

## Fas and Activation-induced Fas Ligand Mediate Apoptosis of T Cell Hybridomas: Inhibition of Fas Ligand Expression by Retinoic Acid and Glucocorticoids

By Yili Yang,\* Mladen Merćep,\* Carl F. Ware,†  
and Jonathan D. Ashwell\*

From the \*Laboratory of Immune Cell Biology, Biological Response Modifiers Program, National Institutes of Health, Bethesda, Maryland 20892-1152; and †Division of Biomedical Sciences, University of California, Riverside, California 92521

### Summary

Activation of T cell hybridomas induces a G<sub>1</sub>/S cell cycle block and apoptosis. We isolated a variant of the 2B4.11 T cell hybridoma that, when activated via the TCR, produced IL-2 and underwent growth inhibition but did not die. Analysis of a variety of cell surface molecules revealed that the variant cell line, termed VD1, expressed very low levels of Fas compared to the wild type cells. Unlike 2B4.11 cells, VD1 cells were not killed by Fas ligand (FasL)-bearing effector cells. To determine if Fas is involved in activation-induced apoptosis, two different reagents that specifically bind Fas without killing the T cell hybridomas, a monoclonal antibody and a soluble Fas:Fc chimeric molecule, were added to activated T cell hybridomas. Both treatments prevented activation-induced apoptosis in a dose-dependent manner, but had no effect on IL-2 production or growth inhibition. Northern blot analysis revealed that unactivated 2B4.11 cells expressed negligible levels of FasL mRNA, but transcripts were detectable as early as 2 h after activation and continued to increase up to 4–6 h after activation. Anti-TCR induced activation of 2B4.11 cells in the presence of a TCR<sup>-</sup> 2B4.11 variant resulted in death of the unactivated “bystander” cells, which was inhibited by anti-Fas antibodies. Finally, treatment of T hybridoma cells with 9-*cis* retinoic acid or glucocorticoids, which are known to prevent activation-induced T cell apoptosis, inhibited the up-regulation of FasL. We conclude that up-regulated expression of FasL and its subsequent interaction with Fas accounts for the apoptotic response of T cell hybridomas to activation, and that retinoic acid and corticosteroids inhibit activation-induced apoptosis by preventing up-regulation of FasL.

**T** lymphocytes undergo apoptosis in response to a variety of stimuli, including glucocorticoids, radiation, withdrawal of growth factors, activation, viral infection, and some chemotherapeutic agents (1). Immature thymocytes and T cell hybridomas die by apoptosis when they were activated via the TCR, as do already activated mature peripheral T cells (2–4). The activation-induced death of thymocytes is an important component of negative selection, and therefore the establishment of tolerance to self antigens. Activation-induced apoptosis of mature T cells is thought to be at least one mechanism of maintaining peripheral tolerance and, perhaps, of limiting an ongoing immune response. In addition to these physiological examples of T cell apoptosis, inappropriate apoptosis of peripheral T cells from individuals infected with HIV has been observed, prompting the speculation that this phenomenon may play a role in the loss of CD4<sup>+</sup> T cells and the immunodeficiency that characterizes AIDS (5–9).

T cell hybridomas have been widely used in the investigation of cell death induced by specific antigen or anti-TCR antibodies. Activation of T cell hybridomas leads to a rapid G<sub>1</sub>/S cell cycle block (growth arrest), followed by apoptosis, which occurs after a period of 4–8 h (2, 3, 10). Although the mechanism of activation-induced apoptosis is not well understood, clues have been gleaned from studies using a variety of chemical and biological agents. Cycloheximide and actinomycin D prevent activation-induced apoptosis in T cell hybridomas, indicating a requirement for new protein biosynthesis (10, 11). Inhibitors of T cell activation and IL-2 production, such as cyclosporin A (CsA)<sup>1</sup> or depletion of extracellular Ca<sup>2+</sup>, also block apoptosis. Dexamethasone, which itself induces apoptosis of T cell hybridomas and thymo-

<sup>1</sup> Abbreviations used in this paper: CsA, cyclosporin A; Dex, dexamethasone; FasL, ligand for Fas.

cytes, paradoxically prevents activation-induced death (12). One can distinguish between activation-induced pathways leading to IL-2 production or cell death, however, with compounds such as retinoic acid and protease inhibitors, which prevent activation-induced apoptosis but have little effect on activation-induced IL-2 production (13–15).

Another cell surface molecule implicated in lymphocyte apoptosis is Fas, also known as APO-1 or CD95. Fas is a transmembrane molecule belonging to the TNF/nerve growth factor receptor superfamily (16, 17), and its multivalent cross-linking with antibody rapidly induces apoptosis in both normal and transformed lymphocytes. The ligand for Fas, FasL, has recently been identified and cloned (18), and is a type II transmembrane protein that is a member of the TNF superfamily. Binding of FasL to Fas results in rapid apoptosis of the Fas-expressing cells (18). T cells from Fas-deficient *lpr* mice and FasL-deficient *gld* mice have a defect in antigen-stimulated apoptosis, strongly supporting the notion that Fas is involved in activation-induced T cell apoptosis (19). We have studied the requirement for Fas and FasL in the activation-induced cell death in T cell hybridomas, and find that these molecules are key components of this process. Both the requirement for cellular activation, and inhibition of death by retinoic acid and glucocorticoids, can be explained by the regulated expression of FasL and its interaction with Fas.

## Materials and Methods

**Cells and Reagents.** 2B4.11 is a murine T cell hybridoma specific for peptide 81-104 of pigeon cytochrome *c* (2). 21.2.2 is a variant of 2B4.11 that lacks TCR  $\alpha\beta$  and therefore does not express cell surface CD3 (20). PC60-d11S (d11S), L1210 (leukemia cell line), and L1210-Fas<sup>+</sup> (L1210 cells transfected with *fas*) cells were kindly provided by Dr. P. Golstein (21). Cells were cultured and assayed in RPMI 1640 (Biofluids Inc., Rockville, MD) supplemented with 10% heat-inactivated FCS, 4 mM glutamine, 100 U/ml penicillin, 150  $\mu\text{g}/\text{ml}$  gentamicin, and  $5 \times 10^{-5}$  M 2-mercaptoethanol. 145-2C11 (2C11) is a hamster monoclonal antibody against mouse CD3- $\epsilon$  (22), and was purified from culture supernatant by affinity chromatography with protein A-sepharose. The anti-mouse Fas antibody Jo2 and PE-labeled Jo2 were purchased from PharMingen (San Diego, CA), and PE-anti human CD8 was purchased from (Biosource International, Camarillo, CA). The anti-mouse Fc receptor antibody 2.4G2 (23) was purified from culture supernatant. Dexamethasone (Dex), PMA, and ionomycin were purchased from Sigma Immunochemicals (St. Louis, MO). 9-*cis* retinoic acid was the generous gift of Dr. R. Heyman (Ligand Pharmaceuticals, La Jolla, CA).

**DNA Fragmentation Assay.** DNA fragmentation was quantitated by adherence to fiberglass filters, as described (14). Briefly, cells were incubated with [<sup>3</sup>H]thymidine (5  $\mu\text{Ci}/\text{ml}$ ) for 3 h and then distributed in triplicate into 96-well microtiter plates. Unless otherwise indicated,  $5 \times 10^4$  T cells were plated per well. After culture for the indicated times the cells were hypotonically lysed and harvested onto fiberglass filters (PHD Cell Harvester; Cambridge Technology, Cambridge, MA). Intact chromatin adheres to the filters, but DNA fragments pass through. The results are expressed as specific DNA fragmentation:

$$\frac{M - E}{M} \times 100$$

where *M* = retained label in cells cultured in medium, and *E* = retained label in cells cultured under experimental conditions,  $\pm$  the standard error of the mean. In cell mixing experiments the number of labeled "target" cells was held constant at  $2.5 \times 10^4$  cells per well and the number of "effector" cells was varied from  $1.25 \times 10^4$  to  $1.25 \times 10^5$  cells per well.

**IL-2 Assay.** Aliquots of supernatant were removed and assayed for IL-2 as described (24). An IL-2 unit is defined as the dilution of supernatant that caused IL-2-dependent CTLL-2 cells to incorporate half-maximal amounts of [<sup>3</sup>H]thymidine.

**Growth Inhibition Assay.** 2B4.11 cells were cultured under the indicated conditions. After 16 h, 1  $\mu\text{Ci}$  of [<sup>3</sup>H]thymidine was added to each well. After another 4 h, the cells were harvested and incorporation of radiolabel determined by liquid scintillation counting. The data are expressed as the cpm incorporated in each experimental condition divided by the cpm incorporated by cells grown in medium alone.

**Activation of d11S Cells.** d11S cells were activated with PMA (10 ng/ml) plus ionomycin (3  $\mu\text{g}/\text{ml}$ ) for 3 h to induce expression of FasL, as described (21).

**Flow Cytometry.** Specific staining was performed with PE-Jo2. Phycoerythrin-conjugated mouse anti-human CD8 was used as a negative control. Flow cytometric analysis was performed with a FACScan (Becton-Dickinson, CA).

**Receptor Fc Constructs.** A cDNA encoding the extracellular domain of Fas was isolated by PCR from the II-23.D7 human T cell hybridoma (25). This fragment was ligated in frame to a 710-bp cDNA fragment encoding the hinge, CH2 and CH3 domains of human IgG1 and subcloned into pDC302. For construction of the baculovirus transfer vector, a double stranded linker was designed with an internal EcoR1 site and a KpnI overhang with the sequence 5'-CACAAATTCGGGTAC-3' and for the bottom strand 5'-CCG-AATTCTC-3'. The bottom strand was treated with T4 polynucleotide kinase before annealing to the linker and subsequent ligation to the Fas:Fc KpnI/NotI site of the baculovirus transfer vector, pV1393 (Invitrogen, San Diego, CA). The TNFR60:Fc chimera, composed of the extracellular domain of the 55–60-kD TNF receptor and the Fc portion of human IgG1, was constructed as described (26). Th5B1-4 cells were infected with recombinant baculovirus in serum-free medium and the proteins purified to homogeneity by affinity chromatography using a protein G-Sepharose 4BCL resin.

**Northern Blot Analysis.** 2B4.11 cells ( $15 \times 10^6$  per point) were cultured in tissue culture dishes (Falcon 3003; Becton Dickinson Labware, Lincoln Park, NJ) that were coated with medium alone or anti-CD3 (10  $\mu\text{g}/\text{ml}$ ). After culture for the indicated times, RNA was prepared by a modified guanidine thiocyanate method (27). cDNA of the extracellular portion of mouse FasL was kindly provided by Dr. B. Niklinska (National Cancer Institute, National Institutes of Health, Bethesda, MD), and was obtained by PCR using the primers GGA ATT CAT GCA GCT CTT CCA GAC AGA AGG A and CGG GAT CCT TAA AGC TTA TAC AAG CCG AAA AAG, and purified from agarose gel using the Genelec II kit (Bio 101, Inc., Viata, CA). The Pst fragment of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was kindly provided by Drs. F. Mushinski and L. King (National Cancer Institute, National Institutes of Health). The probes were labeled with <sup>32</sup>P using the random primer labeling kit from Stratagene (La Jolla, CA). 15  $\mu\text{g}$  of total RNA, as determined by UV absorption, was loaded into each well and electrophoresed through a 1.0% agarose gel. After transfer to nitrocellulose, hybridization was carried out

using QuikHyb solution and procedures suggested by the manufacturer (Stratagene Inc., San Diego, CA). After exposure to detect FasL, the blots were stripped twice with boiling  $0.1\times$  SSC and  $0.1\%$  SDS buffer, and then reblotted for GAPDH.

## Results

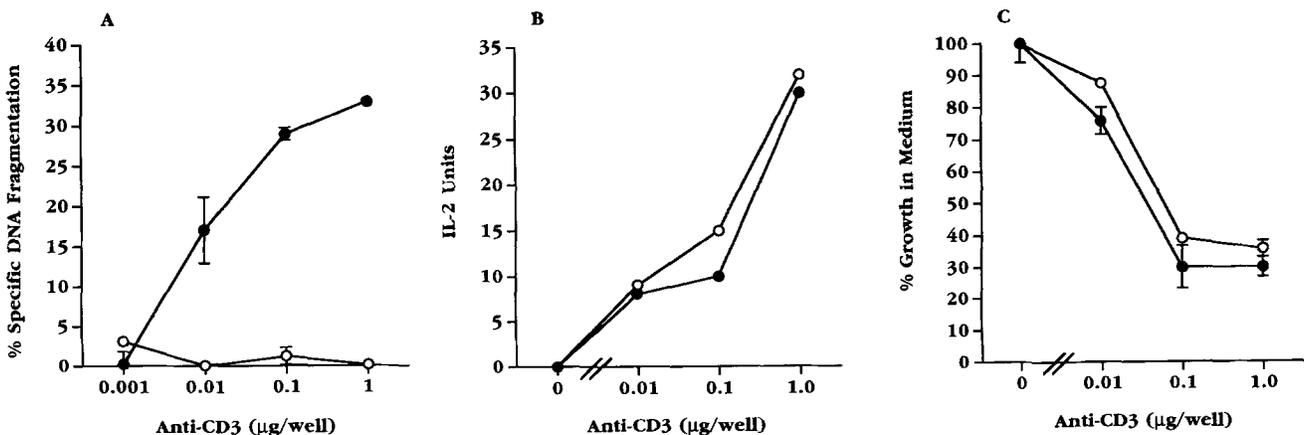
**Characterization of a "Deathless" Variant of the 2B4.11 T Cell Hybridoma.** Activation of T cell hybridomas via the TCR results in IL-2 production, a  $G_1/S$  cell cycle block, and apoptosis (2, 3, 10, 28). There appears to be more than one signaling pathway for these responses, since activation-induced apoptosis and IL-2 production require extracellular  $Ca^{2+}$  and are inhibited by CsA, while the cell cycle block does not require extracellular  $Ca^{2+}$  and is CsA resistant (28). In an attempt to obtain T cell hybridoma variants that do not die when activated, 2B4.11 T hybridoma cells were cultured at a density of  $10^4$  per microtiter well with  $5 \times 10^5$  B10.A irradiated spleen cells (as a source of antigen-presenting cells) and  $30 \mu\text{M}$  pigeon cytochrome *c* fragment 81-104. As shown in Fig. 1, cells that grew from one well, termed VD1 cells, did not die when restimulated with anti-CD3 (Fig. 1 A). VD1 cells also did not die when the antigen for which it is specific, pigeon cytochrome *c*, was used as a stimulus (data not shown). In contrast to the failure to undergo activation-induced apoptosis, stimulation of VD1 cells with anti-CD3 resulted in two other discrete biological responses that were quantitatively indistinguishable from those of the wild-type cells: IL-2 production and growth inhibition, the latter being mainly a manifestation of the  $G_1/S$  cell cycle block (Fig. 1, B and C) (28). Therefore, although biological responses that are mediated by several distinct TCR-mediated signaling pathways are intact in VD1 cells, activation-induced apoptosis is not.

**Analysis of Fas in 2B4.11 and VD1 Cell.** 2B4.11 and VD1 cells were analyzed for a variety of functionally important cell surface molecules, and were found to express comparable

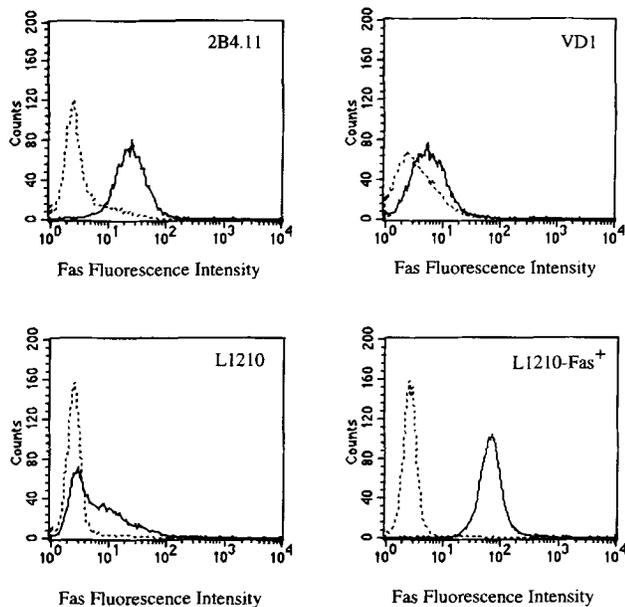
levels TCR, Ly-6, Thy-1, CD45, CD4, and CD8 (both cells being negative for the latter two). There was a difference, however, in expression of Fas. As shown in Fig. 2, unactivated 2B4.11 cells expressed easily detectable levels of Fas, with virtually all of the cells constituting a distinct peak in the fluorescence profile. No change in Fas expression was detected 1, 2, 4, or 6 h after anti-TCR activation of 2B4.11 cells (data not shown). Although positive, VD1 cells were very dull for Fas compared to the staining control. For comparison, Fas expression on L1210 cells, which are insensitive to FasL-mediated killing, and L1210-Fas cells, which die when cultured with FasL-bearing effector cells (21), was determined. L1210 cells are heterogeneous for Fas expression, with roughly half the cells appearing to be negative and the other half expressing variable amounts of Fas (Fig. 2). L1210-Fas cells are uniformly positive for Fas, with a mean fluorescence intensity approximately twice that of 2B4.11 cells.

Since Fas is a receptor that transduces signals leading to death, we asked how 2B4.11 and VD1 cells would respond when cultured with FasL<sup>+</sup> effector cell. d11S is a subclone of a rat-mouse T cell hybridoma whose expression of cell surface FasL increases after activation with PMA and ionomycin (21). As previously shown (21), unactivated d11S caused a small amount of apoptosis in 2B4.11 cells, and its activation with PMA and ionomycin greatly enhanced this activity (Fig. 3 A). The killing was mediated by Fas, since the Jo2 anti-Fas monoclonal antibody blocked the killing of the 2B4.11 cells (note that soluble Jo2 does not kill 2B4.11 cells since it is an IgG, and bivalent binding of Fas is insufficient to induce death [29]). In contrast to 2B4.11 cells, VD1 cells were not killed by d11S, even when the effector cells were activated with PMA and ionomycin (Fig. 3 B). Thus, just as when they are activated via the TCR, VD1 cells do not die when signaled through Fas.

**Activation-induced Death of T Cell Hybridoma Is Blocked by Anti-Fas Antibody and Soluble Fas Protein.** The failure of VD1



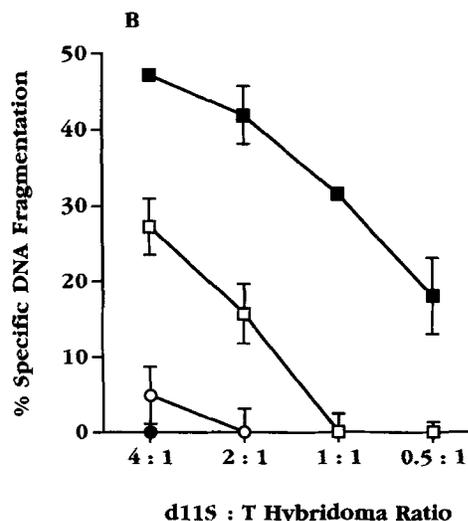
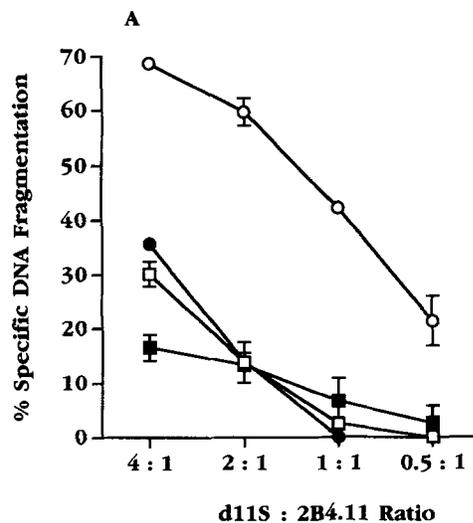
**Figure 1.** Activation of VD1 cells results in growth arrest and IL-2 production but not DNA fragmentation. [ $^3\text{H}$ ]Thymidine-labeled 2B4.11 and VD1 cells were stimulated with plastic-coated anti-CD3 antibody in microtiter wells for 18 h, and the percentage DNA fragmentation determined (A). Unlabeled cells were cultured in parallel under identical conditions and after 18 h assessed for IL-2 production (B) and incorporation of [ $^3\text{H}$ ]thymidine (C). —●—, 2B4.11; —○—, VD1.



**Figure 2.** Flow cytometric analysis of Fas expression on 2B4.11, VD1, L1210, and L1210-Fas cells.  $10^6$  cells were incubated with the anti-mouse Fc receptor antibody 2.4G2 and then stained with either PE-labeled anti-mouse Fas antibody Jo2 (solid line) or PE-labeled anti-human CD8 antibody (dashed line).

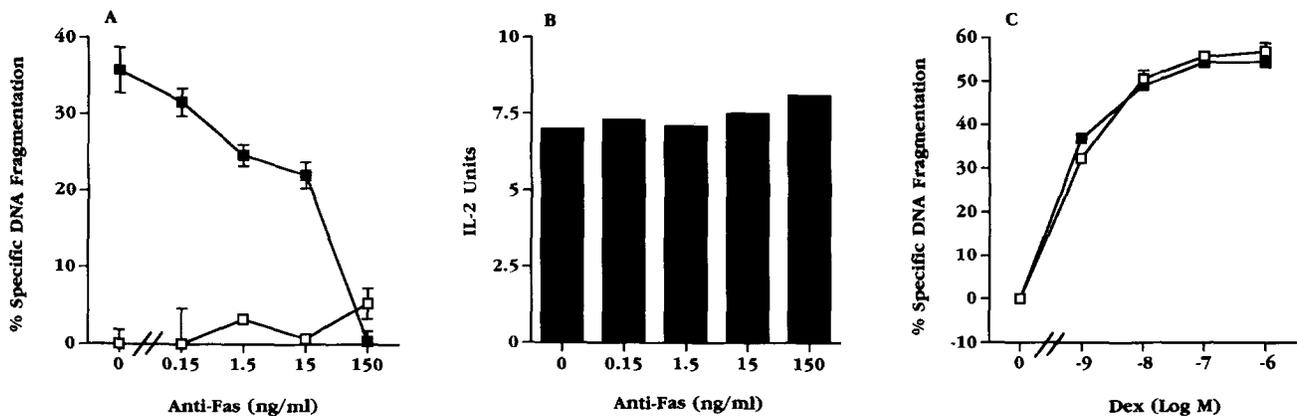
cells to be killed by FasL-bearing effector cells raised the possibility that activation-induced death, to which VD1 is resistant, is mediated by Fas. To determine if Fas is involved in activation-induced apoptosis, the Jo2 anti-Fas antibody was added to 2B4.11 cells that were or were not activated with anti-CD3 (Fig. 4). Immobilized anti-CD3 antibody alone caused ~35% specific DNA fragmentation of 2B4.11 cells. The activation-induced DNA fragmentation was completely prevented by anti-Fas antibody in a dose-dependent fashion, with complete inhibition achieved at 150 ng/ml (Fig. 4 A). In contrast to its inhibition of apoptosis, the Jo2 antibody had no effect on activation-induced IL-2 production (Fig. 4 B). Glucocorticoids also induce apoptosis in T cell hybridomas, but by a pathway that is distinct from that of activation (12). To determine the specificity of anti-Fas inhibition of apoptosis, Jo2 was added to 2B4.11 cells treated with increasing concentrations of Dex (Fig. 4 C). Unlike activation via the TCR, addition of anti-Fas antibody had no effect on DNA fragmentation caused by Dex. Another response to activation, growth arrest, was also measured. Anti-Fas antibodies did not prevent activation-induced inhibition of transformed growth (Fig. 5). Together with the failure to block IL-2 production, these results indicate that anti-Fas did not interfere with activation per se, just with the apoptotic response to activation.

Another means of blocking an interaction between Fas and FasL is to use a soluble form of Fas to bind the FasL on the effector cells. A chimeric molecule consisting of the extracellular region of human Fas and the Fc portion of human IgG1 (Fas:Fc) has been generated. Given that mouse and human Fas and FasL bind to one another (30), we asked what effect



**Figure 3.** 2B4.11 cells but not VD1 cells are sensitive to Fas-mediated killing. [ $^3$ H]Thymidine-labeled 2B4.11 cells with or without anti-Fas Jo2 (250 ng/ml) (A) or 2B4.11 and VD1 cells (B) were cocultured with activated or unactivated d11S cells at the indicated cell ratios, and DNA fragmentation measured 6 h later. (A)  $\square$ —, unactivated d11S;  $\blacksquare$ —, unactivated d11S + Jo2;  $\circ$ —, activated d11S;  $\bullet$ —, activated d11S + Jo2. (B)  $\square$ —, 2B4.11 + unactivated d11S;  $\blacksquare$ —, 2B4.11 + activated d11S;  $\circ$ —, VD1 + unactivated d11S;  $\bullet$ —, VD1 + activated d11S.

this soluble molecule would have on activation-induced T cell apoptosis (Fig. 6). Anti-CD3 induced ~55% specific DNA fragmentation, which was inhibited by soluble Fas:Fc in a concentration-dependent manner, with maximal inhibition achieved at 10  $\mu$ g/ml. As a control, a chimeric molecule consisting of the extracellular portion of the Fas-related TNF receptor and the Fc portion of human IgG1 (TNFR60:Fc) was added to activated 2B4.11 cells. Unlike Fas:Fc, this molecule had no effect on activation-induced death. The effect of Fas:Fc on T cell death was stimulation specific, in that glucocorticoid-induced apoptosis was unaffected by Fas:Fc,

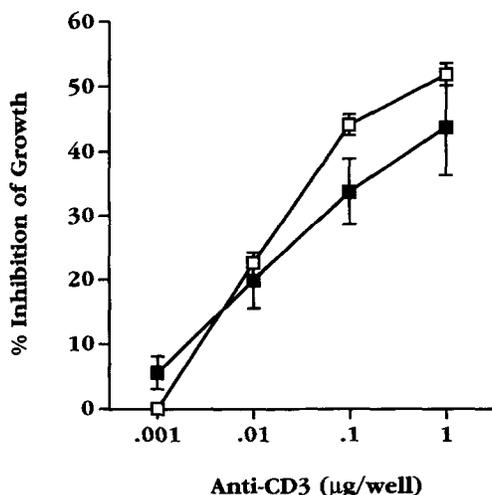


**Figure 4.** Effect of anti-Fas on killing of activated or glucocorticoid-treated T cell hybridomas. (A) [ $^3\text{H}$ ]Thymidine-labeled 2B4.11 cells ( $5 \times 10^4$  per well) were cultured in microtiter wells that had not (□) or that had (■) been coated with anti-CD3 ( $1 \mu\text{g}/\text{well}$ ). Jo2 was added at the indicated concentrations, and DNA fragmentation measured 18 h later. □, medium; ■, anti-CD3. (B) Supernatant from unlabeled 2B4.11 cells cultured as in (A) was removed after 4 h and assayed for IL-2. (C) Labeled 2B4.11 cells ( $5 \times 10^4$ ) were cultured with the indicated concentrations of Dex in the absence (□) or presence (■) of Jo2 ( $150 \text{ ng}/\text{ml}$ ). □, medium; ■, anti-Fas.

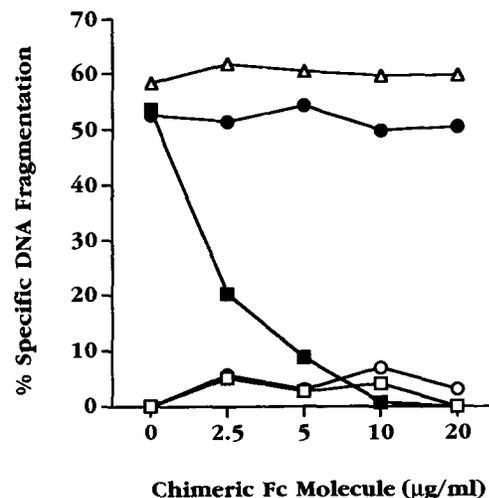
even at a concentration of  $20 \mu\text{g}/\text{ml}$ . As with the anti-Fas antibody, Fas:Fc did not prevent activation-induced growth inhibition or IL-2 production (data not shown).

**Expression of Fas and FasL on T Cell Hybridomas.** FasL has been reported to be highly expressed on activated but not resting T cells (18). Since Fas itself is constitutively expressed on 2B4.11 cells and its levels were unaffected by activation, it seemed likely that the activation-regulated expression of FasL might account for the apoptotic response to stimulation via the TCR. FasL mRNA expression was measured by Northern blot analysis. No FasL mRNA was detected in unstimulated 2B4.11 cells (Fig. 7 A). However, as early as

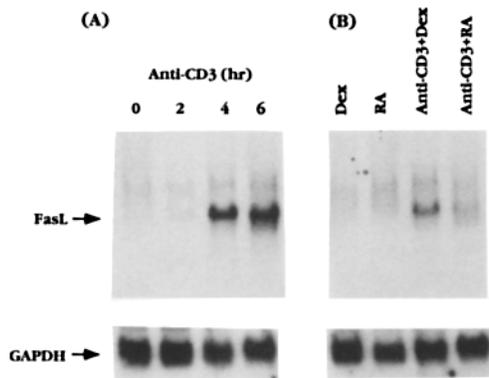
2 h after anti-TCR mediated activation, a small amount of FasL mRNA was detected (the FasL band at 2 h is readily detected on longer exposure of the blot). The level of FasL mRNA continued to increase up to 4–6 h. FasL mRNA levels were not assessed beyond this time point because of the increasing amount of cell death, which is accompanied by degradation of RNA (31), in these activated cultures. To determine if the increase in FasL mRNA was reflected in the expression of functional cell surface FasL, the ability of activated 2B4.11 cells to induce Fas-mediated cell death was assessed (Fig. 8). Unactivated 2B4.11 cells did not kill either L1210 cells or L1210-Fas cells. However, 2B4.11 cells that had



**Figure 5.** Anti-Fas does not prevent activation-induced growth inhibition. 2B4.11 cells ( $2.5 \times 10^4$  per well) were incubated in wells coated with the indicated concentrations of anti-CD3 or medium alone in the absence (□) or presence (■) of Jo2 ( $250 \text{ ng}/\text{ml}$ ). The data are expressed as percent inhibition of growth compared to cells cultured in the absence of anti-CD3. In this experiment, anti-CD3 caused 53.0% specific DNA fragmentation; anti-CD3 plus anti-Fas ( $250 \text{ ng}/\text{ml}$ ) induced 0.9% specific DNA fragmentation. □, medium; ■, anti-Fas.



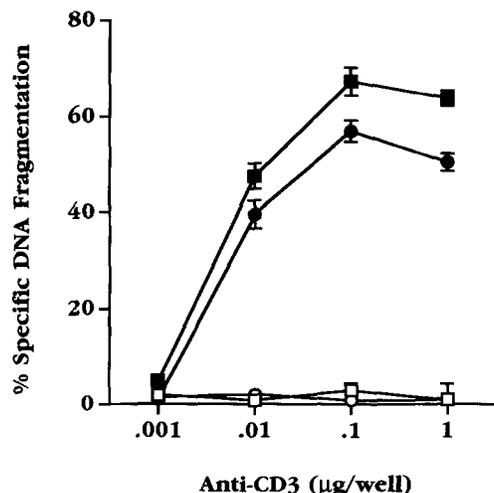
**Figure 6.** Soluble Fas:Fc prevents activation- but not glucocorticoid-induced apoptosis. Labeled 2B4.11 cells ( $5 \times 10^5$  per well) were cultured in medium, wells coated with anti-CD3 ( $1 \mu\text{g}/\text{well}$ ), or in medium containing Dex ( $10^{-7} \text{ M}$ ) in the presence of the indicated concentrations of either Fas:Fc or TNFR60:Fc. DNA fragmentation was assessed after 18 h of culture. The standard errors of the mean were  $\leq 5\%$  for each experimental point. □, medium + Fas:Fc; ■, anti-CD3 + Fas:Fc; ○, medium + TNFR60:Fc; ●, anti-CD3 + TNFR60:Fc; △, Dex + Fas:Fc.



**Figure 7.** Northern blot analysis of FasL expression in 2B4.11 cells. (A) RNA was prepared from 2B4.11 cells cultured for the indicated times in plates coated with anti-CD3. (B) RNA was prepared from 2B4.11 cells cultured for 6 h in the presence of either 9-*cis* retinoic acid ( $10^{-6}$  M) or Dex ( $10^{-6}$  M). The cells were cultured in plates that had or had not been coated with anti-CD3, as indicated. The 6 h time point in A represents the amount of FasL induced in the absence of these reagents. After probing with  $^{32}$ P-labeled FasL cDNA, the blot was stripped and reprobed with  $^{32}$ P-labeled GAPDH cDNA. FasL is  $\sim 2$  kb, and is indicated by the arrow.

been activated by immobilized anti-CD3 specifically lysed the Fas<sup>+</sup> L1210-Fas cells, but not the Fas<sup>-</sup> L1210 cells, in a dose-dependent manner (Fig. 8). Thus, activated 2B4.11 cells express functional FasL.

Since 2B4.11 cells express Fas constitutively and are susceptible to killing by FasL<sup>+</sup> d11s cells, one would expect that activated, FasL<sup>+</sup> 2B4.11 cells would cause the death of unactivated 2B4.11 bystander cells. To test this, we used a variant of 2B4.11, 21.2.2, that does not express cell surface



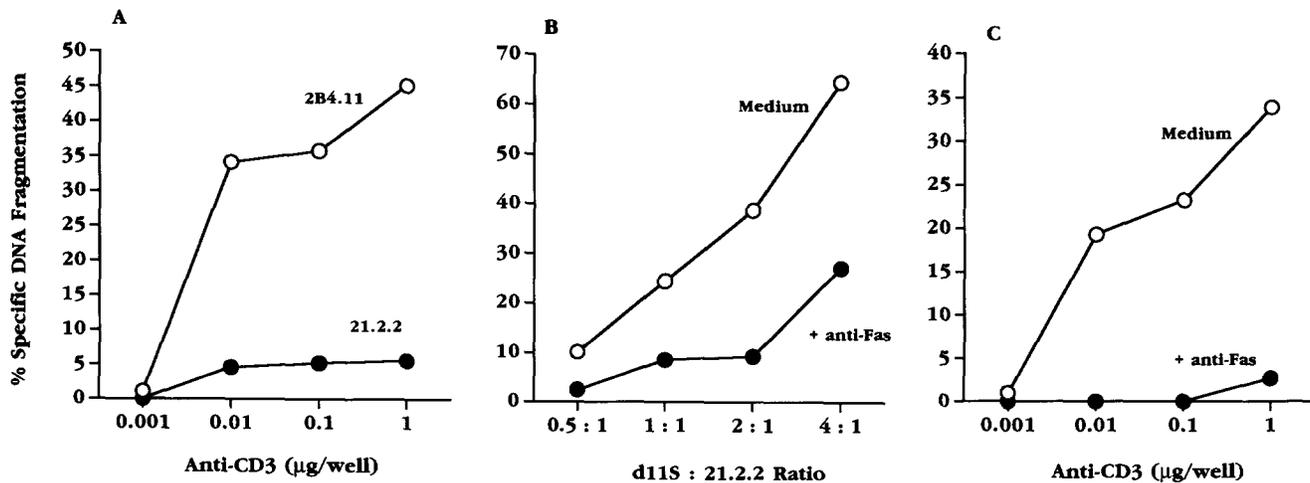
**Figure 8.** Activated VD1 cells express FasL. 2B4.11 or VD1 cells ( $5 \times 10^4$ /well) were cultured for 4 h in the presence of the indicated amounts of anti-CD3. After 4 h, [ $^3$ H]thymidine-labeled L1210 or L1210-Fas cells ( $5 \times 10^4$ /well) were added. After another 6 h of culture, DNA fragmentation of the labeled cells was determined. There was no specific DNA fragmentation detected when L1210 cells or L1210-Fas cells were cultured in parallel under identical conditions except that no T cell hybridomas were present (data not shown).  $\square$ —, 2B4.11 + L1210;  $\blacksquare$ —, 2B4.11 + L1210-Fas;  $\circ$ —, VD1 + L1210;  $\bullet$ —, VD1 + L1210-Fas.

TCR because it lacks TCR  $\alpha$  and  $\beta$  chains (20). As shown in Fig. 9 A, 2B4.11 but not 21.2.2 cells died in a stimulus-dependent manner when cultured in wells coated with anti-CD3 antibodies. As shown for 2B4.11, TCR<sup>-</sup> 21.2.2 are also susceptible to Fas-mediated killing, since coculture with d11s cells resulted in their death, which was inhibited by anti-Fas antibodies (Fig. 9 B). In Fig. 9 C, unlabeled 2B4.11 cells were cultured with labeled 21.2.2 cells for 16 h in the presence of anti-CD3. As the amount of anti-CD3 per well increased there was a corresponding increase in specific DNA fragmentation of 21.2.2 cells. This cell death required Fas, since the Jo2 anti-Fas antibody blocked 21.2.2 cell death in this mixed culture. Thus, activated, FasL<sup>+</sup> 2B4.11 cells can kill unactivated bystander cells via a Fas-dependent mechanism.

**Retinoic Acid and Dexamethasone Prevent Activation-induced FasL Up-regulation.** Both retinoic acid, especially the 9-*cis* stereoisomer, and glucocorticoids inhibit activation-induced apoptosis of T cell hybridomas (12–14). Experiments were performed to determine if the biological activity of either of these reagents is due to effects on the regulation of FasL expression. 9-*Cis* retinoic acid completely prevented, and Dex greatly reduced, the activation-induced increase in FasL mRNA in 2B4.11 cells (Fig. 7 B). These decreases in FasL mRNA were reflected in the biological activity of this molecule, since 2B4.11 cells that were stimulated with anti-CD3 antibody in the presence of 9-*cis* retinoic acid ( $10^{-6}$  M) or Dex ( $1 \mu$ M) did not induce the death of L1210-Fas cells (Fig. 10). This effect on cytotoxicity was at the level of effector 2B4.11 cell generation, because addition of retinoic acid or Dex to L1210-Fas cells at the time of adding activated 2B4.11 cells had no effect on Fas-mediated cell death (Fig. 10).

## Discussion

Fas is a 48-kD transmembrane molecule that belongs to the TNF/nerve growth factor receptor gene superfamily, which also includes CD27, CD40, CD30, and OX40 (16, 17, 32). Mouse Fas mRNA is expressed in thymus, liver, heart, lung, and ovary; it is undetectable in spleen, brain, kidney, and bone marrow (32). Fas is not expressed by the majority of human peripheral blood T cells unless they have been activated (33–35). It is expressed at high levels on human CD4<sup>+</sup>CD8<sup>+</sup> TCR<sup>intermediate</sup> thymocytes, decreasing in level as the cells mature to the TCR<sup>hi</sup> stage (36). Two groups independently identified Fas as a signaling molecule whose cross-linking with antibody can lead to death (33, 37). Until recently little was known about how ligation of Fas leads to death. Mutational analysis has shown that an intracellular domain that is homologous to a region in the TNF receptor is required for Fas-mediated killing (29). However, the intracellular portion of the molecule has no consensus sequences for kinases or phosphatases. It has recently been demonstrated that cross-linking of Fas on a variety of human cell lines induces the activity of an acid sphingomyelinase, which results in the generation of ceramide, and that exogenous addition of the cell-permeable C<sub>2</sub>-ceramide induces apoptosis in these cells (38). This mechanism has also been suggested for cell



**Figure 9.** Activated 2B4.11 cells kill unactivated bystander cells by a Fas-dependent mechanism. (A) Labeled 2B4.11 (○) or VD1 (●) cells ( $2.5 \times 10^4$ /well) were cultured for 16 h in the presence of the indicated concentrations of anti-CD3 and then assayed for DNA fragmentation. (B) Labeled 21.2.2 cells were cultured with activated d11S cells at the indicated cell ratios for 16 h in the absence (○) or presence (●) of Jo2 (250 ng/ml), at which time DNA fragmentation was measured. (C) Unlabeled 2B4.11 ( $5 \times 10^4$ /well) were cultured with equal numbers of [ $^3$ H]thymidine-labeled 21.2.2 cells in the presence of the indicated concentrations of anti-CD3 and without (○) or with (●) Jo2 (250 ng/ml). After 16 h the cells were assayed for DNA fragmentation. Standard errors were typically  $\leq 5\%$  for each experimental point.

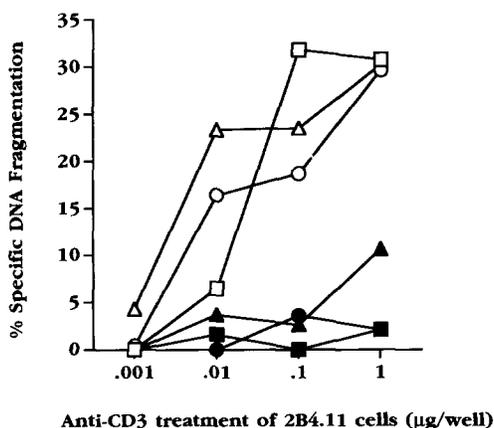
death mediated by the TNF receptor (39). How ceramides might initiate a program leading to cell death remains to be elucidated.

FasL has recently been identified (18). The FasL gene encodes a transmembrane protein with a predicted molecular mass of 31 kD. The molecular mass of the expressed mole-

cule is 38,000–42,000, the larger molecular size probably being due to *N*-linked glycosylation. FasL is homologous to the TNF family of molecules. Cells transfected with FasL cDNA, but not control cells, were able to kill Fas<sup>+</sup> targets. Interestingly, supernatants of COS cells transfected with FasL cDNA were also able to kill Fas<sup>+</sup> targets, suggesting that FasL can be shed in an active form. Rat FasL mRNA has a very different tissue distribution from Fas, being expressed in small intestine, testis, lung, and spleen. Of particular interest is that activation of spleen cells or thymocytes induces expression of FasL, indicating that activated T cells can express both Fas and FasL (18). Human and mouse FasL are highly homologous, and human and mouse FasL bind to Fas from either species (30).

Our data demonstrate that, as for the majority of thymocytes, the 2B4.11 T cell hybridoma constitutively expresses Fas, and cellular activation induces expression of FasL. The interaction of this receptor-ligand pair is required for activation-induced apoptosis of these cells. VD1 cells, which were selected by virtue of their resistance to TCR-mediated activation-induced death, have very low levels of Fas but normal levels of functional FasL upon activation. We believe that the low level of Fas on VD1 is sufficient to account for its resistance to FasL- or activation-induced death, since the level of Fas on these cells is comparable to, or even less than, the levels of Fas on L1210 cells. It is possible, however, that in addition to lower than normal expression, Fas may be mutated and nonfunctional in VD1 cells, or there may be defects in other components of the Fas-mediated signaling pathway. Further evaluation of Fas and its signaling pathway(s) in VD1 cells should allow us to distinguish between these possibilities.

Pharmacological agents such as retinoic acid and glucocorticoids are able to block activation-induced apoptosis of T cells. The data in the present paper show that both of these



**Figure 10.** Retinoic acid and glucocorticoids block expression of functional FasL on activated T cell hybridomas. 2B4.11 cells ( $5 \times 10^4$ /well) were cultured in wells coated with the indicated amounts of anti-CD3 without or with  $10^{-6}$  M Dex (●) or  $10^{-6}$  M 9-*cis* retinoic acid (▲). After 6 h, [ $^3$ H]thymidine-labeled L1210 or L1210-Fas cells ( $2.5 \times 10^4$ ) were added to these wells. In some cases,  $10^{-6}$  M Dex (○) or  $10^{-6}$  M 9-*cis* retinoic acid (△) was added to wells that initially contained medium alone. After 4 h of coculture the amount of DNA fragmentation of the labeled cells was measured. The parentheses in the boxed legend indicate at what time, in the first culture or in the mixed cell culture, Dex or retinoic acid was added. The standard errors of the mean were  $\leq 5\%$  for each experimental point. □, 2B4 + L1210-Fas; ■, 2B4 L1210; ●, (2B4 + Dex) + L1210-Fas; ▲, (2B4 + RA) + L1210-Fas; ○, 2B4 + (Dex + L1210-Fas); △, 2B4 + (RA + L1210-Fas).

agents prevent the up-regulation of FasL, but have no effect on killing signaled through Fas by effector cells that are already FasL<sup>+</sup>. Both of these molecules mediate their effects by binding ligand-regulated nuclear transcription factors. Therefore, the simplest mechanism by which they might affect FasL expression would be for their respective receptors/transcription factors to directly regulate FasL gene transcription. An analysis of ~300 bp 5' of the ATG initiation codon of human FasL has found regulatory elements for SP-1, NF- $\kappa$ B, and IRF-1 (30). There are no obvious retinoic acid response elements or glucocorticoid response elements in this region. Such response elements may be found in more distal flanking regions of the gene, or possibly in intronic sequences. Alternatively, glucocorticoids and/or retinoic acid may exert their effects on FasL by an indirect mechanism. We have also found that CsA, which prevents Ca<sup>2+</sup>-dependent activation events by inhibiting the phosphatase calcineurin (40, 41) and inhibits activation-induced apoptosis but not the G<sub>1</sub>/S cell cycle block (28), also prevents activation-induced up-regulation of FasL (our unpublished observation). Therefore, a variety of agents that mediate their protective effects by interacting with different DNA-regulatory sites or proteins have in common the ability to suppress FasL expression.

In the present study we found that activated 2B4.11 cells were able to kill unactivated 2B4.11 cells in a Fas-dependent manner. We have previously shown that when T cell hybridomas with different antigen specificities are cocultured in the presence of one antigen, only the growth of the activated cell is inhibited (2). That is consistent with the finding in the present report in that the G<sub>1</sub>/S block induced by activation does not seem to be Fas dependent: unlike apoptosis, growth inhibition was not reversed by anti-Fas antibodies. Another study, in which only one of two cocultured T cell hybridomas was specifically activated, detected only a small amount of bystander killing (of 2B4.11 cells) as measured by release of fragmented DNA, which was interpreted as indicating a lack of bystander killing (10). However, in that experiment the total time of stimulation was only 8 h, compared to 16 h of coculture in the present report. FasL must be up-regulated before activated cells can act as effectors, and

by Northern blot analysis it takes 4–6 h to achieve high levels of expression. We have found that at shorter times of coculture Fas-dependent bystander killing is more difficult to detect (data not shown), indicating that killing in trans may be less efficient than killing in cis. This may be because Fas–FasL interactions are more effective if both molecules are present on the same cell. Alternatively, just as with FasL-expressing COS cells (18), FasL may be shed from activated cells and preferentially interact with Fas molecules on the same cell.

The biological importance of Fas and FasL in normal T cell biology is demonstrated by the phenotypes of *lpr* and *gld* mice, which have defects in expression of Fas and FasL, respectively (42, 43). The major pathological changes in these mice are accumulation of CD4<sup>-</sup>CD8<sup>-</sup>TCR<sup>+</sup> T cells (lymphoproliferation) and autoimmunity (44). It has been suggested that the accumulating aberrant T cells originate from “neglected” CD4<sup>+</sup>CD8<sup>+</sup>TCR<sup>dull</sup> thymocytes that escape to the periphery due to absence of Fas (45). However, although the thymus expresses the highest level of Fas mRNA among normal tissues, and activation of thymocytes induces FasL mRNA, the development of thymocytes appears to be normal in *lpr* mice (46, 47). More recently, using transgenic TCR crossed with MRL-*lpr/lpr* or control MRL +/+ mice, it has been possible to demonstrate a role for Fas in peripheral but not thymic deletion of T lymphocytes (48). This observation is supported by experiments that show activation-induced apoptosis of peripheral T cells depends on Fas and FasL (19, 49–51). Thus, at this time, it appears that Fas/FasL plays a critical role in peripheral T cell deletion. It is interesting to note that degree of T cell hybridoma apoptosis induced with a mitogenic anti-Thy-1 antibody varies with the maturity of the T cell fused to the BW5147 murine thymoma: fusions made with immature thymocytes are resistant to killing, while those made with peripheral T cells are sensitive (11). This may suggest that the T cell hybridoma phenotype, with regard to activation-induced apoptosis at least, reflects a pathway present in mature, but not immature, T cells, which is consistent with our present understanding of the biological role of Fas and FasL in regulated T cell death.

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We thank B. Niklinska for the FasL cDNA, P. Golstein for cell lines, and A. Weissman for critical review of this manuscript. We are also grateful to R. Walker Force, P. D. Crowe, and B. Walter for technical contributions.

This work was supported, in part, by grants (to C. F. Ware) from the American Cancer Society (IM663) and National Institutes of Health (AI33068).

Address correspondence to Dr. J. D. Ashwell, Laboratory of Immune Cell Biology, Biological Response Modifiers Program, National Institutes of Health, Bldg. 10, Rm. 1B-40, Bethesda, MD 20892.

Dr. Merćep's current address is Ciba Molecular Biology Resources, R-1056.P.23, 4002 Basel, Switzerland.

Received for publication 12 December 1994 and in revised form 19 January 1995.

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