Trop. Med., 38 (3/4), 91－99, December, 1996

Immunopathological Characterization of Iiposome Adjuvant Coated with Mannan

Kohdo SHIKATA[1,2], Kazue OHISHI[1], Hiromi KAWAI[1], Masashi FUKASAWA[1], Ryuzo SAKAKIBARA[2], Masatsune ISHIGURO[2], Hideyo ITAKURA[3], Masakazu HATANAKA[1], and Masanobu SUGIMOTO[1,4]

[1]*Department of Preventive Medicine, Institute of Tropical Medicine, Nagasaki University, 1－12－4 Sakamoto Nagasaki 852*

[2]*Faculty of Pharmaceutical Science, Nagasaki University, Bunkyo-machi 1－14, Nagasaki 852*

[3]*Department of Pathology, Institute of Tropical Medicine, Nagasaki Uniersity*

[4]*Agene Research Institute, 200 Kajiwara Kamakura Kanagawa 147, Japan.*

Abstract: The adjuvant activity of liposomes coated with mannan-cholesterol was studied in mice. Ovalbumin (OVA) was reconstituted into liposomes as a model antigen. The adjuvant activity was assessed by the following two immunological responses: delayed-type hypersensitivity (DTH) footpad swelling responses and *in vitro* release of interferon-γ and interleukin-4 by regional lymph node cells. First, we studied dose effects on DTH responses of total lipid, mannan-cholesterol and OVA used for liposomes. The minimal doses per mouse required for the induction of optimal responses were as follows; 1μg of OVA, 10μg of mannan-cholesterol and 336μg of total lipid. Second, immunological and histopathological studies showed the following two points: 1) mannan-coated liposomes induced a tuberculin-type DTH response while non-coated liposomes elicited a Jones-Mote reaction, and 2) mannan-coated liposomes induced obvious microabscesses but non-coated liposome did not. Third, the inoculation of mannan-coated liposomes rendered the regional lymph node cells to release a large amount of interferon-γ with little IL-4 against OVA while non-coated liposome released neither of the lymphokines. These results indicated that mannan-coated liposomes are a potent adjuvant to induce type 1 helper T cells but have a disadvantage to form microabscesses at the inoculation sites.

Key words: liposome, adjuvant, cell－mediated immunity, mannan

INTRODUCTION

Adjuvant is essential for the development of vaccines against various infectious diseases including those endemic in tropical area such as acquired immunodeficiency syndrome (AIDS) and malaria. Liposomes are considered to satisfy various criteria necessary for successful

Received for publication, December 17,1996.

Correspondent author, Dr. Masanobu Sugimoto, Agene Research Institute, 200 Kajiwara Kamakura, Kanagawa 147, Japann. Contribution No. 3337 from the Institute of Tropical Medicine, Nagasaki University.

adjuvants such as being biodegradable, non-toxic and non-immunogenic (Gregoriadis, 1995). They also have the advantage of being able to induce cell-mediated immunity: Noguchi *et al.* (1991) reported that liposomes coated with yeast mannan induced a cytotoxic T-cell (CTL) response against a virus-related protein reconstituted in them. In the present experiment, we investigated the characteristics of liposome adjuvant coated with mannan in detail, and evaluated their potential as adjuvant. Liposomes reconstituted with ovalbumin (OVA) as a model antigen were used, and their immunogenicity in mice to induce a DTH footpad swelling response and *in vitro* release of lymphokines by regional lymph node cells was assessed in mice. Part of preliminary results of the present investigation is reported previously (Sugimoto *et al.*, 1995).

Materials and Methods

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

Reagents: Dipalmitoylphosphatidylcholine (DPPC) and cholesterol were purchased from Nichiyu Liposome Co. Ltd. (Tokyo). OVA and mannan-cholesterol (Cholesterol-AECM Mannan) were obtained from Wako Purechem. Inc. Ltd. (Osaka) and Dojin Laboratories (Kumamoto), respectively.

Preparation of liposomes: Liposomes (multilamellar vesicles) reconstituted with OVA were prepared according to the modified method by Bangham *et al.* (1965): 500μl of 10mM DPPC in chloroform-methanol (2:1) solution and 250μl of 10mM cholesterol in the same solvent were mixed in a conical flask, and then the organic solvent was removed by evaporation. 200μl of 10mg/ml OVA solution was added to the dried lipid film, and then multilamellar vesicles were prepared by vortex dispersion. The amount of OVA in the liposomes was determined by BCA protein assay reagent (Pierce, Rockford IL) after dissolution in 2% sodium dodecylsulfate solution at a boiling temperature. Liposomes were coated with mannan-cholesterol by incubating liposomes overnight with mannan-cholesterol solution at 4℃. More than 95% of mannan-cholesterol was adsorbed to liposomes. The amounts of cholesterol and carbohydrate were determined by the assay kit of Cholesterol CII Test (Wako Purechem. Inc., Ltd.), and anthrone-sulfate method, respectively. The total lipid of liposomes was calculated from the amount of cholesterol (the ratio of DPPC and cholesterol was 2:1 in mole ratio).

Immunological assays: A DTH response was assessed by footpad swelling response as follows (Sugimoto *et al.*, 1995): Mice were immunized subcutaneously at two sites on the dorsal skin on day 0 with liposomes suspended in 200μl of phosphate buffered saline (PBS). Unless otherwise mentioned, mice were immunized with liposomes with the following doses of each component per mouse; 5-10μg of OVA, 50-100μg of mannan-cholesterol and about 350μg of total lipid. At various days after immunization, they were challenged subcutaneously in the right hind footpad with 10μg OVA with 25μg alum adjuvant in 25μl PBS and in the left footpad with the same solution without OVA as a control. The specific footpad swelling responses were expressed as the difference between the thickness of right and left footpad. Footpad thickness was measured by a Peacock dial thickness gauge (Osaki MGF Inc., Ltd., Tokyo). Unless otherwise mentioned, the footpad

swelling response was assayed 24 hours after the challenge in the footpad. Usually 5 animals were used in each gorup.

IFN-γ and IL-4 assays: The release of IFN-γ and IL-4 in vitro was assayed as follows: On day 0, two groups of mice were immunized subcutaneously in both footpads with mannan-coated or non-coated liposome, and on day 7 they received an additional immunization with the same antigen. On day 11, popliteal lymph nodes of right and left hind legs were removed. Cell suspensions of lymph node cells were made, and cultured with a 96-well plate (5×10^5 cells/100μl/well) in RPMI1640 medium containing 10% fetal calf serum with or without OVA at 37℃ for 3 days. The amount of IFN-γ or IL-4 released in the culture medium was determined using a kit for mouse IFN−γ (Genzyme, Cambridge, MA), or a kit for mouse IL-4 (Endogen Inc., Boston, MA), respectively.

Histopathological observation: Specimens of the skin of footpad challenged with OVA and back skin inoculated with various liposomes were fixed in 10% formalin and their paraffin sections were prepared by conventional methods. They were stained with haematoxylin and eosin, and were observed under a light microscope.

RESULTS

The time courses of DTH responses: Previously we reported that mannan-coated liposomes (+OVA) induced a strong footpad swelling response in mice with a peak at 24 to 48 hours after challenge with OVA, whereas non-coated liposomes (+OVA) induced a weak response at 12 hours after challenge (3, see also Fig. 1). Fig. 2 shows the time courses of 12-hour and 24-hour responses induced by non-coated liposomes as well as mannan-coated liposomes. The 24-hour response with mannancoated liposomes continued to increase toward 18 days after immunization whereas the 12-hour response with non-coated liposomes had a peak 7 days after immunization and tended to decrease thereafter. These results together support the idea that the 24-hour response with mannan-coated liposomes is a tuberculin-type DTH reaction while the 12-hour response with non-coated liposome is a Jones-Mote reaction (Klein, 1982).

Dose effects of mannan-cholesterol, OVA and total lipid: When the dose of mannan-cholesterol was changed while the doses of OVA and lipid being fixed at 3.9 and 336μg/mouse respectively, a maximal response was attained at 10μg/mouse of mannan-cholesterol or above (Fig. 3). For the dose effect of OVA with fixed doses of total lipid and mannan-cholesterol, nearly plateau levels of responses were attained at 0.9μg/mouse or above. Under the conditions by fixing the dose of OVA at 10μg/mouse, the increase of total lipid content of liposomes from 29 to 326μg/mouse led to dose-dependent augmentation of the footpad response. A near plateau level was attained at total lipid contents above 326μg/mouse (data not shown). It is noted that the effect of coating with mannan-cholesterol was much more obvious at the higher lipid contents.

Histological observation: The histological profile of the footpad swelling responses was investigated, and summarized in Table 1. The skin lesions of 24- and 48-hour responses by mannan-coated liposomes manifested infiltration by numerous mononuclear cells and fewer

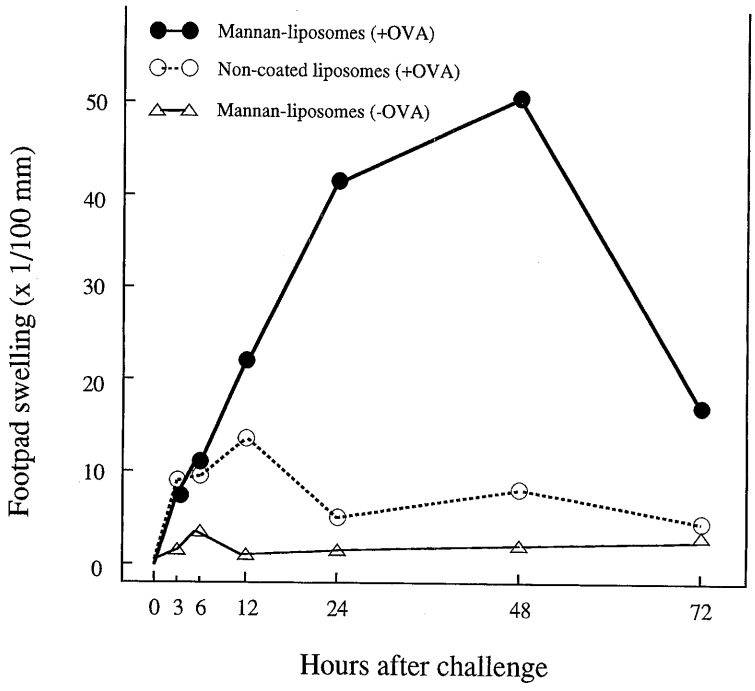

Fig. 1. Footpad swelling responses of mice immunized with mannan-coated liposomes with or without OVA and non-coated liposomes with OVA. Mice were challenged at footpad with OVA 7 days after immunization, and the footpad swellingg responses were assessed after challenge. This figure was drawn based on the reference of 3.

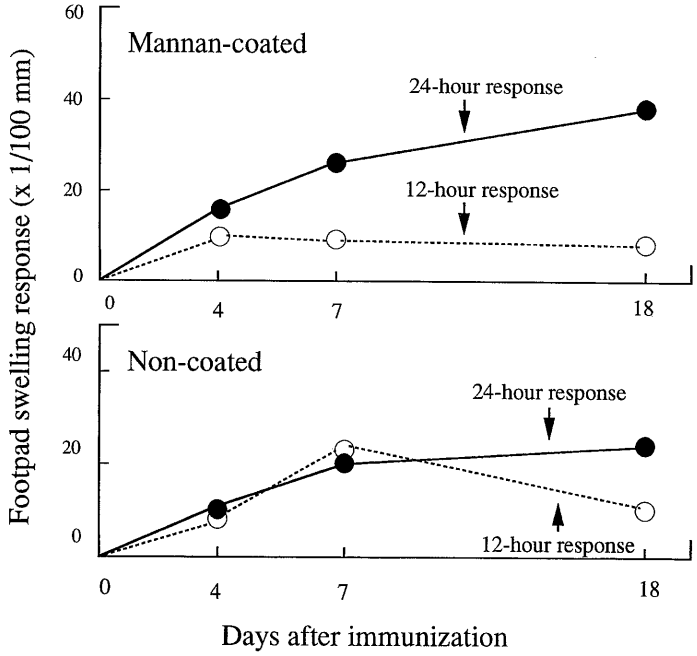

Fig. 2. Footpad swelling responses after immunization. Mice were immunized with mannan-coated or non-coated liposomes with OVA, and footpad responses were assessed various days after immunization. See also the text.

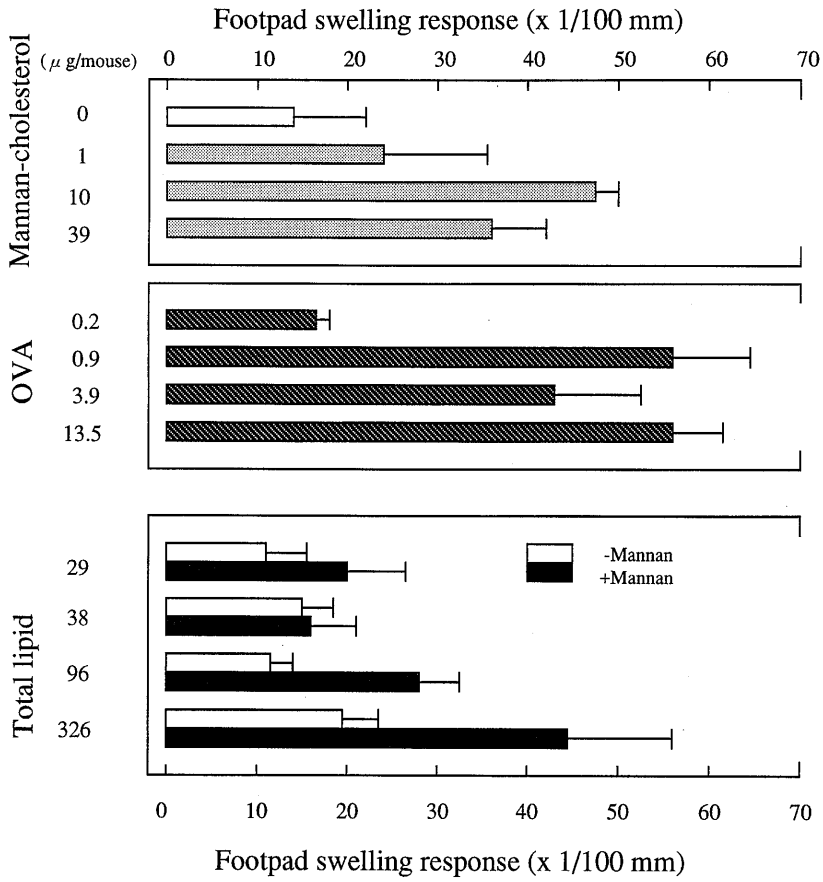

Fig. 3. The dose effects of mannan-cholesterol, OVA and total lipid of liposomes on footpad swelling responses. Mice were immunized with various types of liposomes and footpad swelling responses were assessed 7 days after immunization. The doses of mannan-cholesterol, OVA or total lipid in liposomes per mouse is indicated at the left of each graph.

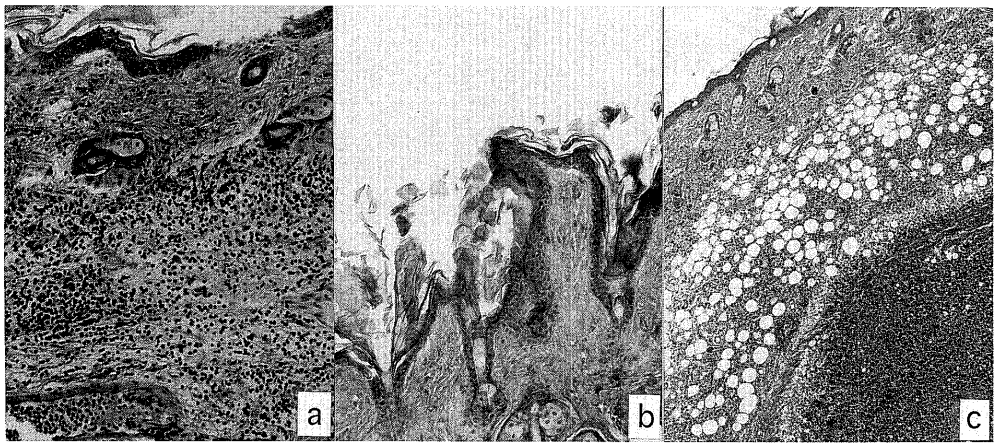

Fig. 4. Histopathological studies of the footpad skin and back skin. Mice were immunized with mannan-coated liposomes with OVA, and 7 days after immunization right footpad was challenged with OVA in alum adjuvant (a), while the left footpad was challenged with alum adjuvant alone (b). Infiltration with mononuclear cells is obvious in the dermis and loose connective tissue of the right footpad, but they are absent in the left footpad. The back skin inoculated with mannan-coated liposomes with OVA 9 days previously formed a microabscess (c). Part of the microabscess is seen in the right/bottom corner with nuclear debris mixed with mononuclear cells and polymorphonuclear cells. There are many mononuclear cells and fibroblast cells surrounding the microabscess. Stained with haematoxylin and eosine. A and B, x40; C, x16.

polymorphonuclear cells (Fig. 4a). The 12-hour 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 (data not shown). These results support the conclusion as mentioned above that the 24- and 48-hours responses with mannan-coated liposomes are a tuberculin-type DTH response whereas the 12-hour response with non-coated liposomes is a Jones-Mote reaction. The lesion of the back skin inoculated with liposomes was also investigated. Sometimes obvious microabscesses were formed at the sites inoculated with mannan-coated liposomes. There were numerous aggregates of nuclear debris inside the microabscess (Fig. 4c). Most of these materials seemed to be derived from leukocytes since many mononuclear cells and polymorphonuclear cells were seen in it. There were many mononuclear cells as well as fibroblast-like cells around the tissue of the microabscess. The size of microabscesses varied depending on individual animals of the same group, and even between two inoculation sites on the back skin of the same animal. Non-coated liposomes rarely formed obvious microabscesses.

In vitro *lymphokine release responses: In vitro* release of both IFN-γ and IL-4 by OVA stimulation was assessed for the regional lymph node cells obtained from mice immunized with mannan-coated liposomes (+OVA) as well non-coated liposomes (+OVA). The lymph node cells of mice immunized with mannan-coated liposomes released a large amount of IFN-γ with negligible amounts of IL-4, whereas non-coated liposomes induced neither of the lymphokine (Table 2).

Table 1. Histological observation of footpad swelling responses.

Infiltrating cells	Challenge with OVA	Liposomes for immunization								
		Mannan-coated (-OVA)			Mannan-coated (+OVA)			Non-coated (+OVA)		
		12*	24	48	12	24	48	12	24	48
Mononuclear cells	+	-	-	-	+	+++	+++	+	+	+
	-	ND	ND	ND	ND	ND	-	-	-	ND
Polymorpho-nuclear cells	+	-	-	-	+	+	+	++	+	+
	-	ND	ND	ND	ND	ND	-	ND	ND	ND

Mice were immunized as shown in the legend of Fig. 1. The footpads were fixed with formalin solution, embedded in paraffin, and thin sections were stained with haematoxilin and eosin.
ND, not done; -, negative; +, weak; ++, moderate; +++, strong.
*Hours after OVA challenge in the footpad.

Table 2. The release of IFN-γ and IL-4 of regional lymph node cells from mice immunized with mannan-coated and non-coated liposomes reconstituted with OVA.

Liposomes	IFN-γ (ng/ml)			IL-4 (ng/ml)	
	OVA (μg/ml)			OVA (μg/ml)	
	0	2	10	0	2
Mannan-coated	0.7	2.5	7.9	<0.01	<0.01
Non-coated	<0.1	<0.1	<0.1	<0.01	<0.01

Mice were immunized twice subcutaneously into the footpad with mannan-coated or non-coated liposomes reconsitituted with OVA. Thereafter, regional lymph node cells were obtained and cultured in vitro with or without OVA, and the amount of IFN-γ or IL-4 in the medium was determined. See in detail for the text.

Discussion

The present results indicated that mannan-coated liposomes reconstituted with OVA induced a strong tuberculin-type DTH responses accompanied by release of IFN-γ by regional lymph node cells against OVA stimulation. From these results together with the fact that mannan-coated liposomes induced CTL responses against the antigens reconstituted into them (Noguchi *et al.*, 1991; Ohishi *et al.*, 1996), it is concluded that mannan-coated liposomes are potent stimulator for type 1 helper T-cells. Recently, a large body of evidence is accumulating that cell-mediated immunity plays an pivotal role in the prevention from infectious diseases by retroviruses such as human immunodeficiency virus (HIV) (Salk *et al.*, 1993) and bovine leukemia virus (BLV) (Ohishi *et al.*, 1991; Sugimoto *et al.*, 1993). These facts raise the possibility that

mannan-coated liposomes are usable as adjuvant against these infectious diseases. In this connection, it is also worthhwhile to note that liposomes coated with oligosaccharides induce natural killer cells (Bezouska *et al.*, 1994). Thus, mannan-coated liposomes may also stimulate innate immunity together with the augmentation of immunity specific to the antigen reconstituted in it.

The dose effects of the components of mannan-coated liposomes indicated minimal doses for the induction of optimal responses. Practically, these data are important, since as small doses as possible are required from an economical standpoint as well as to reduce side effects such as the formation of microabscesses, as discussed next.

One potential problem may be the formation of microabscesses (Fig. 4c). This process seems to be composed of the infiltration of leukocytes followed by formation of a small granuloma. To deal with this problem, the following things are considered. 1) Since non-coated liposomes did not form obvious microabscesses, the reduction of the amount of mannan-cholesterol to the levels as small as possible may be effective. 2) From the fact that mode of microabscess formation varied depending on individual animals as well as on inoculation sites, it may be that choice of the inoculation sites or of the depth of subcutaneous injection reduces the side effect. 3) The use of oligomannoses instead of mannan may also be effective (Sugimoto *et al.*, 1995).

As for the effectiveness of the adjuvant activity of mannan-coated liposomes, their assessment in animal infectious disease models is essential.

ACKNOWLEDGMENTS

The authors thank professor Tatsuji Yasuda of Okayama University Medical School for his valuable suggestion for the preparation of liposomes. We also thank Miss Masako Sasaki of Institute of Tropical Medicine, Nagasaki University for her technical assistance.

REFERENCES

1) Bangham, A. D., Standish, M. M., and Watakins, J. C. (1965): Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol., 13, 238–252.
2) Bezouska, K., Yuen, C.-T., O'Brien, J., Childs, R. A., Chai, W., Lawson, A. M., Drbal, K., Fiserova, A., Posisil, M. and Feizi, T. (1994): Oligosaccharide ligands for NKR–P1 protein activate NK cells and cytotoxicity. Nature 372, 150–157.
3) Gregoriadis, G. (1995): Liposomes as immunological adjuvants. In "*The Theory and Practical application of Adjuvants.*" (Ed. by D. E. S. Stewart-Tull) pp 145–169. John Wiley & Sons Ltd.
4) Klein, J. (1982): In "Immunology, The science of Self-nonself Discrimination." pp 467. Johns Wiley & Sons, New York, Chichester, Brisbane, Toronto, Singapore
5) Noguchi, Y., Noguchi, T., Sato, T., Yokoo, Y., Itoh, S., Yoshida, M., Yoshiki, T., Akiyoshi, K., Sunamoto, J., Nakayama, E. and Shiku, H. (1991): Priming for in vitro ;and in vivo anti-human T lymphotropic virus type 1 cellular immunity by virus-related protein reconstituted into liposomes. J. Immunol., 146, 3599–3603.
6) Ohishi, K., Suzuki, H., Yamamoto, T., Maruyama, T., Miki, K., Ikawa, Y., Numakunai, S., Okada, K., Ohshima, K. and Sugimoto, M. (1991): Protective immunity against bovine leukaemia virus (BLV) induced

in carrier sheep by inoculation with a vaccinia virus-BLV env recombinant: association with cell-mediated immunity. J. Gen. Virol., 72, 1887–1892.

7) Ohishi, K., Kabeya, H., Amanuma, H. and Onuma, M. (1996): Induction of bovine leukaemia virus Env-specific Th-1 type immunity in mice by vaccination with short synthesized peptide-liposome. Vaccine 14, 1143–1148.

8) Salk, J., Bretcher, P. A., Salk, P. L., Clerici, M. and Shearer, G. M. (1993): A strategy for prophylactic vaccination against HIV. Science 260, 1270–1272.

9) Sugimoto, M, Ohishi, K. and Ikawa, Y. (1993): Cell-mediated immunity to BLV replication; analogous to HIV immunopathogenesis? Immunol. Today 14, 190–191.

10) Sugimoto, M., Ohishi, K., Fukasawa, M., Shikata, K., Kawai, H., Itakura, H., Hatanaka, M., Sakakibara, R., Ishiguro, M., Nakata, M. and Mizuochi, T. (1995): Oligomannose-coated liposomes as an adjuvant for the induction of cell-mediated immunity. FEBS Letters 363, 53–56.