

Liposomal Retinoic Acids Modulate Asthma Manifestations in Mice^{1,2}

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Abstract

Signaling of all-trans retinoic acid (ATRA) through nuclear retinoid acid (RA) receptors regulates several biological functions in airway epithelial cells, eosinophils, and immune cells, yet its impact on different in vivo aspects of pulmonary allergic reaction remains elusive. We compared the effect of a treatment with liposomally encapsulated ATRA (Lipo-ATRA) in a mouse model of ovalbumin (OVA)-induced T helper (Th) 2-type responses and airway remodeling. Daily intraperitoneal injections of 10 mg/kg Lipo-ATRA, at the time of each of the 2 systemic sensitizing injections, increased OVA-induced Immunoglobulin E synthesis, bronchoalveolar lavage (BAL) eosinophilia, and accumulation of IL-5, transforming-growth factor β 1, fibronectin, eotaxin/chemokine (C-C motif) ligand 11 (eotaxin/CCL11) and regulated upon activation, normal T expressed and secreted chemokine (C-C motif) ligand 5. In contrast, Lipo-ATRA, administered during each of the 4 intranasal OVA challenges, did not affect these variables. Regardless of the treatment regimen, Lipo-ATRA augmented mucin levels in BAL fluid and reduced lung total collagen content. In vitro incubation of mouse splenocytes or purified spleen cluster of differentiation (CD) 4-positive T lymphocytes, with ATRA, increased, respectively, OVA- and anti-CD 3 antibody-induced IL-4 and IL-5 production and inhibited IFN γ release. These findings demonstrate that, when given during systemic sensitization, Lipo-ATRA exacerbates allergic immune and inflammatory responses, most likely by promoting Th2 development. J. Nutr. 137: 2730–2736, 2007.

Introduction

Asthma is a chronic inflammatory disease associated with IgE synthesis and infiltration of the bronchial wall by inflammatory cells, particularly T cells and eosinophils (1,2). Airway inflammation is tightly regulated by IL-4 and IL-5, which are primarily involved in T helper (Th)² switching, in the growth, differentiation, survival, and activation of eosinophils and in IgE synthesis by B cells (3). Eosinophil attraction and persistence

to inflammatory sites are also sustained by (C-C motif)-chemokines, including eotaxin-1/chemokine CC ligand 11 (eotaxin/CCL11) and regulated upon activation and normal T cell expressed and secreted chemokine CC ligand 5 (RANTES)/CCL-5 (4,5). These chemokines are synthesized by structural cells such as the bronchial epithelium (6) and by inflammatory cells, including eosinophils (4,5). Asthma is also characterized by airway remodeling, which is defined, among others, by mucus overproduction and subepithelial fibrosis, with an enhanced synthesis of fibrogenic cytokines, chemokines, and growth factors and an excessive extracellular matrix deposition in the connective tissue (1).

Dietary vitamin A is an essential precursor of retinol, which, in the liver, is either stored as retinyl esters or metabolized by sequential oxidations into all-trans retinoic acid (ATRA). Retinol and ATRA are secreted and transported to target cells associated to retinol-binding protein, lipoproteins, or chylomicrons. In target cells, retinol is oxidized to ATRA, which in turn is reversibly converted to other retinoid acids (RA), 9-*cis* RA and 13-*cis* RA (7,8). RA are the biological active metabolites of vitamin A. They interact with 2 groups of receptors, with 3 subtypes (α , β , and γ) for each: the RA receptors (RAR), which mainly bind ATRA and 9-*cis* RA with high affinity, and the retinoid X receptors (RXR), which bind 9-*cis* RA but not ATRA (9). The RAR/RXR form homodimers and/or heterodimers that bind to *cis*-acting response elements of retinoid target genes and

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⁸ Abbreviations used: Ab, antibody; ATRA, all-trans retinoic acid; BAL, bronchoalveolar lavage; CD, cluster of differentiation; DMSO, dimethylsulfoxide; (eotaxin/CCL11), eotaxin-1/chemokine (C-C motif) ligand 11; i.n., intranasal; i.p., intraperitoneal; Lipo-ATRA, liposomally encapsulated all-trans retinoic acid; OVA, ovalbumin; OVA/OVA, ovalbumin-immunized and ovalbumin-challenged; OVA/saline, ovalbumin-immunized and 0.9% NaCl-challenged; RA, retinoic acid; RANTES/CCL5, regulated upon activation normal T expressed and secreted chemokine (C-C motif) ligand 5; RAR, RA receptor; RXR, retinoid X receptor; saline, 0.9% NaCl; Th, T helper; TGF β 1, transforming-growth factor β 1.

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interact with a variety of coactivators and repressors (9). Of note, ATRA has a very short half-life in liver and tissues, because it is rapidly catabolized by a specific cytochrome P450, named CYP26, which is induced by ATRA itself (7).

RA regulate cell growth, differentiation, and matrix formation in various cell types and play a major role in the maintenance of a normal epithelial mucociliary phenotype (10,11). Besides their modulatory activities on airway epithelial cells, retinoids suppress eosinophil and basophil differentiation from bone marrow precursors (12), most likely by down-regulating the expression of IL-5 receptor on their surface (13), and prevent mitogen-induced proliferation and IL-4-dependent IgE production from murine splenocytes (14,15). However, retinoids also promote in vitro Th2 development both directly, by enhancing transcription of IL-4 by T cells (16,17), and indirectly, by influencing antigen-presenting cell functions (18,19). These in vitro effects of retinoids involve their interaction with either RXR or RAR, or both (14–19). A recent study demonstrated that a selective pan-RXR antagonist given to antigen-sensitized mice during the immunization phase prevented allergic airway inflammation by biasing the immune response in a Th1 direction (20). The possibility that retinoids also control the whole responses (inflammatory and remodeling) to the allergenic challenge remains to be established.

In this study, we examined the effect of pharmacological doses of intraperitoneally (i.p.) injected, liposomally encapsulated ATRA (Lipo-ATRA) in a mouse model of ovalbumin (OVA)-induced asthma manifestations. We used this strategy because it provides advantages over the ATRA oral formulation, notably by circumventing ATRA liver catabolism and clearance (21) and allowing an efficient delivery to target tissues (22,23). To discriminate between potential regulatory effects on primary immune responses and on airway inflammation, Lipo-ATRA was administered either at the time of antigen sensitization or during intrapulmonary antigen challenges. In addition, the ability of ATRA to modulate the synthesis of Th1- and Th2-type cytokines by OVA- and anti-cluster of differentiation (CD)3 antibody (Ab)-stimulated splenocytes and purified spleen CD4+ T lymphocytes was investigated. Finally, because airway remodeling is considered one of the major events involved in asthma severity and chronicity (1), the impact of Lipo-ATRA on antigen-induced extracellular matrix protein deposition and transforming growth factor β 1 (TGF β 1) production in the lung was determined.

Materials and Methods

Mouse immunization and challenge. Male BALB/c mice aged 6 wk (Centre d'Élevage R. Janvier) were sensitized (23) by 2 i.p. injections, 1 wk apart, of 0.9% NaCl (saline) containing 10 μ g OVA (Sigma) adsorbed in aluminum hydroxide (VWR International). Seven days after the second immunization, mice were challenged 4 times, at 24-h intervals, by intranasal (i.n.) instillations of 20 μ g OVA in sterile saline (OVA/OVA mice). Control mice received the same volume of sterile saline (OVA/saline mice). Mice were fed with RO3–40 diet (Safe) containing 22% crude protein, 3% crude fat, 4% crude fiber, 5.5% ash, 12,000 UI/kg vitamin A, 2000 UI/kg cholecalciferol, 30 mg/kg vitamin E, and 25 mg/kg copper. Protocols were approved by the International Review Board at our institution, Inserm.

Assessment of serum levels of retinoids by HPLC. Naïve mice received a single i.p. injection of 1, 10, or 100 mg/kg Lipo-ATRA containing 10% (wt:wt) ATRA (Antigenics) in 0.2 mL of sterile saline (23) and they were used 1, 2, or 24 h thereafter (Fig. 1, Protocol 1). In the next set of experiments, 100 mg/kg Lipo-ATRA was given as a single i.p. injection 1 h before the first i.p. OVA immunization (Fig. 1, Protocol 2)

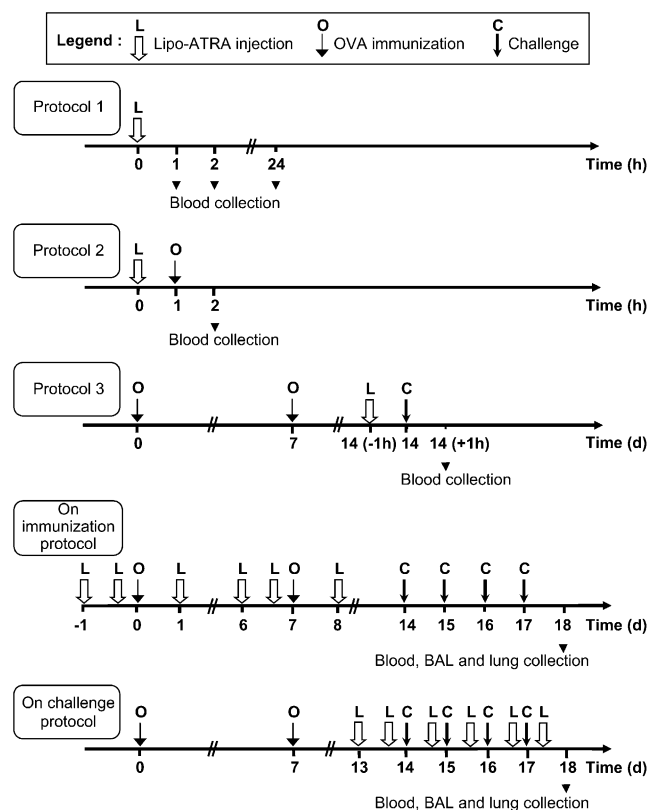


FIGURE 1 The different protocols used to evaluate the circulating retinoid levels and the effects of RA on asthma manifestations in vivo.

or before the first i.n. OVA challenge (Fig. 1, Protocol 3) and were used 2 h after Lipo-ATRA administration. All experiments were performed in duplicate. At the selected time points, mice were killed by an i.p. injection of 2.4 g/kg ethyl-carbamate (Sigma) and blood was collected (24). We measured concentrations of serum ATRA, 13-*cis* RA, and 9-*cis* RA by HPLC (25) using an Alliance HPLC system with a 2690 separation module and 996 photodiode detector (Waters). The sensitivities were 0.0075 μ mol/L for ATRA and 0.0045 μ mol/L for both 13-*cis* RA and 9-*cis* RA.

Drug treatment to assess retinoid effects on asthma manifestations. Two protocols of treatment were applied (Fig. 1, On immunization and On challenge protocols). In both protocols, we treated mice for 6 consecutive days with 1, 10, or 100 mg/kg $\cdot$ d $^{-1}$ Lipo-ATRA in 0.2 mL of sterile saline (23); therefore, the total dose of ATRA administered per mouse in both protocols was 1.5 mg. In the “on immunization” protocol, Lipo-ATRA was injected 24 h before, 1 h before, and 24 h after the 2 immunizations. In the “on challenge” protocol, Lipo-ATRA was administered 24 h before the first saline or OVA challenge, 1 h before each of the 4 challenges, and 6 h after the final challenge. Two control groups consisting of OVA/saline and OVA/OVA mice received i.p. injections of empty liposomes following the same 2 administration regimens described above. At least 2 independent series of experiments with each regimen were conducted. Twenty-four hours after the final challenge, mice were euthanatized and blood, bronchoalveolar lavage (BAL), and saline-washed lungs were collected as previously described (24).

Determination of OVA-specific IgE. The levels of OVA-specific IgE were measured by specific enzyme immunometric assay (26) using a rat anti-mouse IgE monoclonal Ab clone R35–72 (Becton Dickinson). Results were expressed in OD.

Cell counts and distribution in the BAL fluid. Total cell counts were evaluated using a hemocytometer and mononuclear cells, neutrophils, and eosinophils were differentially counted (24).

Mediator assessment in the BAL fluid. IL-4 was measured in the supernatant of BAL fluid by specific ELISA (24). Recombinant murine IL-4 (R&D Systems Europe) was used to generate standard curves. IL-5, IFN γ , eotaxin/CCL11, RANTES/CCL5, and TGF β 1 were assayed using Quantikine Murine kits (R&D Systems).

We assessed fibronectin release by enzyme immunometric assay (27). Plates were coated with 2 mg/L murine fibronectin (Biogenesis). BAL samples (100 μ L) were incubated with a polyclonal rabbit anti-mouse cellular fibronectin Ab (1:5,000; Biogenesis) and then transferred to the fibronectin-coated wells. After washings, excess Ab that did not react with soluble fibronectin was revealed by sequentially adding a biotinylated secondary anti-rabbit IgG (1:25,000; Jackson ImmunoResearch) and the ExtrAvidine peroxidase solution (1:20,000; Sigma). A ready-to-use solution of 3,3', 5,5'-tetramethyl-benzidine (Sigma) was then added for 15 min, the reaction was stopped by adding 20% H $_2$ SO $_4$, and the OD was measured at 450 nm.

Mucin levels were measured by a colorimetric assay (28). Plates were coated with serial dilution of the standard type I-S mucin from bovine submaxillary glands (Sigma) or BAL samples diluted at 1:100 for OVA/saline mice or 1:500 for OVA/OVA mice. We then washed, blocked, rewashed, and incubated plates for 1 h at 37°C with biotinylated jacalin (Vector Laboratories). Plates were washed again and mucin-jacalin-biotin complexes were revealed by incubation with ExtrAvidine peroxidase solution (1:40,000). After a final wash, plates were further processed as described above.

Sensitivities were of 7.5 ng/L for IL-4, RANTES/CCL5, and IFN γ ; 15.6 ng/L for IL-5 and eotaxin/CCL11; 31.2 ng/L for TGF β 1; 0.5 mg/L for fibronectin; and 500 ng/L for mucin.

Total collagen content in the lung tissue. Total soluble collagen content was determined in homogenates of saline-washed right lung lobes using the Sircol assay (Biocolor). The sensitivity of the assay was 6 μ g.

In vitro experiments with isolated splenocytes and purified spleen CD4 $^+$ T cells. Spleen cells from OVA/OVA untreated mice were dispersed using a Dounce homogenizer and erythrocytes were lysed using Mouse Erythrocyte lysing kit (R&D Systems). Whole spleen mononuclear cells were isolated by centrifugation in the Mouse NycoPrep 1.077A separation medium (Axis Shield). CD4 $^+$ T lymphocytes were then magnetically enriched using an anti-CD4 monoclonal Ab (clone L3T4; Miltenyi Biotec) as described (29). Whole spleen mononuclear cells ($0.375 \times 10^6/200 \mu$ L) and CD4 $^+$ T lymphocytes ($0.2 \times 10^6/200 \mu$ L) were cultured as described (29) with or without 100 mg/L OVA. Alternatively, cells were incubated in plates previously coated with 4.5 mg/L of activating anti-CD3 monoclonal Ab (clone 145-2C11; a kind gift of Pr. Michel Goldman) or with its isotype control, i.e. hamster IgG (Becton Dickinson). In both types of experiments, cells were treated with 0.01 and 1 μ mol/L ATRA or 9-*cis* RA (both from Sigma) with their vehicle, i.e. 0.1% dimethylsulfoxide (DMSO) as final concentration or with the medium alone. These concentrations of RA were selected based on previous in vitro studies showing their ability to alter T cell functions (16). After 84 h, plates were centrifuged ($80 \times g$; 5 min, 4°C) and the supernatants were collected and stored at -80°C until the measurements of IL-4, IL-5, and IFN γ were performed as described above. The cell pellets were incubated at 37°C in a 5% CO $_2$ atmosphere for 4 h with 0.5 g/L of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (Sigma) in medium, plates were centrifuged, and supernatants were replaced by a solution of 0.04 mol/L HCl in isopropanol. After vigorous stirring, the OD, corresponding to cell number, was 550 nm.

Statistical analysis. We examined the in vivo effects of OVA challenge, empty liposome injection, and Lipo-ATRA administration on BAL cell number, cytokine, chemokine, TGF β 1, fibronectin, and mucin levels in BAL fluids and lung collagen content and the in vitro effects of ATRA and 9-*cis* RA on cytokine production. Log-transformed values were analyzed using 2-way ANOVA followed by Fisher's protected least significant difference test with StatView SE+Graphics program for Macintosh (Abacus Concepts). $P \leq 0.05$ was considered significant. Values are expressed as means $\pm$ SEM of the indicated number of mice or cell preparations.

Results

Serum retinoid concentrations. Assessment of retinoids by HPLC in Lipo-ATRA powder used to treat mice revealed the presence of ~82% ATRA, 12% of its stereoisomer 13-*cis* RA, and traces of 9-*cis* RA.

Endogenous ATRA, 13-*cis* RA, and 9-*cis* RA were undetectable in the serum of naïve mice (data not shown). A single i.p. injection of 100 mg/kg Lipo-ATRA to nonimmunized mice led to the detection of elevated serum levels of ATRA, 13-*cis* RA, and 9-*cis* RA at 1 h ($n = 2$; Fig. 2A). These levels decreased by >250% at 2 h and became undetectable at 24 h (Fig. 2A). Dose-dependent studies performed at 1 h showed a lower concentration of ATRA following the administration of 10 mg/kg Lipo-ATRA, whereas the levels of 13-*cis* RA and 9-*cis* RA were below the detection limit of the assay ($n = 2$). Neither ATRA nor 13-*cis* RA or 9-*cis* RA were detectable 1 h after the i.p. injection of 1 mg/kg Lipo-ATRA to naïve mice (data not shown).

Two hours after a single i.p. injection of 100 mg/kg Lipo-ATRA, the serum levels of ATRA, 13-*cis* RA, and 9-*cis* RA were similar in naïve (Fig. 2A) and OVA-immunized or OVA-challenged mice (Fig. 2B).

Effect of Lipo-ATRA on OVA-induced specific IgE production. OVA/OVA empty liposome-treated mice had higher serum levels of OVA-specific IgE compared with OVA/saline empty

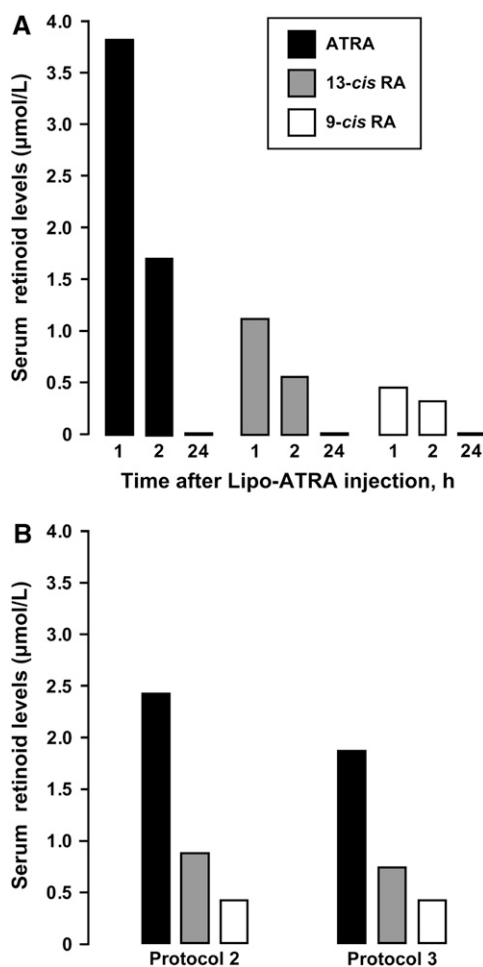


FIGURE 2 Serum levels of ATRA, 13-*cis* RA, and 9-*cis* RA in protocol 1 (A) and in protocols 2 and 3 (B) after a single i.p. injection of 100 mg/kg Lipo-ATRA. Values are means of $n = 2$ mice per group.

liposome-treated mice (Fig. 3). Treatment with Lipo-ATRA during challenges did not modify IgE production, whereas administration at the time of sensitization phase resulted in higher levels of IgE in OVA/OVA mice (Fig. 3).

Effect of Lipo-ATRA on OVA-induced changes in BAL cell composition. OVA-OVA mice treated with empty liposomes, either during challenge or immunization, showed substantially greater numbers of BAL total cells, macrophages, and eosinophils compared with OVA/saline empty liposome-treated mice (Table 1). Lipo-ATRA administered at the time of each OVA challenge reduced the number of macrophages without modifying that of total BAL cells and of eosinophils (Table 1). In contrast, the administration of Lipo-ATRA during the sensitization phase augmented OVA-induced total cell, alveolar macrophages, and eosinophil accumulation in the BAL fluid (Table 1). The number of BAL neutrophils did not vary in the different study groups (Table 1).

Effect of Lipo-ATRA on OVA-induced cytokine and chemokine release in the BAL fluid. OVA/OVA mice treated with empty liposomes, either at the time of OVA challenge or during the immunization phase, had higher concentrations of IL-4 and IL-5 in their BAL fluid than OVA/saline empty liposome- or Lipo-ATRA-treated mice (Fig. 4A,B). The levels of eotaxin/CCL11 and RANTES/CCL5 were very low, near the detection limit of the assays (Fig. 4C,D). Lipo-ATRA administered at the time of challenges did not alter IL-4, IL-5, eotaxin/CCL11, and RANTES/CCL5 release (Fig. 4). In addition, Lipo-ATRA upregulated IL-5, eotaxin/CCL11, and RANTES/CCL5 release in the BAL fluid of OVA/OVA mice, without altering the levels of IL-4 (Fig. 4). BAL levels of IFN γ were below the threshold of the sensitivity of the assay in all groups of mice (data not shown).

Effect of Lipo-ATRA on OVA-induced features of lung remodeling. Significantly higher amounts of fibronectin, TGF β 1, and mucin in the BAL fluid and greater collagen amounts in lung homogenates were found in OVA/OVA com-

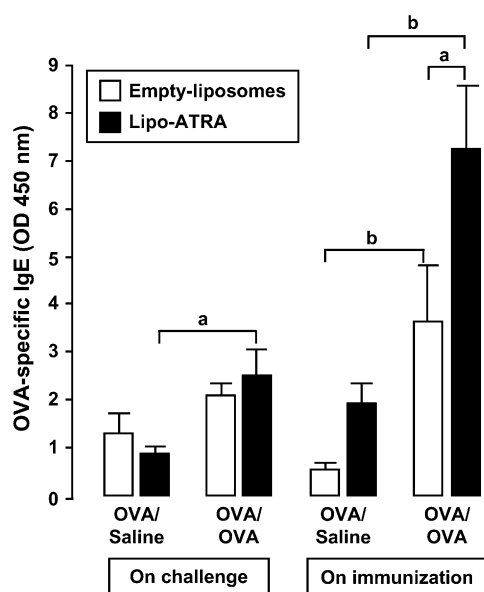


FIGURE 3 Effect of Lipo-ATRA (100 mg·kg⁻¹·d⁻¹) on serum IgE levels. Values are means $\pm$ SEM, $n = 4$ OVA/saline mice or $n = 6$ OVA/OVA mice. ^a $P < 0.05$; ^b $P < 0.01$.

TABLE 1 Effect of Lipo-ATRA on BAL cell composition in mice¹

Cell type	Empty liposomes	Lipo-ATRA
<i>On challenge protocol</i> $n \times 10^6/L$ BAL		
Total cells		
OVA/saline	2.0 $\pm$ 0.3	1.6 $\pm$ 0.2
OVA/OVA	18.4 $\pm$ 3.2*	16.4 $\pm$ 2.7*
Macrophages		
OVA/saline	1.9 $\pm$ 0.3	1.5 $\pm$ 0.2
OVA/OVA	4.7 $\pm$ 1.2*	2.4 $\pm$ 0.3*
Eosinophils		
OVA/saline	0.02 $\pm$ 0.00	0.02 $\pm$ 0.01
OVA/OVA	13.6 $\pm$ 2.2*	13.9 $\pm$ 2.6*
Neutrophils		
OVA/saline	0.0 $\pm$ 0.0	0.6 $\pm$ 0.6
OVA/OVA	0.2 $\pm$ 0.1*	0.2 $\pm$ 0.2*
<i>On immunization protocol</i>		
Total cells		
OVA/saline	1.1 $\pm$ 0.1	1.6 $\pm$ 0.4
OVA/OVA	10.6 $\pm$ 1.6*	34.2 $\pm$ 5.3 ^a
Macrophages		
OVA/saline	0.8 $\pm$ 0.2	1.4 $\pm$ 0.3
OVA/OVA	2.7 $\pm$ 0.6*	6.1 $\pm$ 1.3*
Eosinophils		
OVA/saline	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
OVA/OVA	7.8 $\pm$ 1.0*	27.8 $\pm$ 4.1 ^a
Neutrophils		
OVA/saline	0.3 $\pm$ 0.3	0.1 $\pm$ 0.1
OVA/OVA	0.2 $\pm$ 0.1*	0.2 $\pm$ 0.1*

¹ Values are means $\pm$ SEM, $n = 5$. *Different from OVA/saline, $P < 0.002$. ^a Different from empty liposomes, $P < 0.001$.

pared with OVA/saline mice treated with empty liposomes either at the time of antigen challenge or of immunization (Fig. 5). BAL fibronectin and TGF β 1 levels were not modulated upon administration of Lipo-ATRA during OVA challenges (Fig. 5A,B). In contrast, these parameters were augmented in mice treated with Lipo-ATRA during the sensitization phase (Fig. 5A,B). Irrespective of the protocol of administration, Lipo-ATRA increased the levels of mucin in the BAL fluid (Fig. 5C) and reduced total collagen content in lung homogenates (Fig. 5D).

Effect of lower doses of Lipo-ATRA administered during the immunization phase. The i.p. injection of 10 mg·kg⁻¹·d⁻¹ Lipo-ATRA to OVA/OVA mice during the immunization phase augmented the levels of OVA-specific IgE (OD mean $\pm$ SEM of 5.3 $\pm$ 1.9, $n = 5$; $P = 0.029$), the number of eosinophils (11.4 $\pm$ 1.9 $\times 10^6/L$ BAL, $n = 5$; $P < 0.05$), and the concentrations of IL-5 in BAL fluids (225 $\pm$ 54 ng/L, $n = 5$; $P < 0.05$). At 1 mg·kg⁻¹·d⁻¹, Lipo-ATRA did not alter OVA-induced IgE production, BAL eosinophilia, and IL-5 levels (data not shown).

Effect of ATRA on whole splenocytes and purified spleen CD4⁺ T lymphocyte activation. OVA-stimulated DMSO-treated splenocytes and anti-CD3-stimulated DMSO-treated CD4⁺ T cells produced higher amounts of IL-4, IL-5, and IFN γ than medium- or hamster IgG-stimulated DMSO-treated cells (Table 2). Adding 1 μ mol/L ATRA (Table 2) or 9-*cis* RA (data not shown) to OVA-stimulated splenocytes augmented the levels of IL-4 and reduced those of IFN γ without modifying IL-5 release. Under the same conditions, ATRA (Table 2) or 9-*cis* RA (data not shown) increased both IL-4 and IL-5 production and decreased IFN γ release in anti-CD3-stimulated CD4⁺ T cells. At 0.01 μ mol/L, RA did not alter OVA-induced cytokine

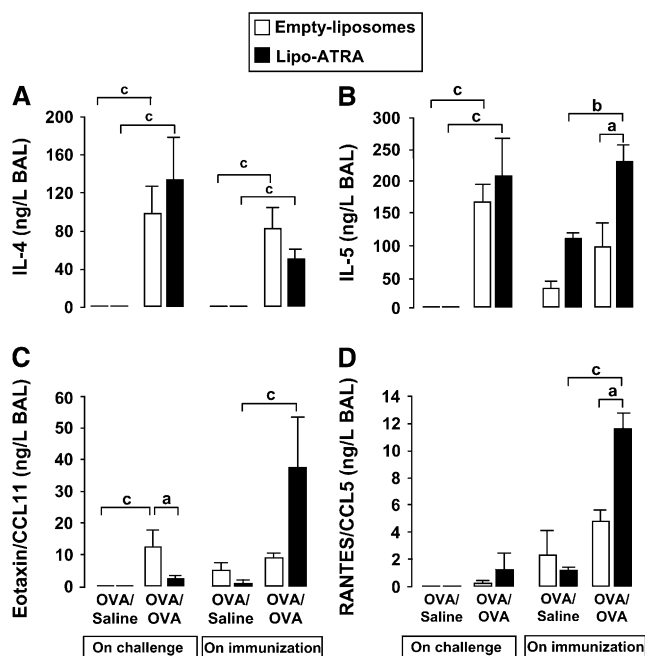


FIGURE 4 Effect of Lipo-ATRA (100 mg·kg⁻¹·d⁻¹) on IL-4 (A), IL-5 (B), eotaxin/CCL11 (C), and RANTES/CCL5 (D) release in BAL fluids. Values are means $\pm$ SEM, $n = 4$ OVA/saline mice or $n = 6$ OVA/OVA mice. ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$.

production, whereas they significantly reduced IFN γ release from anti-CD3-stimulated CD4⁺ T-cells (Table 2). Retinoids did not modulate cytokine levels in medium-stimulated cells (data not shown). Finally, DMSO- or medium-treated cells released similar amounts of cytokines either spontaneously or in response to OVA or anti-CD3 Ab (Table 2).

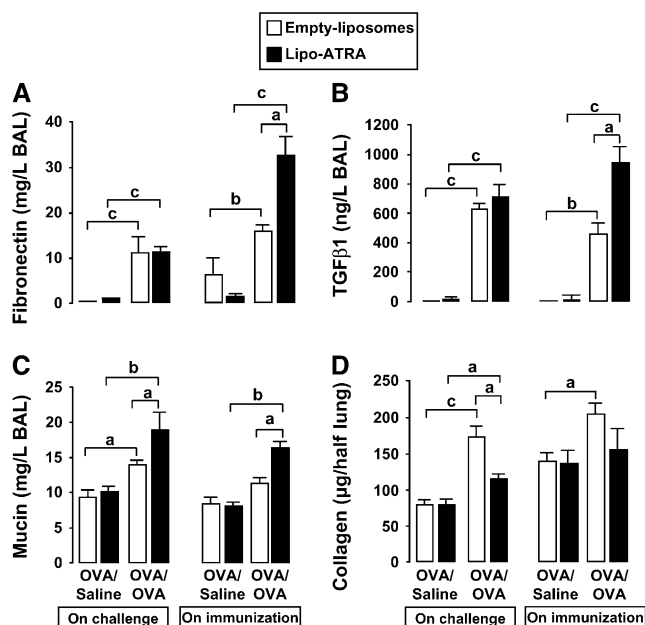


FIGURE 5 Effect of Lipo-ATRA (100 mg·kg⁻¹·d⁻¹) on fibronectin (A), TGF β 1 (B), and mucin (C) release in BAL fluids and on lung collagen accumulation (D). Values are means $\pm$ SEM, $n = 4$ OVA/saline mice or $n = 6$ OVA/OVA mice. ^a $P < 0.05$; ^b $P < 0.01$; ^c $P < 0.001$.

At the concentration used, RA did not alter proliferation of activated splenocytes and CD4⁺ T lymphocytes (data not shown).

Discussion

To determine whether retinoids would alter the onset of allergic asthma, we examined the effectiveness of liposome-encapsulated ATRA in an in vivo mouse model characterized by antigen-specific IgE production, BAL eosinophilia, Th2-type cytokine, and eosinophilotactic chemokine synthesis and features of airway remodeling.

Using a treatment regimen previously described in mice (23), we demonstrated that, depending on its timing of administration, supraphysiological doses of Lipo-ATRA either exacerbated or altered minimally OVA-induced asthma manifestations. The quasi-absence of effects of Lipo-ATRA in the on challenge protocol is unlikely to be the consequence of OVA i.n. administration on ATRA clearance, because challenge did not alter the levels of circulating ATRA. These results extend those recently reported showing that the administration of a retinoid receptor antagonist during the sensitization phase downregulated antigen-induced allergic airway inflammation (20). The similarities of results between these 2 studies suggest that high doses of Lipo-ATRA are required to achieve physiological concentrations of ATRA at the level of target cells. Indeed, despite ATRA encapsulation into liposomes, a strategy known to limit its clearance, ATRA concentrations rapidly decreased in serum, most probably as a result of its catabolism in the liver and other tissues.

We also noticed that Lipo-ATRA powder used to treat mice contained its natural stereoisomers, 13-*cis* RA and 9-*cis* RA, indicating that the effects presently reported result most likely from the activation of both RAR and RXR.

When given at the time of the 2 immunizing injections, Lipo-ATRA upregulated OVA-induced Th2-type responses, including peripheral IgE synthesis, eosinophilia in BAL fluid, and the associated production of IL-5. In contrast, Lipo-ATRA did not modify IgE levels, BAL eosinophilia, and IL-5 release when administered during the 4 i.n. OVA challenges. Using in vitro-cultured splenocytes, we showed that ATRA and 9-*cis* RA augmented OVA-induced IL-4, but not IL-5, production and decreased the levels of the Th1-type cytokine, IFN γ . Because RA-mediated inhibition of IFN γ synthesis may result from a direct effect on the antigen-presenting cells contained in the whole spleen cell populations, experiments were conducted using anti-CD3-stimulated CD4⁺ T cells. Under these conditions, RA reduced IFN γ production, increased IL-4 synthesis, and, contrary to the data obtained with OVA-stimulated splenocytes, augmented the levels of IL-5. The similarities of IL-4 and IFN γ and the discrepancy of IL-5 production by mononuclear splenocytes and purified CD4⁺ lymphocytes suggest that retinoids alter Th2 responses by modulating the activation of both antigen-presenting cells and T cells. These results corroborate a previous finding showing that retinoids enhance the synthesis of Th2 cytokines and decrease the production of Th1 mediators directly by activating naïve Th0 cells and indirectly by preventing the release of Th1-promoting factors, such as IL-12, by antigen-presenting cells (30). Together, the in vitro and in vivo results showing a shift of RA-treated mononuclear cells toward Th2 phenotype and an upregulation of allergic responses obtained in mice treated with Lipo-ATRA at the time of the 2 systemic sensitizing injections corroborate previous reports demonstrating that in vivo vitamin A dietary

TABLE 2 Effect of ATRA on cytokine release from splenocytes and CD4+ T cells of mice¹

Cell type	Stimulus	Treatment	IL-4	IL-5	IFN γ
				ng/L	
Splenocytes	Medium	Medium	16 $\pm$ 7	88 $\pm$ 22	11 $\pm$ 3
Splenocytes	Medium	DMSO	18 $\pm$ 11	91 $\pm$ 61	15 $\pm$ 6
Splenocytes	OVA	Medium	727 $\pm$ 111 ^a	3972 $\pm$ 432 ^a	1157 $\pm$ 104 ^a
Splenocytes	OVA	DMSO	767 $\pm$ 146 ^a	4050 $\pm$ 571 ^a	1203 $\pm$ 175 ^a
Splenocytes	OVA	ATRA (0.01 μ mol/L)	942 $\pm$ 166 ^a	3712 $\pm$ 569 ^a	1191 $\pm$ 203 ^a
Splenocytes	OVA	ATRA (1 μ mol/L)	997 $\pm$ 183 ^{a,b}	3840 $\pm$ 615 ^a	1032 $\pm$ 152 ^{a,b}
CD4+ T cells	IgG	Medium	48 $\pm$ 9	56 $\pm$ 21	23 $\pm$ 7
CD4+ T cells	IgG	DMSO	59 $\pm$ 16	63 $\pm$ 37	28 $\pm$ 9
CD4+ T cells	Anti-CD3	Medium	2080 $\pm$ 318 ^c	11,107 $\pm$ 2124 ^c	2189 $\pm$ 341 ^c
CD4+ T cells	Anti-CD3	DMSO	2160 $\pm$ 619 ^c	10,907 $\pm$ 3323 ^c	2277 $\pm$ 542 ^c
CD4+ T cells	Anti-CD3	ATRA (0.01 μ mol/L)	2500 $\pm$ 512 ^c	12,155 $\pm$ 3004 ^c	1323 $\pm$ 353 ^{c,d}
CD4+ T cells	Anti-CD3	ATRA (1 μ mol/L)	2790 $\pm$ 529 ^{c,d}	15,248 $\pm$ 4157 ^{c,d}	1355 $\pm$ 430 ^{c,d}

¹ Values are means $\pm$ SEM, $n = 10$ – 12 . ^aDifferent from medium-incubated DMSO-treated cells, $P < 0.05$. ^bDifferent from OVA-stimulated DMSO-treated cells, $P < 0.05$. ^cDifferent from hamster IgG-stimulated DMSO-treated cells, $P < 0.05$. ^dDifferent from anti-CD3 Ab-stimulated DMSO-treated cells, $P < 0.05$.

supplementation biased the immune response in a Th2 direction, whereas its deficiency shifted this response toward a Th1 phenotype (18,31–33). Supporting these observations, *in vivo* retinoid treatment improved experimental allergic encephalomyelitis, a Th1-mediated disease in which IL-4 administration delays the onset and reduces the severity (34,35).

The ability of Lipo-ATRA to exacerbate Th2-type responses when given during the immunization phase was accompanied by increased BAL levels of eotaxin/CCL11 and RANTES/CCL5, 2 chemokines that sustain eosinophil homing and accumulation in the airways (4). Of interest, eotaxin/CCL11 and RANTES/CCL5 are produced mainly by the airway epithelium in response to several stimuli, including IL-4 (6,36). However, the finding that Lipo-ATRA is effective on eotaxin/CCL11 and RANTES/CCL5 production only when it is administered at the time of sensitization suggests that, in our model, retinoids influence airway epithelial cell activation, indirectly, through upregulation of Th2-type responses.

To determine the impact of Lipo-ATRA on airway remodeling, we next examined TGF β 1, fibronectin, and mucin release in the BAL fluid and collagen deposit in the lung tissue. We found that Lipo-ATRA administration at the time of the immunization phase, but not during intrapulmonary antigen challenges, upregulated OVA-induced TGF β 1 and fibronectin generation in the BAL fluid. These results also demonstrated that TGF β 1 and fibronectin release, on one hand, and BAL eosinophilia, on the other hand, were similarly modulated by Lipo-ATRA and substantiate previous *in vivo* observations showing that eosinophils control TGF β 1 and fibronectin production in the lung (37–42). It is thus likely that increased TGF β 1 and fibronectin production in OVA/OVA Lipo-ATRA-treated mice is the consequence of the upregulation of Th2 responses and the subsequent accumulation and activation of eosinophils in the lungs. Regardless of the treatment regimen, Lipo-ATRA downregulated OVA-induced total soluble collagen accumulation in the lung, suggesting that this phenomenon is independent from the extent of Th2-type responses. In a recent study, ATRA was able to prevent collagen biosynthesis in the mouse lung during bleomycin- and radiation-induced pulmonary fibrosis (43). This effect resulted from a selective inhibition of fibroblast activation, ATRA being ineffective against bronchial epithelial and endothelial cells, 2 other important sources of extracellular matrix proteins in the airways (43). Collectively, these results suggest

that Lipo-ATRA may reduce OVA-induced total lung collagen accumulation by inhibiting directly the activation of mesenchymal cells.

Finally, mucus cell metaplasia has been associated with airway eosinophilia in some (38–40) but not all (40) studies and previous reports have established the central role played by IL-4 in this process (44). Here, we provide evidence that OVA-induced mucin release in BAL fluid was similarly augmented by Lipo-ATRA, irrespective of the administration regimen and despite dissimilar modulation of IL-4 release. These findings suggest that these events may be dissociated and that the increased mucus production results from the well-known ability of retinoids to promote epithelial cells differentiation toward a mucociliary phenotype (10,11).

In conclusion, this study demonstrates that the administration of Lipo-ATRA to OVA-immunized mice at the time of antigen sensitization exacerbates IgE synthesis and allergic airway inflammation, whereas it did not substantially alter these responses when given at the time of intrapulmonary OVA challenges. *In vitro*, RA augmented the synthesis of Th2-type cytokines and reduced the production of IFN γ from antigen- or anti-CD3-activated splenocytes and spleen CD4+ T lymphocytes, suggesting that Lipo-ATRA aggravates *in vivo* allergic responses most likely by increasing early Th2 differentiation. Regardless of its administration regimen, Lipo-ATRA reduces lung collagen deposition while it increases mucin production, indicating that Th2-type inflammation, on one hand, and certain features of airway remodeling, on the other hand, are parallel yet independent events in this model. These observations also suggest that the *in vivo* regulatory properties of retinoids extend to cell types other than immune cells, most likely fibroblasts and airway epithelial cells.

Together, the current results corroborate the hypothesis that targeting retinoid receptors potently modulates Th2-type asthma manifestations in mice. Whether or not diet-derived retinoids are also involved in the development of asthma in humans requires further investigation.

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