C with propidium iodide (50 µg/ml) in the presence of RNaseA (300 U/ml) (16), and 5–10 × 10³ cells were analyzed in an EPICS Elite cytofluorograph (Coulter Immunology, Hialeah, FL). Controls for antilymphocyte antibodies were included, with no evidence for specific adherence of sera, diluted 1:20, to autologous lymphocytes.

Apoptosis was recognized or quantitated in viable, fluorescence-positive cells by two flow cytometric methods: detection of depressed forward scatter and increased right angle (side) scatter characteristic of apoptotic cells (17) and computer-assisted DNA histogram analysis of propidium iodide-labeled cells with calculation of A₀ peaks (16,

18). All experiments involved at least two patient samples obtained ≥ 2 mo apart, with A₀ values usually varying by ≤15%.

Parallel cell aliquots were analyzed for fragmentation of genomic DNA. Low molecular weight DNA was prepared by lysis of 2 × 10⁶ cells per condition in 0.4 ml of lysis buffer, consisting of 10 mM Tris-HCl, pH 7.5, 0.2% Triton X-100, and 1 mM EDTA, followed by centrifugation and precipitation of the supernatant with 0.1 ml 5 M NaCl and 0.5 ml isopropanol overnight at –20°C (10, 19, 20). Samples were microcentrifuged, pellets washed with 70% ethanol, and air dried. The DNA was then resuspended in Tris-EDTA buffer and treated with RNase A (50 µg/ml) for 1 h at 37°C. Samples were electrophoresed through 1% agarose gels at 50 V/25 mA for 3 h, with bands visualized by ethidium bromide (0.5 µg/ml) staining and photography.

Features of apoptotic cells were also directly visualized. Staining of nucleic acid with acridine orange at low pH can discriminate among viable, apoptotic, and necrotic cells (17). This metachromatic dye intercalates into double-stranded DNA to fluoresce green, and denatured (single-stranded) DNA and RNA to fluoresce red (17). Cells were fixed as described for FACS[®] analysis, washed, resuspended in 0.1 ml PBS, and transferred onto glass slides by cytocentrifugation. Slides were then fixed overnight at room temperature in a 1:9 solution of glacial acetic acid and absolute ethanol, air dried, and treated with 2–3 drops of a 2% solution of Triton X-100 for 2 min. After washing with PBS, 2–3 drops of an 8 µg/ml solution of acridine orange in 1 N NaOH was added for 1 min, and the slides were washed with PBS, and cells observed and photographed using an ultraviolet light-illuminated microscope.

Fas and Fas ligand expression. Fas (APO-1, CD95) and Fas ligand (FasL) expression was determined by reverse transcriptase (RT)-PCR. Oligodeoxynucleotide primers capable of amplifying a segment from nucleotides 271–820 of Fas cDNA (21) or nucleotides 392–1182 of FasL cDNA (22) were used: Fas: Fas-1: 5'-CAAGTGACTGACATCAACTCC and Fas-2: 5'-CCTTGGTTTTCCTTCTGTGC; FasL: No. 13203: 5'-CAGCTCTTCCACCTACAG and No. 13206: 5'-TCATGCTTCTCCCTTTCACATGG.

Total cellular RNAs were isolated from 2 × 10⁶ cells/sample by the TriZOL (GIBCO BRL, Gaithersburg, MD) method (21). RNAs were treated with RNase-free DNase, then reverse transcribed into cDNA, using an MuLV reverse transcriptase (GIBCO BRL). Ali-

quots of the cDNA were then amplified by PCR, as described from our lab (14), using Fas, FasL, or β -actin (sense: 5'-TGACGGGGT-CACCCACACTGTGCCCATCTA; antisense: 5'-CTAGAAGCA-TTTGCGGTGGACGATGGAGGG; Stratagene Inc., La Jolla, CA) primers. Briefly, 50 μ l of reaction volume, including 1 \times reaction buffer (Perkin-Elmer), optimized concentrations of MgCl₂, dNTP and primers, and 2 units of Taq polymerase were used. The cDNAs were denatured for 2 min at 97°C before 35 runs in a thermal cycler, with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min in each cycle. A final extension at 72°C for 5 min was included. PCR products were separated by electrophoresis in a 1.4% agarose gel, visualized by ethidium bromide staining under ultraviolet illumination, and photographed. The expected sizes of the amplicons were: Fas, 549 nucleotides; FasL, 790; and β -actin, 661.

The identity of the FasL product was confirmed by southern blotting (22) using an internal probe (5'-CAGCCCAGTTTCATTGATC). No cross-over in terms of detection of TNF- α receptor or TNF- α expression was seen with these Fas and FasL primers, respectively.

Both Fas and FasL determinations were performed on nonadherent PBMC, prepared as described (14). Control populations of neutrophils and monocytes constitutively express high levels of Fas (23, 24), whereas resting CD4+ T lymphocytes have much less Fas expression (25). FasL is expressed on normal antigen-presenting cells (26).

Fas function. The lysis of a clone of ⁵¹Cr-labeled, CD4+, Fas+ Jurkat T cells, having very low baseline FasL expression, was quantitated following exposure to ICL or control PBMC. These Jurkat cells are highly sensitive to lysis following Fas cross-linking but are resistant to other potential confounding factors such as cytokine-mediated apoptosis (24). The functional meaning of changes in FasL was explored using a panel of mAbs directed to Fas, generated by immunizing mice with a purified fusion protein consisting of the extracellular domain of human Fas and the constant region of human IgG₁ (13). When added in solution, Fas mAb M3 blocks Fas-mediated cell lysis, whereas Fas mAb M31 binds to the antigen but has no agonistic or antagonistic properties (24, 27). 1 \times 10⁴ Jurkat cells, labeled for 3 h with ⁵¹Cr as described (24, 27), were added to varying numbers of effector cells (1:1 to 25:1) in 0.2 ml of culture medium in 96-well round bottom plates. All conditions were established in triplicate. Anti-Fas mAbs were included in certain experiments. After overnight culture at 37°C, 100 μ l of supernatant were harvested from each well, and ⁵¹Cr release determined in a gamma counter. Percent specific killing was calculated according to the formula: $100 \times [(experimental\ cpm) - (spontaneous\ cpm)] / [(maximum\ cpm) - (spontaneous\ cpm)]$, where spontaneous cpm is the counts per min released in medium alone, and maximum cpm is the radioactivity released in the presence of 1% Triton X-100. Spontaneous release represented 7–15% of maximal counts released.

Histone antibody analyses. An ELISA for antihistone antibodies in sera of control and ICL patients, using H1, H2A, and H2B histone preparations from calf thymus (Worthington Biochemicals, Malvern, PA), was performed by standard techniques. Human sera were diluted 1:200, rabbit anti-human Ig diluted 1:2,000 and adsorbed overnight with all histone preparations, and horseradish peroxidase-conjugated goat anti-rabbit Ig used at a dilution of 1:500 (Kirkegaard and Perry, Gaithersburg, MD). Each microtiter well of the ELISA plates was coated with \sim 10 ng of the appropriate histone preparation.

Results

Apoptosis in CD4+ T lymphocytes from ICL patients. Fluorescent-labeled CD4+ T lymphocytes in PBMC derived from seven of eight ICL patients with a history of opportunistic infections exhibited apoptosis exceeding control levels. This was first noted by the criterion of low DNA staining, represented by a pre-G₁ A₀ peak that occupied from 19 to > 40% of the

DNA histogram (Table I). Three control donors gave no evidence for such cell death. These data were obtained after a 72-h culture of PBMC in the absence of exogenous stimuli. At least 12 h of culture was required to detect an A₀ peak. Extended culture periods resulted in an increase in A₀ area for all patients, as described in a subsequent experiment. The one individual, ICL-3, who did not display accelerated apoptosis still had an A₀ peak in the control range after prolonged culture (< 10% at 120 h).

Examples of the DNA histograms used to derive the A₀ values of Table I are presented in Fig. 1. The A₀ peaks for two representative patients, ICL-2 and ICL-7, with profound CD4+ T cell depletion accompanied by normal numbers of CD8+ T cells, are outlined for both total PBMCs and labeled CD4+ T cells. These histograms illustrate the fact that apoptosis appears to be limited to the CD4+ T cell population in these two individuals, with a marked disparity between the A₀ peak of CD4+ cells vs. unselected cell populations (< 4%, Fig. 1) or isolated CD8+ cells (< 5%, data not shown). Three of three controls gave < 3% apoptosis in all cell populations. In contrast, ICL-1, with decreases in absolute numbers of both CD4+ and CD8+ T cells, showed equivalent levels of accelerated apoptosis in both lymphocyte subpopulations (data not shown).

Fig. 2 presents another criterion for apoptosis, changes in light scatter, along with DNA histograms for a control and two ICL patients. In these experiments, a longer period of culture was used so as to be able to determine the impact of mitogenic stimulation on apoptosis. The majority of unstimulated ICL CD4+ T cells demonstrate an apoptotic pattern with depressed forward light scatter (Fig. 2), secondary to cell shrinkage, and elevated side scatter (not shown) due to chromatin condensation with resultant increased granularity. Propidium iodide staining of genomic DNA from fixed aliquots of the same cell populations revealed prominent A₀ regions of low DNA staining, comprising up to 70% of the Leu-3a+ (CD4+) T cell population in patients but < 20% in controls. (Note that the additional culture period led to a general increase in apoptotic index [compare data for patients ICL-4 and ICL-5 in Table I and Fig. 2].) A₀ rose to > 80% in some samples after exposure to the mitogen phytohemagglutinin (PHA, 5 μ g/ml; Fig. 2) or anti-CD3 mAb (100 ng/ml; data not shown) in patient but not control samples. This is consistent with the fact that T cell receptor triggering of cells preprogrammed for apoptosis facilitates endonucleosomal cleavage (18).

Apoptosis was confirmed by direct observation of differential staining of double-stranded vs. denatured, condensed DNA after exposure to acridine orange (data not shown). It was recognized before disruption of plasma membranes, with trypan blue dye exclusion viability > 85% in all samples.

In contrast to these results, apoptosis above control levels was not seen in unstimulated or PHA-activated PBMC from a donor with transient T lymphopenia ("case 5" [1]), tested when his CD4 counts were in the 300/mm³ range as well as after their rise to > 600/mm³. The A₀ region of CD4+ cells was 11.5% at 96 h of culture, similar to that of controls at this time point (< 10%). Comparable values were obtained for two other individuals diagnosed as having ICL based solely upon stable CD4+ T cell counts < 300/mm³ over several years (not shown). As for case 5, their CD4/CD8 ratios were > 1, and they had no clinical signs or symptoms of immune deficiency. This suggests a physiologic T cell lymphopenia, rather than an ongoing pathologic process, in these donors (5).

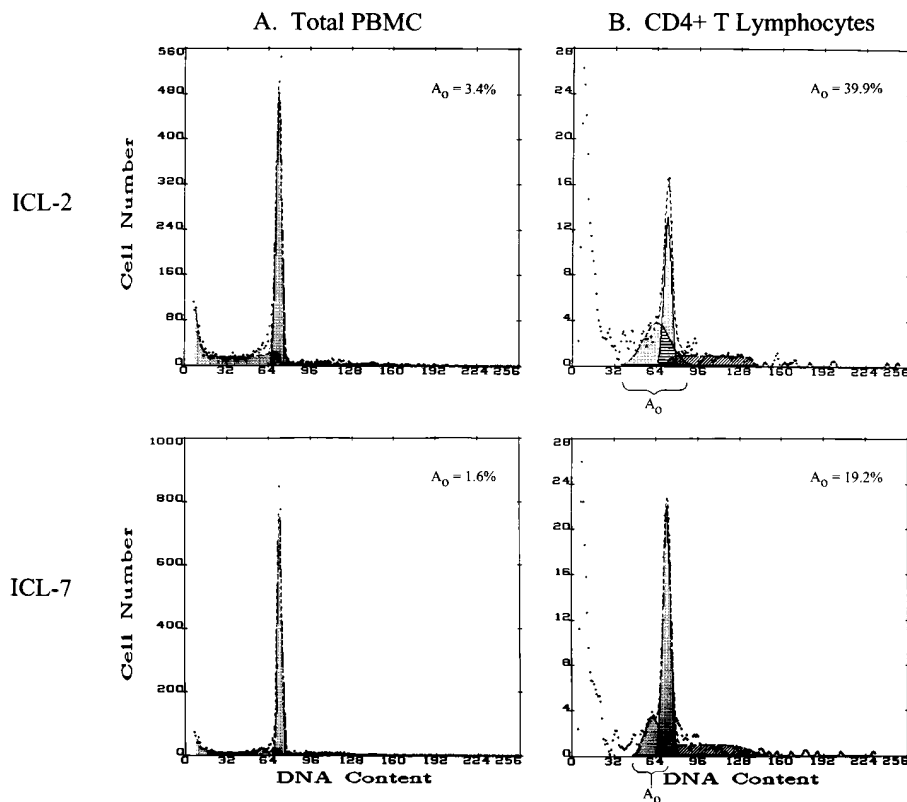


Figure 1. Comparison of DNA histograms for CD4 negative cells vs. CD4+ T lymphocytes in PBMCs derived from ICL patients. < 4% of CD4 negative cells from patient ICL-2 and < 2% of such cells from patient ICL-7 were in the apoptotic, A_0 portion of the cell cycle (lightly shaded, computer-generated peak) after 72 h of culture, while 39.9% of CD4+ cells from ICL-2 and 19.2% from ICL-7 were apoptotic by this criterion at this time point.

DNA fragmentation was further documented by electrophoresis of low molecular weight DNA extracted from ICL PBMCs. Oligonucleosomal units were visible as bands whose molecular sizes are approximate multiples of 180 nucleotides in only one sample, where sufficient cell numbers were available to obtain an adequate quantity of DNA. In the remaining samples, an increase in low molecular weight DNA was seen, but distinct laddering could not be visualized.

Inhibition of ICL associated apoptosis in vitro. We examined the susceptibility of these apoptotic processes to potential inhibitors of two major pathways for programmed cell death: Ca^{2+} -dependent endonucleases that cleave host chromatin in the nucleosomal linker regions, and nuclease induction via a Ca^{2+} -independent, cAMP-directed mechanism (28). Two molecules with divergent mechanisms of action and potential clinical utility were selected. At 50–100 μ M, monomeric and polymeric aurantricarboxylic acid ($C_{22}H_{14}O_9$) inhibits a variety of enzymes that process nucleic acids, including RNA and DNA polymerases, reverse transcriptase, integrase, and exo and endonucleases (29). At lower concentrations (< 3 μ M), ATA continues to suppress Ca^{2+} -mediated endonuclease induction and, as such, has proven a potent inhibitor of apoptosis in cells as disparate as thymocytes (20) and TNF- α stimulated mammary adenocarcinoma (30). Tamoxifen citrate (1-[*p*-dimethylamineorthoxyphenyl]-1, 2-diphenyl-1-butene) is an estrogen antagonist having no effect on nucleases but capable of suppressing calmodulin, cAMP, and protein kinase A (PKA) induction at clinically attainable doses (1–2.5 μ M) (16, 31). cAMP/PKA inhibitors block several triggers to apoptosis in T lymphocytes, independent of Ca^{2+} flux (32). TMX, as its 5-hydroxy derivative, has recently been shown to block apoptosis in some cell types (33).

PBMC from four of the seven ICL patients exhibiting accelerated apoptosis, when exposed to ATA or TMX or both, had a $\geq 50\%$ decrease in apoptotic index, with improvement in light scatter patterns, concomitant with suppression of the A_0 peak (Fig. 2 and Table II). Together with IL-2, concentrations of ATA as low as 0.01 μ M suppressed A_0 by > 40%, with maximal effect at 0.1 μ M (60–90% inhibition). TMX gave an IC_{50} for inhibition of the A_0 peak in both unstimulated and PHA-treated ICL CD4+ T cells of 0.25 μ M, with maximal suppression at 1 μ M (Fig. 2). These results were confirmed by DNA electrophoresis. DNA extracted from ICL PBMC treated with ATA or TMX showed suppression of the low molecular weight fraction of DNA as compared to samples from cells cultured in the absence of drugs (data not shown).

Preexistence of apoptotic mechanisms in ICL. Activation-induced death of CD4+ T cells from HIV-1+ individuals is enhanced by calcium ionophores (34) and blocked by the protein synthesis inhibitor cycloheximide (10, 34), while spontaneous apoptosis in unstimulated HIV+ cultures is unaffected by suppression of protein or RNA synthesis (34). We investigated these phenomena in ICL. Ca^{2+} mobilization after exposure of ICL PBMC to A23187 (1 μ M) for 24 h. led to an increase in DNA fragmentation, while phorbol myristate acetate (5 ng/ml) and cycloheximide (0.5 μ g/ml) did not alter spontaneous apoptosis (data not shown). This is similar to effects in PBMC from HIV+ patients and suggests that T cells from ICL patients possess the complete machinery necessary for accelerated cell death.

Fas and FasL expression. RT-PCR amplification of Fas and Fas ligand mRNA in unstimulated normal donor and ICL cells was performed on aliquots of RNA. Fas was present at very low levels in unstimulated, adherent cell-depleted control

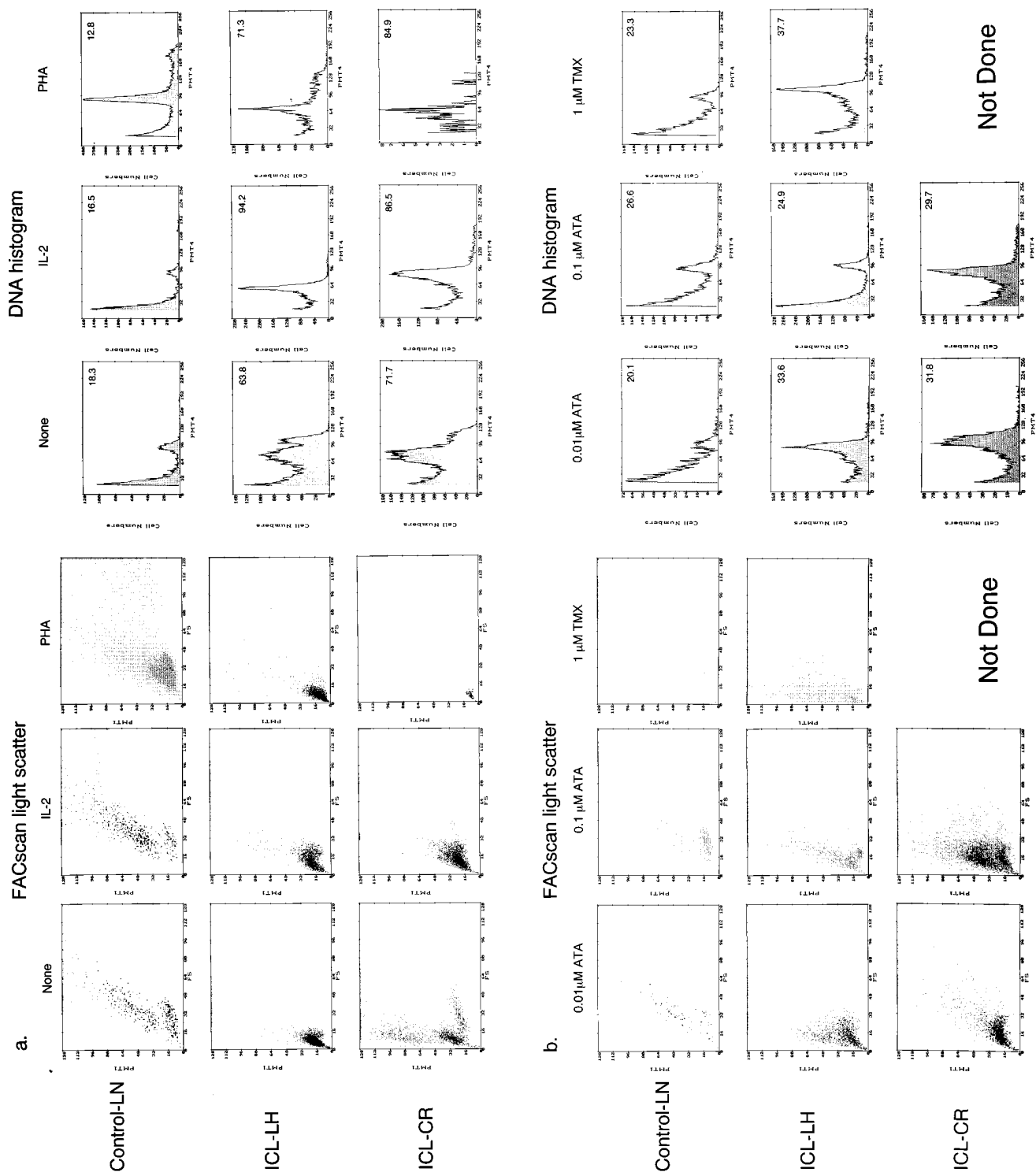


Figure 2. Spontaneous and mitogen-induced apoptosis in CD4⁺ T lymphocytes from ICL patients. Flow cytometric data profiles depict propidium iodide DNA staining (*PMT1*, *y* axis) versus forward light scatter (*FS*, *x* axis), as well as DNA histograms (cell number, *y* axis, vs. propidium iodide DNA staining [*PMT4*], *x* axis), of FITC-labeled CD4⁺ T cells. PBMCs were obtained from two ICL patients, ICL-4 (*LH*) and ICL-5 (*CR*), and donor Control-1 (*LN*), and examined in the absence of stimuli and after culture with IL-2 (20 U/ml) or PHA (5 μg/ml). All cultures were maintained for 120 h. The percent DNA staining in the A₀ region is given in the upper right quadrant of each DNA histogram. Experiments were performed in the absence (*a*) or presence (*b*) of ATA or TMX. The latter also contain IL-2 (20 U/ml).

PBMC samples (Fig. 3 *A*), but was present in high levels in all of eight ICL patients evaluated (Fig. 3 *B*), despite no observable difference in levels of β-actin expression in these samples (Fig. 3, *A* and *B*). Two bands, the larger of anticipated size and

the smaller, confirmed as a Fas product by southern blotting and presumably representing a transmembrane domain-truncated form, was also noted in all ICL samples (Fig. 3 *B*). Using 1 μg of total RNA, no or very weak signals for FasL were seen

Table II. Effect of Various Agents on ICL-associated CD4+ T Cell Apoptosis In Vitro

Subject	Condition* percent apoptosis (A ₀ peak)					
	Buffer	IL-2	ATA + TMX	IL-2 + ATA + TMX	IL-2 + ATA	IL-2 + TMX
Control-1	0	0	0	0		
Control-2	0.6	0.7	0	0		
Control-3	0	0	ND	0		
ICL-1	43.8	8.6	2.5	3.8		
ICL-2	39.0	45.4	54.7	54.9		
ICL-3	3.8	2.0	0	0		
ICL-4	63.8	94.2	ND	ND	24.9	37.7
ICL-5	71.7	86.5	ND	ND	29.7	ND
ICL-6	19.4	13.3	9.7	2.9		
ICL-7	19.2	8.8	14.1	12.0		
ICL-8	30.7	18.5	—	15.4		

* IL-2: 20 U/ml nonrecombinant; ATA: 0.1 μM; TMX: 1 μM. PBMC placed in culture for 3 d before staining with anti-CD4 mAb and propidium iodide, except for ICL-4 and ICL-5 which were cultured for 5 d.

in adherent cell-depleted PBMC from two normal control donors (Control-1 and -2, Fig. 3, C and A, respectively) and from a control donor with physiologic CD4 lymphopenia (Control-3, Fig. 3 A). In contrast, in six of eight ICL patients, intense bands for FasL were seen (Fig. 3 C and additional data not shown).

Neither ATA nor TMX had any impact on expression of Fas (not shown), but they did block FasL expression (Fig. 3 C).

Fas-mediated apoptosis. An assay of FasL function was next performed. As shown in Fig. 4, PBMC from two ICL patients expressing FasL by RT-PCR, ICL-4 and ICL-6, but not

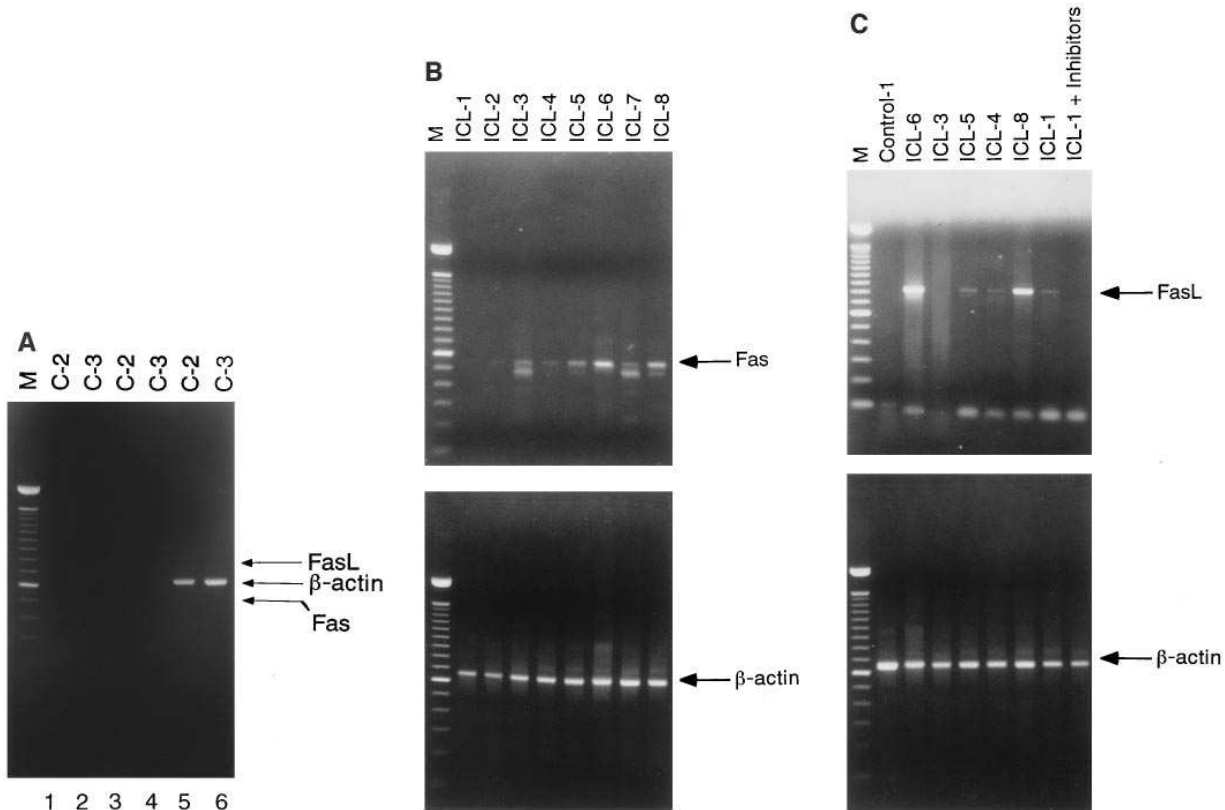


Figure 3. RT-PCR for expression of Fas and Fas ligand. Control (C; A and C) and ICL (B and C) PBMC were maintained in culture for 72 h in the absence or presence of 1 μM ATA + 1 μM TMX + IL-2 (inhibitors). RNA derived from these cultures was reverse transcribed into cDNA, and the products amplified by PCR using the Fas or FasL primers described in the text. β-actin mRNA levels were simultaneously determined as a control for RNA integrity in these samples. (A) M, markers; lanes 1,2: FasL amplification; lanes 3,4: Fas amplification; lanes 5,6: β-actin amplification.

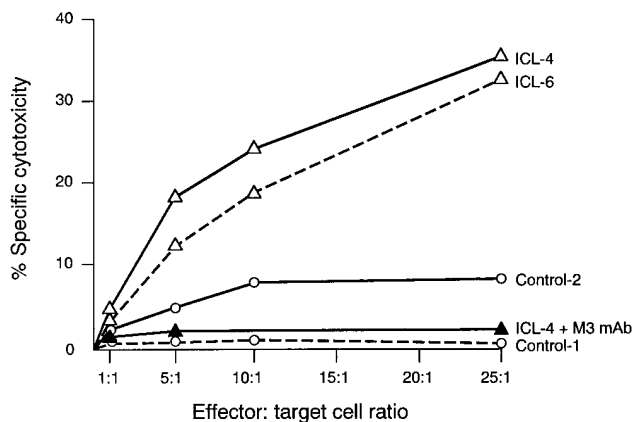


Figure 4. Fas ligand-expressing cells from ICL patients are active in a bioassay for FasL activity. ^{51}Cr -labeled Jurkat CD4⁺ T cell targets were cultured for 18 h with ICL patient or control PBMCs, either alone or in the presence of soluble Fas mAb M3 (solid triangles) or Fas mAb M31 (not shown).

PBMC from Control-1, who did not express elevated FasL (Fig. 3 C), gave a high degree of specific killing of ^{51}Cr -labeled CD4⁺ Jurkat T cell targets. This killing was blocked by soluble anti-Fas mAb M3, but not by anti-Fas mAb M31, which does not interfere with Fas cross-linking.

In agreement with these data, soluble anti-Fas mAb M3 decreased the A_0 peak of spontaneous apoptosis when added to ICL PBMC cultures on their initiation, while an anti-TNF- α polyclonal rabbit anti-human neutralizing antiserum (Genzyme Corp., Cambridge, MA) had no or little effect. For example, apoptosis occurring after 72-h cultures, as measured by percent A_0 peak, was 31.2% for ICL-6 PBMC alone, 30.8% in the presence of 1,000 neutralizing U/ml of anti-TNF- α , but 18.2% in the presence of 10 $\mu\text{g}/\text{ml}$ M3 mAb. Similarly, ICL-2 gave 26.6% baseline apoptosis, 23.4% in the presence of anti-TNF- α , and < 1% with M3 mAb.

Antihistone reactivity in ICL sera. We detected IgG antihistone antibodies to histone H2B in sera from three of our eight ICL patients (ICL-1,4,6), with optical density readings > three times the standard deviation of controls. 10 control sera and sera from two donors with physiologic lymphopenia showed no reactivity above background with H2B (mean OD 0.10 ± 0.01).

Discussion

Accelerated apoptosis of CD4⁺ T lymphocytes was documented in seven of eight patients with a subtype of ICL, as characterized by persistent CD4 lymphopenia, CD4/CD8 ratios < 1, and evidence for clinical immune deficiency. Apoptosis was demonstrated by three different methods: laser-illuminated light scatter; DNA labeling and quantitation of the pre-G₁ A_0 peak; and direct visualization of chromatin fragmentation by DNA agarose gel electrophoresis. The use of multiple confirmatory assays for apoptosis was undertaken as there may not be a direct correlation between any single assessment of apoptosis and all the morphologic and genomic criteria for this type of programmed cell death (35).

Evidence for an *in vivo* role for apoptosis in this subset of ICL comes from three types of data. First, the low CD4 counts

in one donor, "case 5," originally classified as having ICL solely on the basis of two CD4 counts < 300/mm³, returned to normal after treatment of an *M. tuberculosis* infection, and this donor, as two others with stable low CD4 counts, showed no evidence for apoptosis at any level of absolute CD4 cell count. Second, we detected antihistone autoantibodies, presumably induced by fragmented chromatin, in some of these sera, with reactivity predominantly to type H2B, a pattern identical to that found in HIV disease (12, 36) and simian immunodeficiency virus infection in macaques (37), both of which have been associated with CD4⁺ T cell apoptosis: *in vitro* in the case of HIV (10, 11), and *in vivo* in macaques (37). Third, detection of apoptosis among CD8⁺ T cells appeared to correlate with their depletion *in vivo*, occurring in those ICL patients with low levels of both CD4⁺ and CD8⁺ T cells.

It is unlikely that a single pathophysiology will be operative in ICL. However, the mechanism of accelerated T cell death in at least some of our patients may relate to the enhanced susceptibility of Fas-expressing T lymphocytes to cross-linking by Fas ligand. Fas, a 48-kD polypeptide belonging to the family of type I plasma membrane receptors, appears to play a central role in the regulation of apoptosis. Quiescent PBMCs express Fas at low levels or not at all (38). While expression is markedly increased after mitogen activation of primary T cells, unlike T cell lines they are not induced to undergo apoptosis when Fas is cross-linked with an anti-Fas mAb or FasL. This process requires restimulation after chronic T cell receptor activation (39). Indeed, rather than initiating cell death, many anti-Fas mAbs act as potent costimulators of normal T cell proliferative responses to mitogen and antigen (39). In contrast to primary cells from normal donors, Fas is increased on T cells from many HIV-1+ individuals (25, 40), and HIV infected cell lines are highly sensitive to the cytolytic activity of certain anti-Fas mAbs (41). The ligand for Fas, a type II transmembrane protein, can induce apoptosis in Fas-expressing targets, as shedding of overexpressed protein actively triggers cell death by Fas binding (21). Fas/FasL interactions apart from cytolytic T cell activity appear to be physiologically important, as point mutations in the FasL gene result in accumulation of large numbers of T cells in the lymphoid tissues of affected mice (22).

Evidence for elevated Fas and FasL in ICL could be interpreted as merely indicating the presence of preactivated cells. The potential functional significance for the sustained expression of FasL in ICL was thus explored. CD4⁺ Jurkat T cells are lysed in the presence of Fas ligand but are resistant to TNF- α -induced apoptosis (21). They were specifically lysed by coculture with unstimulated PBMC from ICL patients expressing elevated levels of FasL mRNA. Killing was blocked by soluble anti-Fas mAb M3. Whether this phenomenon occurs via receptors and ligands expressed by the same cell (*in cis*) or by different cells (*in trans*) and whether cell contact or soluble FasL or both is involved was not addressed.

The single patient showing little apoptosis also did not express FasL (ICL-3). However, Fas/FasL interactions cannot be the only mechanism of apoptosis in our ICL patients. For example, ICL-4, with high levels of apoptosis, had minimal expression of FasL mRNA. It should also be noted that ICL samples gave not only the Fas product of expected size (arrow, Fig. 3 B) but also a distinct smaller fragment (Fig. 3 B). By size this appears to be a truncated, soluble form of Fas, normally seen at low levels, but reported to be present in high levels in sys-

temic lupus erythematosus (42). We are currently investigating the relative levels of these two products in ICL. It should be emphasized, however, that there was no evidence in our ICL patients for retroviral infection, active infection with other viruses known to cause apoptosis, or for autoimmune diseases such as systemic lupus, which have been linked to both CD4+ T cell loss (43) and accelerated apoptosis in vitro (44).

There are several similarities between apoptosis occurring among CD4+ T cells from HIV+ and ICL patients. These include its enhancement by T cell activators, its independence from new protein synthesis, and its susceptibility to general inhibitors of programmed cell death. In addition, both Fas and FasL appear to be elevated on T lymphocytes from many patients with both disorders. One obvious difference is the direct involvement of HIV in some apoptotic T cells (12). This finding has been challenged, however, by recent data indicating that it is predominantly bystander T cells, and not productively infected cells, that undergo apoptosis in HIV/AIDS (11). In addition, many studies show equivalent levels of apoptosis among both CD4+ and CD8+ T cells from HIV+ donors, regardless of their absolute levels of CD8+ peripheral T cells. In contrast, the two ICL patients we investigated with normal absolute numbers of CD8+ T cells had evidence for apoptosis only among the CD4+ T subset. Our limited sample size precludes an absolute statement in this regard, however.

12–24 h of culture were required before changes in A_0 peak or fragmentation of DNA could be visualized. This lag has also been seen with PBMC isolated from AIDS patients (11, 45) and may relate to the fact that fully apoptotic cells would be rapidly and efficiently removed from the circulation, whereas cells programmed for such death require some interval for expression of the full phenotype.

The exact process by which ATA and/or TMX, in conjunction with IL-2, blocked apoptosis in T cells from some ICL patients, most prominently in ICL-1, ICL-4 and ICL-5, but not others is unclear. Given the ability of ATA and TMX to suppress two activities integral to endonucleosomal DNA cleavage in apoptosis, Ca^{2+} -mediated endonuclease activation and cAMP/PKA induction, respectively, these represent possible mechanisms of action. It should be noted, however, that ATA is a potent antiapoptotic agent even in anucleate CD4+ T cells (46), so that its mechanism of action cannot yet be established with any certainty. In addition, TMX can drive cells into G_0 , and may thus protect them simply by reducing their activation state (16). We have also shown that ATA plus TMX can block FasL, but not Fas, expression, and this may be related to the apoptotic suppression observed. There is precedent for such a mechanism, as two agents which can inhibit apoptosis in murine T lymphocyte hybridomas, retinoic acid and glucocorticoids, block upregulation of FasL (47). IL-2 has also been associated with partial suppression of apoptosis linked to a variety of factors, from glucocorticoid exposure (48) to HIV infection (49).

Finally, the severity of disease in subsets of individuals with ICL, such as those presented here, should not be underestimated. Albeit the prevalence of ICL among unselected populations is very low, usually transient, and most often associated with a recent illness (5, 50), opportunistic infections and other disorders frequent in or pathognomonic of AIDS caused the death of three of our original five ICL patients (1). A similar high death rate was reported by the Italian Study Group on Non-HIV AIDS (51). The ability of ATA or TMX or both, in

combination with IL-2, to inhibit common pathways for apoptosis in ICL in vitro should encourage studies of such agents in the treatment of clinically significant idiopathic CD4+ T lymphocytopenias.

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