Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6170–6174, July 1993
Immunology

9-*cis*-Retinoic acid inhibits activation-driven T-cell apoptosis: Implications for retinoid X receptor involvement in thymocyte development

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Communicated by Herman N. Eisen, April 5, 1993 (received for review December 24, 1992)

ABSTRACT **Retinoic acid is a morphogenetic signaling molecule derived from vitamin A and involved in vertebrate development. Two groups of receptors, retinoic acid receptors and retinoid X receptors (RXRs), have been identified. All-*trans*-retinoic acid is the high-affinity ligand for retinoic acid receptors, and 9-*cis*-retinoic acid additionally binds RXRs with high affinity. Here we report that although retinoic acid has little inhibitory effect on activation-induced T-cell proliferation, it specifically prevents activation-induced apoptosis of T-cell hybridomas and antigen-specific deletion of immature $CD4^+CD8^+$ thymocytes from $\alpha\beta$ T-cell receptor transgenic mice. 9-*cis*-Retinoic acid was ≈10-fold more potent than all-*trans*-retinoic acid, suggesting that RXRs participate in this process. Thus, although 9-*cis*-retinoic acid has little immunosuppressive activity, it is a potent negative regulator of activation-induced T-cell apoptosis, raising the possibility that RXRs may take part in regulating T-cell development.**

Retinoids are a group of natural and synthetic vitamin A (retinol) derivatives that modulate the growth and differentiation of many different cells *in vivo* and *in vitro* (1, 2). Many effects of vitamin A are mediated by retinoic acid (RA), and several lines of evidence have shown that RA is a necessary morphogenetic signaling molecule that is produced in local tissues during vertebrate development (3, 4). RA also has a profound influence on immune cells and immunological responses (5). Similar to steroid/thyroid hormones, RA binds to intracellular receptors that in turn bind specific responsive elements (RAREs) and regulate gene transcription (6–8). Two families of retinoid receptors, RA receptors (RARs) and retinoic X receptors (RXRs), have been identified (9–11). All-*trans*-RA binds RARs with high affinity, and its 9-*cis* stereoisomer is a high-affinity ligand for both RARs and RXRs (12, 13). RXRs can form homodimers in the presence of 9-*cis*-RA (14) or can heterodimerize with RARs. Such heterodimers bind and trans-activate responsive elements much better than RAR or RXR homodimers (15–17). Although in transient transfection assays RA-responsive genes can usually be regulated by both RARs or RXRs, there are RAREs that have been found to respond to all-*trans*-RA much better when the responsive elements are cotransfected with RXRs rather than RARs. Studies on one such RARE, from the *CRABPII* gene, found that although the binding of RAR–RXR heterodimers can mediate transactivation, 9-*cis*-RA is more effective than all-*trans*-RA at stimulating a response (18). It is not yet clear how these molecular interactions are related to the physiological and pharmacological effects of RA.

Activation of T-cell hybridomas and thymocytes via the T-cell antigen receptor (TCR) induces programed cell death (PCD), which is thought to be the mechanism of thymocyte negative selection (19–21). Glucocorticoids prevent this response (22, 23) and it has been speculated that this antagonism may be mediated by functional or physical interactions between the glucocorticoid receptor and nuclear transcription factors (24). As retinoid receptors represent another example of intracellular receptors/transcription factors, we investigated the effect of RA on activation-induced death of T-cell hybridomas and thymocytes.

MATERIALS AND METHODS

Mice. Mice expressing transgenic TCR $\alpha\beta$ chains specific for H-Y antigen presented by $H\text{-}2D^b$ (25) were maintained in our mouse facility (a breeding pair was kindly provided by Wendy Shores and Al Singer, National Institutes of Health). Male and female C57BL/6 mice were obtained from the Animal Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health.

Cells, Antibodies, and Reagents. 2B4.11 is a murine T-cell hybridoma specific for the antigen pigeon cytochrome *c* (26). 145-2C11 (2C11) is a hamster anti-mouse CD3-ε monoclonal antibody (27). Cytotoxic T-cells specific for H-Y in the context of $H\text{-}2D^b$ were kindly provided by Polly Matzinger and John Ridge, National Institutes of Health). All-*trans*- and 9-*cis*-RAs were kindly provided by Keiko Ozato (National Institutes of Health) and Joseph Grippo (Hoffmann–La Roche).

DNA Fragmentation Assay. DNA fragmentation was quantitated by adherence to fiberglass filters, as described (28, 29). Briefly, the pigeon cytochrome *c*-specific 2B4.11 T-cell hybridoma (26) was incubated with [^{3}H]thymidine (5 μCi/ml; 1 Ci = 37 GBq) for 3 hr. After thorough washing, 5×10^4 cells were distributed in triplicate into 96-well microtiter plates that had been coated with purified 2C11 at 1 μg per well as described (19) or treated with complete medium [RPMI medium 1640 supplemented with 2 mM glutamine, 50 μM 2-mercaptoethanol, antibiotics, and 10% (vol/vol) fetal calf serum]. After 16 hr of culture at 37°C, the cells were hypotonically lysed and harvested onto fiberglass filters (PHD Cell Harvester, Cambridge Technology, Cambridge, MA). Intact chromatin adheres to the filters, but DNA fragments pass through (28, 29). The results are expressed as specific DNA fragmentation: $[(M - E)/M] \times 100$, where M is the retained label in cells cultured in medium and E is retained label in cells cultured under experimental conditions.

Growth Inhibition Assay. 2B4.11 cells (2×10^4 cells) were cultured in complete medium in either normal or 145-2C11-coated (1 μg per well) 96-well plates in the presence of various concentrations of 9-*cis*-RA. After 16 hr of culture at 37°C, 1

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Abbreviations: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; TCR, T-cell antigen receptor; PCD, programed cell death; IL-2, interleukin 2; APC, antigen-presenting cell; Dex, dexamethasone.

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μCi of [^{3}H]thymidine was added to each well. After another 4 hr, the cells were harvested and incorporation of radiolabel was determined by liquid scintillation counting. The data are expressed as the amount of cpm incorporated in each experimental condition divided by the cpm incorporated by cells grown in medium alone.

T-Cell Proliferation Assay. C57BL/6 spleen cells (2×10^5 cells) were cultured in triplicate in microtiter wells coated with the indicated amounts of 2C11 and the indicated concentrations of all-*trans*-RA or 9-*cis*-RA. After 24 hr, an aliquot of supernatant was removed for interleukin 2 (IL-2) assay, and after 48 hr, 1 μCi of [^{3}H]thymidine was added to each well. The cells were harvested 16 hr later and incorporation of radiolabel was determined by liquid scintillation counting.

Measurement of Thymocyte Deletion. *In vitro* antigen-specific deletion of TCR αβ transgenic CD4$^+$CD8$^+$ thymocytes was assayed by the method of Swat *et al.* (30). Briefly, 4×10^6 thymocytes from *H-2^b* female αβ transgenic mice were cultured on confluent monolayers of plastic-adherent male or female C57BL/6 antigen-presenting cells (APCs) made from thymus fragments, as described (30), in the presence of the indicated concentrations of all-*trans*- or 9-*cis*-RA. After 36 hr, the cells were harvested and stained with fluorescein isothiocyanate-labeled anti-CD8 antibodies and biotin-labeled anti-CD4 antibodies. The latter were developed with phycoerythrin-conjugated avidin. Fluorescence staining was determined with a FACScan flow cytometer (Becton Dickinson); forward scatter and side scatter were used to gate out debris but not dead cells. The data were analyzed with FACScan software.

Cytolytic Assay. C57BL/6 thymic male or female APCs were cultured for 36 hr with either 9-*cis*-RA (1 μM) or medium alone. They were then labeled with $Na_2{}^{51}CrO_4$ (ICN) and plated in triplicate in flat-bottom 96-well microtiter plates at 5×10^3 cells per well. A cytotoxic T-cell clone specific for H-Y in the context of H-2D^b was added at the indicated effector-to-target ratios, and 16 hr later specific release of ^{51}Cr was determined as described (19).

RESULTS

RA Prevents Activation-Induced T-Cell Hybridoma Death. To determine whether RA could influence activation-induced T-cell PCD, all-*trans*-RA was added to cultures of the 2B4.11 T-cell hybridoma in the presence or absence of an activating stimulus. All-*trans*-RA by itself had no effect on T-cell viability as assessed by either specific fragmentation of DNA (Fig. 1*A*) or release of ^{51}Cr (data not shown). Immobilized anti-CD3 antibodies alone caused ≈50% specific DNA fragmentation in the T-hybridoma cells. However, activation-dependent PCD was prevented by all-*trans*-RA in a dose-dependent fashion, almost complete inhibition being achieved at 10 μM. Identical results were obtained using release of ^{51}Cr as a measure of cell death (data not shown).

9-*cis*-RA Is More Potent Than All-*trans*-RA in Preventing PCD. Studies using receptor and reporter gene cotransfection have shown that although all-*trans*-RA and 9-*cis*-RA are equipotent for stimulation of RARs, 9-*cis*-RA is 10- to 30-fold better than all-*trans*-RA at activating RXRs (11, 13). To assess which type of RA mediates inhibition of PCD, we compared the potency of retinol, all-*trans*-RA, and 9-*cis*-RA in the DNA fragmentation assay. None of these reagents induced PCD by themselves. 9-*cis*-RA was 10-fold more potent than all-*trans*-RA at preventing anti-TCR-mediated PCD in three experiments, one of which is shown in Fig. 1*B*. Retinol itself had only a small effect on this process and then only at the highest concentration (perhaps due to the conversion of retinol to 9-*cis*-RA by the cells). 13-*cis*-RA was also 10-fold less potent than 9-*cis*-RA in preventing 2B4.11 PCD (data not shown). This hierarchy is typical for phenomena mediated by RXRs (11, 13). 9-*cis*-RA also blocked DNA fragmentation caused by activation with antigen (pigeon cytochrome *c*) or the combination of phorbol ester plus a Ca^{2+} ionophore, ionomycin (data not shown). To determine whether 9-*cis*-RA could block PCD caused by a nonactivating stimulus, the synthetic glucocorticoid dexamethasone (Dex) was used (Fig. 1*C*). As described (22), overnight treatment with Dex caused substantial DNA fragmentation in 2B4.11 cells in a dose-dependent manner. Unlike activation via the TCR, however, addition of 1 μM 9-*cis*-RA did not prevent Dex-induced PCD.

Effects of RA on Other Measures of T-Cell Activation. Activation-induced T-cell hybridoma growth inhibition is largely the consequence of a G_1/S-phase cell-cycle block (26). This response is distinct from activation-induced PCD as judged by kinetics (growth inhibition occurs within 1 hr and PCD is first detected at 4–6 hr) and by the observations that PCD but not growth inhibition is prevented by removing Ca^{2+} from the medium and that cyclosporin A prevents PCD but not the cell-cycle block (31). To determine whether 9-*cis*-RA globally prevents T-cell hybridoma activation, proliferation of 2B4.11 cells activated with anti-CD3 antibodies in the presence or absence of 9-*cis*-RA was measured. Unlike its effect on PCD, 9-*cis*-RA had little effect on anti-TCR-induced growth inhibition (Fig. 2). IL-2 production by activated 2B4.11 cells was also tested and was consistently found to be partially inhibited (typically ≈50%) by all-*trans*-RA and

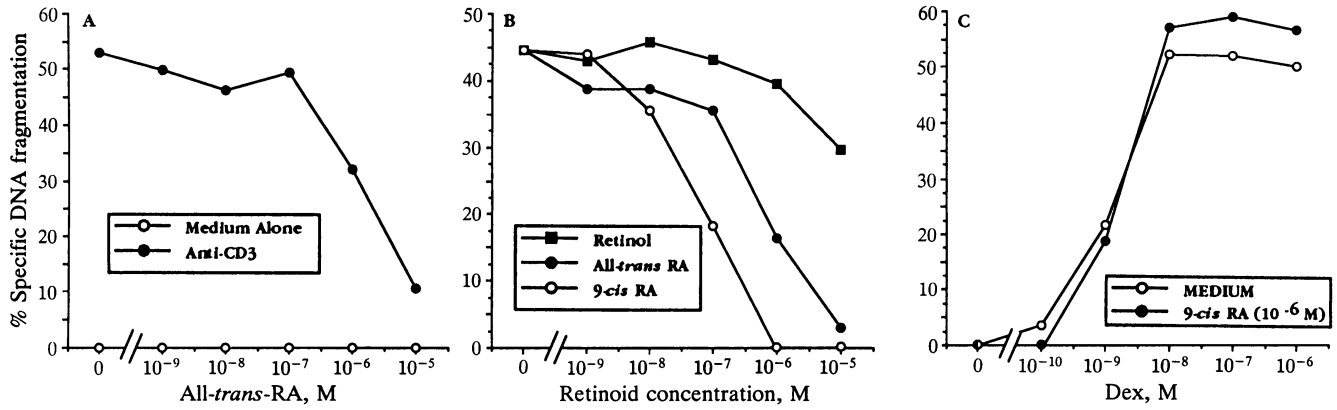


FIG. 1. RA prevents activation-induced but not glucocorticoid-induced PCD. [^{3}H]Thymidine-labeled 2B4.11 T hybridoma cells were cultured with the indicated concentrations of retinoids in anti-CD3 antibody (2C11)-coated plastic microtiter wells (*A* and *B*) or in Dex (*C*). Specific DNA fragmentation was measured after 16 hr. None of the retinoids caused DNA fragmentation by themselves (*A*, open circles; *B*, data not shown). The SEM for each experimental point was always ≤5%.

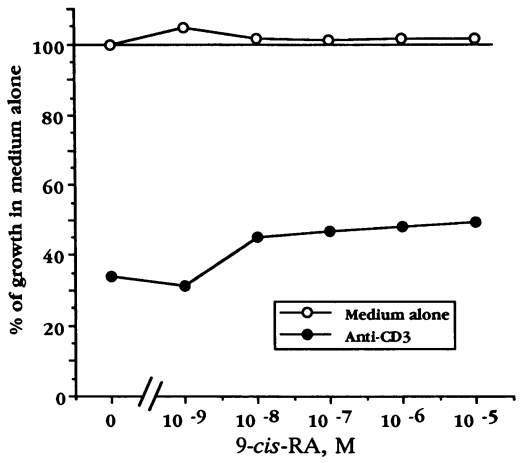


FIG. 2. RA does not prevent activation-induced T-cell hybridoma growth inhibition. Approximately 2 × 10^4 2B4.11 cells were cultured in complete medium in normal or 2C11-coated (1 μg per well) 96-well plates in the presence of various concentrations of 9-*cis*-RA. After 16 hr of culture at 37°C, incorporation of [^{3}H]thymidine was measured and growth inhibition was determined.

9-*cis*-RA (data not shown). To determine whether RA is immunosuppressive for normal cells, splenocytes were stimulated *in vitro* with anti-CD3 antibodies in the presence of various amounts of all-*trans*- or 9-*cis*-RA (Fig. 3). All-*trans*-RA had virtually no effect on T-cell proliferation, and 9-*cis*-RA had only a modest inhibitory effect and then only at lower levels of anti-CD3 stimulation. The supernatant from these wells contained IL-2 (10–15 units/ml); no inhibition of IL-2 production was detected at any concentration of either RA stereoisomer (data not shown). Thus, RA has only a small inhibitory effect on activation of normal T cells.

RA Prevents Antigen-Specific Deletion of Thymocytes. TCR-mediated PCD occurs in thymocytes and is thought to be the major mechanism for eliminating cells bearing TCRs with high affinity for self antigens during T-cell ontogeny (negative selection) (21, 32). Experiments were performed to determine whether the effects of RA on TCR-mediated PCD could be extended to the process of negative selection. Swat *et al.* (30) have shown that culture of thymocytes from antigen-specific TCR αβ transgenic mice with the appropriate antigen results in a reduction in the levels of CD4 and CD8 on $CD4^+CD8^+$ thymocytes; these $CD4^{lo}CD8^{lo}$ cells were shown to be undergoing PCD (30). We performed experiments in which thymocytes from female TCR αβ transgenic mice, for which >90% of the $CD4^+CD8^+$ cells express a TCR that recognizes the male-specific H-Y antigen, were cultured on a monolayer of either female or male thymic APCs. After 36 hr, most of the $CD4^+CD8^+$ cells from female thymocytes cultured with female APCs expressed normal levels of CD4 and CD8, although a small number manifested the $CD4^{lo}CD8^{lo}$ phenotype of dying cells (Fig. 4*A*) (30). Culture of thymocytes from female mice with male thymic APCs ($H\text{-}Y^+$) resulted in a loss of two-thirds of the $CD4^+CD8^+$ cells, with a concomitant increase in the $CD4^{lo}CD8^{lo}$ cells undergoing PCD (Fig. 4*B*). Culture with 0.1 μM all-*trans*-RA resulted in partial recovery of $CD4^+CD8^+$ cells, and 1 μM all-*trans*-RA completely blocked antigen-specific deletion (Fig. 4 *C* and *E*). 9-*cis*-RA (10 nM) also caused partial recovery of normal $CD4^+CD8^+$ cells (≈40% reversal of deletion) and at 0.1 μM was as effective as 1 μM all-*trans*-RA (Fig. 4 *D* and *F*). Further increases in all-*trans*-RA or 9-*cis*-RA concentrations did not result in further increases in the number of $CD4^+CD8^+$ cells (data not shown). The 10-fold difference in potency between 9-*cis*-RA and all-*trans*-RA suggests that activation of RXRs is a potent means of inhibiting activation-induced negative selection. As originally described in this system (30), H-Y-specific female thymocytes cultured with male APCs exhibited DNA fragmentation; this was also prevented by culture with RA (data not shown).

It is possible that RA prevented antigen-specific thymocyte deletion by downregulating expression of the TCR's ligand ($H\text{-}Y/H\text{-}2D^b$) on the thymic APCs. To test this, male and female APCs were cultured with or without 9-*cis*-RA for 36 hr and used as targets for an $H\text{-}Y/H\text{-}2D^b$-specific cytotoxic T-lymphocyte clone (Fig. 4*G*). 9-*cis*-RA did not inhibit antigen-specific T-cell lysis of these APCs, indicating that it does not affect expression of $H\text{-}Y/H\text{-}2D^b$.

DISCUSSION

RARs and RXRs have overlapping but distinct functions and are responsible for the activity of RA. RXRs can form homodimers in the presence of 9-*cis*-RA and bind to target gene response elements (14). In addition, RXRs can form heterodimers with RARs, and the resultant dimers bind response elements at higher affinities than either RAR or

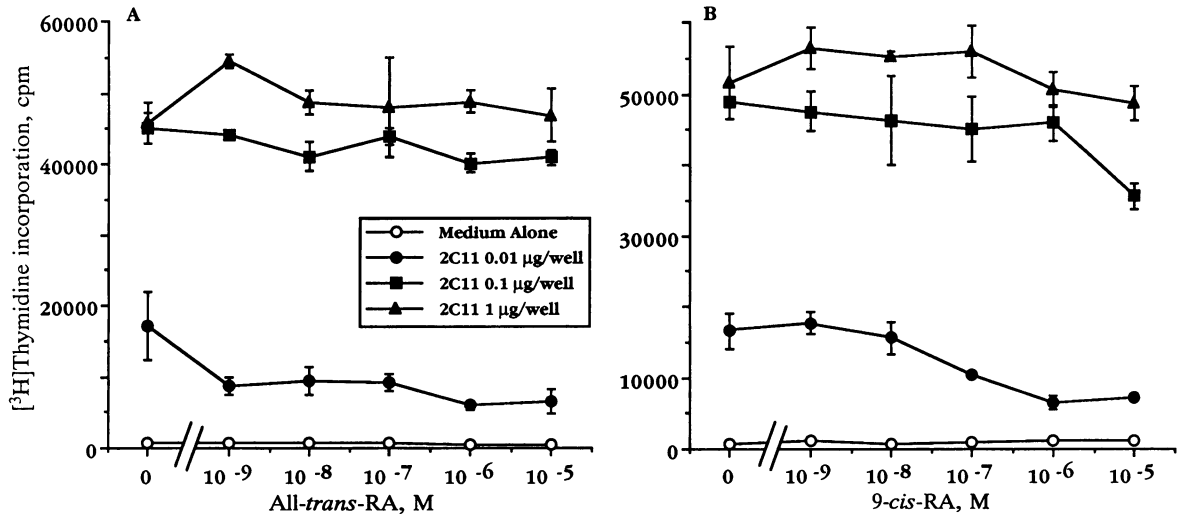
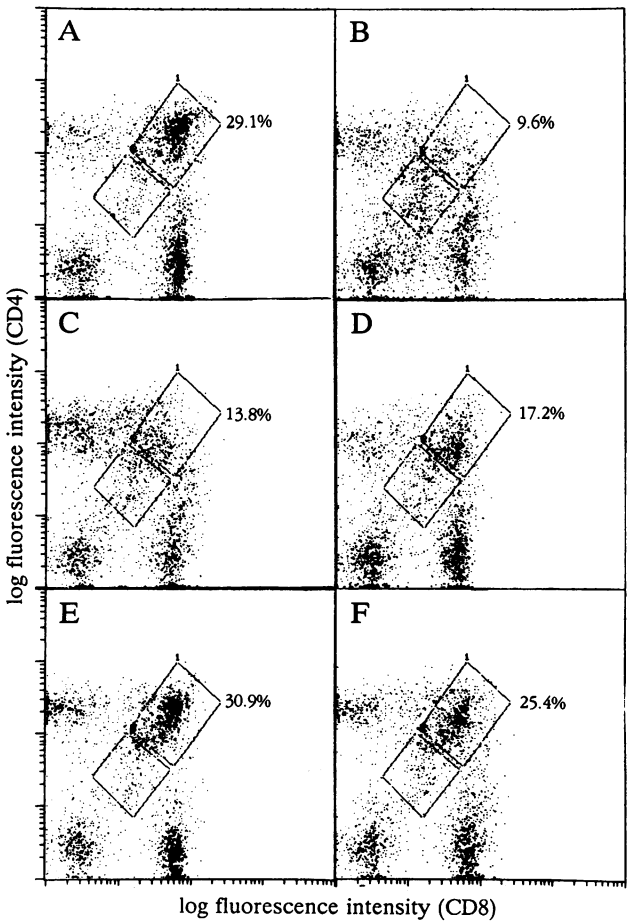


FIG. 3. RA has little effect on splenic T-cell proliferation. Approximately 2 × 10^5 C57BL/6 spleen cells were cultured in triplicate in microtiter wells coated with the indicated amounts of 145-2C11 (2C11) and the indicated concentrations of all-*trans*-RA (*A*) or 9-*cis*-RA (*B*). After 24 hr, an aliquot of supernatant was removed for IL-2 assay, and after 48 hr, 1 μCi of [^{3}H]thymidine was added to each well and its incorporation was measured 16 hr later.



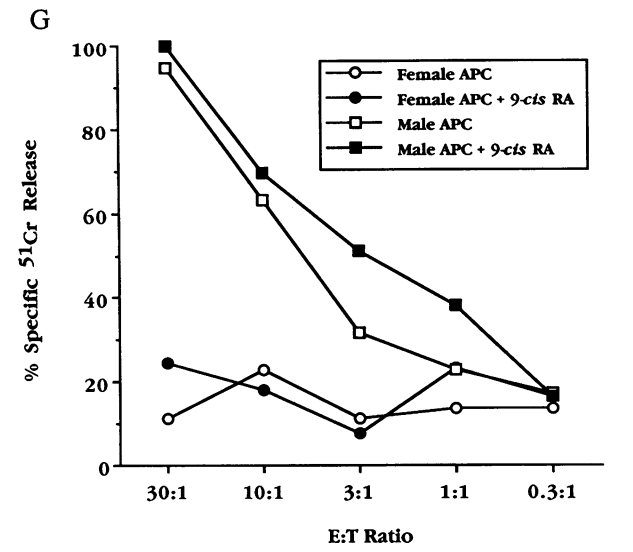


FIG. 4. RA prevents antigen-driven deletion of $CD4^+CD8^+$ thymocytes. (*A–F*) Thymocytes from female mice expressing transgenic TCR $\alpha\beta$ chains specific for H-Y antigen presented by $H\text{-}2D^b$ were cultured with either male ($H\text{-}Y^+$) or female ($H\text{-}Y^-$) APCs from C57BL/6 thymuses. After 36 hr, the CD4/CD8 phenotype of the thymocytes was determined. The population in box 1 represents normal viable $CD4^+CD8^+$ thymocytes. The cells in the other box ($CD4^{lo}CD8^{lo}$) are undergoing PCD (30). The numbers next to box 1 are the calculated fractions of $CD4^+CD8^+$ viable cells. (*A*) Female APCs. (*B*) Male APCs. (*C*) Male APCs plus all-*trans*-RA (0.1 μM). (*D*) Male APCs plus 9-*cis*-RA (10 nM). (*E*) Male APCs plus all-*trans*-RA (1 μM). (*F*) Male APCs plus 9-*cis*-RA (0.1 μM). (*G*) C57BL/6 thymic male or female APCs were cultured for 36 hr with either 9-*cis*-RA (1 μM) or medium alone. Cells were then assayed for susceptibility to lysis by an $H\text{-}Y/D^b$-specific cytolytic T-cell clone. E:T, effector to target.

RXR homodimers (33). RXRs can also heterodimerize with nuclear receptors, such as those for thyroid hormone, vitamin D_3, and others (15–17, 34). Although many RA-responsive genes can be regulated both by RARs and RXRs (8), there may be genes for which expression is regulated predominantly by RXRs (35, 36). It is not yet clear how those molecular interactions relate to the physiologic and pharmacologic effects of retinoids, but it is presumably by regulation of gene transcription that 9-*cis*-RA exerts its effects on PCD.

As these studies were being completed, another group (37) reported that all-*trans*-RA prevented activation-induced T-cell hybridoma death and thymocyte death in response to the combination of anti-TCR plus anti-lymphocyte function-associated antigen antibodies. In addition to these observations, the present work demonstrates that 9-*cis*-RA is much more potent than all-*trans*-RA, strongly implicating the RXR in this phenomenon, and that 9-*cis*-RA prevents antigen-driven deletion in immature thymocytes. Previous data on the effects of 9-*cis*-RA have dealt with its effects on DNA binding and transcriptional regulation of certain genes (12–14, 38). To our knowledge, the data in the present report are the first to demonstrate a specific effect of 9-*cis*-RA on a physiologic immune function. It is of interest that RXRs can heterodimerize with vitamin D_3 receptor *in vitro* (33) and that RXRs are necessary cofactors for vitamin D_3 receptor binding to its response element (39). It may be pertinent that 1,25-dihydroxyvitamin D_3 also blocks activation-induced PCD in 2B4.11 cells (unpublished observation), although the significance of this phenomenon and a possible role for the RXR need further investigation.

Vitamin A deficiency has been associated with many immune function abnormalities (2), some of which may be due to inappropriate and/or deleterious lymphocyte apoptosis. The results with the H-Y-specific TCR transgenic mice indicate that RA can prevent TCR-mediated thymocyte deletion. Would lack of endogenous RA result in enhanced negative selection? In preliminary studies, we have analyzed the effect of RA depletion on thymocyte development. The RA-deficient animals had markedly reduced thymocyte numbers, largely due to loss of $CD4^+CD8^+$ cells, with little change in the number of $CD4^-CD8^-$ cells (unpublished observation). It is an intriguing speculation that in the absence of RA even low-affinity TCR–self antigen interactions are sufficient to result in $CD4^+CD8^+$ cell PCD. Although these observations are consistent with the notion that RA might be involved in T-cell selection, it is also possible that the effect of RA depletion is indirect, affecting thymocyte development by altering, for example, the levels of other biologically active molecules such as glucocorticoids. Studies on the *in vivo* effects of RA in TCR transgenic animals may help elucidate what role this retinoid might play in development of the T-cell antigen-specific repertoire.

We are grateful to Drs. Joseph Grippo and Keiko Ozato for providing all-*trans*-RA and 9-*cis*-RA, Dr. Luigi De Luca and Nadine Darwiche for tissues from vitamin A-deficient mice, Drs. Polly Matzinger and John Ridge for the gift of the anti-H-Y CTL clone, and Dr. Charles Zacharchuk for helpful discussions. We also thank Drs. Zacharchuk, Allan Weissman, and Keiko Ozato for critical review of this manuscript.

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