FULL PAPER

Immunology

Induction of Immune Responses against Glycosphingolipid Antigens: Comparison of Antibody Responses in Mice Immunized with Antigen Associated with Liposomes Prepared from Various Phospholipids

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ABSTRACT. The immune responses of mice against glycosphingolipid (GSL) antigens and the effect of the phospholipid composition of liposomes on the immunogenicity in mice of liposome-associated GSL antigens were examined. The immunization with GSL antigen alone was unable to induce any detectable anti-GSL antibody responses. On the other hand, the immune responses against GSL antigens were detected after immunization with liposomes composed of dipalmitoylphosphatidylcholine (DPPC) (0.5 µmol), cholesterol (Chol) (0.5 µmol), Salmonella minnesota R595 lipopolysaccharides (LPS) (10 µg) and GSL (0.05 µmol) (DPPC-liposome). However, the administration with liposome composed of dimyristoylphosphatidylcholine (DMPC) (0.5 µmol), Chol (0.5 µmol), S. minnesota R595 LPS (10 µg) and GSL (0.05 µmol) and with liposomes composed of distearylphosphatidylcholine (DSPC) (0.5 µmol), Chol (0.5 µmol), and S. minnesota R595 LPS (10 µg) and GSL (0.05 µmol) was ineffective for the induction of the immune responses against GSL antigens. These results suggest that DPPC-liposome would serve effectively as a delivery vehicle for inducing immune responses against GSL antigen.

KEY WORDS: glycosphingolipid, GSL antigen, immunogenicity, liposomes.

Glycosphingolipids (GSLs) are ubiquitous components of the plasma membrane of cells. It is well known that GSLs not only function as cell markers and antigens, but also relate to cell to cell interaction, adhesion, signal transmission, and cell growth regulation without antigenic variation [7]. In particular, gangliosides, the GSLs containing sialic acids, on cell surfaces are of importance as antigen determinants and mediators of immune responses [6]. As a result, they have been considered attractive targets for immunotherapy of cancer [3, 11]. In general, however, the poor immunogenicity of GSL antigens is well-documented [12]. This is particularly serious for attempts to immunize against GSL antigens. Thus, it is important to clarify the induction method of immune responses against GSL antigens for establishing GSL vaccines.

Liposomes are artificial model membranes and have been exploited as experimental tools in a number of biological subjects including immunological phenomena. Applications of liposomes in immunology, as immunogens, have demonstrated that liposomes may have considerable practical utility as carriers of antigens and adjuvants [5]. Previous study has demonstrated that modulation of liposomal immunogenicity by lipid A was an effective method for enhancing antibody production against lipid antigens [2]. In addition, it has also been reported that antigenic expression in liposomal model membranes was influenced by phospholipid composition [1]. However, the respective immunogenicity of GSL antigens incorporated into liposomes made of various phospholipids has not been comparatively studied as of yet. Thus, it is necessary to examine whether changes in the phospholipid composition of liposomes have a pronounced effect on the ability of these model membranes to induce immune response against liposomal membrane-associated GSL antigens.

In order to reveal the immunogenicity of GSL antigens and the phospholipids composition suitable for increasing the immunogenicity of GSL antigens incorporated into liposomes, we examined the immune responses against GSL antigens and the influence of the phospholipid composition of liposomes on the immunogenicity in mice of liposome-associated GSL antigens.

MATERIALS AND METHODS

Lipids: Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidic acid (DPPA) were commercially obtained from Nippon Oil & Fats, Tokyo, Japan, and Nippon Fine Chemical, Osaka, Japan, respectively. Dipalmitoylethanolamine and DPPA were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Mice: Female BALB/c mice (6-wk-old) were purchased from Charles River Japan (Tokyo, Japan) and used for immunization.

Liposome preparation: Liposomes containing GSL
GSL (GlcCer, LacCer, GM3 or GM1) alone (0.05 µg) was intraperitoneally immunized as follows: group I, mice, were divided into 5 groups (5 mice per a group). Each group was suspended in gelatin veronal-buffered saline (1 mM Tris buffer (pH 7.4), and 145 mM NaCl). The final pellet of liposome composed of DSPC (0.5 µmol) was prepared according to the method of Watarai [13–15]. Briefly, 25 µg of the antibody solution and 5 µl of the immunogen per mouse. All seven days after immunization, sera were collected and used for antibody assay.

Antibody assay: Anti-GSL antibodies in sera were detected by LILA according to the method described previously [13–15] and used for detection of anti-GSL antibodies. As the release marker, 0.05 M carboxyfluorescein (CF; Eastman Kodak, Rochester, NY, U.S.A.) was used. Unencapsulated CF was removed by repeated centrifugation at 20,000 × g for 20 min in gelatin veronal-buffered saline [0.1% gelatin, 10 mM veronal buffer (pH 7.4), and 145 mM NaCl]. The final pellet of liposomes was suspended in gelatin veronal-buffered saline (1 mM) and stored at 4°C.

Immunization of mice: Mice, 6-week-old female BALB/c mice, were divided into 5 groups (5 mice per a group). Each group was intraperitoneally immunized as follows: group I, GSL (GlcCer, LacCer, GM3, or GM1) alone (0.05 µmol); group II, liposome composed of DMPC (0.5 µmol), Chol (0.5 µmol), S. minnesota R595 LPS (10 µg) and GSL (0.05 µmol) (DMPC-liposome); group III, liposome composed of DPPC (0.5 µmol), Chol (0.5 µmol), S. minnesota R595 LPS (10 µg) and GSL (0.05 µmol) (DPPC-liposome); group IV, liposome composed of DSPC (0.5 µmol), Chol (0.5 µmol), and S. minnesota R595 LPS (10 µg) and GSL (0.05 µmol) (DSPC-liposome). All were immunized with 0.5 ml of the immunogen to a mouse.

Immunogenicity of GSL antigens: In order to elucidate immunogenicity of GSL antigens, mice were administered intraperitoneally with GSL antigen, such as GlcCer, LacCer, GM3, and GM1, alone, and antibodies against GSLs were evaluated with LILA at 7 days after immunization. The serum antibody activities against GSL antigens were not detected in any mice administered with GSL antigen alone (group I) (Fig. 1).

Immunogenicity of liposome-associated GSL antigens: In order to examine the influence of the phospholipid composition of liposomes on the immunogenicity of liposome-associated GSL antigens, GSL antigen-containing liposomes prepared from various phospholipids were administered intraperitoneally to mice, antibody response to liposomal antigen was evaluated at 7 days after immunization.

Figure 2 shows the antibody responses to GSL antigen associated with DMPC-liposome (group II) after immunization. Production of anti-LacCer antibody was demonstrated in sera from mice receiving DMPC-liposome containing LacCer, but no serum anti-GSL antibody responses could be detected in mice immunized with DMPC-liposome containing GlcCer, GM3, and GM1, respectively.

Figure 3 shows the results of an experiment in which mice were immunized with DPPC-liposome having GSL antigen (group III). Serum antibody activity against GlcCer, LacCer, GM3, and GM1 could be seen in mice administered with DPPC-liposome having GlcCer, LacCer, GM3, and GM1, respectively.

DISCUSSION

GSLs on the cell surface act as cell-surface antigens. However, the immunogenicity of GSL antigen is often too weak to provoke sufficient immune responses [12]. In this study, in fact, immune responses against GSL antigens could not be induced by immunization of GSL antigen alone (Fig. 1). This seems attributable to a poor immunogenicity of GSL antigens [12].

It has been reported that the induction of an immune response against antigen in liposomes is influenced by the bilayer phospholipids composition of liposomal membranes [2]. In this study, the intraperitoneal administration of DPPC-liposome containing GSL antigens (GlcCer, LacCer, GM3 and GM1) was effective for induction of the antigen-specific antibody responses (Fig. 3). On the other hand, when GSLs were reconstituted in DMPC- or DSPC-liposome and administered to mice, immune responses against GSL antigens were lower than that induced by DPPC-liposome administration (Figs. 2 and 4). These results suggest that the induction of an immune response against liposomal GSL antigen is also influenced by the bilayer phospholipids composition of liposomes. Because DMPC, DPPC, and DSPC possess an identical polar head group (i.e., phosphati-
**Fig. 1.** Immune responses in mice immunized with GSL antigens. Mice were immunized intraperitoneally with GSL antigen (GlcCer, LacCer, GM3 and GM1) alone (group I). Sera were assayed by LILA using (A) liposomes composed of DMPC (0.5 \( \mu \)mol), Chol (0.5 \( \mu \)mol), DPPA (0.05 \( \mu \)mol) and GlcCer (0.05 \( \mu \)mol), (B) liposomes composed of DMPC (0.5 \( \mu \)mol), Chol (0.5 \( \mu \)mol), DPPA (0.05 \( \mu \)mol) and LacCer (0.05 \( \mu \)mol), (C) liposomes composed of DMPC (0.5 \( \mu \)mol), Chol (0.5 \( \mu \)mol), DPPA (0.05 \( \mu \)mol) and GM3 (0.05 \( \mu \)mol), and (D) liposomes composed of DMPC (0.5 \( \mu \)mol), Chol (0.5 \( \mu \)mol), DPPA (0.05 \( \mu \)mol) and GM1 (0.05 \( \mu \)mol), before ( ) and after ( ) immunization. Results are expressed as the mean ± S.E. in 5 different mice.

**Fig. 2.** Immune responses in mice immunized with GSL antigen-containing DMPC-liposome. Mice were immunized intraperitoneally with GSL antigen (GlcCer, LacCer, GM3 and GM1) incorporated into DMPC-liposome (group II). Sera were assayed by LILA using (A) liposomes composed of DMPC (0.5 \( \mu \)mol), Chol (0.5 \( \mu \)mol), DPPA (0.05 \( \mu \)mol) and GlcCer (0.05 \( \mu \)mol), (B) liposomes composed of DMPC (0.5 \( \mu \)mol), Chol (0.5 \( \mu \)mol), DPPA (0.05 \( \mu \)mol) and LacCer (0.05 \( \mu \)mol), (C) liposomes composed of DMPC (0.5 \( \mu \)mol), Chol (0.5 \( \mu \)mol), DPPA (0.05 \( \mu \)mol) and GM3 (0.05 \( \mu \)mol), and (D) liposomes composed of DMPC (0.5 \( \mu \)mol), Chol (0.5 \( \mu \)mol), DPPA (0.05 \( \mu \)mol) and GM1 (0.05 \( \mu \)mol), before ( ) and after ( ) immunization. Results are expressed as the mean ± S.E. in 5 different mice.
Fig. 3. Immune responses in mice immunized with GSL antigen-containing DPPC-liposome. Mice were immunized intraperitoneally with GSL antigen (GlcCer, LacCer, GM3 and GM1) incorporated into DPPC-liposome (group III). Sera were assayed by LILA using (A) liposomes composed of DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol) and GlcCer (0.05 µmol), (B) liposomes composed of DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol) and LacCer (0.05 µmol), (C) liposomes composed of DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol) and GM3 (0.05 µmol), and (D) liposomes composed of DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol) and GM1 (0.05 µmol), before (○) and after (●) immunization. Results are expressed as the mean ± S.E. in 5 different mice.

Fig. 4. Immune responses in mice immunized with GSL antigen-containing DSPC-liposome. Mice were immunized intraperitoneally with GSL antigen (GlcCer, LacCer, GM3 and GM1) incorporated into DSPC-liposome (group IV). Sera were assayed by LILA using (A) liposomes composed of DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol) and GlcCer (0.05 µmol), (B) liposomes composed of DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol) and LacCer (0.05 µmol), (C) liposomes composed of DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol) and GM3 (0.05 µmol), and (D) liposomes composed of DMPC (0.5 µmol), Chol (0.5 µmol), DPPA (0.05 µmol) and GM1 (0.05 µmol), before (○) and after (●) immunization. Results are expressed as the mean ± S.E. in 5 different mice.
dycholine), furthermore, the difference in immunogenicity of liposome-associated GSL antigens may be due to a difference in the non-polar region of phospholipid in liposomal lipid bilayers (i.e., fatty acid). Phosphatidylcholines which contain longer chain fatty acids are generally characterized by a higher transition temperature (Tc) than those with shorter chain fatty acids [10]. Tc of DMPC, DPPC, and DSPC is 23.0°C, 41.5°C, and 58.0°C, respectively. The membrane fluidity of liposomes composed of phosphatidylcholine having a high Tc is usually low. Thus, liposomes containing DPPC or DSPC should have a greater solidity than liposomes containing DMPC. Consequently, the membrane permeability of liposomes containing DPPC or DSPC may be low and they show greater bilayer stability at mouse body temperature. Indeed, it has been previously reported that liposomes were very rapidly solubilized above body temperature. It has been reported that moderate fluidity of liposomal membrane fluidity of liposomes composed of DPPC having a high Tc elicited higher antibody response against GSL antigens than administration of liposomes prepared with DMPC having a low Tc (Figs. 2 and 3). The above explanation is, however, difficult to reconcile with the observation that when GSLs were reconstituted in liposomes made with DSPC having higher Tc than DPPC and administered to mice, immune responses against GSL antigens were lower than that induced by DPPC-liposome administration (Figs. 3 and 4). The reason for a lower immunogenicity of DSPC-liposome is unknown. However, it has been reported that moderate fluidity of liposomal membrane, such as DPPC-liposome, is essential for exertion of immunogenic activity [8, 9]. Thus, additional studies are required to elucidate that administration of GSL antigen containing liposomes prepared with DSPC having a high Tc was ineffective for induction of immune responses against GSL antigen.

In this study, anti-LacCer antibody was generated by immunization with DMPC- and DSPC-liposome containing LacCer, but antibody responses against other GSL antigens, such as GlcCer, GM3, and GM1, were not obtained with DMPC- and DSPC-liposome (Figs. 2 and 4). These results suggest that liposome-associated LacCer is immunogenic, irrespective of liposomal phospholipid composition. At the moment, however, the reason for this is unknown. In the future, it needs to be studied in detail about the immunogenicity of LacCer-containing liposomes.

Taking the findings obtained here into consideration, DPPC-liposome would be useful for the effective carrier of antigens. In addition, GSL antigen was virtually non-immunogenic by itself (Fig. 1), but was highly immunogenic in DPPC-liposome (Fig. 3). Thus it would have potential in establishing the active immunotherapy with antigens exhibiting poor immunogenicity, including GSL antigens.

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REFERENCES