Interleukin-10 Inhibits Apoptotic Cell Death in Infectious Mononucleosis T Cells

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Abstract

T lymphocytes from patients with acute EBV-induced infectious mononucleosis rapidly die by apoptosis in vitro. Because human and viral IL-10 are likely to be induced during acute EBV infection and display a variety of functions on human T cells, we examined IL-10 effects on infectious mononucleosis T cell death. After 12 h of incubation in medium alone, only 35.6 (±8.2%) of the originally seeded infectious mononucleosis T cells were viable. Addition of human IL-10 (100 U/ml) to T cell cultures significantly improved recovery of viable cells (71.3±6.2%, P = 0.0156). Viral IL-10 had comparable effects to human IL-10 in this system. Protection from death by human and viral IL-10 (100 U/ml) was dose dependent and continued over a 6-d culture period. The human IL-10 effect was neutralized by the anti-human IL-10 mAb 19F1. Morphology and analysis of DNA after separation on agarose gels showed that IL-10 inhibits loss of cell volume, chromatin condensation, and DNA fragmentation, characteristics of death by apoptosis. As assessed by [^{3}H]thymidine incorporation, the T cells were not induced to proliferate by IL-10 above the level exhibited when first removed from blood. T cells protected from death by IL-10 proliferated to IL-2 and spontaneously killed sensitive targets as effectively as medium-precultured T cells. Thus, IL-10 promotes the survival of infectious mononucleosis T cells otherwise destined to die by apoptosis and may be critical for the establishment of immunologic memory after resolution of the illness. (*J. Clin. Invest.* 1994. 94:251–260.) Key words: Epstein-Barr virus • programmed cell death • BCRF-1 • cell survival • DNA fragmentation

Introduction

Acute infectious mononucleosis (Mono),[1] a manifestation of primary infection with EBV, is characterized by virus replication in the oropharynx, latent virus infection in a proportion of the B cells, and reactive T cell lymphocytosis (1–3). The T cells activated during EBV-induced Mono suppress and kill EBV-infected B cells in vitro, functions that are believed to be important for control of the viral infection in vivo (4–8).

A striking feature of Mono T cells is that they rapidly die by apoptosis when incubated in vitro (9, 10). Although there is no direct evidence for the occurrence of substantial T cell death in vivo, we know that lymphadenopathy and T cell lymphocytosis are generally transient during EBV-induced Mono (11). Upon resolution of the illness, the lymphadenopathy disappears, and the absolute number of circulating T cells, as well as the relative proportion of $CD4^+$ and $CD8^+$ cells, returns to normal (12). This points to a rapid clearance of activated T cells from the lymphoid organs and from the circulation, features that characterize death by apoptosis in vivo (13). However, a balance must exist between death and survival of immune reactive T cells because immunologic memory is established after resolution of acute EBV-induced Mono (14, 15).

The presence of EBV replication in the oropharynx during Mono suggests that the late viral gene BCRF-1 is expressed in the productively infected cells and that its protein product, viral IL-10 (vIL-10), is secreted by these cells (11, 16). There is also evidence to suggest that viral and human IL-10 (hIL-10) are expressed in EBV-infected B cells (17). Thus, the oropharyngeal epithelium and lymphoid tissues should be sites of IL-10 production during EBV-induced Mono. The structurally homologous viral and human IL-10 share a number of biological properties, including inhibition of IFN-γ production (16), suppression of T cell proliferation in response to antigen and mitogen (18), inhibition of cytokine production by activated monocytes (19), and stimulation of B cell growth and immunoglobulin production (20, 21). This has raised the possibility that EBV might have captured, during evolution, the IL-10 gene for its own advantage (22).

The present experiments were undertaken to evaluate the role of IL-10 on T cell immunity during acute EBV-induced Mono and to specifically address the possibility that this cytokine is an important regulator of T cell survival.

Methods

Study population. The study population consisted of 23 patients diagnosed as having acute EBV-induced Mono at Georgetown University Student Health Center. The diagnosis of EBV-induced Mono was based upon the presence of fever, pharyngitis, lymphadenopathy, lymphocytosis with atypical lymphocytes, and the presence of heterophile antibodies as determined by a positive monospot test (Monosticon Dri-Dot; Organon Teknika, Durham, NC). The patients ranged from 18 to 23 yr; 14 were male, and 9 were female. No patient had received steroids before the study.

Reagents, antibodies, and cytokines. The murine mAb anti-CD25 (anti-Tac) was purified from hybridoma ascites by using protein A-Sepharose (a gift of Dr. T. Waldmann, National Institutes of Health, Bethesda, MD; reference 23). A purified murine anti-hIL-2 neutralizing

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1. *Abbreviations used in this paper:* hIL-10, human IL-10; mIL-10, murine IL-10; Mono, infectious mononucleosis; MTT, 3-(4,5 dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide; vIL-10, viral IL-10.

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mAb was purchased from Genzyme Corp. (Cambridge, MA); a concentration of 4 μg/ml was found to neutralize 50% of the proliferation of activated peripheral blood T cells induced by 10 U recombinant hIL-2 (Cetus Corp., Emeryville, CA). TCRδ1, an mAb against the T cell γδ receptor, was obtained as FITC-conjugated from T Cell Sciences, Inc. (Cambridge, MA). The mAbs anti-CD3 (anti-Leu-4), anti-CD4 (anti-Leu-3a), anti-CD5 (anti-Leu-1), anti-CD8 (anti-Leu-2a), anti-CD20 (anti-Leu-16), anti-CD25 (anti-hIL-2 receptor), anti-CD38 (anti-Leu-17), anti-CD71 (anti-transferrin receptor), anti-TCRαβ (anti-T cell αβ receptor), and anti-HLA-DR were purchased as FITC-conjugates from Becton Dickinson Immunocytometry Systems (Mountain View, CA). The neutralizing anti-hIL-10 mAb was a gift of Dr. J. Abrams (DNAX Research Institute Palo Alto, CA; reference 19).

hIL-10 was purified from culture supernatants of Chinese hamster ovary cells stably transfected with the hIL-10 gene and contained 10^7 U/mg. Murine IL-10 (mIL-10) was baculovirus derived and contained ~ 14,000 U/ml. Both hIL-10 and mIL-10 were a kind gift of Dr. K. Moore (DNAX Research Institute; references 16 and 24). A unit of IL-10 is defined as the amount inducing half-maximal stimulation of MC/9 mast cells, as described (25). Recombinant hIL-2 was from Cetus Corp.

Cells and culture conditions. T lymphocytes were obtained from PBMC by density centrifugation after rosetting with 2-aminoethylisothiouronium-treated sheep red blood cells (26). To study spontaneous cell death, T lymphocytes were cultured in RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal calf serum (Rehatuin Intergen Co., Purchase, NY), 2 mM L-glutamine (Gibco Laboratories, Grand Island, NY), and 5 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO) at a density of 2×10^6 cells/ml in flat-bottom microtiter plates (Costar Corp.) containing 200 μl/well. The culture period ranged between 6 h and 6 d.

Cell viability and cell morphology. Cell viability was determined by light and fluorescent microscopy after cell staining with acridine orange (4 μg/ml; Sigma Chemical Co.) and trypan blue (0.02%; Sigma Chemical Co.). As described (27, 28), viable cells were easily identified as trypan blue-negative cells displaying bright green nuclear chromatin fluorescence with organized structure. Cell morphology was evaluated by light microscopy examination of May-Grünwald Giemsa-stained cells subjected to cytocentrifugation. A cell was considered morphologically apoptotic when it displayed loss of cell volume, chromatin condensation along the nuclear membrane with intensely basophilic staining, and/or nuclear fragmentation into spherical structures containing condensed chromatin, still surrounded by membrane (29).

DNA electrophoresis. DNA fragmentation analysis was performed essentially as described (30). Briefly, 4×10^6 cells, which had been incubated at 37°C for 12 h (2×10^6/ml), were centrifuged and washed twice in PBS. The pellets were suspended in 40 μl of lysing buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA containing 0.5% laurylsarkosinate and 1.25 mg/ml proteinase K; all from Sigma Chemical Co.). After incubation at 50°C for 1 h, 100 μg of RNase A (Life Technologies, Inc., Grand Island, NY) was added, and the samples were incubated at 50°C for 1 h, followed by the addition of 10 μl stop solution (10 mM EDTA, pH 8.0, 0.25% bromophenol blue, 30% Ficoll 400). Then, the samples were heated at 70°C for 10 min before loading onto the dry well of a 2% neutral agarose gel. Electrophoresis was carried out at 30 V for 12 h in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.3), and the gel was stained with 0.5 μg/ml ethidium bromide (Sigma Chemical Co.).

Proliferation and MTT assays. Proliferation assays were carried out in 96-well flat-bottom plates (Costar Corp.) in triplicate cultures of 4×10^5 cells in 200 μl/well. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. DNA synthesis was determined by [^{3}H]-thymidine deoxyribose uptake (0.5 μCi/well, 6.7 Ci/mmol; New England Nuclear, Boston, MA) during the last 2–6 h of cultures.

MTT (3-[4,5 dimethylthiozol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma Chemical Co.) assays were carried out in 96-well flat-bottom plates in triplicate cultures of 8×10^5 cells/well in 200 μl, essentially as described (31). MTT was dissolved in PBS at 5 mg/ml and added (25 μl) to each well, followed by incubation at 37°C for 2 h. After incubation, an aliquot (100 μl) of a solution containing 10% SDS in 0.01 N HCl was added, and the cultures were incubated overnight at 37°C in a 5% CO_2 incubator. The absorbance at 570 nm was then measured on a multiwell scanning spectrophotometer (Titertek Multiskan MCC/340; Flow Laboratories, Inc., McLean, VA).

Cytotoxicity assay. Peripheral blood T cells from patients with acute EBV-induced Mono were incubated for 12 h either in medium alone consisting of RPMI 1640 medium (Biofluids, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Reheis), 2 mM L-glutamine (Gibco Laboratories), and 5 μg/ml gentamicin (Sigma Chemical Co.), or in medium supplemented with 100 U/ml hIL-10 at a cell density of 2×10^6 cells/ml in T25 tissue culture flasks (Costar Corp.). After culture, the cells were washed, and viable cells were counted (trypan blue-negative cells, staining brightly with acridine orange) and tested for cytotoxicity essentially as described (32). EBV-immortalized cells (VDS-0 and TB cell lines; reference 33) and the natural killer-sensitive K562 cell line (American Type Culture Collection, Rockville, MD) were used as ^{51}Cr-labeled targets (ICN Biomedicals, Inc., Irvine, CA). The percentage of specific cytotoxicity was calculated as: 100 × ([ex-

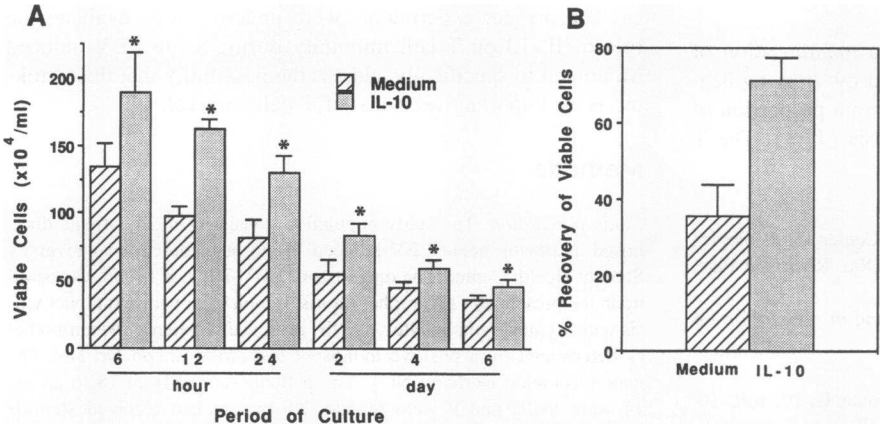

Figure 1. Effects of hIL-10 on viability of Mono T cells in vitro. Mono T cells were cultured (4×10^5 viable T cells in 0.2 ml/well) in either medium alone or in medium containing 100 U/ml hIL-10. Viable cells were counted visually after staining with trypan blue and acridine orange. A cell was considered viable when it excluded trypan blue and exhibited bright green nuclear fluorescence with characteristic organized structure. (*A*) Time course of T cell viability with or without hIL-10. The results are expressed as total number of viable cells per milliliter and reflect the arithmetic mean (±SD) of quadruplicate determinations. Immediately after seeding, the cells were found to be at 240.0 (±9.2) × 10^4 cells/ml. * Significant difference ($P < 0.05$) between cultures with or without hIL-10, as determined by Mann-Whitney U test. Representative experiment of three performed. (*B*) Recovery of viable cells after 24 h of culture with or without hIL-10, a mean of seven determinations with distinct blood samples. The results are expressed as a percentage of the total number of viable cells counted in the well immediately after seeding.

Table I. Neutralization of the Viability-promoting Effect of hIL-10 by an Anti-hIL-10 mAb

Experiment*	Additions	Viable cells ($\times 10^4$/ml)‡
1	Medium	78.8 (5.3)
	hIL-10 (20 U/ml)	160.0 (15.6)§
	hIL-10 (20 U/ml) + 19F1 mAb (2 μg/ml)	87.0 (4.7)
	19F1 mAb (2 μg/ml)	84.8 (8.5)
2	Medium	74.8 (6.7)
	hIL-10 (20 U/ml)	135.0 (7.6)§
	hIL-10 (20 U/ml) + 19F1 mAb (2 μg/ml)	85.0 (5.0)
	hIL-10 (20 U/ml) + anti-Tac mAb (5 μg/ml)	132.0 (6.5)§
	anti-Tac mAb (5 μg/ml)	74.3 (2.6)
3	Medium	60.3 (6.4)
	hIL-10 (20 U/ml)	99.5 (6.5)§
	hIL-10 (20 U/ml) + anti-hIL-2 mAb (100 μg/ml)	99.0 (11.0)§
	Anti-hIL-2 mAb (5 μg/ml)	61.8 (9.4)

* Mono T cells (4×10^5 cells in 0.2 ml/well) were cultured for 12 h in medium alone or in medium containing 20 U/ml hIL-10 with or without 19F1 anti-hIL-10 mAb, anti-Tac mAb, or anti-hIL-2 mAb. Immediately after seeding into culture, the cells were found to be at 209.0±8.0, 202.0±11.9, and 122.3±9.3 $\times 10^4$ cells/ml in experiments 1–3, respectively. ‡ Viable cells were counted after staining with trypan blue and acridine orange. The results are expressed as mean number of viable cells per milliliter (±SD) of quadruplicate cultures. § Number of viable cells significantly different ($P < 0.05$, by Mann-Whitney U test) from that of T cells cultured in medium alone.

perimental release − spontaneous release]/[total release − spontaneous release]). In all experiments the spontaneous release was < 20% of the maximum release.

Immunofluorescence analysis. Immunofluorescence staining of cell surface markers was performed as described (34). After the cells were stained with the appropriate FITC-conjugated mAb, the cells were analyzed using a FACScan® cytometer (Becton Dickinson Immunocytometry Systems) equipped with a 488 nm air-cooled argon laser and linked to a computer (Hewlett-Packard Co., Palo Alto, CA). Detection of FITC was carried out with detectors set at 530 nm. 20,000 cells were analyzed. Data were acquired using Consort 30 and analyzed using Lysis I software (Becton Dickinson Immunocytometry Systems).

Statistical analysis. Group differences were analyzed for significance by Wilcoxon signed-rank test or Mann-Whitney U test.

Results

IL-10 protects Mono T cells from spontaneous death by apoptosis. It has been reported previously that Mono T cells rapidly die when incubated in vitro (9, 10). As shown in a representative experiment (Fig. 1 *A*), only 40.8 and 33.8% of the originally seeded Mono peripheral blood T cells were still alive after 12- and 24-h incubations, respectively. A cell was considered viable when it both excluded trypan blue on light microscopy and exhibited bright green nuclear fluorescence when stained with acridine orange, as described (27, 28). This contrasts with normal peripheral blood T cells that generally

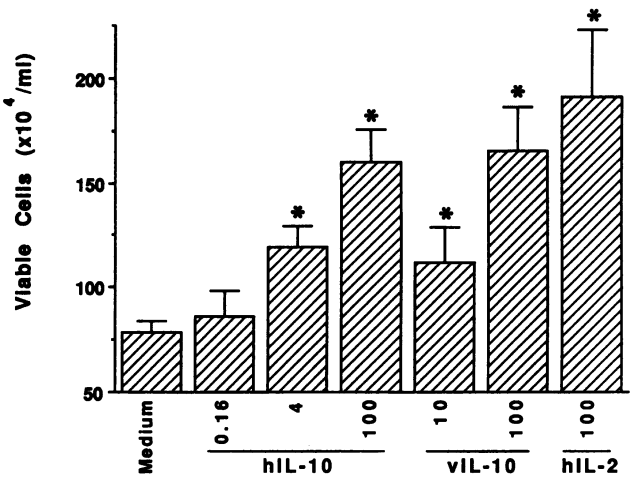

Figure 2. Dose-dependency of IL-10 viability-promoting effect. Mono T cells were cultured (4×10^5 cells in 0.2 ml/well) for 12 h in medium alone, medium with hIL-10 or vIL-10 at various concentrations (Units per milliliter), or medium with 100 U/ml hIL-2. Immediately after seeding into the culture well, the cells were found to be at 209.0±8.0 $\times 10^4$ cells/ml. Viability was assessed as described in the legend to Fig. 1. The results reflect the arithmetic mean (±SD) of quadruplicate determinations. Representative experiment of three performed. * Significant difference ($P < 0.05$) between cultures with or without cytokine, as determined by Mann-Whitney U test.

are > 90% viable after 24 h of incubation under identical conditions (not shown). We examined whether hIL-10 had an effect on Mono T cell survival in vitro. As shown (Fig. 1 *A*), hIL-10 (100 U/ml) enhanced cell viability of Mono T cells throughout the 6-d culture period. In the presence of hIL-10, 67.5 and

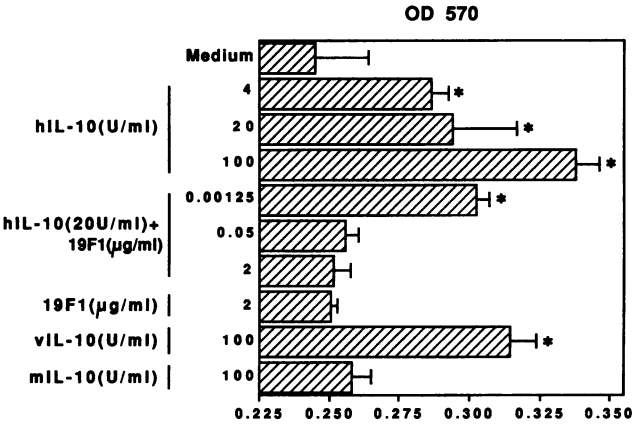

Figure 3. Effects of IL-10 on MTT conversion to MTT formazan in Mono T cells. T lymphocytes were cultured (8×10^5 cells in 0.2 ml/microwell) in either medium alone, medium with hIL-10 at the indicated concentrations, medium with 2 μg/ml 19F1 anti-hIL-10 mAb, medium with hIL-10 plus 19F1 mAb at the indicated concentration, medium with 100 U/ml vIL-10, or medium with 100 U/ml mIL-10. At the end of 12 h of culture, the MTT assay was performed as described in Methods, and the optical absorbance was determined at 570 nm. The results reflect the arithmetic mean (±SD) of triplicate cultures. * Significant difference ($P < 0.05$) between cultures with or without cytokine, as determined by Mann-Whitney U test.

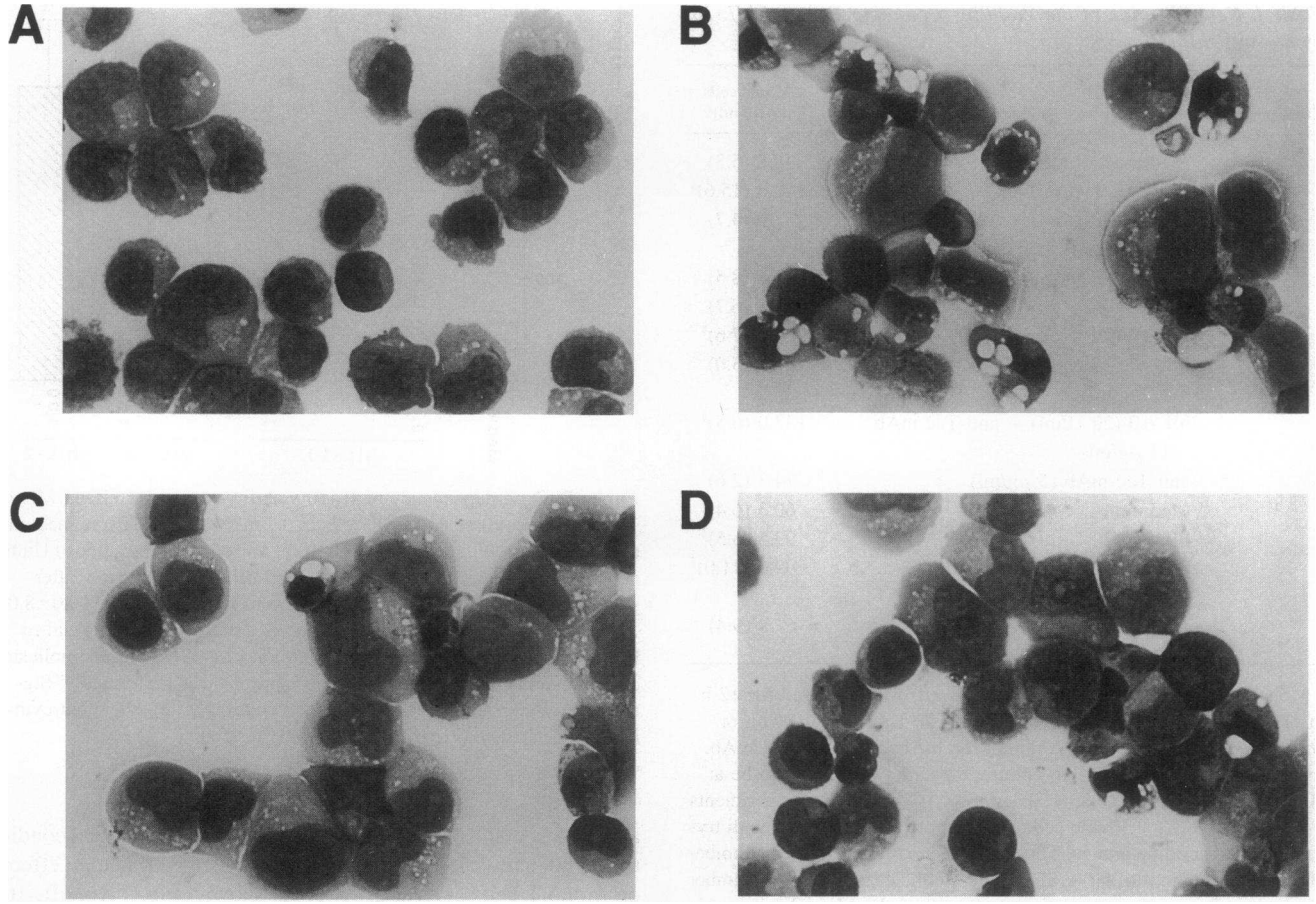

Figure 4. Cytocentrifuged preparations of Mono T cells incubated in vitro with or without IL-10. (*A*) T cells immediately after separation from peripheral blood; (*B*) T cells after 12 h of incubation at 37°C in medium alone; (*C*) T cells after 12 h of incubation at 37°C in medium with 100 U/ml hIL-10; and (*D*) T cells after 12 h of incubation at 37°C in medium with 100 U/ml vIL-10. May-Grünwald Giemsa stain (×1,000).

54.2% of the originally seeded Mono T cells were still alive after 12 and 24 h of incubation, respectively (Fig. 1 *A*).

In seven separate experiments with T cells from different patients with acute EBV-induced Mono, only 35.6±8.2% of the originally cultured T cells were viable after incubation in medium alone for 12 h, but 71.3±6.2% were viable when incubated under identical culture conditions plus 100 U/ml hIL-10 ($P = 0.0156$, by Wilcoxon signed-rank test) (Fig. 1 *B*).

The viability-promoting effect of hIL-10 was reversed by the neutralizing effect of 19F1, an anti–hIL-10 mAb, but not by anti-Tac (CD25), an anti–hIL-2 receptor mAb (Table I, *Experiments 1* and *2*). Also, when added alone to Mono T cells (Table I, *Experiment 1*), 19F1 mAb alone had little or no effect on T cell viability, suggesting that death and survival of Mono T cells in this system are not influenced by IL-10 endogenously produced during incubation. In addition, the finding that anti-Tac mAb had no effect on the viability-promoting effect of IL-10 suggests that endogenously produced hIL-2 does not have a substantial effect on T cell viability in this system. To test this further, a neutralizing mAb to hIL-2 was also used. As shown (Table I, *Experiment 3*), anti–hIL-2 mAb had little or no effect on the viability-promoting effect of IL-10.

In this system, increased T cell viability induced by hIL-10 is dose dependent. Although some effect was observed at 4 U/ml, a much greater induction of cell viability by hIL-10 occurred at 100 U/ml (Fig. 2).

vIL-10 also promoted cell viability in Mono T cells. When added at 100 U/ml, the magnitude of the effect was comparable with that induced by hIL-10 at the same concentration. As expected, hIL-2 also promoted T cell viability (Fig. 2).

Although microscopic examination permits us to distinguish live from dead cells, its accuracy may be limited by the subjective nature of the evaluations. To confirm the viability-promoting effect of IL-10 by a more objective method, we have used the property of metabolically active cells to enzymatically convert the yellow tetrazolium salt MTT into a dark blue formazan salt. The amount of MTT formazan produced, measured as absorbance at 570 nm, is proportional to the number of viable cells, regardless of the presence of nonviable cells, and is also related, in part, to the degree of cell activation (31). As shown in Fig. 3, hIL-10 dose-dependently enhanced the conversion of MTT to MTT formazan by Mono T cells. This stimulatory effect of hIL-10 was specific in that it was neutralized by the anti–hIL-10 mAb 19F1 added at 0.05 or 2 μg/ml. vIL-10 (100 U/ml) also stimulated MTT conversion into MTT formazan by Mono T cells, but mIL-10 (100 U/ml) did not (Fig. 3). These findings are consistent with the observation that hIL-10 and vIL-10 promote the viability of Mono T cells in culture.

Table II. Proliferation of Mono T Cells in Response to hIL-2 and hIL-10

Additions* (total culture time)	Units per milliliter	[^{3}H]Thymidine deoxyribose uptake	Viable cells $\times 10^4$ cells/ml‡	[^{3}H]Thymidine deoxyribose uptake per cell $\times 10^{-3}$ cpm/cell§
		counts per minute/well‖		
Medium (3 h)		6,338 (159)	166.5 (9.9)	31.7
Medium (24 h)		1,173 (60)	50.5 (5.7)	19.3
hIL-2 (24 h)	1	1,264 (83)		
	10	2,577 (368)		
	100	12,552 (346)	160.7 (13.3)	65.1
hIL-10 (24 h)	0.16	1,102 (29)		
	0.8	1,344 (64)		
	4	2,091 (93)		
	20	3,057 (138)		
	100	3,900 (198)	121.0 (8.8)	26.9
mIL-10 (U/ml) (24 h)	10	1,060 (78)		
	100	1,180 (31)		

* Mono T cells (120 μl/well) were cultured either in medium alone or in medium supplemented with hIL-2 (1–100 U/ml), hIL-10 (0.16–100 U/ml), or mIL-10 (10 or 100 U/ml). Total culture time was either 3 or 24 h. ‖ [^{3}H]Thymidine was added to the cultures during the final 2 h of incubation. The results reflect the mean (±SD) of quadruplicate determinations. Representative experiments of four performed. ‡ Viable cells were counted after staining with trypan blue and acridine orange. Mean (±SD) of triplicate determinations. § [^{3}H]Thymidine uptake per cell was calculated as the ratio of the mean [^{3}H]thymidine uptake per well and the mean number of viable cells per well.

Effects of IL-10 on DNA replication of Mono T cells. We measured cell replication in Mono T cells incubated in vitro with hIL-10 or hIL-2 by assessing [^{3}H]thymidine uptake during the last 2 h of a 24-h total culture period (Table II). As expected, hIL-2 enhanced the replication of Mono T cells; at 100 U/ml, cell replication (measured in counts per minute per well) was enhanced by 10.7-fold over T cell replication in medium alone. In contrast, hIL-10 induced significantly less [^{3}H]thymidine incorporation compared to hIL-2; at 100 U/ml hIL-10, T cell replication was enhanced by 3.3-fold over T cell replication in medium alone. This could be the result of hIL-10 inducing activation of Mono T cells in vitro or, alternatively, could be the result of hIL-10 preventing cells activated in vivo from dying. To differentiate between these possibilities, we assessed the amount of [^{3}H]thymidine uptake per viable cell during the last 2 h of a 24-h incubation in medium, hIL-10, or hIL-2 and compared it with that found during the last 2 h of a 3-h incubation in medium alone. During the final 2 h of a 24-h culture, the average [^{3}H]thymidine incorporation by viable Mono T cells was 19.3×10^{-3} cpm/cell in medium alone, 65.1×10^{-3} cpm/cell in the presence of hIL-2, and 26.9×10^{-3} cpm/cell in the presence of hIL-10. In addition, the average [^{3}H]thymidine uptake by Mono T cells incubated in medium alone for only 3 h after separation from peripheral blood was 31.7×10^{-3} cpm/cell. This indicates that Mono T cells present initially in culture incorporated [^{3}H]thymidine more readily than the T cells still living after 22–24 h of incubation in medium alone. The level of [^{3}H]thymidine incorporation by viable Mono T cells cultured for 24 h with hIL-10 (26.9×10^{-3} cpm/cell) is intermediate between the level of incorporation by Mono T cells cultured in medium alone for 3 (31.7×10^{-3} cpm/cell) and 24 h (19.3×10^{-3} cpm/cell). In contrast to hIL-10, hIL-2 promoted [^{3}H]thymidine incorporation by viable Mono T cells (65.1×10^{-3} cpm/cell) above the level detected in cells cultured in medium alone for either 3 or 24 h. These findings strongly suggest that hIL-10 increases T cell viability in this system by preventing cell death rather than by stimulating cell growth.

IL-10 inhibits apoptosis in Mono T cells. Previous studies have demonstrated that T cells from patients with acute EBV-induced Mono rapidly die by apoptosis as demonstrated both by cell morphology and DNA fragmentation (9, 10). We examined the effects of IL-10 on T cell death as assessed both by morphology and DNA fragmentation.

As shown in Fig. 4, whereas > 90% of the Mono T cells just purified from peripheral blood appeared morphologically viable on cytocentrifuged smears (*A*), only a small proportion of these T cells appeared viable after 12 h of incubation in medium alone (*B*). A number of these cells show loss of cell volume, nuclear condensation, and fragmentation, characteristic of apoptosis, while other cells appeared as cell ghosts. Addition of hIL-10 (Fig. 4 *C*) or vIL-10 (Fig. 4 *D*) during the 12-h incubation visibly enhanced the number of morphologically viable cells and reduced the number of apoptotic and ghostic cells.

As shown in Fig. 5, DNA electrophoresis of Mono T cells incubated for 12 h in medium alone resulted in a ladder pattern, characteristic of DNA fragmentation in oligonucleotide bands (lane *6*). In contrast, DNA electrophoresis of the same Mono T cells incubated under otherwise identical culture conditions except for the addition of 100 U/ml hIL-10 (Fig. 5, lane *5*) or vIL-10 (Fig. 5, lane *2*) showed a reduced level of DNA fragmentation. A similarly reduced degree of DNA fragmentation was noted in T cells incubated with 100 U/ml hIL-2 (Fig. 5, lane *1*). No evidence of fragmentation was seen in DNA from fresh Mono T cells (Fig. 5, lane *7*). Together, these findings confirm the rapid occurrence of death by apoptosis in Mono T cells ex vivo and further demonstrate that IL-10 inhibits this process.

Phenotype and function of Mono T cells. Expression of the T cell marker CD3 and of the histocompatibility class II antigen HLA-DR (Fig. 6), and expression of CD25, CD4, CD16, CD56,

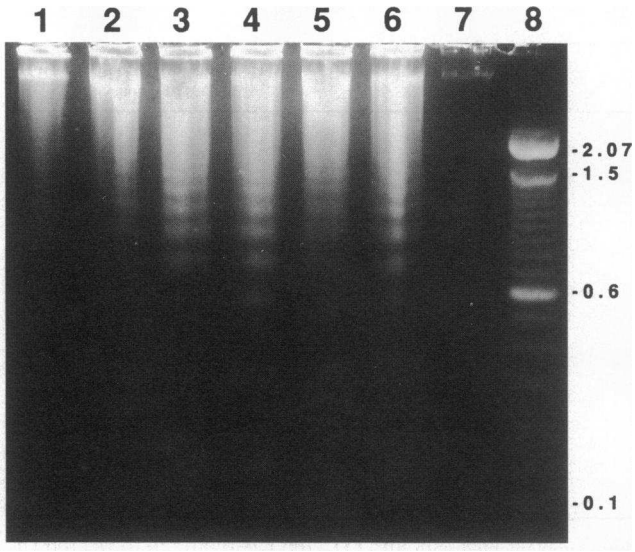

Figure 5. Effects of IL-10 on DNA fragmentation in Mono T cells. Electrophoresis of DNA extracted from 4×10^6 Mono T cells. Lane *1,* T cells cultured for 12 h with 100 U/ml IL-2, recovery 96.5%; lane *2,* T cells cultured for 12 h with 100 U/ml vIL-10; lanes *3–5,* T cells cultured for 12 h with 4, 20, and 100 U/ml hIL-10, respectively, recovery 72.9% at 100 U/ml; lane *6,* T cells cultured for 12 h in medium alone, recovery 30.3%; lane *7,* T cells just separated from peripheral blood; and lane *8,* molecular weight markers (kbp).

or of transferrin receptor (not shown) were not significantly different in Mono T cells incubated for 12 h in medium alone or hIL-10. In addition, both populations (incubated with or without hIL-10) exhibited a similar reduction in expression of these determinants when compared with fresh autologous Mono T cells just separated from peripheral blood (Fig. 6, *dotted profile*). In contrast, surface expression of CD8 was significantly different in Mono T cells cultured in medium alone or with hIL-10 (Fig. 6, *middle*). hIL-10–treated populations contained significantly more cells staining brightly with CD8 (51.1 vs 15.9%) and significantly fewer cells staining dully with CD8 (33.9 vs 68.0%). The CD8 bright and dull cells were gated and analyzed separately by forward and side light scatter (not shown). The dull cells exhibited a decrease in forward light scatter when compared with the bright cells, characteristic of the changes in size and complexity seen in apoptotic cells (35). This demonstrates that apoptosis in Mono T cells is associated with a markedly diminished surface expression of CD8. Thus, treatment with hIL-10 promotes cell viability and consequently prevents loss of CD8 surface expression. In contrast, surface expression of CD3 was found not to be distinguishable in Mono T cells with differing light scattering properties. Together, these findings demonstrate that Mono T cells undergoing apoptosis exhibit reduced expression of CD8 but not other surface antigens such as CD3 and HLA-DR. These findings further demonstrate that Mono T cells protected from death by IL-10 are phenotypically similar to the T cells that have not undergone as yet the phenotypic changes associated with apoptosis.

In further experiments, we examined whether T cells protected from death by IL-10 are functional and can kill targets such as natural killer–sensitive K562 cells and allogenic EBV-immortalized cells which are susceptible to killing by Mono T cells (4, 5). To this end, equal numbers of viable Mono T cells obtained from cultures incubated for 12 h with or without hIL-10 were tested for their ability to kill these two target cell populations. As shown in a representative experiment (Fig. 7), Mono T cells precultured with hIL-10 were more effective than cells precultured in medium alone at killing each of the targets. The apparent greater level of killing by hIL-10–precultured T cells might be a consequence, in part, of increased survival of the activated, more cytotoxic T cells induced by IL-10.

Proliferation in response to hIL-2 was also measured. Equal numbers of viable cells were obtained from cultures of Mono T cells treated for 12 h with or without hIL-10. As shown in Table III, hIL-10–precultured T cells proliferated in response to hIL-2 at varying concentrations at least as well as medium-precultured T cells. These functional studies suggest that cells spared from death by IL-10 can still proliferate and exert cytotoxic activity.

Discussion

In this study we have found that hIL-10 and vIL-10 promote the survival of Mono T cells that are otherwise destined to die rapidly by apoptosis. Over a 6-d period of incubation, cultures of Mono T cells contained significantly more viable cells when exogenous IL-10 was initially added to the culture medium. In addition, IL-10 treatment was associated with a reduction in the number of T cells with condensed and fragmented nuclei and in the degree of DNA fragmentation in T cells.

This survival effect of IL-10 was not due to T cell growth stimulation by IL-10, as monitored by [^{3}H]thymidine incorporation. Rather, IL-10 appeared to delay the death in vitro of T cells activated in vivo. When added together with IL-2 and IL-4, or at least IL-2, IL-10 can stimulate T cell growth (36, 37). However, IL-2 was not required for IL-10 to promote T cell survival in the present system, because neutralization of IL-2 or of the IL-2 receptor alpha chain failed to inhibit the survival effect of IL-10. It is possible that the effect of IL-10 on T cell survival is indirect and mediated through IFN-γ. Unlike IL-10, IFN-γ was reported to both promote T cell death by apoptosis (38) and to be required during thymocyte death by apoptosis (39). By inhibiting IFN-γ production by Mono T cells, IL-10 might cause increased T cell survival.

It is important to note that Mono T cells spared from death by IL-10 appeared similar to viable, medium-treated cells in the expression of a number of T cell–specific and activation-related markers, including CD3, CD4, CD8, CD25, HLA-DR, and transferrin receptor. Furthermore, the IL-10–protected T cells continued to be functional, as demonstrated by their ability to effectively kill targets sensitive to cytotoxicity by Mono T cells and to subsequently proliferate in response to IL-2. Thus, IL-10 acts to ensure the persistence of functional T cell clones that would otherwise spontaneously die by apoptosis.

We have demonstrated recently that hIL-10 promotes the survival of IL-2–dependent T cells that have been starved of IL-2 and are destined to die by apoptosis (34). The data presented here extend these earlier observations and further demonstrate that the viability-promoting effect of hIL-10 is shared by vIL-10 and includes T cells activated in vivo after EBV infection.

What might be the reason for large-scale T cell expansion

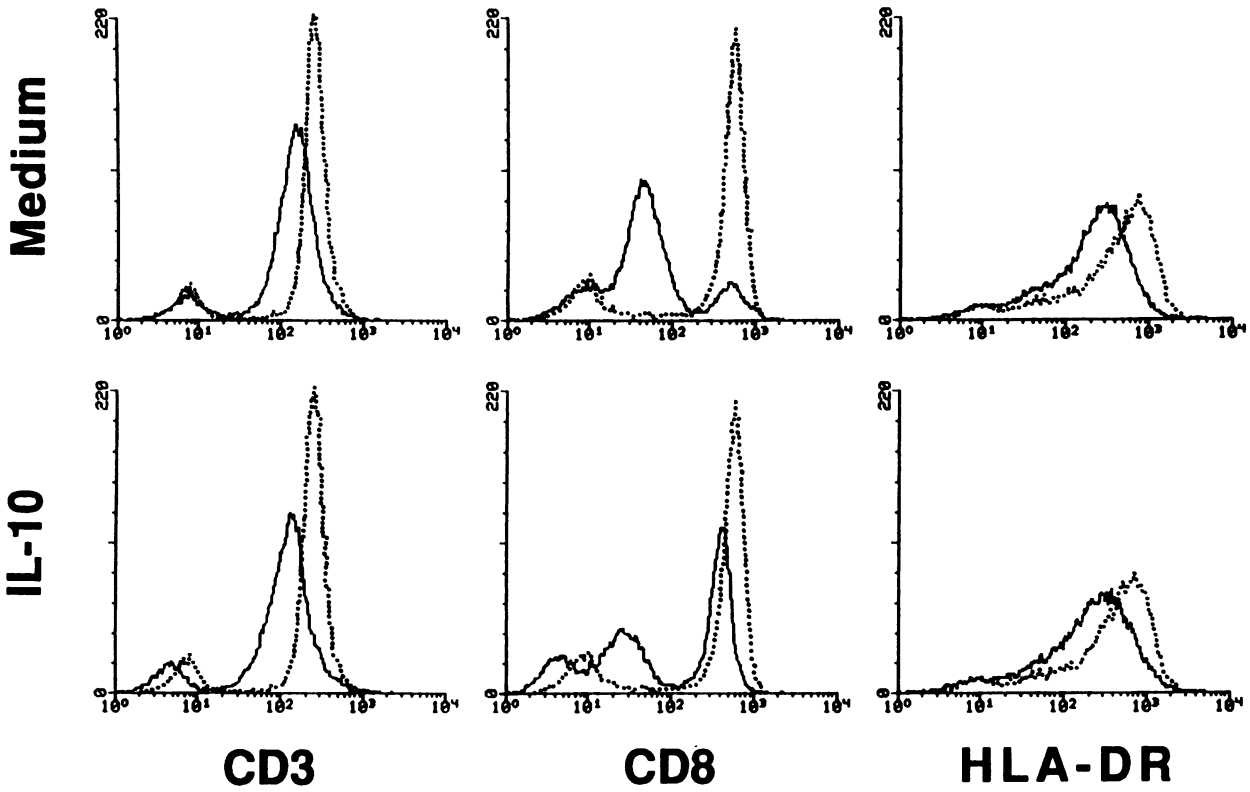

Figure 6. Effects of hIL-10 on expression of selected surface antigens on Mono T cells. T lymphocytes separated from peripheral blood were either stained with FITC-conjugated mAbs and then fixed with 1% paraformaldehyde in PBS or incubated (2×10^6 cells/ml) for 12 h in either medium alone or medium with 100 U/ml hIL-10. Recovery of viable T cells was 58.9% with 100/ml hIL-10 and 20.9% without it. Subsequently, the cultured cells were stained with FITC-conjugated mAbs and fixed with 1% paraformaldehyde in PBS. All cells were analyzed by flow cytometry; 20,000 cells were analyzed for each surface marker. Dotted lines identify results with fresh T cells, solid lines identify cells incubated for 12 h with or without hIL-10. Representative experiment of four performed.

followed by rapid cell death during acute EBV-induced Mono? What might be the role of IL-10 in regulating these processes? It is now believed that death by apoptosis occurs in most tissues at some stage of development (40–43) and that this process continues throughout life even though it often goes unrecognized because death by apoptosis can be histologically inconspicuous (44, 45). A system based upon overproduction of cells followed by selection might appear wasteful, but would provide a mechanism for ensuring that necessary functions are fulfilled by the most appropriate candidates. In the case of EBV-induced Mono, where the host is infected with a virus that can immortalize B cells by providing them with the capacity of unlimited growth, it is not surprising that evolution pressure has yielded an immune system that mounts an effective and massive response to this potentially life-threatening event. During this response, many immune T cells are generated with various properties such as help, suppression, and cytotoxic function (4–8). Subsequently, either because EBV infection is rapidly controlled by the cytotoxic effector cells or because some of the immune T cells localize where they are not needed or are functionally unfit, the need for many of the originally induced immune T cells may soon be over, and death by apoptosis ensues (9, 10). A balance must exist, however, between death and survival of EBV-immune T cells because after acute infection a highly effective memory population is retained, exhibiting much of the properties of T cells during the acute disease (14, 15).

The mechanism for the selective and regulated death by apoptosis of unwanted T cells after resolution of the acute disease is not understood. Previous studies in other systems have raised the possibility that survival of many cells is dependent upon extracellular survival signals (44, 46). In the absence of such signals, the cells would be normally programmed to die. It is possible that EBV-infected cells play a critical role in controlling the survival of T cells. By producing IL-10 (16, 17, 21) and a variety of other factors that can regulate T cell growth and survival, including IL-12 (47), IL-6 (48), lymphotoxin (49), and TNF-α (50), B cells and epithelial cells infected with EBV may regulate initial growth and survival of immune T cells. Once EBV replication in the oropharynx diminishes or subsides, and the number of circulating EBV-infected B cells is reduced, the availability of growth and survival factors for T cells may also be reduced, and most of the immune T cells may subsequently die.

The molecular basis for programmed cell death is also not understood. The mammalian gene bcl-2 has been shown to suppress programmed cell death in many cells (51). Interestingly, bcl-2 expression is low in circulating activated Mono CD45RO$^+$ T cells of patients with acute viral infections, including EBV-induced Mono, and a significant correlation was seen

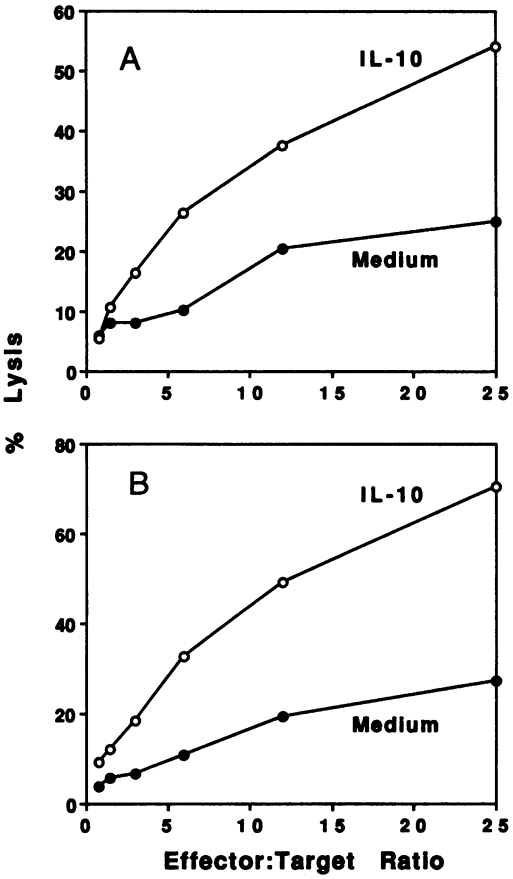

Figure 7. Effects of hIL-10 on cytotoxicity on Mono T cells. T cells were cultured (2×10^6 cells/ml) for 12 h at 37°C in either medium alone or with 100 U/ml hIL-10. Subsequently, the cells were washed, counted, and tested in triplicate for cytotoxicity against ^{51}Cr-labeled (*A*) K562 cells and (*B*) EBV-immortalized cells (VDS-0 line). Recovery of T cells cultured with or without hIL-10 was 74.9 and 37.0%, respectively. Effector to target ratios relate to viable cells only. Representative experiment of three performed.

between low bcl-2 expression by activated T cells and their death in vitro (52). The effect of IL-10 on bcl-2 expression by Mono T cells was not investigated here. It was reported, however, that survival of activated CD45RO$^+$ Mono T cells was promoted by IL-2, which resulted in a concomitantly increased bcl-2 expression, as well as exposure to fibroblasts which did not induce bcl-2 expression (52). This fibroblast-derived survival factor could be IL-10.

EBV is a successful pathogen that has managed to infect most adults worldwide and has done so by periodically replicating in the oropharynx and establishing latency in B cells (53). Successful infiltration of EBV in the human species has occurred in the face of effective and complex T cell regulation (54). Because EBV has probably acquired the BCRF-1 gene during evolution, it is important to ask whether vIL-10 expression may have contributed to virus survival. It was proposed that by inhibiting IFN-γ production and T cell growth IL-10 suppresses T cell immunity and promotes B cell immortalization by EBV (16). IFN-γ is known to inhibit B cell immortalization by EBV in vitro (55). Infection of normal mice with recombinant vaccinia virus expressing IL-10 resulted in reduced natural killer and specific cytotoxic activity (56). However, infection of immunodeficient mice with IL-10–expressing vaccinia virus resulted in increased natural killer function (56). Also, IL-10 enhanced T cell cytotoxicity of EBV-infected cells in vitro (57). Recently, EBV recombinants with specifically mutated BCRF-1 gene were found to immortalize normal B cells, excluding a critical role of vIL-10 in initiating and maintaining B cell immortalization by EBV (58). However, IL-10 promotes B cell growth, including growth of EBV-immortalized B cells (59), and increases B cell survival by inhibiting cell death (60). Thus, the effects of IL-10 on EBV infection appear to be more complex than had been anticipated.

Very little is known about the mechanisms for the establishment and persistence of long-lived memory T cells directed at EBV. The principal growth and survival factor for T cells, IL-2, is not widely available for T cells in vivo, and various mechanisms are believed to operate in vivo to guarantee compartmentalization of IL-2, thus avoiding systemic effects (49). In the absence of IL-2, T cell survival may depend upon other factors, including IL-10.

Table III. Function of T Cells Protected from Death by hIL-10

Additions to secondary culture*	[^{3}H]Thymidine deoxyribose incorporation‡	
	Medium-precultured	hIL-10–Precultured
	mean counts per minute/well	
Medium	125 (12)	59 (6)
hIL-2 (1 U/ml)	77 (13)	144 (23)
hIL-2 (10 U/ml)	1,452 (29)	4,449 (532)
hIL-2 (100 U/ml)	13,344 (485)	28,910 (866)
hIL-2 (1,000 U/ml)	19,265 (436)	37,152 (2,297)

* Mono T cells were cultured (2×10^6 cells/ml in T25 tissue culture flasks) for 12 h either in medium alone or in medium with 100 U/ml hIL-10. Subsequently, the cells were washed, and equal numbers of viable cells were incubated in secondary cultures either in medium alone or with varying amounts of hIL-2 (1–1,000 U/ml). Recovery of viable cells precultured with or without hIL-10 was 74.9 and 37.0%, respectively. ‡ Proliferation was measured by [^{3}H]thymidine incorporation during the final 6 h of a 3-d secondary culture. The results reflect the mean (±SD) of triplicate determinations. Representative experiment of three performed.

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