

T Cell-Independent Regulation of IgE Antibody Production Induced by Surface-Linked Liposomal Antigen¹

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Control of IgE Ab production is important for the prevention of IgE-related diseases. However, in contrast to the existing information on the induction of IgE production, little is known about the regulation of the production of this isotype, with the exception of the well-documented mechanism involving T cell subsets and their cytokine products. In this study, we demonstrate an alternative approach to interfere with the production of IgE, independent of the activity of T cells, which was discovered during the course of an investigation intended to clarify the mechanism of IgE-selective unresponsiveness induced by surface-coupled liposomal Ags. Immunization of mice with OVA-liposome conjugates induced IgE-selective unresponsiveness without apparent Th1 polarization. Neither IL-12, IL-10, nor CD8⁺ T cells participated in the regulation. Furthermore, CD4⁺ T cells of mice immunized with OVA-liposome were capable of inducing Ag-specific IgE synthesis in athymic nude mice immunized with alum-adsorbed OVA. In contrast, immunization of the recipient mice with OVA-liposome did not induce anti-OVA IgE production, even when CD4⁺ T cells of mice immunized with alum-adsorbed OVA were transferred. In the secondary immune response, OVA-liposome enhanced anti-OVA IgG Ab production, but it did not enhance ongoing IgE production, suggesting that the IgE-selective unresponsiveness induced by the liposomal Ag involved direct effects on IgE, but not IgG switching in vivo. These results suggest the existence of an alternative mechanism not involving T cells in the regulation of IgE synthesis. *The Journal of Immunology*, 2002, 169: 4246–4252.

Immunoglobulin E is believed to play a key role in allergic diseases. Binding of IgE Abs to surface receptors on a wide variety of cells, mainly mast cells and eosinophils, and cross-linking of receptor-bound IgE with Ag result in the release of inflammatory mediators that lead to symptoms of allergic diseases, namely type I hypersensitivity reactions. Consequently, finding a way to control IgE Ab production is one of the major goals of allergy research. At present, more information is available regarding the induction than regarding the inhibition of IgE synthesis. In the induction of IgE synthesis, cytokines such as IL-2 (1), IL-5 (2), IL-6 (3), and TNF- α (4) are known to act in concert with IL-4; furthermore, interaction between CD40L on activated T cells and CD40 on B cells is required for the production of mature ϵ mRNA and IgE protein (5–7). CD23 expressed on lymphocytes, macrophages, and many other cell types has been reported to also play a role in this process (8). In contrast, in the inhibition of IgE synthesis, the participation of cytokines such as IFN- γ (9–11), IFN- α (12), IL-10 (13), and IL-12 (14), and, in some cases, PGs (9) has been reported. Unfortunately, a method of controlling ongoing IgE

synthesis, despite its potential value in the treatment of allergic diseases, is currently unavailable.

Today, the balance between Th1 and Th2 is conceptually used to explain the regulation of IgE synthesis, because Mosmann et al. (15) have reported that CD4⁺ Th cells can be divided into two subsets with contrasting and cross-regulating cytokine profiles, namely, the Th1 and Th2 subsets. Th1 cells produce IL-2, TNF- β , and IFN- γ and induce cell-mediated immunity, whereas Th2 cells produce IL-4, IL-5, IL-10, and IL-13, and are associated with humoral immunity. In mice, the production of selective Ig isotypes is known to be regulated by contrasting cytokines; IL-4 induces IgG1 and IgE Ab production, while IFN- γ induces IgG2a production, but inhibits IgE production (16). Differentiation of either the Th1 or Th2 subset is affected by various factors such as the dose of Ag at immunization (17), costimulatory molecules (18), altered peptide ligands (19, 20), and adjuvants used for immunization (21–23). Regarding adjuvants, CFA is known to activate Th1 and to induce IgG2a Ab production without inducing IgE Ab production, while aluminum hydroxide activates Th2 and induces IgE and IgG1 production (22). In addition to these two adjuvants, liposomes are also known to act as powerful adjuvants (24). However, their effects on immune response vary according to the nature of the linkage. Surface-linked and liposome-encapsulated Ags are known to possess distinct properties (25). This difference is thought to be due to the differential activation of Th cell populations; surface-linked Ags appear to preferentially stimulate CD4⁺ T cells to proliferate and mature into typical Th1 cells, while encapsulated Ags do not.

We have previously reported that OVA-liposome conjugates induced IgE-selective unresponsiveness in mice (26). The IgE-selective unresponsiveness was induced by Ag-liposome conjugates, regardless of the coupling procedure of Ag and liposomes (27),

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using liposomes of different lipid formulations (28), or using different Ags such as tetanus toxin (29) or Shiga-like toxin (30). Thus, Ag-liposome conjugates are expected to be applicable as part of a novel protocol for the development of vaccines that would induce minimal IgE synthesis.

We conjectured that the mechanism of this IgE regulation can be attributed to the balance between Th1- and Th2-type immune responses; liposome-coupled Ags preferentially activate Th1, whereas alum-adsorbed Ags, which induce production of IgG1 and IgE Abs, activate Th2. To test this hypothesis, cytokine production by splenic CD4⁺ cells was investigated in mice immunized with either OVA-liposome or OVA-alum. The results did not show any polarized Th1- or Th2-type immune responses induced by distinct adjuvants. Further investigations made use of IL-12-deficient mice and CD4⁺ T cell transfer experiments. A possible explanation for IgE-selective unresponsiveness induced by the surface-linked liposomal Ag is discussed.

Materials and Methods

Mice

BALB/c mice (female, 8 wk of age) were purchased from Charles River Breeding Laboratories (Kanagawa, Japan). Breeding pairs of IL-12-deficient mice (BALB/c-IL-12b^{tm1 Jm}) were purchased from Charles River Breeding Laboratories and bred in the animal care unit at the National Institute of Infectious Diseases (Tokyo, Japan). Mice were maintained in sterile cages under specific pathogen-free conditions at the Division of Experimental Animals Research (National Institute of Infectious Diseases).

Antigen

OVA (grade VII) was purchased from Sigma-Aldrich (St. Louis, MO).

Monoclonal Abs

Cytotoxic anti-CD8 mAb 2.43 (rat IgG2b) was a kind gift from Dr. T. Mizuuchi (National Institute of Infectious Diseases).

OVA-liposome conjugates

Liposomes and OVA-liposome conjugates were prepared as previously described (26). Briefly, 0.5 ml 2.5% glutaraldehyde was added, drop by drop, to a mixture of liposome (90 mg lipid) and 6 mg OVA in 2.5 ml phosphate buffer (pH 7.2) and was gently stirred for 1 h at 37°C. To block excess aldehyde groups, 3 M glycine-NaOH (pH 7.2) was added, and the mixtures were kept at 4°C overnight. Liposome-coupled and uncoupled OVA were separated using CL-4B column chromatography (Pharmacia, Uppsala, Sweden). The resulting conjugate of OVA-liposome contained an average of 0.50 ± 0.04 mg OVA/10 mg liposome, as quantified using radiolabeled OVA.

Immunization

Mice were immunized i.p. with 200 µl OVA-liposome solution, 10 µg OVA adsorbed with 3 mg alum (Alhydrogel; Superfos Biosector, Vedback, Denmark), or 10 µg OVA emulsified in CFA (Difco, Detroit, MI). After immunization, blood samples were taken weekly from the tail vein.

Detection of Abs

Anti-OVA IgG Abs in the sample sera were determined by an ELISA using peroxidase-labeled rabbit Abs against mouse IgG or mouse Ig subclasses (Zymed Laboratories, San Francisco, CA). For the quantitative analysis of Ag-specific Ab, murine mAbs against OVA with IgG1 and IgG2a subclasses were produced and affinity purified in our laboratory and used as the assay standard. The levels of Ag-specific IgE were determined by mAb-captured ELISA using mAb against murine IgE produced by clone B1E3.

Preparation of splenic adherent cells

Splenic adherent cells were obtained from naive BALB/c mice and were used as APCs in the T cell cytokine culture. Spleen cell suspensions were prepared in RPMI 1640 containing 10% FCS. Cells (5 × 10⁷) in 5 ml medium containing 10% FCS were plated into 50-mm plastic tissue culture dishes (number 3002; BD Labware, Franklin Lakes, NJ) and were incubated at 37°C in a humidified 5% CO₂ atmosphere for 2 h. After culture,

nonadherent cells were removed by vigorous washing in warm medium, and adherent cells were then harvested with a cell scraper.

Preparation of CD4⁺ T cells

CD4⁺ T cell purification from spleen cells of mice immunized with OVA-alum or with OVA-liposome was performed with the magnetic cell sorter system MACS, according to the manufacturer's protocol, using anti-CD4 Ab-coated microbeads (Miltenyi Biotec, Auburn, CA; 492-01). CD4⁺ T cells were suspended in RPMI 1640 containing 10% FCS at a cell density of 2 × 10⁶/ml. In the preliminary examinations, OVA at final concentrations of 1–1000 µg/ml yielded similar patterns of cytokine production. The highest magnitude of cytokine production was observed at 1000 µg/ml. In the present study, OVA at a final concentration of 1000 µg/ml with 24- and 96-h culture periods was used for Th1 and Th2 cytokine production, respectively. The CD4⁺ T cell suspension was plated at 250 µl/well onto 48-well culture plates (number 3047; BD Labware), and 500 µl 2 mg/ml OVA solution and 250 µl 8 × 10⁵/ml splenic adherent cells in the same medium were added to the plates. After incubation in a CO₂ incubator, the culture supernatants were collected and assayed to determine the concentration of cytokines.

Cytokine assays

IL-2, IL-4, IL-5, IL-10, and IFN-γ in the culture supernatant were measured using the Biotrak mouse ELISA system (Amersham, Little Chalfont, U.K.). All test samples were assayed in duplicate, and the SE in each test was always less than 5% of the mean value.

Administration of mAbs

To neutralize endogenously produced IL-10, mice were injected i.p. three times/wk, starting 24 h before the primary immunization until 6 wk after the primary immunization; neutralizing rat IgG1 anti-mouse IL-10 Ab, designated as JES5-2A5, was used for the injections (31) (1.0 mg/injection). The control group received equivalent volumes (200 µl) of PBS. The JES5-2A5 Abs were obtained from serum-free hybridoma supernatants, and were purified using HiTrap protein G columns (Amersham Pharmacia Biotech, Uppsala, Sweden). For in vivo depletion of CD8⁺ T cells, 200 µg anti-CD8 mAb 2.43 (32) was injected twice/week, starting 3 days before the primary immunization until 6 wk after the primary immunization. Treated sentinel mice were used to evaluate the efficiency of CD8⁺ T cell depletion by Ab staining and flow cytometric analysis of the spleen cell samples. The efficiency of this depletion was >95% for the target T cell population. The control group received equivalent volumes (100 µl) of PBS.

Cell transfer experiments

BALB/c mice were immunized with OVA-liposome, OVA-alum, or nothing at all at 0 and 3 wk. Four weeks after the primary immunization, splenic CD4⁺ cells of these mice were transferred i.v. into naive BALB/c *nu/nu* mice, at 1.5 × 10⁷ cells/mouse. Immediately after the cell transfer, recipient mice received immunization either with OVA-liposome or OVA-alum.

Results

Ab production in mice immunized with OVA using three different adjuvants

BALB/c mice were immunized with OVA using three different adjuvants, liposome, alum, or CFA, and serum anti-OVA Abs were monitored. As shown in Fig. 1a, comparable levels of anti-OVA IgG Ab production were observed in the three immunization groups. In contrast, anti-OVA IgE production was not observed in mice immunized either with OVA-liposome or OVA-CFA, whereas a substantial amount of anti-OVA IgE was produced in mice immunized with OVA-alum (Fig. 1b). Table I shows titers of two anti-OVA IgG isotypes, IgG1 and IgG2a, in the sera 6 wk after primary immunization. In mice immunized with OVA-liposome, no significant difference was observed in the amounts of IgG1 and IgG2a. In contrast, the IgG1 level was significantly ($p < 0.01$) higher than the IgG2a level in mice immunized with OVA-alum, and IgG2a was significantly ($p < 0.01$) higher than IgG1 in mice immunized with OVA-CFA. OVA-liposome solution inoculated into mice contained 100 µg OVA/injection.

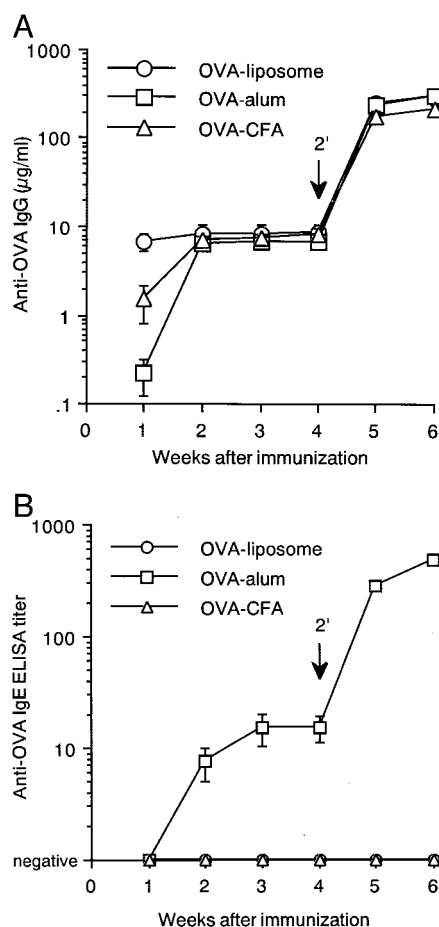


FIGURE 1. Anti-OVA Ab production in mice immunized with OVA-liposome, OVA-alum, or OVA-CFA. BALB/c mice were immunized i.p. with OVA-liposome (○), OVA-alum (□), or OVA-CFA (△) on days 0 and 28. A, Anti-OVA IgG. B, Anti-OVA IgE ELISA titers. Data represent the mean and SE of five mice/group.

Cytokine production by splenic CD4⁺ T cells from mice immunized with OVA using three different adjuvants

The results of the above experiment suggested that immunization with OVA-alum and OVA-CFA induced typical Th2- and Th1-type immune responses, respectively. Splenic CD4⁺ cells were taken from mice of the above experimental groups, and in vitro cytokine production was investigated. Because no cytokine production was observed in the absence of OVA under the culture conditions described in *Materials and Methods*, the data shown in Table II are considered to represent Ag-specific cytokine production. The levels of all cytokines tested were comparable in groups of mice immunized with OVA-liposome or OVA-alum. Both in the OVA-liposome-immune group, in which anti-OVA IgE Ab production was not induced, and in the OVA-alum-immune group, in which a substantial production of anti-OVA IgE was observed, not only Th1 cytokines (e.g., IL-2 and IFN-γ), but also Th2 cytokines (e.g., IL-4 and IL-5) were produced. Thus, T cell cytokine production did not appear to correlate with IgE Ab production in mice immunized with OVA-liposome or OVA-alum. In contrast, IL-4 and IL-5 were undetectable in the group of mice immunized with OVA-CFA, suggesting that OVA-CFA induced a typical Th1-type immune response.

Table I. Anti-OVA Ig isotype production in mice immunized with OVA-liposome, OVA-alum, or OVA-CFA^a

Immunization	Anti-OVA Abs (μg/ml)	
	IgG1	IgG2a
OVA-liposome	260.2 ± 72.7	239.4 ± 80.8
OVA-alum	252.8 ± 70.4	32.6 ± 5.4 ^b
OVA-CFA	29.2 ± 11.5	132.7 ± 21.7 ^b

^a Anti-OVA IgG1 and IgG2a Abs in the sera of mice 6 wk after primary immunization in the experiment shown in Fig. 1. Data represent the mean and SE of five mice/group.

^b Significant difference ($p < 0.01$) compared with IgG1 in the same immunization group.

Anti-OVA Ab production in IL-12-deficient mice immunized with OVA using three different adjuvants

To investigate whether or not the IgE-selective unresponsiveness induced by OVA-liposome is dependent on IL-12, IL-12-deficient mice were immunized with OVA-liposome, OVA-alum, or OVA-CFA, and anti-OVA Ab production was monitored. Similar to the results shown in Fig. 1a, OVA-liposome and OVA-alum induced comparable levels of anti-OVA IgG Ab production (Fig. 2a). In contrast, OVA-CFA induced significantly lower levels of anti-OVA IgG production throughout the observation period than those induced by OVA-liposome or OVA-alum, suggesting that the anti-OVA IgG Ab response induced by OVA-CFA was dependent on IL-12. Anti-OVA IgE Ab production was significantly induced by OVA-alum, whereas OVA-liposome induced IgE-selective unresponsiveness (Fig. 2b). In mice immunized with OVA-CFA, anti-OVA IgG production remained at low levels, and anti-OVA IgE production was undetectable.

Effects of in vivo inoculation with mAbs

The effects of in vivo inoculation with neutralizing anti-mouse IL-10 or cytotoxic anti-CD8 mAbs on anti-OVA Ab production were investigated in mice immunized with OVA-liposome. As shown in Table III, neither of the two Abs affected anti-OVA IgG production nor IgE unresponsiveness induced by OVA-liposome.

Anti-OVA Ab production in BALB/c nu/nu mice after CD4⁺ T cell transfer

We next evaluated the ability of CD4⁺ T cells of mice immunized either with OVA-liposome or OVA-alum to induce anti-OVA Ab production in athymic nude mice. As shown in Table IV, CD4⁺ T cells of mice immunized with OVA-liposome induced anti-OVA IgE Ab production upon immunization of recipient mice with OVA-alum. In contrast, CD4⁺ T cells of mice immunized with OVA-alum failed to induce anti-OVA IgE Ab production when recipient mice were immunized with OVA-liposome. Thus, IgE-selective unresponsiveness was induced by immunization of the recipient with OVA-liposome, irrespective of how the T cell donor was immunized.

Effect of secondary immunization with OVA-liposome on an ongoing IgE response

Anti-OVA IgG and IgE production were induced in mice by primary immunization with OVA-alum, and the effects of secondary immunization with OVA-liposome or OVA-alum were investigated. The control group received no secondary immunization. As shown in Fig. 3, secondary immunization with OVA-alum enhanced both IgG and IgE production. In contrast, secondary immunization with OVA-liposome enhanced IgG production, but not IgE production. Moreover, IgE production was even suppressed by

Table II. Cytokine production by splenic CD4⁺ T cells of OVA-immune mice^a

Immunization of T Cell Donor	Cytokines (pg/ml)				
	IL-2	IL-4	IL-5	IL-10	IFN- γ
OVA-liposome	1068.1 $\pm$ 115.3	146.6 $\pm$ 21.2	389.3 $\pm$ 103.3	707.9 $\pm$ 128.7	1335.9 $\pm$ 126.9
OVA-alum	1256.2 $\pm$ 66.5	205.5 $\pm$ 144.8	557.2 $\pm$ 72.2	1172.1 $\pm$ 151.0	1269.8 $\pm$ 192.7
OVA-CFA	946.3 $\pm$ 252.1	N.D.	N.D.	172.3 $\pm$ 26.7	1094.0 $\pm$ 132.2

^a Cytokine production by splenic CD4⁺ cells of mice immunized with OVA-liposome, OVA-alum, or OVA-CFA. Six weeks after primary immunization in the experiment shown in Fig. 1, splenic CD4⁺ cells were taken and cultured, as described in *Materials and Methods*. Data represent the mean cytokine concentration and SE of five mice/group. N.D., not detected.

secondary immunization with OVA-liposome 2 wk after the immunization.

Discussion

Although both OVA-liposome and OVA-CFA induced IgE-selective unresponsiveness (Fig. 1), CD4⁺ cells of mice immunized with OVA-liposome produced significant levels of Th2 cytokines, IL-4 and IL-5, whereas those of mice immunized with OVA-CFA did not produce Th2 cytokines (Table II). These results suggested that OVA-CFA, but not OVA-liposome induced IgE-selective unresponsiveness via the induction of a typical Th1-type immune response. In fact, anti-OVA IgG2a was predominant over IgG1 in the sera of mice immunized with OVA-CFA, whereas no signifi-

cant difference between anti-OVA IgG1 and IgG2a was observed in mice immunized with OVA-liposome (Table I). It is unlikely that the lack of IgE response was due to the high dose of OVA contained in the OVA-liposome solution, because the same dose of OVA solution induced production of far less IgG than that induced by OVA-liposome, but induced significant production of IgE (27). In general, Th1 is involved in the suppression of IgE production induced by high doses of Ag (17). However, in the present study, a polarized Th1 response was not observed in mice immunized with OVA-liposome. The experiment using IL-12-deficient mice further confirmed these results (Fig. 2): OVA-liposome induced IgE-selective unresponsiveness in IL-12-deficient mice, suggesting that the IgE-selective unresponsiveness induced by OVA-liposome was independent of IL-12, which is known to play a central role in promoting Th1 (14) and in suppressing IgE secretion via induction of IFN- γ (33, 34). In contrast, anti-OVA IgG production in IL-12-deficient mice immunized with OVA-CFA remained at low levels, suggesting that the immune response induced by OVA-CFA was primarily due to Th1. Further investigation using CD4⁺ T cell transfer to athymic nude mice demonstrated that CD4⁺ T cells of mice immunized with OVA-liposome were capable of inducing Ag-specific IgE synthesis in the recipients (Table IV). Moreover, CD4⁺ T cells of mice immunized with OVA-alum failed to induce anti-OVA IgE Ab production when recipient mice were immunized with OVA-liposome, indicating that the IgE-selective unresponsiveness induced by OVA-liposome did not involve CD4⁺ T cells. Similar results were obtained when splenic non-B cells, instead of CD4⁺ T cells, were used in the same experimental conditions (data not shown), suggesting that B cells might play a key role in the induction of IgE-selective unresponsiveness by OVA-liposome.

The results of this study demonstrated that different patterns of Ab response against the same Ag were induced by the use of three different adjuvants. It is known that OVA is allergenic by itself (35); however, the use of CFA or liposome as an adjuvant resulted

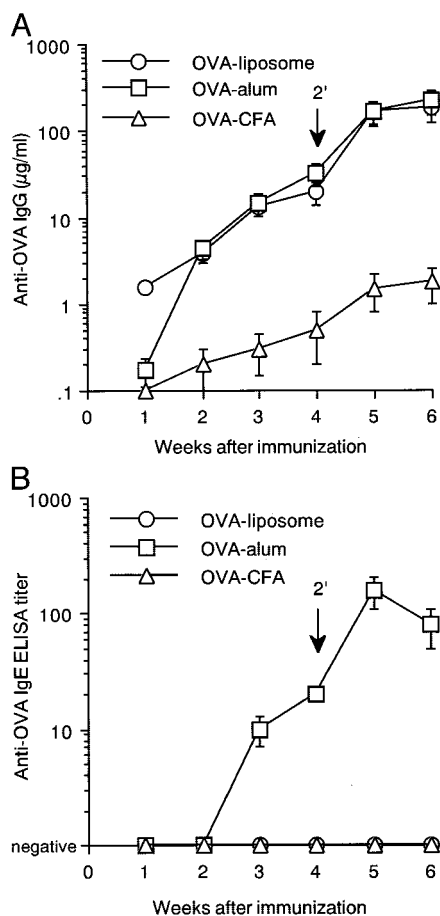


FIGURE 2. Anti-OVA Ab production in IL-12-deficient mice immunized with OVA-liposome, OVA-alum, or OVA-CFA. IL-12-deficient mice were immunized i.p. with OVA-liposome (○), OVA-alum (□), or OVA-CFA (△) on days 0 and 28. A, Anti-OVA IgG. B, Anti-OVA IgE ELISA titers. Data represent the mean and SE of five mice/group.

Table III. Effect of in vivo administration with mAbs on anti-OVA Ab production in mice immunized with OVA-liposome^a

Expt.	mAb	In Vivo Administration with mAb	Anti-OVA Abs	
			IgG (μg/ml)	IgE
1	Anti-IL-10	No	179.8 $\pm$ 22.0	N.D.
		Yes	176.3 $\pm$ 18.7	N.D.
2	Anti-CD8	No	192.7 $\pm$ 28.3	N.D.
		Yes	178.6 $\pm$ 23.8	N.D.

^a BALB/c mice were immunized with OVA-liposome at 0 and 4 wk. During the immunization, mice received inoculation with mAb, as described in *Materials and Methods*. Six weeks after primary immunization, mice were bled and serum anti-OVA Abs were monitored. Data represent the mean Ab titers and SE of five mice/group. N.D., not detected.

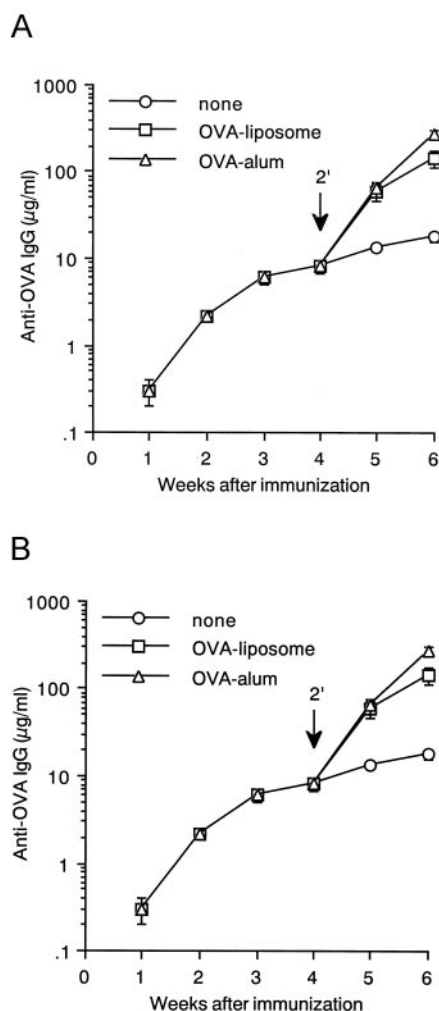


FIGURE 3. Effect of a booster injection with OVA-liposome on an ongoing IgE response. Mice were first immunized with OVA-alum, and 4 wk later, they received a booster injection with OVA-liposome (□) or OVA-alum (△). The control group did not receive the second immunization (○). A, Anti-OVA IgG. B, Anti-OVA IgE ELISA titers. Data represent the mean and SE of five mice/group. *, Significant ($p < 0.01$) as compared with the control group.

in IgE-selective unresponsiveness, which in the former case involved a polarized Th1-type immune response, but in the latter case did not. Thus, the results demonstrated the presence of an IgE-regulatory mechanism other than that due to a balance between Th1 and Th2. Because Mosmann et al. (15) reported the presence of Th1 and Th2 cells, the role of Th1 and Th2 cells has, to a significant extent, become dogma, with categorical statements now appearing in immunology textbooks. However, a number of reports have suggested the presence of another Th subset whose cytokine profile does not agree with that of Th1 or Th2 (36–38). In addition, at the single cell level, most Th cell clones produce only one cytokine (39). Moreover, regulation of IgE Ab production not due to polarization toward Th1 and Th2 cell subsets has also been reported (40). OVA-coupled liposomes might also induce IgE-selective unresponsiveness via a mechanism not due to polarization toward Th1.

OVA-alum induced significant production of anti-OVA IgE and a predominant production of IgG1. In contrast, OVA-liposome induced production of a level of IgG1 comparable with that of IgG2a without detectable IgE, suggesting that anti-OVA IgE and IgG1

Table IV. Anti-OVA Ab production in BALB/c nu/nu mice transferred splenic $CD4^+$ cells of BALB/c mice^a

Immunization of T Cell Donor	Immunization of Recipient Mice	Anti-OVA Abs	
		IgG (μg/ml)	IgE ELISA titer
No immunization	OVA-liposome	12.3 ± 8.7	N.D.
	OVA-alum	17.7 ± 5.2	N.D.
OVA-liposome	OVA-liposome	144.7 ± 24.3	N.D.
	OVA-alum	124.3 ± 12.8	105.6 ± 10.7
OVA-alum	OVA-liposome	178.0 ± 28.3	N.D.
	OVA-alum	246.8 ± 29.4	139.3 ± 12.3

^a BALB/c mice were immunized with OVA-liposome, OVA-alum, or not at all, and splenic $CD4^+$ cells of these mice were transferred i.v. into naive BALB/c nu/nu mice, as described in *Materials and Methods*. Data represent the mean Ab titers and SE of five mice/group. N.D., not detected.

Ab production were regulated independently in mice immunized with OVA-liposome. The discrepancy between the presence of IgG1 and IgE suggests the switching of B cells to the production of IgG1, but not to IgE. In fact, in humans, production of IgE and IgG4 is reported to be regulated differentially by IL-10 (41) or by conformational Ag variants (42). Also in mice, differential regulation of IgE and IgG1 has been reported (43). In addition, contrary to the general conception, Kolbe et al. (44) described a case in which no reciprocal regulation of IgE and IgG2a Ab formation was observed.

Although $CD4^+$ T cells of mice immunized with OVA-liposome produced a significant level of IL-4 upon in vitro stimulation with OVA, OVA-liposome did not induce anti-OVA IgE production in vivo, suggesting that the presence of IL-4 is not an adequate condition for the induction of switching to IgE. Although IL-4 is a critical requirement for the induction of switching to IgE, as demonstrated in early in vivo studies (16, 45, 46), evidence for the requirement of costimuli, i.e., additional B cell activators, has been provided by subsequent investigation (47, 48). Snapper et al. (47) reported that, in the presence of IL-4, stimulation of B cells with dextran-conjugated anti-IgD mAbs induced production of IgG1, but failed to induce IgE synthesis, while anti-IgD conjugated to Sepharose beads costimulated a strong IgE response, suggesting that a modification of B cell activators affects the induction of IgE synthesis. OVA-liposome, in which OVA is modified by coupling to liposomes, might activate OVA-specific B cells in a manner different from that activated by native OVA, and induced IgE-selective unresponsiveness in the present study.

IL-10, produced by activated T cells and macrophages, is known to inhibit IgE production (13). In addition, Tr1, a recently reported subset of $CD4^+$ T cells (49), is known to inhibit the Th2 response by producing IL-10 (50). However, it is unlikely that either Tr1 or IL-10 participated in the IgE-selective unresponsiveness induced by OVA-liposome, because $CD4^+$ cells of mice immunized with OVA-liposome produced a significant amount of IL-10 (Table II); furthermore, in vivo inoculation with IL-10-neutralizing mAb did not affect IgE-selective unresponsiveness (Table III).

Although originally known as cytotoxic cells, $CD8^+$ T cells have been reported to play a role in IgE regulation (51–53). However, in the present study, we did not seriously consider the possibility that $CD8^+$ T cells participated in the IgE-selective unresponsiveness induced by OVA-liposome, because cytokines produced by $CD8^+$ cells of mice immunized with OVA-liposomes were undetectable in the in vitro culture with OVA and APC (data not shown); furthermore, in vivo depletion of $CD8^+$ T cells, which was achieved by inoculating mAbs against CD8, had no effect

whatsoever on the immune response in mice immunized with OVA-liposome (Table III).

IFN- γ is known to inhibit IgE synthesis by counteracting IL-4 (9–11, 16). In addition, IFN- α is known to suppress IgE production via down-regulation of Th2 responses. This was shown by treatment of mice with IFN- α , which led to a decrease in production of IL-4 (12). However, in the present study, a significant level of IL-4 was produced by CD4⁺ T cells of mice immunized with OVA-liposome (Table II), and these CD4⁺ T cells induced IgE production in recipient mice in the transfer experiment (Table IV). It is unlikely that IFN- γ or IFN- α produced by other cell types suppressed IgE production because splenic non-B cells of mice immunized with OVA-liposome also induced IgE production in the transfer experiment (data not shown).

Booster immunization with OVA-liposome enhanced anti-OVA IgG Ab production, but did not enhance ongoing IgE production (Fig. 3), suggesting that the IgE-selective unresponsiveness induced in vivo by the liposomal Ag involved direct effects on IgE, but not IgG switching.

In conclusion, we investigated the mechanism of IgE-selective unresponsiveness induced by OVA-liposome. It was demonstrated that IgE production was regulated by a mechanism that did not involve polarization of Th1 and that was independent of IL-12 in mice immunized with OVA-liposome. OVA-liposome induced an IgG, but not an IgE class switch. Because CD4⁺ T cells derived from mice immunized with OVA-liposome produced IL-4 and induced IgE synthesis when transferred to athymic nude mice, it is likely that OVA-liposome may not have induced an additional B cell activator that participated in the induction of the IgE class switch. Thus, these results suggest the existence of an alternative mechanism not involving T cells in the regulation of IgE Ab production.

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