Mucosal immunoadjuvant activity of liposomes

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INDUCTION OF A SECRETORY IgA RESPONSE IN THE MURINE FEMALE UROGENITAL TRACT BY IMMUNIZATION OF THE LUNGS WITH LIPOSOME-SUPPLEMENTED VIRAL SUBUNIT ANTIGEN

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ABSTRACT

This study demonstrates that liposomes administered to the lower respiratory tract of mice have the capacity to stimulate secretory IgA (s-IgA) antibody production in the female urogenital system. Total respiratory tract immunization of mice with influenza virus subunit antigen simply mixed with negatively charged liposomes induced antigen-specific s-IgA in vaginal secretions, in addition to systemic IgG and s-IgA in the respiratory tract. Immunization of the upper respiratory tract alone or oral immunization were ineffective. These observations demonstrate that, upon stimulation with liposomes, the lymphoid tissue associated with the lung can act as an inductive site for migration of IgA-committed B cells to distant mucosal tissues, including the female urogenital tract. It is concluded that liposomes administered to the lower respiratory tract provide a promising adjuvant system for stimulation of both systemic and mucosal antibody responses against coadministered antigen, including production of s-IgA at distant mucosal sites.

INTRODUCTION

Mucosal surfaces are protected against infections by the mucosal immune system, of which secretory IgA (s-IgA) is the major humoral defense factor (19, 91). s-IgA is a polymeric immunoglobulin, which is actively transported to mucosal secretions (91). The s-IgA response is initiated in the Mucosal-Associated Lymphoid Tissue (MALT). It involves stimulation of IgA-committed B cells, migration of these cells to local lymph nodes, and systemic circulation and homing of IgA plasma cells to the mucosal sites where the antigen was first encountered (91). An interesting feature of the common mucosal immune system is that IgA-producing B cells also home to distant mucosal sites where, subsequently, IgA synthesis and secretion occur (19, 87, 91).

There is an increasing interest in the development of vaccines that will elicit a mucosal s-IgA response as a first line of defense, not only in the respiratory tract and the gut, but also in the mucosa of the female urogenital tract, where an s-IgA response may be beneficial in preventing sexually transmitted diseases. However, the induction of mucosal s-IgA responses in the female urogenital tract, following local administration, has proven to be difficult. Therefore, alternative vaccination strategies are being developed, involving immunization of other IgA-inductive compartments of the MALT, preferably in combination with an adjuvant, in order to induce an urogenital s-IgA response (59, 89, 161).
In this paper, we show that liposomes, administered to the lower respiratory tract, can serve as a versatile mucosal adjuvant system for stimulation of s-IgA responses in distant mucosal tissues, including the mucosa of the female urogenital system.

**MATERIALS AND METHODS**

**Antigen and liposomes**

Influenza virus subunit vaccine, containing the major virus surface antigen hemagglutinin (HA), was prepared from the influenza A virus NIB26 (H3N2), and was provided by Solvay Duphar B.V., Weesp, The Netherlands. Liposomes consisted of egg-yolk phosphatidylcholine (PC), cholesterol (Chol) and dicetylphosphate (DCP), in a molar ratio of 4:5:1, and were prepared in 10 mM Tris, 150 mM NaCl (pH 7.5), as described previously (33). Before use the liposomes were diluted in phosphate-buffered saline (PBS) and mixed with appropriate amounts of influenza subunit antigen in PBS.

**Immunization and sample collection**

Groups of 5 female Balb/c mice (8-10 weeks old) were immunized by i.n. instillation of 50 µl of the (liposomal) vaccine formulations under light ether or pentobarbital anesthesia (0.06 mg per g body weight, injected intraperitoneally). This immunization protocol results in deposition of the fluid inoculum in the total respiratory tract, including the lungs (163). Immunization of the upper respiratory tract only was achieved by instillation of 20 µl (at the same total dose of antigen and liposomes) in the nostrils of unanesthetized mice (163). Control groups were vaccinated intramuscularly (i.m.) or perorally. For intratracheal administration, mice were anesthetized with pentobarbital as above. Subsequently, a 1-cm incision was made in the throat parallel to the trachea. The trachea was exteriorized and 50 µl of the vaccine was injected towards the lungs using a 30-gauge needle, during which the ventral aspect of the trachea was clamped off to prevent back flow of the vaccine. The incision was closed with simple interrupted sutures.

Vaginal washes were taken by pipetting 50 µl of PBS into the vaginal tract using a Gilson pipet. The same volume was withdrawn and reintroduced 10 times. The washes were performed on 3 consecutive days and pooled for each mouse. Following the final vaginal wash protocol the mice were bled. Broncho-alveolar washes were performed as described before (33). Sera and washes were stored at -20°C.

**Antibody assays**
Influenza-specific serum IgG and s-IgA were determined by ELISA, as described before (33). Antibody titers represent the reciprocal serum dilution with an $A_{492}$ value $>0.2$, after subtraction of the background value of a nonimmune serum at a matching dilution. Since some vaginal washes contained mucus, these samples were not serially diluted. Therefore, the level of s-IgA within a group is given as the mean $A_{492}$ value of undiluted samples, pooled for each individual animal as described above. Serum antibody levels are expressed as geometric mean titers (GMT). Levels of lung s-IgA are given as mean $A_{492}$ values of undiluted samples. Differences in antibody levels between groups were analysed using Student's $t$ test, in which a $p$-value of $<0.05$ was considered to represent a statistically significant difference.

RESULTS

We have shown previously that, upon i.n. administration to mice, liposomes simply mixed with influenza subunit vaccine not only stimulate serum antibody responses, but also induce s-IgA antibodies in the lungs and in the nasal cavity (33, 158). Our present study demonstrates that i.n. immunization of mice with such a liposome-supplemented influenza subunit vaccine, but not i.n. immunization without liposomes or i.m. immunization, induces significant vaginal s-IgA antibodies (Table 1). In agreement with our earlier observations (33, 158), this response was paralleled by an s-IgA response in the respiratory mucosa and an enhanced serum IgG response (Table 1). Western blot analysis indicated that the observed vaginal s-IgA antibodies, as well as the lung s-IgA and serum IgG, were primarily directed against the viral HA (results not shown). Although levels of lung and vaginal s-IgA antibodies in Table 1 are both indicated as absorbance values of undiluted washes, these values can not be compared directly since the surface areas of the lung and vaginal tissues are different, while also, different volumes of PBS were used to sample the lung and vaginal secretions.
weeks after immunization with free or liposomal influenza subunit antigen

<table>
<thead>
<tr>
<th>Route</th>
<th>Liposomes</th>
<th>Serum IgG log_{10} titer</th>
<th>s-IgA (A_{492})^{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>&lt; 1</td>
<td>0.03±0.004</td>
</tr>
<tr>
<td>i.m</td>
<td>-</td>
<td>3.1±0.1</td>
<td>0.04±0.002</td>
</tr>
<tr>
<td>i.n.</td>
<td>-</td>
<td>&lt; 1</td>
<td>0.03±0.001</td>
</tr>
<tr>
<td>i.n. +</td>
<td>+</td>
<td>3.0±0.3</td>
<td>1.50±0.030</td>
</tr>
</tbody>
</table>

In both the i.m. and i.n. immunization the antigen dose was 5 µg of HA
Liposome dose was 1 mg of total lipid

GMT ± s.e.m.; titers in the group that received the liposomal antigen i.n. were significantly higher than the titers in the group that received the antigen i.n. without liposomes (p<0.01, Student’s t test)

Mean absorbance values ± s.e.m. of undiluted samples; levels of lung wash and vaginal wash s-IgA in the group that received the liposomal antigen i.n. were significantly higher than the levels in the control group or groups that received the antigen i.n. or i.m. without liposomes (p<0.0001 and p<0.05, respectively; Student’s t test)

The induction of a vaginal s-IgA response was not restricted to influenza subunit antigen. We also observed induction of antigen-specific s-IgA antibodies in the urogenital tract (and in the lungs) after i.n. administration of whole inactivated measles virus, supplemented with liposomes, to anesthetized mice (results not shown).

To determine the optimal route of immunization for induction of an s-IgA response in the vaginal mucosa, we immunized mice with liposome-supplemented influenza subunit antigen using different immunization protocols. I.n. immunization under anesthesia, resulting in deposition of antigen and liposomes throughout the respiratory tract including the lungs (163), induced significant levels of s-IgA in vaginal washes (Figure 1A, bars b and c). Consistent with our earlier results (33, 158), a significant s-IgA response was also observed in the respiratory tract of the animals, particularly in the lungs (Figure 1B, bars b and c). Intratracheal immunization with the liposomal antigen also induced s-IgA responses in the vagina and the lungs (Figure 1A and 1B, bars d), although the antibody levels were somewhat lower than in the groups immunized i.n. under anesthesia. This is probably due to the ineffective intra-tracheal immunization of one animal, which did not respond to the vaccine. Administration of antigen orally or to the upper respiratory tract only (i.n. immunization while awake) was found to be ineffective in inducing mucosal s-IgA responses.
(Figure 1A and 1B, bars a and e). Obviously, immunization with liposomes alone did not elicit an antigen-specific mucosal antibody response (Figure 1A and 1B, open bars), irrespective of the route of administration.

**DISCUSSION**

In this paper, it is demonstrated that antigen-specific s-IgA is secreted not only in the respiratory tract, but also in the female urogenital system, following i.n. immunization of mice with a liposome-supplemented viral antigen.

Liposomes have been shown to exhibit potent immunoadjuvant activity in a variety of experimental vaccine formulations (7, 52, 146). This adjuvant activity is generally thought to be due to the natural targeting of liposomes to cells belonging to the mononuclear phagocytic system, including macrophages, which would mediate an improved presentation of antigen-derived peptides to T helper cells. Obviously, this requires the antigen to be physically associated with the liposomes. Recently, we (33, 158) and others (1, 38) have shown that liposomes, upon i.n. administration to mice, stimulate both systemic IgG as well as s-IgA production in the respiratory tract. In our studies this immune stimulation occurred when liposomes and antigen were simply mixed or even when they were administered separately in time, indicating that liposomes have the capacity to act as nonspecific immune stimulators, independent of their putative function as an antigen-carrier system (7, 52, 146). Our present results confirm this conclusion. Immune stimulation did not require association of the antigen with the liposomes.
Figure 1  
Induction of s-IgA in vaginal and pulmonary secretions following i.n. immunization of mice with a liposome-containing influenza subunit vaccine. Groups of mice were immunized i.n. while awake (bars a), i.n. while under anesthesia using ether (bars b) or pentobarbital (bars c), intratracheally (bars d) or orally (bars e) with 5 µg of HA mixed with liposomes at 1 mg of total lipid per dose (hatched bars). Control groups received the same amount of liposomes without antigen according to the same immunization protocols (open bars). Washes were taken 4 weeks post-immunization and screened for antigen-specific s-IgA by ELISA. Bars represent mean absorbance values (± SEM) for undiluted pooled vaginal washes (panel A) and lung washes (panel B). The mice that received HA supplemented with liposomes i.n., under ether or pentobarbital anesthesia, exhibited significantly higher vaginal s-IgA levels (p<0.05 and p<0.01, respectively; Student's t test) and lung s-IgA levels (p<0.01, for both types of anesthesia) than the control groups that received liposomes alone.

It is likely that the liposomes exert their mucosal adjuvant activity primarily at the level of the lungs. Immune stimulation was observed only when antigen and liposomes were given i.n. in a relatively large volume (50 µl) to anesthetized mice, while the same dose of antigen and liposomes given i.n. in small volume (20 µl) to unanesthetized animals did not induce an s-IgA response. Studies by Yetter et al. have demonstrated that administration of a relatively large fluid inoculum to anesthetized mice
results in deposition of the preparation throughout the respiratory tract, whereas a small volume given to unanesthetized animals remains confined to the nasal cavity (163). Therefore, the nasal mucosa does not appear to be the primary target for the liposomal adjuvant action. It is also unlikely that stimulation of the Gut-Associated Lymphoid Tissue (GALT), due to swallowing of the liposomal antigen, was involved, since oral administration was completely ineffective (Figure 1).

The appearance of antigen-specific s-IgA in secretions of the vagina following immunization of the lower respiratory tract demonstrates that the lymphoid tissue associated with the lungs, of which the BALT is a major constituent, has the capacity to act as an inductive site for migration of IgA-committed B cells to distant mucosal tissues. Therefore, it appears that the s-IgA response, induced in the respiratory tract, disseminates throughout the common mucosal immune system (19, 91). It is well established that the GALT may serve as an inductive site for migration of IgA-committed B cells to other mucosal tissues (87), while a recent study by Wu and Russell indicates that also lymphoid tissue associated with the nasal mucosa has the capacity to prime the common mucosal immune system (161).

Our results demonstrate that a single i.n. immunization of mice with liposomal antigen induces significant levels of s-IgA in vaginal secretions. Although, obviously, further investigation of liposome-mediated s-IgA responses remains necessary, particularly using antigens more relevant to infections of the urogenital tract, it would appear that liposomes provide an effective and versatile mucosal adjuvant system for potential use in i.n. vaccines against mucosal infections, including urogenital infections. In addition we note that liposomes are nontoxic and biodegradable, while, also, pulmonary administration of aerosolized liposomes is feasible and safe (125).