HTLV-I Tax Induces Cellular Proteins That Activate the κB Element in the IL-2 Receptor α Gene

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Jurkat T cell lines constitutively expressing Tax, the 40-kilodalton transactivator protein of human T lymphotropic virus type I (HTLV-I), were used to investigate the mechanism by which this viral product deregulates the expression of the interleukin-2 receptor α gene (IL-2Rα, Tac). Transfection of deleted forms of the IL-2Rα promoter and in vitro DNA-binding studies revealed that a 12–base pair promoter segment, which has homology with the binding site for NF-κB, was required for Tax-induced activation of the IL-2Rα promoter in vivo. An 18–base pair oligonucleotide containing this κB-like regulatory element proved sufficient to confer Tax inducibility upon a heterologous promoter. DNA affinity precipitation assays showed that Tax, like mitogenic stimuli, induced the expression of the 86-kilodalton cellular protein HIVEN86A, which specifically binds to the IL-2Rα κB element in vitro. Furthermore, DNA/protein cross-linking studies revealed that several polypeptides interact with this sequence motif. Thus, the deregulation of IL-2Rα gene expression encountered in HTLV-I leukemias appears to involve Tax activation of one or more cellular proteins that are normally induced by mitogens and that directly contribute to transcriptional activation of this receptor gene.

MALIGNANT T CELL GROWTH MANifested as the adult T cell leukemia (ATL) is etiologically associated with type 1 human T-cell leukemia virus (HTLV-I) infection (*1*). The pX region of the HTLV-I genome encodes a 40-kD polypeptide termed Tax ($p40^x$, X-lor, *tat*-1) (*2*), which functions in trans to activate enhancer sequences located within the retroviral long terminal repeat (LTR) (*3*). Recent interest has focused on the possible interplay of Tax with cellular genes that regulate T cell growth and whose altered expression may play a role in HTLV-I–induced transformation (*4*). Specifically, transient cotransfection studies showed that Tax activates the IL-2Rα gene promoter (*5*). These findings provided an explanation for the deregulated display of IL-2Rα on HTLV-I–infected T cells (*6*). However, the molecular mechanism by which Tax alters the expression of this receptor gene remained unknown.

We have used Jurkat T cell clones stably expressing either sense or antisense *tax* cDNAs (designated J-*tax* and J-anti-*tax*, respectively) (*7*) to explore the biochemical basis for Tax-induced activation of the IL-2Rα gene. In the J-*tax* lines only, functional Tax protein is produced and the endogenous IL-2Rα gene is transcriptionally active (*7*). Plasmids containing various 5′-deleted forms of the IL-2Rα promoter linked to the chloramphenicol acetyltransferase (CAT) gene were transfected into both of these cell lines. Deletion end points for these plasmids encompassed sequences located between nucleotides −271 and −179, a region previously found to be required for transient Tax induction of the IL-2Rα-CAT plasmids in normal Jurkat cells (*8*). As shown in Fig. 1A, IL-2Rα promoter sequences positioned between nucleotides −266 and −248 were required for optimal CAT induction in J-*tax*-9 (closed bars). In contrast, none of the promoter deletion mutants were significantly activated in either J-anti-*tax*-10 (open bars) or unstimulated normal Jurkat cells (not shown). Notably, this critical IL-2Rα promoter region contains a 12-bp element (GGGGAATCTCCC, −267 to −256) with striking similarity (*9*) to the κB enhancers (NF-κB binding sites) present in the κ immunoglobulin gene intron (*10*) and human immunodeficiency virus (HIV-1) LTR (*11*). This IL-2Rα promoter motif interacts with mitogen-inducible proteins and plays an important role in transcriptional activation of the IL-2Rα gene mediated by various inducers including phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) (*8, 9*).

To examine whether this κB-like element was required for Tax inducibility of the IL-2Rα promoter, we used site-directed mutagenesis to effect its selective deletion from a fully Tax-inducible IL-2Rα-CAT plasmid terminated at −317 (Fig. 1A). The unmodified −317 plasmid was readily induced in J-*tax*-9 (closed bar) but not in J-anti-*tax*-10 (open bar) (Fig. 1B). Similarly, this promoter construct was activated by PMA after transfection into normal Jurkat T cells. In contrast, deletion of the κB element (plasmid −317Δ) was associated with a marked decrease of both Tax- (78%) and PMA- (88%) induced activation in J-*tax*-9 and normal Jurkat T cells, respectively. These findings suggest that this regulatory motif is necessary for maximal IL-2Rα activation induced by either Tax or PMA.

Synthetic oligonucleotide duplexes encompassing this Tax-responsive region were cloned immediately upstream of the thymidine kinase promoter–CAT (TK-CAT) transcription unit (*12*) to examine effects of this element on a heterologous promoter (*8, 9*). As shown in Table 1, plasmids containing a single copy of either a 47-bp (IL-2R III) or an 18-bp (IL-2R VII) IL-2Rα promoter segment spanning the κB motif proved sufficient to confer Tax inducibility

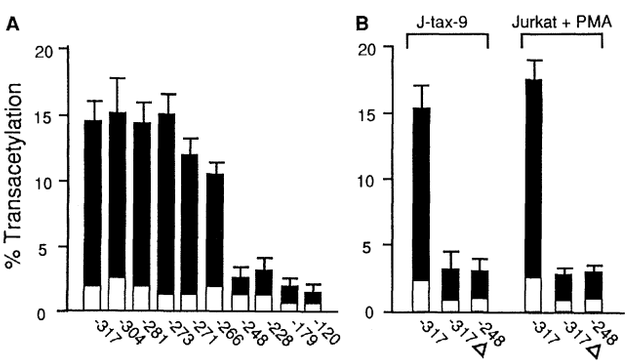

Fig. 1. (**A**) Tax stimulation of 5′-deleted forms of the IL-2Rα promoter linked to the CAT reporter gene. IL-2Rα-CAT deletion mutants, terminated upstream by Bal 31 exonuclease treatment (*8*) at the nucleotide positions indicated (relative to major cap site, +1), were transfected by the DEAE-dextran method (5 μg of DNA per 10^7 cells) into either J-*tax*-9 (closed bars) or J-anti-*tax*-10 cells (open bars). CAT activity (expressed as percentage chloramphenicol transacetylation) was measured after 48 hours as described (*20*). (**B**) Effect of selective deletion of the κB site from a fully functional IL-2Rα promoter on Tax and mitogen inducibility. Nucleotides −269 to −254 were deleted from the −317 IL-2RΔ-CAT construct by in vitro mutagenesis (*21*) to create the −317Δ IL-2Rα-CAT construct. Parallel transfections of these plasmids (including the −248 IL-2Rα-CAT mutant; see Fig. 1A) and CAT activity measurements were carried out on Jurkat *tax* lines (J-*tax*-9, closed bars; J-anti-*tax*-10, open bars), or parental Jurkat cells stimulated with medium (open bars) or PMA (50 ng/ml, closed bars). Results shown represent mean values from three independent experiments.

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(seven- to tenfold) to the TK promoter. These stimulatory effects were independent of insert orientation and were amplified when either segment was reiterated. Point mutations introduced within the κB motif (IL-2R III M1 and IL-2R VII M1) markedly diminished Tax responsiveness to levels comparable to that present in J-anti-*tax*-10 transfected with either wild-type or mutated constructs. Consistent with our previous findings (*8, 9*), these recombinant TK-CAT plasmids were also induced by PMA.

Nuclear extracts were analyzed in gel retardation assays (*13*) for the presence of Tax-inducible proteins that specifically bind to the IL-2Rα promoter element identified in our functional studies. As shown in Fig. 2A, nuclear extracts prepared from both mitogen-stimulated Jurkat cells (lane 2) and HTLV-I–infected HUT 102B2 cells (lane 3) contain proteins that specifically bind to a 47-bp IL-2Rα promoter segment spanning the κB site (IL-2R III, −291 to −245), retarding probe mobility to the two positions indicated (*8, 9*). Incubation of this probe with nuclear extracts from the J-*tax*, but not J-anti-*tax*, cell lines also produced two discrete DNA/protein complexes with similar electrophoretic mobility (Fig. 2B, lanes 1 to 4). In competition studies (Fig. 2C), formation of these complexes was completely blocked by preincubation of J-*tax*-9 nuclear extracts with unlabeled oligonucleotides corresponding to the 3′ half (lane 3), which contains the κB element, but not the 5′ half (lane 2) of the radiolabeled IL-2R III probe. Mutation of three bases within the κB motif of IL-2R III abolished its inhibitory properties (lanes 4 and 5). Unlabeled oligonucleotides containing NF-κB binding sites from the HIV-I LTR (*11*) also effectively blocked specific protein binding (lane 6).

Direct physical evidence for DNA/protein contacts within the IL-2Rα κB motif was obtained by subjecting these radiolabeled IL-2R III/protein complexes to in situ DNA footprinting with 1,10-phenanthroline-copper (*14*). Compared to that of free DNA, the cleavage pattern of the coding strand from complexed DNA revealed a single strong protection within the 12-bp IL-2Rα promoter element (Fig. 2D, lanes 4 and 5). Similar results were obtained when nuclear extracts from mitogen-stimulated normal Jurkat cells were used (lane 3). Collectively, these studies confirmed that Tax, like mitogens, induces the expression of a cellular factor (or factors) that specifically binds to this IL-2Rα promoter element in vitro. However, the precise requirements for Tax and mitogen activation of the IL-2Rα promoter are somewhat different, as both we (*8*) and Cross *et al.* (*5*) have found that additional sequences located 5′ of the κB element are required for maximal mitogen, but not Tax, activation.

To directly identify protein candidates responsible for this DNA-binding activity, we incubated [^{35}S]methionine-labeled extracts with biotinylated forms of the IL-2R III footprint probe. Proteins from the resultant complexes were recovered by precipitation with avidin-agarose beads and analyzed on two-dimensional polyacrylamide gels (*15*). Fluorography of proteins from J-*tax*-9 (Fig. 3B) revealed the presence of an 86-kD protein with isoelectric point heterogeneity indistinguishable in electrophoretic mobility from HIVEN86A, a mitogen-in-

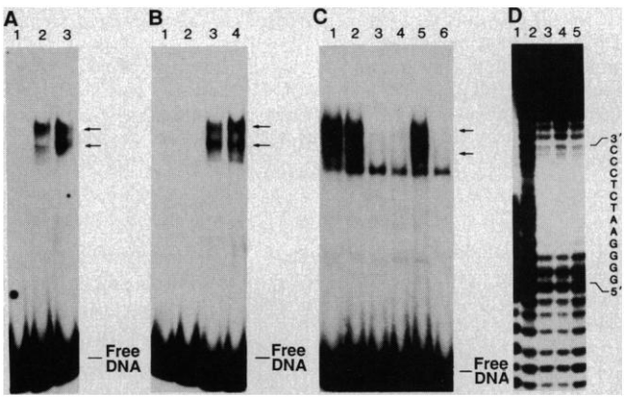

Fig. 2. (**A** and **B**) Tax induction of DNA-binding proteins that bind to the IL-2Rα promoter. Nuclear extracts were prepared from J-*tax* (B, lanes 3 and 4) and J-anti-*tax* (B, lanes 1 and 2) lines, HTLV-I–infected HUT 102B2 cells (A, lane 3), and normal Jurkat cells before (A, lane 1) and after (A, lane 2) stimulation with PMA (50 ng/ml) and PHA (1 μg/ml) as described (*8, 9*). These extracts were incubated with a 47-bp ^{32}P-labeled DNA probe (IL-2R III) spanning nucleotides −291 to −245 of the IL-2Rα promoter, and ^{32}P-labeled DNA/protein complexes were resolved on 5% polyacrylamide gels (*8, 9*). The migration of the ^{32}P-labeled complexes (arrows) and free ^{32}P-labeled DNA are indicated. (**C**) Specificity of DNA-binding proteins induced by Tax. J-*tax*-9 nuclear extracts were incubated with a 100-fold molar excess (relative to ^{32}P-labeled DNA) of various competitors, and then ^{32}P-labeled IL-2R III was added and the extracts were subjected to polyacrylamide gel electrophoresis. Competitor oligonucleotide duplexes included IL-2R III (−291 to −245, lane 4), IL-2R I (−293 to −270, lane 2), and IL-2R II (−267 to −243, lane 3) from the IL-2Rα promoter, IL-2R III M1 (an IL-2R III mutant with the three-base substitution GGG→CTC at positions −266 to −264 within the kilobase site, lane 5), and HIV-DR (−106 to −81, lane 6) from the enhancer region of the HIV-1 LTR (*9*). No competitor was added to binding reactions analyzed in lane 1. (**D**) Chemical footprinting of IL-2Rα promoter DNA complexed with Tax-induced DNA-binding proteins. ^{32}P-labeled IR-2R III/protein complexes formed with nuclear extracts from J-*tax*-9 (lane 4), J-*tax*-19 (lane 5), or mitogen-stimulated Jurkat cells (lane 3) were resolved by gel retention and partially digested in situ with 1,10-phenanthroline-copper (*14*). Recovered products, including free probe (lane 2), were electrophoresed on a 10% polyacrylamide/urea sequencing gel. The same probe (labeled on the coding strand) was independently cleaved with piperidine (A+G ladder, lane 1) for sequence alignment of the IL-2Rα κB motif denoted at right.

Table 1. Relative CAT activities directed by TK-CAT plasmids containing IL-2Rα promoter segments spanning the κB element. Oligonucleotide duplexes designated in column 1 (IL-2R III, −291 to −245; IL-2R III M1, GGG→CTC substitution at −266 to −264 in IL-2R III; IL-2R VII, −272 to −255; IL-2R VII M1, G→A at −267, A→T at −262, T→A at −259, and C→T at −256 in IL-2R VII) were inserted immediately upstream of the TK promoter linked to the CAT gene (*12*). J-*tax*-9, J-anti-*tax*-10, and parental Jurkat cells were transfected with plasmids as described in Fig. 1; the Jurkat cells were stimulated with PMA (50 ng/ml) 24 hours after transfection. For comparison, transacetylation values (*20*) were normalized relative to that obtained with the use of the unmodified TK-CAT vector (1 to 2% basal conversion).

Insert	Orientation	Relative CAT activity		
		J-*tax*-9	J-anti-*tax*-10	Jurkat + PMA
None		1.0	1.0	1.0
IL-2R III	→	7.2	0.9	4.7
	←	10.8	1.1	5.2
	←→	34.5	1.1	21.1
IL-2R III M1	→	1.4	1.3	1.3
	←	0.9	0.9	1.0
	→→	1.2	0.8	1.2
IL-2R VII	→	9.5	1.0	5.1
	←	7.1	0.9	4.5
	→→	19.2	1.2	14.6
	←←	22.8	1.1	16.2
IL-2R VII M1	→	1.1	0.8	1.1
	→→	0.8	0.9	1.1
	←←	1.0	0.8	0.9

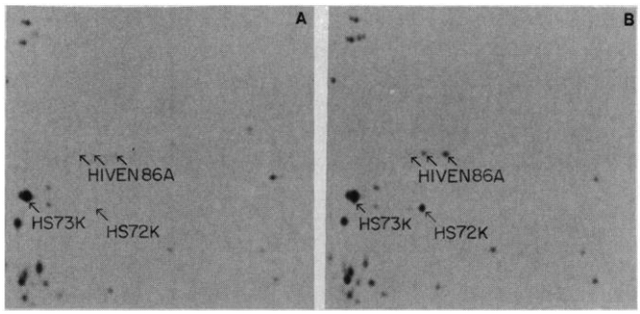

Fig. 3. Microscale DNA affinity precipitation assay of Tax-induced cellular DNA-binding proteins that interact with the IL-2Rα promoter κB element. Biotinylated IL-2R III DNA from the IL-2Rα promoter (−291 to −245) was incubated with [^{35}S]methionine-labeled nuclear proteins isolated from J-anti-*tax*-10 (**A**) or J-*tax*-9 (**B**), followed by DNA-protein complex precipitation with avidin-agarose and two-dimensional gel electrophoresis (*15*). The different isoelectric forms of the HIVEN86A protein (*9, 15*), and two heat shock proteins (HS72K and HS73K), are identified by arrows. HS73K is coprecipitated in these assays independent of Tax expression and DNA probe sequence; HS72K represents a Tax-inducible protein with similar nonspecific DNA-binding properties (*22*).

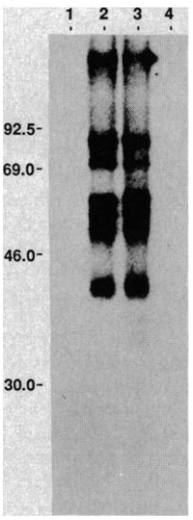

Fig. 4. Cross-linking of DNA-binding proteins to the IL-2Rα κB site. A 27-bp sequence (−275 to −249 of the IL-2Rα promoter coding strand) was annealed with two complementary oligonucleotides (−275 to −266, −258 to −249); bromodeoxyuridine and [α^{32}P]dNTPs were incorporated into the noncoding strand of this gapped duplex as described (*16*). DNA/protein complexes formed by incubation with J-*tax*-9 nuclear extracts were resolved by retardation on low-melting agarose gels, cross-linked in situ with UV-irradiation, and analyzed on SDS-polyacrylamide gels (*16*). Samples shown were excised from retardation gel regions corresponding to free ^{32}P-labeled DNA (lane 1), complexed ^{32}P-DNA (lane 2), or the equivalent of the latter in DNA binding reactions containing competitor DNA fragments derived from the −317 (−275 to −249, lane 4) or −317Δ (−290 to −238, lane 3) IL-2Rα promoter constructs (see Fig. 1B). Positions and relative molecular weights (in kilodaltons) of protein standards are indicated.

ducible polypeptide with identical binding specificity (*9, 15*). In contrast, this protein was not detectable in the two-dimensional gel patterns of extracts from J-anti-*tax*-10 (Fig. 3A), nor was this protein identified with biotinylated IL-2R III probes bearing a 3-base substitution within the κB motif. These data suggest that Tax activates the synthesis or DNA-binding activity of HIVEN86A, which is similarly stimulated by PHA and PMA (*9*).

In an attempt to correlate HIVEN86A DNA-binding activity with the protein components of the DNA/protein complexes resolved by gel retardation (Fig. 2B), we incubated J-*tax*-9 nuclear extracts with a photoreactive IL-2Rα κB probe containing bromodeoxyuridine. The resultant complexes were separated from free DNA by gel retardation and covalently cross-linked in situ by ultraviolet (UV) irradiation (*16*). Analysis of gel slices corresponding to free probe and retarded complexes by SDS–polyacrylamide gel electrophoresis (Fig. 4, lanes 1 and 2) revealed seven distinct DNA-protein adducts, including a species migrating between 80 and 90 kD. Formation of these covalent complexes was completely inhibited by preincubation of extracts with κB DNA fragments derived from a functional −317 IL-2Rα promoter (lane 4), but not by κB-deleted DNA fragments from the corresponding region of an unresponsive −317Δ promoter (lane 3) (see also Fig. 1B). Consistent with previous results from DNA affinity precipitation assays with tandem κB elements being used as a probe (*15, 17*), these UV cross-linking data suggest that other nuclear proteins, in addition to HIVEN86A, may also specifically associate with the IL-2Rα κB site. However, it remains to be determined whether these polypeptides represent distinct gene products or related proteolytic fragments of a larger precursor protein.

Our results indicate that Tax activation of the IL-2Rα gene involves the induced expression of a cellular protein, HIVEN86A, and the subsequent interaction of this and possibly other cellular factors with an IL-2Rα promoter motif homologous to the NF-κB binding site. These same cis and trans elements appear to play an important role in normal IL-2Rα gene activation induced by mitogens (*9*). The finding that Tax induces HIVEN86A provides a unifying explanation for Tax activation of the HIV-I LTR (*19*), because the enhancer region of this LTR contains NF-κB binding sites (*11*) that specifically interact with HIVEN86A (*9, 15*). Since Tax activation of the HTLV-I LTR involves different cis sequences [21-bp repeats (*3*)] and an apparently different set of cellular proteins (*18*), it seems likely that Tax is capable of interfacing with multiple cellular DNA-binding proteins.

Note added in proof: Since submission of this paper, two other groups (*23*) have reported findings that implicate the IL-2Rα κB motif as a Tax-responsive promoter element.

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Effects of Cyclosporine A on T Cell Development and Clonal Deletion

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Cyclosporine A (CsA) is an important immunosuppressive drug that is widely used in transplantation medicine. Many of its suppressive effects on T cells appear to be related to the inhibition of T cell receptor (TCR)–mediated activation events. Paradoxically, in certain situations CsA is responsible for the induction of a T cell–mediated autoimmunity. The effects of CsA on T cell development in the thymus were investigated to elucidate the physiologic events underlying this phenomenon. Two major effects were revealed: (i) CsA inhibits the development of mature single positive ($CD4^+8^-$ or $CD4^-8^+$) TCR-$\alpha\beta^+$ thymocytes without discernibly affecting $CD4^-8^-$ TCR-$\gamma\delta^+$ thymocytes and (ii) CsA interferes with the deletion of cells bearing self-reactive TCRs in the population of single positive thymocytes that do develop. This suggests a direct mechanism for CsA-induced autoimmunity and may have implications for the relative contribution of TCR-mediated signaling events in the development of the various T cell lineages.

CYCLOSPORINE A (CsA) IS AN IMMUNOSUPPRESSIVE drug that inhibits lymphokine production by helper T cells in vitro and has been widely used to alleviate tissue allograft rejection in vivo (*1*). It is also effective in preventing graft-versus-host disease (GVHD) secondary to allogeneic bone marrow transplantation (*2*). Surprisingly, irradiated hosts transplanted with syngeneic bone marrow and then treated with, and withdrawn from, CsA actually develop autoimmunity (*3*). This phenomenon, observed in rats (*3, 4*), mice (*5, 6*), and humans (*7*), is characterized by a GVHD-like syndrome that is transferable to naïve recipients by T cells. Removal of the thymus from transplanted animals prevents the induction of autoimmunity (*4*), thus, the thymus is required for the development of the autoreactive T cells. Several mechanisms have been postulated to explain these results, including repertoire alterations secondary to reduced class II major histocompatibility complex (MHC) molecule expression in the thymic medulla (*5*) or effects on the development of suppressor cells that normally regulate autoreactive T cell clones (*4, 6*). However, the direct effects of CsA on thymocyte development have not been fully explored.

Advances in the delineation of the molecular and cellular events involved in T cell differentiation enabled us to directly examine the effect of CsA on specific developmental stages. A critical early event in thymocyte differentiation is the progressive rearrangement of T cell receptor (TCR) gene segments leading to surface expression of either a TCR-γδ or a TCR-αβ (*8–10*). Thymocytes that productively rearrange and express TCR-γδ maintain a $CD4^-8^-$ phenotype and appear to represent a stable distinct lineage from TCR-αβ–bearing thymocytes (*10–12*). Cells that fail to productively rearrange their γ or δ genes probably continue to rearrange their α and β genes and also initiate expression of the accessory molecules CD4 and CD8, resulting in a $CD4^+8^+$ TCR-$\alpha\beta^{lo}$ intermediate stage thymocyte (*10, 11, 13*). Deletion of potentially self-reactive T cells occurs at this stage (*14–16*). A small subset of $CD4^+8^+$ thymocytes ("double positive") undergoes a subsequent differentiation step characterized by an increase in surface TCR density and down-regulation of either CD4 or CD8 resulting in the "single positive" TCR-$\alpha\beta^{hi}$ phenotype of mature T cells (*11, 13*). There is an additional "positive selection" step resulting in the skewing of the T cell repertoire toward self-MHC restriction (*17*). The developmental step at which this occurs is unknown.

We initially investigated the effects of CsA on the various developing thymocyte subsets as defined by CD4, CD8, and TCR expression (Table 1 and Fig. 1). C57BR mice were lethally irradiated, reconstituted with autologous bone marrow cells, and injected intraperitoneally with CsA at 20 mg/kg daily for 25 days; at this time full reconstitution of the predominant thymocyte subsets with donor-derived cells is complete (*18*). Thymocytes from control mice analyzed by two-color flow cytometry (FC) had the normal subset distribution: 4% $CD4^-8^-$, 78% $CD4^+8^+$, 11% $CD4^+8^-$, and 7% $CD4^-8^+$ (Fig. 1C). In contrast, thymocytes from CsA-treated mice were depleted of single positive cells ($CD4^+8^-$ and

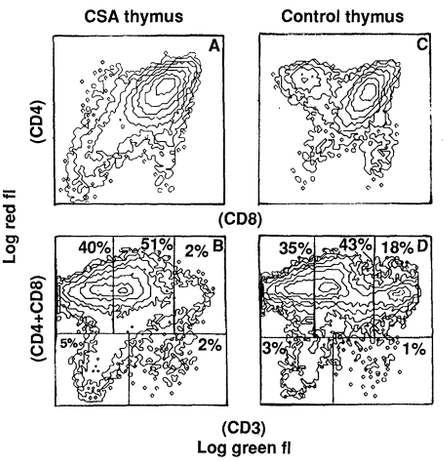

Fig. 1. Effect of CsA on developing thymocyte subsets. C57BR mice (Jackson Laboratory) were irradiated with 850R from a Gammacell 40 irradiator, reconstituted with 10^7 autologous bone marrow cells, and maintained in autoclaved cages on antibiotic water. Beginning 3 days after reconstitution and continuing for the next 23 days, mice received daily intraperitoneal injections of 20 mg of CsA per kilogram dissolved in olive oil (20 mice per experiment, referred to as CsA-treated) or an equal volume of olive oil alone (10 mice per experiment, referred to as controls). Age- and sex-matched experimental and control mice were treated and analyzed simultaneously within each experiment. Unfractionated thymocytes, pooled from all mice from CsA-treated (panel A) or control (panel C) groups were stained with fluorescein isothiocyanate (FITC)–labeled antibody to CD8 (Becton Dickinson) followed by biotinylated antibody to CD4 (H129.19) (*27*) plus allophycocyanin-avidin. Alternatively, unfractionated thymocytes from CsA-treated (panel B) or control (panel D) mice were stained with MAb to CD3-ε (500-A2) (*28*) plus FITC-labeled goat antibody to hamster immunoglobulin (Ig) (Kirkegard and Perry) and then with biotinylated anti-CD4 and biotinylated MAb to CD8 (Becton Dickinson) plus allophycocyanin-avidin. Samples (2×10^5 cells) were analyzed by FC on a FACS 440 (Becton Dickinson). Plots shown are representative of three separate experiments. Standard errors of the mean were less than 10%.

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