Oligomannose-coated liposomes as an adjuvant for the induction of cell-mediated immunity

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Abstract The effect of the coating of ovalbumin-reconstituted liposomes with various oligosaccharides on their immunogenicity was investigated in mice. The coating of liposomes with oligomannose or yeast mannan drastically enhanced their ability to induce an ovalbumin-specific delayed-type footpad swelling response with a peak at 24 to 48 h post-challenge. Among various oligosaccharides tested, only those with mannose residue at the non-reducing termini manifested the activity when applied to liposomes. Since such oligosaccharides are ubiquitously found in the body, these results suggested the usefulness of oligomannose-coated liposomes as a safe adjuvant for the induction of cell-mediated immunity.

Key words: Liposome; Adjuvant; Oligomannose; Delayed-type hypersensitivity; Cell-mediated immunity

1. Introduction

Cell-mediated immunity (CMI) including delayed-type hypersensitivity (DTH) and cytotoxic T-lymphocyte responses is implicated to be deeply involved in protection against such pathogens as human immunodeficiency virus [1], bovine leukemia virus [2], *Leishmania major* [3] *Mycobacterium leprae* [4] and *Treponema pallidum* [5]. Thus, activation of the cell-mediated, rather than the antibody-mediated, arm of the immune system is considered to be required in a vaccine against these pathogens [6]. The only adjuvant available at present to the human is alum adjuvant made of aluminum hydroxide or aluminum phosphate. This adjuvant, however, induces a poor CMI response, if any at all. In order to design an adjuvant which is able to stimulate effective CMI and is applicable to the human, we have undertaken a series of experiments to study the adjuvant activity of liposomes coated with oligosaccharides from the following reasons: (i) The surface of various infectious agents such as viruses [7], bacteria [8], yeast [9] and protozoa [10] is covered with carbohydrate moieties including those rich in mannose, and it is possible that the immune system employs these chemical structures for its activation. (ii) Noguchi *et al.* [11] reported that liposomes coated with yeast mannan induced a cytotoxic T-lymphocyte response against a virus-related protein reconstituted in them, although mannan is considered not to be suitable for practical use since it is immunogenic and toxic [12,13]. In the present study, liposomes reconstituted with ovalbumin (OVA) as a model antigen were coated with various oligosaccharides ubiquitously found in the human body or with mannan as a reference and their immunogenicity was studied in terms of the induction of DTH responses.

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Abbreviations: CMI, cell-mediated immunity; DTH, delayed-type hypersensitivity; OVA, ovalbumin; DPPC, dipalmitoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; Mal5, mannopentaose; IsoMal5, isomaltopentaose; Lami5, laminaripentaose; GlcNAc5, penta-*N*-acetylchitopentaose; Man5, mannopentaose; Man3, mannotriose; Man2, mannobiose; Man1, mannose.

2. Materials and methods

Female inbred Balb/c mice of 6-week old were obtained from Charles River Japan, Inc. (Yokohama), and were used at the ages of 7–12 weeks old.

Maltopentaose (Mal5), isomaltopentaose (IsoMal5), laminaripentaose (Lami5), penta-*N*-acetylchitopentaose (GlcNAc5) and mannose (Man1) were purchased from Seikagaku Corporation, mannopentaose (Man5), mannotriose (Man3) and mannobiose (Man2) were from Dextra Laboratories Ltd. and high mannose-type oligosaccharides (RN) were prepared by hydrazinolysis from bovine pancreatic ribonuclease B as described previously [14]. The detailed chemical structure of these oligosaccharides is shown in Fig. 1. Mannan-cholesterol (Cholesterol-AECM Mannan) was obtained from Dojin Laboratories (Kumamoto), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE) and cholesterol from Nichiyu Liposome Co. Ltd. (Tokyo), and OVA from Wako Purechem. Inc. Ltd. (Osaka).

Neoglycolipids were constructed from DPPE and oligosaccharides listed in Fig. 1 as reported previously [15]. Liposomes (multilamellar vesicles) reconstituted with OVA were prepared with DPPC and cholesterol (2:1 in mol) according to the modified method by Banghm *et al.* [16]. Liposomes were coated with mannan-cholesterol and various oligosaccharide-DPPE conjugates except for Man1-, Man2- and Man3-DPPE by incubating liposomes with one of these neoglycolipids at 4°C for 3 days. More than 95% of neoglycolipid was adsorbed to liposomes. Since Man1-, Man2- and Man3-DPPE conjugates were water-insoluble, they were added to the solution of cholesterol and DPPC dissolved in chloroform and methanol when liposomes were prepared.

A DTH response was assessed by footpad swelling according to the method as previously reported [17]. The specific footpad swelling responses were expressed as the difference between the thickness of right (challenged with OVA in alum adjuvant) and left (challenged with alum adjuvant alone) footpads.

3. Results

A preliminary experiment indicated that the coating of OVA-liposomes with mannan-cholesterol or Man5-DPPE signifi-

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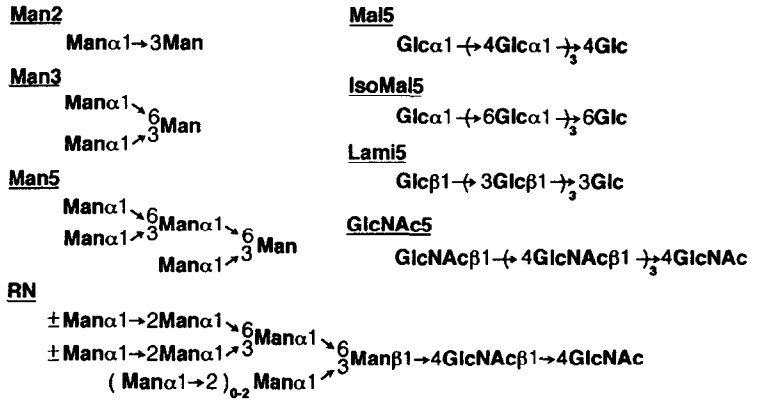

Fig. 1. Chemical structure of oligosaccharides used in the construction of various neoglycolipids.

cantly augmented the immunogenicity of liposomes to induce a DTH response as assessed by footpad swelling in Balb/c mice. At first, we roughly determined following experimental conditions (data not shown). The optimal dose of liposomes expressed as the amount of cholesterol was found to be between 60 to 70 μg per mouse. If the amount of liposomes was fixed in these conditions, the footpad responses increased dose-dependently from 0.2 to 0.9 μg OVA per mouse, attainig a maximal level at the above doses. Maximal levels of the effect of coating liposomes with neoglycolipids were observed at the dose of 10 μg per mouse or more for mannan-cholesterol, and 20 μg per mouse or more for Man5-DPPE. Most experiments were done using enough amount of OVA or neoglycolipids.

Mice were subcutaneously inoculated with OVA together with various adjuvant and the footpad swelling response was assessed by challenging the footpad with OVA 7 days later. Fig. 2 shows the time course of footpad responses post-challenge. OVA-liposomes coated with either mannan-cholesterol or Man5-DPPE conjugate induced a remarkable DTH response with a peak around 48 h post-challenge (g and h). Position of the peak varied between 24 and 48 h depending on the experiment. Either Man5- or mannan-coated liposomes induced apparently stronger and longer-lasting DTH responses than OVA in Freund's complete adjuvant (FCA) (d), while non-coated OVA-liposomes induced a weak response with a peak around 12 h post-challenge (f), which was very similar to the response elicited by immunization of OVA in alum adjuvant (c). The immunization of OVA in saline induced only poor responses at 12, 24 and 48 h (b). Both non-immunized mice and those immunized with mannan-coated liposomes without OVA failed to give any significant response (a and e). These results formally excluded the possibility that the anti-OVA footpad swelling responses were the consequence of a cross-reaction between OVA and compounents of liposomes such as mannan, oligomannose or lipids.

The time course of footpad responses between 4 and 18 days post-immunization was investigated with the following results (data not shown): (i) significant 24- or 48-h responses were induced as early as 4 days post-immunization by either Man5- or mannan-coated liposomes, and the responses were maintained until or even increased by 18 days, and (ii) non-coated liposomes induced a weak temporal 12-h response on day 7 post-immunization with virtually no response at 18 days when anti-OVA antibodies as assessed by enzyme-linked immunosorbent assay appeared.

The histological profile of the footpad swelling responses revealed the following facts (data not shown): the skin lesions

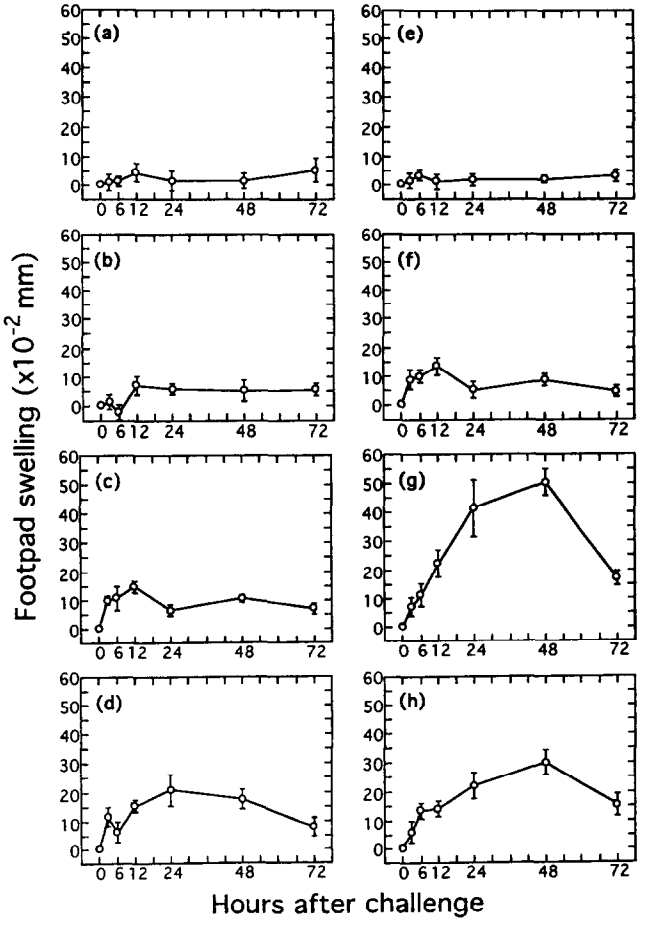

Fig. 2. Footpad swelling responses after challenge with OVA. (a) Non-immunization, (b) OVA in saline, (c) OVA in alum adjuvant, (d) OVA emulsified in FCA (1;1), (e) mannan-coated liposomes without OVA (92 μg mannan-cholesterol per mouse), (f) OVA-liposomes, (g) mannan-coated OVA-liposomes (92 μg mannan-cholesterol per mouse), (h) Man5-coated OVA-liposomes (186 μg Man5-DPPE/mouse). The dose of OVA and that of liposomes expressed as the amount of cholesterol were 8.5 μg and 70 μg per mouse, respectively.

of 24- and 48-h responses by mannan- or Man5-coated liposomes manifested infiltration by numerous mononuclear cells and fewer polymorphonuclear cells. The 12-h response lesion of non-coated liposomes, on the other hand, was characterized by mild infiltration by polymorphonuclear leukocytes with a relatively small number of mononuclear cells.

Table 1 shows the effect of various neoglycolipids (artificial glycolipids) on the immunogenicity of OVA-liposomes: liposomes were coated with one of these neoglycolipids and their ability to induce a 24-h DTH response was studied. Mal5-DPPE was not effective, while IsoMal5-, Lami5- and GlcNAc5-DPPE tended to stimulate the immunogenicity but with effects which were not statistically significant (Exp. 1 of Table 1). RN-DPPE as well as Man5-DPPE were significantly effective (Exp. 2). Among the three neoglycolipids of Exp. 3, Man2-DPPE and Man3-DPPE revealed a significant effect. It is noted that the pyranose ring of mannose of Man1-DPPE is opened, which may be the reason why this compound was not effective. In conclusion, among various oligosaccharide-DPPE conjugates tested, only those with mannose residues at the non-reducing termini were effective.

4. Discussion

The present study demonstrated that the coating of OVA-liposomes with oligomannose or mannan drastically augmented their immunogenicity to mount an OVA-specific DTH response (Fig. 2). DTH against a conventional protein antigen can be classified into tuberculin-type and Jones–Mote-type hypersensitivity in the human and guinea pig, although the difference is not always clear-cut [18]. Jones–Mote-hypersensitivity is characterized by: (i) the peak response post-challenge appears earlier (24 h), (ii) immunity is temporal, that is, it disappears when antibody appears, (iii) polymorphonuclear cells and basophils (in the guinea pig) are the major cells infiltrating in the lesion. On the other hand in tuberculin-type hypersensitivity, (i) the peak post-challenge appears later (48 to 72 h), (ii) the immunity is longer-lasting and (iii) mononuclear cells are the dominant infiltrating cells in the lesion. As judged from the above criteria, the 24- and 48-h responses in mice induced by Man5- and mannan-coated liposomes are considered to correspond to tuberculin-type hypersensitivity in the human and guinea pig whereas the 12-h response by non-coated liposomes is very likely to be Jones–Mote-type hypersensitivity.

Table 1
Effect of various neoglycolipids on the adjuvant activity of OVA-liposomes

Group	Neoglycolipids	OVA	Animal number	Footpad swelling ($\times 10^{-2}$ mm ± S.E.)	%
Exp. 1					
1	nothing	−	6	6.7 ± 2.0	36
2	nothing	+	6	18.4 ± 4.9	100*
3	mannan-cholesterol	+	5	38.9 ± 3.7[a]	211
4	Mal5-DPPE	+	5	19.0 ± 4.4	103
5	IsoMal5-DPPE	+	5	23.5 ± 3.3	128
6	Lami5-DPPE	+	5	23.4 ± 2.8	127
7	GlcNAc5-DPPE	+	5	27.2 ± 5.4	148
Exp. 2					
1	nothing	+	5	18.9 ± 2.7	100*
2	mannan-cholesterol	+	5	58.1 ± 5.6[b]	307
3	Man5-DPPE	+	5	47.9 ± 7.5[c]	253
4	RN-DPPE	+	5	35.3 ± 3.1[c]	188
Exp. 3					
1	nothing (+DPPE)	−	5	10.5 ± 4.4	70
2	nothing (+DPPE)	+	5	14.9 ± 3.9	100*
3	Man1-DPPE	+	5	26.2 ± 5.7	176
4	Man2-DPPE	+	5	32.9 ± 3.4[d]	221
5	Man3-DPPE	+	5	32.0 ± 1.9[d]	215

*Group 2 of Exp. 1, group 1 of Exp. 2 and group 2 of Exp. 3 were used as a control (100). S.E., standard error. [a]$P < 0.05$ against group 2, [b]$P < 0.001$ and [c]$P < 0.005$ against group 1, [d]$P < 0.001$ against group 2. Exp. 1: the doses of OVA and neoglycolipids were 2.1 μg and 200 μg per mouse. Exp. 2: the dose of OVA was 3.9 μg while the doses of mannan-cholesterol, Man5-DPPE and RN-DPPE were 70 μg, 197 μg and 76 μg per mouse, respectively. Exp. 3: the dose of OVA was 6–10 μg per mouse. The mol ratio of DPPC and DPPE or DPPE conjugates added to liposome preparations was 10:1 (see also section 2). DPPE was added to groups 1 and 2 (+DPPE) as a control.

The effect of carbohydrate on CMI may have an evolutionary background in the immune system. It is conceivable that the defense system of the body has established a refined mechanism to recognize the carbohydrate structure of various infectious agents and employs it to activate the immune system. The mannose receptors of macrophages [19–21] may be one such mechanism: these receptors are known to bind molecules containing non-reducing terminal carbohydrates with the following order of potency: D-mannose = L-fucose > D-GlcNAc = D-glucose >> D-galactose. This order is roughly consistent with the present results that among the various neoglycolipids tested only those with mannose at the non-reducing termini rendered a statistically significant effect when applied to liposomes (Table 1). Further experiments, however, are necessary to clarify the sugar specificity. It is possible that the receptor-mannose interaction of oligomannose- or mannan-coated liposomes augmented the processing of OVA reconstituted in these liposomes. In addition, the mannose residues may possess some other activity such as the stimulation of IL-12 release culminating in the activation of T-lymphocytes.

From practical standpoint of view, mannan is considered not to be suitable for adjuvant from the following reasons. First, mannan is known to be highly immunogenic: it can elicit antibody and B-cell mitosis [12]. Second, mannan is toxic to mice when it is intravenously injected [13]. One main difference in chemical structure between mannan and Man5 is that mannan possesses different glycosidic linkages, far more branches, higher molecular weight, and, as a consequence, much larger clusters of non-reducing termini of mannose than Man5 does. In contrast, the structure of either Man5 or DPPE exists as part of natural components of the body and thus Man5-DPPE is thought to be neither immunogenic nor toxic. Therefore, Man5 is considered to be superior to mannan in safety. The present experimental system of liposomes coated with various oligosaccharides has provided a tool for elucidation of the role of carbohydrate in the immune system and has paved the way for the design of a safe adjuvant with which to induce effective CMI.

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