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Key Words

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Induction of Protection against Tetanus Toxin in Mice by Tetanus Toxoid-Liposome Conjugate

Abstract

Tetanus toxoid (Ttd) was coupled to liposomes via glutaraldehyde. Intraperitoneal injection in BALB/c mice with Ttd-liposomes induced a substantial amount of anti-Ttd IgG antibody production and an extremely low level of anti-Ttd IgE antibody production. Mice immunized with Ttd-liposomes were successfully protected against a subsequent challenge with a lethal dose of tetanus toxin (Ttx). On the other hand, aluminum hydroxide-adsorbed Ttd (Ttd-alum) and plain Ttd solution induced the production of both IgG and IgE antibodies against Ttd. Moreover, secondary immunization with Ttd-liposomes in mice, in which anti-Ttd IgE antibody production was induced by Ttd-alum led to enhanced anti-Ttd IgG and a limited anti-Ttd IgE antibody production. When Ttd-liposome preparation was lyophilized, the efficacy of Ttd-liposomes was maintained for 6 months at 37°C, suggesting that this vaccine preparation would be stable without refrigeration. These results demonstrate the potential ability of Ttd-liposome conjugates to produce a tetanus vaccine which provides protection against (Ttx) while inducing the least amount of anti-Ttd IgE antibodies.

Introduction

Allergic reactions such as local swelling and an immediate-type reaction upon human vaccination are frequently observed phenomena [1, 2], and the formation of IgE upon vaccination is thought to participate in these reactions. Diphtheria-pertussis-tetanus vaccine has been shown to induce a significant amount of IgE antibodies in mice, in addition to the induction of IgG antibody production [3]. We have previously reported that ovalbumin (OVA) coupled with liposomes induces anti-OVA IgG but not IgE antibody production [4]. An antigen-liposome conjugate is expected to be a novel protocol of vaccine preparation which induces the production of a very small quantity of IgE antibodies.

In this study, liposomal conjugates with tetanus toxoid (Ttd) were produced, and the induction of anti-Ttd antibody production was investigated in mice. Ttd-liposome conjugates induced the least amount of IgE antibodies while resulting in a fairly high level of IgG antibody production against Ttd. Further investigations were made to explore the effects of secondary immunization with Ttd-liposomes in mice which had been primed for anti-Ttd IgE antibody production. We also examined the efficacy of Ttd-liposome conjugates after storage at high temperature, and the possible clinical uses of liposomal conjugates with Ttd will be discussed.

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Materials and Methods

Mice

BALB/c mice (female, 8 weeks of age) were purchased from Charles River, Kanagawa, Japan.

Tetanus Toxin and (Ttd)

For the preparation of tetanus toxin (Ttx), culture supernatant of *Clostridium tetani* (Harvard A-47 strain) was concentrated by ultrafiltration, salted out twice with ammonium sulfate and purified by ion-exchange chromatography. Ttd was prepared by detoxifying the purified Ttx, using formaldehyde followed by dialysis against phosphate-buffered saline (PBS, pH 7.0).

Liposomes

Liposomes (45 mg lipid/ml), used for the coupling with Ttd, were composed of dipalmitoyl phosphatidylcholine, dipalmitoyl phosphatidyl ethanolamine (DPE), cholesterol, and palmitic acid in 4:3:7:2 molar ratios. The liposome preparation used in this study contained DPE, since this amino group was essential on the surface of the liposome to couple the antigen with the liposome via the aldehyde group.

Coupling of Ttd to Liposomes

Liposomal conjugates with Ttd (Ttd-liposomes) were prepared as previously described [4]. Briefly, 0.5 ml of 2.5% glutaraldehyde solution was added in drops to a mixture of 90 mg of liposomes and 0.85 mg of Ttd in 2.5 ml phosphate buffer (pH 7.2). 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 liposome-coupled Ttd and the uncoupled Ttd 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 by using a phospholipid content assay kit (Phospholipid Test Wako; Wako Pure Chemical Industries, Osaka, Japan). The Ttd-liposome solution was adjusted to 10 mg lipid/ml and kept at 4°C until use.

Immunization, Measurement of Serum Antibodies, and Ttx Challenge in Mice

Mice were immunized intraperitoneally (i.p.) with Ttd-liposomes, aluminum hydroxide-absorbed Ttd (Ttd-alum, 2 µg Ttd with 3 mg alum), or 2 µg plain Ttd solution in PBS, and bled weekly from the tail vein. IgG antibodies against Ttd in the sample sera were measured using ELISA. IgE antibodies against Ttd were measured using monoclonal-antibody-captured ELISA. Six weeks after immunization, mice immunized with Ttd-liposomes and age-matched naive mice were challenged subcutaneously (s.c.) with 100 LD₅₀ of Ttx, i.e. 150 ng of purified Ttx in 0.1 ml PBS.

Results

Anti-Ttd Antibody Production in Mice

Mice were immunized i.p. on days 0 and 28 with Ttd-alum, Ttd-liposomes, or with plain Ttd solution. As shown in figure 1a, serum anti-Ttd IgG in mice immunized with ei-

ther Ttd-alum or Ttd-liposome reached almost the same level after secondary immunization. In contrast, a very low level of anti-Ttd IgE antibodies was detected in the sera of mice immunized with Ttd-liposomes, while a substantial amount of anti-Ttd IgE antibodies was detected in the Ttd-alum-immune group (fig. 1b). Plain Ttd solution, which induced a lower level of IgG production than Ttd-alum or Ttd-liposomes (fig. 1a), induced the production of a fairly large amount of anti-Ttd IgE antibodies (fig. 1b). Production of IgG1 predominated in both Ttd-alum-immune group and Ttd-liposome-immune group (fig. 1c).

Effect of Secondary Immunization with Ttd-Liposomes in Mice Immunized Primarily with Ttd-Alum

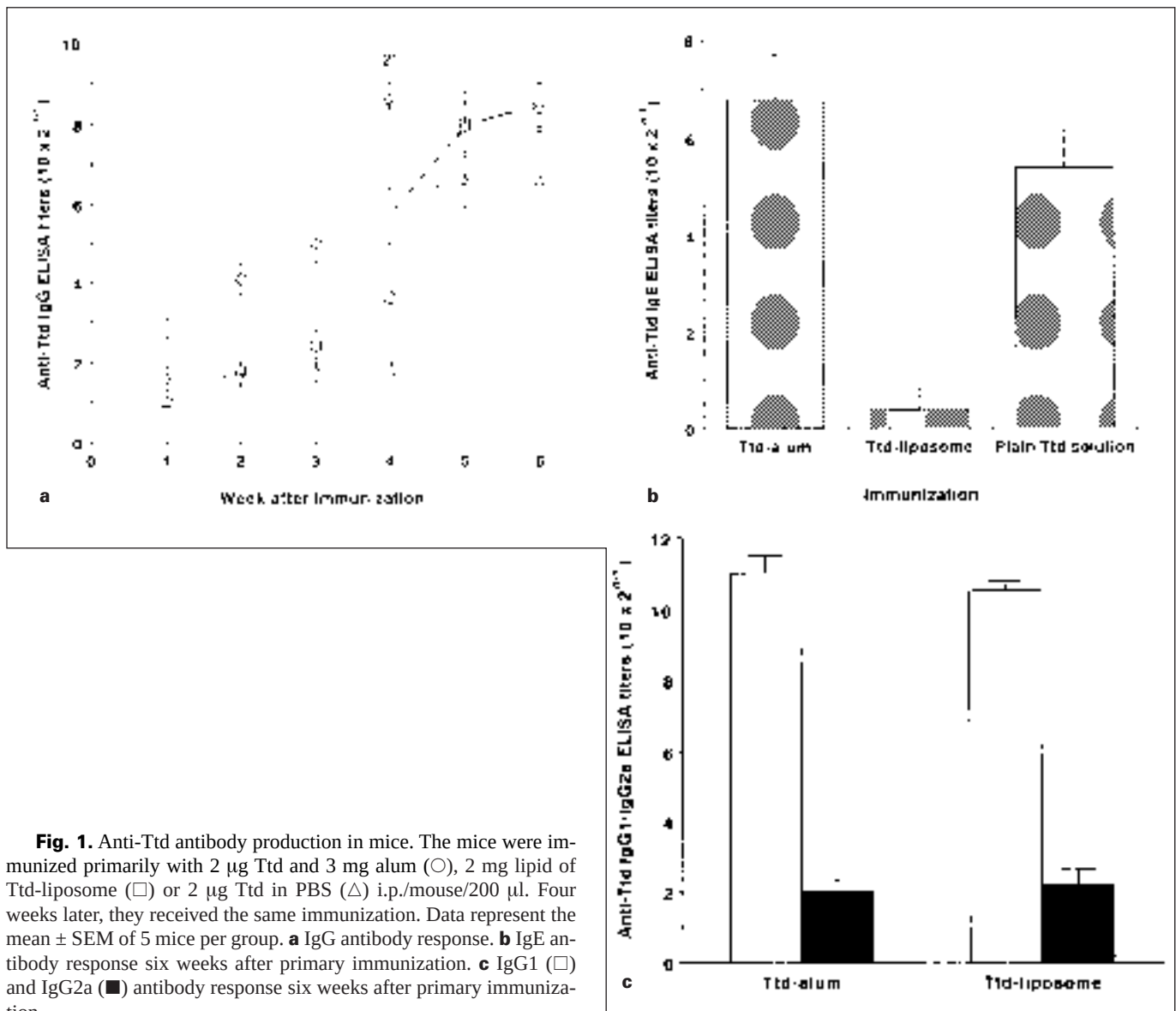
Primary immunization of mice with Ttd-alum induced a significant production of both IgG and IgE antibodies against Ttd (fig. 2). Four weeks later, the mice were immunized secondarily with Ttd-alum, Ttd-liposomes, or nothing. The enhancement of anti-Ttd IgG antibody production was comparable in the Ttd-alum-immune and Ttd-liposome-immune groups at six weeks, after the primary immunization. However, the enhancement of anti-Ttd IgE production by Ttd-liposomes was significantly less ($p < 0.01$) than that by Ttd-alum.

Maintenance of Efficacy in Ttd-Liposome Conjugates after Storage for 6 Months

Ttd-liposomes, either liquefied or lyophilized, were kept at 4°C or 37°C for 6 months. After a storage of 6 months, they were inoculated in mice, and the inducibility of anti-Ttd IgG antibody production was compared. As shown in figure 3, all the Ttd-liposome preparations maintained their efficacy for 6 months except for liquefied Ttd-liposomes, which had been stored at 37°C.

Anti-Ttd Antibody Production in Ttd-Liposome-Immune Mice

Mice were immunized primarily with 2 mg lipid or 0.25 mg lipid of Ttd-liposomes, and 4 weeks later, mice were immunized secondarily with 2 mg lipid or 0.25 mg lipid of Ttd-liposomes. As shown in figure 4a, mice immunized primarily with either 2 mg lipid or 0.25 mg lipid of Ttd-liposomes produced almost the same amount of anti-Ttd IgG antibodies. Secondary immunization with Ttd-liposomes enhanced anti-Ttd IgG production significantly in all groups, and the amount of anti-Ttd IgG in the sera of mice at 6 weeks after primary immunization seemed to be dependent on the dose of Ttd-liposomes at the secondary immunization; serum anti-Ttd IgG titer reached the same level in groups of mice receiving a booster injection with 2 mg lipid



of Ttd-liposomes, regardless of the dose at the primary immunization. Anti-Ttd IgE antibodies were not detected in all groups after primary immunization and were only limited after secondary immunization (fig. 4b). All of the mice immunized with Ttd-liposomes in the above experiment (fig. 4) were protected against an s.c. injection with 100 LD₅₀ of (Ttx) without any apparent symptoms, while non-immunized control mice died within 24 h (data not shown).

Discussion

In this study, Ttd-liposome conjugates were inoculated into mice, and the induction of Ttd-specific antibody production and its protection against a (Ttx) challenge were evaluated. Mice immunized with Ttd-liposomes produced a substantial amount of anti-Ttd IgG antibodies (fig. 1a, 4a), and they were successfully protected against a subsequent challenge with a lethal dose of (Ttx), suggesting that immunization with Ttd-liposomes induces the production of protective antibodies in mice. In addition, the level of serum anti-Ttd IgE antibodies induced by Ttd-liposomes was very low, if present at all (fig. 4b), while both Ttd-alum and plain

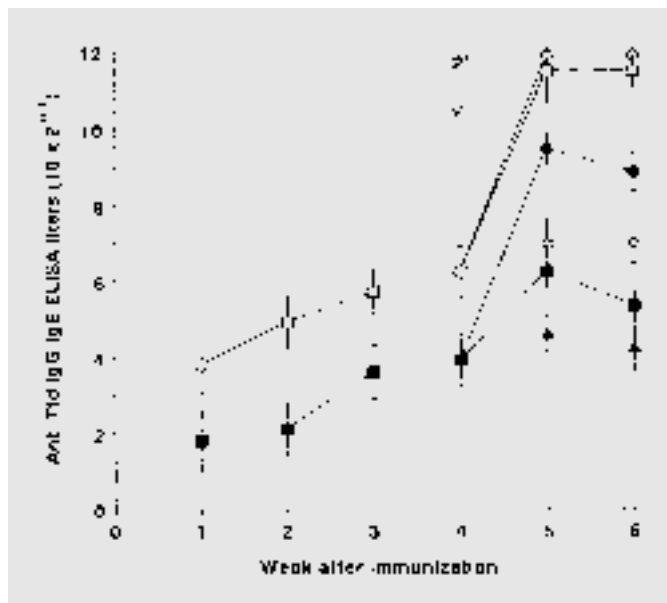


Fig. 2. Anti-Ttd antibody production in mice immunized primarily with Ttd-alum. The mice were immunized i.p. with 2 μ g Ttd and 3 mg alum. Four weeks later, they were immunized with the same dose of Ttd-alum ($\circ$, $\bullet$), 2 mg lipid of Ttd-liposome ($\square$, $\blacksquare$), or nothing ($\triangle$, $\blacktriangle$). Serum IgG ($\circ$, $\square$, $\triangle$) and IgE ($\bullet$, $\blacksquare$, $\blacktriangle$) antibody titers are shown. Data represent the mean $\pm$ SEM of 5 mice per group.

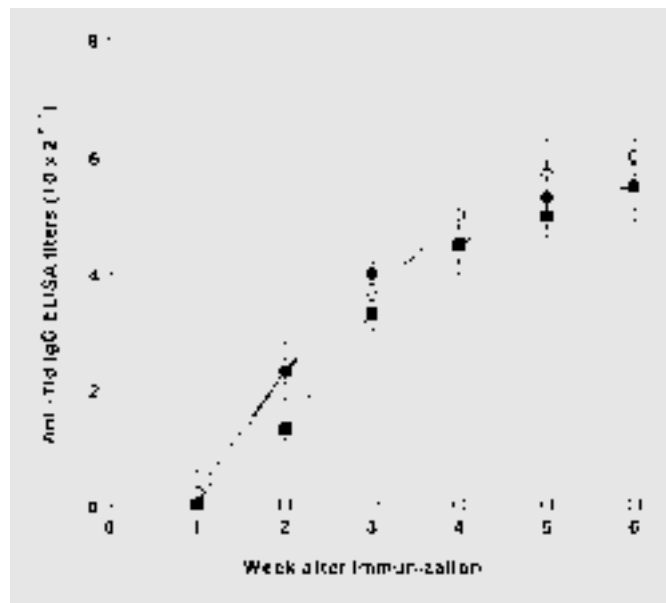


Fig. 3. Anti-Ttd IgG antibody production in mice immunized with Ttd-liposomes. The mice were immunized i.p. with 2 mg lipid of Ttd-liposome conjugates which had been stored, liquefied ($\circ$, $\square$), or lyophilized ($\bullet$, $\blacksquare$) for 6 months at 4°C ($\circ$, $\bullet$) or 37°C ($\square$, $\blacksquare$). Data represent the mean $\pm$ SEM of 5 mice per group.

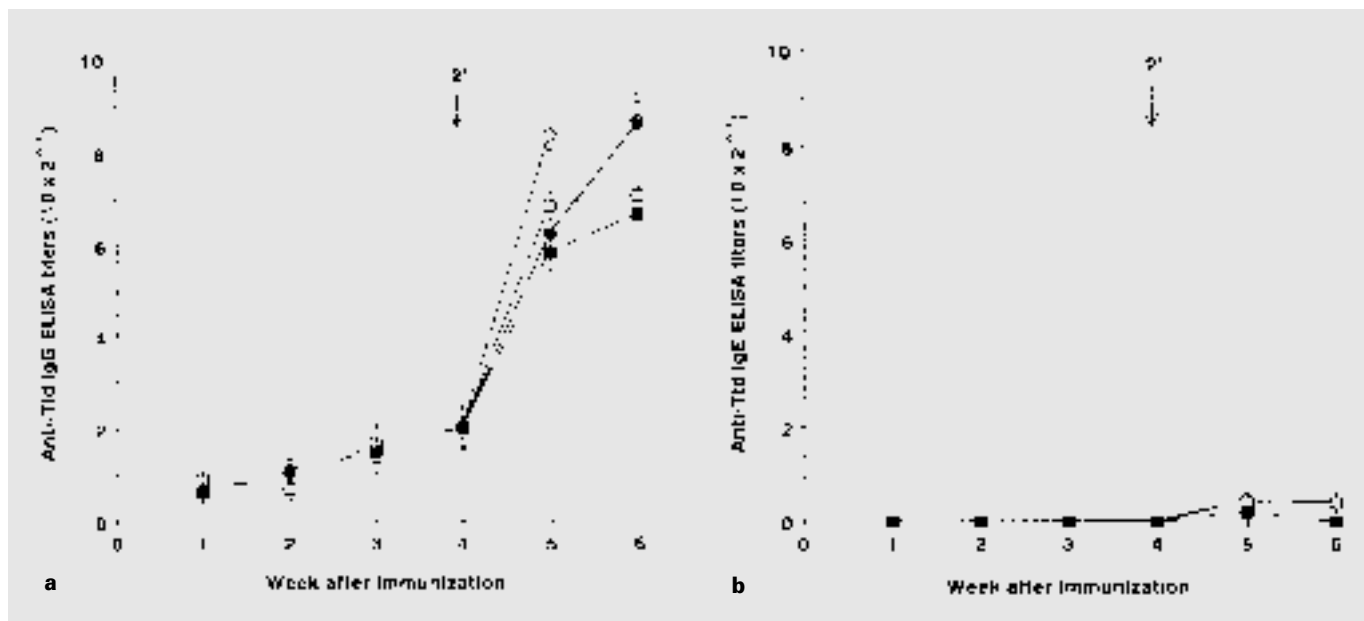


Fig. 4. Anti-Ttd IgG antibody production in mice immunized with Ttd-liposomes. The mice were immunized primarily with 2 mg lipid ($\circ$, $\square$) or 0.25 mg lipid ($\bullet$, $\blacksquare$) of Ttd-liposomes. Four weeks later they were immunized secondarily with 2 mg lipid ($\circ$, $\bullet$) or 0.25 mg lipid ($\square$, $\blacksquare$) of Ttd-liposomes. Data represent mean ELISA titer $\pm$ SEM of 5 mice per group. **a** Anti-Ttd IgG antibody production. **b** Anti-Ttd IgE antibody production.

Ttd solution induced the production of a substantial amount of anti-Ttd IgE antibodies (fig. 1b). When Ttd-liposomes were used for booster injection in mice that had been primed with Ttd-alum for anti-Ttd IgE antibody production, Ttd-liposomes enhanced the production of anti-Ttd IgG antibodies, but the enhancement of anti-Ttd IgE production was only limited (fig. 2), suggesting that if Ttd-liposomes are used as a secondary immunization in a high-IgE recipient, they may boost anti-Ttd IgG antibody production without affecting IgE synthesis. Secondary immunization with OVA-liposome conjugates did not affect anti-Ttd antibody production (data not shown), suggesting that the effects of antigen-liposomes are antigen-specific. The fact that the efficacy of Ttd-liposome conjugates was maintained for 6 months when they were lyophilized and kept at 37°C (fig. 3) may be another advantage in using liposomal conjugates for vaccine preparation.

Adjuvants have been used to augment the immune response to antigens [5]. Although many adjuvant formulations have been developed, most of them have never been applied to vaccines, mainly due to their toxicity and side effects [6]. Mechanisms of adjuvant action include stimulation of the cells of the immune system to secrete a variety of cytokines which lead to systemic side effects. In a previous study [7], alum-adsorbed OVA has been shown to induce IL-4-producing T cells which are characteristic of the so-called Th2 cells that are known to induce a dominant production of IgG1 antibodies and IgE [8]. On the other hand, OVA-liposome conjugates have been demonstrated to in-

duce IFN- γ -producing T cells which are characteristic of the so-called Th1 cells that are known to induce a dominant production of IgG2a antibodies without inducing IgE antibody production. However, in this study, Ttd-liposome conjugates were shown predominantly to induce anti-Ttd IgG1 antibody production while resulting in a selective unresponsiveness of anti-Ttd IgE antibody production, suggesting that this selective unresponsiveness of IgE antibody production does not necessarily go in parallel with a dominant production of IgG2a antibody production. Thus, the mechanism of IgE-selective unresponsiveness induced by antigen-liposome conjugates is hardly explained by using the context of Th1-Th2 cells. Efforts to clarify the mechanism are now in progress.

A clinically applicable adjuvant should be confirmed as safe with regard to local and systemic reactions, autoimmune diseases, hypersensitivity reactions, and carcinogenicity [9]. It should be chemically defined, biodegradable, and nonimmunogenic by itself, and should elicit a protective immune response [6]. Liposomes used for the coupling with antigens in this study, which consist of chemically defined lipids that are the constituents of mammalian cell membranes, are known to be biodegradable and nonimmunogenic by themselves [10]. In this study, Ttd-liposome conjugates were demonstrated to induce protection against the subsequent challenge with (Ttx). Thus, the Ttd-liposomal conjugates appear to have the potential to serve as a novel protocol for vaccination which induces the production of protective antibodies without IgE synthesis.

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