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EFFECTS OF SYNTHETIC RETINOIDS AND RETINOIC ACID ISOMERS ON THE EXPRESSION OF ALKALINE PHOSPHATASE IN F9 TERATOCARCINOMA CELLS

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Expression of ALP in F9 teratocarcinoma cells is induced by all-trans retinoic acid (ATRA) (Gianni' et al., Biochem. J., 274: 673-678, 1991). The specific ligand for retinoic acid related receptors (RXRs), 9-cis retinoic acid (9-cis RA), and three synthetic analogs binding to the α, β and γ forms of the retinoic acid receptors (RARs), AM580, CD2019, and CD437, were used to study their effects on alkaline phosphatase (ALP) enzymatic activity and mRNA levels. At concentrations close to the Kd for their respective receptors, 9-cis RA, AM580 (the RAR α agonist) and CD437 (the RAR γ agonist) clearly upregulate the expression of the ALP gene, whereas the effect of CD2019 (the RAR β agonist) is very modest. A specific inhibitor of the RAR α, Ro 41-5253, completely blocks the induction of ALP triggered by AM580, while it has minor effects on the upregulation caused by ATRA, 9-cis RA, CD437 and CD2019. The induction of ALP observed with the various retinoids is inhibited by the contemporaneous treatment with dibutyryl cAMP. The levels of the RAR α and γ transcripts are unaltered, while RAR β mRNAs are induced by ATRA, AM580, CD437 and to a lower extent by 9-cis RA and CD2019. © 1993 Academic Press, Inc.

All-trans retinoic acid (ATRA) exerts many of its pleiotropic activities through binding and activation of nuclear receptors (RARs) that positively or negatively modulate the expression of specific sets of genes. RARs belong to the superfamily of nuclear hormone receptors and are subdivided into three classes of proteins, α, β and γ, which are differing in their primary structure and tissue distribution. Each class of RARs is constituted of several members that result from the translation of alternatively spliced mRNAs (1). To exert their regulatory function on responsive genes, RARs heterodimerize with accessory proteins known as RXRs (2,3). Besides their ability to heterodomerize with RARs, RXRs homodimerize (4) and bind specifically to 9-cis retinoic acid (9-cis RA) (5).

To understand the mechanism of action of ATRA, it is important to establish whether the various RAR isoforms are controlling the same or different sets of genes. In faci, certain types of cells and tissues express predominantly one class of RARs, whereas others contain all the three classes (6). Although there are some exceptions (7), genes regulated by ATRA are, in general, equally responsive to the α, β and γ RARs in transient transfection experiments with appropriate reporter genes (8). However, few studies have approached this problem using selected genes regulated by ATRA in their native context.

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The expression of the mouse liver/bone/kidney-type alkaline phosphatase (ALP) gene is induced by ATRA in various cell lines (9-11). In mouse F9 teratocarcinoma cells, the induction of the expression of the ALP gene is transcriptional in nature, it does not require *de novo* protein synthesis and it is correlated with differentiation towards the primitive endoderm. Cotreatment of F9 cells with ATRA and agents that increase intracellular cAMP leads to further differentiation towards the parietal endoderm. In these experimental conditions, the increase in cAMP levels dramatically inhibits the upregulation of ALP mRNA by the retinoid (9).

ATRA has almost the same affinity for the three classes of RARs. Recently, retinoid derivatives that bind specifically and selectively to RAR α, β and γ (12-14) were developed and they permit to study the involvement of receptor subtypes in the biological effects triggered by the retinoid. In this report, 9-cis RA, a specific agonist of both RXRs and RARs (5), and three specific agonists of the RAR α, β and γ, respectively, were used to study the regulation of ALP in F9 teratocarcinoma cells.

MATERIALS AND METHODS

Cell lines and reagents

Mouse F9 is a teratocarcinoma cell line, obtained from Dr. B. Terrana (Sclavo Labratories, Siena, Italy). Cells were seeded in Dulbecco Modified Eagle's medium (DMEM) containing delipidated (stripped of retinoid) 10% (v/v) fetal calf serum, at a concentration of 10^5 cells/ml in 24 well plates or 25 cm^2 Falcon culture flasks (Becton Dickinson, Franklin Lakes, N.J.) and grown for 24 hours before appropriate treatments were performed. Cultures were free from mycoplasma as assessed using the Hoechst 33258 fluorescent dye system (Farbwerke Hoechst AG, Frankfurt, Germany).

{[4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid} [AM580] {6-[3-(1-methylcyclohexyl)-4-methoxyphenyl]-2-naphthoic acid} [CD2019] and {6-[3-(1-adamantyl)-4-hydroxyphenyl]-2-naphthoic acid} [CD437] were kind gifts of Dr. B. Shroot (CIRD Galderma, Sophia Antipolis, Valbonne, France), p-[(E)-2-[3', 4'-Dihydro-4', 4'-dimethyl-7'-(heptyloxy)-2' H-1-benzothiopyran-6'-yl]propenyl] benzoic acid 1', 1'-dioxide [Ro 41-5253] (15) was kindly provided by Dr. M. Klaus (Hoffmann LaRoche, Basel, Switzerland). 9-cis RA was synthesized by oxidation of 9-cis retinaldehyde (16). The purity of the compound was 98 % as assessed by HPLC analysis using a standard of pure 9-cis retinoic acid, kindly provided by Dr. A. Levin (Hoffmann LaRoche, Nutley, NJ). ATRA and dibutyryl-cAMP (db cAMP) were from Sigma (St. Louis, MO). Solutions of db cAMP were directly prepared in DMEM. Stock solutions of the retinoids (10^{-3} M for 9-cis RA and 10^{-2} M for all the other compounds) were prepared in dimethylsulfoxide under dimmed light and stored at -80° C protected from light until use.

Measurement of ALP activity

Cell monolayers were washed twice with 0.9% NaCl, harvested using a rubber policeman and pelleted by centrifugation at 400 g for 10 minutes. Measurement of ALP enzymatic activity was performed on cell homogenates as described (9) One unit of ALP activity is defined as the amount of enzyme capable of transforming 1 μmole of substrate in 1 min at 25°C.

RNA extraction and Northern-blot analysis

Total RNA was prepared from F9 teratocarcinoma cells according to a modification of the guanidium isothiocyanate/CsCl method (9) and used for Northern-blot analysis. RNA samples (10μg) were fractionated on 1% agarose gels containing 6% formaldehyde and blotted onto synthetic nylon membranes (Gene ScreenPlus; New England Nuclear, Boston, MA). These membranes were hybridized with a 2 Kb EcoRI-Bgl II fragment of mouse placental cDNA (17), and mouseRAR α1,β2 and γ1 c-DNAs (18). The various probes were labelled to a specific radioactivity of (1-2) x 10^9 c.p.m./μg by using hexanucleotide primers and [^{32}P] dCTP(19). Hybridization and washing of membranes were performed according to the instruction of the manufacturer. The membranes were dried and exposed to Kodak X-Omat X-ray films with two intensifying screens (Dupont Cronex, Dupont de Nemours, Bad Homburg, Germany) at -70°C.

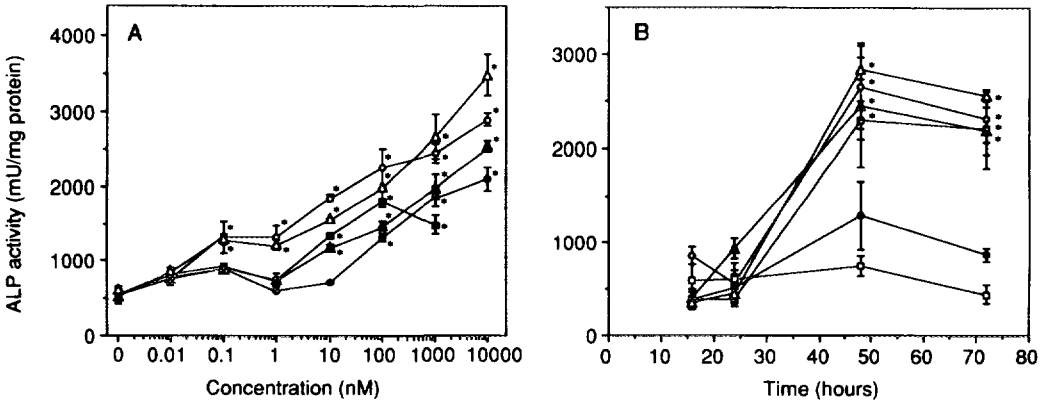

Fig.1. Concentration and time dependent induction of ALP activity by ATRA, 9-cis RA and the specific agonists for the three classes of RARs in F9 teratocarcinoma cells.

A) ALP activity was measured in cell homogenates obtained from F9 teratocarcinoma cells cultured in the presence of increasing concentrations of ATRA (○), 9-cis RA (△), AM580 (▲), CD2019 (●) and CD437 (■) for 48 hours. Each experimental value is the mean ± S.D. of three culture dishes.

B) F9 cells were treated with medium alone (□), or medium containing 10 nM ATRA (○), 10 nM 9-cis RA (△), 8 nM AM580 (▲), 25 nM CD2019 (●), 75 nM CD437 (■), for the indicated amount of time. At the end of each treatment, cells were collected, homogenized and ALP enzymatic activity was measured. Each experimental value is the mean ± S.D. of three separate culture dishes.

* Significantly higher relative to medium alone ($p < 0.01$ after one-way ANOVA and post hoc comparison with the Tukey's test).

RESULTS

When F9 cells are incubated for 2 days in medium alone, the level of ALP enzymatic activity is 547 ± 42 mU/mg protein (Mean ± S.E., n=3). As shown in Fig. 1A, at concentrations between 0.1 and 1 nM, only ATRA and 9-cis RA significantly stimulate the expression of ALP relative to control conditions. Between 100 nM and 10 μM, ATRA, 9-cis RA, AM580 (the RAR-α agonist) and CD2019 (the RAR-β agonist) are capable of inducing ALP activity at least two folds relative to control conditions, whereas CD437 (the RAR-γ agonist) is toxic above 1 μM, therefore its effect above this concentration is not presented. When F9 cells are treated with CD437 and AM580 at 10 nM, ALP activity is significantly higher than in undifferentiated control cells, whereas treatment with CD2019 does not lead to a statistically significant increase in the levels of the enzyme. This concentration is equal to the mean Kd of ATRA for the three RARs (14) and similar to the Kd of 9-cis RA to RXR α and of the specific agonists for their respective receptors (5, 14).

Since the different levels of upregulation of ALP might be due to differences in the kinetics of induction of the enzyme by the various retinoic acid analogs, a time course study was performed. Cells were treated with the various retinoids at concentrations equal to the Kd of each compound for its respective receptor(s). As shown in Fig. 1B, ATRA, 9-cis RA, AM580 and CD437 upregulate ALP by 3-4 folds, while CD2019 augments the levels of the enzyme by less than 2 fold at 48 and 72 hours (in this case, the increase produced by CD2019 does not reach statistical significance). Its effect is much less pronounced than that of all the other compounds tested even though the time course of induction of the ALP enzymatic activity is similar for all the retinoids. In fact, maximal induction of the enzyme is always observed around 48 hours and remains constant at least up to 72 hours.

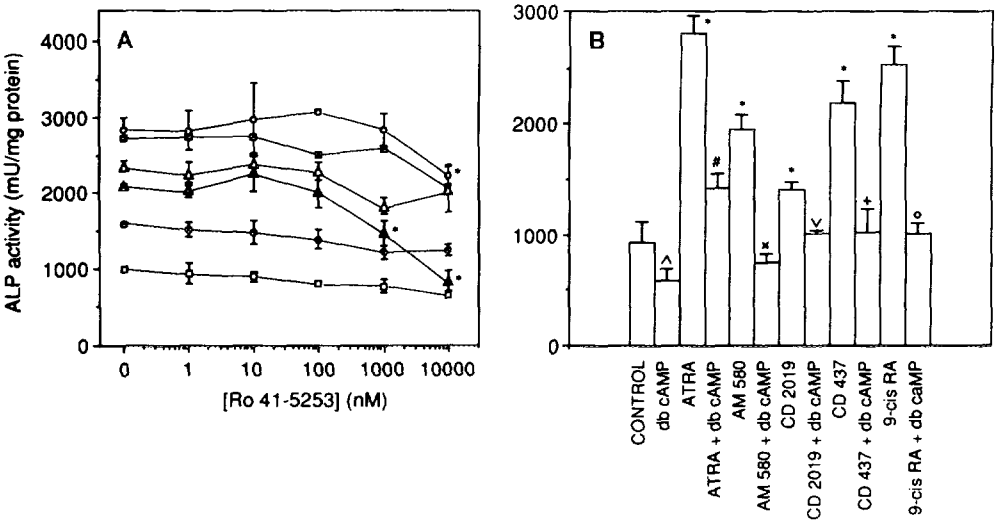

Fig.2. Effect of Ro 41-52-53 and db-cAMP on the induction of ALP activity by ATRA, 9-cis RA and the specific agonists for the three classes of RARs in F9 teratocarcinoma cells.

A) ALP activity was measured in cell homogenates obtained from F9 teratocarcinoma cells cultured with medium alone (□) or medium containing 10 nM ATRA (○), 10 nM 9-cis RA (△), 8 nM AM580 (▲), 25 nM CD2019 (●), 75 nM CD437 (■) in the presence of increasing concentrations of Ro-41-5253 for 48 hours. Each experimental value is the mean ± S.D. of three culture dishes.

* Significantly lower relative to AM 580 or relative to ATRA alone ($p < 0.01$ after one-way ANOVA and post hoc comparison with the Tukey's test).

B) ALP activity was measured in cell homogenates obtained from F9 teratocarcinoma cells cultured with medium alone (control) or medium containing 10 nM ATRA, 10 nM 9-cis RA, 8 nM AM580, 27 nM CD2019, 77 nM CD437 in the absence or in the presence of 1 mM db-cAMP for 48 hours. Each experimental value is the mean ± S.D. of three culture dishes.

* Significantly higher relative to control ($p < 0.01$ after one-way ANOVA and post hoc comparison with the Tukey's test).

Significantly lower ($p < 0.01$ after one-way ANOVA and post hoc comparison with the Tukey's test) relative to control (^), ATRA (#), AM 580 (x), CD 2019 (v), CD 437 (+) and 9-cis RA (○).

To evaluate the specificity of the interaction between retinoids and their receptors, F9 teratocarcinoma cells were treated with ATRA, 9-cis RA and the three specific agonists for the various forms of RAR in the presence of increasing concentrations of Ro 41-5253, a RAR α antagonist. As shown in Fig. 2A, Ro 41-5253 at a concentration of 10 μM slightly decreases the levels of ALP present in undifferentiated F9 cells, however, this inhibition does not reach statistical significance. As expected, the RAR α antagonist inhibits the AM580 mediated induction of ALP by 50% at 1 μM and by more than 90% at 10 μM, while the compound marginally inhibits the ALP upregulation triggered by ATRA, 9-cis RA, CD437 and CD2019 (only the 15 % inhibition observed with ATRA reaches statistical significance). The IC50 for the inhibition of ALP induction by Ro 41-5253 is 1 μM for AM580 and more than 10 μM for ATRA, 9-cis RA, CD2019, and CD 437.

As illustrated in Fig. 2B, the augmentation of the levels of ALP enzymatic activity caused by ATRA, 9-cis RA, AM580 and CD437 are blocked by cotreatment with db-cAMP, demonstrating that the cross-talk between the cAMP and the retinoid dependent intracellular pathways is active regardless of the type of RAR or RXR involved.

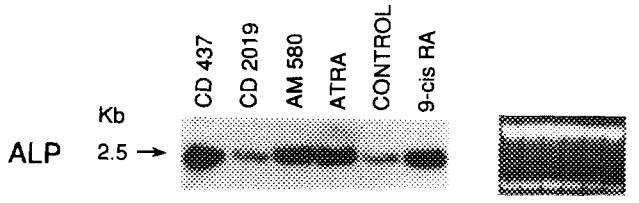

Fig.3. Induction of ALP mRNA by ATRA, 9-cis RA and the specific agonists for the three classes of RARs in F9 teratocarcinoma cells.
Total RNA (20 µg for each lane) was extracted for Northern-blot analysis from F9 cells after 48 hrs incubation in medium alone (control), or in the presence of 10 nM ATRA, 10 nM 9-cis RA, 8 nM AM580, 25 nM CD2019, and 75 nM CD437. The position of the ALP mRNA as well as is indicated by an arrow on the left along with the size in Kb of the transcript. A picture of the ethidium bromide staining of the RNA is shown on the right, demonstrating that approximately equal amounts of RNA were loaded in each lane.

In order to demonstrate that ATRA and the other derivatives are all acting through the same molecular mechanisms, Northern-blot experiments using a specific probe for the mouse ALP were performed. As shown in Fig. 3, ATRA, 9-cis RA, AM580 and CD437 remarkably induce the expression of the ALP mRNA, whereas CD2019 is ineffective in this respect. These data are consistent with the results obtained on ALP enzymatic activity.

To test possible interactions among the various subclasses of RARs and RXRs in the induction of ALP enzymatic activity, F9 teratocarcinoma cells were simultaneously treated with various combinations of the retinoids, each at a concentration equal to one half of the Kd for the respective

TABLE I
Interactions among ATRA, 9-cis RA, AM580, CD2019 and CD437 on the induction of ALP activity in F9 teratocarcinoma cell

Treatment	ALP activity (mU/mg protein)
medium	514 ± 70
ATRA	2321 ± 306 °
9-cis RA	2003 ± 59 °
AM580	1156 ± 92 °
CD2019	751 ± 63
CD437	1814 ± 214 °
ATRA + 9-cis RA	3417 ± 285 °
ATRA + AM580	1674 ± 310 °*
ATRA + CD2019	1881 ± 480 °
ATRA + CD437	1486 ± 228 °*
9-cis RA + AM580	3223 ± 65 °^
9-cis RA + CD2019	2493 ± 295 °
9-cis RA + CD437	5408 ± 592 °^
AM580 + CD2019	1277 ± 49 °
AM580 + CD437	1951 ± 159 °*
AM580 + CD2019 + CD437	2207 ± 161 °
CD2019 + CD437	1673 ± 132 °

F9 cells were treated for 48 hours with medium alone (medium) or medium containing 5 nM ATRA, 5 nM 9-cis RA, 4 nM AM580, 12 nM CD2019, and 38 nM CD437 or various combinations of the compounds as described. ALP activity was measured on cell homogenates and each value represents the mean ± S.D. of three replicate cultures.
° Significantly higher relative to medium ($p < 0.01$ after one-way ANOVA and post hoc comparison with the Tukey's test).
(*) Significantly lower and (^) significantly higher relative relative to the sum of the two independent treatments ($p < 0.01$ after two-way ANOVA and post hoc comparison with the Tukey's test).

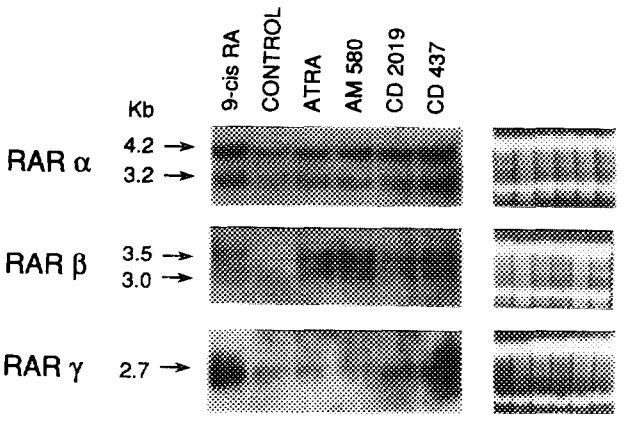

Fig.4. Regulation of α, β and γ RAR mRNAs by ATRA, 9-cis RA and the specific agonists for the RARs in F9 teratocarcinoma cells.
Total RNA was extracted for Northern-blot analysis from F9 cells after 48 hrs incubation in the medium alone (control) or in the presence of one of the compounds indicated blow (10 nM ATRA, 10 nM 9-cis RA, 8 nM AM580, 25 nM CD2019, and 75 nM CD437). The RNA (20 μg for each lane) was used to prepare three sets of blot that was separately hybridized to ^{32}P-labelled RAR α, β and γ cDNAs, respectively. The position of the RAR mRNAs is indicated by arrows on the left along with the size in Kb of the transcripts. A picture of the ethidium bromide staining of the RNA is shown on the right, demonstrating that approximately equal amounts of RNA were loaded in each lane.

receptors. At these concentrations, all the retinoids tested, except for CD2019, induce ALP activity relative to control conditions (Table I). As expected , ATRA and 9-cis RA are the most powerful agents, followed by CD437 and AM580. Whereas 9-cis RA and ATRA show an additive interaction, 9-cis RA synergistically increases ALP activity when given in combination with AM580 or CD437, however, the synergism between 9-cis RA and AM580 is less remarkable than the interaction between 9-cis RA and CD437. AM580 and CD437 interact negatively with each other when they are present simultaneously in the culture medium. The same negative interactions are observed if F9 cells are treated simultaneously with ATRA and AM580 or CD437. The combination of CD2019 with all the other retinoids does not lead to inhibition or potentiation of the effects caused by each compound separately. Similarly CD2019 given in combination with AM580 and CD437 does not lead to an induction of ALP activity that is higher than that observed after simultaneous treatment of F9 cells with the RAR α and γ agonists. Interactions among the various retinoids were verified also at concentrations equal to the Kd of each compound for its receptor(s) (data not shown).

To correlate the induction of ALP with the state of the various RAR mRNAs, Northern-blot analysis was perfomed on RNA extracted from F9 cells cultured in the absence and in the presence of the two retinoic acid isomers and the three synthetic RAR agonists. As illustrated in Fig. 4, two RAR α mRNAs are expressed in F9 cells in basal conditions and the levels of the two transcripts are not modulated by any of the 5 compounds tested. RAR β mRNA species are not expressed in basal conditions, however, the expression of two transcripts is evident after 48 hours of treatment with each of ATRA, 9-cis RA, AM580, CD437 and CD2019. The effect of CD2019 is lower than that of the other retinoids. RAR γ is expressed as a single mRNA in undifferentiated conditions and it is regulated neither by ATRA, AM580, CD2019 nor by CD 437. However, the transcript is induced approximately 3 folds after treatment of F9 cells with 9-cis RA.

DISCUSSION

The results presented in this study demonstrate that induction of ALP in F9 teratocarcinoma cells is the result of receptor mediated mechanisms. In fact, two agonists specific for the RAR α and γ as well as 9-cis RA (the specific RXR ligand) are capable of inducing ALP activity and the respective mRNA. The specific agonist for RAR β is much less effective than the other compounds. The different levels of induction of ALP by the the agonists of RAR α and γ relative to the agonist for RAR β is evident at concentrations between 10 and 100 nM, but it is lost at higher concentrations. The low level of induction of ALP by the RAR β selective ligand between 10 and 100 nM is not due to poor affinity for its receptor or to lack of activity relative to the other two analogs. In fact, CD2019 has a Kd for RAR β (27 nM) that is slightly higher than that of AM580 for RAR α (8 nM) and lower than that of CD437 for RAR γ (77 nM). Furthermore, the transactivation potential of this compound measured in transient transfection assays using a model retinoic acid responsive promoter is lower than that of AM580 but higher than that of CD437 (12). Finally, in the presence of db-cAMP, treatment of L929 cells with CD2019 induces ALP activity to the same extent as after incubation with AM580 and CD437 (unpublished data). Taken together, these facts imply that the low efficacy of CD2019 in increasing ALP levels is due to the very low levels of expression of RAR β relative to RAR α and γ in F9 teratocarcinoma cells. They also suggest that the upregulation of RAR β by retinoids, albeit preceeding induction of ALP (20 and results not shown), does not play any role in the observed phenomenon. Furthermore, they indicate that induction of ALP by ATRA is mediated by RAR α and/or RAR γ. Data obtained with a specific inhibitor of RAR α suggest that the γ isoform is probably the one predominantly mediating the induction of the enzyme after treatment of F9 cells with ATRA. In fact the RAR α antagonist is only marginally inhibiting the induction of ALP triggered by ATRA at concentrations that are readily inhibiting the RAR α agonist mediated response.

In terms of possible cross-talk among the retinoid receptors, the observed interactions between the various RAR and RXR ligands are complex and not fully understandable. The synergism between the RAR α or γ agonists and 9-cis RA demonstrates that RAR and RXR are cooperating in the induction of ALP gene expression and it is in line with data obtained in transient transfection experiments with the promoters of other ATRA responsive genes (21). The negative interaction between RAR α and γ is not easy to explain even though similar results have been reported in other experimental systems (22). The inhibitory effect of AM580 and CD437 on the induction of ALP gene expression caused by ATRA is probably the consequence of a competition between the two synthetic ligands and the natural retinoid with the same binding site on the RAR α and γ. In fact, the affinity of the two syenthetic derivatives of retinoic acid for the respective receptors is of the same order of magnitude as that of ATRA, but the transactivation potential is lower, thus explaining the observed inhibition. ATRA and 9-cis RA are endogenous retinoids and at least ATRA is present both in the plasma and in tissues at significant concentrations (5). This fact should be considered, since both AM580 and CD437 might present unexpected anti-vitamin A effects when administered *in vivo*.

Whereas synergism between 9-cis RA and CD437 correlates with the induction of RAR γ mRNA by the former compound and it might be easily explained, other interactions between the various retinoids are more difficult to interpret. For example, 9-cis RA is a weak inducer of the expression of

RAR β, whereas it has no effect on the levels of RAR α. However, in terms of ALP induction, 9-cis RA does not interact with CD2019 while it synergizes with AM580. In this context, it is interesting to notice that the amounts of RAR β mRNA are increased by the selective activation of all the three classes of receptors by their specific ligands, even though the effect triggered by CD2019 is quantiatively lower relative to that induced by AM580 and CD437.

Although the major aim of this study was to evaluate the involvment of RXRs and the various classes of RARs in the induction of ALP by retinoids in F9 cells, our data may have more general implications. For instance, although selectivity of AM 580, CD 2019 and CD437 for their respective receptors is good (12-14), our data on their effects on the expression of RAR β mRNAs as well as on their interaction with ATRA suggest that the action of these compounds may be complex and difficult to predict *in vivo* under conditions of normal vitamin A supply.

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