Immunology 1996 **89** 488–493

Mucosal immunoadjuvant activity of liposomes: role of alveolar macrophages

A. DE HAAN,*† G. GROEN,† J. PROP,† N. VAN ROOIJEN‡ & J. WILSCHUT* **Department of Physiological Chemistry, University of Groningen, †Cardiopulmonary Surgery Research Division, University Hospital Groningen, Groningen, and ‡Department of Cell Biology and Immunology, Free University, Amsterdam, the Netherlands*

SUMMARY

Previously, we have reported on a liposomal adjuvant system for stimulation of both systemic IgG and mucosal s-IgA responses against viral antigens (influenza virus subunit antigen or whole inactivated measles virus) administered intranasally to mice. Immune stimulation is observed with negatively charged, but not with zwitterionic, liposomes and is independent of a physical association of the antigen with the liposomes. Furthermore, liposome-mediated immune stimulation requires deposition of the liposomes and the antigen in the lower respiratory tract. In the present study, it is shown that alveolar macrophages (AM) are the main target cells for negatively charged liposomes administered to the lungs of mice. AM isolated from animals, to which negatively charged liposomes were administered beforehand, showed large intracellular vacuoles, suggestive of massive liposome uptake. Under *ex vivo* conditions, both AM and RAW 264 cells exhibited a high capacity to take up negatively charged liposomes. The deposition of negatively charged liposomes, but not zwitterionic, liposomes in the lung reduced the phagocytic and migratory behaviour of AM, as assessed on the basis of transport of carbon particles to the draining lymph nodes of the lungs. Depletion of AM *in vivo* with dichloromethylene diphosphonate, facilitated an enhanced systemic and local antibody response against influenza subunit antigen deposited subsequently to the lower respiratory tract. In conclusion, these data provide support for the hypothesis that uptake of negatively charged liposomes blocks the immunosuppressive activity of AM, thereby facilitating local and systemic antibody responses.

INTRODUCTION

In previous studies, we have shown that liposomes, formulated in an experimental influenza virus subunit vaccine or an inactivated measles whole virus vaccine, stimulate systemic and local antibody responses upon intranasal administration to mice.[1–3] This immune-stimulatory activity of liposomes was found to be independent of a physical association of the antigen with the liposomes and required administration of the liposomes and antigen to the lower respiratory tract, including the lungs.[1–3] Furthermore, a negative surface charge on the liposomes was found to be essential for immune stimulation.[1] It is known that cells belonging to the mononuclear phagocytic system (MPS), including alveolar macrophages (AM), have a natural tendency to take up liposomes.[4–6] In the present study, we specifically investigated the role of AM in the mucosal immunoadjuvant activity of liposomes.

Macrophages are abundantly present in the alveolar spaces (AM) and lung interstitium (interstitial macrophages, IM).[7] AM function as scavenger cells clearing the lung of foreign (antigenic) material.[8] In this respect, it has been reported that AM play a role in particle translocation from the lung to draining lymph nodes.[9] In addition, AM have been suggested to perform immunoregulatory functions such as cytokine production and antigen presentation.[10–12] However, there is increasing evidence to indicate that AM are not particularly efficient stimulators of immune responses when compared to IM, other macrophages, or monocytes of the same species.[7,11–13] In fact, in many cases, AM appear to suppress local immune responses, and thus, may play an important role in maintaining immunological homeostasis and preventing allergic sensitization of the healthy lung.[12,14] Studies of Holt and co-workers have demonstrated that AM constitutively suppress immune responses in the lung by inhibition of T-cell proliferation and down-regulation of antigen-presenting functions of pulmonary dendritic cells (DC).[15,16] Additionally, AM may down-regulate immune responses by rapid uptake and degradation of antigen, thus preventing other antigen-presenting cells, like DC, to process and present the antigen. In agreement with the notion that AM down-regulate immune responses in the lung, Thepen *et al.* have shown that the formation of antigen-specific antibody-forming cells is enhanced in the lungs of mice which were previously depleted of their AM population.[17] In these experiments, selective elimination of AM was achieved by intratracheal injection of liposomes containing dichloromethylene diphosphonate (CL_2MDP), a technique described earlier by Van Rooijen.[18] Contrary to AM, IM seem to augment DC function by processing of antigen and release of antigenic fragments which are then presented by DC to T cells.[13]

We hypothesized, in agreement with Van Rooijen,[19] that uptake

Received 31 July 1996; accepted 1 September 1996.

Correspondence: Dr J. Wilschut, Department of Physiological Chemistry, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands.

of negatively charged liposomes by AM could inhibit AM-mediated immune suppression temporarily, thereby stimulating antibody responses against a coadministered antigen. In the present study we demonstrate that, *in vivo* and *ex vivo*, AM are target cells for negatively charged liposomes. In addition, it is shown that uptake of negatively charged liposomes affects AM function with respect to their phagocytic and migratory behaviour. Furthermore, it is demonstrated that functional depletion of AM facilitates an increased systemic IgG and local secretory IgA (s-IgA) antibody response against influenza subunit antigen. These data support the hypothesis that liposomes interfere with the AM-mediated immune suppression, thereby facilitating an increased immune response against a coadministered antigen.

MATERIALS AND METHODS

Animals

Female BALB/c mice (8–10 weeks) were used throughout. For immunization studies, groups consisted of five animals each. For carbon phagocytosis and transport studies, groups consisted of three animals each.

Influenza subunit vaccine

Influenza virus subunit vaccine, as used for human flu vaccination, was provided by Solvay Duphar B.V. in Weesp, the Netherlands. This subunit vaccine consists primarily of aggregates of viral haemagglutinin (HA) and neuraminidase. The antigen preparation was derived from the influenza virus A/PR/8/34 (H1N1). The potency of the preparation, expressed in μg of HA per ml, was determined by the single-radial-diffusion test.[20]

Liposomes

Egg-yolk phosphatidylcholine (PC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol and dicetylphosphate (DCP) were from Sigma Chemical Co. (St. Louis, MO). Fluorescently labelled BODIPY-phosphatidylcholine (BODIPY-PC) was from Molecular Probes (Eugene, OR).

Liposomes were prepared as reported earlier.[1] Liposomes used in the liposomal vaccine were extruded oligolamellar vesicles and consisted of PC, cholesterol and DCP in a molar ratio of 4 : 5 : 1. For *in vitro* liposome uptake studies, liposomes consisted of BODIPY-PC, PC, cholesterol and DCP in a molar ratio of 2 : 2·5 : 4·5 : 1 (negatively charged liposomes), or BODIPY-PC, PC and cholesterol in a molar ratio of 2 : 3 : 5 (zwitterionic liposomes). Lipid content of the liposomal preparations was determined by phosphorus analysis as described by Böttcher *et al.*[21]

Liposome uptake by macrophages

Broncho-alveolar lavage (BAL) cells were obtained by 10 consecutive *in situ* washes of the lungs of anaesthetized mice using 1 ml RPMI-1640 medium (Gibco BRL, Breda, the Netherlands) supplemented with 5% fetal calf serum (FCS) (Gibco BRL) and 0·25% (w/v) lidocaine (Sigma) at 37°. Routinely, cytospins were prepared and stained with May–Grunwald Giemsa (MGG) for differentiation and counting of harvested cells. BAL cells obtained from normal untreated animals consisted almost exclusively of AM (>98%), in agreement with observations by Holt *et al.*[12] The yield of AM was approximately 4×10^5 cells per mouse. The cells were washed twice with serum-free RPMI-1640 at 4°, resuspended in RPMI-1640 supplemented with 5% FCS and placed in 96-well cell culture plates (4×10^5 cells/well). RAW 264 cells (a macrophage-like cell line) in RPMI-1640 medium with 5% FCS were placed in 96-well cell culture plates at the same cell density. The plates were incubated for 24 hr at 37° in a humidified CO_2-incubator for adherence of the cells. Subsequently, the plates were washed with phosphate-buffered-saline (PBS) to remove non-adherent cells.

To assess liposome uptake by the adherent cells, fluorescently labeled liposomes (0·5 μmol of total lipid per ml), in RPMI-1640 supplemented with 1% FCS, were added to the cells in a volume of 200 μl in triplicate. After 24 hr of incubation at 37° in a humidified CO_2-incubator, the plates were washed three times with PBS and the cells were lysed by the addition of 100 μl of 0·5% Triton per well. The amount of liposomes taken up by the cells was determined on the basis of the fluorescence intensity associated with the (lysed) cells, measured in an SLM/Aminco AB2 fluorometer at an exitation wavelength of 500 nm and an emission wavelength of 520 nm. The uptake of liposomes is expressed as arbitrary units of fluorescence, after subtraction of the background fluorescence of cells which were incubated in medium alone.

AM depletion

Liposomes containing Cl_2MDP in PBS were prepared as described by Van Rooijen.[18] Control liposomes were prepared accordingly but contained no Cl_2MDP. For *in vivo* AM depletion, mice were anaesthetized (0·06 mg pentobarbital per g body weight, injected intraperitoneally) and instilled intratracheally with the CL_2MDP–liposome suspension.[17] For intratracheal administration, an incision was made in the throat parallel to the trachea. The trachea was exteriorized and the liposome-suspension was instilled towards the lungs using a 30-gauge needle, during which the cranial aspect of the trachea was clamped off to prevent back-flow of the liposomes. The skin incision was closed with interrupted sutures. A separate group of mice, given liposomes without CL_2MDP using the same protocol, is referred to hereafter as sham-depleted mice.

Immunization and sample collection

Mice were immunized by intranasal instillation of influenza subunit antigen alone or influenza subunit antigen mixed with empty liposomes, consisting of PC, cholesterol and DCP (molar ratio, 4 : 5 : 1). The immunization was performed under light ether anaesthesia, resulting in deposition of the fluid inoculum in the total respiratory tract, including the lungs.[22]

Mice were bled 2 weeks postimmunization and serum samples were prepared. Lung washes were taken by gently injecting 1·5 ml PBS into the lungs with a syringe connected to the trachea, followed by aspiration of 1 ml of the lung wash fluid. Cellular components were removed by low-speed centrifugation. Sera and washes were stored at −20°.

Antibody assays

Influenza-specific serum IgG and s-IgA were determined by enzyme-linked immunosorbent assay (ELISA) as described before.[21] Antibody titres represent the reciprocal serum or lung wash dilution with an A_{492} value > 0·2, after subtraction of the background value of a non-immune serum or lung wash at a matching dilution. Serum and lung wash antibody levels are expressed as geometric mean titres (GMT).

Uptake and transport of carbon by AM

Mice were inoculated intranasally under ether anaesthesia with carbon particles (India ink) diluted 10 times with PBS, in the

presence or absence of liposomes. The volume of the inoculum was 50 μl and each preparation contained the same amount of carbon particles. Two days later, the mice were sacrificed and two lung-associated lymph nodes (LALN) from the mediastinal area were excised. Detection of carbon-containing AM was performed as described by Wang *et al.*[23] Briefly, paraffin sections were stained with nuclear fast red and examined microscopically for determination of the number of cells containing carbon particles. One animal, in which no deposition of carbon could be seen in the lungs due to an apparent technical failure of the carbon administration, was excluded from the experiment.

Statistical analyses

Differences in antibody levels and numbers of cells containing carbon-particles were analysed using Student's t test, in which a p-value of $<0{\cdot}05$ was considered to represent a statistically significant difference.

RESULTS

In vivo uptake of liposomes by AM

Previously, we have observed that negatively charged liposomes, but not zwitterionic liposomes, have the capacity to stimulate systemic and local antibody responses against an admixed antigen upon administration of the liposome/antigen mixture to the murine lower respiratory tract, and we have hypothesized that AM may be involved in this immune stimulation by negatively charged liposomes.[1] In the present study, we have addressed this issue in a direct manner.

We first examined BAL cells isolated from the lungs of mice treated 2 days or 2 weeks beforehand with negatively charged or zwitterionic liposomes. An untreated control group was also included. On day 2, AM isolated from mice which received negatively charged liposomes appeared enlarged compared to AM isolated from untreated normal animals (Fig. 1, panel B versus panel A). Moreover, many of these macrophages contained large intracellular vacuoles suggestive of massive liposome uptake. Among the BAL cells, some polymorphonuclear cells (PMN) could also be observed (Fig. 1B). Some of the BAL cells isolated from animals which received zwitterionic liposomes were also enlarged (Fig. 1, panel C), but to much lesser extents than in the case of pretreatment with negatively charged liposomes. Furthermore, no PMN were observed in these preparations. BAL cell preparations obtained 14 days after the treatment with negatively charged liposomes still showed occasional enlarged AM, whereas those obtained from animals treated with zwitterionic liposomes had returned to a normal appearance (results not shown). These observations suggest that, *in vivo,* AM are target cells for negatively charged liposomes.

Subsequently, we investigated the uptake of negatively charged and zwitterionic liposomes by AM *in vitro*. Figure 2 shows that negatively charged liposomes were taken up efficiently by AM. By contrast, uptake of zwitterionic liposomes was not detectable under the conditions of the experiment. We also investigated liposome uptake by RAW 264 cells, a macrophage-like cell line. Although these cells demonstrated an overall higher capacity to take up liposomes, again, the uptake of negatively charged liposomes was markedly higher than that of zwitterionic liposomes (Fig. 2).

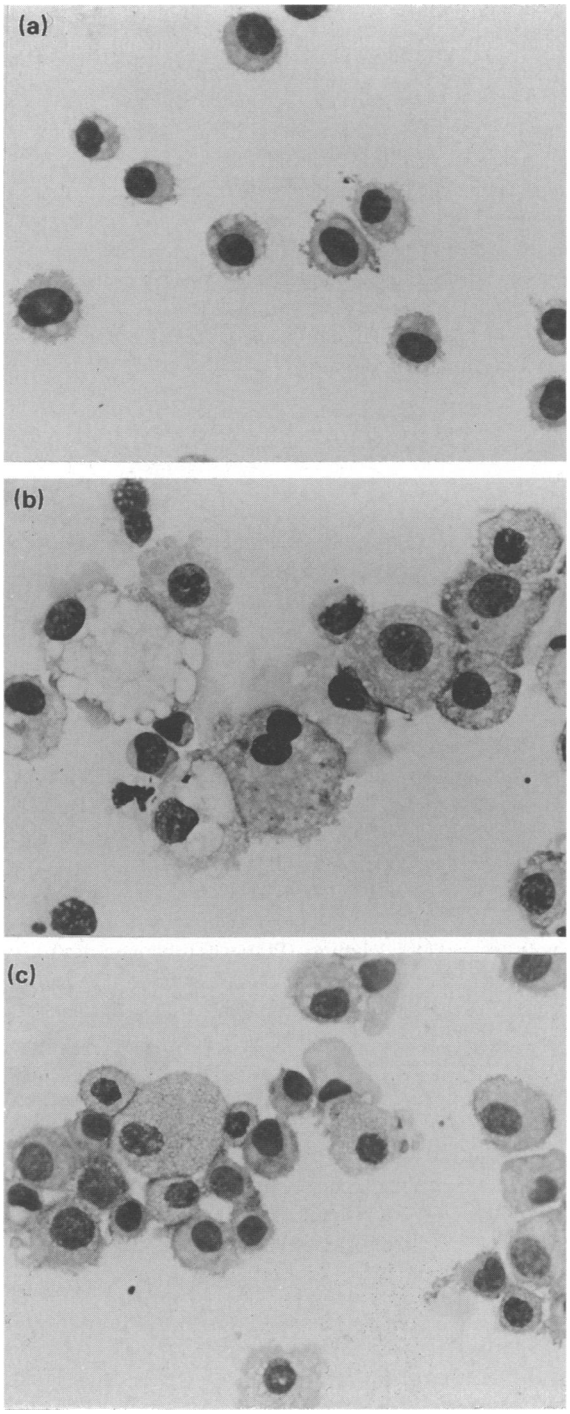

Figure 1. The effect of negatively charged and zwitterionic liposomes on the morphology of murine AM. Anaesthetized mice were inoculated intranasally with zwitterionic or negatively charged liposomes. BAL cells were isolated 2 days later, spun onto microscope slides and stained with MGG. (a) BAL cells of normal mice; (b) BAL cells from mice after administration of negatively charged liposomes; (c) BAL cells from mice after administration of zwitterionic liposomes. Magnification, ×30. Liposome dose was 1 mg of total lipid.

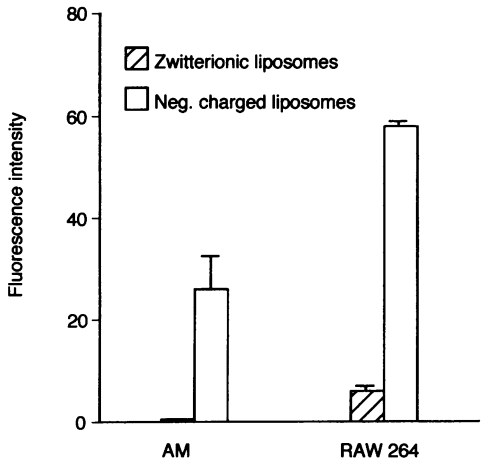

Figure 2. *In vitro* uptake of zwitterionic (hatched bars) and negatively charged (open bars) liposomes by AM or RAW 264 cells. Triplicate cultures of adherent AM or RAW 264 cells (4×10^5 cells per well) were incubated for 24 hr with fluorescently labelled liposomes at a concentration of 0·5 μmol of total lipid per ml. Uptake of liposomes is expressed in arbitrary units as the mean fluorescence intensity ± SEM associated with the cells, after subtraction of the fluorescence of AM or RAW 264 cells incubated in medium alone.

Effect of liposomes on the uptake and transport of carbon by AM

AM are scavenger cells that have been shown to participate in particle translocation from the lung to draining lymph nodes.[9] Since the above data showed that AM have a high affinity for negatively charged liposomes both *in vivo* and *in vitro*, we studied the effect of liposomes on the phagocytic and migratory function of AM, using carbon particles as a marker.

Negatively charged liposomes were coadministered intranasally with carbon particles to anaesthetized mice. Control groups received carbon particles alone or mixed with zwitterionic liposomes. Two days later, in the control animals, carbon-positive AM were observed in the lung-associated lymph nodes (Table 1). On the other hand, in the animals which received carbon particles mixed with negatively charged liposomes, the number of carbon-positive AM was significantly lower. Coadministration of zwitterionic liposomes had no effect on the number of carbon-containing cells in the lymph nodes. These data suggest that negatively charged liposomes interfere with the phagocytic uptake of carbon particles and/or the migratory behaviour of AM.

Table 1. Effect of coadministered liposomes on the uptake and transport of carbon by AM

Treatment*	Number of cells†
Carbon	41 ± 9
Carbon + zwitterionic liposomes	34 ± 11
Carbon + negatively charged liposomes	6 ± 2‡

* Intranasal instillation under light ether anaesthesia.

† Relative mean number of carbon-positive cells ± SEM in LALN; combined results of two individual experiments.

‡ Significantly reduced number relative to that after instillation of carbon alone or carbon mixed with zwitterionic liposomes; $P < 0.01$ and $P < 0.05$, respectively, Student's t test.

AM depletion and antibody responses against influenza subunit antigen administered intranasally subsequently

The above results suggest that, *in vivo*, AM are major target cells for lung-deposited negatively charged liposomes, and that these liposomes affect the phagocytic capacity of the AM. Previously, we have hypothesized that AM, as a result, lose their immune-suppressive activity temporarily, thereby facilitating local and systemic antibody responses against coadministered antigen. In order to investigate in a direct manner whether AM do suppress antibody responses against influenza virus subunit antigen, we depleted mice of their AM population using the technique described by Van Rooijen[17] involving administration of zwitterionic liposomes containing CL_2MDP. Controls groups were treated similarly with zwitterionic liposomes without CL_2MDP (sham-depleted), or were left untreated altogether. Subsequently, the mice were immunized intranasally with influenza virus subunit antigen.

Two days after the AM-depletion protocol, the number of AM isolated from the lungs of the mice was markedly reduced (Fig. 3), which is in agreement with observations by Thepen *et al.*[17] The sham-depleted animals had normal numbers of AM. At day 14 after the treatment, AM-depleted mice showed normal numbers of AM, indicating that complete repopulation of AM had occurred within this timespan (Fig. 3).

Mice were treated as described in Fig. 3 and immunized intranasally two days later with influenza virus subunit antigen alone or subunit antigen supplemented with negatively charged liposomes. Immunization of the AM-depleted animals with subunit antigen alone not only resulted in a substantial serum IgG response, it also induced an s-IgA response in the lungs of the animals (Fig. 4a). By contrast, immunization of normal or sham-depleted animals with subunit antigen alone did neither elicit a serum IgG nor a local s-IgA response (Fig. 4a). As expected, intranasal administration of influenza subunit antigen supplemented with negatively charged liposomes induced high levels of serum IgG and lung s-IgA antibodies in both untreated and sham-depleted mice (Fig. 4b). Interestingly, addition of liposomes to the antigen administered to the AM-depleted animals stimulated the antibody responses (Fig. 4, panel b versus panel a).

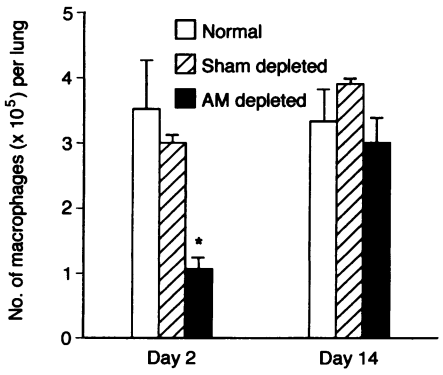

Figure 3. Number of AM isolated in BAL from the lungs of normal (open bars), sham-depleted (hatched bars) or AM-depleted (solid bars) mice. Two or 14 days after the AM-depletion protocol, BAL preparations were isolated and the numbers of macrophages were determined. Bars represent the mean number ± SEM.

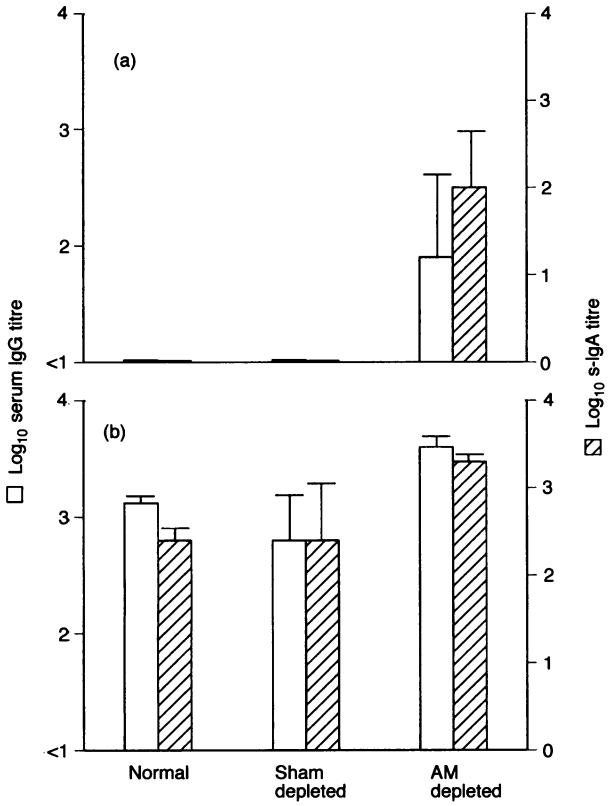

Figure 4. Serum IgG and lung s-IgA responses against intranasally administered influenza subunit antigen alone or supplemented with liposomes in normal, sham-depleted and AM-depleted mice. Two days after the depletion protocol, mice were immunized intranasally with influenza virus subunit antigen (5 μg of HA) alone (a) or subunit antigen supplemented with liposomes at a dose of 1 mg of lipid (b). Blood samples and lung washings were taken 2 weeks postimmunization. Serum IgG (open bars) and lung wash s-IgA levels (hatched bars) are given as GMT $\pm$ SEM. Levels of serum IgG and s-IgA in the AM-depleted animals which received subunit antigen alone were significantly enhanced relative to the levels in sham-depleted or untreated animals ($P<0{\cdot}05$ and $P<0{\cdot}01$ for serum IgG and s-IgA levels, respectively). Supplementation of liposomes to the subunit antigen further significantly enhanced the s-IgA response in control and AM-depleted animals ($P<0{\cdot}0001$ and $P<0{\cdot}05$, respectively).

DISCUSSION

In this study, it is shown that AM are target cells for negatively charged liposomes deposited in the lungs of mice. AM isolated from animals which received negatively charged liposomes showed large intracellular vacuoles, suggestive of liposome uptake *in vivo*, while *ex vivo* liposome uptake by AM and RAW 264 cells demonstrated that these cells have a high affinity for negatively charged liposomes. The uptake of negatively charged liposomes, but not zwitterionic liposomes, affected AM function with respect to their phagocytic and migratory behaviour. Furthermore, depletion of the AM population in mice facilitate an enhanced systemic and local antibody response against influenza virus subunit antigen deposited subsequently to the lower respiratory tract, supporting the notion that AM down-regulate local immune responses in the lung.

The immunopotentiating effect of liposomes is generally thought to be the result of increased uptake of liposome-associated antigen by macrophages, due to natural targeting of liposomes to these cells.[4–6] Increased uptake of antigen would then result in an improved presentation of antigen-derived peptides to T cells, facilitating the humoral immune response.[4–6,24,25] In addition, liposomes have been suggested to function as a depot for associated antigen.[26] It is likely that uptake of liposome-associated antigens by macrophages and subsequent improved presentation to T cells as well as slow release of antigen from liposomes are indeed the major mechanisms by which liposomes exert their adjuvant activity upon parenteral administration of liposome-associated antigen. However, as demonstrated by us previously, upon administration to the lungs liposomes may exert an additional immunoadjuvant activity, different from that described above.[1–3] This adjuvant activity is independent of a physical association of the antigen with the liposomes and results in induction of a mucosal s-IgA response and in a stimulation of the systemic IgG response. The present study provides evidence to indicate that this mode of liposomal immunoadjuvanticity is also mediated by macrophages, in particular AM.

AM have been demonstrated to perform immunoregulatory functions that are important in prevention of allergic sensitization in the healthy lung.[12,14] For example, AM down-regulate the antigen-presentation functions of dendritic cells (DC) that, *in vivo*, form a contiguous network within the respiratory epithelium and lung interstitium.[16,27] The mechanisms by which AM influence the antigen-presentation functions of DC remain largely unknown, but may well be mediated by AM-produced tumour necrosis factor α (TNF-α) and/or nitric oxide (NO).[16] In addition, since AM are scavenger cells that are inefficient in antigen presentation,[7,11–13] it is likely that AM suppress antibody responses by early uptake and degradation of antigen, thus preventing other, more potent, APCs to process and present the antigen to the immune system. Contrary to AM, however, IM have been proposed to play apositive role in processing of (particulate) antigens. For example, studies by Gong *et al.*[13] suggest that IM may augment interstitial DC function by the release of antigenic fragments derived from particulate antigens, which would then be presented by DC to T cells.

In agreement with Thepen *et al.*,[17] we observed an increase of the antibody response against an influenza subunit antigen, administered to the lower respiratory tract, in AM-depleted animals. This increase was most prominent at the level of the local s-IgA response. In addition, we found that AM, but also RAW 264 cells, have a high affinity for negatively charged liposomes, which is in agreement with reports by other investigators.[5,28] The high affinity of AM for negatively charged liposomes was also reflected in the altered morphology of the AM, isolated from animals which had received this type of liposomes. AM from mice which had received zwitterionic liposomes did not exhibit a substantially altered morphology. Previously, we have demonstrated that zwitterionic liposomes are ineffective in immune stimulation upon intranasal coadministration with influenza subunit antigen.[1] Therefore, it is likely that the uptake of negatively charged liposomes impairs the (normal) immunosuppressive activity of AM temporarily, resulting in an increased antibody response. As discussed above, this may involve a relief of the down-regulation of antigen-presentation functions of local DC. In addition, it is likely that a high uptake of negatively charged liposomes reduces the phagocytic activity and migratory behaviour of AM. In this respect, it can be argued that the reduced phagocytic capacity of AM would rescue antigen coadministered with the liposomes from uptake by the AM and early degradation. As a consequence, more antigen would be

available for DC, resulting in a more efficient antigen presentation, and hence, an increased antibody response.

We observed that the levels of serum IgG and s-IgA antibodies induced by immunization of AM-depleted mice with the subunit antigen alone did not reach the levels observed in control animals which received the liposome-supplemented antigen. In addition, administration of antigen supplemented with negatively charged liposomes to AM-depleted animals resulted in significantly higher levels of s-IgA than after administration of antigen alone. This additional stimulation of local immune responses could result from the uptake of liposomes by the residual AM that were not depleted (Fig. 3), resulting in a further interference with the residual AM-mediated immunosuppression.

ACKNOWLEDGMENTS

The investigations reported in this paper were supported by the Netherlands Organization for Scientific Research NWO (Research Grant 349–2301, under the auspices of the Technology Foundation STW, supporting AdH) and by Solvay Duphar B.V. in Weesp, the Netherlands (Research Grant to the University of Groningen).

REFERENCES

1. De Haan A., Geerligs H.J., Huchshorn J.P., Van Scharrenburg G.J.M., Palache A.M. & Wilschut J. (1995) Mucosal immunoadjuvant activity of liposomes: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with an influenza subunit vaccine and coadministered liposomes. *Vaccine* **13,** 155.
2. De Haan A., Renegar K.B., Small Jr. P.A. & Wilschut J. (1995) Induction of a secretory IgA response in the murine female urogenital tract by immunization of the lungs with liposome-supplemented viral subunit antigen. *Vaccine*, **13,** 613.
3. De Haan A., Tomee J.F.C., Huchshorn J.P. & Wilschut J. (1995) Liposomes as an immunoadjuvant system for stimulation of mucosal and systemic antibody responses against inactivated measles virus administered intranasally to mice. *Vaccine* **13,** 1320.
4. Van Rooijen N. (1990) Liposomes as carrier and immunoadjuvant of vaccine antigens. In: *Bacterial Vaccines* (ed. A. Mizrahi) p. 255. Alan R. Liss, Inc., New York.
5. Gregoriadis G. (1990) Immunological adjuvants: a role for liposomes. *Immunol Today* **11,** 89.
6. Alving C.R. (1992) Immunologic aspects of liposomes: presentation and processing of liposomal protein and phospholipid antigens. *Biochim Biophys Acta* **1113,** 307.
7. Brain J.D. (1988) Lung macrophages: how many kinds are there, what do they do?. *Am Rev Respir Dis* **137,** 507.
8. Reynolds H.Y. (1988) Normal and defective respiratory host defenses. In: *Respiratory Infections: Diagnosis and Management* (ed. J.E. Pennington), p. 1. Raven Press, New York.
9. Harmsen A.G., Muggenburg B.A., Snipes M.B. & Bice D.E. (1985) The role of macrophages in particle translocation from lungs to lymphs nodes. *Science* **230,** 1277.
10. Hunninghake G.W. (1987) Immunoregulatory functions of human alveolar macrophages. *Am Rev Respir Dis* **136,** 253.
11. Jeffrey T.J.F., Kern J.A., Elias J.A., Kamoun M., Daniele R.P. & Rossman M.D. (1987) Alveolar macrophages, blood monocytes, and density-fractionated alveolar macrophages differ in their ability to promote lymphocyte proliferation to mitogen and antigen. *Am Rev Respir Dis* **135,** 682.
12. Holt P.G. (1994) Down-regulation of immune responses in the lower respiratory tract: the role of alveolar macrophages. *Clin Exp Immunol* **63,** 261.
13. Gong J.L., McCarthy K.M., Rogers R.A. & Schneeberger E.E. (1994) Interstitial lung macrophages interact with dendritic cells to present antigenic peptides from particulate antigens to T cells. *Immunology* **81,** 343.
14. Holt P.G. & McMenamin C. (1989) Defence against allergic sensitization in the healthy lung: the role of inhalation tolerance. *Clin Exp Allergy* **19,** 255.
15. Strickland D.H., Thepen T., Kees U.R., Kraal G. & Holt, P.G. (1993) Regulation of T-cell function in lung tissue by pulmonary alveolar macrophages. *Immunology* **80,** 266.
16. Holt P.G., Oliver J., Bilyk N., McMenamin C., McMenamin P.G., Kraal G. & Thepen, T. (1993) Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells *in vivo* by resident alveolar macrophages. *J Exp Med* **177,** 397.
17. Thepen T., Van Rooijen N. & Kraal G. (1989) Alveolar macrophage elimination *in vivo* is associated with an increase in pulmonary immune response in mice. *J Exp Med* **170,** 499.
18. Van Rooijen N. (1989) The liposome-mediated macrophage 'suicide' technique. *J Immunol Methods* **124,** 1.
19. Van Rooijen N. (1993) Immunoadjuvant activities of liposomes: two different macrophage mediated mechanisms. *Vaccine* **11,** 1170.
20. Wood J.M., Schild G.C., Newman R.W. & Seagroatt V. (1977) An improved single radial immunodiffusion technique for the assay of influenza haemagglutinin antigen: application for potency determination of inactivated whole virus and subunit vaccines. *J Biol Stand* **5,** 237.
21. Böttcher C.J.F., Van Gent C.M. & Pries C.A. (1961) A rapid and sensitive submicro phosphorus determination. *Anal Chem Acta* **24,** 203.
22. Yetter R.A., Lehrer S., Ramphal R. & Small Jr., P.A. (1980) Outcome of influenza infection: effect of site of initial infection and heterotypic immunity. *Infect Immun* **29,** 654.
23. Wang F., Winter J.B., Dam M., Wildevuur C.R.H. & Prop J. (1992) Influence of interrupted pulmonary lymph drainage on antibody responses in hilar-stripped lungs. *J Heart Lung Transplant* **11,** s215.
24. Dal Monte P.R. & Szoka F.C. (1989) Antigen presentation by B cells and macrophages of cytochrome C and its antigenic fragment when conjugated to the surface of liposomes. *Vaccine* **7,** 401.
25. Dal Monte P.R. & Szoka F.C. (1989) Effect of liposome encapsulation on antigen presentation *in vitro*. Comparison of presentation by peritoneal macrophages and B cell tumors. *J Immunol* **142,** 1437.
26. Fortin A. & Therien H.M. (1993) Mechanism of liposome adjuvanticity: an *in vivo* approach. *Immunobiology* **188,** 316.
27. Holt P.G., Degebrodt A., O'Leary C., Krska K. & Plozza T. (1985) T-cell activation by antigen-presenting cells from lung tissue digests: suppression by endogenous macrophages. *Clin Exp Immunol* **62,** 586.
28. Monkkonen J., Valjakka R., Hakasalo M. & Urtti A. (1994) The effects of liposome surface charge and size on the intracellular delivery of chlodronate and gallium *in vitro*. *Int J Pharm* **107,** 189.