Inhibition of activation-induced apoptosis of thymocytes by all-*trans*- and 9-*cis*-retinoic acid is mediated via retinoic acid receptor α

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Thymocytes can be induced to undergo apoptotic cell death by activation through the T-cell receptor (TCR). This process requires macromolecular synthesis and has been shown to be inhibited by retinoic acids (RAs). Two groups of nuclear receptors for RAs have been identified: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). All-*trans*-RA is the high-affinity ligand for RARs, and 9-*cis*-RA additionally binds to RXRs with high affinity. Because 9-*cis*-RA is much more potent in inhibiting TCR-mediated death than all-*trans*-RA, it was suggested that RXRs participate in the process. In the present study various synthetic retinoid analogues were used to address this question further. The results presented suggest that the inhibitory effect of RAs on activation-induced death of thymocytes is mediated via RARα, because (1) it can be reproduced by various RARα analogues both *in vitro* and *in vivo*, (2) the effect of RAs can be inhibited by the addition of an RARα antagonist, (3) CD4+CD8+thymocytes, which die on TCR stimulation, express RARα. Stimulation of RARγ, in contrast, enhances the activation-induced death of thymocytes and inhibits its prevention by RARα stimulation. RXR co-stimulation suspends this inhibitory effect of RARγ and permits the preventive function of RARα on activation-induced death. Our results suggest a complex interaction between the various isoforms of retinoid receptors and demonstrate that low (physiological) concentrations of all-*trans*-RA do not affect the activation-induced death of thymocytes because the RARα-mediated inhibitory and the RARγ-mediated enhancing pathways are in balance, whereas if 9-*cis*-RA is formed, additional stimulation of RXRs permits the inhibitory action of RARα.

INTRODUCTION

Apoptosis has an essential role in shaping the T-lymphocyte repertoire of the body. One form of it is seen after 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 of thymocytes [1], peripheral deletion of mature T-cells [2], and loss of non-infected T-cells in AIDS [3]. Much of our understanding of the molecules and pathways involved in T-cell receptor (TCR)-mediated death comes from studies with murine T-cell hybridomas, which are made by fusing normal peripheral T-cells with a thymoma cell line. The signal transduction pathway involves both protein kinase C activation and an increase in cytosolic Ca^{2+} concentration, and can be mimicked by the addition of phorbol dibutyrate and calcium ionophore [4]. The process is dependent on RNA and protein synthesis *de novo* [5], and involves transcription factors such as *c-myc* [6] and *nur77* [7]. The effector part of the program includes protease activation [8], genomic DNA fragmentation [5] and induction and activation of the tissue transglutaminase [9].

Among many independently acting agents, all-*trans*- and 9-*cis*-retinoic acid (RA) were shown to inhibit activation-induced death of thymocytes [10–12]. T-cell hybridomas [10,11] and peripheral blood lymphocytes from patients infected with human immunodeficiency virus [13]. RAs are physiological ligands for retinoic acid receptors (RARs) and retinoid X receptors (RXRs) that belong to the steroid/thyroid/retinoid nuclear receptor family [14]. All-*trans*- and 9-*cis*-RAs are equipotent in activating RAR, whereas activation of RXR by all-*trans*-RA is 1/50 that by 9-*cis*-RA [15]. Although all-*trans*-RA does not bind to RXRs, the observed activation at high concentrations is explained by the conversion of all-*trans*-RA to 9-*cis*-RA within the cells by unknown mechanisms. RA receptors function in the form of RAR/RXR heterodimers or RXR/RXR homodimers in the presence of RAs [16]. In addition, RXR can form heterodimers with various other members of the steroid/thyroid/retinoid receptor family (e.g. thyroid receptor, vitamin D_3 receptor, COUP-TF, peroxisome proliferator-activated receptor [17–19]). The presence of RXR in most of the heterodimers is needed to enhance the co-operative binding of these receptors to the DNA; the activation requires only the presence of the cognate vitamin D_3, thyroid receptor, peroxisome proliferator-activated receptor or RAR ligands, but can be modulated by the simultaneous binding of the RXR ligand [18,19]. These complex interactions and the existence of multiple 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 cell.

Because 9-*cis*-RA was found to be more potent in inhibiting the activation-induced death of thymocytes and T-cell hybridomas than all-*trans*-RA [10–12], it was suggested that RXRs are involved in the process. Indeed, studies performed on T-cell hybridomas transfected with cDNA encoding RXRβ or dominant-negative RXRβ have shown that cells that overexpressed RXRβ were more sensitive to 9-*cis*-RA rescue from activation-induced death. In contrast, cells expressing the dominant-negative RXRβ could not be rescued from death with 9-*cis*-RA

Abbreviations used: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; TCR, T-cell receptor.

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[20]. Additionally, in wild-type cells, an RAR-selective synthetic ligand had little effect, whereas an RXR-selective agonist prevented activation-induced apoptosis but only at concentrations about 10-fold those required for 9-*cis*-RA. However, simultaneous addition of the RAR- and RXR-selective retinoids completely prevented activation-induced apoptosis at concentrations where either alone had relatively little protective effect [20,21]. These results suggest that the binding of not only RXRs but also RARs is required for efficient inhibition of activation-induced death.

In our experiments this phenomenon was studied further by using various synthetic retinoids and mouse thymocytes. In this paper we provide evidence that RARα alone is involved in the prevention of activation-induced death, this inhibitory function is inhibited by RARγ co-stimulation, and the role of RXR receptor co-stimulation is to suspend this RARγ-mediated inhibition and permit the preventive function of RARα.

MATERIALS AND METHODS

Chemicals

All retinoid compounds used for 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-adamantyl)-4-hydroxyphenyl]-2-naphthoic acid}, CD2425 (AGN 191701) [24], {(*E*)-5-[2-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethylnaphthalen-2-yl) propen-l-yl]-3-thiopene carboxylic acid}, CD2503 (Ro41-5253) [25], {4-[(E)-2-(3,4-dihydro-4,4-dimethyl-1, 1-dioxide-2*H*-benzothiopyran-6-yl)propenyl]benzoic acid}, CD2665 [4-(6-methoxyethoxymethoxy-7-adamantoyl-2-naphthyl)benzoic acid] [26], CD2081 [2-hydroxy-4-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydronaphthalene-2-carboxamido)benzoic acid] [26] and CD2314 [2-(5,6,7,8-tetrahydromethyl-2-anthryl)-4-thiophenecarboxylic acid] [26]. Anti-CD3 monoclonal antibody was purchased from Pharmingen (San Diego, CA, U.S.A.). Anti-transglutaminase monoclonal antibody was a gift from Dr. Paul Birckbichler (Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.). Horseradish peroxidase-labelled goat anti-(mouse IgG), FITC-conjugated anti-CD8, phycoerythrin-conjugated anti-CD4 antibodies and *N*,*N*′-dimethylcasein were from Sigma Chemical (St. Louis, MO, U.S.A.). Luminol and BSA were from Reanal (Budapest, Hungary), PVDF membrane was from Millipore (Bedford, MA, U.S.A.), and [^{3}H]putrescine (26 Ci/mmol) was purchased from Amersham International (Little Chalfont, Bucks., U.K.). 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 with [^{3}H]CD367 as radiolabelled 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 trans-activate RXR (U. Reichert, J. M. Bernardon and S. Michel, unpublished work). The assays were performed as previously described [27], by 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, U.S.A.).

Trans-activation assay

Because no radiolabelled RXR-specific ligand was available for binding studies, interaction of the retinoids with this receptor type was assessed by a functional trans-activation assay as previously described [30]. In brief, HeLa cells were co-transfected with an expression vector for RXRα (provided by Dr. M. Pfahl) and with a CRABP$\frac{1}{2}$-tk-CAT reporter plasmid (provided by C. Gerst, CIRD Galderma, Sophia Antipolis, France). Cells were grown for 24 h in the presence of various retinoids. CAT activity was determined in lysates and expressed as a percentage of maximum induction after background CAT activity had been subtracted. The retinoid concentration giving half-maximum activation (AC_{50} value) was calculated by non-linear regression analysis.

The trans-activation abilities of the RARα-, RARβ- and RARγ-selective compounds were tested as described previously [31].

Experimental animals

Male NMRI mice 4 weeks old, purchased from LATI (Gödöllő, Hungary), were used. For the induction of thymic apoptosis mice received 80 μg of anti-CD3 antibody intraperitoneally, whereas for its prevention 0.4 mg of CD336 was used, dissolved in a mixture of 40 μl of DMSO and 0.4 ml of physiological saline. Control animals were injected with the same amount of vehicle.

Thymocyte preparation

Thymocyte suspensions were prepared from thymus glands of 4-week-old male NMRI mice by mincing the glands in RPMI 1640 media (Sigma) supplemented with 10 % (v/v) charcoal-treated foetal calf serum (Gibco, Grand Island, NY, U.S.A.), 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin. Thymocytes were washed three times and diluted to a final concentration of 10^7 cells/ml before incubation at 37 °C in a humidified incubator under an air/CO_2 (19:1) atmosphere. Cell death was measured by Trypan Blue uptake. A total of 95–98 % of cells routinely excluded Trypan Blue after the isolation procedures.

Determination and characterization of DNA fragmentation

These were performed as described previously [32]. In brief, thymocytes were incubated in 24-well plates in the presence of various agents. After 6 h, 0.8 m 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, 100 mM Tris and 20 mM EDTA, pH 8.0, before centrifugation for 15 min at 13000 ***g***. DNA content in the supernatant (DNA fragments) and pellets (intact chromatin) was precipitated with an equal amount of 10 % (w/v) trichloroacetic acid, resuspended in 5 % (w/v) trichloroacetic acid and quantified with diphenylamine reagent [33]. Alternatively, a rapid hypotonic technique with DNA staining by propidium iodide was used. For staining, cells were washed and redissolved in distilled water containing 50 μg/ml propidium iodide, 1 % (w/v) sodium citrate and 1 % (v/v) Triton X-100. With this technique the percentage of cells carrying a decreased amount of DNA owing to apoptosis, and cells in the G_0–G_1, S or G_2–M phases, could be detected on DNA histograms by flow cytofluorimetry. The degree of fragmentation was well correlated with the number of Trypan Blue-positive dead cells throughout the experiments.

For DNA gel electrophoresis the cells were lysed. The 13000 ***g*** supernatants were precipitated overnight in ethanol containing 0.15 mM NaCl. The pellets were redissolved in a buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0, treated with RNase,

sequentially extracted with equal volumes of phenol and chloroform/3-methylbutan-1-ol (24:1, v/v) and precipitated in ethanol before electrophoresis for 3 h at 60 V in a 1.8% (w/v) agarose gel. DNA fragments were then revealed by UV light after the gel had been stained with ethidium bromide.

Tissue transglutaminase activity

Thymus was collected from control or treated animals at 1 day 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 PMSF. 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/ml *N*,*N*′-dimethylcasein and 0.2 mM putrescine, with 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 on Whatman 3 MM filter paper moistened with 20% (w/v) trichloroacetic acid. Free [^{3}H]putrescine was eliminated by washing with large volumes of cold 5% (w/v) trichloroacetic acid containing 0.2 M KCl before counting for radioactivity. Activity was calculated as nmol of [^{3}H]putrescine incorporated into protein/h.

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% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 0.02% Bromophenol Blue] and subsequently incubated at 100 °C for 10 min. SDS/PAGE [10% (w/v) gel] was performed by the method of Laemmli [34]. The separated proteins were electroblotted on a PVDF membrane.

The blot was first saturated with 1% (w/v) BSA in Tween-containing Tris-buffered saline. Transglutaminase antibody, diluted 1:100, was then added and incubated at 4 °C overnight, followed by overnight incubation with horseradish peroxidase-labelled affinity-purified goat anti-(mouse IgG). Transglutaminase bands were detected by enhanced chemiluminescence, with H_2O_2 and luminol as substrates.

Characterization of thymocyte subpopulations

Thymocytes were isolated from control thymuses and after 24 h of treatment *in vivo* with anti-CD3 antibody, CD366 or both. Cells were washed twice and resuspended in ice-cold PBS containing 0.1% sodium azide before staining either with phycoerythrin-labelled anti-CD4 or FITC-conjugated anti-CD8. The cells were agitated, incubated for 30 min at 4 °C, washed twice with ice-cold PBS supplemented with 10% (v/v) foetal calf serum 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 non-reactive FITC-conjugated goat IgG_1 and phycoerythrin-conjugated goat IgG_1 antibodies served as controls for non-specific staining. Dual fluorescence was analysed on a Becton Dickinson FACScan (Le Pont de Claix, France) with excitation at 488 nm. Logarithmically integrated green fluorescence (emission at 530 nm) and logarithmically integrated red fluorescence (emission at 585 nm) were collected after combined gating on forward angle light scatter and 90° light scatter. The overlap in the green and red emissions was corrected by using an electronic compensation network.

RESULTS

Inhibition of activation-induced apoptosis of thymocytes by retinoic acids and RARα-selective compounds

It was shown previously that activation-induced death of thymocytes can be mimicked by simultaneous addition of the protein kinase C activator phorbol dibutyrate and calcium ionophore [4]. At optimum concentration this treatment induced an 18–25% or 35–40% increase in DNA fragmentation of cultured mouse thymocytes isolated from various mice during a 6 h or 24 h culture period (Figure 1 and Table 1) respectively. Part of the freshly isolated thymocytes entered the apoptotic programme spontaneously owing to the removal of the protective thymic environment [35], resulting in approx. 10–15% and 30–35% DNA fragmentation in control cultures after a 6 h or 24 h culture period respectively. The same treatment induced proliferation in

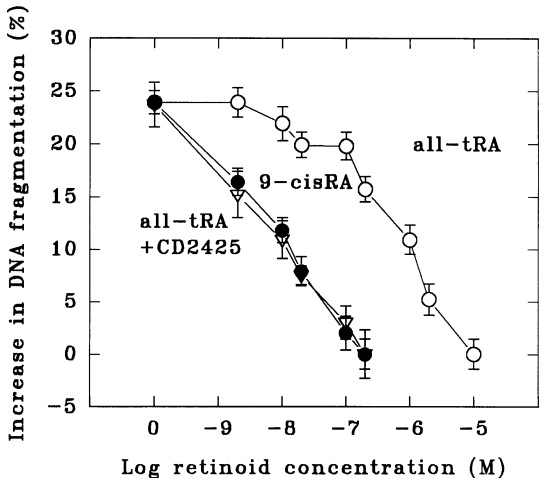

Figure 1 Effect of increasing concentrations of various retinoids on the activation-induced death of mouse thymocytes

Thymocytes (10^7/ml) were separated and cultured in RPMI solution supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin. They were induced to die by the addition of 5 ng/ml phorbol dibutyrate and 0.5 μM A23187. 9-*cis*-RA (●) or all-*trans*-RA (all-tRA) in the absence (○) or presence of 0.1 μM CD2425 (▽) were added at the indicated concentrations at the beginning of culturing freshly separated thymocytes. At 6 h, thymocytes were harvested and tested for the amount of fragmented DNA as described. Results are means ± S.D. for three determinations.

Table 1 DNA histogram of propidium iodide-stained thymocytes after 24 h in culture treated with various compounds

Thymocytes (10^7/ml) were separated and cultured in the absence and in the presence of 5 ng/ml phorbol dibutyrate (PdBu) and 0.5 μM A23187 without or with an RARα agonist (CD2081 or CD336; 0.1 μM). After 1 day the cells were stained with propidium iodide, as described in the Materials and methods section.

	Thymocytes at each stage (%)		
Treatment	Apoptosis	G_0–G_1 phase	S–G_2–M phase
Control	12.2	54.9	2.0
PdBu + A23187	47.1	19.6	12.7
PdBu + A23187 with CD336	19.9	46.5	6.6
PdBu + A231897 with CD2081	24.1	49.1	7.2

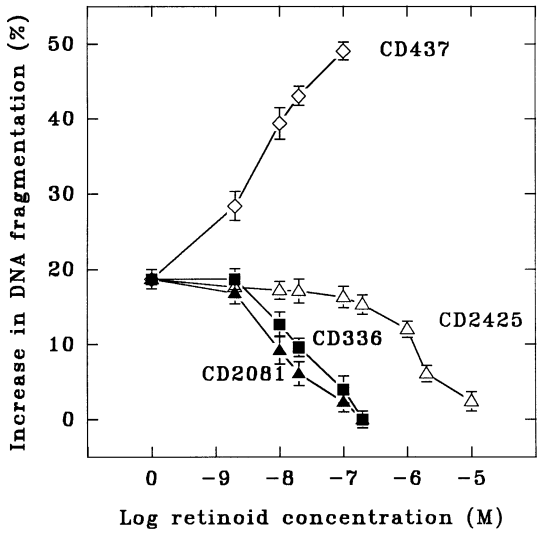

Figure 2 Effect of increasing concentrations of RXR, RARγ or RARα agonists on the activation-induced death of mouse thymocytes

Thymocytes (10^7/ml) were separated and cultured in RPMI solution supplemented with 10% (v/v) foetal calf serum, 2 mM glutamine, 100 i.u./ml penicillin and 100 μg/ml streptomycin. They were induced to die by the addition of 5 ng/ml phorbol dibutyrate and 0.5 μM A23187. CD2425 (an RXR agonist), CD437 (an RARγ agonist), CD2081 or CD336 (RARα agonists) were added at the indicated concentrations at the beginning of culturing freshly separated thymocytes. At 6 h, thymocytes were harvested and tested for the amount of fragmented DNA as described. Results are means $\pm$ S.D. for three determinations.

a small proportion of thymocytes, resulting in an increase in the number of S–G_2–M phase cells (Table 1). Both all-*trans*- and 9-*cis*-retinoic acid inhibited the DNA fragmentation induced by this treatment in a dose-dependent manner, but were unable to prevent the spontaneously occurring death (Figure 1). The number of Trypan Blue-positive cells decreased proportionally (results not shown), indicating that the decrease in DNA fragmentation was indeed due to an increased cell survival and not to an increased rate of necrotic death. 9-*cis*-RA was much more effective than all-*trans*-RA, suggesting that RXR receptors selectively stimulated by 9-*cis*-RA might participate in the phenomenon. Indeed, CD2425 (0.1 μM), an RXR-specific analogue, effectively lowered the concentration of all-*trans*-RA required to inhibit the activation-induced death of thymocytes (Figure 1). The RXR-specific analogue CD2425, however, alone prevented the activation-induced apoptosis of mouse thymocytes, only at much higher concentrations than 9-*cis*-RA (Figure 2). Because CD2425 and 9-*cis*-RA have very similar AC_{50} values for RXRα (Table 2), this observation suggests that not only the stimulation of RXRs has a role in the phenomenon. Therefore further receptor-specific compounds were used to analyse which RAR is involved in the inhibition of activation-induced apoptosis by retinoic acids; binding constants and transactivation potentials of the compounds are shown in Table 2.

There is no RARβ receptor in the thymus [36,37] and, in agreement with these results, we found that the RARβ-selective compound (CD2314) tested cannot inhibit the activation-induced apoptosis of thymocytes (results not shown). An RARγ-binding compound (CD437) with a very weak affinity for RARα, in contrast, stimulated rather than inhibited activation-induced death (Figure 2). These results suggested that neither RARβ nor RARγ is a good candidate for inhibiting activation-induced death.

Table 2 Binding constants and trans-activation properties of retinoids used in this study

Binding constants and transactivation potentials of retinoids were determined as described in the Materials and methods section. The property stated relates to the receptor(s) whose values are printed in bold.

Compound	Binding K_d(nM) RARα	RARβ	RARγ	Trans-activation AC_{50} (nM) RXRα	Property
all-*trans*-RA	**16**	**7**	**3**	> 1000	Agonist
9-*cis*-RA	**30**	**11**	**20**	**24**	Agonist
CD336	**8**	131	450	> 1000	Agonist
CD2081	**6**	147	753	> 1000	Agonist
CD2314	> 3760	**145**	No binding	> 1000	Agonist
CD437	6500	2480	**77**	> 1000	Agonist
CD2425	1000	1467	712	**54**	Agonist
CD2503	**6**	964	> 1000	> 1000	Antagonist
CD2665	> 1000	**306**	**110**	> 1000	Antagonist

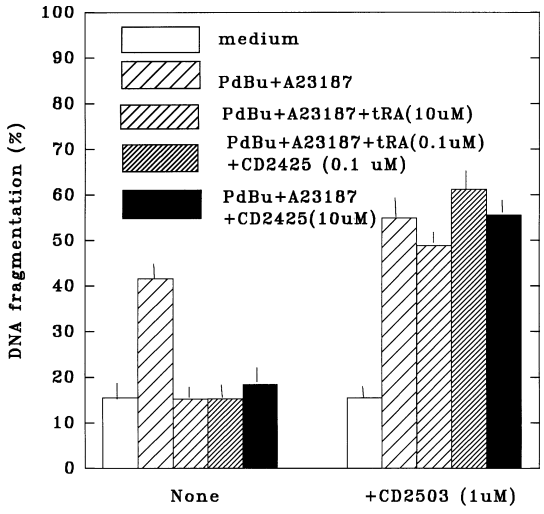

Figure 3 Effect of an RARα receptor antagonist on the prevention of activation-induced cell mediated by various retinoids

Column 1, spontaneous apoptosis in the culture; column 2, cell death induced by the addition of 5 ng/ml phorbol dibutyrate (PdBu) and 0.5 μM A23187; column 3, prevention of activation-induced cell death by 10 μM all-*trans*-RA; column 4, prevention of activation-induced cell death by all-*trans*-RA (0.1 μM) with CD 2425 (0.1 μM), an RXR receptor agonist; column 5, prevention of activator-induced cell death by CD2425 (10 μM), in the presence or absence of CD 2503, an RARα receptor antagonist (1 μM). The compounds were added at the beginning of culturing of isolated thymocytes. At 6 h, thymocytes were harvested and tested for the amount of fragmented DNA as described. Results are means $\pm$ S.D. for three determinations. Abbreviation: uM, μM.

Two RARα-selective agonists, however, effectively inhibited activation-induced death (Figure 2, Table 1). The EC_{50} values for inhibition of apoptosis by the compounds were 10.1 and 30 nM respectively for CD2081 and CD336. These results suggested that RARα stimulation alone might be responsible for the observed inhibition by all-*trans*- and 9-*cis*-RA. To prove this, CD2503, an RARα antagonist, was added to the thymocyte culture. Increasing concentrations of CD2503 alone had no effect on the rate of spontaneous apoptosis (results not shown) but slightly stimulated activation-induced death (Figure 3). This

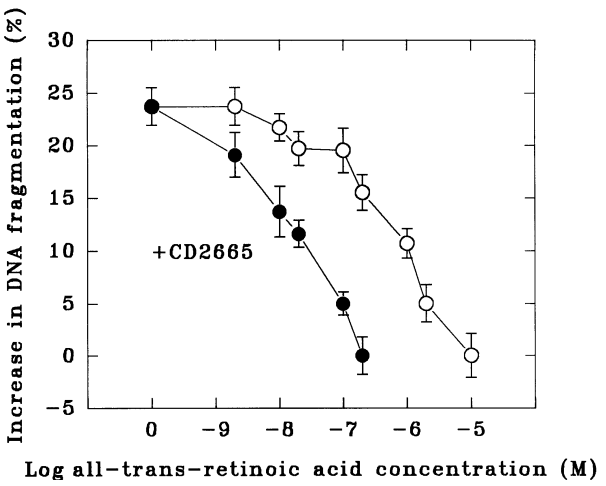

Figure 4 Inhibition of activation-induced apoptosis of thymocytes by simultaneous addition of all-*trans*-RA and an RARγ antagonist

Thymocytes (10^7/ml) were separated and induced to die by the addition of 5 ng/ml phorbol dibutyrate and 0.5 μM A23187. They were cultured without (○) or with (●) 0.1 μM CD2665, an RARγ antagonist, in the presence of increasing concentrations of all-*trans*-RA. At 6 h, thymocytes were harvested and tested for the amount of fragmented DNA as described. Results are means ± S.D. for three determinations.

Table 3 RARγ agonist inhibits prevention of activation-induced death by an RARα agonist

This inhibition is suspended by RXR co-stimulation. Thymocytes (10^7/ml) were separated cultured alone or induced to die by addition of 5 ng/ml phorbol dibutyrate (PdBu) and 0.5 μM A23187. The inhibitory effects of CD336 (0.3 μM) (an RARα agonist) alone, in the presence of CD437 (0.3 μM) (an RARγ agonist) or in the combined presence of CD437 (0.3 μM) (an RARγ agonist) alone and CD2425 (0.3 μM) (an RXR agonist) on activation-induced death are shown. Results are means ± S.D. for three determinations. * Significantly different from the PdBu + A23187 + CD336-treated culture as determined by Student's paired *t* test, $P < 0.05$. † Significantly different from the PdBu + A23187 + CD336 + CD437-treated culture as determined by Student's paired *t* test, $P < 0.05$.

Treatment	DNA fragmentation (%)
Control	11.1 ± 2.6
PdBu + A23187	40.5 ± 4.1
PdBu + A23187 with CD336	12.7 ± 2.4
PdBu + A23187 with CD336 + CD437	39.6 ± 3.5*
PdBu + A23187 with CD336 + CD437 + CD2425	13.9 ± 3.1†

observation suggests that freshly isolated thymocytes might contain a certain amount of endogenous all-*trans*-RA, and when RARα is inhibited by CD2503 the RARγ pathway stimulated by the endogenous all-*trans*-RA enhances activation-induced death. If an effective (1 μM) concentration of CD2503 was added to the culture it completely prevented inhibition of activation-induced death by retinoids (Figure 3), suggesting that retinoids mediate their anti-apoptotic effect via the RARα receptor.

Prevention of activation-induced apoptosis by RARα is modulated by co-stimulation of RARγ and RXR

Because CD336, CD2081 and all-*trans*-RA are nearly equally potent inducers of RARα, the difference in their dose–response curve related to apoptosis inhibition suggests that other nuclear receptors co-stimulated by the panagonist retinoic acids modulate the effect of retinoids on the RARα inhibitory pathway. One likely candidate is RARγ, because the comparison of K_d(RARα)/K_d(RARγ) values of the RAR-selective compounds (Table 2) with their effective concentrations inhibiting apoptosis shows that the more specific a compound is for RARα, the higher is its potential for inhibiting activation-induced apoptosis. This assumption is strongly supported by the observation that the addition of an RARγ antagonist (CD2665) significantly decreases the concentration of all-*trans*-RA required to inhibit activation-induced death (Figure 4). Additionally, co-stimulation of RARγ can inhibit the prevention of activation-induced death by RARα (Table 3).

It looks, however, as though the inhibitory effect of the activated RARγ receptor on the RARα preventive pathway can be suspended by RXR co-stimulation, because (1) 9-*cis*-retinoic acid is a more effective inhibitor of activation-induced apoptosis than all-*trans*-retinoic acid (Figure 1), (2) co-stimulation of RXR decreases the concentration of all-*trans*-RA required to prevent cell death (Figure 1), (3) the protective function of all-*trans*-RA can be observed at higher concentrations when a higher amount of 9-*cis*-RA conversion from all-*trans*-RA can be expected and this might co-stimulate RXRs (Figure 1), and (4) in the presence of RXR agonist, co-stimulation by CD437 (an RARγ agonist) cannot inhibit the RARα preventive pathway (Table 3).

If the prevention of activation-induced death is mediated via RARα, why can an RXR analogue alone prevent activation-induced death (Figure 2)? The experiments done with CD2503 suggested that primary isolated thymocytes might contain some endogenous all-*trans*-RA that is not lost during the fast isolation procedures. If this is so, the role of the RXR analogue could be to decrease the concentration of all-*trans*-RA required to inhibit cell death. This reduced required concentration of all-*trans*-RA in the presence of RXR analogue might then be close to the endogenous concentration. To test this possibility we applied CD2503 (RARα antagonist) to see whether it is able to inhibit the prevention of activation-induced death by the RXR analogue. As is shown in Figure 3, this was indeed so. This suggests that the prevention of activation-induced death by the RXR analogue also involved RARα.

Table 4 CD336, an RARα agonist, prevents anti-CD3 induced thymic involution and increase in the tissue transglutaminase activity

Mice were treated with 80 μg of anti-CD3 antibody with or without 0.4 mg of CD336 intraperitoneally and killed 1 day later. The thymus was removed and its weight was measured. Tissue transglutaminase activity was determined, as described in the Materials and methods section. Results are changes in thymus weight and tissue transglutaminase activity after apoptosis induction *in vivo*, as means ± S.D. from determinations on three mice. * Significantly different from values of the control animals as determined by Student's paired *t* test, $P < 0.05$.

Treatment	Thymus weight (mg)	Tissue transglutaminase activity (nmol/h per mg of protein)
Control	88.0 ± 6.7	1.45 ± 0.8
CD336	87.1 ± 6.5	2.32 ± 0.7
anti-CD3	41.1 ± 5.7*	16.10 ± 1.2*
anti-CD3 + CD336	84.3 ± 6.1	3.17 ± 0.5

Prevention of anti-CD3-induced apoptosis in the thymus by the RARα-selective retinoid analogue CD336

Induction of apoptosis *in vivo* through the well-defined pathway of TCR/CD3 stimulation results in thymus involution, selective death of the CD4+CD8+ population [1], endonuclease ac-

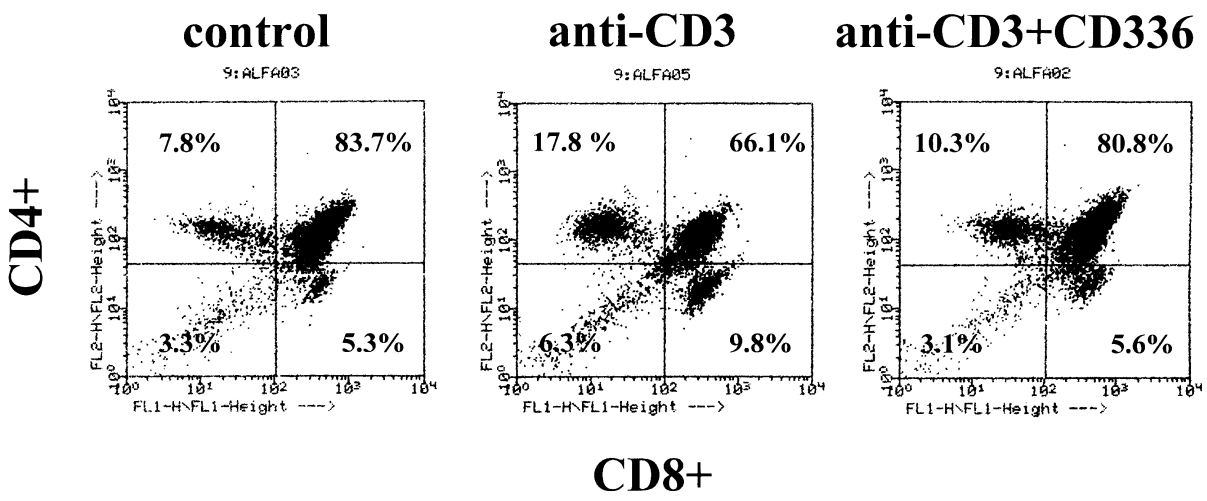

Figure 5 Effect of CD336 treatment on the survival of different subpopulations of thymocytes induced to die *in vivo* with anti-CD3 antibody with respect to the expression of CD4/CD8 receptors

Mice (control) treated with 80 μg of anti-CD3 antibody without or with 0.4 mg of CD336 were killed 24 h after treatment. The thymocytes were isolated and stained with FITC-labelled anti-CD8 and phycoerythrin-labelled anti-CD4 as described in the Materials and methods section.

Figure 6 Prevention of induction of tissue transglutaminase expression *in vivo* via anti-CD3 treatment in thymocytes by an RARα agonist

Mice, control (lane 1), treated with 0.4 mg of CD336 (lane 2), 80 μg of anti-CD3 antibody with 0.4 mg of CD336 (lane 3) or 80 μg of anti-CD3 antibody alone (lane 4), were killed 24 h after treatment. Western blots for transglutaminase (tTG) expression were performed as described in the Materials and methods section. Lane L, tissue homogenate from mouse liver in which tTG is constitutively expressed. tTG indicates the position of the electrophoretic migration of mouse tTG (77 kDa).

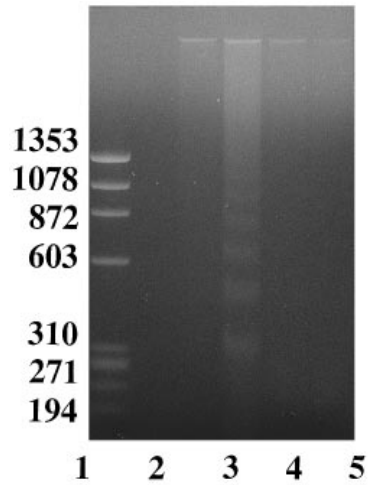

Figure 7 Electrophoretic fractionation of DNA extracted from freshly isolated mouse thymocytes after apoptosis induction *in vivo*

Thymocytes were freshly isolated from mice that had been treated with various compounds for 24 h. The DNA was extracted, electrophoresed on a 1.8% (w/v) agarose gel as described in the Materials and methods section and revealed after staining with ethidium bromide. Lane 1, DNA molecular mass markers (molecular masses shown in bp at the left); lane 2, freshly isolated thymocytes from non-treated animals; lane 3, freshly isolated thymocytes from anti-CD3 (80 μg)-treated animals; lane 4, freshly isolated thymocytes CD336 (0.4 mg)-treated animals; lane 5, freshly isolated thymocytes from anti-CD3 (80 μg) and CD336 (0.4 mg)-treated animals.

tivation [5] and induction and activation of the tissue transglutaminase [9]. To test whether an RARα agonist could also function under *in vivo* conditions, 4-week-old NMRI mice were treated with 80 μg of anti-CD3 antibody; and the possible anti-apoptotic effect of CD336 was tested by analysing changes in thymic weight, tissue transglutaminase expression and activity, endonuclease activity and thymocyte subpopulations 24 h later. The dose of CD336 was chosen as 0.4 mg because preliminary studies showed that this amount was required for the complete prevention of anti-CD3-induced thymic involution.

As shown in Table 4, anti-CD3 treatment induced a 50% decrease in thymic weight 1 day after antibody injection. The decrease in thymic weight was related to a selective decrease in the CD4+CD8+ subpopulation, from 83% to 66% (Figure 5). CD336, at the dose used, did not affect thymic weight or subpopulations, but prevented both the decrease in thymic weight and the loss of the CD4+CD8+ subpopulation induced by anti-CD3 treatment. Additionally, anti-CD3 treatment induced an 8-fold increase in tissue transglutaminase activity, which was prevented by the addition of CD336 (Table 4). The activation of tissue transglutaminase was related to an increased expression of the enzyme protein, which again was inhibited by CD336 (Figure 6). Finally, endonuclease activation with the characteristic 'DNA ladder' formation was also prevented by CD336 (Figure 7). Our results suggest that RARα analogue can also protect CD4+CD8+ thymocytes *in vivo* from TCR-mediated death.

DISCUSSION

The complexity of the differential expression and interaction of RARs and RXRs makes understanding the physiology of action of retinoic acids extremely difficult. There are two possible approaches that might dissect the complexity of retinoid biology.

The first is either knocking out each receptor by homologous recombination [38–40] or expressing their dominant-negative variants [21]. The second uses receptor-selective retinoid analogues and antagonists to stimulate or block one or more of the RARs. In our experiments the second method was used to study the involvement of various RARs in inhibiting activation-induced death of thymocytes. The results presented above demonstrate that 9-*cis*-RA is more effective than all-*trans*-RA in inhibiting TCR-mediated death, and both RAR and RXR stimulation achieved by the panagonist 9-*cis*-RA is required for the observed effect at physiological concentrations. These results are in good agreement with previous studies done on activation-induced apoptosis of T-cell hybridomas [20,21]. Our study also demonstrates that retinoic acids mediate their effect on the activation-induced death of thymocytes via the RARα receptor. This is suggested because (1) the phenomenon can be reproduced by various RARα agonists both *in vitro* and *in vivo*, (2) the effect of RAs can be inhibited by addition of an RARα antagonist, and (3) CD4+CD8+ thymocytes that die on TCR stimulation express RARα [37].

Interestingly, we have also found that RARγ stimulation enhances the rate of activation-induced death (Figure 2) and counteracts the protective action of RARα stimulation (Table 3). Although we have shown that RARγ stimulation induces death in mouse thymocytes [37], this enhancement does not seem to be a simple additive effect because the RARγ agonist did not, for example, stimulate the dexamethasone-induced death (results not shown). The observations that RARγ stimulation enhances the activation-induced death (Figure 2) and counteracts the inhibitory effect of RARα (Table 3) suggest that the balance between stimulation by RARα and RARγ will decide whether a retinoid will enhance or inhibit TCR-mediated death. At physiological concentrations of all-*trans*-RA the stimulation of RARα and RARγ are in balance, with no change in the net rate of activation-induced death. If, however, RARγ is inhibited (Figure 4) or RXR is co-stimulated (Figure 1), the RARα-mediated pathway starts to operate and a strong decrease in the rate of activation-induced death can be detected. These results suggest that the role of RXR co-stimulation, which is achieved under physiological conditions by the appearance of 9-*cis*-RA within the cells, is to facilitate the RARα-mediated pathway.

We have previously shown that retinoic acids can induce apoptosis in mouse thymocytes by stimulating RARγ [37]. This pathway was also inhibited by RARα co-stimulation, suggesting that a balance between RARα and RARγ stimulation regulates the apoptosis induction as well. Interestingly, however, RXR co-stimulation in this context facilitated the availability of the RARγ and not the RARα-mediated signal transduction pathway. These results suggest that not only the retinoic acid receptors stimulated, but also interaction with other transcription factors activated via TCR stimulation or TCR-mediated modifications of the retinoic acid receptors might affect the outcome of the interactions between RARs and RXRs. In this context it is worth noting that the α, β and γ subfamilies both in the RAR and in the RXR receptor families exist. However, whereas RARs show a time- and tissue-specific expression during mouse development, and various RAR-null mice have characteristic malformations [38,39], the RXRα and β receptors have a widespread (possibly ubiquitous) expression pattern and $RXR\alpha^{+/-}/RXRB^{-/-}/RXR\gamma^{-/-}$ mutant mice are viable [40]. These observations suggest that the specificity of the RAR receptors present and stimulated are critical in the retinoid signalling pathways, whereas one copy of RXRα is sufficient to perform most of the functions of the RXRs. The naturally occurring retinoids are pan-RAR agonists with nearly equal K_d values for the various RARs (Table 2). Because of this lack of selectivity it was at first difficult to explain how natural retinoids can regulate antagonistic RARα and RARγ-mediated signalling pathways in thymocytes. Our results suggest a possible new way through which RXR co-stimulation by affecting the balance between the antagonistic RAR-mediated pathways can modulate the outcome of the retinoid signal transduction pathway.

Our studies are not sufficiently detailed to decide the precise mechanism by which the stimulation of RARα leads to the observed inhibition of apoptosis in mouse thymocytes. The main reason for this is that the specific proteins responsible for the initiation of either RARγ-mediated or TCR-mediated death have not yet been determined. It is worth noting, however, that the expression of tissue transglutaminase, an effector element of apoptosis [41], was increased in thymocytes if apoptosis was induced via either the RARγ-mediated [37] or the TCR-mediated pathway [9] *in vivo*, whereas it was not if RARα was co-stimulated. The promoter of this protein contains a 30 bp retinoid response element (mTGRRE1) that consists of three hexanucleotide half-sites in the DR7/DR5 motif. This response element binds both RAR/RXR heterodimers and RXR homodimers, can be partly activated by RAR or RXR agonists, but for the full activation the panagonist 9-*cis*-RA is required [42]. It is conceivable that genes with such regulatory elements might be targets of the complex effects of retinoids described above.

What might the biological significance of these findings be? The concentration of all-*trans*-RA needed to inhibit activation-induced apoptosis in thymocytes is much higher than its physiological level *in vivo*, which is estimated to be 12 nM [43–45] (although its concentration might vary around the cells owing to local retinoid production [46]). However, the apoptosis-regulating effect of 9-*cis*-RA occurs at a much lower concentration. 9-*cis*-RA was shown to be generated by the isomerization of all-*trans*-RA, being in equilibrium with and depending on the concentration of all-*trans*-RA [47]. Therefore, if the circulating all-*trans*-RA is converted to the 9-*cis* ligand in sufficient amounts in thymocytes, the RA-mediated protection against TCR-mediated death can be observed.

Glucocorticoids, RAs and the TCR might regulate the positive selection of thymocytes in a co-ordinated manner. Glucocorticoids that also inhibit TCR-induced cell death were suggested to increase the threshold at which an antigen is recognized as a high-affinity ligand and initiates negative selection [48]. At low concentrations of glucocorticoid, retinoids proved to be additive in inhibiting TCR-mediated cell death [10], suggesting that retinoids and glucocorticoids might simultaneously affect the number of positively selected thymocytes. Additionally, RAs might modulate other TCR-mediated differentiation processes because selective overexpression of the RARγ receptor in the T cell was shown to affect the CD8+/CD4+ T-cell ratio [49].

We thank Ms. Jolán Csató for technical assistance, and Dr. Jozsef Szentmiklóssy for providing analytical computer programs. This study was partly supported by Hungarian grants from OTKA (T 022 705) and the Ministry of Welfare T (1997).

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Received 11 November 1997/16 December 1997; accepted 14 January 1998