

## Original Article

# Cytokine production by spleen cells from mice with ovalbumin-specific, IgE-selective unresponsiveness induced by ovalbumin–liposome conjugate

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### ABSTRACT

Ovalbumin coupled with liposomes (OVA–liposome) induced selective unresponsiveness of anti-OVA IgE antibody production in BALB/c mice, whereas OVA adsorbed with aluminum hydroxide (OVA–alum) induced a substantial amount of anti-OVA IgE antibody production. Ovalbumin–liposome and OVA–alum predominantly induced IgG<sub>2a</sub> and IgG<sub>1</sub> anti-OVA production, respectively. These results suggest that OVA–liposome and OVA–alum induce type 1 and type 2 T helper (Th) immune responses, respectively. To further investigate this issue, we examined the cytokine production induced by these two distinct adjuvants. Spleen cells taken from mice immunized with either OVA–liposome or OVA–alum were cultured *in vitro* with OVA and the cytokine production from each culture was analyzed. It was demonstrated that spleen cells from mice immunized with OVA–liposome produced more interferon (IFN)- $\gamma$  than those immunized with OVA–alum and, furthermore, interleukin (IL)-4 was produced only by spleen cells from mice immunized with OVA–alum. These results favor the notion that OVA–liposome and OVA–alum induce Th1 and Th2 cytokines, respectively. Interestingly, the production of IL-2, a Th1 cytokine, was higher in the OVA–alum-immunized group and

the production of IL-10, a Th2 cytokine, remained at low levels in both groups after primary immunization; levels of IL-10 increased in the OVA–liposome-immunized group after secondary immunization. These results do not agree with the above notion and, thus, suggest that it may be important to consider the balance between IFN- $\gamma$ -producing cells and IL-4-producing cells rather than that between Th1 and Th2 cells for the regulation of IgE antibody production.

**Key words:** cytokine, IgE unresponsiveness, ovalbumin–alum, ovalbumin–liposome, Th1/Th2, vaccine.

### INTRODUCTION

Vaccines are nowadays regarded as the most efficient tool for the control or elimination of severe infectious diseases. However, there have been a number of reports regarding unwanted side effects of vaccines, such as local swelling, fever and anaphylaxis caused by vaccinations.<sup>1–3</sup> Many cases are diagnosed as immediate-type allergic reactions and it is well established that the IgE antibody induced against the vaccine antigen or vaccine components is responsible for the reactions. Adjuvants are commonly used in vaccine preparations to elicit an immune response because most vaccine antigens, particularly purified, subunit and synthetic antigens, possess weak immunogenicity by themselves. Aluminum (alum) adjuvants, the most commonly used adjuvants in modern vaccine preparations, are well known to induce IgE synthesis.<sup>4</sup> We have been focusing on the establishment of an immunization protocol for inducing the production of protective antibodies, mainly associated

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with the IgG isotype, without the induction of IgE antibody production. We have recently reported that, in mice, intraperitoneal (i.p.) administration of ovalbumin coupled with murine red blood cells (OVA-MRBC) induced anti-OVA IgG antibody production without IgE synthesis.<sup>5</sup> For the practical use of this system, liposomes were chosen for coupling with the antigen as they are biodegradable and are composed of non-toxic and immunologically inert phospholipids that are common to all mammalian cell membranes. Ovalbumin-liposome successfully induced a substantial amount of IgG antibody production with least IgE antibody production.<sup>6</sup> Although the application of this protocol for practical use is currently in progress, the mechanism for the regulation of IgE antibody production remains unclear. The aim of the present study was to elucidate the mechanism for IgE-selective unresponsiveness induced by immunization with OVA-liposome.

## METHODS

### Mice

BALB/c mice (8 weeks of age; female) were purchased from SLC (Shizuoka, Japan).

### Antigen

Ovalbumin (grade IV) was purchased from Sigma Chemical Co. (St Louis, MO, USA).

### OVA-liposome conjugate

The preparations of liposomes and OVA-liposome conjugates were made as described previously.<sup>6</sup> Briefly, 0.5 mL of 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 mol/L glycine-NaOH (pH 7.2) was added and kept at 4°C overnight. Liposome-coupled and -uncoupled OVA were separated using CL-4B column chromatography (Pharmacia Fine Chemical Co., Uppsala, Sweden). The resulting conjugate of OVA-liposome contained an average of  $0.47 \pm 0.01$  mg OVA per 10 mg liposome, as quantified using radiolabeled OVA.

### Immunization

Mice were immunized i.p. with 2  $\mu$ g OVA adsorbed with 3 mg aluminum hydroxide (alum) in 200  $\mu$ L phosphate-buffered saline (PBS), pH 7.2. Ovalbumin-liposome was injected i.p. at a dose of 2 mg lipid/200  $\mu$ L per mouse.

## Quantification of Ig levels

Mice were bled weekly from the tail vein. Levels of antigen-specific IgG and IgG<sub>1</sub> and IgG<sub>2a</sub> subclasses in the sera were determined by ELISA using peroxidase-labeled rabbit antibodies against mouse IgG or mouse Ig subclasses (Zymed Lab., San Francisco, CA, USA). For the quantitative analysis of antigen-specific antibody, murine monoclonal antibodies against OVA with IgG<sub>1</sub> and IgG<sub>2a</sub> subclasses produced in our laboratory were used as the assay standard. Levels of antigen-specific IgE were determined by a monoclonal antibody captured ELISA.

## Spleen cell culture

Spleen cells were taken from mice immunized with OVA-alum or OVA-liposome and were suspended in RPMI-1640 containing 10% fetal calf serum (FCS) at a cell density of  $5 \times 10^6$  /mL. In preliminary examinations, OVA at a final concentration of 1 mg/mL and a 24 h culture period were found to be the optimal conditions for cytokine production by spleen cells. The cell suspension was plated at 100  $\mu$ L per well onto a 96-well culture plate (No. 3072; Becton-Dickinson Labware, Franklin Lakes, NJ, USA) and 100  $\mu$ L of 2 mg/mL OVA solution in the same media was added. After incubation for 24 h in a CO<sub>2</sub> incubator, the culture supernatants were assayed for cytokines.

## Cytokine ELISA

Concentrations of interleukin (IL)-4, IL-10 and interferon (IFN)- $\gamma$  in the supernatants were measured using InterTest™-4X, -10X and - $\gamma$  (Genzyme, Cambridge, MA, USA), respectively. The Biotrak™ mouse ELISA system for IL-2 (Amersham International plc, Buckinghamshire, UK) was used for the measurement of IL-2. All test samples were assayed in duplicate and the SEM in each test was less than 5% of the mean value.

## RESULTS

### Anti-OVA antibody production

As shown in Fig. 1a, both OVA-liposome and OVA-alum induced comparable anti-OVA IgG antibody production more than 2 weeks after immunization. In contrast, only OVA-alum but not OVA-liposome induced anti-OVA IgE antibody production (Fig. 1b). Serum taken from mice 4 weeks after immunization was assayed for Ig subclasses. As shown in Fig. 1c, IgG<sub>2a</sub> antibody production was

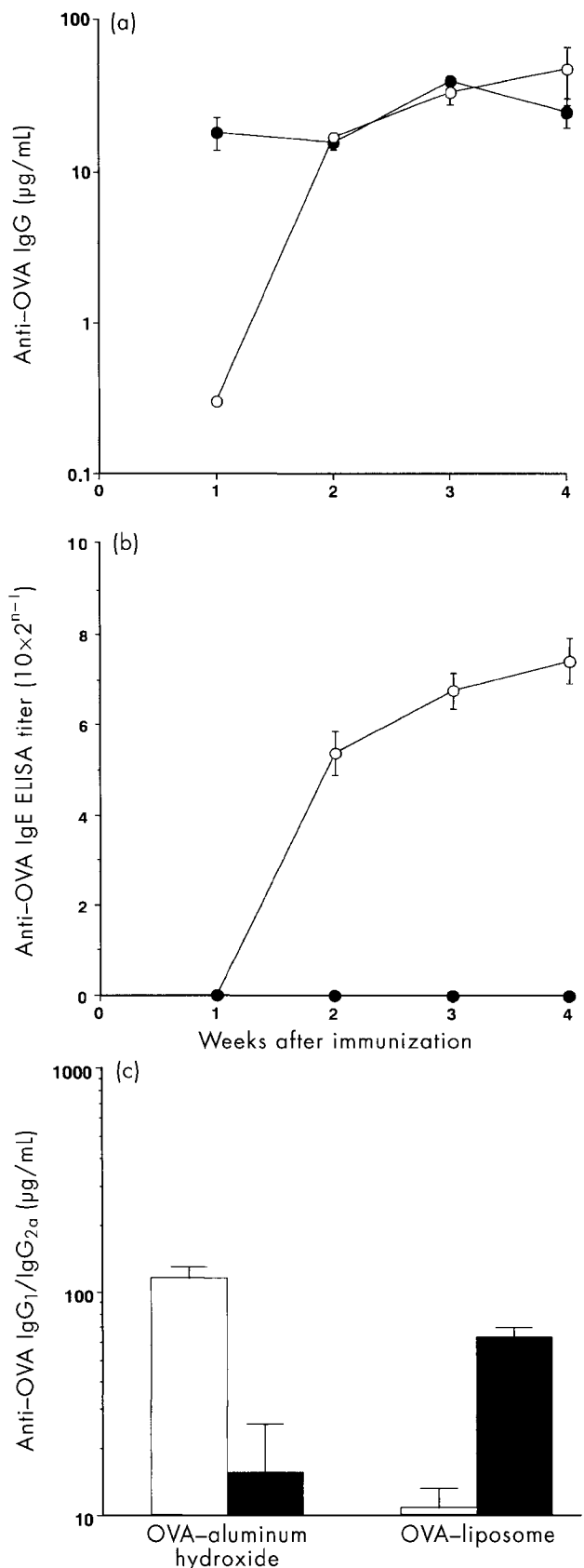
dominant in mice immunized with OVA-liposome, whereas IgG<sub>1</sub> antibody production was dominant in mice immunized with OVA-alum.

### Cytokine production by spleen cells from OVA-immunized mice

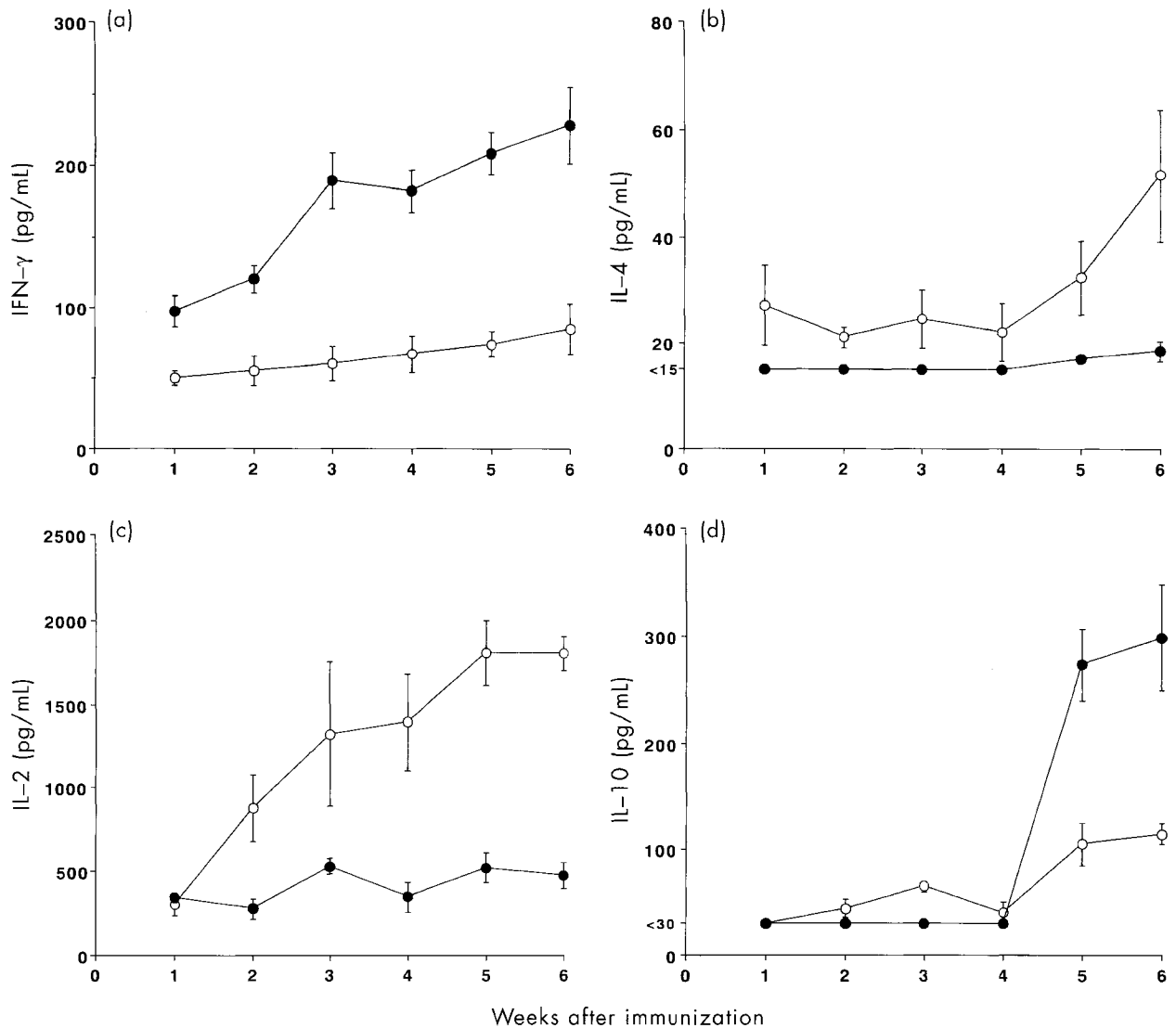
Spleen cells were taken from mice immunized with OVA-alum or OVA-liposome and were stimulated *in vitro* with OVA to observe OVA-specific cytokine production. Four weeks after primary immunization, mice were boosted in the same manner as for primary immunization. As shown in Fig. 2a, the amount of IFN- $\gamma$  in the supernatants of spleen cells from OVA-liposome-immunized mice was higher than that from OVA-alum-immunized mice throughout the observation period. In contrast, IL-4 was detected only in the supernatants of spleen cells from OVA-alum-immunized mice (Fig. 2b). Figure 2c shows the amount of IL-2 in the same sample. More IL-2 was detected in cultures of spleen cells from OVA-alum-immunized mice. In addition, levels of IL-10 remained low in both groups after primary immunization, but increased and was higher in the OVA-liposome-immunized group at 5 weeks or later after immunization than in the OVA-alum-immunized group (Fig. 2d).

### DISCUSSION

Mosmann *et al.*<sup>7</sup> have established two types of T helper cell clones with distinct patterns of cytokine production, namely Th1 and Th2 cells. Interferon- $\gamma$  secreted from Th1 cells induces IgG<sub>2<sub>a</sub></sub> antibody production without IgE synthesis and counteracts IL-4, which is secreted from Th2 cells and induces the production of IgG<sub>1</sub> and IgE.<sup>7</sup> In the present study we demonstrated that liposome-coupled OVA induced the predominant production of IgG<sub>2<sub>a</sub></sub> but not IgE anti-OVA antibodies, whereas alum-adsorbed OVA induced IgG<sub>1</sub> as well as IgE anti-OVA antibody production. These results imply that two distinct immunization protocols (i.e. OVA-liposome and OVA-alum) induced two distinct T helper subsets (i.e. Th1 and Th2). This notion was further supported in the present study by the fact that IFN- $\gamma$  and IL-4 were



**Fig. 1** Anti-ovalbumin (OVA) antibody production in BALB/c mice immunized with ovalbumin coupled with either aluminum hydroxide (○) or liposomes (●). (a) IgG, (b) IgE, (c) IgG<sub>1</sub> (□) and IgG<sub>2<sub>a</sub></sub> (■). Data represent the mean  $\pm$  SEM of five mice per group.



**Fig. 2** Cytokine production by spleen cells from mice immunized with ovalbumin coupled with either aluminum hydroxide (O) or liposomes (●). Four weeks after primary immunization, mice were boosted in the same manner as for primary immunization. Spleen cells were cultured *in vitro* in the presence of ovalbumin and cytokines of the supernatants were measured thereafter. (a) Interferon- $\gamma$ , (b) interleukin (IL)-4, (c) IL-2, (d) IL-10. Data represent the mean  $\pm$  SEM of five mice for every time point, representative of three independent experiments.

produced by spleen cells of mice immunized with OVA-liposome and OVA-alum, respectively, suggesting that mice immunized with OVA-liposome preferentially induced Th1, while those immunized with OVA-alum preferentially induced Th2. We tried to ascertain whether this explanation was correct by examining the production of both IL-2 and IL-10, rather than Th1 and Th2 cytokines, respectively, from the spleen cells of mice immunized with either adjuvant following stimulation *in vitro* with OVA. However, the results were inconsistent with the theory, as the production of IL-2 was much higher in mice immunized with OVA-alum than in those

immunized with OVA-liposome and IL-10 production remained at low levels in both groups after primary immunization and was observed to be higher in the OVA-liposome-immunized group than in the OVA-alum-immunized group after the secondary immunization, although anti-OVA IgE antibody production was undetectable in the OVA-liposome-immunized group, even after secondary immunization (data not shown). Similar results were obtained when T cells were enriched by passing spleen cells through a nylon wool column and cultured with OVA and antigen-presenting cells (data not shown). The antigen-presenting cells used in the

experiment were cloned macrophage hybridoma<sup>8</sup> that did not produce detectable levels of IL-10, IL-2, IL-4 or IFN- $\gamma$ , either constitutively or even after stimulation with lipopolysaccharide (data not shown), suggesting that the cytokine production observed in cultured spleen cells was due to T cells. Miyajima *et al.*<sup>9</sup> have reported the IL-2 suppression of IgE production by B cells stimulated with IL-4, demonstrating that IL-2, together with IFN- $\gamma$ , participates in the regulation of IgE. However, in the present study, IL-2 was produced even more in the OVA-alum-immunized group in which a significant level of IgE production was observed. These results suggest that the patterns of cytokine production induced by these two immunization protocols do not necessarily agree with Th1/Th2 cytokine patterns.

Although there are many well-documented Th1 and Th2 responses,<sup>10-13</sup> they are not the only cytokine patterns possible.<sup>14</sup> In fact, Bucy *et al.*<sup>15</sup> have reported that in primary responses only a minority of cytokine-positive cells coexpressed two cytokines and, even in secondary responses, double-positive cells remained a minority of the total, suggesting that T helper cells expressing Th1 and Th2 phenotypes are rare. Studies on cytokine phenotypes of T helper cells at the single cell level have demonstrated that IL-4 and IL-5 are often coexpressed, IL-4 and IL-10, IL-10 and IFN- $\gamma$  are expressed in some cases,<sup>16</sup> but IL-4 and IFN- $\gamma$  are exclusively expressed.<sup>16,17</sup> Thus, for the regulation of IgE antibody production, we may have to consider the balance between IFN- $\gamma$ - and IL-4-producing cells rather than the balance between Th1 and Th2 cells. We are currently investigating the cytokine production at the single cell level in order to clarify the correlation between IgE unresponsiveness and the patterns of cytokine production induced by certain immunization protocols. These studies would be of value in the development of novel vaccine preparations free of IgE antibody production.

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