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Lipid Vesicle Size Determines the Th1 or Th2 Response to Entrapped Antigen

James M. Brewer, Laurence Tetley, James Richmond, Foo Y. Liew, and James Alexander

Understanding the factors that control the differential induction of Th1 and Th2 responses is a key immunologic objective with profound implications for vaccination and immunotherapy of infectious and autoimmune diseases. Using Ag formulated in lipid vesicles prepared from nonionic surfactants, we describe a novel mechanism influencing the balance of the Th1 or Th2 response. Our results indicate that inoculation of BALB/c mice with vesicles with a mean diameter ≥225 nm preferentially induces Th1 responses, as characterized by increased titers of IgG2a in plasma and elevated IFN-γ production by lymph node cells. However, preparation of the same quantity of Ag in vesicles with mean diameter of ≤155 nm induces a Th2 response, as identified by IgG1 in the absence of IgG2a production and elevated lymph node IL-5 production. Although large (≥225 nm) vesicles could induce IL-12 production, smaller vesicles (≤155 nm) could not. However, small vesicles did induce higher levels of IL-1β production by macrophages than larger vesicles. The role of IL-12 in this response was confirmed in IL-12-deficient mice, whose spleen cells failed to produce IFN-γ following in vivo priming with Ag prepared in large vesicles. Our results therefore indicate that macrophages respond to endocytosis of large or small vesicles by producing different patterns of cytokines that can subsequently direct the immune response toward a Th1 or a Th2 phenotype. The Journal of Immunology, 1998, 161: 4000–4007.

The reciprocal nature of the regulation of cell-mediated and humoral immune responses was first proposed in the early 1970s as a result of studies with chemically modified Salmonella flagellin (1). More recently, control of this relationship has been attributed to mutually antagonistic subsets of CD4+ Th lymphocytes. Activation of the Th1 subset is associated with the production of IFN-γ and IL-2 and the development of a classical cell-mediated immune response, such as delayed-type hypersensitivity, whereas activation of the Th2 subset and the subsequent production of cytokines such as IL-4, IL-5, IL-6, and IL-10 are associated with the development of classical humoral immune responses (2). Accordingly, the generation of a predominantly Th1 response is essential for the development of a protective immune response against obligate intracellular organisms such as Leishmania major (3), while the induction of a predominately Th2 response is more appropriate for the effective control of certain helminth infections (4). Although the available evidence indicates that Th1- and Th2-type cells develop from a common precursor (5), the elements that influence the preferential expansion of one subset rather than the other remain ill defined. Postulated factors include the population of accessory cells presenting the Ag (6, 7) and the presence of different costimulatory molecules (8) and cytokines in the cell microenvironment (9, 10). Using Ag formulated in lipid vesicles (11–13), we have identified a novel mechanism by which the balance of the Th1/Th2 response to an Ag can be influenced. Our data indicate that vesicles with a mean diameter ≥225 nm preferentially induce Th1-type responses, while the same quantity of Ag entrapped in vesicles with a mean diameter ≤155 nm induces Th2-type responses, as characterized by in vivo Ab subclass production and in vitro cytokine production. Further studies have revealed that the macrophage appears to play the central role in orchestrating this effect.

Materials and Methods

Lipid vesicle preparation

All glassware was heated at 180°C for 5 h to inactivate endotoxin, and autoclaved Elgastat Ultra High Purity water (Elga, Bucks, U.K.) was used to prepare solutions. Vesicles were prepared under aseptic conditions by the methods described previously (12) and were tested as endotoxin negative by the Limulus amoebocyte assay (Sigma, Poole, U.K.) Briefly, 150 μmol of 1-monopalmitoyl glycerol, cholesterol, and dicetyl phosphate (Sigma) were mixed in a 15-ml Pyrex test tube in the molar ratio 5:4:1, and then heated at 130°C in a dry-block (Grant Instruments, Cambridge, U.K.) until melted. Vesicles were formed when 2.5 ml of aqueous buffer (PBS; pH 7.4) was added, and the resulting suspension was vortexed vigorously for 1 min. After shaking the suspension at 60°C for 2 h, OVA (grade V, Sigma) was entrapped by freezing the Ag vesicle mixture in liquid nitrogen and thawing to 60°C five times. After an additional 2 h of shaking at 60°C, vesicle preparations were extruded through decreasing pore size polycarbonate filters (Costar, Bucks, U.K.) at 60°C in a thermostable extruder (Lipex Biomembranes, Vancouver, Canada) as described previously (14). After removing nonentrapped Ag by centrifuging at 100,000 × g for 45 min, the protein concentrations of the vesicle suspensions were determined using a modified ninyhydrin assay (15). Protein concentrations in the various vesicle preparations were then adjusted so all inoculations contained the same quantity of protein.

Electron microscopy

Small samples (∼10 μl) of the extruded vesicle preparations were sandwiched between the cleaned surfaces of pairs of copper support plates (Balzers, Furstentum, Leichtenstein). The vesicle suspension was then flash-frozen by immersion in liquid propane at −180°C and then transferred under liquid nitrogen into a fracturing device. After fracturing at −90°C under a vacuum of 4 × 10−6 Torr, the samples were shadowed...
immediately with evaporated platinum/carbon at 45° to the fracture surface and strengthened by coating with carbon evaporated at 90° to the fracture surface. The vesicle preparations were removed from the replicas by sequential washing with acetone/distilled water solutions of decreasing acetone concentration. After washing the replicas several times in distilled water, they were collected onto copper grids, dried, and examined by transmission electron microscopy.

Animals and inoculations

Female BALB/c mice were in-house bred at the University of Strathclyde and used when they were 8 to 10 wk old. Groups of five mice were inoculated s.c. with 0.1 ml of vesicle suspension containing 100 μg of OVA in PBS or prepared in vesicles. Inoculations were repeated after 2 wk, and blood was sampled for Ab determination 2 wk subsequently. For lymph node cytokine assays, groups of five animals were inoculated in each footpad with 10 μl of OVA (10 μg) in PBS or prepared in vesicles. Inguinal and popliteal lymph nodes were collected 10 to 14 days later, although previous studies using later time points have produced similar results (12), consistent with the observation that cytokine profiles following inoculation with Ag prepared in adjuvant are rapidly induced and stable (16).

IL-12 (p40)-deficient BALB/c mice (17) were donated by Dr. J. Ma-gran of Hoffmann-La Roche, Nutley, NJ. Normal BALB/c mice and control Bagg albino mice were bred and maintained at the Central Research Facility, University of Glasgow (Glasgow, Scotland). Groups of five mice were immunized s.c. with 10 μg of OVA in vesicles, and appropriate booster doses were administered 2 wk later. Spleens were collected after an additional 4 wk.

Plasma Ab determination

ELISAs were performed as described previously (11) to detect Ag-specific IgG, IgG1, and IgG2a in plasma. Briefly, flat-bottom polystyrene plates (Dynatech, Alexandria, VA) were coated overnight at 4°C with 100 μl of OVA (64 μg/ml in PBS; pH 9.0), and following blocking (18), 100-μl samples of plasma serially diluted in PBS/Tween were added to duplicate wells and incubated for 1 h at 37°C. One hundred microliters of HRP-conjugated goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates, Birmingham, AL) was added to each well at dilutions of 1/8000, 1/8000, and 1/800, respectively, in 75% PBS/25% sheep serum (Sigma). Alkaline phosphatase-streptavidin conjugate (PharMingen) was added to wells, and detection was performed using biotinylated polyclonal Abs (anti-mouse IgG1, 11B11, and 9A5, respectively) at predetermined concentrations. One hundred microliters of 1,1′-diotoxoyacetyl-3,3′,3′-tetramethylindocarbocyanine perchlorate (Molecular Probes, Eugene, OR)-labeled lipid vesicles (24) by J.774 macrophages was performed as described above, although the fluorescence intensities were corrected for surface-bound vesicles by subtracting the fluorescence intensity at time zero.

Peritoneal macrophage cultures

Female BALB/c mice were housed in-house bred at the University of Strathclyde and used when they were 8 to 10 wk old. Groups of five mice were inoculated s.c. with 0.1 ml of vesicle suspension containing 100 μg of OVA in PBS or prepared in vesicles. Inoculations were repeated after 2 wk, and blood was sampled for Ab determination 2 wk subsequently. For lymph node cytokine assays, groups of five animals were inoculated in each footpad with 10 μl of OVA (10 μg) in PBS or prepared in vesicles. Inguinal and popliteal lymph nodes were collected 10 to 14 days later, although previous studies using later time points have produced similar results (12), consistent with the observation that cytokine profiles following inoculation with Ag prepared in adjuvant are rapidly induced and stable (16).

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Cytokine assays

Levels of cytokines (IL-2, IL-5, and IFN-γ) were determined in cell culture supernatants by capture ELISA under the conditions described previously (12). Reagents for IL-1β and IL-4 analysis were purchased from Genzyme (Cambridge, MA) and used according to the manufacturer’s directions. IL-12 reagents were generously donated by Dr. Horst Bluethmann, Roche (Basel, Switzerland). Briefly, flat-bottom polystyrene plates were coated with 50 μl of IL-1β, IL-4, and IL-12 (p70) neutralizing mAbs (B122, 11B11, and 9A5, respectively) at predetermined concentrations. One hundred microliters of supernatants from standards and wells were added in duplicate to wells, and detection was performed using biotinylated polyclonal anti-mouse cytokine Abs (IL-1β and IL-4) or HRP-labeled mAb (IL-12; POD-5C3). Alkaline phosphatase-streptavidin conjugate (PharMingen) was used at a dilution of 1/2,000, and substrate (paranitrophenyl-phosphosphate, 1 mg/ml; Sigma) in glycine buffer (0.1 M; pH 10.4) was added. Absorbances were read at 405 nm on a Titer-Tek Multiskan plate reader (Flow Laboratories, Irvine, U.K.). For IL-12 analysis, the secondary Ab, HRP-labeled anti-p40 POD-5C3, was detected by incubation with substrate buffer prepared as described for determination of plasma Ab titers. Cytokine concentrations in the cell cultures were determined from the standard curve (regression coefficient, r = 0.970 or better). Comparisons between groups were made using Student’s t test.

Results

Electron microscopy

Analysis of vesicle preparations extruded through 800 nm (Fig. 1a), 400 nm (Fig. 1b), 200 nm (Fig. 1c), and 100 nm (Fig. 1d) pore size polycarbonate membranes was performed by electron microscopy. The micrographs demonstrate that vesicle characteristics are retained following extrusion and that extrusion through successively smaller pore size membranes results in correspondingly smaller vesicles. Mean vesicle diameters were estimated from the micrographs as being approximately 560 ± 60 nm (Fig. 1a) and 100 ± 10 nm (Fig. 1d). Photon correlation spectroscopy indicated that the mean size of preparation B was 225 ± 25 nm, that of preparation C was 155 ± 10 nm, and that of nonextruded vesicles was 3100 ± 660 nm in diameter.

Abbreviation used in this paper: HRP, horseradish peroxidase.
Effect of vesicle size on Th1/Th2-type responses

Reducing the size of the vesicle preparations did not significantly affect their overall adjuvant activity compared with that in nonextruded control vesicles as assessed by OVA-specific IgG titers (data not shown). Similarly, vesicle size had no significant effect on the production of OVA-specific IgG1, the vesicle preparations again inducing significantly higher IgG1 titers in mice than those detected following inoculation with OVA alone (Fig. 2A). In contrast, the production of OVA-specific IgG2a was very much a function of vesicle size (Fig. 2B). While no difference could be observed in IgG2a production in groups treated with 3100-, 560-, or 225-nm vesicles, significantly reduced titers of IgG2a were observed when OVA was prepared in NISV extruded through smaller pore size membranes (560 nm > 100 nm or 155 nm, p < 0.025; 225 nm > 100 nm or 155 nm, p < 0.025).

IFN-γ and IL-5 production in Con A- and OVA-stimulated lymph node cells isolated from mice 10 to 14 days after administration of Ag alone or prepared in different sizes of vesicle preparations was also compared (Fig. 2, C–E). Ag-stimulated production of IFN-γ increased with increasing vesicle size, such that only vesicles with mean diameters of 560 nm (p < 0.025) and 225 nm (p < 0.01), but not 155 nm, stimulated significantly higher levels of IFN-γ compared with inoculation of Ag alone. Furthermore, both 560- and 225-nm vesicle preparations produced significantly higher concentrations of IFN-γ than inoculation with 155-nm vesicles (Fig. 2D; p < 0.05 and p < 0.01, respectively). Similar results were obtained when Con A was employed as the in vitro stimulus (Fig. 2F). In contrast, IL-5 production by lymph node cells appeared to be preferentially stimulated by immunization with smaller vesicles, with 155-nm diameter vesicles producing significantly more IL-5 than 225-nm diameter vesicles (p < 0.025) or OVA alone (p < 0.05) following in vitro stimulation with Con A (Fig. 2E). Levels of this cytokine in Ag-stimulated cultures were not significantly greater than background levels (Fig. 2C). Thus, increasing vesicle size results in a switch from predominantly Th2-associated IL-5 production to Th1-associated IFN-γ production, with the switch in response occurring between 155- and 225-nm vesicle preparations.
Role of vesicle size in determining uptake by B cells and macrophages

Reducing vesicle size from 225 to 155 nm did not affect the ability of 1,774 macrophages or B cells to internalize lipid vesicles (Fig. 3). While B220-positive spleen cells did not accumulate either size of lipid vesicle compared with the pinocytic marker, FITC-dextran (Fig. 3A), 1,774 macrophages avidly ingested both sizes of lipid vesicle tested (Fig. 3B). The data shown are representative of three experiments, and in each experiment the number of positive J774 macrophages was in excess of 90%.

Role of the protein/lipid ratio in determining Th1/Th2 responses

Because reducing the size of lipid vesicles will effectively reduce the amount of protein delivered to APCs per vesicle, we examined how Th1/Th2 responses were affected by this parameter, known as the protein/lipid ratio, in vesicles with constant size (Fig. 4). Varying the protein/lipid ratio above and below normally employed levels (typically 1:30) had little effect on titers of OVA-specific IgG1 or IgG2a in mice inoculated twice with each preparation. In fact, as demonstrated in Figure 4, altering this parameter over a 50-fold range did not significantly affect the IgG1/IgG2a response in any fashion and certainly not in a manner similar to reducing vesicle size.

Effect of vesicle size on in vitro macrophage cytokine production

The ability of Ag-containing vesicle preparations to initiate macrophage cytokine production was compared with that of LPS from Salmonella abortus equi. Significant IL-1β production could be induced by each of preparations tested compared with cells incubated with medium alone (Fig. 5A; p < 0.01). However, cells incubated with 560-nm vesicles produced less IL-1β than cells treated with either 155-nm vesicles (p < 0.01) or LPS (p < 0.02). The levels of IL-1β induced by the 155-nm vesicles was not significantly different from those induced following incubation with LPS.
Although 560-nm vesicles did induce IL-12 production, no detectable IL-12 production was observed following incubation of cells with smaller, 155-nm diameter vesicles (Fig. 5B). The levels of IL-12 detected following incubation with 560-nm vesicles were significantly greater than those detected in cultures of untreated cells (p < 0.01) or cells treated with 155-nm vesicles (p < 0.01) or LPS (p < 0.01). As previously described (26), treatment of resident peritoneal macrophages with LPS did not induce detectable levels of IL-12 p70.

The role of IL-12 in lipid vesicle determined Th1/Th2 responses in vivo

To evaluate the in vivo significance of the in vitro-driven IL-12 response, we compared cytokine production by spleen cells of IL-12-deficient BALB/c mice with those of wild-type control mice (Fig. 6A). In agreement with the data presented above, spleen cells from wild-type mice inoculated with OVA entrapped in 560-nm vesicles produced significantly higher concentrations of IFN-γ than cells from mice immunized with OVA prepared in 155-nm vesicles following in vitro stimulation with Ag (p = 0.01). In contrast, although cells from the IL-12-deficient mice exhibited significant proliferation (Fig. 6B), they only produced low levels of IFN-γ regardless of whether they were from mice immunized with OVA entrapped in 560- or 155-nm vesicles (Fig. 6A).

Discussion

Inoculation of BALB/c mice with OVA entrapped in lipid vesicles of different sizes alters the subsequently induced immune response in a qualitative fashion. As has been described previously (14) and as characterized here by electron microscopy, the effect of extrusion of lipid vesicles through polycarbonate membranes was to reduce the diameter of the lipid vesicles in a manner proportional to the pore diameter of the membrane. When vesicles containing OVA were extruded through successively smaller sized pore membranes and inoculated s.c. into BALB/c mice, a size-dependent and significant reduction in the Ag-specific IgG1 detected 2 wk after secondary inoculations. B. Similarly, no significant alteration in IgG2a titer could be observed by varying the protein/lipid ratio in these studies. Results are expressed as the mean reciprocal end-point dilution ± SEM (n = 5).

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Th2-type cells (27, 28). Therefore, these results indicate that as vesicle size is reduced, the adjuvant effect produced by the vesicles switches from inducing a mixed Th1/Th2-type response characterized by B cell IgG1 and IgG2a production to the production of a Th2-dominated response in the absence of IgG2a. The critical factor in this switch appears to be the change in particle size that lies between mean diameters of 155 and 225 nm.

To further study this phenomenon, we have analyzed cytokine production by draining lymph node cells isolated from BALB/c mice 10 days after footpad inoculation with differently sized vesicles containing OVA. The production of the Th1-associated cytokine, IFN-γ (2, 29), was only elevated in the lymph node cells of mice inoculated with vesicles with a mean diameter of 225 nm or greater. Conversely, elevated levels of the Th2-associated cytokine, IL-5 (2, 29), could only be detected in mitogen-stimulated cultures of lymph nodes taken from mice following inoculation with smaller vesicles with a mean diameter of 155 nm or less. In agreement with the Ab data presented above, this switch in response appears to lie in between the mean vesicle diameters of 155 and 225 nm. However, the cytokine data also indicate that rather than a mixed Th1/Th2 response, vesicles with a diameter ≥225 nm induce a predominantly Th1-type response. Thus, the Th1 and Th2 responses induced by large and small vesicles, respectively, are more polarized than indicated by the Ab data. While we could not detect IL-4 (<50 pg/ml) in Ag-stimulated spleen cells from BALB/c mice immunized with either large or small vesicles (data not shown) this may merely reflect the sensitivity of the assay. However, we and others have demonstrated that Th2-type responses can still be generated in the absence of IL-4 (30–33) or its receptor.4

Previous studies have indicated that, unlike administration of soluble Ag alone, which induces neither a Th1- nor a Th2-biased

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response, high m.w. glutaraldehyde-polymerized OVA induces a strong Th1-like response in vivo (21). This change in response was associated with the increased ability of polymerized OVA to induce IFN-γ production and decreased uptake of the immunogen by B cells (34). While the physical size of the polymerized OVA particles was not determined and the effect of the change in composition of the formulation by the addition of glutaraldehyde could not be assessed (34), the data presented in our present report demonstrate that size is an important factor in altering the Th1 or Th2 balance of an immune response. Furthermore, the present study indicates that the critical cut-off in the ability to stimulate Th1-like as opposed to Th2-like responses lies between 155 and 225 nm. Intriguingly, this figure also corresponds to the lower limit of the macrophage phagocytic response (35, 36).

The ability to phagocytose large particles is one of the features that distinguishes macrophages from other APCs (37, 38), although some capacity for ingestion of particulate Ags has been demonstrated for dendritic cell progenitors in vitro (39, 40). In contrast, other APCs, such as B lymphocytes, cannot phagocytose (37, 38) and must presumably ingest Ag by pinocytic mechanisms, which effectively means they cannot internalize particles greater than approximately 150 nm (35, 36). A number of studies in vivo and in vitro demonstrate an essential role for phagocytic cells in both Th1 cell expansion (6, 7, 10, 41–44) and the processing of exogenous Ag via the endogenous pathway to stimulate CD8+ T cell expansion (36, 45–47). Similarly, B cells have been implicated in preferentially stimulating Th2 cell expansion (42, 48, 49).

In vivo studies have previously demonstrated that entrapment of Ag within liposomes, which requires phagocytic ingestion, totally inhibits the ability of B cells to present that Ag (37, 38). Therefore, it is possible that preparation of Ag in vesicles of different sizes alters the distribution of Ag among the APC populations. The crucial factor in this arrangement would then be whether the size of the particle made it more likely to be phagocytosed or pinocytosed. However, in vivo localization of horseradish peroxidase-labeled vesicles up to 24 h after administration demonstrated similar patterns of distribution for both large (560 nm) and small (155 nm) vesicles (data not shown). Furthermore, the ability of B220+ splenic B cells to acquire FITC-dextran, a marker of fluid phase endocytosis (23), could be totally inhibited by preparation of FITC-dextran in any size of vesicle. Alternatively, larger vesicles may be more avidly phagocytosed by macrophages than smaller vesicles; however, our experiments with J.774 macrophages demonstrate that this is clearly not the case. These observations suggest that the mechanism by which different sizes of vesicles induce Th1 or Th2 responses does not involve differential distribution of the vesicles among APC populations, and therefore that the ultimate destination for vesicles in vivo is most likely the macrophage.

A number of studies have suggested that the density of T cell epitopes on APCs can influence the Th1/Th2 bias of the developing T cell response (50, 51). It is possible, therefore, that the varying abilities of different sizes of vesicle to deliver protein to APCs may affect this parameter. To explore this possibility we prepared vesicles of constant size with different amounts of entrapped Ag. By manipulating the ratio of protein to lipid we demonstrated that across a large range of ratios the relative Th1/Th2 response was not affected. We would conclude from these data that the ability of different sizes of lipid vesicle to influence the Th1/Th2 response while not being mediated through variations in the amount of Ag per vesicle, is more likely to be influenced by the direct effects of vesicles on macrophages.

To determine the potential role of macrophages in the mechanism of Th1 or Th2 induction by vesicles we analyzed the abilities of different sizes of vesicles to induce the production of the T cell stimulatory cytokines IL-12 (52) and IL-10 (53) by macrophages in vitro. Our results indicate that while small vesicles induce high levels of IL-12 in macrophages, they cannot induce IL-12 production. In contrast, when macrophages were treated with large vesicles, both IL-12 and IL-10 were produced, although IL-10 levels were significantly lower than those observed for small vesicles. In agreement with this result, we have previously demonstrated the ability of large nonextruded vesicles administered i.p. to potentiate splenocyte IL-12 production upon in vitro restimulation (12). As macrophage-derived IL-12 is known to be an essential factor for the development of Th1 responses (53–56), these results would suggest a mechanism for the preferential generation of Th1 responses by large vesicles via induction of macrophage IL-12. The importance of IL-12 in the Th1 response induced by large vesicles was further confirmed in vivo in studies using IL-12 (p40)-deficient mice. These results demonstrated that the elevated production of IFN-γ observed in vitro with spleen cells from BALB/c mice inoculated with OVA prepared in large vesicles (560 nm) was absent in IL-12-deficient mice.

The endocytic pathways through which large and small vesicles are most likely to be sequestered may determine the differential responses of the macrophage to these stimuli. As large vesicles have a mean diameter above the lower limit of the phagocytic response (150 nm), they are likely to act as a phagocytic stimulus to macrophages. This would not be the case with smaller vesicles, which have a mean diameter below this limit. In this context, it is relevant that the ability of chitin particles to induce the production of IL-12 by spleen cells has recently been shown to be ablated by incubation with cytochalasin D (25), an inhibitor of phagocytosis (57). Collectively, these observations indicate a relationship between phagocytosis and the production of IL-12 and, consequently, the induction of Th1-type responses in vivo.

In conclusion, the study described here indicates that the same adjuvant can be physically manipulated to preferentially stimulate either a Th1- or a Th2-type response to an Ag. This capacity is a function of the size of the adjuvant particle, and our further analyses indicate a central role for macrophages in distinguishing these stimuli and influencing the generation of Ag-specific Th1- or Th2-type responses by the respective secretory or nonsecretion of IL-12.

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References


