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9-*cis* Retinoic Acid Inhibition of Activation-Induced Apoptosis Is Mediated via Regulation of Fas Ligand and Requires Retinoic Acid Receptor and Retinoid X Receptor Activation

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T-cell hybridomas, thymocytes, and T cells can be induced to undergo apoptotic cell death by activation through the T-cell receptor. This process requires macromolecular synthesis and thus gene expression, and it has been shown to be influenced by factors regulating transcription. Recently, it has been shown that following activation, T-cell hybridomas rapidly express the Fas/CD95 receptor and its ligand, Fas ligand (FasL), which interact to transduce the death signal in the activated cell. Retinoids, the active metabolites of vitamin A, modulate expression of specific target genes by binding to two classes of intracellular receptors, retinoic acid receptors (RARs) and retinoid X receptors (RXRs). They are potent modulators of apoptosis in a number of experimental models, and they have been shown to inhibit activation-induced apoptosis in T-cell hybridomas and thymocytes. Particularly effective is the prototypic pan-agonist 9-*cis* retinoic acid (9-*cis* RA), which has high affinity for both RARs and RXRs. We report here that 9-*cis* RA inhibits T-cell receptor-mediated apoptosis in T-cell hybridomas by blocking the expression of Fas ligand following activation. This inhibition appears to be at the level of FasL mRNA, with the subsequent failure to express cell surface FasL. RAR-selective (TTNPB) or RXR-selective (LG100268) ligands alone were considerably less potent than RAR-RXR pan-agonists. However, the addition of both RAR- and RXR-selective ligands was as effective as the addition of 9-*cis* RA alone. This demon-strates that the inhibitory effect requires the ligand-mediated activation of both retinoid receptor signaling pathways.

Apoptosis, or active cell death, plays a number of essential roles in normal development, homeostasis, and pathology. One interesting form of apoptosis is seen in lymphocytes following stimulation through antigen receptors, a signal that is usually associated with lymphoid activation and proliferation. Such activation-induced apoptosis is thought to be the mechanism of negative selection in thymocytes (28, 55), peripheral deletion in mature T cells (30), and loss of noninfected T cells in AIDS (38, 58).

Activation-induced apoptosis in T-cell hybridomas has been a useful model for elucidating the molecular mechanisms underlying this process. This particular form of apoptosis requires macromolecular synthesis (51, 59) and therefore gene expression and transcriptional regulation. Transcription factors which have been shown to be important in this process include c-Myc (50), Myc-Max heterodimers (4), and the orphan receptor nur77a (33, 64), the last encoded by an early response gene induced immediately following activation. Activation-induced apoptosis is inhibited by several different agents, including the immunosuppressive drugs cyclosporine and FK506 (3, 13, 37, 51, 52). In addition, although dexamethasone can by itself induce death in these cells (24, 70), glucocorticoids can also block activation-induced apoptosis in this system, and thus these two pathways of apoptosis are mutually antagonistic in both T-cell hybridomas and thymocytes (25, 26, 69). One possible mechanism for this may be via glucocorticoid receptor-mediated regulation of gene expression, suggesting that this phenomenon may be relevant to normal T-cell development (62).

The surprising observation that glucocorticoids regulate activation-induced cell death led to investigations of other members of the intracellular receptor superfamily for the potential to antagonize T-cell receptor (TCR)-mediated apoptosis. Retinoic acid is an active metabolite of vitamin A and is critically important in vertebrate development (23), influencing the function of a variety of cell types (20), including lymphocytes. Vitamin A deprivation is associated with reduced immune function and increased susceptibility to infection, as well as thymic and splenic atrophy, and moderate doses of all-*trans* retinoic acid (ATRA) can increase thymus weight and cellularity (reviewed in reference 44). These observations and those discussed above led two laboratories to investigate the effects of ATRA on activation-induced apoptosis, and both found potent inhibition of this process in T-cell hybridomas and thymocytes (27, 68). Unlike glucocorticoids, however, ATRA by itself does not cause apoptotic cell death (68).

Retinoids modulate the specific expression of target genes by binding to and activating two distinct subfamilies of intracellular receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs) (reviewed in reference 34). Like other members of the nuclear receptor superfamily, RARs (α, β, and γ) and RXRs (α, β, and γ) become transcriptionally active upon ligand binding and transactivate their target genes by binding to hormone response elements that generally consist of two direct repeat half-sites of the consensus sequence AGGTCA. ATRA is a high-affinity ligand for RAR α, β, and

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γ (1) only, whereas 9-*cis* retinoic acid (9-*cis* RA), the active stereoisomer of ATRA, is a high-affinity ligand for both RARs and RXRs (1). RAR-RXR heterodimers activate transcription in response to ATRA or 9-*cis* RA, while RXR-RXR homodimers transactivate in response to 9-*cis* RA. The observation that 9-*cis* RA is 10-fold more potent than ATRA in blocking activation-induced apoptosis suggests that this inhibition involves retinoic X receptors (68).

Recently, we and others have demonstrated that activation-induced apoptosis in T-cell hybridomas occurs via induction of expression of two molecules, Fas (CD95) and Fas ligand (FasL), which then interact to transduce an apoptotic signal (8, 12, 29). Using several different T-cell hybridomas, we found that both molecules are induced within 4 h of activation and that competitive inhibition of their interaction blocks the induction of apoptosis. Further, we found that this interaction can occur even in a single cell. Thus, activation-induced apoptosis in T-cell hybridomas proceeds via a cell-autonomous (single-cell) Fas-FasL interaction. Here, we show that 9-*cis* RA blocks activation-induced apoptosis via its ability to inhibit expression of FasL following activation. We present data indicating that both RAR- and RXR-mediated pathways are essential for this process. These results are discussed in the context of the potential physiological role of FasL-induced apoptosis and its regulation.

MATERIALS AND METHODS

Cell lines and antibodies. All cells were maintained in RPMI 1640 or Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), glutamine, and β-mercaptoethanol. The murine T-cell hybridoma lines A1.1 (51) and 2B4 (2) were obtained from Arun Fotedar and Jon Ashwell, respectively, and have been described in detail elsewhere. Both cell lines express the TCR-CD3 surface receptor complex and undergo activation-induced apoptosis when exposed to anti-TCR antibodies (37, 51). Jurkat cells were obtained from the American Type Culture Collection. L1210 mouse T leukemia cells transfected to constitutively express human Fas (L1210.fas) were provided by Pierre Golstein (45). The cells were periodically reselected for high-level Fas expression. The B-cell hybridoma clone 145.2C11 secreting a monoclonal hamster anti-mouse CD3 antibody (32) was obtained from the American Type Culture Collection. 145.2C11 culture supernatants were clarified, and the antibody was affinity purified by protein A-Sepharose chromatography (Pharmacia, Piscataway, N.J.). The interleukin 2 (IL-2)-dependent cell line CTLL-2 (16) was obtained from the American Type Culture Collection. Monoclonal immunoglobulin M (IgM) and IgG antibodies to human Fas protein were obtained from Kamiya Biomedical (Thousand Oaks, Calif.). Monoclonal IgG antibody specific for mouse Fas antigen (Jo2) was purchased from Pharmingen (San Diego, Calif.).

Retinoids. All retinoids were synthesized at Ligand Pharmaceuticals. The syntheses of the pan-agonist 9-*cis* RA and the RAR-selective retinoid TTNPB {(*E*)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid} have been described in detail elsewhere (6). A description of the synthesis of the RXR-selective retinoid LG100268 has been submitted for publication (7). All compounds were solvated to 1 mM in dimethyl sulfoxide-ethanol.

Retinoid binding and transfection assays. Ligand competition-binding assays were performed by using receptors prepared with a baculovirus expression system. Receptors were obtained from lysis extracts (1) of SF21 cells infected with baculovirus transfer vectors expressing human RAR α, β, and γ or human RXR α, β, and γ. The receptor extracts were incubated for 2 h at 0°C with [^{3}H]9-*cis* RA (5) in the presence or absence of 200-fold excess unlabeled ligand. Specific ligand binding to receptors was determined by a hydroxyapatite assay according to the method of Wecksler and Norman (63).

The cotransfection assay to determine ligand activation of receptor transcriptional activity has been described in detail elsewhere (21). The retinoid receptor expression vectors used in this study were pRS-hRAR γ (human RAR γ) (15) and pRS-hRXR α (human RXR α) (35). The RAR reporter construct used, ΔMTV-TRE-Luc (60), contains two copies of the thyroid hormone response element (TRE) palindromic response element inserted into the basal reporter construct ΔMTV-Luciferase. The RXR reporter construct TK-CRBPII-Luc (60) contains one copy of the DR1 response element from cellular retinol-binding protein II linked to the herpes simplex virus thymidine kinase minimal promoter upstream of the luciferase gene. Briefly, CV-1 cells were seeded into 96-well plates and transiently cotransfected by the calcium phosphate method with 10 ng of either receptor expression vector, 50 ng of reporter plasmid, and 50 ng of pRS-β-GAL (β-galactosidase) internal control for 6 h. The cells were washed, incubated in the presence of ligand for an additional 36 h, and assayed for luciferase and β-galactosidase activity as described previously (21). All determinations were done in triplicate and normalized for transfection efficiency with the β-galactosidase internal control.

Apoptosis assays. DNA fragmentation quantitated by recovery of radiolabeled DNA was performed as follows: cells were labeled for 2 h at 37°C with 5 μCi of [^{3}H]thymidine deoxyribonucleotide ([^{3}H]TdR) per ml in RPMI 1640–10% FCS. The cells (50×10^3 per well) were washed twice in warm Hanks balanced salt solution, resuspended in IMDM–10% FCS, and seeded into 96-well plates coated with anti-CD3 antibody. Twelve to 16 h later, the plates were extracted with a 96-well plate harvester (Skatron, Sterling, Va.) onto glass fiber filter mats (Skatron) and the unfragmented, high-molecular-weight [^{3}H]TdR-labeled DNA retained was counted in a liquid scintillation counter (Pharmacia LKB). The difference between the amount of radioactivity recovered in experimental cultures and the amount recovered in control cultures is a measure of the loss of low-molecular-weight, fragmented DNA and thus apoptosis. The data are expressed as percents DNA fragmentation, i.e., by the formula $100 \times (1 -$ cpm in experimental group/cpm in unstimulated cells). Each datum point represents the mean for six wells with the standard deviation.

The quantitation of apoptosis by terminal deoxytransferase UTP nick end labeling (18) (ApopTag; Oncor, Gaithersburg, Md.) of fragmented DNA was performed as follows: cells were fixed in paraformaldehyde, washed several times, and then incubated with the enzyme terminal deoxynucleotide transferase (TdT) in the presence of labeled (digoxigenin) UTP, which results in the incorporation of label at points of DNA strand breakage. The cells were then incubated with a fluorescein isothiocyanate-labeled antidigoxigenin antibody to detect the amount of UTP label incorporated and thus the amount of DNA fragmentation present. The cells were then counterstained with propidium iodide for DNA content and analyzed on a FACScan device with Macintosh-based CELLQuest software (Becton Dickinson, San Jose, Calif.).

Flow cytometric detection of FasL. For FasL expression, 2B4 and A1.1 cells were activated with anti-CD3 antibody for 6 h, removed from culture, and washed twice with phosphate-buffered saline (PBS)–1% FCS–0.1% sodium azide. The pellets, containing approximately 10^6 cells, were resuspended in 50 μg of normal mouse IgG in PBS–0.1% sodium azide–1% FCS and incubated for 15 min at room temperature to saturate nonspecific binding sites. To detect Fas, the cells were stained with monoclonal anti-Fas antibody (Jo2) added directly to the unwashed suspensions, incubated for 30 min on ice, washed once in PBS–0.1% sodium azide–1% FCS, and then stained with an anti-hamster antibody–fluorescein isothiocyanate conjugate (Southern Biotechnology Associates, Birmingham, Ala.) for 30 min on ice. The cells were washed twice in PBS–0.1% sodium azide and fixed in 1% paraformaldehyde in PBS. To detect FasL, a chimeric Fas-Fc protein recognizing FasL expressed in cells following activation was utilized (8). The Fas-Fc chimeric protein was produced in a baculovirus expression system (10). The Fas-Fc insert was constructed from the cDNA encoding the extracellular domain of Fas, ligated to the cDNA encoding the hinge, CH2, and CH3 domains of human IgG1 (8). Tn5B1-4 cells were infected with a baculovirus transfer vector containing the Fas-Fc cDNA, and the Fas-Fc protein was protein G purified from cells grown in serum-free medium (8). A 15-μg portion of purified Fas-Fc was added as described above to unwashed cells suspended in PBS–0.1% sodium azide–1% FCS–normal mouse IgG, and the mixture was incubated for 30 min on ice. The cells were washed once in PBS–0.1% sodium azide–1% FCS, incubated for 30 min with anti-human IgG–biotin, washed, and stained with Streptavidin TRICOLOR (Caltag, San Francisco, Calif.) or Streptavidin-PerCP (Becton Dickinson) for 30 min on ice. The cells were fixed and analyzed by FACScan analysis. The specificity of the Fas-Fc reagent used in the experiments described above has been studied in detail by our laboratory (i.e., that of D.R.G.) as well as others (8, 10, 67). Fas-Fc does not bind to cells which do not express Fas ligand, activated or otherwise, including cells from *gld* mice, which are Fas ligand null. Experiments were performed (8) with a control chimeric protein reagent constructed from the extracellular domain member of the tumor necrosis factor receptor and the Fc portion of human IgG1 (TNFR-Fc) (10) expressed and produced in a fashion identical to that for the Fas-Fc protein.

Fas ligand functional activity. Functional activity of FasL was determined by the ability of cells expressing FasL to induce apoptosis in the Fas$^+$ L1210 target cell line transfected to constitutively express mouse Fas (L1210.fas [45]). L1210 wild-type and Fas$^+$ L1210.fas cells were labeled for 2 h at 37°C with 5 μCi of [^{3}H]TdR per ml in RPMI 1640–10% FCS. The cells were washed twice in warm Hanks balanced salt solution and resuspended in IMDM–10% FCS. The target cells (25×10^3 per well) were added to 96-well plates containing T hybridoma cells which had been seeded into uncoated or anti-CD3-coated wells at densities yielding the final ratios of effector cells to target cells indicated in Fig. 2 and 5A. The cultures were incubated for 8 h at 37°C, and the unfragmented, high-molecular-weight DNA was extracted and counted as described above. Determinations of amounts of incorporated label from control (without effector cells) cultures of L1210 and L1210.fas target cells at the beginning and end of the assay reveal very little significant loss (plus or minus 5%) of incorporated [^{3}H]TdR label (59,922 dpm versus 62,446 dpm for L1210 cells and 55,643 dpm versus 54,936 dpm for L1210.fas cells). The data are expressed as percents DNA fragmentation, i.e., by the formula $100 \times (1 -$ cpm in experimental group/cpm in

targets alone). Each datum point represents the mean for six wells with the standard deviation.

Detection of mRNA expression. mRNAs expressed following activation were detected by reverse transcriptase PCR (RT-PCR) or RNase protection assay. Total RNA was obtained from cells by acid-guanidium thiocyanate-phenol extraction (65). Reverse transcriptase synthesis of cDNA was performed with oligo(dT) primers and a commercial kit (cDNA Cycle Kit; Invitrogen, San Diego, Calif.). The resulting cDNA-RNA hybrids were phenol-chloroform extracted, precipitated, and serially diluted for PCR amplification with *Taq* polymerase (Gibco/BRL, Grand Island, N.Y.) and the following primer pairs: glyceraldehyde-3-phosphate dehydrogenase, sense (5′ GTGAAGGTCGGAGTCAACG) and antisense (5′ TGAAGACGCCAGTGGACTC); nur77a, sense (5′ GGAAC ACCAGCAACGAGC) and antisense (5′ CATCTGGAGGCTGCTTGG); and FasL, sense (5′ TTCTCTGGAGCAGTCAGCGT) and antisense (5′ TAAGGA CCACTCCATGGACC). Alternatively, FasL mRNA expression was determined by RNase protection using RT-PCR-generated murine Fas and FasL probes and a human γ-actin which has 100% homology with the corresponding murine γ-actin sequence. For the construction of the Fas and FasL probes, RNA obtained from activated 2B4 cells was reverse transcribed and amplified with primer pairs specific for Fas (sense, 5′ GGCCGAATTCCTCGAGAAAATAAC ATCAAGGAGG, and antisense, 5′ GGCCAAGCTTGTTGATTTTCCAAGG TCCTTCTGG) and FasL (sense, 5′ GGCCGAATTCAGATGGAAGGAGGTC TGTGA, and antisense, 5′ GGCCAAGCTTAACGGCCTCTGTGAGGTAGT), producing *Eco*RI and *Hin*dIII linker ends, which were used for insertion into pGEM4Z (Promega). A similar procedure was employed to construct the control γ-actin probe (sense, 5′ TTGATGCTGCAGGTCACCAACTGGGACGAC ATG, and antisense, 5′ AACCCTAAGCTTCGCAGCTCGTTGTAGAAGG). The probes were sequenced to ensure identity with target sequences. [α-^{32}P] UTP-labeled RNA transcripts were prepared with a kit (MAXIscript; Ambion) using T7 RNA polymerase. RNase protections were performed on total RNA samples according to the kit (RPA II; Ambion) manufacturer's instructions. The samples were resolved on 8 M urea–6% polyacrylamide sequencing gels, and the gels were dried and exposed to Kodak Biomax AR film. The developed films were scanned and analyzed with a PDI (Huntington Station, N.Y.) scanner and Quantity One software.

IL-2 assay. Supernatants were assayed for IL-2 activity as described previously (16). Briefly, the supernatants were titrated into cultures of the IL-2-dependent cell line CTLL-2, which had been starved of IL-2 for 4 h prior to the addition of the IL-2-containing supernatants. The cells were cultured for 16 h and then pulsed for 6 h with 5 μCi of [^{3}H]TdR per ml. The labeled DNA was harvested, and the amount of label incorporated was determined. Units of IL-2 per milliliter were calculated by using a standard curve obtained with recombinant IL-2 and linear regression analysis.

RESULTS

Retinoid-mediated inhibition of activation-induced DNA fragmentation (apoptosis) in T-cell hybridomas. Our studies of the mechanism by which retinoic acid inhibits activation-induced apoptosis employed two T-cell hybridomas, A1.1 (51) and 2B4 (2), two cell lines which have been used extensively to examine the phenomenon of activation-induced apoptosis. To assess apoptosis, we used an assay based on the ability of the enzyme TdT to label DNA strand breaks associated with apoptosis (18). As shown in Fig. 1, 9-*cis* RA effectively inhibited activation-induced apoptosis in anti-CD3-stimulated A1.1 (from 82 to 27.1%) and 2B4 (from 77.0 to 28.6%) cells. The concentration of 9-*cis* RA required to produce a 50% inhibition of this response was 150 nM (data not shown). Similar results were obtained by other methods (data not shown), and these observations are consistent with those reported by others (68) and demonstrate that the RAR-RXR pan-agonist 9-*cis* RA is a potent modulator of activation-induced apoptosis.

Inhibition of 2B4-mediated cytotoxicity in Fas$^+$ L1210.fas cells. We have recently demonstrated that activation-induced apoptosis in T-cell hybridomas proceeds via a Fas-FasL interaction. Therefore, we envisioned three nonexclusive possibilities for the mechanism of inhibition by 9-*cis* RA: inhibition of FasL expression, inhibition of Fas expression, and/or inhibition of Fas-transduced apoptosis. These possibilities were examined functionally in the experiments whose results are shown in Fig. 2. L1210 T-cell leukemia cells normally express neither FasL nor CD3 and only barely detectable levels of Fas, and they are therefore resistant to FasL-induced cell death. L1210

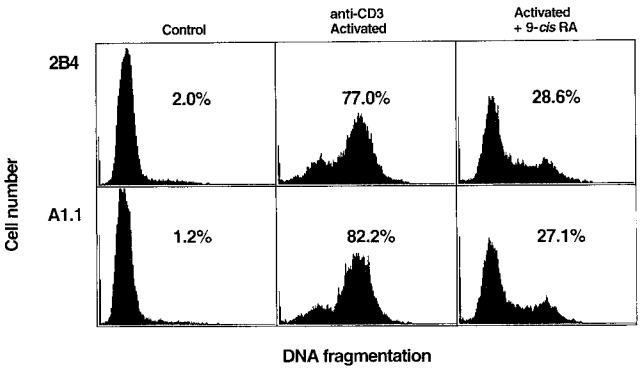

FIG. 1. 9-*cis* RA inhibits activation-induced apoptosis in T-cell hybridomas. The T-cell hybridomas 2B4 and A1.1 were activated in anti-CD3-coated six-well microtiter plates for 12 h at 37°C in the presence or absence of 1 μM 9-*cis* RA. The cells were collected, fixed in paraformaldehyde, assayed for DNA fragmentation by TdT UTP nick end labeling according to the kit manufacturer's instructions, and analyzed by flow cytometry. Cells undergoing apoptosis with accompanying DNA fragmentation incorporated label (UTP-digoxigenin-antidigoxigenin-fluorescein isothiocyanate) and can be detected by flow cytometry as a peak to the right of that for the nonapoptotic cells. The percentage of apoptotic cells (percent TdT positive) is indicated in each panel.

cells transfected with mouse fas (L1210.fas) cells express high levels of Fas constitutively and are sensitive to the induction of apoptosis either by cells expressing FasL or by anti-Fas antibody. Therefore, they can be used as target cells to detect FasL expression when mixed with 2B4 or A1.1 effector cells activated via the TCR to express FasL (and Fas). Activation of either of the T-cell hybridomas with anti-CD3 antibody resulted in apoptosis in L1210.fas target cells, as measured by DNA fragmentation (Fig. 2A). The induction of apoptosis in this well-characterized reaction is mediated by Fas-FasL interactions, since no apoptosis is seen in L1210 targets lacking Fas expression and since the apoptosis is blocked with a soluble Fas-Fc chimeric protein which acts as a competitive inhibitor of the FasL (data not shown; also reference 8). Addition of 9-*cis* RA significantly inhibited the cytotoxic activity of the T-cell hybridomas activated with anti-CD3 antibody (Fig. 2A) or with agents capable of mimicking TCR-mediated activation, i.e., phorbol ester plus ionomycin (data not shown).

Inhibition of 2B4 cytotoxic (FasL) activity by 9-*cis* RA is not mediated via a block in Fas signal transduction. The ability of retinoid to inhibit 2B4 cytotoxic (FasL) activity was clearly not due to a blockade of Fas receptor signaling per se (i.e., retinoid did not interfere with the ability of Fas to transduce a death signal once it had interacted with FasL). To make this determination, we simply delayed the addition of either L1210.fas target cells or retinoid for 6 h, thus allowing FasL to be expressed prior to retinoid treatment (8). Control cultures received retinoid only at time zero. After the delay, both retinoid and target cells were added, and the cultures were incubated for a further 6 h to allow the induction of apoptosis in the L1210.fas targets, as measured by DNA fragmentation. If retinoids inhibit activation-induced cell death via a block in Fas signal transduction, delaying the addition of retinoid should nevertheless produce comparable levels of inhibition. As shown in Fig. 2B, when 9-*cis* RA was added to cultures of 2B4 or A1.1 cells at the time of exposure to anti-CD3, the ability to induce apoptosis in L1210.fas was completely inhibited. Delaying the addition of the target cells had no effect on the outcome. More importantly, delaying the addition of retinoid completely removed the inhibitory effect normally seen when retinoid is added at time zero, suggesting that retinoids inhibit

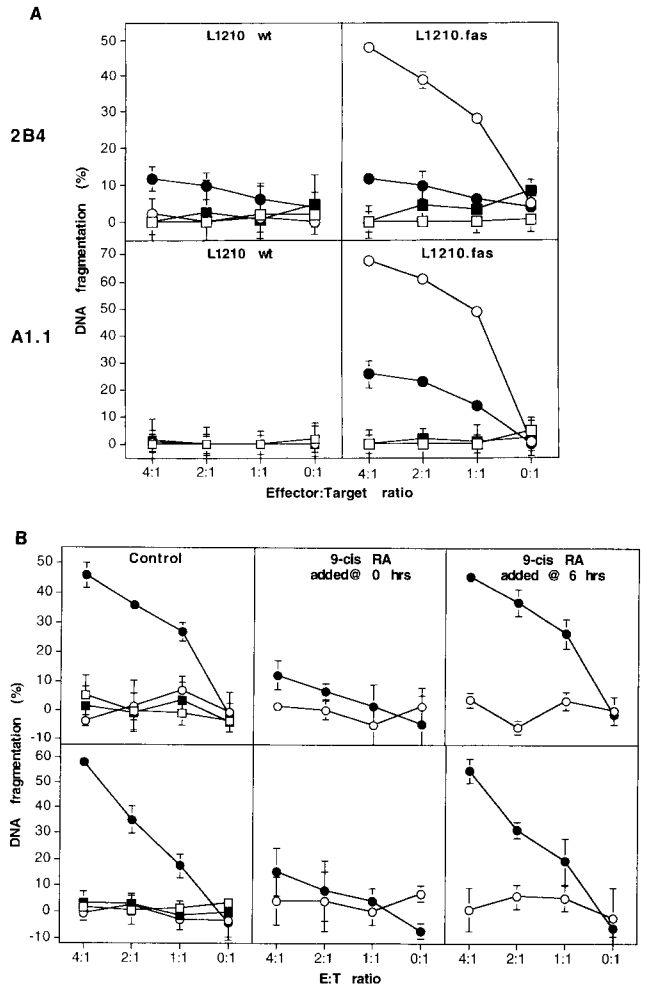

FIG. 2. 9-*cis* RA inhibits activation-induced cell death by modulating the induction of FasL and the expression of FasL functional activity (A) and not by blocking apoptotic signal transduction by the Fas antigen (B). FasL functional activity was measured as the cytotoxic activity of 2B4 and A1.1 cells against Fas^+ target cells. (A) L1210 and L1210.fas target cells were labeled with [^{3}H]TdR and then plated with either 2B4 or A1.1 cells, at the ratios indicated, into 96-well plates coated with anti-CD3 antibody. The cultures were incubated in the presence or absence of 9-*cis* RA for 10 h at 37°C and extracted, and the radioactivity retained on glass fiber mats was counted in a scintillation counter. The results shown are expressed as percents DNA fragmentation relative to control wells (absence of effector cells) and represent the means for six replicates with standard deviation (bars). Symbols: □, unstimulated effector cells (A1.1 and 2B4); ○, anti-CD3-stimulated cells; ■, unstimulated effector cells plus 1 μM 9-*cis* RA; ●, anti-CD3-stimulated effector cells plus 1 μM 9-*cis* RA. (B) 2B4 and A1.1 cells were plated at time zero into 96-well plates coated with anti-CD3 antibodies in the presence or absence of 9-*cis* RA. The L1210 and L1210.fas target cells were not added at this time. The T hybridoma cells were then cultured for 6 h to allow the expression of FasL, at which time the [^{3}H]TdR-labeled L1210 and L1210.fas target cells (as for Fig. 1) were added. A parallel set of cultures which had not received retinoid did so at +6 h. All plates were cultured for a further 6 h and extracted as described above for Fig. 1. The results shown are expressed as percents DNA fragmentation relative to control wells (absence of effector cells) and represent the means for six replicates with standard deviations (bars). Symbols: □, unstimulated effector cells (A1.1 and 2B4) mixed with L1210 cells; ○, unstimulated effector cells mixed with L1210.fas cells; ■, anti-CD3-stimulated effector cells plus L1210 cells; ●, anti-CD3-stimulated effector cells plus L1210.fas cells.

activation-induced apoptosis not via a block in Fas signal transduction but rather by some event(s) taking place immediately following activation. The data shown in Fig. 2A and B, together with the observation that 9-*cis* RA failed to block the induction of apoptosis in Fas^+ Jurkat cells by an anti-Fas antibody (data not shown), support the interpretation that 9-*cis* RA does not interfere with Fas signaling but that it appears to inhibit at least FasL expression.

Analysis of FasL mRNA following activation in the presence of 9-*cis* RA. The apparent ability of 9-*cis* RA to regulate FasL expression was examined at the RNA level. Total RNA was isolated from 2B4 cells at 4 h postactivation (we had previously observed that optimum FasL expression is seen at this time [8]) and analyzed for FasL expression by RT-PCR and RNase protection. As shown in Fig. 3A, FasL expression in 2B4 cells as measured by RT-PCR was induced as a result of anti-CD3 stimulation. This expression was inhibited by the presence of 9-*cis* RA. Similar results were obtained for RNA isolated from A1.1 cells (data not shown). Simultaneously we monitored the expression of nur77a, an orphan intracellular receptor known to be induced in T-cell hybridomas following activation (33, 64). As expected, nur77a expression was induced following activation. However, it was not altered by the addition of 9-*cis* RA to the cultures (Fig. 3A). As measured by RNase protection (Fig. 3B), FasL expression in 2B4 cells was induced more than 50-fold following anti-CD3 stimulation, and this expression was profoundly inhibited by the presence of 9-*cis* RA in both the 2B4 (to 20% of the control level) and A1.1 (similar results [data not shown]) cell lines. Because glucocorticoids have also been shown to inhibit cell death in activated T-cell hybridomas, the effect of dexamethasone on FasL mRNA expression was tested in parallel with that of 9-*cis* RA. The results (Fig. 3B) demonstrate that dexamethasone is also able to inhibit the expression of FasL following activation (to less than 4% of the control level). Finally, we confirmed that these results correlate with cell surface expression of FasL by using a chimeric Fas-Fc protein to stain cells for FasL (8). As shown in Fig. 3C, both 2B4 and A1.1 cells express significant levels of surface FasL following activation, as reported previously (8), and 9-*cis* RA inhibits this surface expression. Thus, activation-induced FasL expression in T-cell hybridomas, measured functionally and at the RNA and protein expression levels, is clearly inhibited by 9-*cis* RA. Finally, the ability of retinoids to inhibit activation-induced cell death in T-cell hybridomas appears to be specific for FasL. When the analyses described above (RT-PCR, RNase protection, and flow cytometric analyses) were performed for Fas expression, we found that retinoids had little, if any, effect (data not shown). Thus, 9-*cis* RA failed to inhibit the expression of Fas mRNA or protein in either 2B4 or A1.1 cells following activation.

The effects of retinoids are known to be mediated through two separate classes of retinoid receptors, the RARs and the RXRs. In the presence of ATRA or 9-*cis* RA, RAR-RXR heterodimeric complexes regulate gene transcription by binding to retinoic acid response elements (17). Additionally, in the presence of 9-*cis* RA, RXRs will form homodimers and activate transcription by binding to retinoid X response elements. Earlier studies showed that 9-*cis* RA induces activation of both retinoic acid response elements and retinoid X response elements and that it is a more potent inhibitor of activation-induced cell death in T-cell hybridomas and thymocytes than is ATRA (which binds RAR only). This suggested a role for the RXR-mediated pathway in this process. To further explore the involvement of the different retinoid receptor subtypes mediating this process, we examined the abilities of synthetic retinoids with receptor-selective binding and activation properties to inhibit activation-induced apoptosis and FasL expression, either alone or in combination.

Receptor selectivity of TTNPB and LG100268. Table 1 shows the comparative abilities (K_d) of the RAR-selective retinoid agonist TTNPB, the RXR-selective LG100268, and the

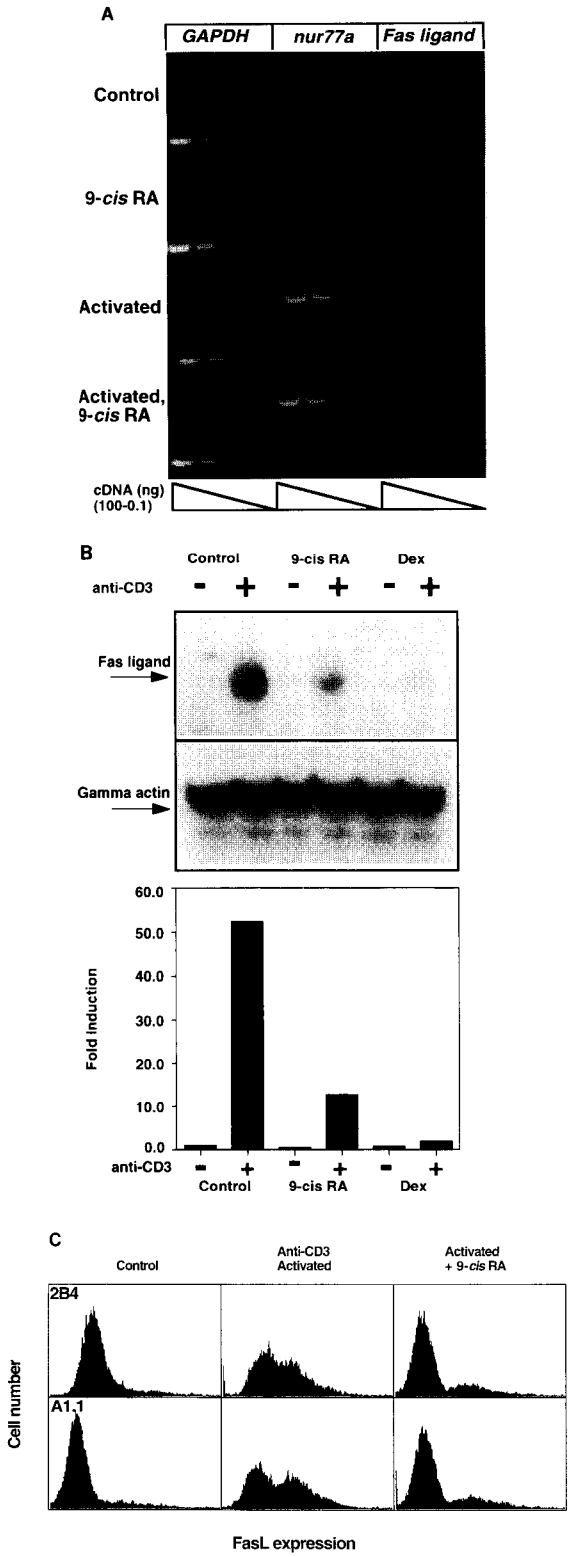

FIG. 3. 9-*cis* RA inhibits activation-induced apoptosis by blocking the expression of FasL mRNA. (A) RT-PCR analysis of total RNA obtained from 2B4 cells activated by anti-CD3 in the presence or absence of 1 μM 9-*cis* RA. RNA was reverse transcribed by using an oligo(dT) primer, and the resulting cDNA was phenol-chloroform extracted. PCR amplification was performed on 10-fold serially diluted cDNA with the primer pairs indicated. The products were electrophoresed on a 1% agarose in TAE buffer (40 mM Tris acetate [pH 8.5]–2 mM EDTA) gel, stained with ethidium bromide, and photographed. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) RNase protection analysis was performed by using [^{32}P]UTP-labeled RNA probes generated with T7 RNA polymerase and the same total RNA preparations used for the RT-PCR described above. The RNase-protected fragments were resolved on a 6% urea–polyacrylamide gel electrophoresis sequencing gel, and the resulting autoradiograph was analyzed by densitometry scanning. The data shown in the graph represent the amount of FasL mRNA expression normalized to the γ-actin in each sample and are expressed as fold induction of FasL signal relative to unactivated 2B4 controls (value of 1). Dex, dexamethasone. (C) For flow cytometric analysis of FasL protein expression, 2B4 and A1.1 cells were incubated for 6 h at 37°C on six-well plates coated with anti-CD3 in the presence or absence of 1 μM 9-*cis* RA. The cells were stained with Fas-Fc, anti-human Fc–biotin, and Streptavidin-PerCP.

TABLE 1. K_d values for receptor-selective ligands[a]

Retinoid agonist	K_d value (nM) for:					
	RAR α	RAR β	RAR γ	RXR α	RXR β	RXR γ
9-*cis* RA[b]	93	97	148	8	15	14
TTNPB[c]	20	39	51	8,113	4,093	2,566
LG100268[d]	10,000	10,000	10,000	3	3	3

[a] Ligand receptor binding (K_d) was determined for each ligand-receptor combination in a competitive binding assay using ^{3}H-labeled 9-*cis* RA and baculovirus-expressed retinoid receptor. Each value represents the mean for three independent determinations performed in triplicate. When the K_d value is >10,000, the default value is 10,000.
[b] Pan-agonist.
[c] RAR selective.
[d] RXR selective.

RAR-RXR pan-agonist 9-*cis* RA to bind the different baculovirus-expressed retinoid receptors. The structures of these compounds are shown in Fig. 4A. Using a competitive binding assay, we have determined that LG100268 binds strongly to RXR α, β, and γ but not to RAR α, β, and γ. Conversely, TTNPB binds with high affinity to RAR α, β, and γ but not at all to RXR α, β, and γ. The pan-agonist 9-*cis* RA binds with high affinity to both RARs and RXRs in this assay. We also determined the abilities of the synthetic receptor-selective retinoids to activate RARs and RXRs to mediate retinoid-dependent transcription by employing a cotransfection assay with either RAR γ or RXR α and the appropriate reporter constructs. As shown in Fig. 4B, the RAR-selective retinoid TTNPB effectively activated RAR-dependent transcription but not RXR-dependent transcriptional responses. Similarly, the RXR-selective LG100268 activated only an RXR-dependent response. 9-*cis* RA, which binds both RARs and RXRs, predictably activated both retinoid-dependent transcriptional responses. In the experiment whose results are shown, expression vectors for RAR γ and RXR α were employed; however, similar results were obtained for the other RAR and RXR subtypes (data not shown), and the data obtained for all receptor subtypes correlate well with the binding data shown in Table 1.

Inhibition of activation-induced apoptosis and functional FasL activity by receptor-selective retinoids. When we examined the abilities of the receptor-selective retinoids to inhibit either activation-induced apoptosis (Fig. 5A) or functional FasL activity (Fig. 5B) in 2B4 cells, the RXR-selective LG100268 was found to be significantly less potent than 9-*cis* RA. Similarly, the RAR-selective TTNPB was much less effective than the pan-agonist 9-*cis* RA. Since RARs and RXRs can form functional heterodimers which can be activated by either TTNPB or 9-*cis* RA and since TTNPB alone was insufficient, these data suggested that the engagement of both RAR-RXR heterodimer and RXR-RXR homodimer receptor pathways was important in mediating the inhibition. To specifically ad-

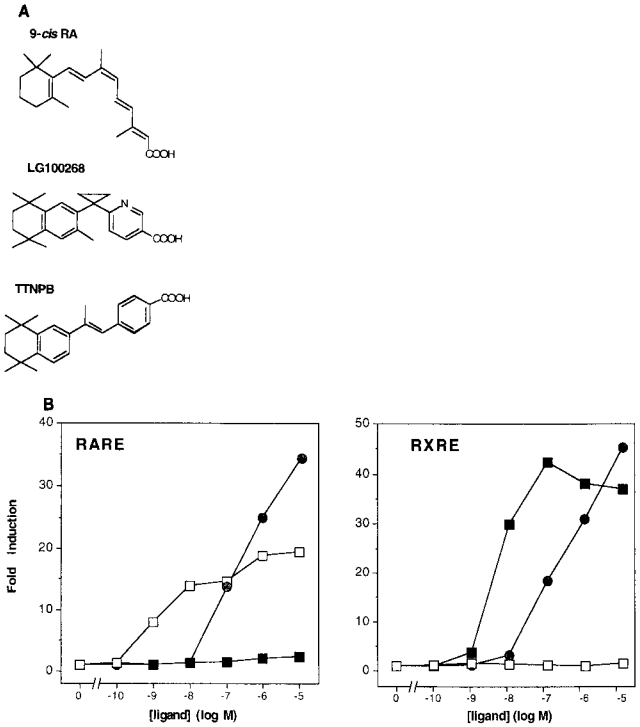

FIG. 4. (A) Structures of retinoid agonists. (B) Receptor activation properties of RAR-selective (TTNPB) and RXR-selective (LG100268) retinoids, as well as 9-*cis* RA. Ligand was added to CV-1 cells transiently cotransfected with receptor expression vectors (RAR or RXR), together with either of the two reporter constructs containing response elements for RAR (DR5-tk-Luc) or RXR (DR1-tk-Luc). Transfection efficiency was monitored by cotransfection with a β-galactosidase expression vector. The data are expressed as fold induction relative to controls without ligand, and they have been normalized relative to the β-galactosidase signal. Symbols: □, TTNPB; ■, LG100268; ◎, 9-*cis* RA.

dress this issue, the two receptor-selective ligands were added together. As shown in Fig. 5A and B, the combination of the RAR- and RXR-selective retinoids was as effective as 9-*cis* RA, inhibiting both anti-CD3-induced apoptosis and FasL functional activity in 2B4 cells. In the mixing experiments described above, 1 μM TTPNB was added with 1 μM LG100268 for a final retinoid concentration in the cultures of 2 μM. To unequivocally demonstrate the requirement in this system for the presence of both RAR and RXR ligand binding, we performed a series of ligand titrations. As shown in Fig. 5C, the addition of progressively higher (10-fold) concentrations of either TTNPB or LG100268 alone produced only minor decreases in DNA fragmentation. Adding both ligands together, however, resulted in an inhibition of DNA fragmentation equal to or greater than that produced by the pan-agonist 9-*cis* RA. These data demonstrate clearly that both RAR- and RXR-selective ligands must be present to effectively block activation-induced cell death in 2B4 cells.

Inhibition of FasL expression correlates with decreased FasL mRNA and requires the presence of both RAR- and RXR-selective ligands. The data shown in Fig. 5 suggest that both receptor pathways must be engaged in order for inhibition of FasL function to be effective. RNase protection was performed to determine the relative levels of both FasL mRNA and Fas mRNA, the latter of which does not appear to be regulated by retinoids. As for Fig. 3, the data shown are normalized relative to a γ-actin internal control. In this experiment, the A1.1 T-cell hybridoma was used as a source of RNA.

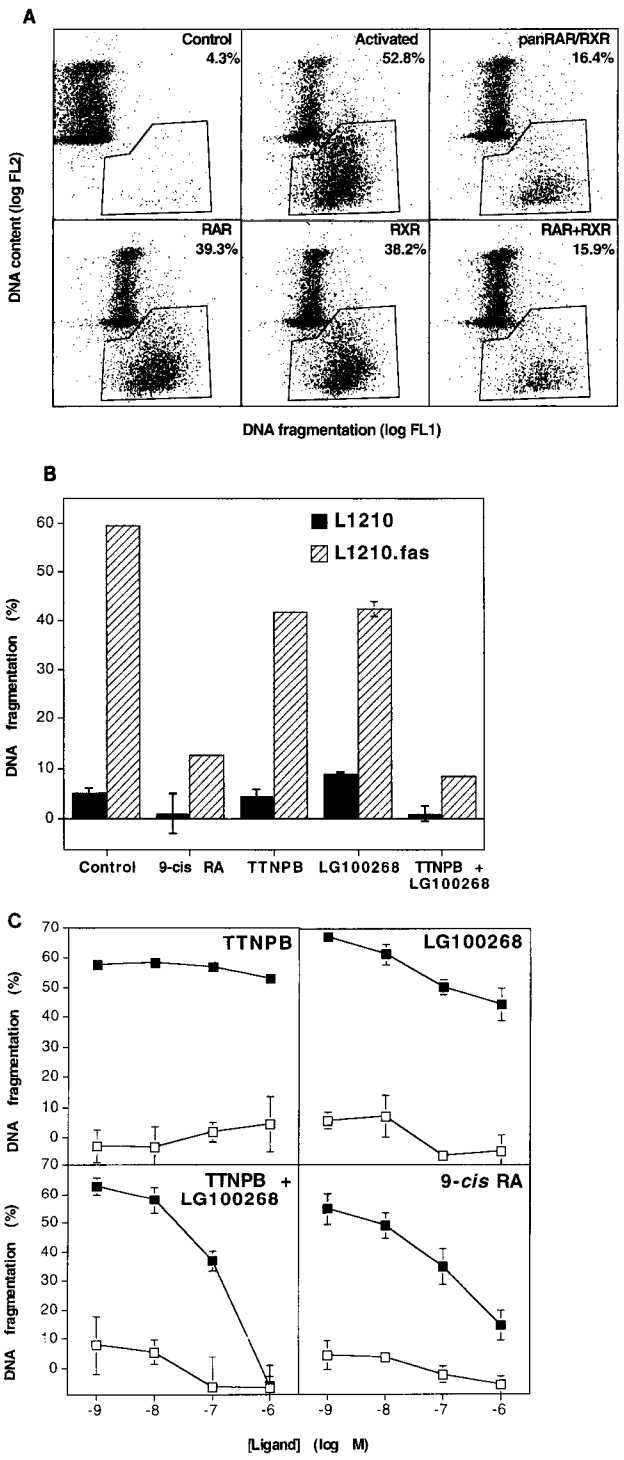

FIG. 5. Retinoid-mediated inhibition of activation-induced apoptosis (A and C) and killing of Fas$^+$ target cells (B) require the ligand-binding-mediated activation of both RAR and RXR pathways. (A) 2B4 cells were cultured in anti-CD3-coated six-well microtiter plates in the presence or absence of the indicated compounds. After 12 h the cells were collected and assayed for DNA fragmentation by TdT UTP nick end labeling with propidium iodide counterstaining. Plotted as DNA content on the *y* axis and TdT positive on the *x* axis, apoptotic cells appear low and to the right (TdT positive and hypodiploid). The numbers shown represent the percentage of apoptotic cells in each sample for the region marker shown in the dot plot. (B) [^{3}H]TdR-labeled L1210 and L1210.fas target cells were exposed to anti-CD3-activated 2B4 and A1.1 cells at the ratios indicated in the text in the presence or absence of 1 μM ligand. (C) The indicated receptor-selective ligands were titrated into cultures of [^{3}H]TdR-labeled 2B4 cells exposed to anti-CD3 antibodies. Symbols: □, control; ■, activated.

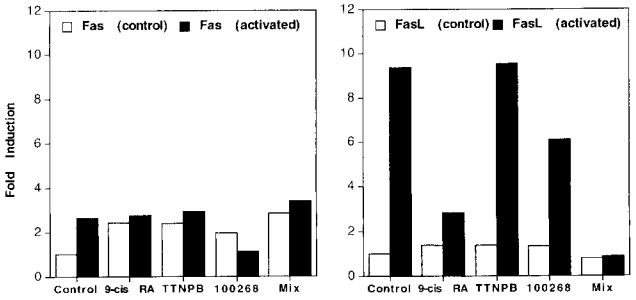

FIG. 6. Expression of Fas and FasL mRNA in activated T-cell hybridomas in the presence of receptor-selective retinoids. Fas and FasL mRNAs were quantitated by RNase protection as described in the legend to Fig. 3. Included in the experiment was a γ-actin internal control. A1.1 cells were activated for 2 h in six-well plates coated with anti-CD3, and the RNA was extracted for RNase protection. Retinoids were added at 1 μM each. The data shown represent the amount of FasL mRNA expression normalized to the γ-actin in each sample and are expressed as fold induction of Fas or FasL signal relative to unactivated controls (value of 1).

Unlike 2B4 cells, which constitutively express both Fas mRNA and protein and show little change after anti-CD3 stimulation, A1.1 cells show a modest increase in both following activation. As shown in Fig. 6, there was a minor (twofold) increase in Fas mRNA levels of activated cells compared with those of unstimulated controls. By immunofluorescent antibody staining, A1.1 cells were found to increase Fas expression to a similarly small degree, and this is consistent with previously published results. As determined by flow cytometry, the expression of Fas receptor protein was unaffected by the addition of retinoids (data not shown). In contrast, activation resulted in a clear increase in FasL mRNA levels compared with unstimulated controls (more than ninefold), which was almost completely inhibited by 9-*cis* RA. Moreover, the data shown in Fig. 6 correlate well with the results of the direct (Fig. 3A and C) and functional (Fig. 3B) assays and demonstrate that maximum inhibition of FasL expression requires the presence of either the pan-agonist 9-*cis* RA or, alternatively, both RAR-selective (TTNPB) and RXR-selective (LG100268) ligands.

Retinoid-mediated inhibition of FasL activity and activation-induced cell death does not involve a block in TCR-mediated activation as measured by the production of IL-2. In addition to undergoing G_1/S cell cycle arrest and apoptosis, T-cell hybridomas activated via the TCR produce IL-2 (2, 36, 37, 59), and the production of this lymphokine is often used as an indicator that activation has indeed taken place (4, 50). Previous reports have suggested that while retinoids block activation-induced apoptosis, they do not interfere with the corresponding cell cycle arrest or production of IL-2 (68). As part of our examination of the mechanism of retinoid-mediated inhibition of this process, we determined IL-2 production by 2B4 cells activated in the presence of the receptor-selective retinoids, as well as the pan-agonist 9-*cis* RA. As shown in Fig. 7, the presence of the retinoids had little, if any, effect, only partially inhibiting IL-2 production, if at all. This is consistent with the notion that retinoids are not globally immunosuppressive, having only minor inhibitory effects on various measures of T-cell activation.

DISCUSSION

Activation-induced apoptosis in T-cell hybridomas is a useful model for the analysis of how a stimulatory signal can trigger the process of active cell death. This occurs through the induced expression of a receptor-ligand pair, Fas and FasL, which then interact to transduce an apoptotic signal (8, 12, 29). Because the process requires de novo synthesis of FasL (and often Fas), the system can be regulated by agents that affect the expression of this protein. Here we have shown that 9-*cis* RA, which is known to block the process of activation-induced apoptosis in T hybridomas (68), does so by the selective inhibition of FasL expression following activation of the cell. This was demonstrated by functional assay, by analysis of FasL mRNA expression using RT-PCR and RNase protection assays, and by detection of the cell surface FasL protein. In contrast, retinoids appear to have no significant effect on the expression of Fas.

The observation that 9-*cis* RA is considerably more potent than ATRA suggested that mechanistically RXR is intimately involved in this process (68). Since 9-*cis* RA binds to members of both the RAR and RXR subfamilies, whereas ATRA binds only to members of the RAR subfamily (1), and since any inhibition by ATRA observed might easily be accounted for by its isomerization to 9-*cis* RA, at least two possible scenarios can be considered. In the first possibility, ligand activation of the RXRs (most probably through an RXR-RXR homodimer) is the critical component in the inhibition of activation-induced cell death (AICD). A second possibility is that bifunctional activation of both the RARs and RXRs (most probably through an RAR-RXR heterodimer) is required to modulate AICD. The design and synthesis of receptor-selective compounds (7) allowed us to specifically address these points, and we have demonstrated that ligand activation of both the RAR and RXR pathways is required to achieve a maximal response. This conclusion is further supported by the demonstration that the addition of both an RAR-selective compound and an RXR-selective compound simultaneously can mimic the addition of the pan-agonist 9-*cis* RA alone. While this paper was in review, another paper demonstrating that the induction of apoptosis in T-cell hybridomas following activation is mediated by the Fas-Fas ligand interaction and that 9-*cis* RA blocks

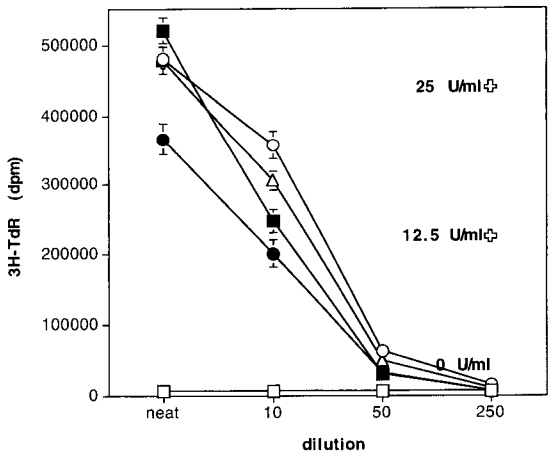

FIG. 7. IL-2 production by 2B4 cells activated in the presence of retinoids. 2B4 cells were cultured for 16 h in 96-well plates coated with anti-CD3 antibody, with the indicated retinoids added at 1 μM. The culture supernatants were collected and centrifuged, and IL-2 unit concentrations were determined by using the IL-2-dependent cell line CTLL-2 and a recombinant IL-2 standard. The CTLL-2 cells were cultured with the supernatants for 16 h and pulsed for 6 h with 5 μCi of [^{3}H]TdR per ml. Units of IL-2 per milliliter were calculated by using a standard curve and linear regression analysis. The data shown represents the means for six determinations with standard deviations. Symbols: □, unstimulated controls (0 U/ml); ■, anti-CD3 activated (138 U/ml); ○, activated plus TTNPB (203 U/ml); ●, activated plus LG100268 (112 U/ml); △, activated plus 9-*cis* RA (160 U/ml); ✣, recombinant IL-2 standard.

activation-induced apoptosis in T hybridoma cells via inhibition of Fas ligand induction was published (67). In addition to these observations, the present study identifies and demonstrates the requirement for both the RAR and RXR pathways for retinoid-mediated inhibition of Fas ligand and activation-induced apoptosis.

Mechanistically, the RARs and RXRs regulate transcription by binding to response elements in target genes that generally consist of two direct repeats of the consensus sequence half-site ATTGCA (61). The binding to DNA can be in the form of (i) an RAR-RXR heterodimer which activates transcription in response to ligand by binding to direct repeats spaced by 5 bp (DR5 elements) such that RAR occupies the downstream half-site or (ii) an RXR homodimer which activates transcription in response to ligand by binding to direct repeats spaced by 1 bp (DR1 elements). In addition, RXR is capable of forming heterodimers with many other intracellular receptors, including the vitamin D receptor, thyroid hormone receptor, the peroxisome proliferation-activating receptor, and nur77 (reviewed in reference 34). Our data suggest that the ligand activation of the RAR-RXR heterodimer is functionally critical to the inhibition of FasL expression and blockade of AICD. Thus, either both DR5 and DR1 elements regulate FasL expression or other RXR heterodimers play additional roles in FasL modulation. With one recent exception (42), the observation that retinoid receptors negatively regulate gene expression is both novel and unusual.

Although expression of FasL is induced upon activation and inhibited by 9-*cis* RA at the mRNA, protein, and functional levels, we have not proven that this occurs at the level of transcription. Analysis of the published promoter regions of Fas or FasL does not reveal the presence of any obvious retinoid receptor response elements, and thus it provides no immediate explanation or mechanism. However, since this process requires the de novo synthesis of Fas ligand mRNA and protein, neither of which is found in unstimulated cells, our data showing a specific decrease in the number of Fas ligand transcripts in 9-*cis* RA-treated cells relative to that in untreated, activated controls imply transcriptional regulation. It is tantalizing to speculate that 9-*cis* RA, either directly or indirectly through intermediates, regulates the transcription of FasL (versus, for example, the half-life of the mRNA). This possibility is made more intriguing by the observation that the functions of at least two classes of transcription factors, Myc-Max (4) and nur77a (33, 64), are required for activation-induced apoptosis in T-cell hybridomas. nur77 is induced immediately upon activation of T cells (33, 64), and retinoids do not interfere with this response (Fig. 3A). The very recent observation that nur77 heterodimerizes and may be regulated by RXRs (14, 43) raises the possibility that FasL expression is regulated in part by the interaction between nur77 and RXR, which may account for a clear requirement for the participation of the RXR pathway.

Studies with dexamethasone have demonstrated that corticosteroids can block AICD and that this block is accompanied by inhibition of FasL expression. This is in contrast to the observation that dexamethasone can by itself induce programmed cell death in T-cell hybridomas and cause involution of the thymus, whereas 9-*cis* RA does not (68). The negative regulation of FasL expression and the inhibition of AICD by both glucocorticoids and retinoids are intriguing in that ligand activation of the cognate receptors of both of these substances has been shown to inhibit AP-1 transcription (48). Ligand-receptor interaction is required to antagonize promoter gene expression, including that of the collagenase and stromelysin promoters (49). Although the glucocorticoid receptor (GR) and the RARs and RXRs almost certainly modulate different target genes, the convergence through the AP-1 pathway might be sufficient to explain their common inhibitory mechanisms. However, it has been reported that 9-*cis* RA does not profoundly inhibit the production of IL-2 following activation (68), which is regulated in part by AP-1. Moreover, our data reveal only small differences in IL-2 expression by activated cells, regardless of which retinoid is added. This includes TTNPB, which is a potent repressor of AP-1 activity, and suggests that IL-2 transcription and FasL transcription are differentially regulated.

Activation-induced apoptosis in T-cell hybridomas can similarly be inhibited by cyclosporine (37, 51) and FK506 (3, 56). We have recently observed that both of these agents inhibit activation-induced expression of FasL (9). Interestingly, like 9-*cis* RA, they do not appear to inhibit the expression of Fas in response to T-cell activation, suggesting that the transcriptional regulation of Fas and that of FasL are significantly different. Activation of T-cell hybridomas is also associated with a cell cycle block, and this block is not affected by 9-*cis* RA (data not shown; also reference 68), cyclosporine (37), or competitive inhibition of Fas-FasL interaction (29). Therefore, this cell cycle block represents yet another level of regulation following activation in these cells.

Activation-induced apoptosis in normal T lymphocytes is known to occur under at least two conditions in vivo. Stimulation of immature thymocytes via the TCR results in apoptosis, and this is likely to be an important mechanism of negative selection during T-cell development (28, 55). Although activation-induced apoptosis in thymocytes is inhibited by both 9-*cis* RA (68) and dexamethasone (26, 69), evidence from mice with defective Fas expression suggests that Fas-FasL interactions are not specifically responsible for these physiological processes (53, 54). However, another form of activation-induced apoptosis occurs in mature T cells in vivo, following expansion of antigen-specific T cells, and this homeostatic process has been called peripheral deletion (47). It appears to be dependent upon normal Fas expression (40, 46). On the basis of the results reported here, it will be interesting to determine whether this process is regulated by retinoic acid or glucocorticoids.

Activation-induced apoptosis in T cells also occurs under pathological conditions, and it has been implicated as a mechanism for the loss of $CD4^+$ and $CD8^+$ T cells in AIDS (19, 38, 58). 9-*cis* RA appears to regulate the levels of both spontaneous and activation-induced apoptosis observed in cultures of T cells from AIDS patients (66). On the basis of reports that there is increased Fas antigen expression in murine retrovirus-induced immunodeficiency syndrome (62) and that there are higher than normal levels of expression of Fas on T lymphocytes from human immunodeficiency virus type 1-infected children (11), together with the data presented here, it is tempting to speculate that the ability of 9-*cis* RA to regulate the levels of apoptosis in T cells from AIDS patients occurs as a consequence of regulation of FasL expression.

In addition, there are several nonlymphoid pathological conditions in which Fas, and possibly FasL, has been implicated (39, 57). One example is the reported disappearance of hepatocytes during chronic hepatitis C virus infections (31). Fas is expressed in normal human and mouse liver tissue (22, 41), and the injection of anti-Fas antibody results in severe apoptosis in the liver (41). Furthermore, Fas has been detected immunohistochemically in chronic hepatitis patients on hepatocytes near infiltrating lymphocytes (22). FasL expressed on these infiltrating T cells (39) may be responsible for the induc-

tion of apoptosis in hepatocytes in this model and may therefore be another target for regulation by 9-*cis* RA.

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