

Epsilon-Polylysine Microparticle Adjuvant Drives Cytokine Production to Th1 Profile

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ABSTRACT. Epsilon-polylysine micro particles (SGEPL) and polyethyleneimine micro particles (SGPEI) were developed by the addition of a hydrophobic group and the immunological characterization of these micro particles and aluminum hydroxide (ALUM) was investigated. BALB/c mice were injected intraperitoneally with ovalbumin (OVA) as an antigen and SGEPL, SGPEI or ALUM as an adjuvant. The results showed that the mice injected with SGEPL produced a significant portion of anti-OVA antibody subclass IgG2a in the sera and suppressed interleukin (IL)-4 and IL-5, but enhanced IL-12 and interferon- γ (IFN- γ) from the spleen cells. Similar results relating to cytokines were also obtained, even without OVA. Direct stimulation with SGEPL to naïve BALB/c mouse spleen cells induced IL-12 and IFN- γ . Both spleen and purified B cells produced IgG1 and IgE after stimulation with IL-4 and the anti-CD40 monoclonal antibody. With the addition of SGEPL, the IgE production from the cells was suppressed as a result of enhanced IFN- γ production. Furthermore, IgE production was also suppressed in the purified B cells without the influence of IFN- γ or IL-12. Thus, we suggest SGEPL drives cytokine production to Th1 profile. It will be a novel promising adjuvant based on this viewpoint.

KEY WORDS: adjuvant, cytokines, epsilon-polylysine, hydrophobize, micro particles.

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Various adjuvants have been developed for practical application for the control of infectious diseases. Among them, aluminum hydroxidesalts (ALUM) have been the most widely used adjuvants because a higher level of IgG titer can be obtained after immunization, which essentially induces Th2 immune responses but poorly induces cell-mediated immunity [2, 5, 6]. To obtain protective immunity against infectious diseases, effective Th1 or Th2 responses are generally required, and their immune balance elicited by vaccination may be crucial [2, 5, 6]. Therefore, the development of very safe and effective adjuvants, which stimulate cellular and humoral immunity, is important for protection against various infectious diseases.

Poly-cationic materials induce a better balance of Th1 and Th2 responses as adjuvants than ALUM [2, 5, 13, 14]. Epsilon-polylysine (EPL), which is a water-soluble polycationic polymer (WSPCP), has been applied as a very safe food preservative for a long time in Japan and is also known to have antimicrobial ability [25]. In addition, polyethyleneimine (PEI) is a WSPCP that has been used in raw materials for various industrial processing methods. Some reports described that these WSPCPs can bind to anionic nucleic acids and proteins around neutral pH and also attach to cell membrane that surface is negative charge, and they penetrate a cell membrane and help to introduce nucleic

acids and proteins into cells [9, 10]. Furthermore, chemically cationized proteins are easily introduced into immunocyte and drive Th1 response [13, 18]. Thus, EPL and PEI were expected to be favorable antigen carrier to immunocyte and induce better balance of Th1 and Th2. Although EPL and PEI may be good candidates for adjuvants to improve up-regulation of Th1 response, WSPCP is very safe by oral ingestion but is toxic by intravenous injection at high doses because WSPCP is easily absorbed into the body by the intravenous route [7]. In this study, in order to reduce their toxicity, EPL and PEI were covalently bound to the hydrophobic group (stearyl glycidylether) to produce hydrophobized EPL (SGEPL) and PEI (SGPEI), and then the immunoresponses of SGEPL, SGPEI and ALUM were characterized and compared with each other.

MATERIALS AND METHODS

Experimental animals: Female BALB/c mice (5–8 weeks of age, Japan Clea, Japan) were maintained in a filter-laminar flow enclosure cage, and provided with autoclaved food and water *ad libitum*. This study was conducted in conformity with the animal study guidelines of Obihiro University of Agriculture and Veterinary Medicine.

Preparation of microparticles (SGEPL and SGPEI): Epsilon-polylysine 25% w/v solution (EPL) (Chisso, Japan) was bound covalently to stearyl glycidylether (SGE) (NOF, Japan). The various mixtures of the SGE, EPL, and Triton-X-405 (Wako, Japan) were stirred and heated at 100°C with

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reflux for 18–24 hr. After cooling down, ethanol was added and microparticles were precipitated by centrifugation (15,000 rpm, 10 min). After repeated washing by ethanol, the precipitations were dialyzed against pure water for 48 hr. Certain volumes were collected, the dry weights were determined, and the slurry was adjusted to 40 mg/ml. Polyethyleneimine (PEI, MW=10000, Wako Pure Chemicals industries, Japan) was diluted with pure water to 25% w/v. The subsequent procedures were the same as for EPL. The reaction molar ratio of EPL and SGE (EPL: SGE) was 1:2, 1:10 and 1:50, respectively, and PEI: SGE was 1:2 alone. The concentration of TritonX-405 was all set at 5% w/v.

Preparation of aluminum hydroxide: Aluminum hydroxide was prepared in accordance with common practice [15]. The dry weight was determined, and the suspensions were adjusted to 10 mg/ml with saline.

Determination of ovalbumin adsorption amount to SGEPL and SGPEI: Two hundred mg of ovalbumin (OVA) (Sigma-Aldrich, U.S.A.) was dissolved in 1 ml of PBS⁻, and 20 μ l of this was mixed with 200 μ l of SGEPL or SGPEI, followed by incubation at 37°C for 1 hr. The amounts of protein remaining in the supernatants were determined using a BCA kit (Pierce, U.S.A.).

Zeta potentials: The characterizations of particles, in terms of particle size and zeta potentials, were measured by a laser analyzer (ELS-8000, OTUKA ELECTRONICS, Japan).

Immunization: OVA was dissolved in PBS⁻ at 20 mg/ml. Each ten parts volume of SGEPL, SGPEI and ALUM were mixed with one part volume of OVA-PBS⁻ solution. Each 20 μ l was injected intraperitoneally into mice (n=8) three times at two-week intervals.

Spleen cell culture of immunized mouse: Single-cell suspensions were prepared from the spleen seven days after the last immunization. The cells were treated with 0.83% NH₄Cl and adjusted to a concentration of 1×10^6 cells/ml in Iscov's modified Dulbecco's medium (Gibco, U.S.A.) containing 4 mM L-glutamine, heat inactivated 10% v/v fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin and 5×10^{-2} mM 2-mercaptoethanol. The cells were cultured at 1 ml each in 24-well flat-bottom culture plates. (SUMITOMO BAKELITE, Japan). Totals of 10 μ l of 1 mg/ml of the OVA- PBS⁻ solutions were pulsed into 1 ml of the cell cultures. The controls were pulsed with 10 μ l of PBS⁻. The cells were cultured at 37°C in a 5% CO₂ incubator for 3 days. The cytokines in the supernatant were determined by ELISA.

Direct addition of SGEPL to cells: The cell suspensions (1×10^6 cells/ml) were prepared from naïve BALB/c (no immunization before test) mouse spleen in the same way as above. B220⁺ B cells were positively collected from naïve BALB/c mice spleen cells by magnetic cell sorting according to the manufacturer's instructions (Miltenyi Biotec, Germany). The purified cells were adjusted to 2×10^6 cells/ml. These cells were anti-CD40+IL-4-stimulated with 1 μ g/ml of anti-mouse CD40 monoclonal antibody (R&D Systems, U.S.A.) and 5 ng/ml of IL-4 (R&D Systems, U.S.A.). Vari-

ous concentrations of SGEPL (0, 25, 50, 100, 200 and 400 μ g/ml) were added and cultured at 37°C in a 5% CO₂ incubator for 3 days for cytokine detection and 9 days for antibody detection. The cytokines and the antibodies in the supernatants were determined by ELISA.

Cytokine assay: IL-4, IL-5, IL-12 and IFN- γ levels were measured using an ELISA kit (Endogen, U.S.A.).

OVA-specific IgG1 and IgG2a assay: OVA-specific antibodies were detected using OVA-immobilized immunoplates (Maxi-sorp, NUNC, Denmark). The collected sera were diluted 1000 times for IgG1 and 25 times for IgG2a detection. Antibody concentrations were determined from the standard curves of IgG1 and IgG2a (ZYMED, U.S.A.) adhered to the plates directly. Horseradish peroxidase-labeled rabbit anti-mouse IgG1 and IgG2a (ZYMED, U.S.A.) were used as secondary antibodies. The reaction products were visualized with TMB (Clinical Science Products, U.S.A.). The absorbance at 450 nm was measured by an immunoplate reader (Multiskan JX, Thermo Electron, U.S.A.). The concentrations of antibodies were described as the quantities in the well (ng/well). The dilution rates did not multiply to the values.

Total IgG1 and IgE assay: IgE levels were measured using an ELISA kit (Shibayagi, Japan). IgG1 levels were measured by a sandwich ELISA using two kinds of rabbit anti-mouse IgG (ZYMED, U.S.A.) and horseradish peroxidase-labeled rabbit anti-mouse IgG1. Immunoplates were coated with anti-mouse IgG. The two-times diluted cell culture supernatants were added. Mouse IgG1 was used as a standard. After incubation, horseradish peroxidase-labeled rabbit anti-mouse IgG1 was added to the wells, and the reaction products were visualized in the same way as above. The data are shown as rates when the mean values of 0 μ g/ml of SGEPL were set at 100%.

Cell proliferation assay: Cells were cultured under various experimental conditions in 96-well culture plates (SUMITOMO BAKELITE, Japan). After 3 days' culture, the cells were assayed using an assay kit (Cell Titer 96 Non-radioactive Cell Proliferation Assay, Promega, U.S.A.). The data are shown as rates when the mean values of 0 μ g/ml of SGEPL were set at 100%.

Statistical methods: Two-tailed Student's *t*-test was performed for statistical analysis of the data, and *P*<0.05 was taken as the level of significance.

RESULTS

Preparation of SGEPL and SGPEI: The effect of the reaction molar ratio on the average particle size, zeta potential, OVA adsorption and anti-OVA antibody values after immunization with OVA are shown in Table 1. The mean particle size for SGEPL and SGPEI was almost the same, except molar ratio 1:50 of SGEPL. According to reaction molar ratio, the OVA adsorption and the antibody titers against OVA decreased, but the zeta potentials did not vary significantly. A possible reason is that even if the amino group reacted with the epoxy group in the SGE, they were

Table 1. Characterizations of microparticles

Molar ratio	Average size (nm)	ζ -potential (mV)	Adsorbed OVA ($\mu\text{g}/\text{mg}$)	IgG1* (ng/well)	IgG2a* (ng/well)
1:2	143 $\pm$ 10	54 $\pm$ 5	375 $\pm$ 20	54.0 $\pm$ 8.0	17.0 $\pm$ 2.0
1:10	133 $\pm$ 8	58 $\pm$ 6	125 $\pm$ 12	2.1 $\pm$ 0.1	0 $\pm$ 0
1:50	418 $\pm$ 21	54 $\pm$ 6	0 $\pm$ 0	3.6 $\pm$ 0.2	0 $\pm$ 0
(reference)					
PEI					
1:2	136 $\pm$ 6	55 $\pm$ 8	368 $\pm$ 13	93.0 $\pm$ 12.0	11.0 $\pm$ 3.0

* Serum antibody titers after immunization of OVA (40 $\mu\text{g}/\text{dose}$).

Values are represented as means of triplicates $\pm$ SE.

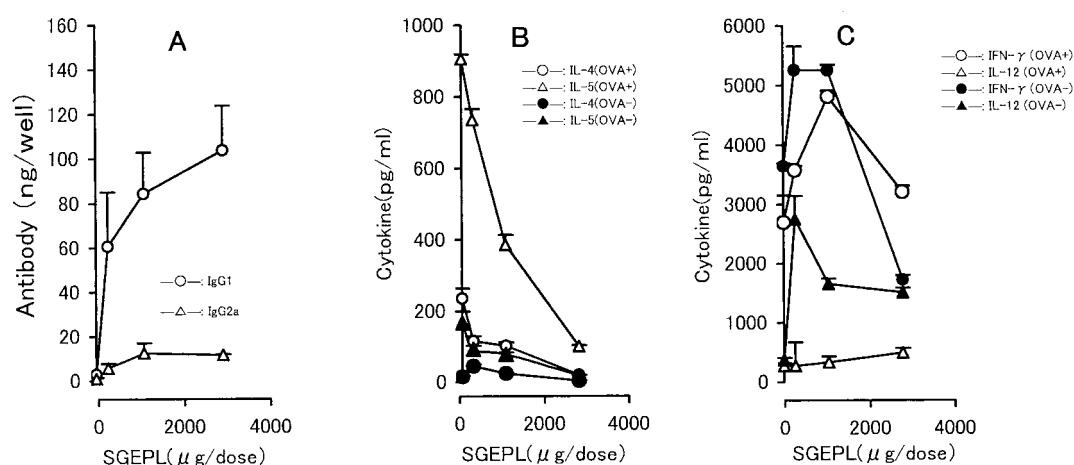


Fig. 1. Production of antibodies in serum and cytokines from spleen cells after immunization of various quantities of SGEPL with OVA to BALB/c mice. n=8. A: Antibodies B: IL-4 and IL-5 C: IFN- γ and IL-12. In cytokines: open symbols; Pulsed with 10 ng of OVA to spleen cells ($1 \times 10^6/\text{ml}$) closed symbols; Pulsed with PBS⁻ to spleen cells ($1 \times 10^6/\text{ml}$).

changed to the cationic amide group. The results suggest that the absorption of OVA on SGEPL is not only a concern regarding the electric charge but also a concern regarding the structure of the particle surfaces. The concentrations of TritonX-405 (1–10% w/v) were not significant for the particle size at a reaction molar ratio of 1:2 (data not shown). The serum anti-OVA IgG1 and IgG2a antibodies were drastically affected by the OVA adsorptions, which were according to the molar ratio. The SGPEI showed quite similar characteristics to SGEPL (1:2) (Table 1). From the above results, SGEPL was used under a molar ratio of 1:2 and 5% TritonX-405 from the following experiments.

Immunoresponses of SGEPL: Each mouse was injected with 0–3200 μg of SGEPL and 40 μg of OVA. All mice did not show any clinical signs during the period of immunity. As a result, the OVA-specific serum IgG1 level dose dependently increased on the concentration of SGEPL, but the IgG2a level reached a plateau over 800 $\mu\text{g}/\text{dose}$ (Fig. 1 A). When the spleen cells from immunized mice were examined to produce cytokines, IL-4 and IL-5 were decreased according to the concentration of SGEPL in OVA-pulsed cells (Fig. 1B). The production of IFN- γ reached a peak at 800

$\mu\text{g}/\text{dose}$ and gradually decreased over 800 $\mu\text{g}/\text{dose}$ in OVA-pulsed and non-pulsed cells, but the production of IL-12 increased gradually according to the concentration of SGEPL at a low level in OVA-pulsed cells (Fig. 1C). However, in OVA-nonpulsed cells, the production of IL-4 and IL-5 decreased, but the production of IL-12 and IFN- γ increased with peaks of production.

Cytokine and antibodies production with various adjuvants: SGEPL, SGPEI and ALUM were intraperitoneally injected into mice with OVA, and then, antibody titers against OVA and cytokine production from spleen cells were compared with each other (Tables 2 and 3).

A low level of antibodies was detected at the injection of only OVA. When ALUM was used as the adjuvant, the highest level of IgG1 was detected ($P < 0.01$) but a lower level of IgG2a was detected ($P < 0.01$) (Table 2). When SGEPL was used as the adjuvant, IgG1 could not be detected as much, but IgG2a was detected more than ALUM. In addition, IgE production was significantly lower on SGEPL ($P < 0.01$). The levels of IgG1 and IgG2a on SGPEI were intermediate among all adjuvants, but IgE as well as ALUM production was significantly higher than that

Table 2. Antibody titers in mice sera

Adjuvants	No. of mice	Mean level of antibody $\pm$ SE (ng/well)				
		IgG1	IgG2a	IgE	IgG1/IgG2a	IgE/IgG2a
None	8	2.8 $\pm$ 0.6	0.9 $\pm$ 0.3	8.1 $\pm$ 0.8	1.9 $\pm$ 0.3	6.5 $\pm$ 0.5
SGEPL	8	53.5 $\pm$ 7.7	16.7 $\pm$ 3.0 ^{b)}	12.1 $\pm$ 1.1	3.8 $\pm$ 0.8	0.9 $\pm$ 0.2 ^{e)}
SGPEI	8	92.8 $\pm$ 11.9	10.9 $\pm$ 3.2 ^{b)}	20.7 $\pm$ 2.1 ^{c)}	10.0 $\pm$ 1.3 ^{d)}	2.6 $\pm$ 0.7
ALUM	8	145.0 $\pm$ 7.2 ^{a)}	3.2 $\pm$ 0.9	18.1 $\pm$ 1.4 ^{c)}	64.7 $\pm$ 18.4 ^{d)}	7.6 $\pm$ 2.4

Quantities (ng/well) of anti-OVA IgG1, IgG2 and total IgE antibodies in sera after inoculation of various adjuvants with OVA (40 μ g/dose) to BALB/c mice. The dilution rates (IgG1: 1/1000, IgG2a: 1/25, IgE: 1/10) are not considered in the results of concentration. a) higher than other IgG1 ($P < 0.01$), b) higher than other IgG2a ($P < 0.05$), c) higher than other IgE ($P < 0.01$), d) higher than other IgG1/IgG2a ($P < 0.05$), e) lower than other IgE/IgG2a ($P < 0.01$).

Table 3. Production of cytokines from spleen cells after immunization of OVA with various adjuvants

Adjuvants	OVA	Cytokine levels (pg/ml)			
		IL-4	IL-5	IL-12	IFN- γ
None	- ^{a)}	15 $\pm$ 5	117 $\pm$ 28	387 $\pm$ 38	3796 $\pm$ 379
	+ ^{b)}	236 $\pm$ 38	859 $\pm$ 26	275 $\pm$ 87	2868 $\pm$ 340
SGEPL	-	7 $\pm$ 3	52 $\pm$ 13	1056 $\pm$ 178 ^{f)}	4173 $\pm$ 454
	+	157 $\pm$ 47 ^{c)}	411 $\pm$ 85 ^{d)}	485 $\pm$ 118 ^{e)}	3970 $\pm$ 345 ^{g)}
SGPEI	-	16 $\pm$ 3	173 $\pm$ 41	768 $\pm$ 178 ^{f)}	3795 $\pm$ 379
	+	410 $\pm$ 60	925 $\pm$ 28	466 $\pm$ 13 ^{e)}	2758 $\pm$ 300
ALUM	-	12 $\pm$ 3	46 $\pm$ 10	433 $\pm$ 27	3191 $\pm$ 493
	+	507 $\pm$ 38	894 $\pm$ 34	164 $\pm$ 40	2100 $\pm$ 230

Cytokines (pg/ml) from spleen cells after immunization of OVA (40 μ g/dose) with various adjuvants to BALB/c mice. None: PBS⁻, SGEPL: 800 μ g/dose, SGPEI: 800 μ g/dose, ALUM: 400 μ g/dose. a) pulsed with PBS⁻. b) pulsed with OVA (10 ng). c) lowest in OVA pulsed ($P < 0.01$). d) lowest in OVA pulsed ($P < 0.01$). e) higher in OVA pulsed ($P < 0.05$). f) higher in OVA non-pulsed ($P < 0.05$). g) highest in OVA pulsed ($P < 0.05$). Values are represented as means $\pm$ SE (n=8).

of SGEPL ($P < 0.01$). In the antibody ratios, ALUM showed the highest ratio of IgG1/IgG2a, whereas IgG1/IgG2a and IgE/IgG2a of SGEPL were lower than those of ALUM and SGPEI.

Only small production of IL-4 and IL-5 was detected in OVA-nonpulsed cells compared with OVA-pulsed cells, but IL-12 and IFN- γ production was repressed in OVA-pulsed cells compared with nonpulsed cells in all adjuvants and the none ($P < 0.05$) (Table 3). In comparison with other adjuvants, SGEPL inhibited the production of IL-4 and IL-5 ($P < 0.01$) in OVA-pulsed cells, but enhanced IFN- γ production in OVA-pulsed cells ($P < 0.05$). IL-12 production was enhanced in SGEPL and SGPEI compared with ALUM (Table 3).

Furthermore, to confirm that the above cytokine kinetics at SGEPL could not have been just due to the combination of SGEPL and OVA, the effect of SGEPL (with no antigens) on cytokine production was examined (Fig. 2). Naïve BALB/c mice were injected with SGEPL (800 μ g/dose) or PBS⁻ three times at two-week intervals. Seven days after the last injection, spleen cells were cultured with or without OVA. There were no significant differences in cytokine production between OVA-pulsed and -nonpulsed cells (Fig. 2). IL-4 and IL-5 production by the injection of PBS⁻ was higher than SGEPL. IFN- γ production was enhanced

slightly by SGEPL, and IL-12 production was more than three times that of PBS⁻, meaning that OVA had not produced or reduced cytokines so much in the spleen cells, whereas SGEPL itself could enhance to produce IL-12 but could not enhance to produce IL-4 and IL-5. This indicates that the results of Table 3 could be due to SGEPL.

Direct effects of SGEPL on naïve mouse spleen cells and B cells: The direct effects of SGEPL on spleen cells and B cells were examined. A dose-dependent inhibition of the cell proliferation of spleen cells and B cells with anti-CD40 and IL-4 antibody stimulations was observed (Fig. 3). The cytotoxic like effect to the spleen cells and B cells was observed from the result of cell proliferation assay (Fig. 3). But since the cytokines and antibodies were produced during the incubation period, the cells would live at least in a certain period.

SGEPL enhanced IgG1 production in the range of 25–200 μ g/ml, while it inhibited the IgE production of spleen cells (Fig. 4A). On the other hand, in B cells, IgG1 levels were almost the same in the range of 25–50 μ g/ml of SGEPL, while IgE production was also inhibited over 25 μ g/ml (Fig. 4B).

IFN- γ production reached a peak at 25 μ g/ml of SGEPL and was inhibited over 50 μ g/ml in stimulated and unstimulated spleen cells (Fig. 4C), but IFN- γ production was

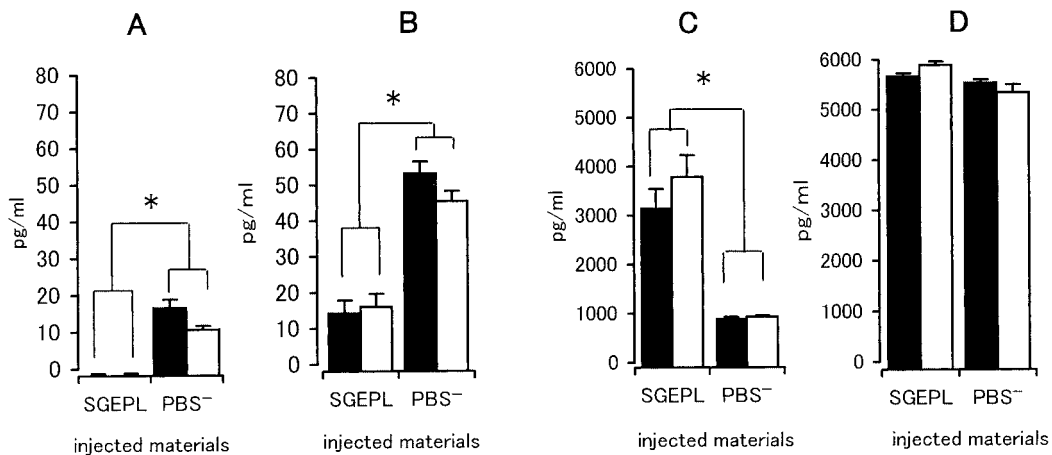


Fig. 2. Cytokine production from spleen cells of SGEPL or PBS⁻ injected mice (n=8). A: IL-4 B: IL-5 C: IL-12 D: IFN-γ. Dark bars: Pulsed with 10 ng of OVA to spleen cells (1×10^6 /ml). Light bars: Pulsed with PBS⁻ to spleen cells (1×10^6 /ml). There were no significant differences between the dark bar and the light bar (OVA pulse and non-pulse) in each SGEPL and PBS⁻ injected mouse in all cytokines. * $P < 0.01$.

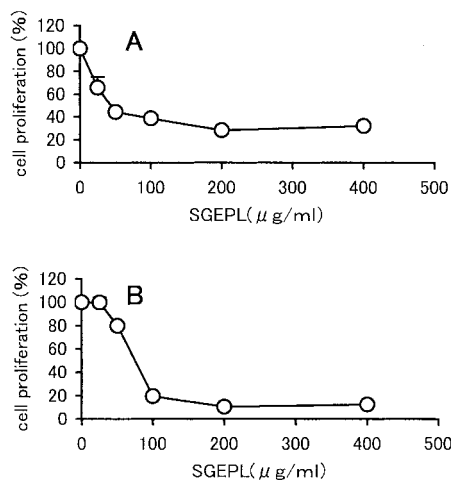


Fig. 3. Effect of SGEPL concentrations on the rates of cell proliferation of spleen cells and B cells. A: spleen cells. B: B cells. Cells were stimulated with IL-4 (5 ng/ml), and anti-CD40 antibody (1 μg/ml). The mean values of cell proliferation at no SGEPL were set at 100%. The values are shown as means of quadruplicates.

almost always lower in stimulated cells than in unstimulated cells, which was assumed to be the effect of IL-4. IL-12 showed only a small change in both stimulated and unstimulated cells (Fig. 4D). Although the data are not shown, IL-4, IL-5, IL-12 and IFN-γ production was detected neither in the B cells nor in the unstimulated cells with the entire range of SGEPL.

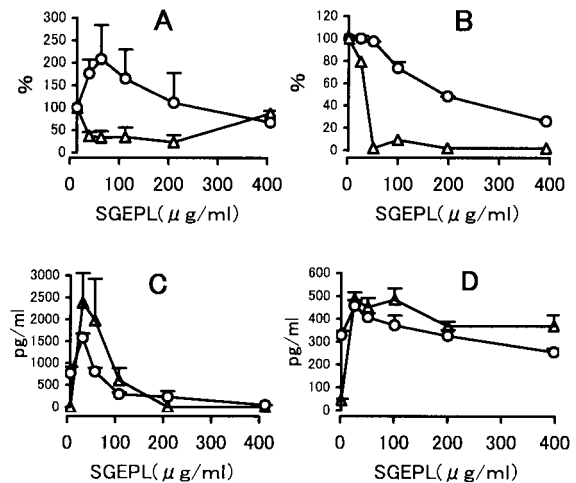


Fig. 4. Effect of SGEPL concentrations on the rates of antibody production of spleen cells and B cells. The cells were stimulated with IL-4 (5 ng/ml), and anti-CD40 antibody (1 μg/ml). The mean values of no SGEPL were set at 100%. A: Antibodies of spleen cells B: Antibodies of B cells. —○—: IgG1. —△—: IgE. C: INF-γ of spleen cells. —○—: stimulated. —△—: unstimulated. D: IL-12 of spleen cells. —○—: stimulated. —△—: unstimulated. The values are represented as means of quadruplicates ± SE.

DISCUSSION

An adoptive immune response is mainly mediated by antigen-specific CD4⁺ Th1 and CD4⁺ Th2 cells. These immune responses can be modulated *in vivo* depending on the adjuvant used for immunization [1, 2, 5–7, 16, 19]. Th1 responses are associated with IFN-γ, IL-2 and IL-12 production, which enhances the production of IgG2a, a hallmark of Th1 immunity [20], and is also correlated with the induction of cell-mediated immunity [3]. IFN-γ, which is mainly pro-

duced by CD4⁺ Th1 cells and NK cells, is a suppressor of activity of Th2 cells and IgE production [24]. IL-12, which is produced by antigen-presenting cells like dendritic cells, macrophages and B cells, is a strong mediator of IFN- γ production [22].

Th2 responses, which control the humoral immune responses through the triggering of B cell proliferation and differentiation [4], are characterized by high levels of circulating antibodies, mainly IgG1 isotypes, and the secretion of cytokines IL-4, IL-5, IL-10 and IL-13 [2, 20]. Excess Th2 responses are associated with occasional allergic reactions. When B cells are stimulated with IL-4 and bound the CD40 on its surface, IgE is released [23]. Although SGEPL and SGPEI more effectively induced the induction of IL-12 and IFN- γ than ALUM, ALUM produced more IL-4 and IL-5, but less IL-12 and IFN- γ in the *in vivo* tests (Table 3). These results relate to the contents of the immunoglobulin subtype in serum, meaning that SGEPL induced the highest IgG2a production (refer to the value of IgG1/IgG2a) and the lowest IgE production compared to the others (refer to the value of IgE/IgG2a) (Table 2). These results did not contradict data that ALUM mostly induces humoral immunity. To the contrary, SGEPL induces the highest level of both IL-12 and IFN- γ , subsequently, it seems to suppress IL-4 and IL-5. IL-12 and IFN- γ had a tendency toward suppression in the OVA-pulsed spleen cells (Fig. 1 and Table 2) in all adjuvants and the none. It was assumed that memory Th2 cells had significantly produced IL-4 and IL-5 with an OVA pulse, and that these cytokines suppress the activity of Th1 and antigen-presenting cells. SGPEI was not very different from ALUM on IL-5 production, and other quantities of cytokines and the antibodies indicated middle values of SGEPL and ALUM. It is interesting that although SGEPL and SGPEI has similar physicochemical characteristics in particle size, zeta potential and OVA adsorption capacity (Table 1), their adjuvant characteristics were different. This suggests that the immune responses were not only affected by these physicochemical characteristics but also affected by other factors.

From the above production data on cytokines and antibodies, at least SGEPL appears to be an adjuvant that elicits more Th1 responses than ALUM, SGPEI and OVA alone in the use of OVA as an antigen.

As mentioned above, since SGPEI indicated middle values of SGEPL and ALUM in antibody and cytokine production, subsequent *in vitro* studies were focused on SGEPL. Surprisingly, IL-12 and IFN- γ were enhanced by only SGEPL in BALB/c mice spleen cells (Fig. 4C). It is reasonable that, since IFN- γ indicated the peak level at 25 μ g/ml of SGEPL in spleen cells (Fig. 4C), IgE production was suppressed about 60% at 25 μ g/ml of SGEPL (Fig. 4A) despite only a 34% decrease in cell proliferation (Fig. 3A). On the other hand, although there was little IFN- γ and IL-12, IgE production in the B cells was strongly inhibited at 50 μ g/ml of SGEPL (Fig. 4B) despite only a 20% decrease in cell proliferation (Fig. 3B). These results suggest, although the reason is not clear, that SGEPL has some specific effect on IgE

suppression rather than on inhibition of cell proliferation. IL-4 is a major factor of IgE class switch [24]. However, since there was little IL-4 adsorption to SGEPL *in vitro* (data not shown), it is difficult to explain that IgE suppression was due to the decrease in IL-4 in the culture medium. One hypothesis is that SGEPL adsorbed on cells changes the surface structure of the cells, and inhibits uptake of IL-4 or other stimulating factors. In fact, it has been observed with microscopy that SGEPL was adsorbed on spleen cells, but that ALUM was not (data not shown). Further investigation of the interaction between SGEPL and cell surfaces is required.

Freund's incomplete adjuvant (FIA) and Freund's complete adjuvant (FCA) are well known that are indeed very efficient in raising high antibody titers. FIA mainly induce humoral immunity, FCA with its mycobacterial component is able to skew a humoral immune response toward the Th1 profile with pronounced IgG2a [8, 11]. SGEPL may not have so higher effects for induction of Th1 response and antibodies production than FCA. FIA and FCA, however, had a profile of adverse strong side effects. Even two times intraperitoneal administration of FIA or FCA developed abdominal adhesions in BALB/c mice [17, 21]. In contrast, there were no clinical signs in SGEPL, SGPEI and ALUM although three times administration in this study (data not shown).

We concluded that SGEPL has ability as an adjuvant to change the balance of IgG1/IgG2a and IgE/IgG2a in comparison with ALUM, SGPEI and no adjuvant. These changes of balance were assumed to be caused by changing the balance of Th1/Th2, especially by enhancing the Th1 response. The Th1 response correlated with the induction of cell-mediated immunity that appears essential to obtain a more efficient response against intracellular pathogens [10, 12]. SGEPL will be a novel promising adjuvant based on this viewpoint.

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