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Programmed Cell Death of T Cells Signaled by the T Cell Receptor and the α_3 Domain of Class I MHC

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As well as being activated or rendered unresponsive, mature T lymphocytes can be deleted, depending on the signals received by the cell. Deletion by programmed cell death (apoptosis) is triggered if a T cell that has received a signal through its T cell receptor complex also receives a signal through the α_3 domain of its class I major histocompatibility complex (MHC) molecule. Such a signal can be delivered by a CD8 molecule, which recognizes the α_3 domain, or by an antibody to this domain. Precursors of both cytotoxic T lymphocytes (CTL's) and T helper cells are sensitive to this signal but become resistant at some point before completing differentiation into functioning CTL's or T helper cells. Because CTL's carry CD8, they can induce cell death in T cells that recognize them. This pathway may be important in both removal of autoreactive T cells and immunoregulation.

ACTIVATION OF A T LYMPHOCYTE requires occupancy of its antigen-specific cell surface receptor (TcR) by its appropriate ligand (processed antigen presented by class I or class II MHC molecules) and a second signal from a growth factor. If the second signal is not provided, the T cell becomes unresponsive (anergic) (*1*). We now present evidence for a pathway that leads to death of T lymphocytes that have been signaled through their TcR and then also receive a signal through the α_3 domain of their class I MHC molecule.

Short-term tissue culture studies of mouse lymphocytes in the mixed lymphocyte reaction (MLR) (*2*) have shown that CTL's can inactivate CTL precursors (CTLp's) that recognize them. This process does not involve the receptor of the CTL being recognized and can occur in the presence of cells and factors that can produce activation (*3*). This implies that, on being recognized, CTL's deliver a signal leading to inactivation; that is, they are acting as specialized antigen presenting cells (veto cells) (*4*) that inactivate T cells that recognize them. CTL's reactive to class I MHC (the majority) also carry the cell surface molecule CD8, which can recognize the α_3 domain of class I MHC (*5*). This binding can strengthen the adhesion of a CTL or CTLp to a cell it recognizes by binding to class I MHC molecules on that cell, thus facilitating T cell responses. We test here whether the CD8 molecule has a second function: Can a CD8 molecule on a cell being recognized by a T cell trigger the inactivation of that T cell by interacting with its class I MHC?

Paired $CD8^+$ and $CD8^-$ CTL lines of mouse origin were derived from two independent MLR with F_1(BALB/c × RNC) as responders and C57BL/6 (B6) as stimulators (*6*). All four lines had an αβ TcR, similar specificity (anti-D^b), and similar cytotoxic activity. The $CD8^+$ lines ($H\text{-}2^{d/k}$), when added to an MLR, reduced development of cytotoxic activity when BALB/c ($H\text{-}2^d$) were used as stimulators (MHC was shared with the added CTL's), but had no effect when SWR ($H\text{-}2^q$) were used as stimulators (no MHC sharing) (Fig. 1A). The $CD8^-$ CTL line had no effect on the response in either MLR.

We reasoned that covering the CD8 molecule on a $CD8^+$ CTL with a monoclonal antibody (MAb) to CD8 should block its ability to inactivate CTLp's. However, a MAb to CD8 would also block response induction (*7*). We therefore used a MAb specific for one of the two allelic forms of the mouse CD8 molecule (Ly-2.1 and Ly-2.2). MLR's were set up in which both responders (SJL and $H\text{-}2^s$) and stimulators (BALB/c and $H\text{-}2^d$) were Ly-2.2. To these were added CTL's from either BALB/c (identical to stimulator) or DBA/2 (same MHC but Ly-2.1) in the presence or absence of MAb to Ly-2.1. Both CTL lines produced equivalent response reduction in the absence of added MAb to Ly-2.1 but, as predicted, the response reduction produced by the Ly-2.1^+ DBA/2 CTL line was partly reversed by addition of the MAb to Ly-2.1 (Fig. 1B).

These results suggested that any cell line might inactivate CTLp's recognizing it if it were transfected with CD8. We compared the ability of a TcR^- $CD4^-$ $CD8^-$ BW 5147 T lymphoma line ($H\text{-}2^k$) to inactivate CTLp's reactive against $H\text{-}2^k$ with that of cells from the same line transfected with either CD8 (*8*) or CD4 (*9*). Appropriate expression of CD4, CD8, and $H\text{-}2^k$ was confirmed by flow cytometry (*10*). Only the $CD8^+$ lymphoma produced inhibition of the anti-$H\text{-}2^k$ MLR (Fig. 1C). This experiment provides direct evidence for the role of CD8 in the inactivation of CTLp's and also demonstrates that cells do not need to have the cytotoxic machinery of a CTL or to carry a TcR to be able to produce inactivation of CTLp's.

The MAb 34-2-12S (*11*), referred to as anti-α3, is known to bind to the α_3 domain of H-2 class I D^d MHC molecules (*12*). In that CD8 also binds to the α_3 domain, addition of this MAb to an MLR in which the responder cells carried D^d might mimic the effect of adding cells expressing CD8. The effects of adding anti-α_3 or control MAb on cell number, CTL activity, and interleukin-2 (IL-2) production in an MLR containing D^d-bearing responder cells were determined (experiment 1, Table 1). The control MAb's, of the same isotype (IgG2a) and generated in the same immunization protocol (*11*), all interact with class I MHC but appear not to interact with the α_3 domain (*12*). Anti-α3 prevented development of cytotoxic activity whereas the other MAb's to MHC had no effect (*13*). Surprisingly, both total cell number and IL-2 production [a measure of T helper cell (Th) activity] were also greatly reduced by anti-α3 but not by the other MAb's. One interpretation is that T helper precursors (Thp's), as well as CTLp's, are inactivated if they also receive a signal through the α_3 domain of their class I MHC molecule.

MAb 28-14-8S (*14*) is a second MAb known to bind to a class I MHC α_3 domain, in this case D^b (*15*). It also produced reduction of both cytotoxic activity and IL-2 production whereas control MAb's (*14, 15*) did not (experiment 2, Table 1A) (*16*).

The inactivation of CTLp's produced by added CTL's occurs early in an MLR; added CTL's have little or no effect if added later than 2 days after initiation (*3*). Similarly,

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anti-α3 completely inhibited cytotoxic activity in the MLR when added on days 0, 1, and 2, but had no effect when added on day 4 (Fig. 2A). The MAb completely inhibited IL-2 production when added on days 0 and 1 but had no effect when added on days 2 or 3 (Fig. 2B).

Access of anti-α_3 to the α_3 domain might be inhibited if MAb's to α_1/α_2 were first added. This, in fact, occurred when MAb 34-5-8S (anti-α_1/α_2) was added 48 hours after MLR initiation and anti-α_3 was added 1 hour later but not when they were added in reverse order (experiment 3, Table 1). This result provides evidence that the MAb to α_3 is not acting in a nonspecific manner and that it is acting through direct binding to MHC.

The TcR is associated with the CD3 molecular complex, thought to transmit signals from the TcR to the cytoplasm. An antibody to CD3 can act as a polyclonal T cell activator (*17*). Simultaneous addition of either MAb specific for α_3, 34-2-12S for H-2^d responders and 28-14-8S for H-2^b responders, but not other MAb's, to MHC produced inactivation as assessed by measurement of total cytotoxic activity (Table 1).

To ensure that anti-α3 spared T cells that had not received an activation signal, we developed a protocol in which, in a first incubation, only a fraction of all T cells would be inactivated by being given a signal through their TcR while being exposed to anti-α3. T cells were activated with the antibody KJ-16 [which recognizes the TcR-Vβ8.1 or −Vβ8.2 gene product present on about 20 percent of T cells (*18*)] in the presence or absence of anti-α3. When cultures were analyzed by flow cytometry, cells underwent blast transformation in response to KJ-16 (assessed by increase in the forward angle, light scatter signal) before undergoing cell death in response to anti-α_3 [assessed by uptake of the fluorescent DNA stain propidium iodide (*19*)]. By 40 hours after adding the KJ-16 alone, there was a significant increase in the total number of viable blasts compared to either control cultures with no added antibody or cultures that also contained anti-α_3 (Table 2). To test the ability of cultures treated with KJ-16 and anti-α_3 to respond to a different stimulus, T cells were again activated with KJ-16 in the presence or absence of anti-α_3. The antibodies were removed after 24 hours of incubation, and the cultures were exposed to MAb's to KJ-16 or CD3. Prior exposure to both KJ-16 and anti-α3 reduced the subsequent response to KJ-16 but had little effect on a subsequent response to MAb to CD3 (Table 2).

Signals that activate mature T cells can lead to programmed cell death of thymocytes (*20*). Death takes place by apoptosis, as indicated by the characteristic fragmentation of DNA. We compared DNA extracted from thymocytes and splenocytes. Exposure to MAb to CD3 plus anti-α3 but not MAb to CD3 alone appeared to induce fragmentation of splenocyte DNA comparable to that seen in DNA from thymocytes exposed

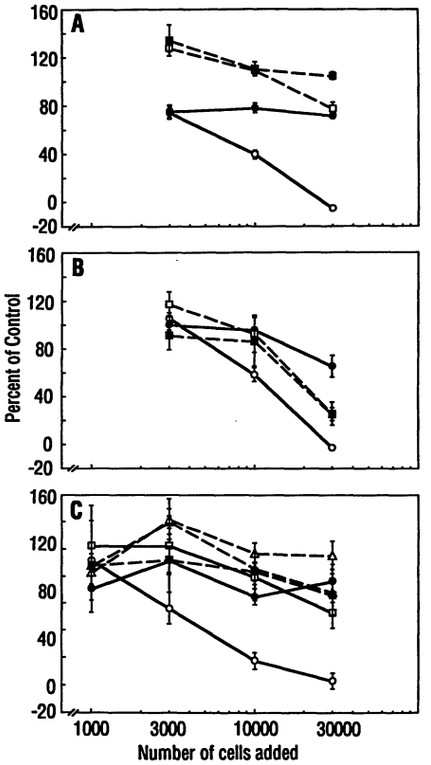

Fig. 1. Three experiments suggesting that CD8 mediates the inactivation of CTLp's by CTL's. (**A**) CD8$^+$, but not CD8$^-$, CTL's inactivated CTLp's. CD8$^+$ CD4$^-$ (○, □) or CD8$^-$ CD4$^-$ (●, ■) CTL's derived from an F_1(BALB/c × RNC) (H-2$^{d/k}$) anti-C57BL/6 (B6, H-2^b) MLR and added to SJL (H-2^s) anti-BALB/c (H-2^d) MLR's (solid line) or to SJL anti-SWR (H-2^q) MLR's (dashed line). (**B**) Covering CD8 molecules on a CD8$^+$ CTL line added to an MLR with a MAb to CD8 reduced the response reduction mediated by the CD8$^+$ CTL. The anti-B6-CTL DBA/2 (H-2^d, Ly-2.1) (○, ●) or the anti-B6 CTL BALB/c (H-2^d, Ly-2.2) (□, ■) were added to a MLR [SJL(Ly-2.2) anti-BALB/c (Ly-2.2)] in the absence (empty) or presence (filled) of antibody to Ly-2.1. (**C**) BW5147 lymphoma cells (H-2^k) transfected with the CD8α chain gene inhibited the generation of a CTL response in SJL anti-C3H (H-2^k) MLR. Untransfected BW cells (□, ■), BW-CD8 transfectants (○, ●), or BW-CD4 transfectants (△, ▲) were added to SJL anti-C3H MLR (empty) or SJL anti-DBA/2 (H-2^d) MLR (filled). MLR contained 1 × 10^5 responder lymph node cells and 3 × 10^5 irradiated (2000 rad) stimulator spleen cells in 200 μl of complete medium [α-minimal essential medium supplemented with fetal bovine serum (10%), 2-mercaptoethanol (10^{-5} M), and Hepes (10 mM) (*27*)]. CTL lines or BW transfectants were added in the numbers indicated on the abscissa. CTL activity was measured on day 5 in a 4-hour ^{51}Cr-release assay with ^{51}Cr-labeled target cells (1000 per culture) sharing MHC with the stimulator cells. Targets were either tumor cell lines or blast cells generated from normal spleen cells by incubation with concanavalin A (con A) at 2 μg/ml for 48 hours (*27*). Specific lysis was calculated and re-expressed as "cytotoxic activity" which is proportional to the number of CTL's present (*32*). A value of 0.1 corresponds to 10% specific lysis; 1.0 to 63% specific lysis. Each value represents the mean ± SD of five replicates and is expressed relative to a control to which CTL lines or BW transfectants were not added. Antibody to Ly-2.1 (Cedarlane Laboratories) was used at 1/40 dilution, the optimal dilution to inhibit an MLR response against C57BL/6 stimulator cells by DBA/2 but not BALB/c responder cells.

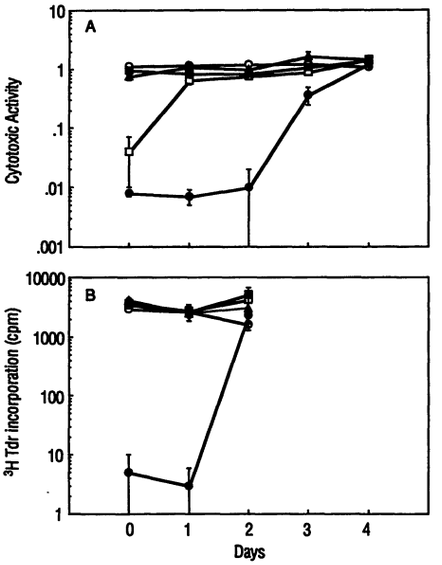

Fig. 2. Inhibition of CTL (**A**) and Th (**B**) responses in an MLR response of BALB/c against B6 by addition of MAb against the α_3 domain of class I MHC added at various times after culture initiation as indicated. Cultures were prepared and assayed as in Fig. 1. Antibodies added were as follows: none (○), anti-α_3 of H-2D^d (34-2-12S, ●), anti α_1 or -α_2 of H-2D (34-5-8S, □), anti H-2D^d (34-4-20S, ▲), or anti H-2K^{d}D^d (34-1-2S, ■) at concentrations indicated in Table 1.

Fig. 3. The anti-α3 signal leads to cell death by apoptosis. DBA/2 thymocytes or spleen cells (10^7 per culture) were cultured in 10 ml of complete medium (see legend to Fig. 1) for 24 hours with antibody to CD3 (145-2C11) or that antibody and anti-α3 (34-2-12S). Viable cells were harvested and total cellular DNA was extracted with phenol-chloroform (*33*). The DNA (3 μg per lane) was subjected to electrophoresis for 7 hours at 30 V in 2% agarose gel with tris-borate buffer (*34*) at 37°C and was stained with ethidium bromide. Left lane, thymocytes cultured with anti-CD3; middle lane, spleen cells cultured with anti-CD3; right lane, spleen cells cultured with anti-CD3 plus anti-α_3.

Table 1. Inhibition of T cell generation by a monoclonal antibody to α_3*. Monoclonal antibodies to MHC were added as indicated to either MLR prepared as in Fig. 1 or spleen cells (10^6 per well) cultured in 200 μl of complete medium with anti-CD3 [145-2C11 (*30*), 2.5 μg/ml]. The MLR cultures were assayed for IL-2 content with a CTLL assay and for cytotoxic activity as described (*27*). The anti-CD3 activated cultures were assayed for cytotoxic activity with ^{51}Cr-labeled EL-4 thymoma cells (10^3) and con A (1 μg/ml); the con A was used to overcome the need for specific recognition in the CTL-target cell interaction and enables the detection of CTL's of all specificities (*31*). All MAb's were from hybridoma culture supernatants. They were included at final concentrations of 1 to 3 μg/ml, which gave equivalent cell surface staining of lymphocytes by fluorescence analysis. All entries are mean ± SD of five replicates. Each experiment was repeated at least three times with the same result.

Antibody added	Determinant recognized	MLR cultures			Anti-CD3 activated cultures cytotoxic activity	
		Cell recovery on day 3 (% of control)	IL-2 on day 3 (units/ml)	Cytotoxic activity on day 5	48 hours*	72 hours*
		Expt. 1: BALB/c (H-2^d) responding to B6 (H-2^b)			*Expt. 1: DBA/2 spleen cells*	
None		100	0.79 ± 0.19	1.19 ± 0.11	1.80 ± 0.23	1.12 ± 0.06
34-2-12S (anti-α_3)	α_3 of D^d	30 ± 10	<0.01	−0.01 ± 0.01	0.30 ± 0.03	−0.01 ± 0.01
34-5-8S	α_1 or α_2 of D^d	96 ± 9	1.33 ± 0.50	1.01 ± 0.11	1.72 ± 0.23	0.97 ± 0.07
34-4-20S	D^d	117 ± 4	1.45 ± 0.60	0.79 ± 0.08	1.63 ± 0.26	ND
34-1-2S	K^{d}D^d	108 ± 4	1.70 ± 0.38	0.79 ± 0.10	1.66 ± 0.42	0.92 ± 0.10
		Expt. 2: B6 (H-2^b) responding to DBA/2 (H-2^d)			*Expt. 2: B6 spleen cells*	
None		100	1.75 ± 0.65	1.43 ± 0.17		1.90 ± 0.25
28-14-8S	α_3 of D^b	50 ± 6	<0.10	−0.03 ± 0.03		−0.04 ± 0.01
20-8-4S	α_1 of K^b	118 ± 11	1.70 ± 0.56	1.89 ± 0.44		ND
28-8-6S	K^b D^b	111 ± 9	1.03 ± 0.09	1.47 ± 0.24		0.93 ± 0.05
		Expt. 3: DBA/2 (H-2^d) responding to B6 (H-2^b)				
None				0.76 ± 0.14		
34-2-12S	α_3 of D^d			0.07 ± 0.08		
34-2-12S plus 34-5-8S (1 hour before)†	α_1 or α_2 of D^d + α_3 of D^d			0.68 ± 0.10		
34-2-12S plus 34-5-8S (1 hour after)†	α_3 of D^d + α_1 or α_2 of D^d			0.03 ± 0.02		

*Independent experiments. †One MAb was added after 48 hours of culture, the second 1 hour later.

to MAb to CD3 alone (Fig. 3).

We conclude that either CD8 or anti-α3 can induce death in either a CTLp or Thp at some point after it has been signaled through its TcR-CD3 complex but before it has become mature (*21*). This appears to be a result of a specific signal induced through the α_3 domain of the class I MHC molecule. Our data are consistent with results with a human CD8$^+$ CTL line and a stable CD8$^-$ antisense transfectant of this line. The CD8$^+$, but not the CD8$^-$ line, could down-regulate proliferative and cytotoxic responses against its own MHC antigens (*22*). A tumor cell line capable of stimulating an MLR down-regulates both CD4$^+$ and CD8$^+$ cells capable of recognizing it if the line is transfected with CD8 (*23*). In CD4$^+$ T cells (*24*), death is induced if CD4 molecules are cross-linked and the cell is then signaled through its TcR.

Programmed cell death may provide a mechanism for deleting self-reactive cells, provided that potentially immunogenic peptides are recognized in association with MHC on CD8$^+$ cells before being recognized on antigen presenting cells that allow activation. CD8-mediated deletion may also play a role in down-regulation of an immune response by deletion of activated Thp. This would require that the CD8$^+$ cell also carry class II MHC so that the Thp could recognize it. Resting T cells do not express class II MHC but at least some activated human T cells can be induced to express class II MHC (*25*) and the same may be true for mouse (*26*). Class II MHC–reactive Thp can be inactivated in vivo within 3 days of injection of allogenic lymphoid cells (*27*).

It is well established that there are CD8$^-$ cells, such as NK cells (*28*) and a Thy-1$^+$ cell subset in bone marrow (*29*) that inactivate CTLp's that recognize them. If these cells act by signaling through the α_3 domain of class I MHC, they must carry some other

Table 2. Deletion of activated T cells by monoclonal antibody to α_3. DBA/2 spleen cells (10^7 per well for cell recovery or 10^6 per well for proliferation) were cultured as in Table 1 (for anti-CD3, activation cultures), and antibodies (as indicated) were included at the start of culture. To assess cell recovery, viable cells (total and blast) were counted after 40 hours. To monitor proliferation, after 24 hours cultures either were washed three times with complete medium, incubated 16 hours, restimulated with either KJ-16 or 145-2C11 and incubated another 24 hours, the last 18 hours with [^{3}H]thymidine, or were left undisturbed except for a final 18-hour incubation with [^{3}H]thymidine (control).

Antibody added initially	Cell recovery* [viable cells (10^4 cells)]		Proliferation [radioactivity incorporated (cpm)]†		
	Total	Blast	+KJ-16 (anti-Vβ8.1 - 8.2)	+145-2C11 (anti-CD3)	Control
None	256 ± 22	27 ± 2	5,179 ± 667	23,147 ± 1,059	3,823 ± 572
KJ-16	768 ± 39	210 ± 11	6,979 ± 368	25,451 ± 2,551	8,114 ± 510
KJ-16 + 34-2-12S (anti-α_3)	350 ± 25	65 ± 5	2,524 ± 264	24,442 ± 1,370	1,331 ± 168
KJ-16 + 34-5-8S (anti-α_1 or -α_2)	ND	ND	6,099 ± 715	23,517 ± 788	6,018 ± 677

*Entries are based on replicate visual counts and percentages based on flow cytometric analysis of 20,000 cells. †Entries are mean (cpm) ± SD of five replicate cultures.

cell surface molecule that can recognize this domain.

The observation that only those CTLp's and Thp's that have been signaled through their antigen-specific surface receptors are killed by exposure to an antibody recognizing all lymphocytes (anti-α3) may prove useful for establishing tolerance in the adult animal to a particular antigen.

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CREB: A Ca^{2+}-Regulated Transcription Factor Phosphorylated by Calmodulin-Dependent Kinases

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The mechanism by which Ca^{2+} mediates gene induction in response to membrane depolarization was investigated. The adenosine 3′,5′-monophosphate (cAMP) response element–binding protein (CREB) was shown to function as a Ca^{2+}-regulated transcription factor and as a substrate for depolarization-activated Ca^{2+}-calmodulin–dependent protein kinases (CaM kinases) I and II. CREB residue Ser^{133} was the major site of phosphorylation by the CaM kinases in vitro and of phosphorylation after membrane depolarization in vivo. Mutation of Ser^{133} impaired the ability of CREB to respond to Ca^{2+}. These results suggest that CaM kinases may transduce electrical signals to the nucleus and that CREB functions to integrate Ca^{2+} and cAMP signals.

DNA ELEMENTS THAT CONTAIN THE consensus sequence TGACGTCA were originally identified as cAMP response elements (CREs) in neuropeptide genes (*1*), but recent studies in neuronal cell lines have mapped inducibility by membrane depolarization and increased intracellular Ca^{2+} concentrations to similar sequences in the c-*fos* (*2–4*) and proenkephalin genes (*5*). Depolarization of PC12 pheochromocytoma cells is correlated with rapid phosphorylation of the CRE-binding protein CREB on amino acid residue Ser^{133} (*4*). Phosphorylation of Ser^{133} stimulates the ability of CREB to activate gene transcription (*6*), suggesting that CREB may mediate transcriptional induction by Ca^{2+} as well as by cAMP. However, because multiple CRE-binding proteins exist (*7, 8*), distinct members of this family may separately confer Ca^{2+} and cAMP inducibility on the CRE (*4*). To determine whether CREB functions as a Ca^{2+}-activated transcription factor and to test if phosphorylation of Ser^{133} is important for this activation, we have targeted CREB to a different DNA regulatory sequence by fusing it to the DNA-binding and dimerization domain of the yeast transcriptional activator GAL4 (*9*) (Fig. 1). The use of a reporter gene that contains GAL4 binding sites allowed a spe-

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