LIPOSOMES AS AN IMMUNOADJUVANT SYSTEM FOR STIMULATION OF MUCOSAL AND SYSTEMIC ANTIBODY RESPONSES AGAINST INACTIVATED MEASLES VIRUS ADMINISTERED INTRANASALLY TO MICE

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Vaccine, in press
Chapter 5

ABSTRACT

This paper reports on the immune-stimulatory activity of liposomes in an inactivated whole measles virus vaccine preparation administered intranasally to mice. Liposomes, simply mixed with inactivated whole measles virus, significantly stimulated the serum IgG response relative to the response to the virus alone. In addition, the liposomal vaccine, but not the free virus, induced a secretory IgA (s-IgA) response in the lungs and nasal cavity. Serum IgG and s-IgA responses persisted up to at least 24 weeks post-immunization. The liposomes induced a moderate increase in the serum IgG response, but no s-IgA response, following intramuscular immunization. It is concluded that liposomes provide a promising adjuvant system for induction of high systemic as well as mucosal antibody responses against inactivated measles virus in an intranasal or inhalation vaccine formulation.

INTRODUCTION

Current measles vaccines used in children generally consist of a live attenuated virus formulation, administered parenterally. These replication-competent vaccines have been shown to be very effective and to confer adequate long-term protection from infection (106, 110, 111). Both humoral and cellular immunity, induced by live virus, appear to be capable of inducing protective immunity against measles infection (106, 110, 111). Although direct evidence is lacking, it is likely that mucosal antibodies, secretory IgA (s-IgA) in particular, provide a first line of defense against invading airborne virus, while a serum IgG response is required for prevention of a systemic infection.

Even though the live attenuated measles virus vaccine is highly efficacious, there is a clear need for development of an inactivated vaccine. First, live vaccines are relatively unstable, and therefore require special conditions for storage and transportation ("cold chain" facilities). This may pose serious problems, particularly in developing countries. Second, the replication of the virus, which is a requirement for the vaccine to be effective, is sensitive to the presence of maternal antibodies. This essentially precludes the use of such vaccines in children below the age of 1 year, while in developing countries measles still causes many deaths among children in this age group (106, 110, 111). Inactivated whole virus or subunit vaccines are effective in the presence of maternal antibodies. Therefore, these preparations have distinct advantages over live vaccines. However, they are usually also considerably less immunogenic and require the use of an adjuvant (106, 110, 111).
Liposomes have been used extensively as stimulators of immune responses in experimental vaccine formulations, and provide a promising, safe and versatile immunoadjuvant system for use in future human vaccines (1, 7, 23, 33, 34, 38, 52, 146, 158). The use of liposomes for enhancement of the immunogenicity of isolated measles virus membrane antigens has been reported by Mougin et al. (98). These investigators observed a stimulated and persisting antibody response in rats following subcutaneous injection of syngeneic peritoneal exudate cells, previously incubated *ex vivo* with measles virus membrane antigens incorporated in liposomes. In addition, Garnier et al. (45) showed that the measles virus hemagglutinin (H) glycoprotein incorporated in liposomes is more potent in priming of T cell responses than the free H glycoprotein. These results demonstrate that association of measles virus antigen to liposomes increases its immunogenicity, as a result of presentation of the antigen in a proper membrane-bound form and/or as a result of targeting of the liposome-associated antigen to antigen-presenting cells, such as macrophages (7, 29, 30, 46, 52, 146).

Recently, using influenza virus subunit antigen, we have demonstrated that liposomes have an immunoadjuvant activity independent of physical association of the antigen with the liposomes (33, 158). This adjuvant activity becomes apparent only upon local administration of the liposomes to the respiratory tract, and mediates not only an enhancement of the serum IgG response but also induction of a mucosal s-IgA response; (33, 158) this mucosal s-IgA response disseminates throughout the common mucosal immune system (34).

In this study we investigated the potential use of coadministered liposomes as a mucosal adjuvant system for induction of high systemic as well as local antibody responses against whole inactivated measles virus. Although, as indicated above, several studies have reported on the use of liposomes in experimental measles virus vaccines before (45, 98), the effect of liposomes on mucosal immune responses has never been explored. We show that liposomes, coadministered intranasally (i.n.) with whole inactivated measles virus to mice, not only efficiently stimulate the serum IgG response but also induce s-IgA antibodies in the respiratory tract.

**MATERIALS AND METHODS**

**Virus and liposomes**

Whole inactivated measles virus (Edmonston B strain) was a gift from Dr. A.D. Plantinga, Laboratory for Live Virus Vaccines, National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The virus, propagated on VERO cells, had been
concentrated and purified essentially according to the procedure described before by Van Wezel et al. (149, 150) for rabies and polio virus. Briefly, in this procedure virus is concentrated from the cell culture supernatant in a hollow-fiber system, purified to > 90% purity by gel filtration, inactivated by treatment with β-propiolactone and finally lyophilized from a lactose-containing dispersion. Lyophilized virus was stored at -20°C. Before use, the preparation was reconstituted in phosphate-buffered saline (PBS), and simply mixed with liposomes. The liposomes (extruded oligolamellar vesicles) consisted of egg-phosphatidylcholine (PC), dicetyl phosphate (DCP) and cholesterol (Chol) in a molar ratio of 4:1:5. The source of the lipids and the liposome preparation protocol have been described in detail elsewhere (33).

**Immunization and sample collection**

Female Balb/c mice (8-10 weeks old) were used throughout this study. Experimental groups consisted of 5 animals each. Mice were immunized, under light ether anesthesia, by i.n. instillation of 50 μl of the (liposomal) antigen resulting in deposition of the inoculum throughout the respiratory tract (163). To achieve deposition of the antigen in the upper respiratory tract only, the antigen was given i.n. to unanesthetized mice in a volume of 25 μl. Oral administration was performed by deposition of 50 μl of the antigen dispersion on the back of the tongue of unanesthetized animals. Intramuscular (i.m.) administration was done by injection of 50 μl of the preparation into the left hind thigh muscle. In all cases the dose of antigen corresponded to 5 μg of total viral protein per mouse. Immunizations were given either in a single-dose or a double-dose regimen. In the double-dose regimen, aimed at achieving a prolonged exposure of the mice to the antigen, the immunization was repeated 4 days after the first immunization.

Four weeks post-immunization (unless stated otherwise), the mice were bled by severing the carotic artery under ether anesthesia, and serum samples were prepared. Also, nasal and lung lavages were taken, pooled separately for each group of mice and concentrated fivefold as described previously (33). Serum samples and concentrated washes were stored at -20°C.

**Antibody assays**

Serum IgG and s-IgA in lung and nasal washes were determined by ELISA. All applied volumes in the ELISA were 100 μl unless stated otherwise. Polyvinyl ELISA-plates were coated for 2 h at 37°C with whole inactivated measles virus (see above) in 0.05 M sodium carbonate buffer, pH 9.6, at 0.1 μg of viral protein per well. After blocking with BSA/PBS/Tween [0.5% bovine serum albumin (BSA) in PBS containing 0.05% Tween-20 (PBS-/Tween)] for 1 h at 37°C, the plates were washed three times with PBS/Tween. Serum samples and washes were transferred
to the plates in twofold serial dilutions in BSA/PBS/Tween and incubated for 1 h at room temperature. The plates were washed and peroxidase-conjugated goat antibody against mouse IgG or IgA in BSA/PBS/Tween was added to the wells and the amount of bound antibody determined by a colour reaction, essentially as described previously (33). Antibody titres are expressed as the reciprocal serum, nasal wash or lung wash dilution with an \( A_{492} \) value > 0.2 after subtraction of the background value of a non-immune serum, nasal wash or lung wash at a matching dilution. Serum IgG antibody levels are expressed as geometric mean titres (GMT) \( \pm \) s.e.m. The concentration of s-IgA is given as the titre in pooled and concentrated washes (see above), corrected for the fivefold concentration (i.e. the starting titre of the undiluted concentrated lavage fluid is 0.2). Therefore, no GMT could be calculated in these cases and titres are given without s.e.m. Differences in serum antibody titres between groups were analysed by Student's t test in which a p-value <0.05 was considered to represent a statistically significant difference.

RESULTS

Mice were immunized i.n., under light ether anesthesia, with whole inactivated measles virus alone or mixed with liposomes. The antigen was also given via the i.m. route, again either alone or mixed with liposomes. In all cases, single-dose as well as double-dose immunizations were performed. Four weeks later the mice were bled and serum samples were screened for the presence of antigen-specific IgG by ELISA.

Figure 1 shows that i.n. immunization with the liposome-supplemented measles virus, in both the single- and the double-dose regimen, resulted in a significant stimulation of the serum IgG response relative to the response to i.n. immunization with the virus alone (bars B vs. A; \( p<0.0001 \) for both single- and double-dose administration).
Figure 1  Serum IgG responses of mice immunized with inactivated measles virus alone or supplemented with liposomes. Groups of 5 mice were immunized i.n. (A,B) or i.m. (C,D) with measles virus alone (A,C) or supplemented with liposomes (B,D), according to the single-dose (open bars) or double-dose (hatched bars) regimen. Each vaccine dose contained 5 µg of viral protein. The dose of supplemented liposomes was 1 mg of lipid. Blood samples were taken 4 weeks post-immunization and assayed for serum IgG by ELISA. Bars represent \( \log_{10} \text{GMT} \pm \text{s.e.m.} \).

In the case of i.m. immunization, liposomes caused an only moderate enhancement of the serum IgG response (bars D vs. C; \( p<0.05 \) and \( p<0.0005 \) for single- and double-dose administration, resp.). Importantly, levels of serum IgG induced by the liposomal virus formulation administered i.n. were superior to the levels obtained with the i.m. injected vaccine, given alone (bars B vs. C; \( p<0.01 \) for both single- and double-dose administration) or supplemented with liposomes (bars B vs. D; \( p<0.0005 \) and \( p<0.05 \) for single- and double-dose administration, resp.).
Figure 2  s-IgA responses in the respiratory tract of mice immunized with inactivated measles virus alone or supplemented with liposomes. Groups of 5 mice were immunized i.n. (A,B) or i.m. (C,D) with measles virus alone (A,C) or supplemented with liposomes (B,D), according to the double-dose regimen, as in the experiment of Figure 1. Four weeks post-immunization, nasal and lung washes were taken, pooled and concentrated. s-IgA titres were determined by ELISA. Bars represent the log₁₀ of the s-IgA titres x 10, the titres being corrected for the fivefold concentration of the pooled washes. Nasal washes, open bars; lung washes, hatched bars.

Figure 2 shows that the i.n. immunization with liposome-supplemented virus induced a distinct s-IgA response both in the lungs and in the nasal cavity (bars D). Neither i.n. immunization with the virus alone (bars C) nor i.m. immunization with or without liposomes (bars B and A) resulted in any detectable s-IgA response. The s-IgA response induced by the liposome-supplemented virus was dependent on the liposome dose; optimal antibody levels were elicited with 1-2 mg of liposomal lipid per inoculation (Figure 3).

Sera from mice immunized i.n. with an irrelevant antigen (influenza virus hemagglutinin), supplemented with liposomes, gave negative readings in the measles-virus-specific ELISA used in this study, indicating that non-specific antibodies possibly induced by liposomes did not contribute to the measles-specific antibody levels observed (results not shown).
Figure 3  
s-IgA responses in the respiratory tract of mice immunized i.n. with inactivated measles virus supplemented with different doses of liposomes. Groups of 5 mice were immunized i.n., according to the double-dose regimen. Each vaccine dose contained inactivated measles virus (5 µg of protein), supplemented with liposomes as indicated. Four weeks post-immunization, nasal and lung washes were taken, pooled and concentrated. s-IgA titres were determined by ELISA and are presented as in Figure 2. Nasal washes, open bars; lung washes, hatched bars.

Consistent with our earlier findings, (33, 34) the liposomal antigen, in order to induce an optimal antibody response, had to be deposited in the lower respiratory tract (as is achieved by i.n. immunization of mice under anesthesia (163). Administration of the same dose of antigen and liposomes to the upper respiratory tract (i.n. immunization of unanesthetized mice) or oral administration did neither elicit a detectable serum IgG response ($\log_{10}$ GMT $<$1) nor an s-IgA response in the respiratory tract ($\log_{10}$ s-IgA titers $<$0.3, for both nasal and lung washes). Since oral immunization was ineffective, it can be concluded that, even though upon i.n. administration of the preparation to anesthetized mice a considerable fraction of the antigen will end up in the gastrointestinal tract, the observed antibody response is not mediated by priming of lymphoid tissue associated with the gut.

In the above experiments an interval of 4 weeks was chosen between immunization and sampling of sera and nasal and lung lavages. To determine the long-term stimulatory effect of
liposomes on serum IgG and s-IgA responses, antigen-specific antibody levels were determined not only four weeks but also 24 weeks after i.n. immunization of mice with liposome-supplemented inactivated measles virus. Figure 4 shows that stimulated serum IgG and s-IgA antibody levels persisted for up to at least 24 weeks post-immunization.

**Figure 4**  Serum IgG and pulmonary s-IgA levels 4 and 24 weeks after i.n. immunization of mice with inactivated measles virus supplemented with liposomes. Groups of 5 mice were immunized i.n., according to the double-dose regimen, with inactivated measles virus (5 µg of protein) supplemented with liposomes (1 mg of lipid). Four and 24 weeks post-immunization, groups of animals were sacrificed. Serum IgG (log$_{10}$ GMT ± s.e.m.; open bars) and s-IgA (log$_{10}$ of (titer x10) in nasal washes (dotted bars) and lung washes (hatched bars) were determined as in the experiments of Figure 1 and 2.
Chapter 5

DISCUSSION

In this study liposomes were used as a mucosal immunoadjuvant system in an experimental inactivated measles virus vaccine administered i.n. to mice. The results demonstrate that liposomes, when coadministered with the virus, have the capacity to not only stimulate the serum IgG response, relative to the response to virus alone, but also induce a significant local s-IgA response in the respiratory tract. At the same time, i.m. administration of the inactivated whole virus preparation, irrespective of the presence of liposomes, failed to induce detectable mucosal s-IgA. Importantly, the serum IgG response upon i.n. immunization with the liposomal antigen compared favorably to the IgG response elicited by free or liposomal antigen administered i.m. Moreover, the serum IgG and s-IgA responses against the i.n. liposomal virus formulation persisted for up to at least 24 weeks post-immunization.

It is generally assumed that the immunopotentiating effect of liposomes primarily involves the uptake of liposome-associated antigen by cells of the mononuclear phagocytic system (MPS), including macrophages, due to natural targeting of liposomes to these cells. Increased uptake of antigen would then result in an improved presentation of antigen-derived peptides to T-cells, facilitating the humoral immune response (7, 29, 30, 52, 146). In addition, exposure of an antigen on a liposomal membrane may enhance its immunogenicity (46). It is likely that targeting of liposome-associated antigen to macrophages represents the main mechanism by which liposomes exert their adjuvant activity in vaccines administered parenterally, including the experimental liposomal measles virus vaccines described previously (45, 98).

Obviously, exposure to the immune system and/or targeting to antigen-processing and-presenting cells, requires the antigen to be physically associated to the liposomes, either through attachment to the surface or by encapsulation within the aqueous compartment of the liposomes (7, 29, 30, 52, 146). By contrast, our previous work on immunization of mice with a liposome-supplemented influenza subunit vaccine has demonstrated that liposomes have an additional, mucosal, immunoadjuvant activity, not related to their role as an antigen-carrier system (33, 34, 158). This adjuvant activity of liposomes became evident upon local administration of the preparation to the lower respiratory tract and required a relatively high dose of liposomes (33, 34, 158). In agreement with these results, in the present study the liposomes had to be deposited in the lungs in order to effectively exert their immune-stimulatory action, since administration to the upper respiratory tract or oral administration were ineffective. Also, a relatively high dose of liposomes (1-2 mg of lipid, similar to the dose scheme used in our previous studies (33, 34, 158)) was required for an optimal response.
Since liposomes are avidly taken up by cells of the MPS (7, 51, 52, 146), it is likely that alveolar macrophages are involved in the mucosal adjuvant activity of liposomes. It has been shown that alveolar macrophages constitutively suppress immune responses in the rodent lung (68, 70). Therefore, in agreement with Van Rooijen (147), we hypothesize that uptake of liposomes by alveolar macrophages affects their immunosuppressive function temporarily, thereby facilitating the immune response against coadministered antigen. In addition, preliminary data from our laboratory indicate that, following i.n. administration, liposomes induce an influx of immune cells into the alveolar space. These cells could further stimulate the immune responses by uptake and presentation of the viral antigen and secretion of cytokines.

The liposomal system presented here provides a promising and versatile adjuvant for potential use in an inactivated measles virus vaccine, inducing high systemic as well as mucosal antibody responses. In comparison with the currently used live virus vaccine, inactivated vaccines have a number of distinct advantages. First, inactivated vaccines are more stable. Second, in contrast to live attenuated virus preparations, inactivated vaccines can potentially be used in young children in the presence of maternal antibodies. These properties are of considerable importance for vaccination against measles, particularly in third-world countries (106, 110, 111). Yet, some concern remains associated with the use of inactivated measles virus vaccines, since atypical measles virus infection has been observed in children vaccinated with an inactivated measles virus vaccine, possibly as a result of a lack of induction of a balanced antibody response against the viral F protein (110, 111). Therefore, with any novel inactivated measles virus vaccine formulation it will be particularly important to establish that it induces a balanced antibody response and solid immunity. In this respect, it is promising that an experimental measles virus subunit vaccine, formulated in an iscom structure, has been shown to confer protective immunity against a measles infection in mice (37). A distinct feature of the liposomal formulation presented here relates to its capacity to induce a mucosal s-IgA response, which is likely to contribute significantly to protection by acting as a first line of defense (87, 154).

ACKNOWLEDGEMENTS

The authors thank Dr. André D. Plantinga for providing the inactivated measles virus and Dr. Petra de Vries for helpful discussions and critical reading of the manuscript.