

## Adjuvant Activity of Synthetic Cationic Amphiphiles for Production of IgG Antibody in Sprague Dawley Rats

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**12GP2 and 14GP2, synthetic cationic amphiphiles, were examined in Sprague-Dawley rats for their adjuvant activity with ovalbumin (OVA) as antigen. Findings were compared with the activity of complete Freund's adjuvant (CFA) and aluminum hydroxide (Alum). Both amphiphiles induced OVA specific IgG comparable to the conventional adjuvants CFA or Alum, and total IgG levels of 12GP2 and 14GP2 groups were lower than the levels of other conventional adjuvants or with no adjuvant. Induction of OVA specific IgA and IgM were not observed. Examination of the effect of these adjuvants on T cell population of spleen lymphocytes showed that. CD4<sup>+</sup>/CD8<sup>+</sup> ratio, CD4<sup>+</sup> populations and CD8<sup>+</sup> populations in rats immunized with the four adjuvants differed. Therefore, these adjuvants have different mechanisms for exerting immunoadjuvant activities.**

Key words: adjuvant, liposome, cationic amphiphiles, complete Freund's adjuvant, aluminum hydroxide

Since 1937 CFA has been used to produce antiserum in laboratory animals. The related toxicity was immediately recognized (Amyx, 1987; Broderson, 1989), but attempts to find a less toxic and equally effective alternative have not been successful. Aluminum-containing adjuvants are the only adjuvants currently approved for human use by the Food and Drug Administration in the U.S.A. (Edleman, 1980; May *et al.*, 1984). The high adjuvant activity of Alum is due to its strong antigen binding activity (Seber *et al.*, 1991; Shirodkar *et al.*, 1990), but some proteins are weakly absorbed by Alum, regardless of the pH (Hirasawa *et al.*, 1997). In addition, aluminum has been found in brains of Alzheimer disease patients (Crapper *et al.*, 1973).

Other authors reported the effectiveness of liposomes for vaccine adjuvants. Liposomes are composed of phospholipids which have no toxicity, and hence may be suitable for a human vaccine adjuvant in place of Alum. The mechanisms of liposome adjuvanticity mainly comprise two functions, one is the efficient targeting of APCs such as macrophages, which incorporate the encapsulated antigen into liposome and present the processed antigen on their cell surface. The helper T lymphocytes were stimulated by the processed antigen, and production of antigen-specific immunoglobulin by B cells followed. The other function of the liposome is a depot for prolonged antigenic stimulation.

Most liposomal adjuvants investigated were composed of

phospholipids and cholesterol (Kato & Goto, 1997; Duits *et al.*, 1993; Bui *et al.*, 1994; Hui, 1994; Alving, 1993; Richards *et al.*, 1996), with a neutral charge. On the other hand, Nakanishi *et al.* (1997) reported that macrophages took up positively charged liposomes, which consisted of phosphatidylcholine, cholesterol and stearylamine, more effectively than neutral or negatively charged liposomes and the positively charged liposomes induced antigen specific cytotoxic T lymphocyte responses and antibody production. A series of amphiphiles with two hydrocarbon tails, a trifunctional glutamate connector, phenylene and methylene spacer, and an ammonium group as the cationic head (Kunitake *et al.*, 1980) were examined for their physicochemical properties and the efficiency of the synthetic amphiphiles in gene transfer into the mammalian cultured cells (Akao *et al.*, 1991; Akao & Ito, 1997). We found a good correlation between their efficiencies in gene transfer and physicochemical characteristics of membrane fluidity and morphology. The effective compound has a  $T_c$  lower than 37°C, the temperature of the culture system, and forms small and stable vesicles (Akao & Ito, 1997).

In the present study, we investigated the effect of the synthetic cationic amphiphiles with different fluidity, regarding adjuvant activities, especially in terms of production of IgG, IgA and IgM.

### Materials and Methods

**Materials** OVA, CFA and Alum were purchased from Wako Pure Chemicals (Osaka). In ELISA, TPBS was used for rinsing, and Block Ace (Dainippon Pharmaceutical Co. Ltd., Osaka) diluted 4 times with distilled water was used for blocking and after being diluted 10 times. Rat IgA, rat IgM, mouse anti-rat IgA and POD-conjugated mouse anti-rat IgA were purchased from Zymed Laboratories (South San Francisco, CA). Rat IgG, goat anti-rat IgG, goat anti-rat IgM, POD-conjugated goat anti-rat IgG and POD-conjugated goat anti-rat IgM were purchased from Organon Teknika Corporation-Cappel Products (Durham,

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Abbreviations: OVA, ovalbumin; CFA, complete Freund's adjuvant; Alum, aluminum hydroxide; APCs, antigen presenting cells; ELISA, enzyme linked immunosorbent assay; 12GP2, didodecyl *N*-[*p*-(2-trimethylammonioethoxy)-benzoyl]-L-glutamate bromide; 14GP2, tetradecyl *N*-[*p*-(2-trimethylammonioethoxy)-benzoyl]-L-glutamate bromide;  $T_c$ , phase transition temperature; TPBS, 0.05% Tween 20 dissolved in phosphate buffered saline pH 7.4; PBS, phosphate buffered saline pH 7.4.

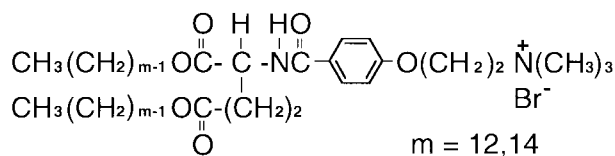


Fig. 1. Structures of 12GP2 ( $m=12$ ) and 14GP2 ( $m=14$ ).

NC). The substrate solution for ELISA was a 10:9:1 mixture of 0.006%  $\text{H}_2\text{O}_2$  dissolved in a 0.2 M citrate buffer (pH 4.0),  $\text{H}_2\text{O}$ , and 6 mg/ml of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Wako Pure Chemicals). Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were used for ELISA procedures.

#### Antigen-adjuvant inclusions

**CFA** A solution of 20  $\mu\text{g}/\text{ml}$  OVA dissolved in PBS and CFA (Wako Pure Chemicals) was mixed and sonicated by VP-60 (Taitec Corporation, Saitama) for several minutes for purposes of emulsification. Stability of the water-in-oil emulsion of CFA was assessed by placing a drop of the emulsion onto the surface of water.

**12GP2 and 14GP2** 12GP2 and 14GP2 (Fig. 1) were synthesized as described previously (Kunitake *et al.*, 1980). PBS solution, including 1.3 mM of these amphiphiles and 10  $\mu\text{g}/\text{ml}$  of OVA was sonicated for 1 min by VP-60.

**Alum** Crystalline aluminum hydroxide in PBS at 20 mg/ml was dispersed and sonicated for several minutes. The solution and 20  $\mu\text{g}/\text{ml}$  of OVA in PBS were mixed, and incubated for 30 min with shaking at 37°C so that the antigen was adsorbed by the surface of Alum.

**No adjuvant** OVA 10  $\mu\text{g}/\text{ml}$  was dissolved in PBS.

**Animals and immunization** Four week old Sprague-Dawley rats were purchased from Seiwa Experimental Animals (Fukuoka) and were fed commercial pellet type NMF (Oriental Yeast Co., Tokyo). After acclimatization for 2 weeks, the rats were separated into groups of 5 and 4 of each group were immunized intraperitoneally with a booster given every 10 days. Each rat was given 10  $\mu\text{g}$  of OVA as the antigen-adjuvant inclusion. Blood was collected from the tail vein 4 days after each injection. Forty days after the first injection, the rats were anesthetized with light diethyl ether anesthesia and whole blood and spleens were removed immediately. This experiment was carried out under the guidelines for Animal Experiments in the Faculty of Agriculture and Graduate Course of Kyushu University, and according to Law No. 105 and Notification No. 6 of the Japanese government.

**Cytotoxicity of amphiphiles** Rat basophilic leukemia (RBL-2H3) cells were cultured in RPMI 1640 (Nissui Pharmaceutical Co., Tokyo) medium supplemented with 10% fetal bovine serum (Intergen Co., Purchase, N.Y.). The cells were inoculated into culture dishes at a cell density of  $10^5$  cells/2 ml, and 10  $\mu\text{l}$  of 0–26 mM of amphiphile solution in PBS was added. After cultivation for 48 h at 37°C, the cell number was counted, using an electronic particle counter Z1 (Coulter Corporation, Miami, FL).

**ELISA** Antigen-specific antibodies were measured using sandwich ELISA. One hundred and fifty microliters of antigen solution (50  $\mu\text{g}/\text{ml}$ ) dissolved in a 50 mM sodium carbonate-sodium bicarbonate buffer (pH 9.6) was added to each well of

the ELISA plates followed by incubation for 1 h. After blocking overnight at 4°C with 300  $\mu\text{l}$  of a blocking solution in each well, we added 100  $\mu\text{l}$  of rat serum diluted with the blocking solution for 1 h. Bound antibodies were detected by reacting with 100  $\mu\text{l}$  of POD-conjugated anti-rat antibodies (diluted 1000 fold for IgA and IgM, and 2000 fold for IgG). Each well was rinsed 3 times with TPBS between each step. After incubating for 15 min with 100  $\mu\text{l}$  of a substrate solution, the reaction was stopped by adding 100  $\mu\text{l}$  of 1.5% oxalic acid, and absorbance at 415 nm was measured using an MPR-A4i ELISA reader (Tosoh Corporation, Tokyo). Total IgG was measured by diluting, anti-rat IgG 1000 times with a sample dilution solution, and 96-well plates were treated with 50  $\mu\text{l}$  of the solution for 1 h, then processed as described above. These reactions were at 37°C, if not otherwise specified. Ig standard curve was prepared for every determination and Ig concentration was calculated from raw absorbance data within the linear range of this curve.

**Spleen lymphocyte analysis of  $\text{CD4}^+$  and  $\text{CD8}^+$  cells** Spleen lymphocytes were put into the RPMI 1640 medium (Lim *et al.*, 1994) followed by incubation for 30 min at 37°C to remove fibroblasts. Then, 5 ml of the cell suspension was layered on 4 ml of Lympholyte-Rat (Cedarlane Laboratories Ltd., Ontario, Canada) and centrifuged at  $1500\times g$  for 30 min. The lymphocyte band at the interface was collected, and the cells were rinsed 3 times with the medium. Spleen lymphocytes were resuspended in PBS containing 10% fetal bovine serum. The cell concentration was then adjusted to  $1\times 10^6$  cells/100  $\mu\text{l}$ , and 5  $\mu\text{l}$  of fluorescein-labeled mouse anti-rat CD4 or phycoerythrin-labeled mouse anti-rat CD8 (Serotec Ltd., Kidlington, Oxford, UK) were added. After incubating for 30 min at 4°C, the lymphocytes were rinsed 3 times with PBS containing 10% fetal bovine serum and then centrifuged at  $1500\times g$  for 5 min. The stained lymphocytes were fixed with 2% paraformaldehyde and used for flow cytometry examination (Epics Profile II, Coulter Electronics Ltd., Luton, Bedfordshire, UK).

## Results

**Cytotoxicity of amphiphiles** Cytotoxicity of RBL-2H3 cells was examined to determine the dose of amphiphiles re-

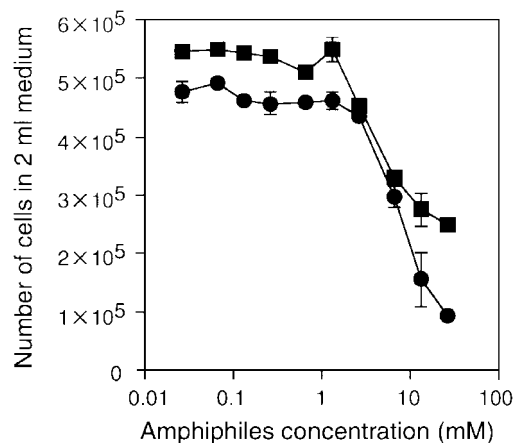


Fig. 2. Toxic effect of amphiphiles on RBL-2H3 cells. RBL-2H3 cells were cultured for 48 h in the presence of various concentrations of 12GP2 (●) or 14GP2 (■) then cells were counted. Data are means  $\pm$  standard deviation ( $n=3$ ).

quired for animal experiments. Ten microliters of amphiphile solution was added to 2 ml of medium and the preparation incubated for 48 h at 37°C. Both 12GP2 and 14GP2 at concentrations below 2.6 mM had little influence on cell growth (Fig. 2). At higher concentrations, these amphiphiles exerted a cytotoxic effect. Thus, a 1.3 mM concentration of amphiphiles as adjuvants was injected.

On day 40, the weight of the no adjuvant group was heaviest, but there were no significant differences in the weight among all groups (data not shown); and at a glance, the rats immunized with 12GP2 or 14GP2 appeared healthy. So we consider that the toxicity of 12GP2 and 14GP2 did not affect this experiment under the conditions used.

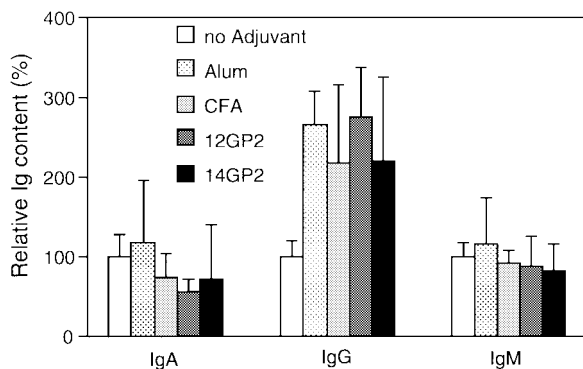
**Induction of OVA specific antibodies** Four groups of rats were injected with 10 µg of OVA emulsified with 12GP2, 14GP2, CFA or Alum, and one group with OVA without adjuvant. The OVA specific antibodies were measured, using ELISA, and expressed as relative values against that of adjuvant free control (Fig. 3).

The specific IgG against OVA of all adjuvant groups was adequately raised. These 4 adjuvants gave 2–3 fold higher OVA specific IgG levels compared with the control group, though there was no statistically significant difference by non-repeated measures ANOVA ( $p < 0.05$ ). In the no adjuvant group, a rise in OVA specific IgG level was observed (Fig. 4), and further studies are needed to elucidate this observation.

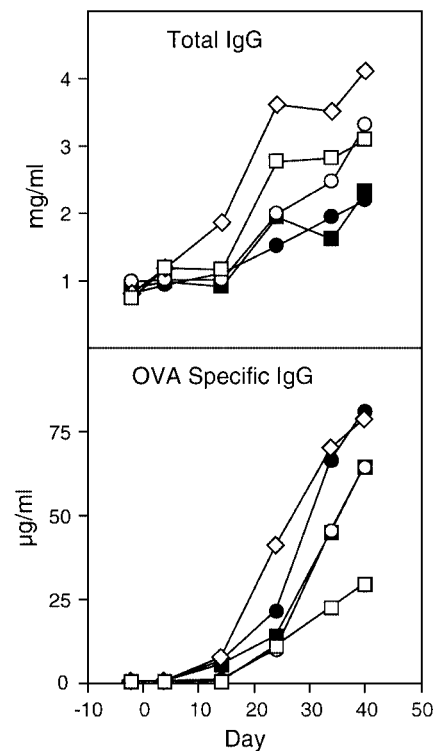
In addition, CFA, 12GP2 and 14GP2 tended to reduce OVA specific IgA levels, though there were no statistically significant difference between adjuvants by non-repeated measures ANOVA ( $p < 0.05$ ).

**Time courses of total and OVA specific IgG levels** Figure 4 shows the time course of total and OVA specific IgG levels. Alum induced specific IgG most rapidly and the level was 2–3 fold higher than other adjuvants on day 24. From days 24 to 34, the specific IgG level of the 12GP2 group was elevated significantly and finally reached the same level as the Alum group. In 14GP2 and CFA groups, OVA specific IgG levels increased at a slower rate than seen with other adjuvant groups.

Alum enhanced the total IgG level most rapidly, with the highest total IgG level. Interestingly, total IgG levels of 12GP2 and



**Fig. 3.** Effects of adjuvants on induction of OVA specific antibodies in Sprague-Dawley rats. Rats were immunized with 10 µg OVA and various adjuvants and the levels of OVA specific antibodies were examined using ELISA on day 40 after first immunization. Data are means  $\pm$  standard deviation ( $n=4$ ).



**Fig. 4.** Effects of adjuvants on total and OVA specific IgG levels in sera of OVA immunized rats. Data are means of 4 rats. □: no adjuvant, ◇: Alum, ○: CFA, ●: 12GP2, ■: 14GP2.

14GP2 groups were lower than levels seen with other conventional adjuvants or no adjuvant. These results suggest that 12GP2 and 14GP2 induce OVA specific IgG more specifically than do CFA and Alum.

**Lymphocyte analysis of immunized rat** The effect of these adjuvants on cell populations of spleen lymphocytes was examined and data was checked by non-repeated measures ANOVA and Dunnett test. The proportion of CD4<sup>+</sup>CD8<sup>-</sup> cells was significantly lower in the Alum and 12GP2 groups than in the control group, but higher in the 14GP2 group, while the proportion of CD4<sup>+</sup>CD8<sup>+</sup> cells was lower in the Alum, CFA and 12GP2 groups than in the control group but higher in the 14GP2 group. The proportion of CD4<sup>-</sup>CD8<sup>-</sup> cells was higher in the Alum, CFA and 12GP2 groups.

## Discussion

A number of approaches have been made to improve immunoadjuvant actions of liposomes. Functional modulators such as ornithine (Kato & Goto, 1997), interleukins (Duits *et al.*, 1993; Bui *et al.*, 1994), muramylpeptide (Hui, 1994) or lipid A (Alving, 1993; Richards *et al.*, 1996) have been used. However, almost all were composed of neutral charged lipids, such as phosphatidylglycerol, phosphatidylcholine and cholesterol. There is little documentation of immunoadjuvant actions of positively charged liposomes. We find that 12GP2 and 14GP2, self-assembled positively charged liposomes, have adjuvant activities.

12GP2 and 14GP2 induced OVA specific IgG equivalent to that seen with a conventional adjuvant such as CFA or Alum (Fig. 3), and the total IgG was suppressed (Fig. 4). Positively charged multilamellar vesicles were taken up by macrophages, as

**Table 1.** Effect of adjuvants on lymphocyte population in Sprague-Dawley rats immunized with OVA.

	CD4 <sup>+</sup> CD8 <sup>-</sup> (%)	CD4 <sup>+</sup> CD8 <sup>+</sup> (%)	CD4 <sup>-</sup> CD8 <sup>-</sup> (%)	CD4 <sup>+</sup> CD8 <sup>-</sup> /CD4 <sup>-</sup> CD8 <sup>+</sup>
No adjuvant	35.70±0.14	21.47±0.59	42.17±0.50	1.66±0.05
Alum	33.37±0.50 <sup>b)</sup>	18.30±0.14 <sup>b)</sup>	47.87±0.45 <sup>b)</sup>	1.82±0.04 <sup>b)</sup>
CFA	37.13±0.65 <sup>a)</sup>	16.87±0.24 <sup>b)</sup>	45.50±0.78 <sup>b)</sup>	2.20±0.02 <sup>b)</sup>
12GP2	29.63±0.66 <sup>b)</sup>	17.90±0.45 <sup>b)</sup>	52.03±0.87 <sup>b)</sup>	1.66±0.06
14GP2	36.73±0.29	24.17±0.34 <sup>b)</sup>	38.57±0.12 <sup>b)</sup>	1.52±0.03 <sup>a)</sup>

Data are means±standard deviation of 4 rats and significantly different at <sup>a)</sup>  $p < 0.05$  and <sup>b)</sup>  $p < 0.01$  compared with No adjuvant group by Dunnett test.

compared with negatively charged or neutral multilamellar vesicles (Nakanishi *et al.*, 1997). The positively charged liposomes may spontaneously interact and fuse with the plasma membrane *in vivo*, and thereby enhance immunopotential activity.

12GP2 and 14GP2 induced OVA specific IgG without affecting the production of specific IgA and IgM. Such class-specific regulation of Ig production is induced by lymphokines such as interleukin 4, interleukin 5, interferon- $\alpha$  and interferon- $\gamma$  (Gauchat *et al.*, 1991; Ochel *et al.*, 1991; Pene *et al.*, 1988; Rousset *et al.*, 1991). A similar type of regulation can be induced by bile acid, lectins (Lim *et al.*, 1994; Lim *et al.*, 1995; Yamada *et al.*, 1993), unsaturated fatty acids (Hung *et al.*, 1999; Matsuo *et al.*, 1996; Yamada *et al.*, 1996; Lim *et al.*, 1996) and some food additives (Kuramoto *et al.*, 1996; Kuramoto *et al.*, 1997). In addition, our cationic amphiphile adjuvants also would induce the class-specific regulation of Ig production.

The cell population of spleen lymphocytes differed with the adjuvant (Table 1) which means that these adjuvants affect the population of lymphocytes in a different manner, though all exert the same result on Ig production, class specific enhancement of IgG production. It is of interest that 12GP2 and 14GP2 had different effects on the T cell and lymphocyte populations, respectively, despite having similar molecular structures.

CD4<sup>+</sup>CD8<sup>-</sup> cells are thought to recognize the MHC class II antigen and mainly act as a helper T cell. And CD4<sup>-</sup>CD8<sup>-</sup> cells in spleen are considered to be mainly B lymphocytes with producing antibodies. Increment in the proportion of CD4<sup>-</sup>CD8<sup>-</sup> cells of the group of Alum, CFA and 12GP2 demonstrates the feasibility of contributing to the production of IgG. In the Alum and CFA groups, the ratio of CD4<sup>+</sup>CD8<sup>-</sup> to CD4<sup>-</sup>CD8<sup>+</sup> was increased, while the ratio of 12GP2 and 14GP2 groups had no significant changes or decreased; these characterize our novel adjuvants.

We reported that 12GP2 has DNA-transfection potential while 14GP2 has little, and their DNA-transfection ability depends on physicochemical characteristics (Akao *et al.*, 1997). The structures of 12GP2 and 14GP2 are subequal except for their length of alkyl chains (Fig. 1), but they have distinct phase transition temperatures ( $T_c$ ), 24.8°C and 41.1°C, respectively. The amphiphiles are in the fluid liquid crystalline state at higher temperatures than their  $T_c$ , and can fuse with the plasma membranes, but at lower temperatures, they are in the solid crystalline state and incapable of fusing. In this case 14GP2 would bind to but not fuse with the plasma membrane surface. Accordingly, it is conceivable that adjuvant activity of the 12GP2 depends on its uptake to macrophages and to depot function. Nakanishi *et al.* (1997) reported that positively charged multilamellar vesicle (composed of egg phosphatidylcholine : cholesterol : stearylamine=4 : 5 : 1) was significantly taken up by macrophages at 37°C, but that there was little uptake at 4°C, a finding which sup-

ports our hypothesis concerning the mechanisms of 12GP2 and 14GP2.

By way of summary, we found that the synthetic cationic amphiphiles 12GP2 and 14GP2 are useful adjuvants to produce the specific IgG. These data suggest that the synthetic cationic amphiphiles may prove to be a safe, effective and chemically defined alternative to CFA.

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