Immunity, Vol. 2, 363–372, April, 1995, Copyright © 1995 by Cell Press

A Signaling Pathway Coupled to T Cell Receptor Ligation by MMTV Superantigen Leading to Transient Activation and Programmed Cell Death

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Summary

Stimulation of T cells by retroviral and bacterial superantigens is followed by specific T cell elimination, in contrast with stimulation of T cells by peptide, which is usually associated with clonal expansion. We show here that this differential response phenotype is apparent at the level of individual T cell clones following TCR ligation with peptide or MTV antigen. We exploited selective coupling of apoptosis to TCR ligation by MTV7 to examine some of the intracellular biochemical events that underlie this response. MTV-dependent activation resulting in apoptosis was associated with activation of phospholipase A_2 and the generation of reactive oxygen intermediates. Inhibition of these biochemical events prevented both MTV-dependent activation and apoptosis without affecting the peptide-dependent response of the same T cell clones. These results indicate that clonal expansion or programmed cell death following TCR ligation may be consequences of distinct TCR-coupled signaling pathways.

Introduction

There is increasing evidence that the relative levels of T cell expansion and apoptosis following T cell receptor (TCR) ligation by antigen can determine the intensity and direction of primary immune responses and regulate the development of long-term immunological memory (Zinkernagel et al., 1993). However, the intracellular signaling events that lead to either clonal expansion or apoptosis are not well understood. Two general explanations have been put forward (Liu et al., 1991). The first holds that quantitative differences in the amount of signaling within a single TCR-linked intracellular signaling pathway can result in these two activation phenotypes. A second explanation holds that apoptosis and clonal expansion may represent alternative activation phenotypes, which result from biochemically distinct signal transduction pathways coupled to the TCR.

One approach to this question is based on earlier observations that the response to retroviral superantigens is characterized by transient cellular activation followed by T cell elimination (Webb et al., 1990), while the response to peptide antigens is associated mainly with T cell expansion (MacDonald et al., 1993). Since these analyses of the in vivo response to superantigen and conventional antigen have relied on comparisons between heterogeneous cell populations, it has not been possible to determine whether they reflect a fundamental difference in TCR-coupled signaling following an interaction with the two types of ligand. Here, we analyze the response of dual-reactive T cell clones that express a TCR specific for a mouse tumor virus (MTV) epitope as well as a peptide. We provide evidence that MTV-dependent ligation of the TCR is coupled to an intracellular signaling pathway that is associated with apoptosis, which is distinct from the signaling pathway coupled to TCR ligation by peptide.

Results

Analysis of Apoptosis after TCR Ligation of Dual-Reactive T Cells

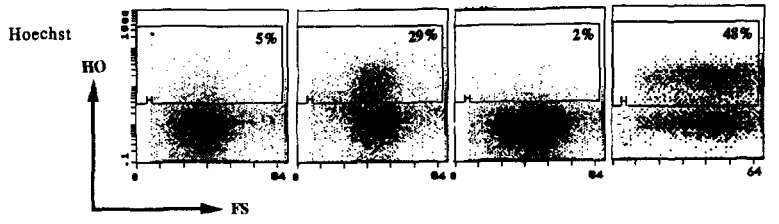
We first determined whether ligation of the TCR on dual-reactive clones by MTV or peptide antigen resulted in differential apoptotic responses. O3 is a $CD4^+$ $V\beta6^+$ clone derived from BALB/c mice that recognizes a peptide derived from ovalbumin (OVA) associated with $I\text{-}A^d$ as well as MTV7 ($Mls\text{-}1^a$) associated with class II, according to proliferation and cytokine release (Patarca et al., 1991; Friedman et al., 1987). Apoptosis was determined by FACS analysis of Hoechst dye uptake (Ormerod et al., 1993; Sun et al., 1992) using concentrations of MTV^+ cells (5×10^6) and OVA (10 μg/ml) plus antigen-presenting cells (APCs) (5×10^6), which induce similar levels of [^{3}H]TdR incorporation and CD25 expression (Figure 1A). Approximately 29%–45% of activated ($CD25^{hi}$) T cells underwent apoptosis 18–20 hr after stimulation by MTV. In contrast, 2%–8% of $CD25^{hi}$ blasts stimulated by OVA underwent apoptosis within the same time frame (Figure 1A). This did not reflect a delayed apoptotic response after stimulation by OVA, since the levels of apoptotic cells remained less than 5% over the next 24 hr (data not shown). Analysis of a second dual-reactive T cell, the AF3.G7 hybridoma, also showed that MTV7-dependent but not peptide-dependent activation resulted in a substantial level of apoptosis (Figure 2A). Apoptosis following MTV7 stimulation might be associated with a high functional dose of this superantigen, rather than a characteristic of MTV7-dependent activation. We therefore measured apoptosis and interleukin-2 (IL-2) release by AF3.G7 cells after stimulation with increasing concentrations of $MTV7^+$ lymphocytes (Figure 2B). These data indicate that substantial levels of apoptosis are noted even after stimulation with low concentrations of $MTV7^+$ cells that are sufficient to induce barely detectable IL-2 release.

Analysis of the Role of Reactive Oxygen Intermediates on the T Cell Response to MTV7 and Peptide Antigen

Since programmed cell death has been associated with an increase in intracellular reactive oxygen intermediates (ROIs) in various cell types (Sandstrom and Buttke, 1993; Hockenberry et al., 1993), we tested whether addition of antioxidant compounds might inhibit MTV-dependent

A.

STIMULUS:	NONE (APC)	MTV/APC	OVA/APC	αCD3
^{3}H-thymidine Incorporation	396	65769	119282	---



B.

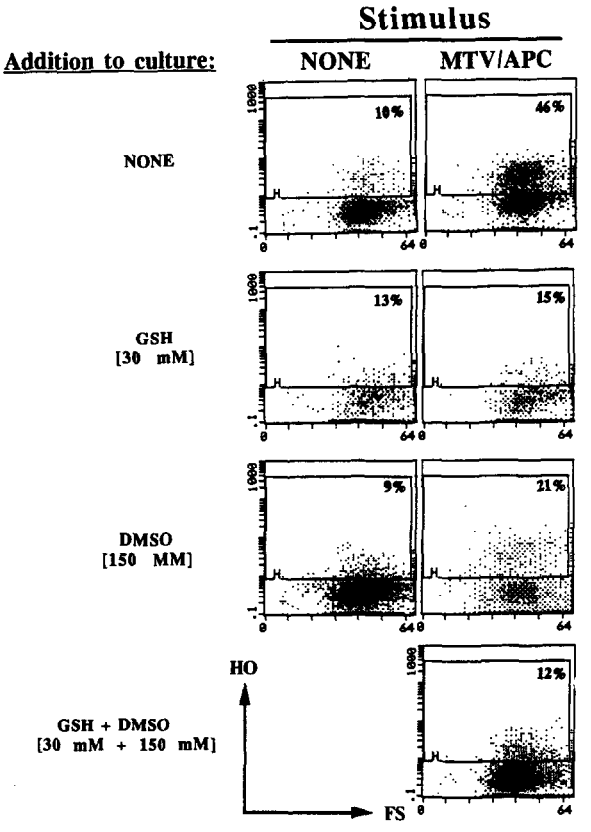


Figure 1. Apoptosis of O3 Cells after TCR Ligation by MTV or OVA

(A) Comparison of cellular apoptosis after activation of O3 cells by MTV, OVA, or plate-bound αCD3. Hoechst-dependent fluorescence intensity (ordinate, $\log_{10}$ scale) versus forward light scatter (abscissa, linear scale) displayed by viable (propidium iodide–negative [PI$^-$]) CD25^{hi} TCRαβ$^+$ O3 cells is shown 18 hr after activation by the indicated antigen associated with T cell–depleted splenic APC (see Experimental Procedures). The number in the top right-hand corner indicates the percentage of cells that display strong Hoechst fluorescence (above the horizontal line) in one of three representative experiments, in which the percentage of Hoechst^{hi} (apoptotic) cells ranged from 29%–45% after MTV stimulation and 2%–8% after OVA stimulation. HO, Hoechst 33342–dependent fluorescence; FS, forward scatter. The levels of [^{3}H]TdR incorporation at 40 hr after stimulation by each antigen are shown. This experiment is representative of a series of eight.

(B) Inhibition of MTV-dependent apoptosis by glutathione (30 mM) and DMSO (150 mM) alone or in combination. T cells (PI$^-$, TCRαβ$^+$) were analyzed according to forward light scatter and Hoechst 33342 dye incorporation after incubation with syngeneic T-depleted APC (left) or MTV7$^+$ (DBA/2) APC, as described in (A), and is a representative experiment from a series of three.

apoptosis. We found that addition of the hydroxyl radical scavengers DMSO or mannitol as well as glutathione at the outset of the T cell response inhibited MTV-dependent apoptosis in a dose-dependent manner for both O3 cells (see Figure 1B) and AF3.G7 cells (Figure 2A, right). Approximately 75% of the MTV7 response was inhibited by the concentrations of DMSO, mannitol, N-acetyl-L-cysteine (NAC), and glutathione used in these experiments. At higher concentrations, these antioxidants began to display nonspecific inhibition of background thymidine incorporation by T cells and APC without antigen. It could be argued that these compounds may exert biochemical effects, in addition to ·OH radical scavenging. This is unlikely to explain the effects of mannitol and DMSO, since the only shared activity of these chemically dissimilar compounds is their ability to scavenge ·OH radicals (Weber, 1990). Possibly, the MTV7-dependent apoptosis of the dual-reactive AF3.G7 hybridoma and O3 clone reflects the relative affinity of these two Vβ6$^+$ T cells for MTV7 and peptide ligand, rather than a more general property of the response following TCR engagement by the two types of ligand. We therefore examined the responses of two additional dual-reactive CD4$^+$ T cell clones. Clones 8-4.G6 and 8-4.E4 were derived from BALB/c and express Vβ8.1 rather than Vβ6$^+$ TCR. Both clones respond to MTV7 as well as a peptide derived from myelin basic protein (Abromson-Leeman et al., 1995). MTV7-dependent activation but not peptide-dependent activation of both 8-4.G6 and 8-4.E4 Vβ8.1 T cell clones results in substantial levels of apoptosis. Moreover, MTV-dependent apoptosis of both

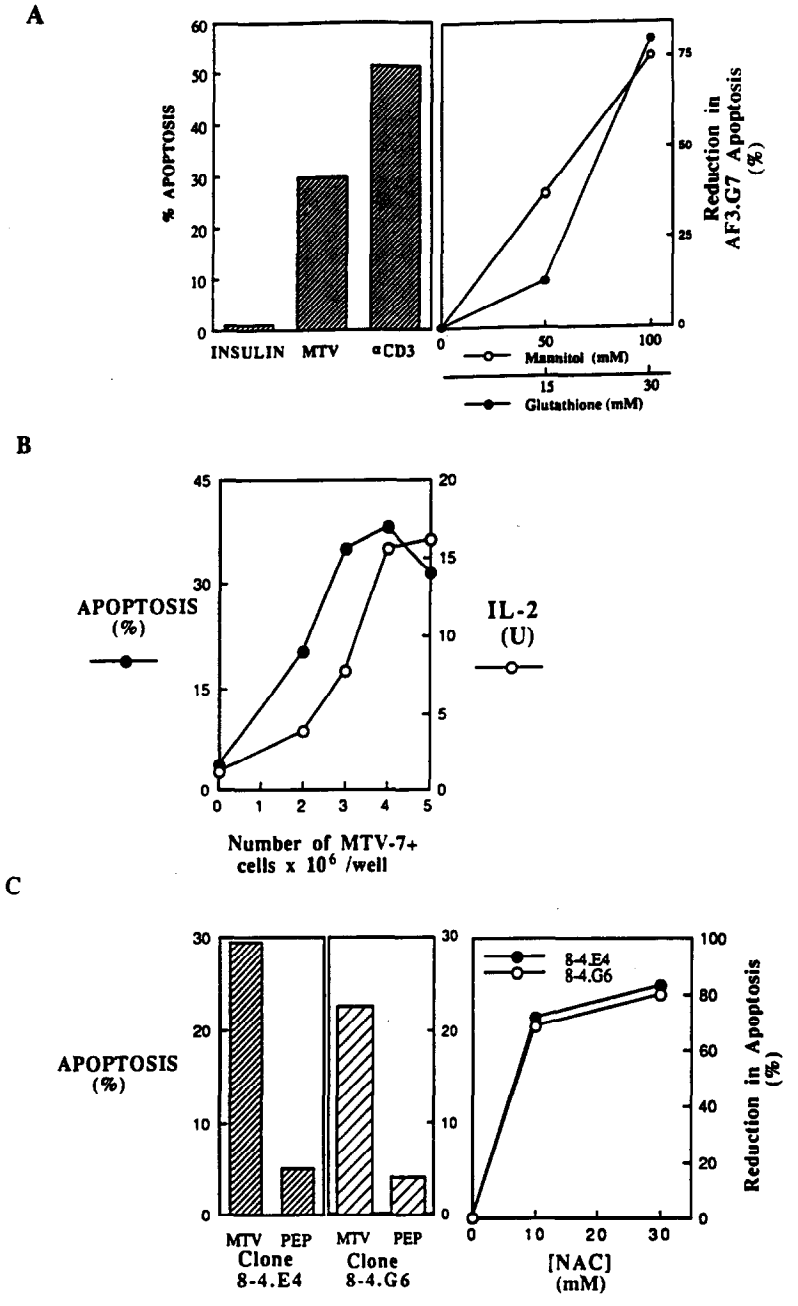


Figure 2. The Apoptotic Response of AF3.G7 Cells after Stimulation by MTV or Cow Insulin: Effect of Glutathione or the Hydroxyl Scavenger Mannitol

(A) The apoptotic response of AF3.G7 cells after stimulation by MTV or cow insulin: effect of glutathione or the hydroxyl scavenger mannitol. (Left) Cow insulin (100 μg/ml), or plate-bound αCD3 resulting in similar levels of IL-2 production (20–30 U/ml), viable T cells (PI^-; $TCR\alpha\beta^+$) were examined 24 hr after stimulation of AF3.G7 cells with MTV^+ DBA/2 cells for Hoechst uptake as described in the legend to Figure 1. The proportion of PI^-, $TCR\alpha\beta^+$ cells that displayed strong Hoechst-dependent fluorescence in one of three similar experiments is shown on the ordinate as percent apoptosis. (Right) AF3.G7 cells were incubated with $MTV7^+$ cells along with the indicated concentrations of mannitol or glutathione for 24 hr before analysis of apoptosis as described in Figure 1.

(B) Dose response analysis of MTV7-dependent IL-2 production and apoptosis by AF3.G7 cells. AF3.G7 cells (5×10^5/well) were incubated with increasing concentrations of $MTV7^+$ DBA/2 cells for 24 hr before viable T cells were tested for Hoechst uptake as in (A). Supernatants from these cultures were also tested for IL-2 activity as described (Liu et al., 1991), and the results are expressed in units/10^6 cells. Similar results were obtained in two additional experiments.

(C) Apoptosis of clones 8-4.E4 and 8-4.G6 after activation by MTV7 or peptide: effect of NAC on MTV-dependent apoptosis. Dual-reactive T cell clones 8-4.E4 and 8-4.G6 (Abromson-Leeman et al., 1995) were examined for Hoechst dye uptake 24 hr after stimulation with either myelin basic protein peptide 59–76 (200 ng/ml) and (syngeneic) BALB/c spleen cells or with ($MTV7^+$) DBA/2 cells as described in the legend to Figure 1 (left). The level of $[^3H]$TdR incorporation (cpm) 24 hr after incubation of clone 8-4.G6 with MTV7 and myelin basic protein peptide was 61,801 ± 4,416 and 49,801 ± 875, respectively; and for clone 8-4.E4, the level was 40,343 ± 5,655 and 45,779 ± 6,044, respectively. In addition, the levels of Hoechst uptake 24 hr after incubation of clone 8-4.G6 (open circle) or 8-4.E4 (closed circle) with $MTV7^+$ cells in cultures containing 10–30 mM of NAC were determined (right).

clones was inhibited in a dose-responsive fashion by the addition of NAC (Figure 2C).

Previous studies have indicated that MTV-dependent activation of O3 cells is not accompanied by detectable changes in $[Ca^{2+}]_i$, phosphatidylinositol synthesis or hydrolysis (Liu et al., 1991; Weber and Cantor, 1994). One possibility suggested from the above analysis of MTV-dependent apoptosis was that MTV-dependent activation also depended on the generation of intracellular ROIs. We therefore asked whether the antioxidant compounds that inhibited MTV-dependent apoptosis (see Figure 1; Figure 2) might also selectively inhibit MTV-dependent T cell activation. These experiments indicated that the hydroxyl radical scavengers DMSO and mannitol, as well as glutathione and its precursors NAC and L-cysteine, efficiently inhibited MTV-dependent activation, as judged by $[^3H]$TdR incorporation. Since, in the same experiments, these compounds had no detectable effect on the response of the same T cell clone to concentrations of OVA that induced levels of $[^3H]$TdR incorporation that were similar to the MTV response (Figure 3A), selective inhibition of the MTV response but not the peptide response of these T cell clones was unlikely to reflect nonspecific cellular toxicity. Moreover, these effects did not reflect differences in APCs that present the two T cell ligands, since both are presented efficiently to O3 cells by a pair of B lymphoma cells, LBB3.4.16 ($I\text{-}A^{d+}/MTV7^+$) and OVA-pulsed LBBIIV.14 ($I\text{-}A^{d+}/MTV7^-$) variant (Weber and Cantor, 1994) and antioxidants

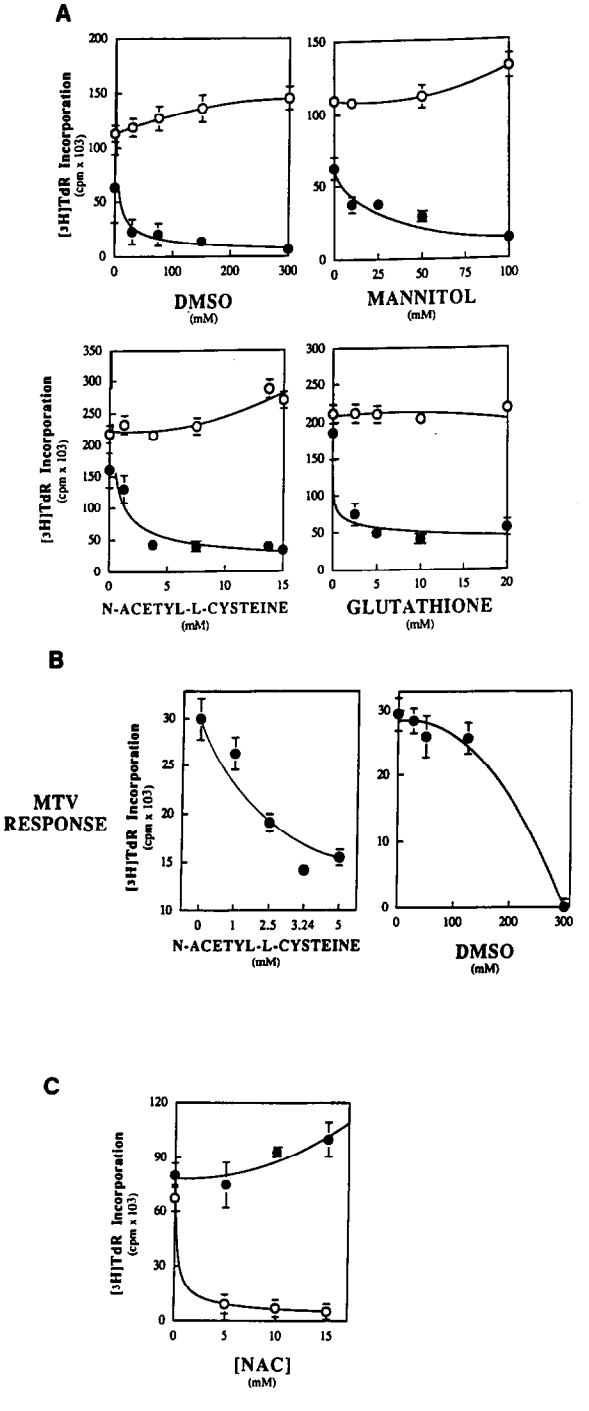


Figure 3. Effects of Antioxidant Compounds on the MTV7 Response

(A) The effects of hydroxyl radical scavengers, glutathione, or a glutathione precursor (NAC) on [^{3}H]TdR incorporation by O3 T cells stimulated with OVA or MTV7 superantigen. O3 cells (2.5 x 10^6/ml) were incubated with 12.5 x 10^5/ml T cell–depleted DBA/2 cells (MTV7$^+$) (closed circle), or with 12.5 x 10^6 BALB/c cells along with 10 μg/ml OVA (open circle) in 200 μl at the same final ratio of O3 cells to APC (1:5) as used for all assays in this study. [^{3}H]TdR incorporation was measured at 40 hr (16 hr pulse with [^{3}H]TdR at 24 hr of stimulation) as described (Friedman et al., 1987). Superoxide dismutase and catalase had no effect on the proliferative response to either stimulus (data not shown).

selectively inhibit [^{3}H]TdR incorporation in response to the MTV7-bearing cell line (data not shown).

It was possible that the inhibitory activity of these antioxidants on the MTV response reflected their effects on stimulator cells (e.g., down-modulation of functional MTV expression). However, preincubation of O3 T cells with NAC or DMSO for 4 hr before washing and addition to culture containing MTV7$^+$ APC resulted in dose-dependent inhibition of the T cell response (Figure 3B). By contrast, preincubation of MTV7$^+$ APC had no effect on the response (Figure 3C).

Potential Role of Phospholipase A_2 and Guanylate Cyclase in T Cell Activation by MTV7 and Peptide Antigen

Early studies of T cell stimulation by mitogens suggested that activation of phospholipase A_2 (PLA_2) might lead to lipoxygenase-mediated generation of hydroxyl radicals and activation of guanylate cyclase (Coffey et al., 1981). Although this activation sequence has not yet been described after TCR ligation (Ashwell and Klausner, 1990; Perlmutter et al., 1993), a similar pathway has been inferred from studies of tumor necrosis factor (TNF) receptor–dependent signaling (Reid et al., 1991; Hoeck et al., 1993; Haliday et al., 1991). According to this model, inhibition of the MTV response by NAC and glutathione noted above might reflect the effects of these compounds on glutathione peroxidase–dependent conversion of hydroperoxyeicosatetraenoic acid (HPETE) to hydroxyeicosatetraenoic acid (HETE), while DMSO- and mannitol-dependent inhibition might reflect direct scavenging of ·OH generated from HPETE.

We therefore measured PLA_2 activity in cell lysates 30 min after stimulation of O3 cells by MTV or OVA according to conversion of ^{14}C-phosphatidylcholine substrate into arachidonic acid. Cell lysates obtained from T cells that had been stimulated by MTV/class II contained substantial PLA_2 activity, while lysates of T cells stimulated by OVA/class II did not contain detectable PLA_2 activity (Figure 4A). We then tested the potential contribution of enhanced PLA_2 activity to MTV-dependent T cell activation using the PLA_2 inhibitor quinacrine. This compound (and the lipoxygenase inhibitor nordihydroguaiaretic acid; data not shown) inhibited both thymidine incorporation and CD25 expression after stimulation of O3 cells with MTV7/class II, but had no detectable effect on the O3 response to OVA/class II. By contrast, the cyclooxygenase inhibitor indomethacin (0–1.5 mM) did not inhibit either the MTV or OVA response (Figure 4B).

Since PLA_2-dependent release of ·OH radicals has been

(B) Cellular target of glutathione precursors and hydroxyl radical scavengers in the T cell MTV response. O3 cells (5 x 10^5/ml) were incubated with the indicated concentrations of NAC or DMSO for 4 hr at 37°C before washing three times and addition to cultures containing irradiated Thy1$^-$ DBA/2 (MTV7$^+$) cells.

(C) Effects of preincubation on O3 cells. The effects of preincubation with NAC x 4 hr on O3 cells (open circle) compared with NAC preincubation of MTV7$^+$ APC (DBA/2 cells) (closed circle) are shown.

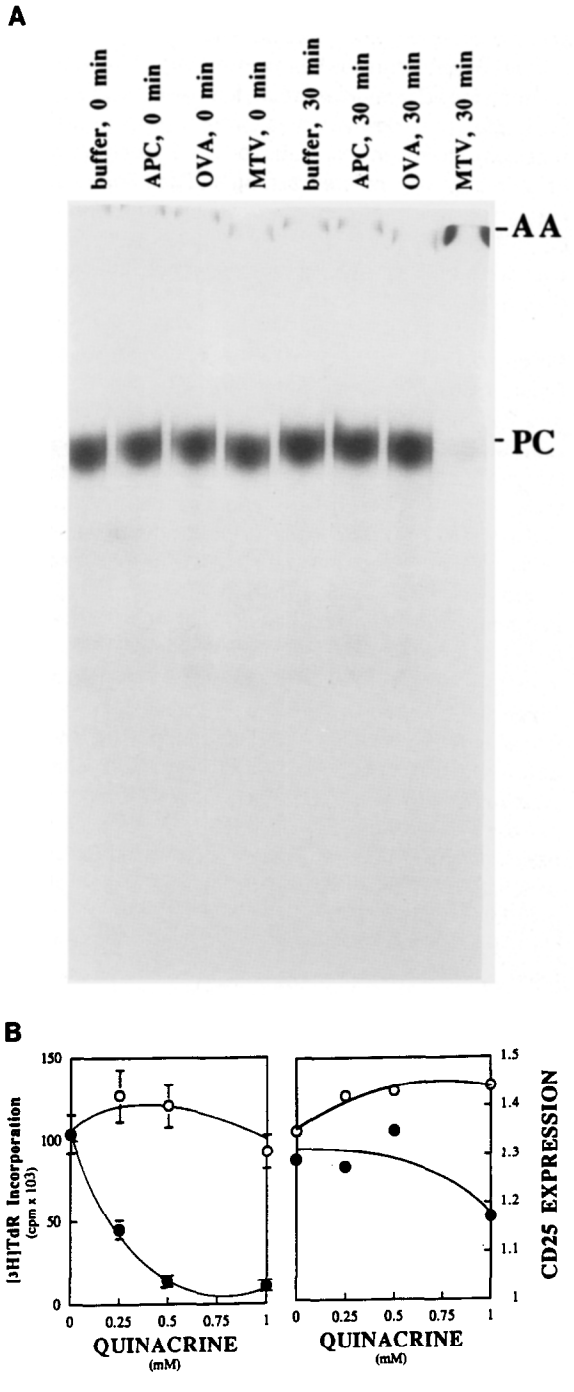


Figure 4. The Role of PLA_2 in MTV-Dependent Activation of T Cells

(A) Intracellular phospholipase A_2 (PLA_2) activity after TCR ligation by OVA/APC and MTV7$^+$/APC. PLA_2 activity in extracts of O3 cells that were incubated with the indicated APC and antigens for 0 min or 30 min was assayed according to conversion of phosphatidylcholine (PC) to arachidonic acid (AA) and was complete in the MTV-stimulated samples at 30 min, while no detectable PC conversion was noted in all other samples (arachidonic acid migrates with the solvent front). Although this enzymatic activity can also reflect a combination of phosphatidylinositol-specific phospholipase C–dependent cleavage followed by diacyl glycerol lipase, phospholipase C–dependent activity has not been detected in O3 cells after MTV activation (Weber and Cantor, 1994). This assay does not distinguish between APC and responders as the origin of PLA_2 activity.

Table 1. Intracellular Guanylate Cyclase Activity after TCR Ligation by Conventional Peptide Antigen and Superantigen

Type of Stimulation	Guanylate Cyclase (fmol/min/mg)
O3 + APC	32.35 ± 17.05
O3 + OVA/APC	47.01 ± 11.5
O3 + MTV/APC	106.2 ± 27.7

O3 cells were incubated with T cell–depleted APC alone (APC), or with OVA (APC/OVA) or with MTV (APC/MTV) for 2 hr followed by separation of O3 cells on Cellect columns and determination of guanylate cyclase activity. The results represent averages from three measurements.

associated with downstream activation of guanylate cyclase (Coffey et al., 1981), we determined guanylate cyclase activity after TCR ligation of O3 cells. Guanylate cyclase activity was induced substantially by MTV (more than 3-fold), while the increase in guanylate cyclase activity after OVA stimulation was not significantly above background levels (Table 1).

Manipulation of the T Cell Response to Superantigen In Vivo

The above analysis of MTV-dependent signaling in vitro suggested that it might be possible to manipulate the T cell response to this retroviral superantigen in vivo using antioxidant compounds. Glutathione and its precursor NAC efficiently inhibit both MTV-dependent activation and apoptosis when present at the outset of the in vitro response (see Figures 1–4). We asked whether administration of NAC might inhibit the in vivo response of Vβ6$^+$ T cells to MTV7. In control animals immunized with MTV7$^+$ cells, the frequency of both total Vβ6$^+$ CD4 cells as well as Vβ6$^+$ CD4 blasts increased by about 50% in the lymph node and spleens 4–5 days after MTV injection, and fell back to baseline levels by day 7 (Figure 5A). Daily administration of NAC beginning at day 0 completely prevented an increase in both total Vβ6$^+$ CD4 cells or Vβ6$^+$ CD4 blasts at day 4–5 (or day 7). This was unlikely to reflect nonspecific toxicity on T cells, because NAC administration had no detectable effect on either the total numbers of viable CD4 T cells or CD4 blasts in BALB/c animals given control injections of syngeneic spleen cells.

In a second approach, we asked whether delayed administration of NAC might allow MTV-dependent activation of Vβ6 cells but prevent subsequent apoptosis. We found that daily administration of NAC beginning 48 hr after immunization with MTV led to a 2-fold enhancement

(B) The effect of PLA_2 inhibition on T cell activation by MTV and OVA. The effect of the indicated concentrations of PLA_2 inhibitor quinacrine on the response to MTV7$^+$ APC or OVA/APC according to thymidine incorporation and levels of CD25 are shown. Assay conditions are described in Experimental Procedures. The MTV7 response but not the OVA response was inhibited by quinacrine and neither response was inhibited by indomethacin at concentrations of 1–15 mM, according to [^{3}H]TdR incorporation (cpm × 10^{-3}): OVA response, 108.6; indomethacin, 105.9–140.0; MTV response, 65.0; indomethacin, 66.5–99.5; APC response, 9.4; indomethacin, 8.2–11.2.

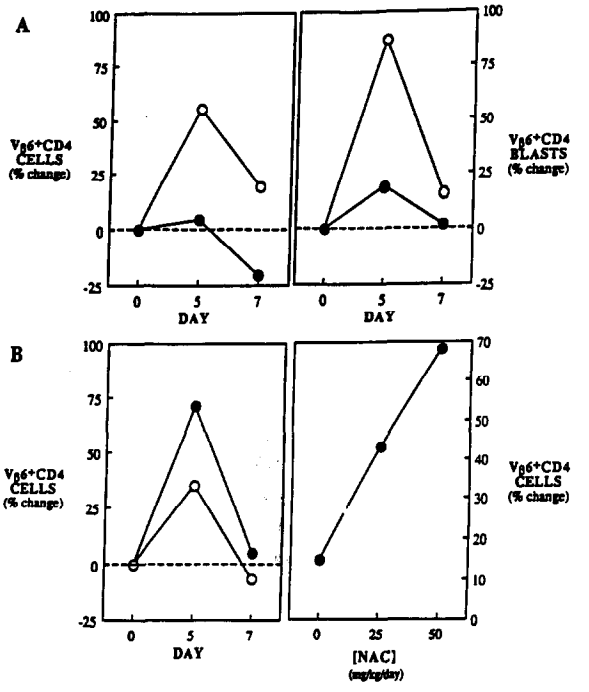


Figure 5. The Effect of NAC Administration on the MTV7 Response In Vivo

(A) Inhibition of the MTV7 response by daily administration of NAC (50 mg/kg/day) beginning at the initiation of the response. (Left) The change in the percentage of CD4 cells in lymph node and spleen that express Vβ6+ CD4 cells at 5 and 7 days after immunization with DBA/2 cells is shown for mice injected daily with (50 mg/kg/day) NAC (closed circle) or PBS (open circle). To avoid nonspecific effects associated with injection of spleen cells on the frequency of Vβ6+ cells and Vβ6+ blasts, the percent increase in Vβ6+ CD4 T cells was calculated from the ratio of Vβ6+ CD4 cells in BALB/c mice injected with syngeneic BALB/c cells compared with BALB/c mice injected with DBA/2 spleen cells, as described in Experimental Procedures. The results shown are representative of three experiments; the actual proportion of Vβ6+ CD4 cells from control (BALB/c-injected) spleen plus lymph node cells in this experiment was 9.4% ± 1.6%. (Right) The increase in Vβ6+ CD4 blast cells according to forward light scatter was determined as described in Experimental Procedures and in Dannecker et al. (1991). Mice injected with DBA/2 cells were coinjected with either NAC (closed circle) or PBS (open circle). The percent increase in Vβ6+CD4 blasts reflects the ratio of these cells in mice injected with DBA/2 cells compared with controls injected with (syngeneic) BALB/c cells. The proportion of Vβ6+ CD4 cells that were blasts according to forward light scatter (Dannecker et al., 1991) in this experiment was 14.2% ± 2.2% in control BALB/c mice 5 days after injection of syngeneic (BALB/c) spleen cells.

(B) Effect of delayed administration of NAC on the MTV response. (Left) The change in the proportion of Vβ6+ CD4 T cells in lymph node and spleen is shown at days 5 and 7. BALB/c mice were injected with DBA/2 spleen cells at day 0, followed by either NAC (50 mg/kg/day) injected intraperitoneally on days 2, 3, and 4 (closed circle) or PBS injected at the same intervals (open circle). The percent change of Vβ6+ CD4 cells represents the ratio of those cells in mice injected with DBA/2 cells compared with mice injected with BALB/c cells, as described above. (Right) The percent increase was calculated from the ratio of Vβ6+ CD4 cells in mice injected with DBA/2 cells compared with mice injected with BALB/c cells.

of the CD4 Vβ6+ T cell response to MTV7, and this was proportional to the concentration of NAC (Figure 5B). It should be noted that NAC-dependent enhancement of the Vβ6+ T cell response noted at day 5 after MTV injections

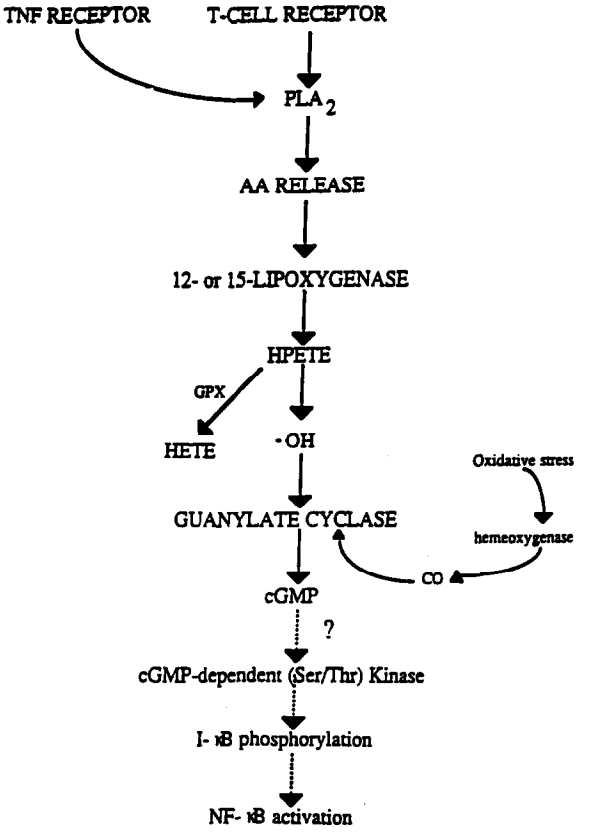


Figure 6. Proposed TCR-Coupled Signaling Pathway that May Be Engaged by MTV Superantigen

TCR ligation by MTV superantigen in association with MHC class II may involve activation of PLA_2, conversion of the generated arachidonic acid to its hydroperoxyl derivative by lipoxygenase, and activation of guanylate cyclase by hydroxyl radicals, which are released from HPETE. This pathway may also be engaged by certain mitogens (Coffey et al., 1981) and by ligation of the TNF receptor (Haliday et al., 1991). Furthermore, oxidative stress leading to heme oxygenase induction and guanylate cyclase activation via generation of CO (Legrand-Poels et al., 1990; Barinaga, 1993) may account for NF-κB inhibition by iron chelators.

was no longer apparent at day 7, consistent with the possibility that continuous presence of this antioxidant is necessary to inhibit new cohorts of MTV-dependent T cell apoptosis, or MTV-dependent activation by day 7 might be down-regulated by additional cellular mechanisms.

Discussion

There is considerable evidence that in vivo stimulation of T cells by retroviral and bacterial superantigens is followed by specific T cell elimination (Webb et al., 1990; Ignatowicz et al., 1992), while in vivo stimulation of T cells by immunogenic concentrations of peptide is usually associated with clonal expansion (MacDonald et al., 1993). We show here that this differential response phenotype is apparent at the level of individual T cell clones following TCR ligation with peptide or MTV antigen. Engagement of the TCR by MTV7 resulted in transient activation followed by apop-

tosis, while engagement of the same clonal TCR by peptide antigen resulted in activation without detectable apotosis. We exploited selective coupling of apoptosis to TCR ligation by MTV7 to examine some of the relevant biochemical events in the absence of intracellular signals generated after TCR ligation by peptide antigen (Liu et al., 1991; Weber and Cantor, 1994). Inhibition of the generation of ·OH radicals, either through activation of glutathione peroxidase by NAC and glutathione or through direct scavenging of ·OH by DMSO and mannitol, completely inhibited MTV-dependent activation without detectably affecting the peptide-dependent response of the same CD4 T cell clone or hybridoma. Previous studies have suggested that PLA_2 activation represents a potential source of hydroxyl radical formation according to the pathway outlined in Figure 6. We found that TCR engagement by MTV7 selectively enhances PLA_2 activity and that inhibition of PLA_2 activity completely and selectively inhibits MTV-dependent activation. The differential effects of these compounds on the response to peptides and MTV following engagement of the TCR on the same T cell clone make it unlikely that inhibition of the MTV response reflected nonspecific effects.

The biochemical events of the signaling pathway proposed in Figure 6 correspond closely to those described for phytohemagglutinin stimulation of human T cells (Coffey et al., 1981; Parker et al., 1979), ligation of the TNF receptor (Reid et al., 1991; Hoeck et al., 1993; Haliday et al., 1991), and the Fas antigen (Itoh et al., 1991). It may be relevant that ligation of the TNF and Fas receptors is also associated with apoptosis. Ligation of the TNF receptor in other cell types may also activate sphingomyelinase-mediated generation of ceramide as an early intracellular signaling event (Schütze et al., 1992). However, we did not detect generation of ceramide in T cells after stimulation by either MTV or peptide antigen (data not shown). Although catalytic activity of PLA_2 has sometimes been associated with elevated $[Ca^{2+}]_i$, the TCR-coupled pathway described here does not appear to depend on a significant increase in $[Ca^{2+}]_i$ (Liu et al., 1991; Weber and Cantor, 1994), consistent with the findings that cytoplasmic PLA_2 activity can also be induced by ligation of the TNF and epidermal growth factor (EGF) receptors without detectable increases in $[Ca^{2+}]_i$ (Hoeck et al., 1993; Hack et al., 1991). Increased intracellular levels of nitric oxide have also been associated with cellular apoptosis. The role of this intermediate is not obvious in the present studies, but nontoxic concentrations of compounds that affect nitric oxide synthetase activity, such as the substrate L-arginine or the inhibitor methylene blue, had no effect on the proliferative response of O3 cells after TCR ligation by either peptide antigen or retroviral superantigen (data not shown).

Earlier studies had initially suggested that TCR ligation by MTV superantigen might be accompanied by biochemical changes that are distinct from those associated with TCR ligation by peptide antigen. MTV-dependent engagement of the TCR leading to incorporation of [^{3}H]TdR did not provoke detectable increases in phosphatidylinositol metabolism or $[Ca^{2+}]_i$, which marked the response of the same dual-reactive T cell clones to peptide-dependent activation (O'Rourke et al., 1990; Oyaizu et al., 1992; Liu et al., 1991; Weber and Cantor, 1994). However, these experiments did not suggest whether alternative biochemical signals might be coupled to the MTV-dependent response and, if so, what these biochemical events might be. The present results indicate that the MTV7 response may depend on activation of PLA_2, increased levels of intracellular ROIs, and activated guanylate cyclase. Although these experiments do not test the causal relationships among several of these individual biochemical events, these data are consistent with the sequence outlined in Figure 6.

Downstream events leading to activation of transcription factors and changes in gene expression have not been investigated for the MTV-dependent response described here. Previous studies suggesting that activation of a dual-reactive clone by MTV7 and peptide results in different patterns of cytokine gene expression are consistent with the hypothesis that the two pathways may result in differential activation of transcription factors (Patarca et al., 1991). Previous studies have suggested that NF-κB can be activated by increased levels of intracellular ROIs (Staal et al., 1990; Kalebic et al., 1991; Schreck et al., 1992; Legrand-Poels et al., 1990) or via activation of kinases leading to serine/threonine phosphorylation of the inhibitor protein I-κB (Ghosh and Baltimore, 1990). We are testing the possibility that increased cyclic guanosine 3′,5′-monophosphate–dependent serine/threonine kinase associated with increased cyclic guanosine 3′,5′-monophosphate levels after MTV-dependent activation (Table I) may lead to NF-κB activation.

The association of this T cell signaling pathway with cellular apoptosis also suggests that it may be relevant to negative selection during T cell development. In this regard, MTV7 is a somewhat unusual example of MTV-dependent superantigens, because it induces both strong T cell proliferative responses as well as thymocyte and peripheral T cell deletion (Subramanyam et al., 1993). Although the majority of MTV-encoded retroviral superantigens also cause thymocyte deletion, they usually stimulate weak or negligible T cell proliferative responses (Acha-Orbea et al., 1993). Indeed, location and detection of MMTV long terminal repeat open reading frame products that can act as superantigens has depended on their ability to cause clonal deletion in the thymus (Simpson et al., 1993). Although the mechanism that accounts for this T cell response phenotype is not well understood, the finding that the interaction between an MTV superantigen and the TCR may be preferentially coupled to a signaling pathway leading to apoptosis may help explain this aspect of MTV immunobiology. Apoptosis of thymocytes can also be induced experimentally by glucocorticoids (Ramakrishnan and Catravas, 1992; Iwata et al., 1992; Buttke and Sandstrom, 1994), although this instance of thymocyte apoptosis may reflect a cellular mechanism that is distinct from TCR-coupled apoptosis. In this regard, inhibition of TCR-coupled apoptosis by low doses of glucocorticoids may reflect glucocorticoid-dependent inhibition of PLA_2 activity (Hoeck et al., 1993; Cohen, 1992).

T cell stimulation by high concentrations of peptides in

vivo (Rocha and von Boehmer, 1991; Critchfield et al., 1994) and in vitro (Ucker et al., 1989) has also been associated with suppression of proliferation, and, in some cases, with apoptosis. We have measured apoptosis of O3 cells after stimulation with increasing concentrations of OVA. Significant levels of apoptosis are detectable only after stimulation by very high concentrations of OVA (500–1000 μg/ml), representing a 10-fold higher dose than is necessary to induce maximal proliferative responses. It is possible that apoptosis of O3 cells under these conditions may reflect engagement of the ·OH-dependent pathway for MTV7 stimulation, because it is reversed in the presence of NAC (10 mM) (G. F. W. and H. C., unpublished data). We speculate that the examples of peptide-dependent T cell elimination noted above may reflect this process.

It is also important to avoid oversimplifying the relationship between MTV7-dependent apoptosis and T cell elimination following in vivo exposure to superantigens. Although it is likely that the signaling pathway described here may play a role in the early phase of T cell elimination by superantigen, other regulatory mechanisms may also contribute to the disappearance of superantigen-specific CD4 cells. For example, Jiang et al. (1995) have recently demonstrated that CD8 T cells can eliminate CD4 T cells within 1–2 weeks after superantigen (staphylococcal enterotoxin B) administration in vivo, and this reflects CD8-mediated Vβ-specific cytotoxicity. Indeed, although delayed administration of NAC potentiated the early response to MTV, it had no effect on the fall-off of $V\beta6^+$ cells by day 7. These considerations suggest that down-regulation of CD4 responses to superantigens may reflect a combination of superantigen-dependent apoptosis early in the response, followed by elimination of CD4 cells by receptor-specific CD8 cells.

We have not studied the potential interaction between the MTV-dependent signaling pathway described here and the one provoked in the same T cell clones by conventional doses of peptides. The original studies of Janeway et al. (1983) and more recent reports (Liu et al., 1991; Janeway, 1993) indicate that simultaneous ligation of the TCR by both MTV and peptide antigen provokes a synergistic response, as judged by either [^{3}H]TdR incorporation or release of IL-2 within 24 hr. Whether this response is followed by apoptosis was not noted. Other investigators have reported that priming T cells to MTV7 in vivo inhibits their subsequent ability to mount a proliferative response (Jacobsson et al., 1975; Lilliehook et al., 1975) or to provoke a graft versus host response to allo-antigens presented in association with MTV7 (Halle-Pannenko et al., 1986). Possibly, the synergistic effects of the two classes of T cell ligand on early [^{3}H]TdR incorporation reflects simultaneous engagement of both pathways, while the inhibitory effects noted after in vivo priming to MTV7 may reflect MTV7-dependent T cell apoptosis upon restimulation with MTV7 and an alloantigen.

In summary, our findings suggest that the prominence of T cell elimination in the response to MTV-encoded retroviral antigens (Webb et al., 1990) may reflect preferential engagement of a signaling pathway that is distinct from the pathway coupled to TCR ligation by peptide antigens. The molecular basis for differential engagement of these signaling pathways by the two classes of TCR ligands is unknown. One possibility is that they may interact with different components of the multichain TCR, in view of studies suggesting that the ζ and ε chains of the receptor may be associated with distinct intracellular signaling events, which result in different patterns of tyrosine phosphorylation (Letourneur and Klausner, 1992; Wegener et al., 1992). Alternatively, the two ligands may cause differential association of the TCR with other cell surface receptors such as Fas, which may modulate the functional effects of TCR-associated signaling. In either case, the data reported here suggest an alternative to the idea that quantitative differences in signaling along a single TCR-connected pathway can account for T cell expansion versus programmed cell death. Instead, these two T cell response phenotypes may be mapped to distinct signaling pathways coupled to the TCR. Additional studies are required to define and distinguish further the biochemical events that comprise these pathways and to establish their respective roles in T cell development and responsiveness to antigen.

Experimental Procedures

Mice

BALB/c and DBA/2 mice (Jackson Laboratory, Bar Harbor, Maine) were used as a source of splenic APC.

Cells

O3 is a $V\beta6^+$ $CD4^+$ Th1 clone derived from BALB/c mice after in vitro selection for proliferation to OVA in association with BALB/c ($I\text{-}A^d$) APCs, which responds to both an OVA peptide and to the retroviral superantigen MTV7 ($Mls\text{-}1^a$) according to proliferation (Leo et al., 1987; Patarca et al., 1991). Two additional dual-reactive $CD4^+$ T cell clones used for these studies are clones 8-4.G6 and 8-4.E4, which were derived from BALB/c and express Vβ8.1 rather than $V\beta6^+$ TCR. Clones 8-4.G6 and 8-4.E4 respond to MTV7 as well as to a peptide derived from myelin basic protein (Abromson-Leeman et al., 1995). The AF3.G7 hybridoma, generated by fusing cow insulin-immune C57BL/6 lymph node cells with the BW5147 thymoma line, expresses a $V\beta6^+/V\alpha3.2^+$ TCR and responds to both a cow insulin peptide and to MTV7 according to IL-2 production (Spinella et al., 1987).

Antibodies

All antibodies were obtained from Promega (Madison, Wisconsin), with the exception of fluorescein isothiocyanate (FITC)-labeled anti-CD25 antibody (Boehringer Mannheim, Indianapolis, Indiana).

Chemicals

The antioxidants (NAC, glutathione, mannitol), Hoechst 33342 dye and propidium iodide (PI) were purchased from Sigma (St. Louis, Missouri). Dimethyl sulfoxide was obtained from Fisher Scientific (Springfield, New Jersey).

Assays of T Cell Responses

To measure in vitro proliferation, O3 cells (2.5×10^5/ml) were incubated with 12.5×10^5/ml T cell–depleted DBA/2 cells ($MTV7^+$), or with 12.5×10^6 BALB/c cells along with 10 μg/ml OVA in 200 μl at the same final ratio of O3 cells to APC (1:5) as used for all assays in this study. Depletion of T cells from APC was performed by complement-mediated lysis with anti-Thy1.2 (TIB 99; American Type Culture Collection, Bethesda, Maryland) and either anti-Lyt1.2 (BALB/c cells) or anti-Lyt1.1 (DBA/2 cells) antibody (Accurate Chemical, Grand Island, New York). [^{3}H]TdR incorporation was measured at 40 hr (16 hr pulse with [^{3}H]TdR at 24 hr of stimulation) as described (Friedman et al., 1987). An IL-2 assay (Collaborative Biomedical Products/Becton Dickinson, Bedford, Massachusetts) was used for analysis of the response of

AF3.G7 cells after stimulation by either cow insulin or T cell–depleted DBA/2 cells.

To measure T cell apoptosis after in vitro activation by antigen, O3 cells (10^6/ml) or AF3.G7 cells (0.5×10^6/ml) were incubated with irradiated (2000 rads) T cell–depleted syngeneic spleen cells (5×10^6/ml) as APC plus OVA (10 μg/ml), cow insulin (100 μg/ml), or 5×10^6/ml T cell–depleted MTV7$^+$ (DBA/2) spleen cells in DMEM plus 5% fetal bovine serum. T cells undergoing apoptosis stain intensely and specifically with Hoechst 33342 dye compared with normal cells (Sun et al., 1992; Ormerod et al., 1993). After 18–20 hr of incubation, the cells were incubated with Hoechst 33342 dye (10 μg/ml for 10 min at 37°C) before FACS analysis. After gating out dead cells according to incorporation of propidium iodide (5 μg/ml), T cells (stained with allophycocyanine-labeled anti-TCRαβ antibody) were divided into CD25^{lo} and CD25^{hi} subpopulations using FITC-labeled anti-CD25 antibody to delineate the activated (CD25^{hi}) fraction. These activated T cells, i.e., propidium iodide–negative (PI$^-$), TCRαβ$^+$ and CD25^{hi}, were analyzed for intensity of Hoechst 33342 fluorescence to measure apoptosis.

To measure in vivo MTV7 responses, BALB/c mice were injected intravenously with either 2×10^7 spleen cells from MTV7$^+$ DBA/2 mice or with 2×10^7 spleen cells from (syngeneic) BALB/c mice in 200 μl phosphate-buffered saline (PBS). Both groups of animals were injected with various concentrations of NAC in 100 μl PBS (pH 7.4, adjusted with NaOH) intraperitoneally daily. Spleens and lymph nodes were removed, and the resulting cell suspensions were subjected to FACS analysis with FITC-anti-Vβ6, allophycocyanine anti-CD4, PI, and Hoechst 33342 dye as described above. Blastogenesis of MTV7-reactive Vβ6$^+$ T cells in spleen and lymph node was measured by forward light scatter as described (Dannecker et al., 1991). To eliminate nonspecific effects of spleen cell injections on levels of Vβ6$^+$ CD4 cells, the percent increase or decrease of Vβ6$^+$CD4 T cells specific to MTV7 activation was determined from the ratio of Vβ6$^+$ CD4 cells in BALB/c mice injected with DBA/2 (MTV7$^+$) cells compared with BALB/c mice injected with syngeneic (BALB/c) spleen cells as follows:

$$100 \times \frac{\text{V}\beta6^+\text{CD4 cells} - \text{BALB/c (DBA/2 i.v.)}}{\text{V}\beta6^+\text{CD4 cells} - \text{BALB/c (BALB/c.i.v.)}} - 100 = \text{MTV7 response (percent change in V}\beta6^+\text{CD4 cells)}$$

In some experiments, levels of CD25 expression were assessed by two-color FACS analysis after 24 hr. O3 cells were identified according to phycoerythrin–anti-Vβ6, and levels of surface CD25 expression were estimated according to the ratio of mean cellular CD25 FITC to mean Vβ6 fluorescence on a double logarithmic graph and expressed as relative fluorescence units.

Phospholipase A_2 Activity

After incubation of T cells with antigen for 30 min, the cells were pelleted and lysed in assay buffer containing 0.1% Triton X-100, 66 mM Tris–HCl (pH 7.4), 395 mM NaCl, 13.2 mM $CaCl_2$ before incubation of 3.2×10^5 cells with 4.4 nmol of the PLA_2 substrate ^{14}C-phosphatidylcholine (Dupont/New England Nuclear, Boston, Massachusetts). After 75 μl of ^{14}C-phosphatidylcholine were dried under N_2 and redissolved in 15 μl Triton X-100 plus 285 μl assay buffer in a total volume of 100 μl for 45 min (Hendrickson, 1991), the reaction was stopped by addition of methanol:HCl (100:1). Lipid soluble material was extracted with chloroform/methanol according to Bligh and Dyer (1959) before separation on thin-layer chromatography (silica gel 60A) with chloroform:methanol:acetic acid:water (50:25:8:4) as eluant.

Guanylate Cyclase Activity

O3 cells (5×10^6/ml) were incubated with irradiated (2000 rads) BALB/c Thy1$^-$ Lyt1$^-$ spleen cells (25×10^6/ml) as APC without antigen or with 75 μg/ml OVA, or with Thy1$^-$ Lyt1$^-$ DBA/2 spleen cells (I-A^d; MTV7$^+$), in 6 ml DMEM, 5% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, penicillin/streptomycin. After 2 hr, O3 T cells were separated on Cellect columns (Biotex, Edmonton, Alberta, Canada), lysed (0.5% Brij 96 in guanylate cyclase assay buffer), and guanylate cyclase activity was measured according to catalytic conversion of guanosine 5′-triphosphate to cGMP as described (Domino et al., 1991). In brief, the reaction in 25 mM Tris–HCl (pH 7.6), 1 mM $MnCl_2$, 0.1 mM 1-methyl-3-isobutylxanthine, 625 μg/ml bovine serum albumin, 1 mM KF, 62.5 μM sodium orthovanadate, and 1 mM dithiothreitol was started by addition of 1 mM guanosine 5′-triphosphate and proceeded for 30 min at 37°C. After the reaction was stopped by protein precipitation with 6% trichloroacetic acid (final concentration), the supernatant was washed four times with 5 vol of water-saturated diethyl ether, and cGMP formation was quantitated using an ^{125}I-based radioimmunoassay (Amersham).

Acknowledgments

The authors wish to thank W. Fu for expert technical assistance and A. Angel for assistance in the preparation of the manuscript. This work was supported in part by National Institutes of Health research grants AI13600, AI12184, AI36105 and an Arthritis Foundation basic research grant to H. C.; a Barr Program Small Research Grant (Dana-Farber Cancer Institute) to G. F. W.; and a National Multiple Sclerosis Society Research Grant to S. A.-L.

Received December 21, 1994; revised February 21, 1995.

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