

Antigen-Specific, IgE-Selective Unresponsiveness Induced by Antigen-Liposome Conjugates

Comparison of Four Different Conjugation Methods for the Coupling of Antigen to Liposome

Yoshio Nakano^a Masahito Mori^a Shouichi Nishinohara^a Yusaku Takita^b
Seishiro Naito^c Atsuko Horino^c Hiroshi Kato^c Maiko Taneichi^d
Yasushi Ami^e Yuriko Suzaki^e Katsutoshi Komuro^c Tetsuya Uchida^c

^aNOF Corp., Tsukuba Research Laboratory, Ibaraki; ^bDepartment of Applied Chemistry, Oita University, Oita;
^cDepartment of Safety Research on Biologics, ^dDepartment of Bacterial and Blood Products, and ^eDivision of Experimental Animals Research, National Institute of Infectious Diseases, Tokyo, Japan

Key Words

IgE unresponsiveness · Antigen-liposome conjugates · Vaccine · Cytokine · Th1/Th2

Abstract

Background: We have previously reported that ovalbumin (OVA) coupled with liposome via glutaraldehyde (GA) induced OVA-specific- and IgE-selective unresponsiveness in mice. **Methods:** In this study, OVA-liposome conjugates were made using four different coupling protocols: via GA, N-(6-maleimidocaproyloxy) succinimide (EMCS), disuccinimidyl suberate (DSS) and N-succinimidyl-3(2-pyridyldithio)propionate (SPDP) and the induction of antigen-specific IgG and IgE antibody production was investigated for each. In addition, antigen-specific cytokine production by spleen cells of mice immunized either with OVA-liposome or with OVA adsorbed with aluminum hydroxide was investigated. **Results:** OVA-liposome conjugates coupled via GA or DSS did not induce anti-OVA IgE antibody production but induced substantial anti-OVA IgG antibody production. On

the other hand, the induction of anti-OVA IgE unresponsiveness by OVA-liposome conjugates coupled via EMCS or SPDP was incomplete. The amount of interleukin 4 (IL-4) produced by spleen cells stimulated in vitro with OVA correlated well with anti-OVA IgE antibody production in donor mice. However, the production of no other cytokine, i.e., IL-2, IL-5, IL-10 or interferon- γ , was correlated with in vivo IgE antibody production. **Conclusion:** OVA-liposome coupled via GA or DSS induced complete suppression of anti-OVA IgE production. The results in this study further suggest that the regulation of IgE antibody production does not necessarily correlate with so-called Th1 cytokine production.

Copyright © 1999 S. Karger AG, Basel

Introduction

Vaccines are currently regarded as the most efficient tool to control or eliminate severe infectious diseases. However, a number of reports have also described the unwanted side effects of vaccinations, including local swelling, fever, and

anaphylaxis [1, 2]. Many of these cases are diagnosed as immediate-type allergic reactions, and it has been well established that these reactions are caused by IgE antibody induced against the vaccine antigen or the vaccine components. Adjuvants are commonly used in the vaccine preparations to elicit an immune response because most vaccine antigens, especially purified, subunit and synthetic antigens, possess weak immunogenicity by themselves [3]. Aluminum adjuvants, the only adjuvants approved for clinical use, are well known to induce IgE synthesis [4, 5]. Improved adjuvants other than currently used aluminum adjuvants are essential for the development of new vaccines.

Among the candidates for vaccine carriers in the improved vaccines, liposomes have been shown to promote humoral and cell-mediated immunity to a wide spectrum of bacterial, protozoan and viral antigens as well as to tumor cell antigens in animal immunization studies [6], and have been successfully used in clinical trials [7, 8]. To date, most liposomal vaccines tested have been made by antigen entrapment [9]. However, antigen-entrapped liposomes and surface-coupled antigens of liposomes are reported to induce different types of immune responses [10]. That is, antigen-entrapped liposomes have been shown to induce antigen-specific IgE antibody production [11], while we have previously reported that immunization of mice with surface-coupled OVA of liposomes (OVA-liposome) induced substantial IgG antibody production with a minimal amount of IgE antibody production [12]. The same results were found when different antigens other than OVA, such as tetanus toxoid [13] or Shiga-like toxin [14], were coupled to liposomes. Therefore, antigen-liposome conjugates are expected to be candidates for novel protocols of vaccination that induce the least IgE antibody production.

The aim of this study was to determine the optimal procedure among four antigen-liposome coupling procedures for producing antigen-liposome conjugates. In addition, antigen-specific cytokine production was investigated using spleen cells (SC) of mice immunized with OVA-liposome or with aluminum-adsorbed OVA. The correlation between T cell cytokine profile and IgE regulation *in vivo* is discussed.

Material and Methods

BALB/c mice (8 weeks of age, female) were purchased from Charles River (Yokohama, Kanagawa, Japan). Antigen: ovalbumin (OVA, grade V) was purchased from Sigma (St. Louis, Mo., USA). Liposomes (45 mg lipid/ml) used for the coupling with OVA were composed of dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidyl ethanolamine (DPPE), cholesterol, and dimyristoyl

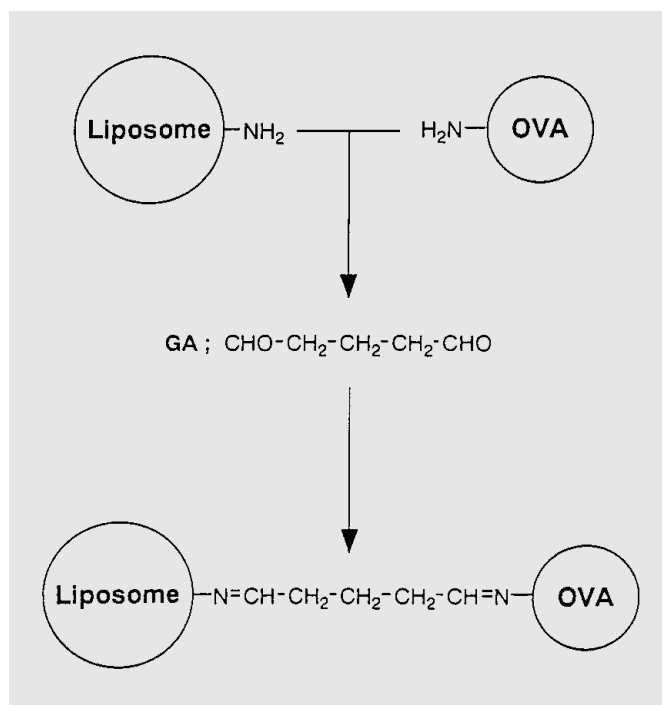


Fig. 1. Coupling of OVA to liposomes via GA.

phosphatidyl glycerol (DMPG) acid in 4:3:7:2 molar ratios. The liposome preparation used in this study contained DPPE since the amino group was essential on the surface of the liposome to couple the antigen with liposomes.

Coupling of OVA to Liposomes via GA

Liposomal conjugates with OVA (OVA-liposome) via glutaraldehyde (GA; fig. 1) were prepared as described previously [5]. Briefly, to a mixture of 90 mg of liposomes and 6 mg of OVA in 2.5 ml phosphate buffer (pH 7.2) was added in drops 0.5 ml of 2.5% GA solution. The mixture was stirred gently for 1 h at 37°C, and then 0.5 ml of 3 M glycine-NaOH (pH 7.2) was added to block excess aldehyde groups. This was followed by incubation overnight at 4°C. The OVA-liposome and uncoupled OVA in the resulting solution were separated using CL-4B column chromatography (Pharmacia Fine Chemical Co., Uppsala, Sweden). The amount of lipid in the liposomal fraction was measured using a phospholipid content assay kit (Phospholipid-Test-Wako: Wako Pure Chemical Industries, Osaka, Japan). The OVA-liposome solution was adjusted to 10 mg lipid/ml and kept at 4°C until use.

Coupling of OVA to Liposomes via N-(6-Maleimidocaproyloxy) Succinimide

Coupling of OVA to liposomes via N-(6-maleimidocaproyloxy) succinimide (EMCS; fig. 2) was performed according to the procedure described by Martin et al. [15] with a modification. Briefly, 11.1 ml of 1% EMCS solution in anhydrous methanol and 45 µl of triethylamine (TEA) were added to 0.2068 g of DPPE dissolved in 10 ml of a 1:1 mixture of anhydrous methanol and chloroform. The

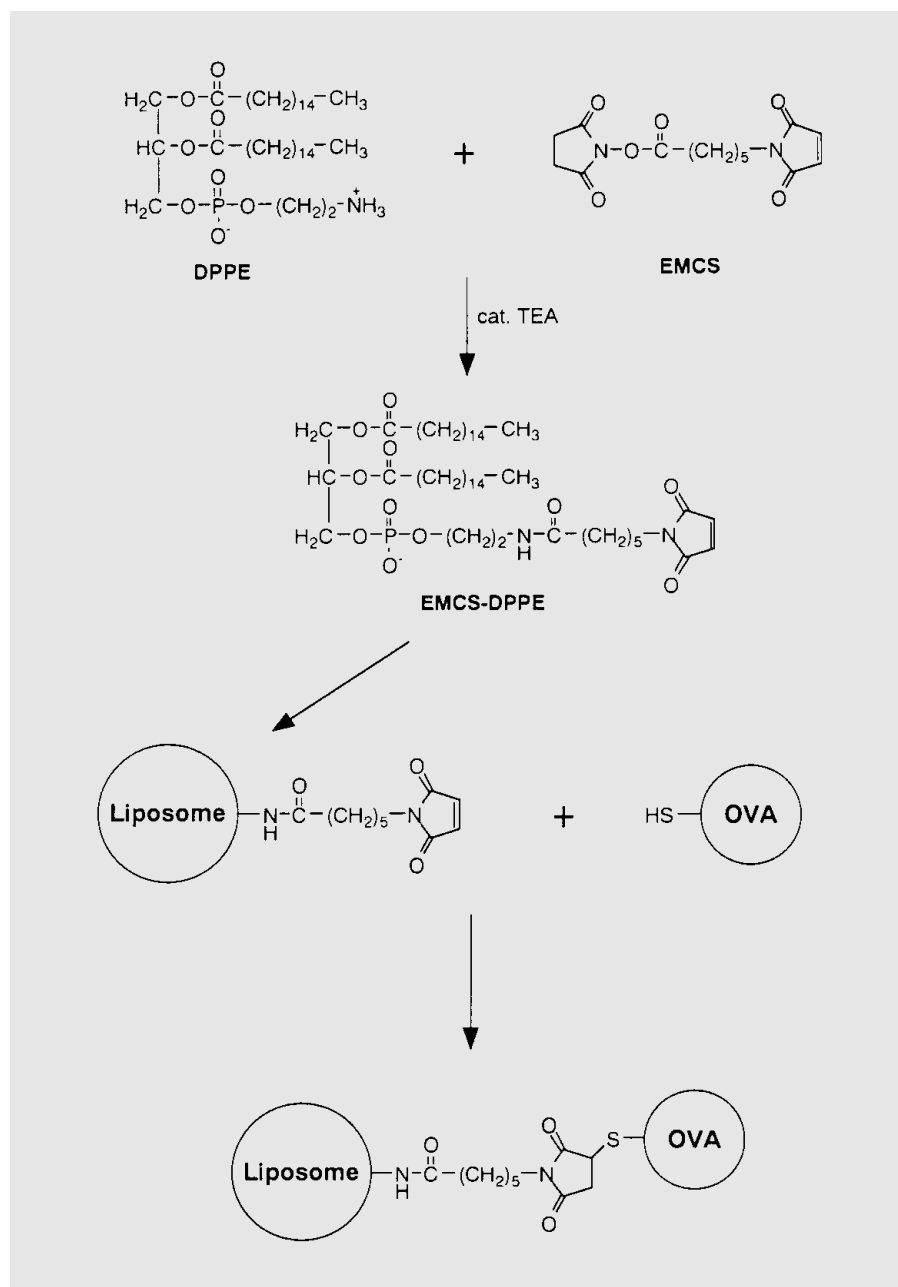


Fig. 2. Coupling of OVA to liposomes via EMCS.

mixture was stirred for 5 h at 40 °C. After evaporating the solvent under reduced pressure, residue was dissolved in 15 ml of acetone and cooled at 4 °C for 2 h to precipitate DPPE-EMCS. 0.188 g of dried crystals of purified DPPE-EMCS was obtained in the vacuum chamber with further recrystallization. 0.6 M DPPC, 0.1 mM DPPE-EMCS, 0.7 mM cholesterol, and 0.2 mM DMPG were dissolved in 10 ml of chloroform/methanol (1/1). The solvent was then removed under reduced pressure and 19.6 ml of phosphate buffer (pH 7.2) was added to make a 4.8% lipid suspension. Vesicle dispersion was extruded through a 0.2-μm polycarbonate filter to adjust liposome size. 1.5 ml

of liposome suspension and 3 ml of 1.25 mM OVA solution were mixed and stirred for 3 days at 5 °C. The liposome-coupled and uncoupled OVA were separated as described above.

Coupling of OVA to Liposome via Disuccinimidyl Suberate

Coupling of OVA to liposomes via disuccinimidyl suberate (DSS; fig. 3) was performed according to the method described by Kinsky et al. [16] with modification. Briefly, a mixture of 10 ml of anhydrous chloroform solution containing 0.136 mM DPPE and 24 μl of TEA was added in drops to 26.6 ml of anhydrous chloroform solution con-

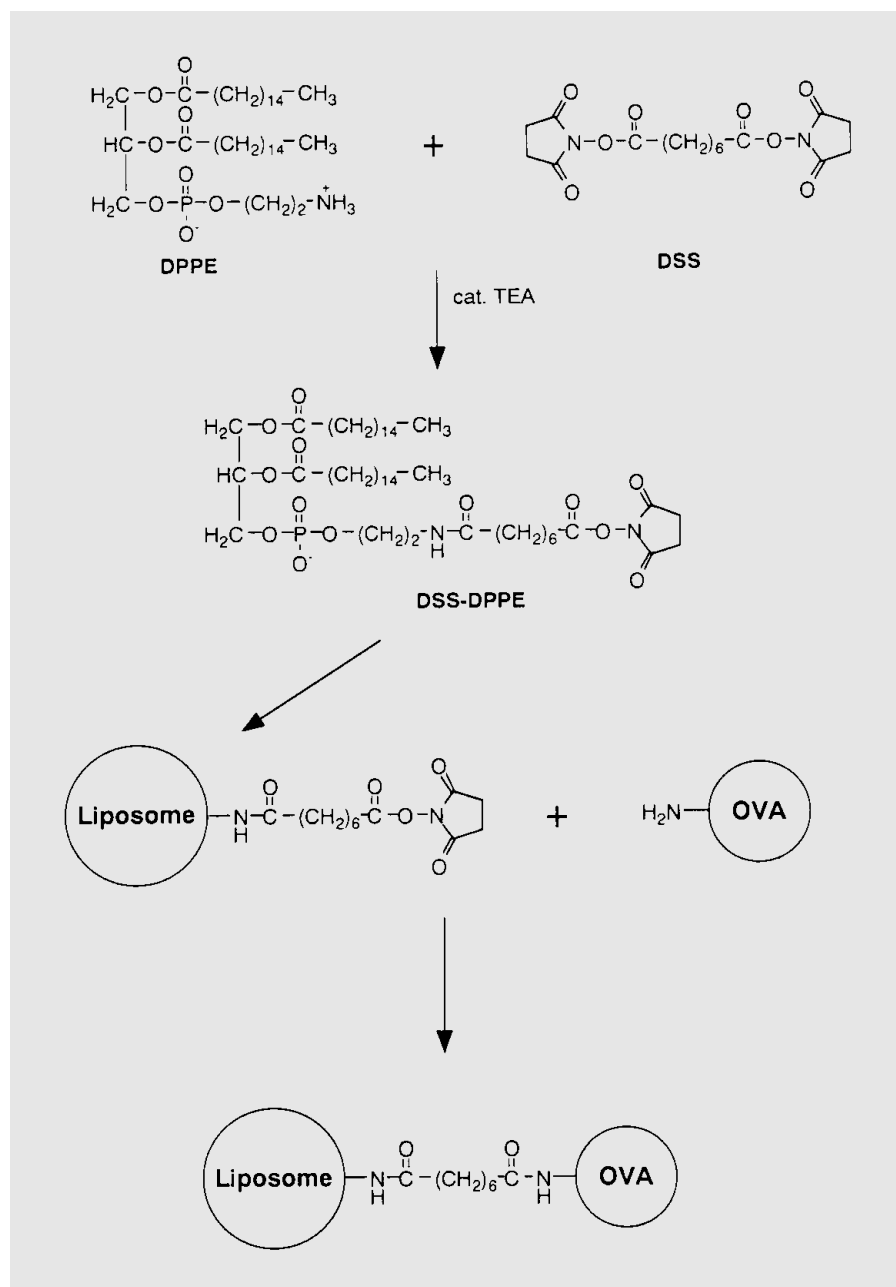


Fig. 3. Coupling of OVA to liposomes via DSS.

taining 0.681 mM DSS and stirred for 5 h at 40°C. The solvent was evaporated under reduced pressure and 18 ml of 2:1 mixture of ethyl acetate, and tetrahydrofuran was added to dissolve the residue. 36 ml of 100 mM sodium phosphate (pH 5.5) and 90 ml of saturated NaCl aqueous solution were added to the solution, shaken for 1 min, and allowed to separate. To remove undesirable materials, the upper layer was washed with the same buffer and, after evaporation of the solvent, 3 ml of acetone was added to dissolve the residue. 100 ml of ice-cold acetone was added in drops and kept on ice for 30 min to precipitate. Crystals were collected and dissolved in 5 ml of chloroform. After evaporation, 34.4 mg of DPPE-DSS was obtained. 0.18 mM

DPPE, 0.03 mM DPPE-DSS, 0.21 mM cholesterol, and 0.06 mM DMPG were dissolved in 10 ml of chloroform/methanol (1/1). The solvent was then removed under reduced pressure and 5.8 ml of phosphate buffer (pH 7.2) was added to make a 4.8% lipid suspension. Vesicle dispersion was extruded through a 0.2- μ m polycarbonate filter to adjust liposome size. 2 ml liposome suspension and 4 ml of 0.76 mM OVA solution were mixed and stirred for 5 days at 5°C. The liposome-coupled and uncoupled OVA were separated as described above.

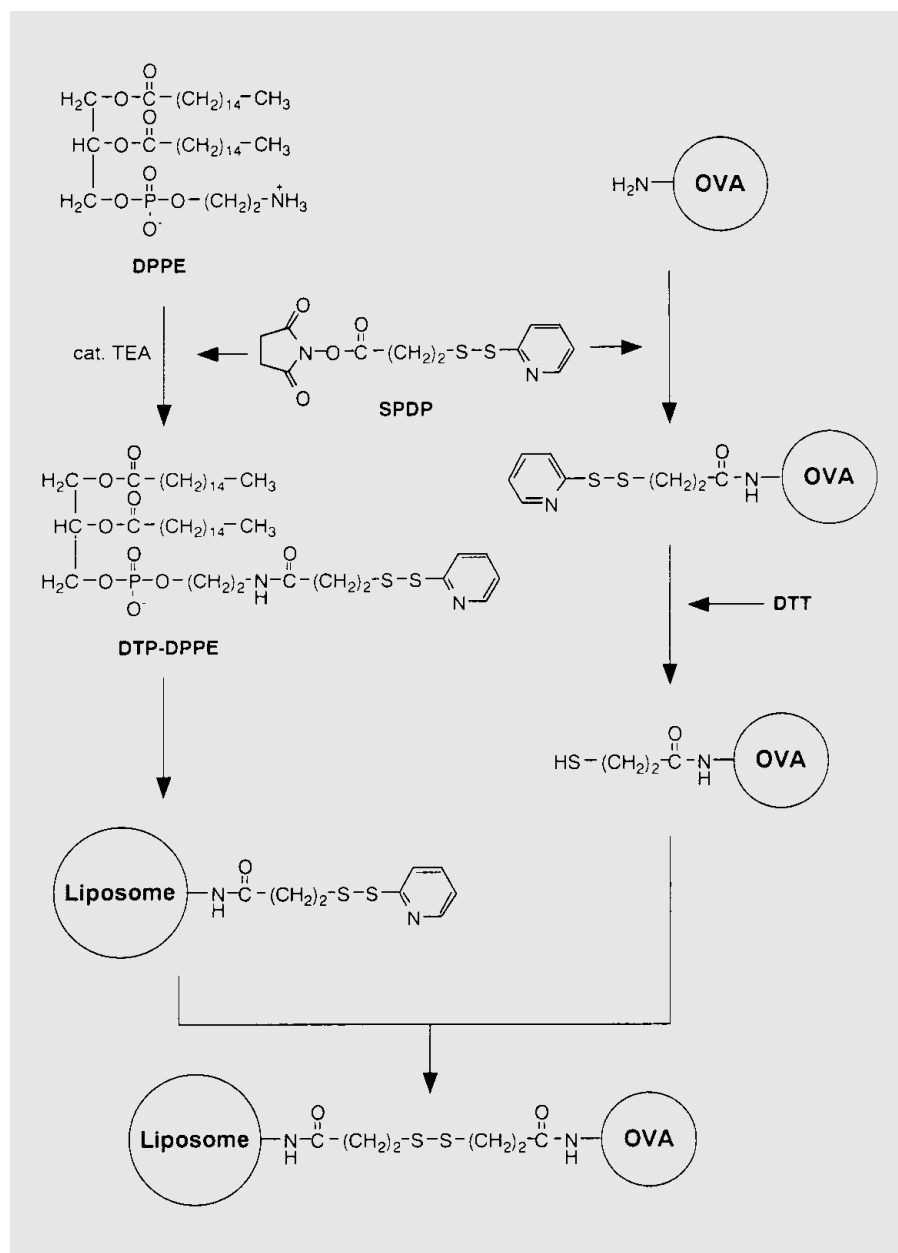


Fig. 4. Coupling of OVA to liposomes via SPDP.

Coupling of OVA to Liposomes via N-Succimidyl-3-(2-Pyridyldithio) Propionate

Coupling of OVA to liposomes via N-succimidyl-3-(2-pyridyldithio) propionate (SPDP: fig. 4) was performed according to the method described by Heath et al. [17] with a modification. Briefly, 10.1 mM DPPE was dissolved in 500 ml of a 5:1 mixture of anhydrous chloroform/methanol, and then 12.1 mM SPDP and 2.8 ml of TEA were added. The mixture was stirred for 2 h at 25°C and then the solvent was evaporated under reduced pressure. The residue was dissolved in 580 ml of acetone and kept on ice for 1 h to precipitate. The collected crystals were further crystallized and dried under reduced pressure. 8.3 g of DPPE-DTP was obtained. 0.6 mM DPPC, 0.1 mM DPPE-DTP, 0.7 mM cholesterol, and 0.2 mM DMPG were dissolved

in 10 ml of chloroform/methanol (1/1). The solvent was then removed under reduced pressure and 19.6 ml of phosphate buffer (pH 7.2) was added to make a 4.8% lipid suspension. Vesicle dispersion was extruded through a 0.2-µm polycarbonate filter to adjust liposome size. To 3.74 ml of 10 mg/ml OVA solution in PBS, 112.5 ml of 30 mM SPDP solution in ethanol was added in drops and stirred for 30 min at 25°C. OVA-DTP and SPDP were separated using G-25 column chromatography. OVA-SH was obtained by mixing 2 ml of OVA-DTP solution and 240 µl of dithiothreitol. Solutions of liposome-DTP and OVA-SH were mixed and stirred for 3 days at 4°C. OVA-liposome and uncoupled OVA-SH were separated using CL-4B column chromatography as described above.

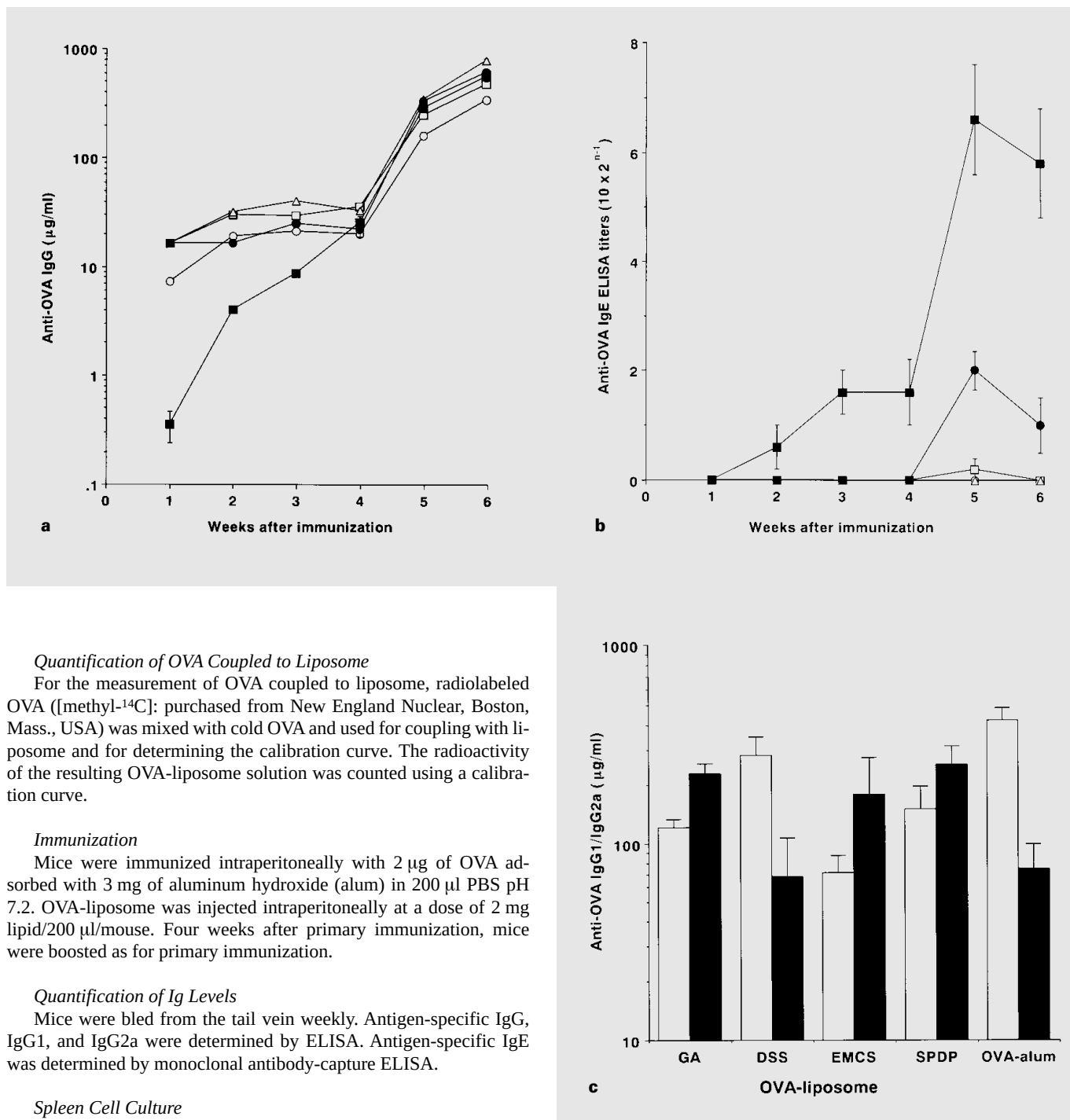


Fig. 5. Anti-OVA antibody production in mice immunized with OVA-liposome conjugate or with OVA-alum. Mice were immunized with OVA-liposome coupled via GA (○), EMCS (□), DSS (△), or via SPDP (●) or with OVA-alum (■). Immunization was done as described in Materials and Methods. Serum anti-OVA IgG and IgE antibody were measured using ELISA. **a** Anti-OVA IgG. **b** Anti-OVA IgE. **c** Anti-OVA IgG isotype antibodies assayed for the serum sample obtained 6 weeks after primary immunization. Data represent mean and SE of 5 mice per group.

Quantification of OVA Coupled to Liposome

For the measurement of OVA coupled to liposome, radiolabeled OVA ([methyl- ^{14}C]; purchased from New England Nuclear, Boston, Mass., USA) was mixed with cold OVA and used for coupling with liposome and for determining the calibration curve. The radioactivity of the resulting OVA-liposome solution was counted using a calibration curve.

Immunization

Mice were immunized intraperitoneally with 2 μg of OVA adsorbed with 3 mg of aluminum hydroxide (alum) in 200 μl PBS pH 7.2. OVA-liposome was injected intraperitoneally at a dose of 2 mg lipid/200 μl /mouse. Four weeks after primary immunization, mice were boosted as for primary immunization.

Quantification of Ig Levels

Mice were bled from the tail vein weekly. Antigen-specific IgG, IgG1, and IgG2a were determined by ELISA. Antigen-specific IgE was determined by monoclonal antibody-capture ELISA.

Spleen Cell Culture

Six weeks after primary immunization, SC were taken from mice and suspended in RPMI-1640 containing 10% FCS at a cell density of $5 \times 10^6/\text{ml}$. In the preliminary examination, OVA at a final concentration of 1 mg/ml and duration of culture for 72 h was found to be optimal for the production of all cytokines tested in this study. The cell suspension was plated at 100 μl per well onto a 96-well culture plate (No. 3072, Becton Dickinson Labware, Franklin Lakes, N.J., USA), and 100 μl of 2 mg/ml OVA solution in the same media was added to each well. After incubation for 72 h in a CO_2 incubator, the culture supernatants were assayed for cytokines.

Cytokine ELISAs

Concentrations of IL-2, IL-4, IL-5, IL-10, and IFN- γ in the culture supernatants were measured using a Biotrak™ mouse ELISA system (Amersham International, UK). All test samples were assayed in duplicate and the standard error in each test was less than 5% of the mean value.

Statistical analysis was done by Student's *t* test.

Results

Comparison of Anti-OVA Antibody Production in Mice Induced by OVA-Liposome Conjugates Made Using Four Different Conjugation Methods

The amount of OVA coupled to liposomes in OVA-liposome conjugates made using GA, EMCS, DSS, and SPDP were 47.0, 43.8, 47.8, and 40.1 $\mu\text{g}/\text{mg}$ lipid, respectively. Mice were immunized with these conjugates or OVA-alum, and anti-OVA antibody production was investigated. As shown in figure 5a, all four conjugates induced a comparable level of anti-OVA IgG antibody production. OVA-liposome induced a high level of anti-OVA IgG production as early as 1 week after immunization. This tendency was consistent among each of the four groups receiving immunization with OVA-liposome conjugates. In the OVA-alum-immune group, serum anti-OVA IgG increased gradually in the 4 weeks after immunization. Anti-OVA IgE antibody production in OVA-liposome-immune mice was undetectable after primary immunization, while OVA-alum induced a significant IgE antibody production as early as 2 weeks after immunization (fig. 5b). After secondary immunization, no anti-OVA IgE antibody was detected in the sera of mice immunized with OVA-liposome coupled via GA or DSS. However, in mice immunized with OVA-liposome coupled via EMCS, a brief and negligible production of IgE was observed, while in those immunized with OVA-liposome coupled via SPDP, a significant production of IgE was seen, although much less than that in the OVA-alum-immune group ($p < 0.001$). Titers of anti-OVA IgG isotype antibodies were assayed for the serum sample obtained 6 weeks after primary immunization. Anti-OVA IgG2a production predominated in groups of mice immunized with OVA-liposome coupled via GA or EMCS ($p < 0.01$) and anti-OVA IgG1 production predominated in the OVA-alum-immune group ($p < 0.001$). However, no significant difference between anti-OVA IgG1 and IgG2a production was observed in mice immunized with OVA-liposome coupled via SPDP and, interestingly, in the group of mice immunized with OVA-liposome coupled via DSS, the production of anti-OVA IgG1 was significantly higher than that of anti-OVA

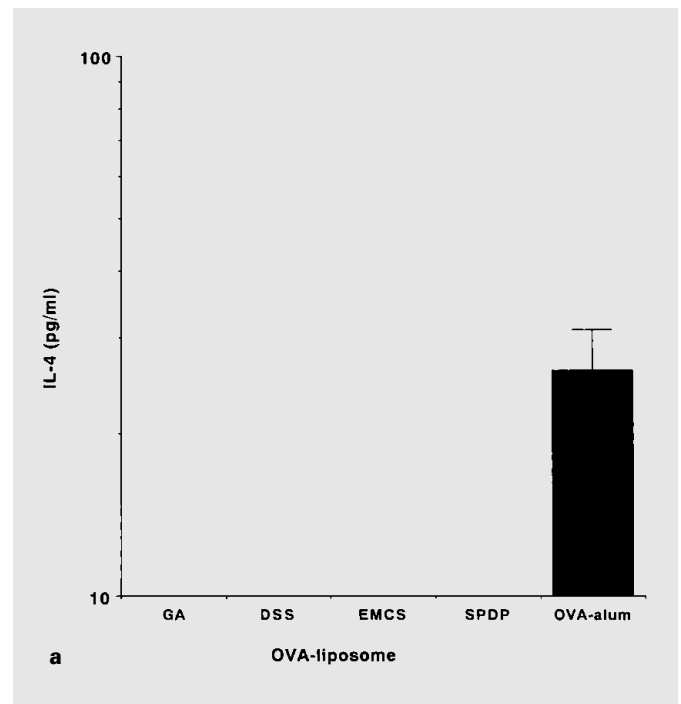


Fig. 6. Antigen-specific cytokine production by spleen cells of mice immunized with OVA. **a** IL-4. **b** IFN- γ . **c** IL-2. **d** IL-5. **e** IL-10. Data represent mean cytokine concentration (pg/ml) and SE of 5 mice per group.

IgG2a ($p < 0.001$). Thus, the results demonstrated that IgE unresponsiveness did not necessarily correlated with a predominant production of IgG2a.

Cytokine Production by SC from OVA-Immune Mice

Six weeks after primary immunization, SC were taken from mice and stimulated *in vitro* with OVA to observe OVA-specific cytokine production. As shown in figure 6a, IL-4 was detected only in the culture supernatant of SC taken from OVA-alum-immune mice. On the other hand, IFN- γ , which is known to counteract IL-4, was detected in all groups (fig. 6b), although the amounts varied from 154 to 1,400 pg/ml. Surprisingly, in the OVA-alum-immune group, in which a substantial IgE antibody was produced, the level of IFN- γ was significantly higher than in the OVA-liposome-immune group ($p < 0.0001$ vs. DSS, EMCS, and SPDP). No correlation was found between the amounts of IFN- γ (fig. 6c) and IL-2 produced within the same group; e.g., IL-2 production by the spleen cells of mice immunized with OVA-liposome coupled via EMCS was significantly higher than in the other groups ($p < 0.01$), but the production of IFN- γ in the same group was significantly less than

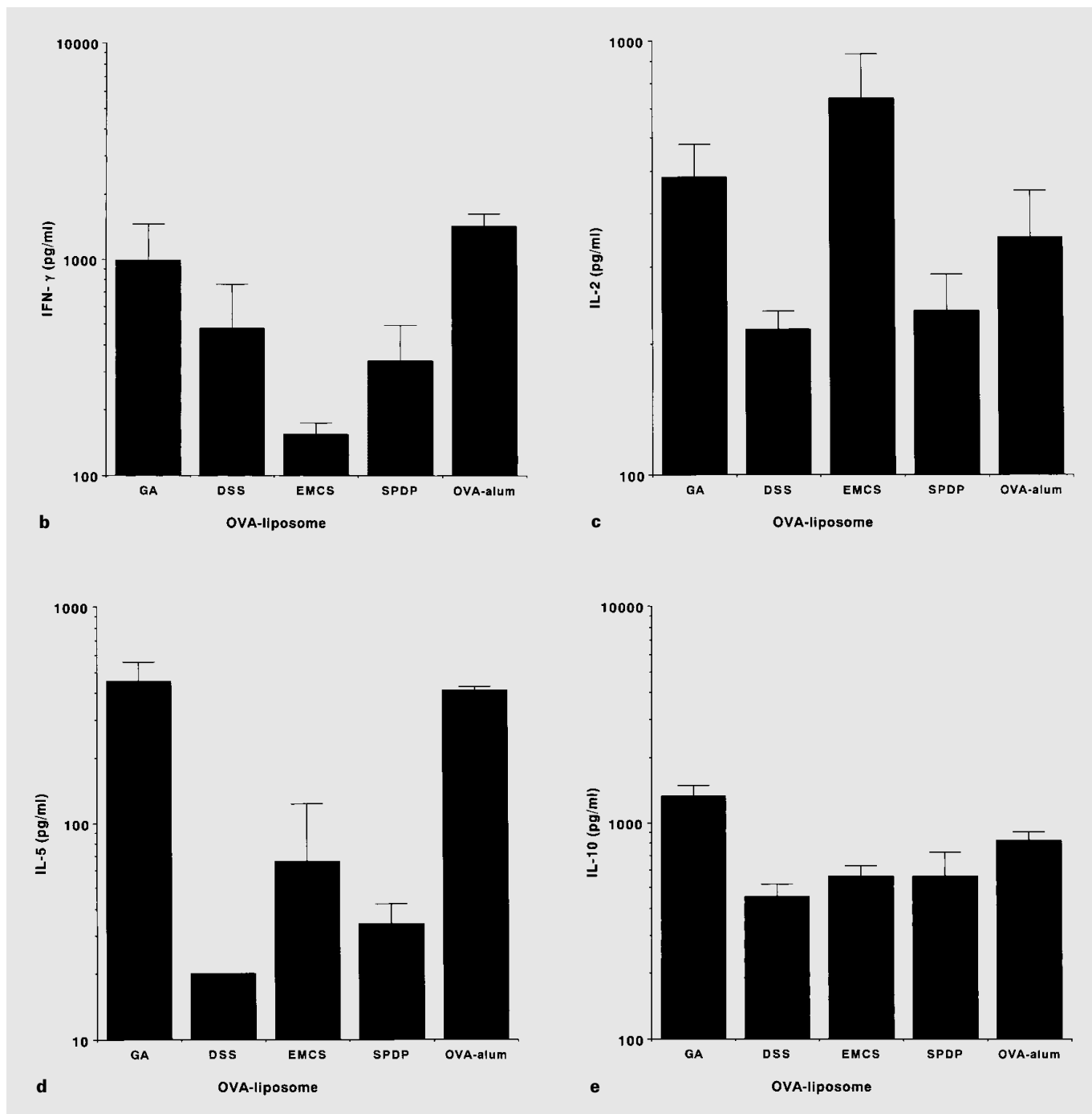


Fig. 6.

in the other groups ($p < 0.01$). IL-5 (fig. 6d) and IL-10 (fig. 6e) were also detected in all groups. The amount of IL-5 produced in the OVA-liposome (coupled via GA)-immune group was almost equivalent to that in the OVA-alum-immune group, suggesting that there was no

correlation between IgE production in vivo and IL-5 in vitro. In addition, the variation among OVA-liposome-immune groups in the production of the cytokines IFN- γ , IL-2 and IL-5 did not correlate with the variations among groups in the production of either IgE or IgG antibodies.

Discussion

In this study, OVA-liposome conjugates coupled via four different conjugation methods were prepared, and induction of anti-OVA antibody production by these conjugates was investigated in mice. A comparable level of anti-OVA IgG antibody production was induced by each of the conjugates (fig. 5a). Among the four OVA-liposome conjugates, the amounts of OVA coupled to liposome ranged from 40.1 to 47.0 $\mu\text{g}/\text{ml}$ lipid. However, this degree of variation in the amount of liposome-coupled antigen did not seem to affect the induction of anti-OVA IgG antibody production. The levels of anti-OVA IgE antibody induced by these conjugates were very low or undetectable, while alum-adsorbed OVA induced substantial production of anti-OVA IgE antibody. In groups of mice immunized with OVA-liposome coupled via GA or DSS, no anti-OVA IgE antibody was produced, while OVA-liposome coupled via SPDP induced the production of a low but significant level of anti-OVA IgE. The coupling of OVA and liposome via SPDP contained a disulfide bond (fig. 4), which might have reduced the stability of the coupling of OVA and liposome, resulting in liposome-free OVA, which in turn might have induced a low level of IgE production. In the coupling via EMCS, thiol residue on OVA was used, while in the coupling via GA and DSS, amino residue on OVA was used. The use of thiol residue on OVA may have caused an unstable conjugation that resulted in the incomplete regulation of IgE production. Hayglass et al., [18] have reported that OVA polymerized with GA induced a selective suppression of IgE production. However, it is unlikely that the induction of IgE-unresponsiveness by OVA-liposome was due to polymerization of OVA, because, in the coupling via EMCS, DSS and SPDP, a spacer was introduced separately to liposome and/or OVA before coupling (fig. 2–4) and thus polymerization of OVA would not have taken place during the coupling procedure. Structural changes of OVA induced by the coupling of OVA with liposomes may lead to a differential antigen presentation that could result in the induction of IgE unresponsiveness although the mechanism is still unclear. Attempts to clarify the mechanism are now in progress.

Mosmann et al. [19] have established two types of T helper cell clones with distinct patterns of cytokine production, namely Th1 and Th2. IFN- γ secreted from Th1 cells induces IgG2a antibody production without IgE synthesis and counteracts IL-4, which is secreted from Th2 and induces the production of IgG1 and IgE. In this study, however, in the group of mice immunized with OVA-liposome coupled via DSS, anti-OVA IgG1 production predominated although anti-OVA IgE production was not induced in the

group (fig. 5b, c). These results suggest that the productions of antigen-specific IgE and IgG isotype do not necessarily correlate with each other. IL-4 was detected only in the culture supernatant of SC from OVA-alum-immune mice, but not in that from OVA-liposome-immune mice. This result correlated well with that of IgE antibody production in vivo; OVA-alum induced the production of a substantial anti-OVA IgE antibody while OVA-liposome did not. On the other hand, IFN- γ , which is known to counteract IL-4 and suppress the production of IgE, was produced not only in the OVA-liposome-immune group but also in the OVA-alum-immune group. The amount of IFN- γ produced by the SC of OVA-alum-immune mice was even larger than that produced by the SC of OVA-liposome-immune mice. In addition, IL-2, another Th1 cytokine, was produced in all groups of mice. Moreover, the largest quantities of IL-5 and IL-10, the other Th2 cytokines, were produced in the group of mice immunized with OVA-liposome coupled via GA. Similar results were obtained when purified CD4⁺ T cells were used for the above experiment (data not shown).

Although there are many well-documented Th1 and Th2 responses [20–23], these are not the only cytokine patterns possible [24, 25], and it remains controversial whether or not the regulation of IgE antibody production can be attributed solely to the balance between Th1 and Th2 cells. The results in this study also suggest that the regulation of IgE antibody production does not necessarily correlate with the so-called Th1 cytokine production.

In conclusion, four different coupling protocols were employed to make OVA-liposome conjugates. Among the four conjugates, only OVA-liposomes coupled via GA or DSS completely suppressed antigen-specific IgE antibody production selectively. Since liposome-encapsulated antigen and the mixture of antigen and liposomes induces IgE antibody production [unpubl. observation], chemical coupling of antigen and liposomes, regardless of the coupling procedure used, was suggested to be essential for the induction of antigen-specific- and IgE-selective unresponsiveness.

Acknowledgment

This work was supported in part by a grant from The Japan Health Sciences Foundation (Research on Health Sciences focusing on Drug Innovation).

References

- Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J: A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. *N Engl J Med* 1996;334:349–355.
- Nothdurft HD, Jelinek T, Marschang A, Maiwald H, Kapaun A, Loscher T: Adverse reaction to Japanese encephalitis vaccine in travelers. *J Infect* 1996;32:119–122.
- Audibert FM, Lise LD: Adjuvants: Current status, clinical perspectives and future prospects. *Immunol Today* 1993;14:281–284.
- Mark A, Bjorksten B, Granstrom M: Immunoglobulin E responses to diphtheria and tetanus toxoids after booster with aluminium-adsorbed and fluid DT-vaccines. *Vaccine* 1995;13:669–673.
- Aggerbeck H, Wantzin J, Heron I: Booster vaccination against diphtheria and tetanus in man. Comparison of three different vaccine formulations. III. *Vaccine* 1996;14:1265–1272.
- Gregoriadis G: The immunological adjuvant and vaccine carrier properties of liposomes. *J Drug Target* 1994;2:351–356.
- Wassef NM, Alving CR, Richards RL: Liposomes as carriers for vaccines. *Immunomethods* 1994;4:217–222.
- Alving CR: Liposomal vaccines: Clinical status and immunological presentation for humoral and cellular immunity. *Ann N Y Acad Sci* 1995;754:143–152.
- Gregoriadis G: Liposomes as immunoadjuvants and vaccine carriers: Antigen entrapment. *Immunomethods* 1994;4:210–216.
- Forthin A, Shahum E, Krzysztyniak K, Therien HM: Differential activation of cell-mediated immune functions by encapsulated and surface-linked liposomal antigens. *Cell Immunol* 1996;169:208–217.
- Arola N, Gangal SV: Efficacy of liposome entrapped allergen in down regulation of IgE response in mice. *Clin Exp Allergy* 1992;22:35–42.
- Naito S, Horino A, Nakayama M, Nakano Y, Nagai T, Mizuguchi J, Komuro K, Uchida T: Ovalbumin-liposome conjugate induces IgG but not IgE antibody production. *Int Arch Allergy Immunol* 1996;109:223–228.
- Naito S, Horino A, Komiya T, Fukuda T, Takahashi M, Ami Y, Suzuki Y, Oka T, Okuma K, Morokuma K, Nakano Y, Mori M, Nishinohara S, Komuro K, Uchida T: Induction of protection against tetanus toxin in mice by tetanus toxoid-liposome conjugate. *Int Arch Allergy Immunol* 1998;116:215–219.
- Fukuda T, Komiya T, Takahashi M, Arakawa Y, Ami Y, Suzuki Y, Naito S, Horino A, Nagata N, Satoh S, Gondaira F, Sugiyama J, Nakano Y, Mori M, Nishinohara S, Komuro K, Uchida T: Induction of protection against oral infection with cytotoxin-producing *Escherichia coli* O157:H7 in mice by Shiga-like toxin-liposome conjugate. *Int Arch Allergy Immunol* 1998;116:313–317.
- Martin FJ, Papahadjopoulos D: Irreversible coupling of immunoglobulin fragments to preformed vesicles. *J Biol Chem* 1982;257:286–288.
- Kinsky SC, Hashimoto K, Loader JE, Benson AL: Synthesis of N-hydroxysuccinimide esters of phosphatidylethanolamine and some properties of liposomes containing these derivatives. *Biochim Biophys Acta* 1984;769:543–550.
- Heath TD: Covalent attachment of proteins to liposomes. *Methods Enzymol* 1987;149:111–119.
- Hayglass KT, Strejan GH: Suppression of the IgE antibody response by glutaraldehyde-modified ovalbumin: Dissociation between loss of antigenic reactivity and ability to induce suppression. *Int Arch Allergy Appl Immunol* 1984;74:332–340.
- Mosmann TR, Coffman RL: Th1 and Th2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989;7:145–173.
- Scott P, Pearce E, Cheever AW, Coffman RL, Sher A: Role of cytokines and CD4+ T-cell subsets in the regulation of parasite immunity and disease. *Immunol Rev* 1989;112:161–182.
- Barnes PF, Lu S, Abrams JS, Wang E, Yamamura M, Mdlin RL: Cytokine production at the site of disease in human tuberculosis. *Infect Immun* 1993;61:3482–3489.
- Oswald IP, Casper P, Jankovic D, Wynn TA, Pearce EJ, Sher A: IL-12 inhibits Th2 cytokine responses induced by egg of *Schistosoma mansoni*. *J Immunol* 1994;153:1707–1713.
- Actor JK, Marshall MA, Eltoun IA, Bulle RM, Berzofsky JA, Sher A: Increased susceptibility of mice infected with *Schistosoma mansoni* to recombinant vaccinia virus: Association of viral persistence with egg granuloma formation. *Eur J Immunol* 1994;24:3050–3056.
- Kelso A: Th1 and Th2 subsets: Paradigms lost? *Immunol Today* 1995;16:374–379.
- Mosmann TR, Sad S: The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996;17:138–146.

Copyright: S. Karger AG, Basel 1999. Reproduced with the permission of S. Karger AG, Basel. Further reproduction or distribution (electronic or otherwise) is prohibited without permission from the copyright holder.