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Induction of Apoptosis by Retinoids and Retinoic Acid Receptor γ-Selective Compounds in Mouse Thymocytes through a Novel Apoptosis Pathway

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SUMMARY

Retinoic acids are morphogenic signaling molecules that are derived from vitamin A and involved in a variety of tissue functions. Two groups of their nuclear receptors have been identified: retinoic acid receptors (RARs) and retinoic acid X receptors (RXRs). All-*trans* retinoic acid is the high affinity ligand for RARs, and 9-*cis* retinoic acid also binds to RXRs with high affinity. In cells at high concentrations, all-*trans* retinoic acid can be converted to 9-*cis* retinoic acid via unknown mechanisms. It was previously shown that retinoic acids prevents activation-induced death of thymocytes. Here, we report that both all-*trans* and 9-*cis* retinoic acid induce apoptosis of mouse thymocytes and purified $CD4^+CD8^+$ cells in *ex vivo* cultures, with 9-*cis* retinoic acid being 50 times more effective. The induction of apoptosis by retinoic acids is mediated by RARγ because (a) the phenomenon can be reproduced only by RARγ-selective retinoic acid analogs, (b) the cell death induced by either retinoic acids or RARγ analogs can be inhibited by RARγ-specific antagonists, and (c) $CD4^+CD8^+$ thymocytes express RARγ. *In vivo* administration of an RARγ analog resulted in thymus involution with the concomitant activation of the apoptosis-related endonuclease and induction of tissue transglutaminase. The RARγ pathway of apoptosis is RNA and protein synthesis dependent, affects the $CD4^+CD8^+$ double positive thymocytes, and can be inhibited by the addition of either Ca^{2+} chelators or protease inhibitors. Using various RAR- and RXR-specific analogs and antagonists, it was demonstrated that stimulation of RARα inhibits the RARγ-specific death pathway (which explains the lack of apoptosis stimulatory effects of all-*trans* retinoic acid at physiological concentrations) and that costimulation of the RXR receptors (in the case of 9-*cis* retinoic acid) can neutralize this inhibitory effect. It is suggested that formation of 9-*cis* retinoic acid may be a critical element in regulating both the positive selection and the "default cell death pathway" of thymocytes.

The cell-autonomous process of apoptosis was originally defined by morphological criteria: cellular shrinkage, chromosome condensation, membrane blebbing, and chromatin fragmentation (1). The intense genetic, biochemical, and cellular studies of recent years have revealed that there are distinct molecular pathways of apoptosis in different cells and even in one cell type. For understanding and utilization of the molecular mechanisms of apoptosis for therapeutic purposes, it is ultimately important to move toward biochemical identification of the various pathways involved, preferably using cells that can undergo apoptosis via distinct molecular pathways. The thymus and *ex vivo* culture of freshly isolated thymocytes provide an excellent model for such studies.

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T cells differentiate into mature T lymphocytes within the thymus. During this differentiation process, T cells proliferate, generate their TCR, and in the $CD4^+CD8^+$ double positive stage of differentiation become selected. The cells that express potentially autoreactive TCR undergo apoptosis (negative selection) after interaction with the APCs (2). Cells that express functionally acceptable TCR can recognize and interact with self-MHC. After interacting with the APC, they become positively selected, escape the cell death pathway, and differentiate into mature single positive thymocytes (3). However, the majority of T cells express functionally unacceptable TCR, they cannot interact with the APC, and will enter the apoptotic program, which is accelerated when cells are exposed to high levels of glucocorticoids (4) or treatments inducing DNA breaks (5). The apoptotic program induced in each of these cases is morphologically indistinguishable, dependent on *de novo* gene expression, and involves the activa-

ABBREVIATIONS: TCR, T cell receptor; RAR, retinoic acid receptor; RXR, retinoic acid X receptor; APC, antigen presenting cells; FCS, fetal calf serum; FITC, fluorescein isothyocyanate; PBS, phosphate-buffered saline; PE, phycoerythrine; RA, retinoic acid; RT, reverse transcription; PCR, polymerase chain reaction.

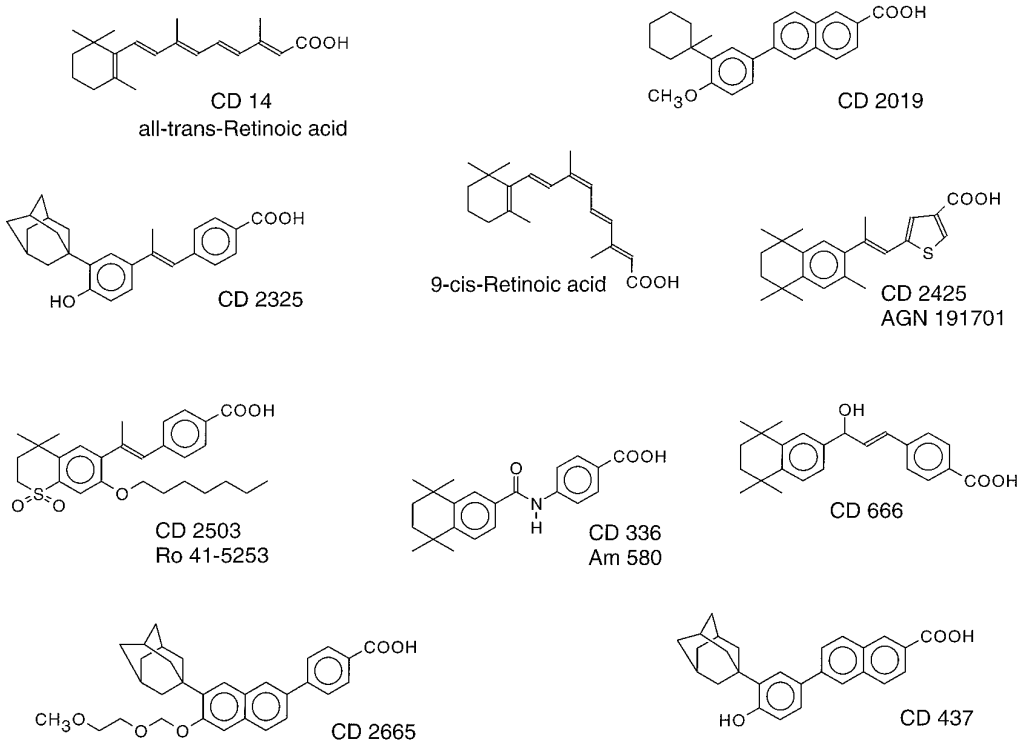

Fig. 1. Chemical structure of retinoids used in the study.

tion of both a Ca^{2+}/Mg^{2+}-dependent endonuclease (6) and a specific protease(s) (7) and the induction of tissue transglutaminase (8).

Recent results show, however, that the induction of apoptosis by these treatments works via distinct signal transduction pathways: TCR and CD3 stimulations induce changes in second messenger systems, such as calcium (9); glucocorticoids bind to cytoplasmic steroid receptors that translocate to the nucleus, and topoisomerase II inhibition by etoposide or irradiation causes direct DNA damage. Each of these pathways seems to induce distinct sets of genes (10). The transcripts RP-2 and RP-8 are expressed in thymocytes after treatment with glucocorticoids. The immediate early gene *nur 77* is induced in response to TCR signals but not by glucocorticoids or ionizing radiation. Antisense inhibition of *nur 77* expression prevents apoptosis in TCR-stimulated cells but not if the death was induced by other stimuli. DNA damage, on the other hand, leads to *p53* induction, and thymocytes lacking *p53* are resistant to the lethal effects of ionizing radiation or etoposide but not to the other treatments. In addition to these forms of apoptosis, which depend on *de novo* gene expression, apoptosis of thymocytes occurs via *Fas* receptor stimulation (11). This type of apoptosis is sensitive to protease inhibitors (7) but not to protein synthesis inhibitors (11) and involves the activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease (11) but does not involve the induction of tissue transglutaminase (8).

It has been reported that all-*trans* and 9-*cis* RAs differentially modulate various forms of thymocyte apoptosis (12–14). All-*trans* and 9-*cis* RAs are vitamin A derivatives formed within most cells. Both are physiological ligands for RARs and RXRs, which belong to the steroid/thyroid/retinoid nuclear receptor family (15). These receptors are ligand-dependent transcription factors that bind to specific hormone response element and transactivate specific target genes. All-*trans* and 9-*cis* RAs are equipotent in activating RAR, whereas activation of RXR by all-*trans* RA is 50-fold less than that by 9-*cis* RA (16). At high concentrations, some of the all-*trans* RA may be converted to 9-*cis* RA within the cells by unknown mechanisms. RARs function in the form of RAR/RXR heterodimers in the presence of RAs (17). In addition, RXR can form heterodimers with various members of the steroid/thyroid/retinoid receptor family (e.g., thyroid receptor, vitamin D_3 receptor, COUP-TF) (18, 19). The presence of RXR in most of the heterodimers is needed to enhance the cooperative binding of these receptors to the DNA; the activation requires only the presence of the cognate vitamin D_3 receptor, thyroid receptor, or RAR ligands but can be modulated by the simultaneous binding of the RXR ligand (20). These complex interactions and the existence of multiple nuclear RARs (RARα, RARβ, and RARγ) as well as RXRs (RXRα, RXRβ, and RXRγ), differentially expressed in various tissues and cell types, account for the pleiotropic effects of retinoids in practically all type of cells.

RARα and RARγ are expressed in the thymus, both maturing thymocytes and thymic stromal cells (21). The most dramatic effect of retinoids on apoptosis of thymocytes observed so far is that RAs inhibit TCR/CD3-mediated (activation-induced) apoptosis; 9-*cis*-RA is 10-fold more potent than all-*trans* RA, suggesting that RXRs participate in this process (12–14). RAs enhanced the effects of glucocorticoids to induce apoptosis (13, 14), and it was also observed that RAs alone can induce a significant rate of thymocyte cell death (14). Further analysis of this latter effect has led to the results presented here.

Materials and Methods

Chemicals. All retinoid compounds (Fig. 1) used in the current study were synthesized at CIRD Galderma. Their chemical names

are CD14 (all-*trans* RA), CD336 (Am580) (22) [4-((5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido)benzoic acid], CD437 (23) [6-(3-(1-adamanthyl)-4-hydroxyphenyl)-2-naphthoic acid], CD666 (24) [(E)-4-(1-hydroxy-1-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl -2-naphthyl)-2-propenyl)benzoic acid], CD2019 (22) [6-(3-(1-methylcyclohexyl)-4-methoxyphenyl)-2-naphthoic acid], CD2325 (25) [4-((*E*)-2- (3-(1-adamanthyl)-4-hydroxyphenyl)propenyl)benzoic acid], CD2425 (AGN 191701) (25) [(E)-5-(2-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalen-2-yl)propen-1-yl)-3-thiophene carboxylic acid], CD2503 (Ro 41–5253) (26) [4-((*E*)-2-(3,4-dihydro-4,4-dimethyl-1,1-dioxid-2*H*-benzothiopyran-6-yl)propenyl)benzoic acid)], and CD2665 [4-(6-methoxyethoxymethoxy-7-adamanthyl-2-naphthyl)benzoic acid].[1] Anti-CD3 and anti-*fas* monoclonal antibody were purchased from PharMingen (San Diego, CA). Anti-transglutaminase monoclonal antibody was a gift from Dr. Paul Birckbichler (Oklahoma Medical Research Fund, Oklahoma City, OK). Horseradish peroxidase-labeled goat anti-mouse IgG, FITC-conjugated anti-CD8, PE-conjugated anti-CD4 antibodies, dexamethasone-21-acetate, etoposide, and *N*,*N*′-dimethyl-casein were from Sigma Chemical (St. Louis, MO). Luminol and bovine serum albumin were from Reanal (Budapest, Hungary). Polyvinylidene difluoride membrane was from Millipore (Bedford, MA). [^{3}H]Putrescine (26 Ci/mmol) was purchased from Amersham International (Buckinghamshire, UK). All other reagents were of analytical grade and obtained from commercial sources.

Binding studies. Equilibrium dissociation constants (K_d values) for the interaction of the different retinoids with the three RAR subtypes were determined by competition binding experiments using [^{3}H]CD367 as radiolabeled reference retinoid. This compound has been characterized recently (27); it binds with high affinity to RARα, RARβ, and RARγ (K_d = 3.7, 4.1, and 1.5 nM, respectively) but does not transactivate RXR.[2] The assays were performed as previously described (27) using nuclear extracts of COS-7 cells transfected with pSG-5-derived expression vectors for RARα (28), RARβ (29), or RARγ (provided by Dr. M. Pfahl, La Jolla Cancer Research Foundation, La Jolla, CA).

Transactivation assay. Because no radiolabeled RXR-specific ligand for binding studies was available, interaction of the retinoids with this receptor type was assessed by a functional transactivation assay as previously described (30). Briefly, HeLa cells were cotransfected with an expression vector for RXRα (provided by Dr. M. Pfahl) and with a CRABP[½]-tk-CAT reporter plasmid (provided by C. Gerst, CIRD Galderma). Cells were grown for 24 hr in the presence of various retinoids. CAT activity was determined in lysates and expressed as percentage of maximum induction after background CAT activity had been subtracted. The retinoid concentration giving half-maximum activation (AC_{50} value) was calculated by nonlinear regression analysis.

Transactivation ability of the RARα-, RARβ-, and RARγ-selective compounds was tested as described previously (24).

Experimental animals. Four-week-old male NMRI mice purchased from LATI (Gödöllô, Hungary) were used. For the induction of thymic apoptosis, mice received 0.5 mg dexamethasone acetate intraperitoneally or 0.5 mg of CD437 intraperitoneally dissolved in a mixture of 0.1 ml of ethanol and 0.4 ml of physiological saline. Control animals were injected with the same amount of vehicle.

Thymocyte preparation. Thymocyte suspensions were prepared from the thymus glands of 4-week-old male NMRI mice by mincing the glands in RPMI 1640 media (Sigma Chemical) supplemented with 10% FCS (GIBCO, Grand Island, NY), 2 mM glutamine, and 100 IU of penicillin/100 μg of streptomycin/ml. Thymocytes were washed three times and diluted to a final concentration of 10^7 cells/ml before incubation at 37° in a humidified incubator under an atmosphere of 5% CO_2/95% air. Cell death was measured by trypan blue uptake. A total of 95–98% of cells routinely excluded trypan blue after the isolation procedures.

Separation of the $CD4^+CD8^+$ thymocyte subpopulation was carried out with the MACS multiparameter magnetic cell-sorting system. Thymocyte suspension in PBS containing 0.5% bovine serum albumin was passed through 30-μm nylon mesh to remove clumps, stained with FITC-labeled anti-CD8 antibody, and then incubated with MACS MultiSort anti-FITC microbeads. $CD8^+$ and $CD4^+CD8^+$ cells were then positively selected on a magnetic column. After selection, microbeads were removed by MACS MultiSort reagent, and cells were further labeled with MACS L3T4 (anti-CD4) microbeads. $CD4^+CD8^+$ thymocytes were then positively selected on a second magnetic column. The population of $CD4^+CD8^+$ thymocytes was 97.5–98.5% in the separated cell fraction.

Determination and characterization of DNA fragmentation. Thymocytes were incubated in 24-well plates in the presence of various agents. After 6 hr, 0.8 ml of cell suspensions was lysed by the addition of 0.7 ml of ice-cold lysis buffer containing 0.5% (v/v) Triton X-100, 10 mM Tris, and 20 mM EDTA, pH 8.0, before centrifugation for 15 min at 13,000 × *g*. DNA contents in supernatant (DNA fragments) and pellets (intact chromatin) were prepared for determination of DNA fragmentation by diphenylamine reagent and for DNA agarose electrophoresis as described previously (31). Because in the experiments carried out with the separated $CD4^+CD8^+$ thymocytes the number of cells were not available for detection of DNA by diphenylamine, a rapid hypotonic technique using propidium iodide DNA staining was applied. For the staining, cells were washed and redissolved in distilled water containing 50 μg/ml propidium iodide, 1% sodium citrate, and 1% Triton X-100. With this technique, the percentage of cells carrying decreased amount of DNA due to apoptosis (gate R2) can be detected on DNA histograms by flow cytofluorometry. The degree of fragmentation correlated well with the number of trypan blue-positive dead cells throughout the experiments.

Tissue transglutaminase activity. Thymus was collected from control or treated animals at various time points after treatment, extensively washed with PBS, and homogenized in 0.1 M Tris·HCl, pH 7.5, containing 0.25 M sucrose, 0.5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. Transglutaminase activity was measured by detecting the incorporation of [^{3}H]putrescine into *N*,*N*′-dimethylcasein. The incubation mixture contained 150 mM Tris·HCl buffer, pH 8.3, 5 mM $CaCl_2$, 10 mM dithiothreitol, 30 mM NaCl, 2.5 mg *N*,*N*′-dimethylcasein/ml, and 0.2 mM putrescine, containing 1 mCi of [^{3}H]putrescine and 0.1 mg of protein in a final volume of 0.3 ml. After 30 min of incubation, the mixture was spotted onto Whatman 3 MM filter paper moistened with 20% trichloroacetic acid. Free [^{3}H]putrescine was eliminated by washing with large volumes of cold 5% trichloroacetic acid containing 0.2 M KCl before counting. Activity was calculated as nmol of [^{3}H]putrescine incorporated into protein/hr.

Western blot of tissue transglutaminase in cell homogenates. Thymus tissue homogenates containing 1 mg/ml protein were mixed with equal volumes of sample buffer (0.125 M Tris·HCl, pH 6.8, containing 4% SDS, 20% glycerin, 10% mercaptoethanol, and 0.02% bromphenol blue) and subsequently incubated at 100° for 10 min. The 10% SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (32). The separated proteins were electroblotted onto a polyvinylidene difluoride membrane. The blot was first saturated with 1% bovine serum albumin in Tween containing Tris-buffered saline. Transglutaminase antibody, diluted 1:100, was then added and incubated at 4° overnight, followed by overnight incubation with horseradish peroxidase-labeled affinity-purified goat anti-mouse IgG. Transglutaminase bands were visualized by ECL using H_2O_2 and luminol as substrates.

Characterization of thymocyte subpopulations. Thymocytes were isolated from control thymuses and after 24 hr of *in vivo* treatment with CD437. Cells were washed twice and resuspended in ice-cold PBS containing 0.1% (w/v) sodium azide before staining with

[1] B. Charpentier and J. M. Bernardon. European patent 658553 (1993).
[2] Z. Szondy, U. Reichert, J.-M. Bernardon, S. Michel, R. Tóth, P. Ancian, E. Ajzner, and L. Fesus, unpublished observations.

PE-labeled anti-CD4 or FITC-conjugated anti CD8. The cells were agitated, incubated 30 min at 4°, washed twice with ice-cold PBS supplemented with 10% FCS and 0.1% sodium azide, and resuspended in PBS containing 0.1% sodium azide. Unstained thymocytes treated similarly served as autofluorescence controls, whereas thymocytes stained with nonreactive FITC-conjugated goat IgG1 and PE-conjugated goat IgG1 antibodies served as controls for nonspecific staining. Dual fluorescence was analyzed on a Becton Dickinson FACScan (Le Pont de Claix, France)with excitation of the incident light at 488 nm. Log integrated green fluorescence (emission at 530 nm) and log integrated red fluorescence (emission at 585 nm) were collected after combined gating on forward angle light scatter and 90° light scatter. The overlap in green and red emission was corrected using an electronic compensation network.

RNA isolation and RT-PCR. RNA from total and $CD4^+CD8^+$ thymocyte population was isolated with the Promega (Madison, WI) RNA isolation kit according to the manufacturer's instructions and treated with 5 units of RNase-free DNase for 20 min at room temperature. Then, 5 μg of total RNA was reverse-transcribed with the Superscript II preamplification kit (Life Technologies, Eggenstein, Germany) according to the manufacturer's instructions. Amplification of the RARs was performed in a total volume of 20 μl with 2 μl of the first-strand cDNAs as template with oligonucleotides 5′-GTCTTTGCCTTCCCAACCAG-3′ and 5′-CATCAGCATCTTGGGGAACA-3′ (sense and antisense for RARα), 5′-CTGGATTTGGTCCTCTGACT-3′ and 5′-CATGTGAGGCTTGCTGGGTC-3′ (sense and antisense for RARβ), and 5′-AAATCACCGACCTCCGGGGC-3′ and 5′-GGGTTCTCCAGCATCTCTCGG-3′ (sense and antisense for RARγ). PCR conditions were 28 cycles of denaturation at 94° for 30 sec, annealing at 55° for 30 sec, and extension at 72° for 30 sec. For negative controls, the RT step was omitted from PCR. For positive PCR controls, first-strand cDNAs were prepared from 5 μg of total RNA of murine F9 cells (for RARα and RARγ) or human dermal fibroblasts (for RARβ). Expected sizes for PCR products are 213, 596, and 150 bp for RARα, RARβ, and RARγ, respectively.

Results

Induction of apoptosis by RAs and RARγ-selective compounds. Both all-*trans* (at concentrations >1 μM) and 9-*cis* (at 0.1–1 μM) RA induced a significant increase in DNA fragmentation in cultured mouse thymocytes during a 6-hr culture period (Fig. 2A). Part of the freshly isolated thymocytes entered the apoptotic program spontaneously due to the removal of the protective thymic environment resulting in ~20% DNA fragmentation in control cultures. Both all-*trans* and 9-*cis*-RA induced further DNA fragmentation (the net increase is shown on Fig. 2), and the number of trypan blue-positive cells increased proportionally (data not shown), indicating the death of an additional cell population. The rate of cell death at optimum concentration was close to that initiated by 1 μM dexamethasone, a known apoptosis inducer, in similar experiments (14). 9-*cis* RA was more effective at lower concentrations than all-*trans* RA, suggesting the possible involvement of RXRs. However, an RXR-specific analog (CD2425) alone did not induce apoptosis in mouse thymocytes (data not shown). Therefore, further receptor-specific compounds were used to analyze which RAR was involved in the induction of apoptosis by RAs; binding constants and transactivation potentials of the compounds are shown in Table 1.

There is no RARβ in the thymus (21), and in agreement with these data, we found that the RARβ-selective compound (CD2314) tested cannot induce apoptosis in thymocytes (data not shown). Although RARα was shown to be present in the

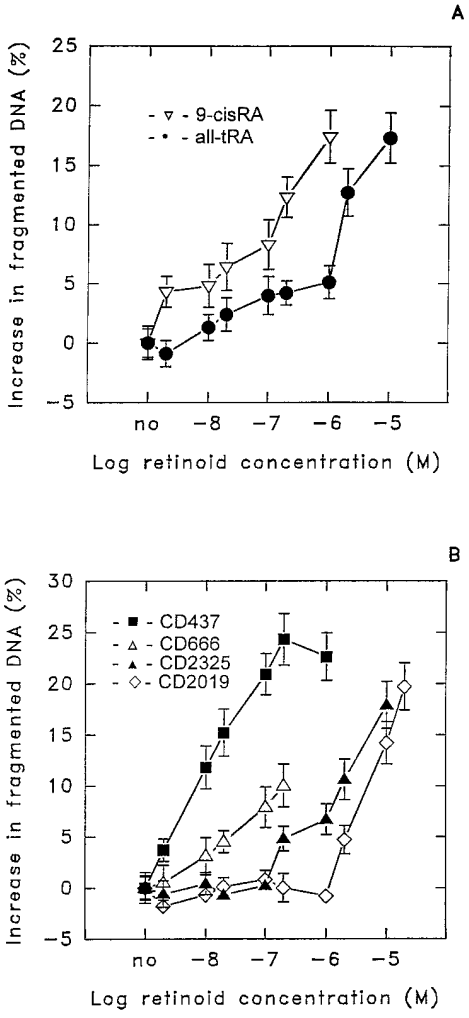

Fig. 2. Effect of increasing concentrations of various retinoids on the DNA fragmentation of mouse thymocytes. Thymocytes (10^7/ml) were separated and cultured in RPMI solution supplemented with 10% FCS, 2 mM glutamine, and 100 IU of penicillin/100 μg of streptomycin/ml. A, 9-*cis* RA (▽) or all-*trans* RA (●) was added at the indicated concentrations at the beginning of the culture of freshly separated thymocytes. B, Various RARγ receptor agonists [CD437 (■), CD666 (△), CD2325 (▲), CD2019 (◇)] were added at the indicated concentrations at the beginning of culturing freshly separated thymocytes. At 6 hr, thymocytes were harvested and tested for the amount of fragmented DNA as described. Data represent mean ± standard deviation of three determinations.

thymus (21), the RARα-selective compound (CD336) tested was not an effective inducer of apoptosis either (data not shown). The RARγ-selective compound CD437, however, can induce apoptosis in the nM range [EC_{10} (concentration leading to 10% DNA fragmentation above base-line level/controls) = 6.3 nM], which suggests that RAs induce apoptosis through the RARγ receptor (Fig. 2B). This assumption is supported by additional studies in which three other RARγ agonists (CD666, CD2325, and CD2019; EC_{10} = 193, 1370, and 4540 nM, respectively) were effective inducers of thymocyte apoptosis (Fig. 2B); the compounds were not toxic in the range of the studied concentrations, except CD666, which induced necrosis at >300 nM. Furthermore, the induction of apoptosis by both RAs and the RARγ-selective CD437 could be blocked by the RARγ antagonist CD2665 (Fig. 3). This compound did not block other forms of thymocyte apoptosis

TABLE 1

Binding constants and transactivation properties of retinoids used in the study

Binding constants and transactivation potentials of retinoids were determined as described in the Materials and Methods. Property is related to the receptor given in bold.

Compound	Binding K_d			Transact EC_{50} RXRα	Property
	RARα	RARβ	RARγ		
			nM		
all-*trans* RA	**16**	**7**	**3**	>1000	Agonist
9-*cis* RA	**30**	**11**	**20**	**24**	Agonist
CD336	**8**	131	450	>1000	Agonist
CD437	6500	2480	**77**	>1000	Agonist
CD666	2240	2300	**68**	>1000	Agonist
CD2019	1100	**26**	**160**	>1000	Agonist
CD2325	1144	1245	**53**	>1000	Agonist
CD2425	>1000	1467	712	**54**	Agonist
CD2503	**6**	964	>1000	>1000	Antagonist
CD2665	>1000	**306**	**110**	>1000	Antagonist

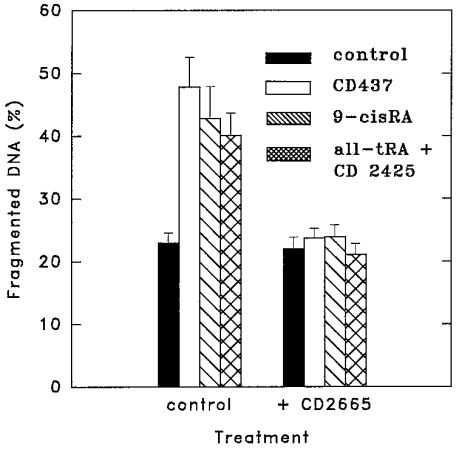

Fig. 3. Effect of an RARγ antagonist on the cell death induced by various retinoids *Column 1*, spontaneous apoptosis in the culture. *Column 2*, CD437, an RARγ agonist (0.3 μM). *Column 3*, 9-*cis* RA (1.0 μM). *Column 4*, all-*trans* RA (*all-tRA*) (0.1 μM) with CD2425 (0.1 μM), an RXR agonist. *Columns 5–8*, same as *columns 1–4* with CD2665, an RARγ antagonist (3 μM). The compounds were added at the beginning of the culture of isolated thymocytes. At 6 hr, thymocytes were harvested and tested for the amount of fragmented DNA as described. Data represent mean ± standard deviation of three determinations.

induced via TCR/CD3, *fas* stimulation or by addition of dexamethasone (steroid pathway) or etoposide (*p53* pathway) (data not shown). One may conclude that there is an RARγ-specific apoptosis pathway in thymocytes that is activated by high concentrations of all-*trans*, physiological concentrations of 9-*cis* RA, and RARγ-specific retinoid analogs.

Modulation of RARγ-dependent apoptosis by co-stimulation of RARα and RXR. Because all-*trans* and 9-*cis* RA are nearly equally potent inducers of RARγ, the difference in their dose-response curve related to apoptosis (Fig. 2A) suggests that other nuclear receptors costimulated by the panagonist RAs modulate the effect of retinoids on the RARγ pathway. One likely candidate is RARα because the comparison of K_d (RARα)/K_d (RARγ) values of the RARγ-selective compounds with their effective concentrations inducing apoptosis shows that the more specific a compound is for RARγ, the higher potential it has for induction of apoptosis (Fig. 4). This assumption is strongly supported by the observation that the addition of increasing concentrations of the RARα-

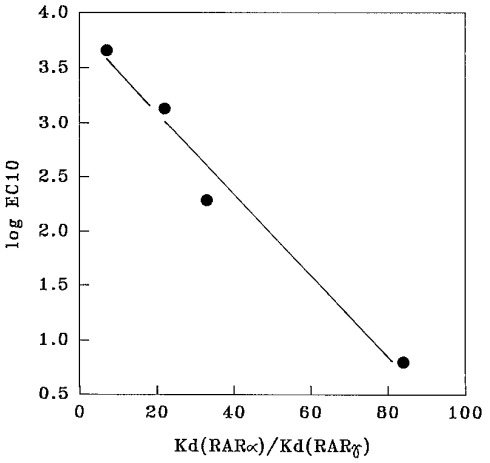

Fig. 4. Relationship between the K_d value for various RARs and the apoptosis-inducing ability of retinoids. K_d (RARα)/K_d (RARγ) values for CD437, CD666, CD2019, and CD2325 were calculated from data presented in Table 1.

selective analog CD336 to the thymocytes together with the strong apoptosis inducer RARγ analog CD437 resulted in a dose-dependent inhibition of RARγ-mediated apoptosis (Fig. 5A). One may presume that the addition of all-*trans* RA to the thymocytes results in the stimulation of both the γ and α receptors and that the latter inhibits apoptosis by shifting the dose-response curve to the right. To test this conclusion further, thymocytes were cultured in the presence of all-*trans* RA in a concentration that is suboptimal for the induction of apoptosis (0.3 μM) combined with increasing concentrations of the RARα antagonist CD2503. This treatment led to the induction of apoptosis in a dose-dependent manner (Fig. 5B); the neutralization of α receptor stimulation during treatment with suboptimal concentration of all-*trans* RA results in RARγ-mediated apoptosis. It seems, however, that the inhibitory effect of the activated RARα receptor on the RARγ pathway can be suspended by RXR costimulation because 9-*cis* RA is a more effective inducer of RARγ-dependent apoptosis than all-*trans* RA (Fig. 2A) and the addition of the RXR analog CD2425 shifted the dose-response curve of all-*trans* RA to the left (Fig. 5C). Furthermore, increasing concentrations of the RARα agonist were not effective in inhibiting the RARγ pathway in the presence of the RXR analog CD2425 (Fig. 5A).

Induction of apoptosis in the thymus by the RARγ-selective retinoid analog CD437. *In vivo* induction of apoptosis through the well-defined pathways of dexamethasone, TCR/CD3, *p53*, and *fas* results in thymus involution. When the RARγ-selective analog was injected into mice, thymus involution was observed, resulting in an almost 60% decrease of thymus weight within 48 hr (Fig. 6), whereas there was no significant change in the thymic weight of the control animals. When samples of the involuting thymus were analyzed, the activation of an apoptotic endonuclease, which cleaves DNA at internucleosomal sites, was observed, similar to the *in vitro* effect of the compound (Fig. 7, *lanes 4 and 6*) and to that of dexamethasone, the well-known apoptosis inducer. FACScan analysis has shown that the majority of cells that disappeared were $CD4^+CD8^+$ double positive immature thymocytes expressing low levels of CD3 receptor (Fig. 8). In addition, the induction of tissue transglutami-

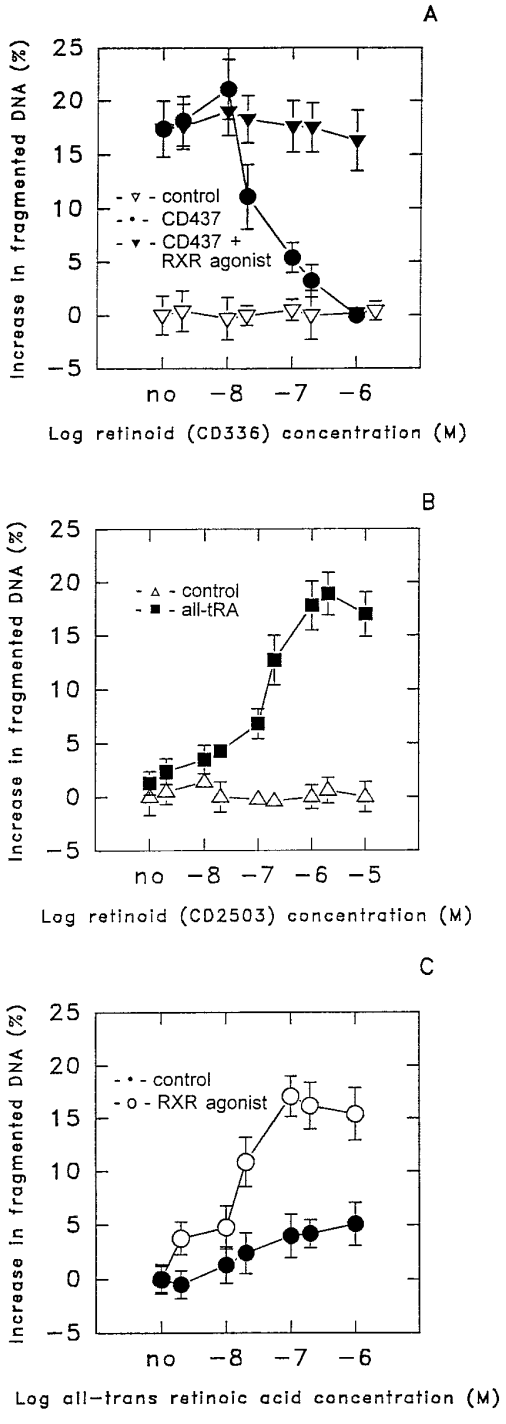

Fig. 5. Interaction between RARα and RARγ activation in regulation of thymocyte cell death A, Effect of RARα activation on RARγ-induced thymocyte death and the influence of RXR costimulation. Thymocytes (10^7/ml) were separated and cultured without (▽), with 0.3 μM CD437 (●), an RARγ agonist, or with 0.3 μM CD437 and 1 μM CD2425 (▼), an RXR agonist in the presence of increasing concentrations of CD366, an RARα agonist. B, Cell death-inducing effect of the simultaneous addition of all-*trans* RA and an RARα antagonist. Thymocytes (10^7/ml) were separated and cultured (△) without or (■) with 0.3 μM all-*trans* RA in the presence of increasing concentrations of CD2503, an RARα antagonist. C, Cell death-inducing effect of the simultaneous addition of all-*trans* RA and an RXR agonist. Thymocytes (10^7/ml) were separated and cultured (●) without or (○) with 0.1 μM CD2425, an RXR agonist, in the presence of increasing concentrations of all-*trans* RA. At 6 hr, thymocytes were harvested and tested for the amount of fragmented DNA as described. Data represent mean ± standard deviation of three determinations.

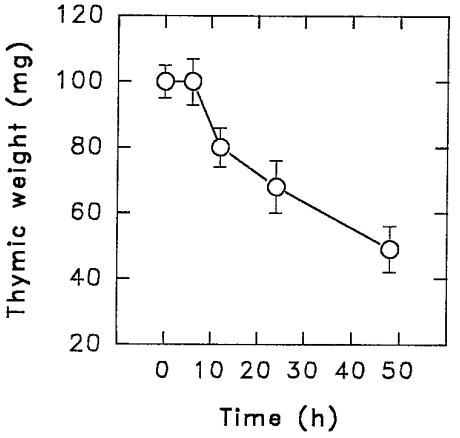

Fig. 6. Changes in thymic weight during *in vivo* apoptosis of thymocytes induced via RARγ. Mice were treated with 0.5 mg of CD437 intraperitoneally and killed at the indicated time points. The thymus was removed, and its weight was measured. Data represent changes in thymic weight at various time points after *in vivo* apoptosis induction and are expressed as mean ± standard deviation of determinations in three mice.

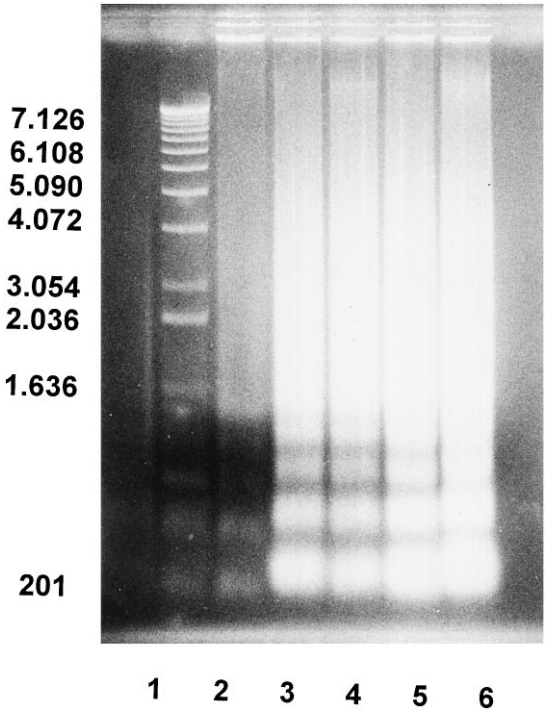

Fig. 7. Electrophoretic fractionation of DNA extracted from freshly isolated and cultured mouse thymocytes after *in vivo* and *in vitro* apoptosis induction. Thymocytes were freshly isolated from mice treated or not treated for 24 hr with dexamethasone (0.5 mg) or CD437 (0.5 mg) or cultured with 10 μM dexamethasone or 0.3 μM CD437 for 6 hr. The DNA was extracted, electrophoresed on a 1.8% agarose gel as described in Materials and Methods, and visualized after staining with ethinium bromide. *Lane 1*, DNA molecular weight marker. *Lane 2*, freshly isolated thymocytes from nontreated animals. *Lane 3*, freshly isolated thymocytes from dexamethasone-treated animals. *Lane 4*, freshly isolated thymocytes from CD437-treated animals. *Lane 5*, thymocytes cultured with dexamethasone. *Lane 6*, thymocytes cultured with CD437.

nase, one of the effector elements of apoptosis shown to be induced and activated in many apoptosis systems (8), was detected by direct measurement of enzyme activity and on the basis of the appearance of the enzyme protein on Western blot analysis (Fig. 9).

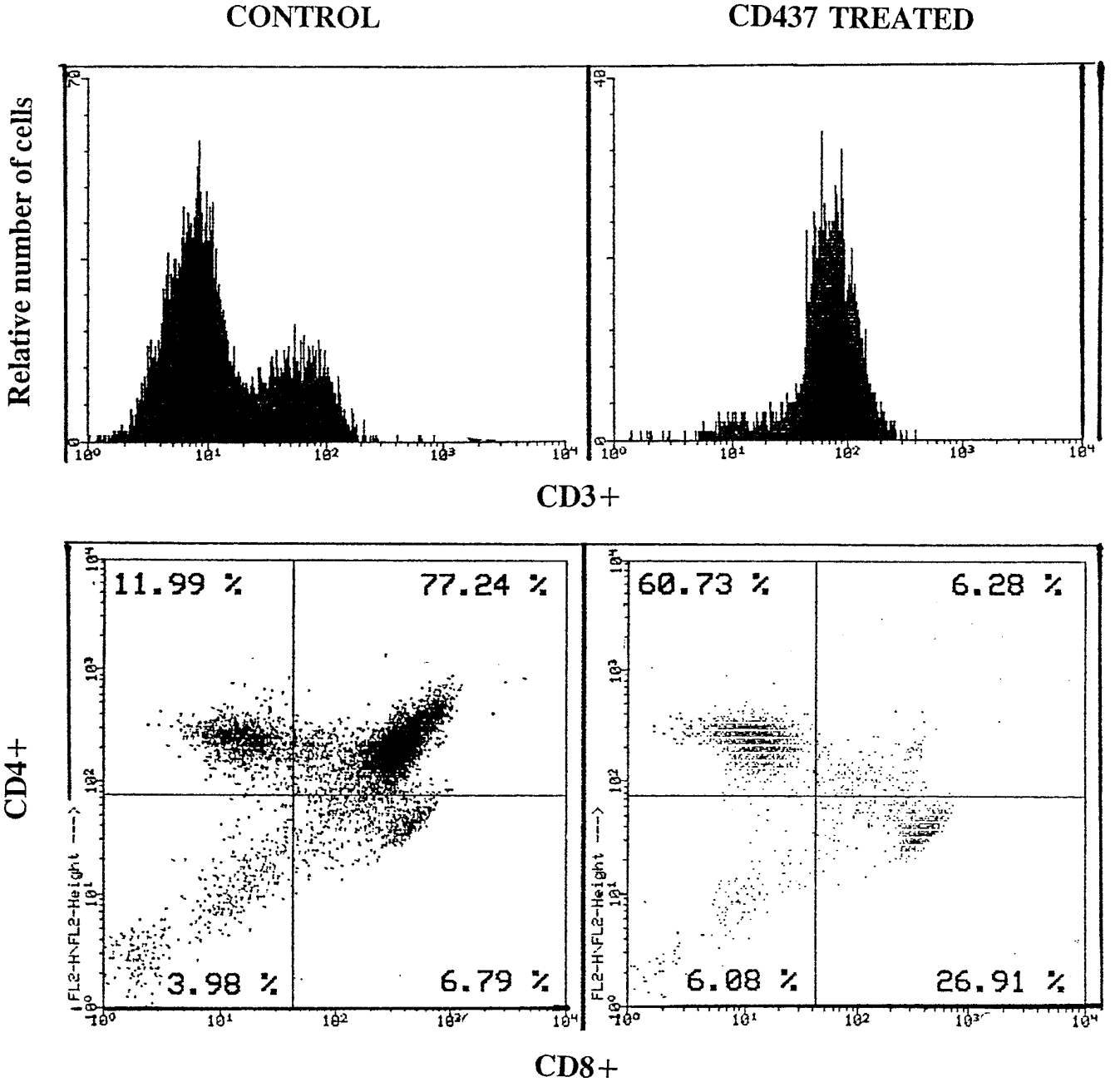

Fig. 8. Effect of CD437 treatment on the survival of different subpopulations of thymocytes with respect to the expression of CD4/CD8 or CD3/T cell receptors. The mice (control and one treated with 0.5 mg of CD437 intraperitoneally) were killed 48 hr after treatment. The thymocytes were isolated and stained with FITC-labeled anti-CD3 or FITC-labeled anti-CD8 and PE-labeled anti-CD4 as described in Materials and Methods.

Retinoids induce cell death in selected $CD4^+CD8^+$ double positive thymocyte populations that express both RARα and RARγ. A number of reports have demonstrated that the stromal cell component of the thymus provides an optimal microenvironment for thymopoiesis and that the thymocyte survival strongly depends on factors produced by the thymic stroma. Because stromal cells also express RARα and RARγ (21), we could not exclude the possibility that retinoids target first stromal cells and that their death or factors or lack of survival factors produced by them affects secondarily the survival of the thymocyte population. For this reason, we also tested the effect of retinoids on isolated $CD4^+CD8^+$ double positive thymocytes. As shown on Fig. 10, retinoids were able to induce a significant amount of DNA fragmentation in the isolated thymocytes as well (the percentage of survival cells containing nonfragmented DNA [gate R3 + R4] in controls and dexamethasone-, all-*trans* RA-, 9-*cis* RA-, and CD437-treated thymocyte cultures was 56.9 ± 4.2%, 5.1 ± 2.7%, 21.8 ± 2.1%, 10.6 ± 4.1%, and 9.8 ± 4.3%, respectively). This suggests that retinoids act directly on $CD4^+CD8^+$ thymocytes. In addition, with RT-PCR, we were able to demonstrate that this population also expresses RARα and RARγ but not RARβ (Fig. 11).

Biochemical characterization of the RARγ-mediated apoptosis pathway. Both actinomycin D and cycloheximide, inhibitors of RNA and protein synthesis, could block

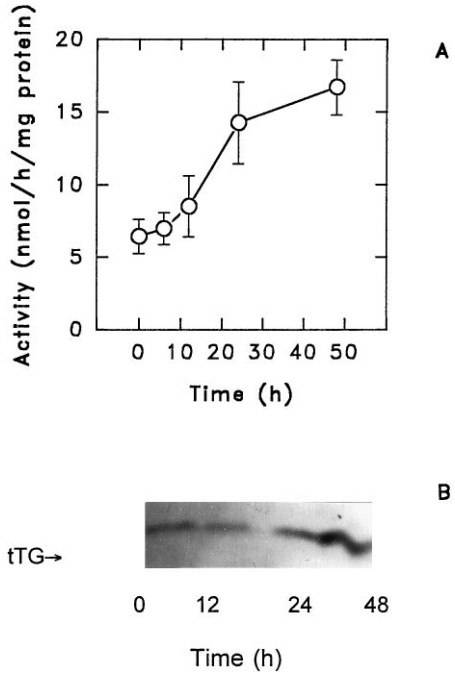

Fig. 9. Changes in tissue transglutaminase activity and expression during *in vivo* apoptosis of thymocytes induced via RARγ. Mice were treated with 0.5 mg of CD437 intraperitoneally and killed at the indicated time points. The thymus was removed, and the tissue transglutaminase (*tTG*) (A) activity and (B) expression were determined at various time points after *in vivo* apoptosis induction as described. Data represent mean ± standard deviation of determinations of three mice.

the apoptosis induced by the RARγ-selective agonist CD437 (Fig. 12, A and B). This inhibition could be observed only if the inhibitors were introduced into the culture at ≤1 and ≤1.5 hr after the addition of the retinoid analog, respectively. These results clearly show that RARγ-mediated apoptosis depends on intact transcriptional and translational activity of the cells and that proteins critical in the induction and execution of apoptosis are synthesized during the first 1.5 hr.

The cysteine and serine protease inhibitor *N*-tosyl-L-lysyl chloromethylketone has been reported to inhibit apoptosis induced by diverse stimuli such as TCR, dexamethasone, *fas*, and *p53* stimulation (7). The RARγ-mediated apoptosis pathway can also be inhibited by this protease inhibitor (Fig. 11C), which was effective even when added 2 hr after the retinoid analog, suggesting the participation of a protease in the molecular events leading to the final stages of the cell death process.

It has been reported that intracellular Ca^{2+} may play a critical role in the initiation of thymocyte apoptotic process (9, 31). Ca^{2+} may act either as a signaling molecule or as an activator of some of the Ca^{2+}-dependent key elements of apoptosis, like endonuclease, calpain, or tissue transglutaminase. When Quin-2 was added with CD437 to the culture media to buffer intracellular Ca^{2+}, apoptosis did not occur (data not shown), demonstrating the requirement of free Ca^{2+} for initiation of the cell death machinery.

Discussion

The complexity of the different expression and interaction of RARs and RXRs makes understanding the physiology of the action of RAs extremely difficult. There are two possible approaches that may dissect the complexity of retinoid biology. The first is either knocking out each receptor by homologous recombination (33) or expressing their dominant negative variants (34). The second uses receptor-selective retinoid analogs and antagonists to stimulate or block one or more of the RARs. The presented results, which were obtained through use of the second methodology, reveal the existence of a novel retinoid response: the induction of apoptosis through stimulation of the RARγ nuclear transcriptional factor. Although there are suggestions that CD437 might affect cellular functions independently of RARγ (35), the following major pieces of evidence support the existence of an RARγ-dependent retinoid effect, at least in thymocytes: (i) all-*trans*, 9-*cis* RA and RARγ-selective RA analogs induce apoptosis; (ii) RARα-, RARβ-, and RXR-selective RA analogs do not induce apoptosis; (iii) the induction of apoptosis by RAs as well as by RARγ-specific compounds could be completely inhibited by an RARγ-antagonist; and (iv) isolated $CD4^+CD8^+$ thymocytes express RARγ and respond with apoptosis to the RARγ-selective retinoids.

It has been clearly demonstrated that at least four independent molecular pathways (initiated by TCR, *fas*, or steroid receptor activation or DNA damage) can lead to the induction of thymocyte apoptosis. The retinoid pathway in thymocytes may represent a new pathway mediated via the RARγ nuclear receptor. Similar to three of the other four pathways so far revealed, this pathway is RNA and protein synthesis dependent and can switch on all the common effector machinery of apoptosis, including the activation of proteolytic, endonuclease, and transglutaminase enzymes.

Previous studies carried out using malignant cells in culture have shown that apoptosis can be induced by retinoids. It was reported that all-*trans* RA is a potent inducer of apoptosis in HL-60 cells (36). In a recent study, it has been shown by using RAR- and RXR-specific ligands that activation of RXRs (very likely in the structure of RAR/RXR heterodimers) is essential and sufficient for the induction of apoptosis (25). In tracheobronchial epithelial cells, the induction of apoptosis by retinoids is mediated through RARα (37). This study is the first to show the induction of apoptosis by retinoids in a well-defined normal cell population both *ex vivo* and *in vivo* and to suggest the direct involvement of the RARγ in the death process. An additional apoptosis-related effect of retinoids has been revealed in our experiments: the inhibition of RARγ-mediated apoptosis when RARα is costimulated and the regulation of this inhibitory effect by RXR stimulation. Our study is not sufficiently detailed to determine the precise mechanism by which stimulation of RARα leads to the observed inhibition of RARγ-mediated apoptosis. Because RARγ analogs that have a higher affinity for RARα require higher concentrations to induce the same rate of apoptosis (e.g., the inhibition is overcome by higher concentrations of retinoids), one possibility is that the costimulation of RARα may compete with RARγ for RXR binding or costimulated RARα/RXR heterodimers may compete with RARγ/RXR heterodimers for DNA binding or transactivation sites. Stimulation of RXR in the same setting might have an opposite effect. Alternatively, RARα may act downstream of RA/RARγ binding, initiating various antiapoptotic processes. These findings suggest that in addition to the type of retinoids available, fine tuning of RAR expression leading to various RARα/RARγ activation ratios in a given cell population

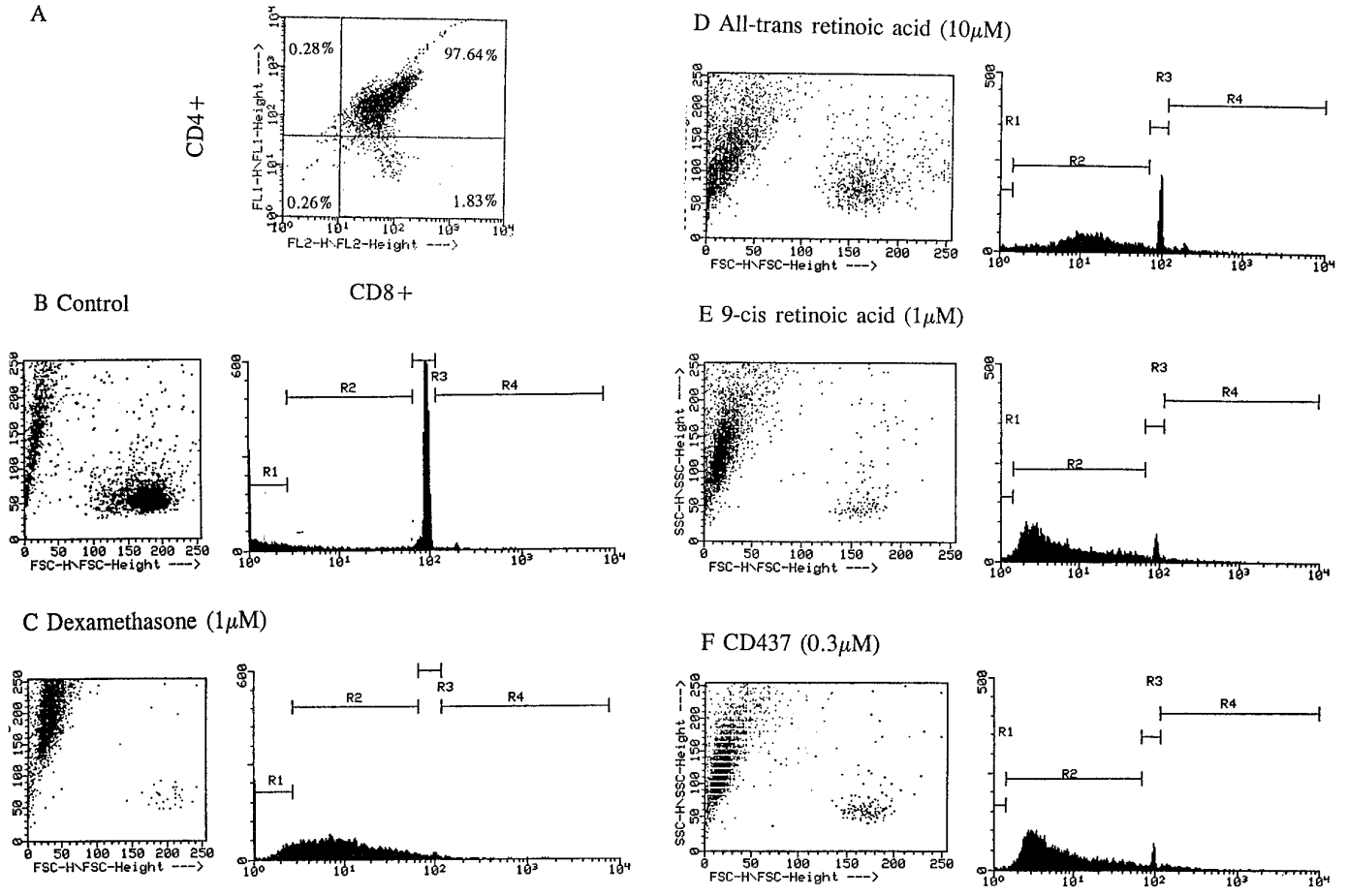

Fig. 10. Induction of apoptosis in isolated $CD4^+CD8^+$ thymocytes by various compounds. A, Proportion of $CD4^+CD8^+$ double positive thymocytes after the isolation procedure. $CD4^+CD8^+$ double positive thymocytes were separated as described in Materials and Methods, and cells in the selected population were further stained with PE-labeled anti-CD4 antibody. B–F, DNA histograms of propidium iodide-stained thymocytes after 24 hr in culture treated with various compounds. Cells gated as *R4*, thymocytes in G2-M phase. *R3*, thymocytes in G0-G1 phase. *R2*, apoptotic thymocytes. *R1*, necrotic thymocytes.

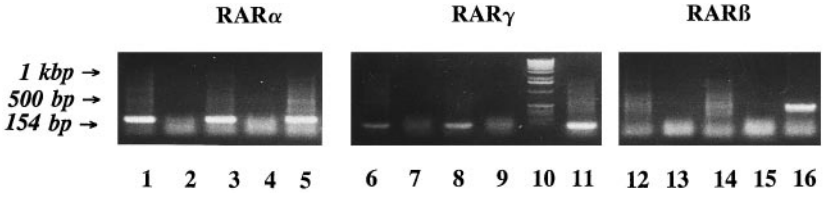

Fig. 11. RAR expression of thymocytes and isolated $CD4^+CD8^+$ cells. RAR expression was detected through RT-PCR as described in Materials and Methods. From the negative controls, RT step was omitted from the PCR reaction. Expected sizes for the PCR products are 213, 596, and 150 bp for RARα, RARβ, and RARγ, respectively. *Lane 1*, RARα in thymocytes. *Lane 2*, thymocytes, negative control. *Lane 3*, $CD4^+CD8^+$ thymocytes. *Lane 4*, $CD4^+CD8^+$ thymocytes, negative control. *Lane 5*, murine F9 cells, positive control. *Lane 6*, RARγ in thymocytes. *Lane 7*, thymocytes, negative control. *Lane 8*, $CD4^+CD8^+$ thymocytes. *Lane 9*, $CD4^+CD8^+$ thymocytes, negative control. *Lane 10*, molecular weight markers: 1kbp DNA ladder (Life Technologies, Grand Island, NY). *Lane 11,* human dermal fibroblasts, positive control. *Lane 12*, RARβ in thymocytes. *Lane 13*, thymocytes, negative control. *Lane 14*, $CD4^+CD8^+$ thymocytes. *Lane 15*, $CD4^+CD8^+$ thymocytes, negative control. *Lane 16*, murine F9 cells, positive control.

may be one of the determining factors for a cell to stay alive or die.

Do the presented results have physiological significance? The concentration of all-*trans* RA needed to initiate apoptosis in thymocytes is much higher than its physiological level *in vivo.* However, the apoptosis-inducing effect of 9-*cis* RA occurs at a much lower concentration because, as we could show, it can neutralize, possibly interacting with one of the RXRs, the inhibitory effect of RARα on the RARγ apoptosis pathway. Therefore, in case the circulating all-*trans* RA is converted to the 9-*cis* ligand in sufficient quantities in thymocytes, the RARγ pathway of cell death is initiated. This may be one of the critical events in the initiation of apoptosis of those $CD4^+CD8^+$ double positive thymocytes that have low affinity TCR and have not been positively selected or eliminated by the negative selection pathway mediated by high affinity TCR/self-antigen interaction. The large majority of thymocytes die through this "default death pathway," but the initiator of apoptosis in these cells has not been clarified. Although dexamethasone certainly accelerates the

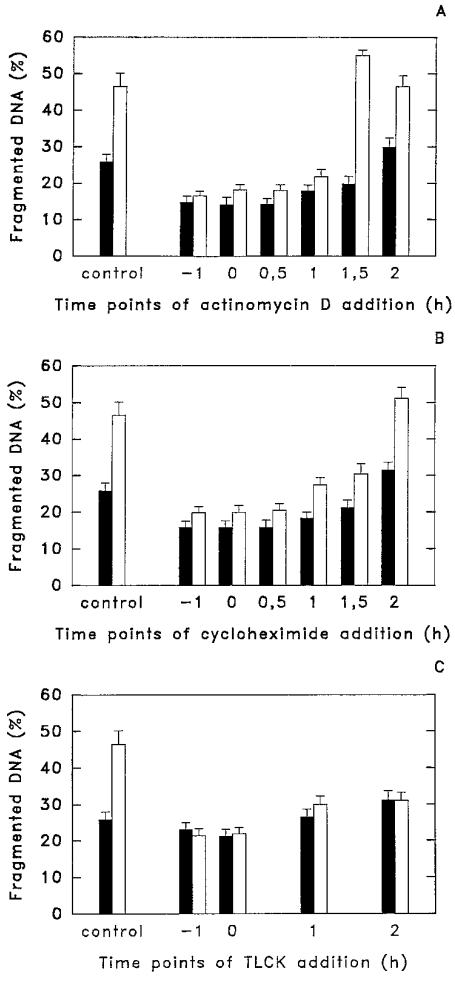

Fig. 12. Time course of inhibition of RARγ-induced thymocyte cell death by actinomycin D, cycloheximide, or the protease inhibitor *N*-tosyl-L-lysil-chloromethylketone (*TLCK*). Thymocytes (10^7/ml) were separated and cultured for 7 hr (*filled column*) without or (*open column*) with 0.3 μM CD437 added at 1 hr of culture. Actinomycin D (12.5 μg/ml), cycloheximide (25 μg/ml), or *N*-tosyl-L-lysil-chloromethylketone (50 μM) was added at the indicated time points. At 7 hr, thymocytes were harvested and tested for the amount of fragmented DNA as described. Data represent mean ± standard deviation of three determinations.

default pathway of apoptosis both *in vivo* and *in vitro*, this does not necessarily mean that that the steroids are the only physiological initiators of the death pathway in these cells. One may speculate that the expression pattern of RARs and RXRs in maturing thymocytes set the stage for the action of physiological concentration of RAs providing RARγ-mediated apoptosis pathway for thymocytes that have not been positively or negatively selected. After the well-documented inhibitory effect of RAs on activation-induced death/negative selection (12–14), this would be the second potential physiological action of RAs in the thymus.

Glucocorticoids, RAs, and the TCR seem to regulate positive and negative selection of thymocytes in a coordinated manner. In addition to RAs, glucocorticoids were shown to inhibit TCR-induced cell death. Suggestions were made that glucocorticoids are required for the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$ cells and may increase the threshold at which an antigen is recognized as high affinity ligand and initiates negative selection (38). At low concentrations of glucocorticoids, retinoids proved to be additive in inhibiting TCR-mediated cell death (12), suggesting that retinoids and glucocorticoids may simultaneously affect the number of positively selected thymocytes.

In addition to affecting TCR-mediated death, retinoids were shown to stimulate glucocorticoid-mediated cell death (12, 14). This raised the possibility that the observed effect of retinoids on the basal apoptosis rate is related to an enhancement of apoptosis initiated by the endogenous glucocorticoids. However, none of the RARγ analogs tested stimulated dexamethasone-induced death (data not shown), suggesting that the RARγ-mediated death is independent of the glucocorticoid-mediated death. Additional experiments also showed that RARα stimulation might be involved in the phenomenon.[3]

The data that we present suggest that a fine tuning of RA concentration- and cell-specific expression of retinoid receptors (including the ratio of RARα to RARγ receptors) may have partially revealed an importance in tissue homeostasis and the immune response. Further studies are required to clarify how the presented observations are applicable to peripheral T cells and other components of the immune system.

The tissue distribution of the RARγ transcript suggests a role for this receptor in morphogenesis, chondrogenesis, and differentiation of squamous epithelia (33). Null mutant mice of all RARγ isoforms exhibit growth deficiency, early lethality, various forms of embryonic malformation, and squamous metaplasia at ectopic locations (33); several of the observed phenotypic changes may be explained by the perturbation of programmed cell death during development. RARγ together with RARα plays a critical role in maintaining keratinocyte differentiation and cornification (34); cornification and apoptosis are closely linked phenomena, and both may occur and be regulated by retinoids, perhaps through RARγ, in the skin (39). These data suggest that the physiological importance of retinoid-induced apoptosis through RARγ may not be restricted to the thymus and the immune system. Furthermore, there are significant therapeutic implications of the existence of a well-characterized, retinoid-initiated apoptosis pathway. If the presence of RARγ in a cell type renders it susceptible to apoptosis, cell death will be initiated by the addition of RARγ-selective compounds such as CD437. If the γ receptor is expressed in malignant or autoreactive cell populations (or introduced into such population through gene transfer), these cells might be eliminated by apoptosis as a part of a new therapeutic strategy against cancer or autoimmune diseases. Furthermore, our data also suggest the the RARγ apoptosis pathway can be potentiated by the administration of either RARα antagonists or RXR agonists, thus providing the basis of appropriately balanced and perhaps cell type-specific therapeutic protocols for retinoids.

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