SYSTEMIC AND LOCAL IgG AND IgA ANTIBODY RESPONSES IN MICE INDUCED BY INTRANASAL IMMUNIZATION WITH LIPOSOME-SUPPLEMENTED INFLUENZA VIRUS SUBUNIT ANTIGEN:

COMPARISON WITH ANTIBODY RESPONSES AFTER CONVENTIONAL IMMUNIZATION AND INFLUENZA INFECTION

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This study reports on the mucosal immunoadjuvant activity of liposomes in an experimental influenza vaccine administered intranasally (i.n.) to mice. Liposome-supplemented influenza virus subunit antigen stimulated IgG responses in serum and pulmonary secretions relative to the responses to subunit antigen alone. Furthermore, the liposome-supplemented antigen, but not the antigen alone, induced a local s-IgA response in the respiratory tract. Immune stimulation by liposomes was observed with negatively charged liposomes, irrespective of the nature of the negatively charged phospholipid incorporated in the liposomal bilayer. Positively charged liposomes did not significantly stimulate antibody responses, while zwitterionic liposomes were completely ineffective. The antibody responses induced by the i.n. liposomal subunit antigen were compared with the corresponding responses induced by an influenza infection or by subcutaneous (s.c.) injection of subunit antigen alone, the conventional route of human vaccination. Infection as well as i.n. or s.c. immunization induced high levels of IgG antibodies in serum and pulmonary secretions. The IgG subclass distribution showed a preferential induction of IgG1 upon i.n. or s.c. immunization with the subunit antigen and preferential induction of IgG2a upon infection. In contrast to s.c. immunization with subunit antigen alone, both i.n. immunization with the liposome-supplemented antigen and infection induced s-IgA secretion in the respiratory tract; moreover, the extents of s-IgA induction in either case were similar. At the same time, both IgA- and IgG-secreting cells appeared in the lungs and lung-associated lymph nodes, suggestive of local antibody production. In conclusion, the liposomal adjuvant system, combined with a mucosal administration protocol, provides a promising strategy for induction of both systemic and local antibody responses against influenza virus subunit antigen.
factor of the mucosal immune system, is of crucial importance in protection of the upper respiratory tract against influenza virus infection (120, 121). Specifically, the authors demonstrated that a monoclonal influenza-specific IgA antibody was actively transported to nasal secretions, acting as a sole mediator of protection of the nasal cavity against infection (121). Furthermore, it was shown that i.n. inoculation of immune mice with an antiserum against the α-chain of IgA abrogated nasal protection (120). These observations point to a key function of s-IgA as a first line of defense against incoming virus. In addition, s-IgA has been shown to have superior cross-reacting properties against different influenza virus variants, as compared to serum IgG (80, 135, 153). Therefore, s-IgA may well mediate heterotypic, and therefore more long-lasting, protection.

In view of the above considerations, new-generation influenza virus vaccines should aim at induction of an s-IgA response in the respiratory tract, in addition to systemic IgG (87). Since parenteral immunization protocols are ineffective in stimulating the mucosal immune system, alternative routes of immunization will have to be explored, including oral or i.n. administration of antigen (39, 77). In addition, effective stimulation of the mucosal immune system is likely to require the use of an antigen-carrier or adjuvant system such as iscoms (13, 14, 83), variants of cholera toxin (CT) or *Escherichia coli* heat-labile enterotoxin (LT) (133, 135, 136), or liposomes (7, 13, 33-35, 38, 52, 158).

This paper focuses on the use of liposomes as a mucosal adjuvant system in an experimental influenza virus subunit vaccine formulation. Liposomes are artificial lipid vesicles which have been shown to have the capacity to stimulate systemic and local antibody responses against associated antigens (7, 52). Previously, we have reported that liposomes, upon i.n. administration to mice, markedly stimulate serum IgG and local s-IgA responses against influenza virus subunit antigen (33, 158). Remarkably, this liposome-induced immune stimulation was found to be independent of a physical association of the antigen with the liposomes: liposomes simply mixed with the antigen exhibited full immunoadjuvant activity. Moreover, i.n. administration of liposomes up to 48 h prior to i.n. administration of antigen also stimulated local and systemic antibody responses (33, 158).

In the present study, we have evaluated local and systemic IgG and IgA responses induced by liposome-supplemented influenza virus subunit antigen administered i.n. to mice. The compartmentalization of the IgG and IgA responses was characterized by determination of the number of antigen-specific antibody-secreting cells in different lymphoid organs. Furthermore, the antibody responses induced by the i.n. liposomal antigen were compared with the corresponding responses induced by an influenza infection, which induces optimal protective antibody responses, or by s.c. immunization with subunit antigen alone, representing the conventional way of human vaccination. It is concluded that i.n. administration of the liposomal antigen is at least as effective as s.c. immunization with subunit antigen alone in terms of induction of serum and pulmonary IgG. However, in con-
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Contrast to s.c. immunization, i.n. immunization with liposome-supplemented subunit antigen has the capacity to induce an s-IgA response in the respiratory tract, the extent of which is equivalent to that induced by an influenza infection.

MATERIALS AND METHODS

Animals

Female BALB/c mice (8-10 weeks old) were used in the experiments in which the immune-stimulatory activity of different liposome compositions on systemic and local antibody responses were compared and in the experiments in which the compartmentalization of the antibody response was investigated. Outbred female NMRI mice (8-10 weeks old) were used in the experiments in which serum and local IgA and IgG (subclass) responses after immunization or infection were evaluated.

Influenza virus

A/PR/8/34 virus (H1N1) was grown for three days in the allantoic cavity of 10-day-old embryonated hen's eggs. The allantoic fluid was collected and stored at -70°C. Influenza subunit antigen, prepared from influenza A/PR/8/34 virus and containing the major virus surface antigen hemagglutinin (HA), was provided by Solvay Duphar in Weesp, The Netherlands. The potency of the preparation, expressed in µg of HA per ml, was determined by the single-radial-diffusion test (159).

Liposomes

Egg-yolk phosphatidylcholine (PC), palmitoyl-oleoyl-phosphatidylglycerol (PG), phosphatidic acid (PA) prepared form egg-PC, and bovine brain phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol, dicetylphosphate (DCP) and stearylamine (SA) were from Sigma Chemical Co. (St. Louis, MO, USA). Liposomes consisted of PC and cholesterol (molar ratio, 1:1) and DCP, PA, PS, PG or SA at either 10 or 30 mol%. Liposomes (extruded oligolamellar vesicles) were prepared as reported earlier (33). Before use in the liposomal vaccine, the liposomes were dialyzed against PBS and appropriate amounts of the influenza subunit antigen were mixed with the liposomes.
Immunizations and sample collection

BALB/c or NMRI mice were immunized by i.n. instillation of 50 µl of (liposomal) antigen under light ether anaesthesia, resulting in immunization of the total respiratory tract (163), or by s.c. injection of 50 µl of the antigen. In some of the experiments, as indicated, a booster immunization was given 4 weeks later according to the same immunization protocol. In order to induce an influenza infection, mice were given a single, nonlethal, dose of A/PR/8/34 virus as an aerosol using a Middlebrook Airborne Infection Apparatus (Tri-R Instruments, Rockville Center, MD, USA). Blood samples, and nasal and lung washes were taken at week 4 (after primary immunization only) or at week 6 (after infection or after primary immunization + booster). Briefly, mice were bled by severing the carotid artery under ether anaesthesia. Lung washes were performed by gentle injection of 1.5 ml of PBS into the lungs with a syringe connected to the trachea, followed by subsequent aspiration of 1 ml of the wash fluid. Nasal washes were done by injection of 0.5 ml PBS retrograde via the trachea into the naso-pharynx, the lavage fluid being collected at the nostrils. Cellular components in the washes were removed by low-speed centrifugation. It is noted that in the experiments comparing antibody responses after s.c. or i.n. immunization or after infection, individual washes were pooled per group and subsequently concentrated 5-fold using Centricon-30 concentrators (Amicon Ltd, Lexington, MA). Sera and washes were stored at -20°C.

Antibody assays

Serum and wash fluid antigen-specific IgG and IgA antibodies were determined by ELISA as described before (33). IgG subclass antibodies were determined, according to the same ELISA protocol, employing peroxidase-conjugated goat antibodies directed against IgG1, IgG2a, IgG2b, or IgG3 (Nordic Immunological Laboratories, Tilburg, The Netherlands). Antibody titers are expressed as the reciprocal serum, nasal or lung wash dilution with an A$_{492}$ value > 0.2 after subtraction of the background A$_{492}$ value of a nonimmune serum, nasal, or lung wash at a matching dilution. Mean serum antibody titers for an experimental group are expressed as geometric mean titers (GMT). Mean s-IgA titers are expressed as GMT or, in cases where washes were pooled per group, as the titer of the pooled and concentrated washes.

ELISA spot assay

BALB/c mice were immunized as described above. An infection was given by inoculation of 25 µl of A/PR/8/34 virus into the nostrils of unanesthetized mice. The groups which received the subunit antigen s.c. or the liposome-supplemented subunit antigen i.n. were given a booster immunization at week 4. Six weeks after the primary immunization or post-infection the mice were anesthetized by i.p. injection of pentobarbital and bled by severing the aorta. Spleen, cervical lymph
nodes (LN) and lung-associated LN were removed and minced with scissors. A single-cell suspension was obtained by passing the tissue through a fine-maze nylon sieve. The cells were collected and red blood cells were lysed by addition of ACK lysing buffer containing 150 mM NH₄Cl, 1 mM KHCO₃ and 0.001 mM EDTA. The cells were subsequently resuspended in RPMI-1640 supplemented with 5% newborn calf serum (NCS).

Lung tissue samples were prepared by cutting off individual lung lobes to ensure that the tissue would not contain lung-associated lymph nodes. Lung tissue was incubated for 1 h at 37°C in RPMI-1640 containing 50 units of collagenase (Sigma) per ml, minced with scissors, and passed through a nylon sieve. Red blood cells were removed by adding ACK lysing buffer (see above) and the collected single cells were resuspended in RPMI-1640 supplemented with 5% NCS.

The cell suspensions were placed in HA-coated ELISA-plates at 5x10⁶, 1x10⁶, 2x10⁵ and 4x10⁴ cells per ml (4 wells for each dilution). Cells were removed after 16 h at 37°C and the plates were washed three times with PBS containing 1% Tween-20. Alkaline phosphatase-labeled goat antibody directed against mouse IgA or mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) was added to the plates in a 1:1000 dilution and incubated for 2 h at 37°C. The plates were washed and spots were developed with the chromogen 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) in 0.1 M Tris buffer, pH 9.5, containing 5 mM MgCl₂, 0.01% Triton and 0.6% agarose during a 2 h incubation at 37°C. The spots, representing individual antigen-specific antibody-secreting cells (ASC) of either the IgA or IgG isotype, were counted and the numbers of ASC per 10⁶ cells were determined.

**Statistical analysis**

Differences in serum or pulmonary antibody titers between different groups were analysed by Student’s t test, p-values <0.05 being considered to represent a statistically significant difference. Antibody levels in pooled washes were not subjected to statistical analysis.

**RESULTS**

**Effect of the surface charge on the mucosal immunoadjuvant activity of liposomes**

Previously, we have shown that liposomes have the capacity to stimulate systemic and local humoral immune responses against an influenza subunit antigen, administered i.n. to mice (33-35, 158). In the present study, we first investigated the effects of liposomal surface charge density and different negatively and positively charged (phospho)lipids on the immune-stimulatory activity of the liposomes. In all cases, groups of mice were immunized by i.n. instillation of the liposome-
supplemented subunit antigen (5 µg of HA) under light ether anesthesia. This immunization protocol results in deposition of the inoculum in the total respiratory tract, including the lungs (163). Liposomes consisted of PC/cholesterol and different concentrations of negatively and positively charged (phospho)lipids. Blood samples and lung washes were taken 4 weeks post-immunization and analyzed for serum IgG and s-IgA by ELISA.

In the experiment shown in Table 1, the liposomes contained in addition 0, 3, 10 or 30 mol% of the negatively charged phospholipid DCP. I.n. administration of the influenza subunit antigen alone did not induce serum IgG or pulmonary s-IgA antibodies (Table 1). Supplementation of the antigen with negatively charged liposomes containing DCP, but not with zwitterionic liposomes, significantly stimulated serum IgG and lung s-IgA responses. The immune stimulation increased with increasing concentrations of DCP in the liposomal bilayer. This effect was reflected in both the serum IgG and the s-IgA responses (Table 1).

The above data show that zwitterionic liposomes without a negatively charged phospholipid do not have the capacity to stimulate antibody responses. In order to investigate if the observed immune-stimulatory effect is a general characteristic of negatively charged liposomes, the DCP was replaced by various other negatively charged phospholipids, such as PG, PA, and PS at 10 or 30 mol%. In addition, positively charged liposomes containing SA were tested.

Table 1 Serum IgG and pulmonary s-IgA responses of BALB/c mice immunized i.n. with influenza virus subunit antigen alone, or supplemented with liposomes containing the negatively charged phospholipid DCP

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Serum IgG log₁₀ titer</th>
<th>Lung wash s-IgA log₁₀ (titer x10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA alone</td>
<td>n.d. *</td>
<td>n.d.</td>
</tr>
<tr>
<td>HA + PC/Chol</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>HA + PC/Chol/DCP (3 mol%)</td>
<td>1.4 ± 0.5 b</td>
<td>n.d.</td>
</tr>
<tr>
<td>HA + PC/Chol/DCP (10 mol%)</td>
<td>3.0 ± 0.5 c</td>
<td>2.1 ± 0.3 c</td>
</tr>
<tr>
<td>HA + PC/Chol/DCP (30 mol%)</td>
<td>3.6 ± 0.3 c</td>
<td>2.9 ± 0.3 c</td>
</tr>
</tbody>
</table>

* n.d.: not detectable.

b GMT ± s.e.m.; serum IgG titers significantly higher than the titers in the group that received no liposomes or liposomes containing PC and cholesterol only (p<0.05).

c GMT ± s.e.m.; serum IgG titers (p<0.05) and lung s-IgA titers (p<0.01) significantly higher compared to titers in the groups which received no liposomes, PC/Chol liposomes or PC/cholesterol liposomes containing 3 mol% DCP.
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Figure 1, upper panel, shows that a significant stimulation of serum IgG responses was observed for all liposome preparations containing different negatively charged phospholipids. There was no significant difference between the effect of liposomes containing 10 or 30 mol% of the negatively charged phospholipid (Figure 1, upper panel, bars C-E), except for the PS-containing liposomes, in which case liposomes containing 30 mol% PS induced markedly lower serum IgG responses than liposomes containing 10 mol% PS (Figure 1, upper panel, bars F). Liposomes containing 10 mol% of the positively charged SA only moderately stimulated the serum IgG response, whereas stimulation was absent at higher concentrations of SA (Figure 1, upper panel, bars G).

Subunit antigen supplemented with PC/cholesterol liposomes containing 10 mol% of a negatively charged phospholipid, but not zwitterionic or positively charged liposomes, induced a significant s-IgA response in the lung (Figure 1, lower panel).
**Figure 1** Serum IgG and pulmonary s-IgA responses of mice immunized i.n. with influenza virus subunit antigen supplemented with liposomes containing negatively or positively charged phospholipids. Groups of 8 BALB/c mice were immunized i.n. with 5 µg of HA alone (bars A), or 5 µg of HA mixed with liposomes (bars B-G), consisting of PC and cholesterol (molar ratio, 1:1) alone (bars B) or containing in addition 10 mol% (open bars) or 30 mol% (hatched bars) of DCP (bars C), PG (bars D), PA (bars E), PS (bars F), or SA (bars G). Liposome dose in each case was 2 µmol of total lipid. Blood samples and lung washes were taken 4 weeks post-immunization. Serum IgG titers (upper panel) and lung s-IgA titers (lower panel) are given as GMT ± s.e.m. Asterisks (* p<0.05, ** p<0.01) indicate groups with significantly higher titers relative to groups immunized i.n. with the subunit alone.
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Again, the response was observed irrespective of the type of negatively charged phospholipid used (bars C-F). Increasing concentrations of PG, PA and PS in the liposomes appeared to suppress the immune-stimulatory activity of the liposomes on lung s-IgA responses (bars D-F), in contrast to the effect of DCP-containing liposomes which induced higher lung s-IgA responses with increasing concentrations of DCP in the liposomes (bars C, see also Table 1).

IgG responses in serum and pulmonary secretions: Comparison with s.c. immunization and infection

The data presented above show that negatively charged liposomes, administered i.n. to mice, stimulate serum IgG and local s-IgA responses against coadministered influenza subunit antigen. In order to evaluate the magnitude of the antibody responses induced by the liposomal vaccine, serum and local antibody levels were compared to corresponding responses to an influenza infection, which induces optimal protection, or to s.c. immunization with influenza subunit antigen alone, representing the conventional way of human vaccination. Groups of mice were given two different doses of subunit antigen (0.5 and 5.0 µg of HA) by either the i.n. or s.c. route of immunization. A booster immunization, following the same immunization protocol, was given 4 weeks later. The antigen given i.n. was supplemented with liposomes, consisting of PC/cholesterol (molar ratio 1:1) and 10 mol% DCP at a dose of 2 µmol of total lipid. The antigen administered s.c. was given without liposomes. A final group received a single, nonlethal, dose of aerosolized A/PR/8/34 influenza virus and was allowed to recover from the infection. The mice were killed 6 weeks after the primary immunization or the infection and serum samples, nasal and lung washes were taken for assessment of antibody levels in blood and respiratory tract secretions.

The higher dose of the liposomal antigen (5.0 µg) administered i.n. induced levels of serum IgG comparable to those observed in the groups immunized s.c. or the infected groups (Table 2). The lower dose of i.n. liposomal antigen induced lower levels of serum IgG (Table 2). It should be noted, however, that in the i.n. immunization protocol, involving deposition of antigen in the total respiratory tract, only about one fifth of the inoculum reaches the lungs (163). Therefore, the effective dose of antigen given i.n. is considerably lower than the amount given s.c. This could explain the relatively low levels of serum IgG detected after i.n. administration of the lower dose of antigen in the presence of liposomes. IgG was detected in the nasal and lung washes of all groups, with the exception of the nasal washes of the group which received the liposomal vaccine i.n. at the lower antigen dose (Table 2). IgG levels detected in the washes were markedly lower than those observed in serum. In this respect, we note that the respiratory tract secretions, containing the IgG, are substantially diluted during sampling. Importantly, however, the levels of nasal and lung wash
IgG after i.n. immunization with the higher dose of antigen were the same as the corresponding levels in the convalescent mice. From the data in Table 2 it can be calculated that transudation of serum IgG to the lung, which is expected to be the sole origin of pulmonary IgG in the case of s.c. immunization, results in a titer in the lung wash corresponding to 1-3% of that in serum. On the other hand, in the convalescent mice and the mice that received the liposomal antigen i.n., the levels of lung wash IgG were comparatively higher (5% and 8-40% of the serum IgG titers, respectively, Table 2).

### Table 2

**IgG responses in serum and respiratory tract secretions of convalescent mice and mice immunized with influenza virus subunit antigen.**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Route</th>
<th>Serum log₁₀ titerᵃ</th>
<th>Nasal wash log₁₀ (titer x10)ᵇ</th>
<th>Lung wash log₁₀ (titer x10)ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 µg HA s.c.</td>
<td>s.c.</td>
<td>3.3 ± 0.8</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td>5.0 µg HA s.c.</td>
<td>s.c.</td>
<td>4.7 ± 0.3</td>
<td>1.5</td>
<td>3.0</td>
</tr>
<tr>
<td>0.5 µg HA / liposomes</td>
<td>i.n.</td>
<td>1.6 ± 0.7</td>
<td>n.d.ᶜ</td>
<td>1.5</td>
</tr>
<tr>
<td>5.0 µg HA / liposomes</td>
<td>i.n.</td>
<td>4.4 ± 0.4</td>
<td>1.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Convalescentᵈ</td>
<td>-</td>
<td>4.6 ± 0.1</td>
<td>0.9</td>
<td>3.6</td>
</tr>
</tbody>
</table>

ᵃ GMT ± s.e.m..
ᵇ IgG levels were determined in pooled and concentrated washes and are therefore indicated as the log₁₀ (titer x10) of the pooled washes without s.e.m..
ᶜ n.d.: not detectable.
ᵈ Mice were given an aerosolized sublethal dose of A/PR/8/34 virus.

This is suggestive of local production of IgG in the lung following infection or i.n. immunization with the liposomal antigen, in addition to transudation of serum IgG. Further evidence for local production was obtained from experiments, in which the compartmentalization of antibody responses was investigated using an ELISA spot assay (see below).

We also investigated the IgG subclass responses following i.n. and s.c. immunization or infection. In all groups, the subclass distribution observed in the pulmonary secretions was similar to the subclass distribution found in the serum (Figure 2). The groups immunized s.c. with the subunit
antigen alone as well those that received the liposomal antigen i.n. developed high titers of IgG1, while also considerable levels of IgG2a and IgG2b, but not IgG3, were detected. In the convalescent mice, the IgG subclass distribution was shifted toward a preferential induction of IgG2a, while in this group production of IgG3 was also more pronounced (Figure 2).

**IgA responses in serum and respiratory tract secretions: Comparison with s.c. immunization and infection**

Next, we compared the serum and local IgA responses after i.n. immunization with liposome-supplemented antigen with the corresponding responses induced by an influenza infection or by s.c. immunization with subunit antigen alone. Groups of mice were either immunized or infected, as described above. I.n. and s.c. immunizations were done with the higher dose of antigen (5 µg of HA), including a booster immunization 4 weeks after the primary immunization.
Figure 2  IgG subclass distribution in serum and pulmonary secretions upon i.n. immunization of mice with influenza virus subunit antigen supplemented with liposomes, s.c. immunization with subunit antigen alone, or infection. Groups of 5 NMRI mice were immunized i.n. with 5 µg of HA per dose supplemented with PC/cholesterol liposomes containing 10 mol% DCP (2 µmol of total lipid) or s.c. with 5 µg of HA alone; a control group was infected with influenza virus by aerosol. The groups which received the subunit vaccine were given a booster immunization at week 4. Blood samples and lung washes were taken 6 weeks post-immunization. IgG levels in serum (upper panel) are presented as GMT ± s.e.m. IgG levels in lung washes (lower panel) are presented as the titer of pooled and concentrated lung washes. S.c. immunization with subunit antigen and i.n. immunization with the liposome-supplemented subunit antigen induced significantly higher titers of serum IgG1 relative to serum IgG2a and IgG2b (p<0.01 for s.c. immunization and p<0.05 for i.n. immunization). Infection induced significantly higher titers of serum IgG2a relative to serum IgG1, IgG2b and IgG3 (p<0.01).

Again, the antigen given i.n. was supplemented with liposomes consisting of PC/cholesterol (molar ratio 1:1) and 10 mol% DCP at a dose of 2 µmol of total lipid. The mice were killed 6 weeks post-immunization or post-infection and serum samples, nasal and lung washes were taken for assessment of antigen-specific IgA.

Table 3  IgA responses in serum and respiratory tract secretions of convalescent mice and mice immunized with influenza virus subunit antigen.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Route</th>
<th>Serum log$_{10}$ titer$^a$</th>
<th>Nasal wash log$_{10}$ (titer x10)$^b$</th>
<th>Lung wash log$_{10}$ (titer x10)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 µg HA</td>
<td>s.c.</td>
<td>n.d.$^c$</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>5.0 µg HA /liposomes</td>
<td>i.n.</td>
<td>0.9 ± 0.4</td>
<td>0.9</td>
<td>2.7</td>
</tr>
<tr>
<td>Convalescent$^d$</td>
<td>-</td>
<td>0.4 ± 0.2</td>
<td>0.3</td>
<td>2.7</td>
</tr>
</tbody>
</table>

$^a$ GMT ± s.e.m.$^b$ IgA levels were determined in pooled and concentrated washes and are therefore indicated as the log$_{10}$ (titer x10) of the pooled washes without s.e.m.$^c$ n.d.: not detectable$^d$ Mice were given an aerosolized sublethal dose of A/PR/8/34 virus.
Consistent with the data shown above, subunit antigen, supplemented with liposomes and administered i.n., induced an s-IgA response in the respiratory tract: the s-IgA responses in lung and nasal washes were similar to or slightly higher than the corresponding responses in convalescent mice (Table 3). By contrast, there was no detectable local s-IgA response in the mice immunized s.c. IgA was also detected in the serum of the i.n. immunized and convalescent mice, but not in the mice immunized s.c. Compared to the serum IgG titers (Table 2), the serum IgA responses were low (Table 3). The concentration of IgA antibodies in the serum was comparatively lower than that in the respiratory tract secretions, even though the respiratory tract secretions are diluted substantially during sampling, which points to local production of IgA.

Detection of antigen-specific antibody-secreting cells

The above data demonstrate that infection as well as immunization of the respiratory tract using liposomes effectively induce IgG and IgA antibodies in respiratory tract secretions. The data are also suggestive of local antibody production, particularly in the lung, lung-associated LN, and the spleen were screened for the presence of antigen-specific ASC using an ELISA spot assay. Figure 3 shows that large numbers of antigen-specific IgG- and IgA-ASC were detected in the lungs of mice immunized i.n. with the liposomal subunit antigen (panel B). To investigate in more detail the exact compartmentalization of IgG and IgA responses, the and in the lungs of convalescent mice (panel C). By contrast, IgG- and IgA-ASC were absent in the lungs of mice immunized s.c. with subunit antigen (panel A). Large numbers of IgG-ASC were also detected in the lung-associated LN of mice immunized i.n. or in convalescent mice.
Figure 3 Number of IgA- and IgG-ASC in lung, lung-associated LN and spleen after i.n. immunization of mice with liposome-supplemented influenza virus subunit antigen, s.c. immunization with subunit alone, or infection. Groups of 4 BALB/c mice were immunized s.c. with 5 µg of HA alone (panel A), i.n. with 5 µg of HA supplemented with PC/cholesterol liposomes containing 10 mol% DCP at a total lipid dose of 2 µmol (panel B) or infected by inoculation of infectious virus into the nose (panel C). The groups which received the subunit antigen were given a booster immunization at week 4. Tissue samples were taken 6 weeks after the primary immunization. The collected single-cell suspensions were pooled per group and tested at different cell numbers in 4 separate cultures. Bars represent mean numbers of 4 cultures ± s.e.m.

In the groups immunized s.c. or i.n. as well as in the convalescent group, IgG-ASC were observed in the spleen (Figure 3), indicating that the systemic immune system was involved in the IgG antibody response. IgA-ASC were observed in the spleens of the convalescent mice and the mice which received the liposomal antigen i.n., but not in the spleens of the mice which received the antigen s.c. (Figure 3).
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DISCUSSION

In this paper it is shown that negatively charged liposomes, but not zwitterionic or positively charged liposomes, significantly stimulate serum IgG and local s-IgA responses against an influenza subunit antigen, coadministered i.n. to anesthetized mice. The liposome-supplemented subunit antigen induced IgG responses in serum and pulmonary secretions which were similar to the corresponding responses observed after an influenza infection or after s.c. immunization with subunit antigen alone. The IgG subclass distribution showed a shift towards a preferential induction of IgG1 upon i.n. or s.c. immunization with the subunit antigen or IgG2a upon infection. I.n. immunization with liposome-supplemented subunit antigen as well as infection, but not s.c. vaccination with subunit antigen alone, induced a local s-IgA response in the respiratory tract. This local IgA antibody response is likely to originate in B-lymphocytes in the lung and lung-associated LN and is paralleled by a local IgG response.

Under the conditions of our experiments, the liposome-supplemented antigen is deposited throughout the respiratory tract of the mice, including the lungs (163). Previously, we found that immunization of the lungs, as opposed to immunization of the upper respiratory tract, is essential for the mucosal immunoadjuvant activity of the liposomes to become apparent (33-35). Accordingly, we have hypothesized that alveolar macrophages, localized in the lumen of lung alveoli, play a role in the mucosal immune stimulation by liposomes (33). Macrophages have a high affinity for liposomes (7, 52). Therefore, uptake of liposomes could down-regulate local immune suppression by alveolar macrophages (147), a function normally performed by these cells to modulate the response against inhaled antigenic material (68, 70). Thus, immune stimulation by negatively charged, as opposed to zwitterionic or positively charged, liposomes would reflect an enhanced uptake by macrophages (52). Our experiments show that the incorporation of a negatively charged phospholipid in the liposomes is essential for immune stimulation. It appears that a relative concentration of 10 mol% of negatively charged phospholipid in the liposomal membrane is optimal. Increasing the concentration of negatively charged phospholipid from 10 to 30 mol% does not result in a further enhancement of the immune-stimulatory capacity of the liposomes (Figure 1).

Protection against influenza infection has been shown to correlate with humoral immunity directed against the viral HA (152). More specifically, HA-specific serum IgG has been shown to protect the lungs against infection by transudation from the circulation (13, 88, 117, 128). In this study, it is shown that i.n. administration of liposome-supplemented subunit antigen effectively induces an antigen-specific serum IgG response. The magnitude of this response is comparable to that of the serum IgG response in convalescent mice or in mice immunized s.c. with subunit antigen alone. As discussed above, in the i.n. immunization protocol only about one fifth of the inoculum
reaches the lungs (163). Considering the fact that in the case of i.n. administration of the higher dose of antigen (5 µg of HA), about 1 µg is the effective dose of HA delivered to the lung, we conclude that the efficacy of the liposomal antigen is at least equivalent to that of the antigen administered s.c.

Neutralization efficiency of influenza virus mediated by IgG has been shown to depend on the distribution of different IgG subclasses that comprise the IgG pool (12, 137). The IgG2a subclass appears to be most effective in vivo [47, 137], although in vitro studies have shown equal neutralizing capabilities for IgG1 and IgG2a subclasses in the BALB/c mouse (64). In the present study, we observed a preferential induction of IgG1 antibodies in serum and lung secretions following immunization with a subunit antigen (non-replicating antigen), while there was also a substantial induction IgG2a and IgG2b. On the other hand, after infection (replicating antigen), the IgG subclass distribution was shifted toward a preferential induction of IgG2a, which is in agreement with earlier findings of Balcovic et al. (12) and Hocart et al. (63-65). In all cases, the IgG subclass distribution in the lung washes was similar to that found in corresponding sera. Although this may suggest that the locally produced IgG only marginally contributes to the IgG pool in pulmonary secretions compared to the transudated IgG, it is more likely that the distribution of the different IgG subclasses is determined primarily by the nature of the antigen, as discussed above (12, 63-65), and does not differ between different compartments. Therefore, the similarity between the IgG subclass distributions in serum and pulmonary secretions does not argue against a local production of IgG (see also below). In conclusion, we emphasize that both the i.n. and s.c. immunization protocols, as described in this study, induce high levels of IgG, including IgG subclass antibodies considered important in protection against influenza (12, 64, 137).

Secretory-IgA acts as a first line of defence against incoming virus and has been shown to correlate with protection of the lower and upper respiratory tract against influenza infection (13, 14, 38, 80, 88, 120, 121, 135, 136). The s-IgA response observed in our experiments was most prominent in the lungs and the extent of the response was comparable between convalescent mice and mice which received the liposomal subunit antigen. The levels of s-IgA in nasal secretions were relatively low, but appeared to be comparable between convalescent mice and mice which received the liposomal antigen. Although a small proportion of the s-IgA antibodies detected in the lungs of the immunized or infected mice could originate from the serum, possibly via transudation of monomeric IgA or due to active transport of polymeric IgA into the secretions (91), it seems likely that most of the s-IgA is produced locally. This notion is supported by the presence of large numbers of IgA-ASC in the lungs following local immunization or infection. Besides IgA-ASC, IgG-ASC also accumulate in the lung. The B-cells that mature into ASC presumably originate from the Bronchus-Associated Lymphoid Tissue (BALT) or lung-associated LN (16-18). This could explain the presence of relative large numbers of ASC in the lung-associated LN after infection or lung immuni-
zation. The BALT is thought to directly take up antigen from the conductive airways via specialized microfold cells (M-cells) (18), whereas the lung-associated LN receive antigen from the lung via lymphatic drainage (16,17). After their priming in the BALT or lung-associated LN, the activated B-cells will enter the circulation and home to the lung (16, 17). The ASC detected in the spleen could originate from the activated B-cells released from the BALT or lung-associated LN into the circulation. Alternatively, these ASC may be generated in the spleen due to spill of antigen from the lung into the blood. In addition to homing of B cells to the lung, recirculating IgA-committed B-cells have the capacity to home to distant mucosal sites, such as the gut-associated lymphoid tissue (GALT) and salivary and urogenital mucosa (16, 18, 87). This linkage of Mucosal-Associated Lymphoid Tissues, including the BALT, is a characteristic property of the common mucosal immune system (18). Recently, we have reported on the detection of antigen-specific s-IgA antibodies in vaginal secretions of female mice following immunization of the lung with liposome-supplemented influenza virus subunit antigen (34). The appearance of s-IgA in vaginal secretions induced by immunization of the lungs is probably the result of migration of IgA-committed B-cells within the common mucosal immune system. Migration of HA-specific IgA-committed B-cells has also been observed after intragastric inoculation, but not after parenteral immunization, of mice with an inactivated whole-virion influenza vaccine (25).

In conclusion, the liposomal adjuvant system described in this paper combined with the mucosal immunization protocol provides a promising alternative to the current influenza subunit vaccines used in human vaccination. The liposome-supplemented experimental vaccine is equally effective as conventional parenterally injected subunit vaccine in terms of induction of a systemic IgG response. In addition, the liposomal vaccine has the capacity to induce an s-IgA response in the respiratory tract acting as a first line of defence. Furthermore, the liposomal vaccine can be administered by inhalation which would provide a more readily acceptable route of administration as compared to injection.