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Virosome-mediated delivery of protein antigens to dendritic cells

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Abstract

Virosomes are reconstituted viral membranes in which protein can be encapsulated. Fusion-active virosomes, fusion-inactive virosomes and liposomes were used to study the conditions needed for delivery of encapsulated protein antigen ovalbumin (OVA) to dendritic cells (DCs) for MHC class I and II presentation. Fusion-active virosomes, but not fusion-inactive virosomes, were able to deliver OVA to DCs for MHC class I presentation at picomolar OVA concentrations. Fusion activity of virosomes was not required for MHC class II presentation of antigen. Therefore, virosomes are an efficient system for delivery of protein antigens for stimulation of both helper and CTL responses.
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1. Introduction

Effective vaccination against a protein antigen requires dendritic cells (DCs), which are essential antigen-presenting cells (APCs) in the induction of primary immune responses [1]. Immature DCs can effectively internalize and process antigens whereas mature DCs are very efficient in presentation of these antigens. Inflammatory signals, such as viral infection, double stranded RNA, bacterial products or cytokines, can induce DC maturation and upregulation of co-stimulatory molecules [2]. The final maturation of DCs is mediated by T cell–DC contact [3]. Uptake of antigen by DCs and its partial proteolysis in endosomes will result in MHC class II presentation of antigenic peptides to CD4+ helper T cells [1]. Stimulation of CD8+ T cells by class I MHC-associated peptides from exogenous antigen requires transport of the antigen to the cytosol of the APCs prior to its translocation to the endoplasmic reticulum for association with nascent MHC class I molecules [4]. Consequently, agents which augment delivery of exogenous antigen into the cytoplasm of APCs and thereby into the classical MHC class I route could be effective in induction of cytotoxic T lymphocyte (CTL) responses [5,6].

We are using virosomes to deliver antigen into the cytosol of APCs. Virosomes are reconstituted viral envelopes, which contain the cell binding and fusion proteins of the native virus but do not contain the genetic material of the virus. Therefore, virosomes made from influenza virus retain the cell entry and membrane fusion capacity of this virus [7,8]. Functionally reconstituted influenza virosomes will bind to sialic acid residues on the surface of cells and enter the cell via receptor-mediated endocytosis [9,10]. Upon endocytosis, the low pH in the endosomes induces fusion of the virosomal membrane with the endosomal membrane, causing the release of the contents of the virosome into the cytoplasm of the cell. The fusion process is mediated by hemagglutinin, the major envelope glycoprotein of influenza virus [11–13].

Previously we have shown that influenza virosomes can deliver whole proteins to the cytoplasm of cells [14,15]. Gelonin or subunit A of diphtheria toxin (DTA) were encapsulated in virosomes and upon incubation of cells with these virosomes cellular protein synthesis was inhibited. We have also shown that virosomes containing the cationic lipid DODAC in their membrane can bind plasmid DNA and deliver this DNA to transfect cells [16]. Both of these effects were dependent on the fusion activity of the virosomes, as they could be inhibited by pre-exposing the virosomes to low pH, resulting in irreversible inactivation of the hemagglutinin. These experiments demonstrate that virosome-encapsulated substances enter the cytosol of target cells, and indicate that fusion of the virosomal membrane with the endosomal membrane is needed for delivery.

Likewise, it is to be expected that protein antigens encapsulated in virosomes can be delivered into the cytosol of an APC and therefore into the classical MHC class I

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presentation pathway. Since not all of the virosomes are likely to fuse with the endosomal membrane, some of the virosomes will continue into the late endosomal/lysosomal route. These virosomes and their contents are expected to be degraded in these compartments and their peptides will thus become available for loading onto MHC class II molecules. Antigen delivered to an APC by a fusogenic virosome is therefore expected to be presented in association with both MHC class I and II molecules, resulting in stimulation of both CD4+ and CD8+ T cells. This property makes virosomes an excellent antigen delivery system for stimulation of both helper and cytotoxic responses.

Liposomes are vesicles composed of lipids, which, unlike virosomes, do not contain viral glycoproteins [17]. "Classical" or conventional liposomes, composed of phospholipids and cholesterol are not able to fuse with the endosomal membrane when taken up by APCs. Thus, in the absence of cellular mechanisms in APCs that may exist for the purpose of mediating cytoplasmic delivery of exogenous protein antigens, liposome contents are not expected to be delivered to the cytoplasm. Liposomes can be associated with ligands, such as antibody, which will increase their binding to and uptake by APCs, at least in vitro. Previously, we have described targeting of antigen-containing liposomes to the FcγR of DCs by opsonizing the liposomes with IgG. This targeting results in uptake of the liposomes and presentation of peptides of the antigen in the context of MHC class II [18]. Uptake of FcγR-targeted liposomes also resulted in the presentation of antigenic peptides in the context of MHC class I, but only when DCs were maintained in culture for longer than about 12 days or at higher antigen concentrations [19].

To investigate whether arrival in the cytoplasm of short-term cultured DCs is sufficient for the presentation of an exogenous antigen in the context of MHC class I, we compare the efficiency of MHC class I and II presentation of a whole protein antigen ovalbumin (OVA) by DCs when delivered by fusion-competent virosomes, fusion-incompetent virosomes or FcγR-targeted liposomes. Only fusion-competent virosomes were capable of inducing potent MHC class I presentation of OVA peptide by these cells. Fusion activity was not required for MHC class II presentation of OVA peptide.

2. Materials and methods

2.1. Mice

OT-1 mice (a kind gift from Matthias Merkenschlager, MRC, London, UK) are transgenic for an $\alpha\beta$ TCR specific for the chicken OVA peptide 257–264 (SIINFEKL) in the context of H-2K^b [20]. They were maintained on the C57BL/6 background and identified by FACS analysis as those mice in which a majority of peripheral blood CD8$^+$ cells express Vα2. T cells obtained from the spleens of 6–12 weeks old transgenic mice were purified by passage over nylon wool columns.

2.2. DCs

DCs were derived from bone marrow of (CBA $\times$ B6) F1 mice (Iffa-Credo, l'Arbresle, France). Bone marrow cells were cultured in DMEM supplemented with 10% FCS, antibiotics, 2 mM glutamine, 50 μM 2-ME and 30% conditioned medium from NIH3T3 cells transfected with the gene for GM-CSF (provided by Jean Davoust, Centre d'Immunologie de Marseille-Luminy, Marseille, France) as described [21]. After 3 days of culture the cells were diluted 1:1 in the same medium and after an additional 3–4 days of culture the plastic non-adherent cells were harvested and washed. These cells were re-suspended in RPMI medium supplemented with 5% FCS, antibiotics, 50 μM 2-ME and 2 mM glutamine (supplemented RPMI) and used in experiments. The percentages of FcγR-, 33D1- and CD11c-expressing cells in these preparations were typically 80–90% as determined by FACS analysis.

2.3. Cell lines

The CD4+ T cell hybridoma OT4H.1D5 is specific for I-A^b plus an undefined OVA peptide [22]. These cells were cultured in supplemented RPMI. IL-2 dependent CTLL cells were incubated in the same medium supplemented with 10 U/ml of recombinant mouse IL-2 (Roche, Basel, Switzerland). The RMA thymoma cell line (H-2^b haplotype) was also cultured in supplemented RPMI.

2.4. Virosomes

Virosomes were prepared from A/Johannesburg/33 influenza virus (gift from Solvay Pharmaceuticals, Weesp, The Netherlands) as described before [7,8]. Briefly, virus (1.5 μmol of viral membrane phospholipid) was solubilized in 100 mM octa (ethyleneglycol)-*n*-dodecyl monoether ($C_{12}E_8$) (Nikkol, Tokyo, Japan) and the nucleocapsid was removed from the preparation by ultracentrifugation. The supernatant containing the phospholipids and glycoproteins of the influenza virus in $C_{12}E_8$ was added to OVA (grade VII) (Sigma) at a concentration of 3 mg OVA/ml (68 μM). OVA–FITC (Molecular Probes, Leiden, The Netherlands) was used in quantitative fluorescence measurements to determine the amount of OVA encapsulated in virosomes (and liposomes, see Section 2.5). Subsequently, the detergent $C_{12}E_8$ was extracted from the supernatant with BioBeads SM2 (Bio-Rad, Hercules, CA) resulting in the formation of virosomes. The virosomes were separated from non-encapsulated OVA on a discontinuous sucrose density gradient and an optiprep flotation gradient. Finally, the virosomes were dialyzed against buffer containing 5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA (HNE buffer) and sterilized by filtration through

a 0.45 μm filter. We determined the amount of virosomal phospholipid phosphate by phosphate analysis [23] to be able to use the same amounts of empty virosomes and OVA virosomes in the experiments. Empty virosomes and OVA containing virosomes were analyzed by negative stain electron microscopy using 2% ammonium molybdate (pH 7.4).

2.5. Liposomes

Liposomes were prepared as described before [19,24]. Briefly, liposomes were composed of 65% (mol/mol) dimyristoyl phosphatidylcholine, 34.5% cholesterol (Sigma) and 0.5% DNP–caproyl-phosphatidylethanolamine (DNP–cap PE) (Molecular Probes). Lipids evaporated from chloroform:methanol (9:1 (v/v)) were exposed to a solution of 30 mg/ml OVA (680 μM) (grade VII) (Sigma) in phosphate buffered saline (PBS), together with an OVA–FITC tracer. After repeated freeze thaw cycles, liposomes were formed by extrusion (Extruder, Lipex Biomembranes, Vancouver, Canada) through polycarbonate filters of 200 nm pore size at 40 °C, followed by gel filtration over Sepharose 4B columns to eliminate unencapsulated solute. The liposomes were sterilized by filtration through 0.45 μm filters and OVA content of the liposomes was determined as described above. Anti-DNP (U7.27.7, mouse IgG2a) was used to target the DNP-bearing liposomes to the FcγR as described [18].

2.6. Fusion assay and fusion inactivation

Virosome fusion with erythrocyte ghosts was measured using a lipid mixing assay based on pyrene excimer fluorescence [14]. The virosomes that were used in this fusion assay were co-reconstituted with 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*syn*-glycero-3-phosphocholine (pyrene PC, 10 mol% with respect to total viral lipid), (Molecular Probes). Fusion was continuously monitored at 37 °C by the decrease of pyrene excimer fluorescence at an excitation wavelength of 345 nm and an emission wavelength of 480 nm in an AB2 fluorometer (SLM/Aminco, Urbana, IL). At $t = 0$ s, fusion was initiated by the addition of 35 μl 0.1 M morpholinoethanesulfonic acid (MES), 0.1 M acetic acid, pre-titrated with NaOH to achieve the final desired pH. At $t = 210$ s, 35 μl of 200 mM $C_{12}E_8$ was added to achieve infinite dilution of the pyrene PC. The extent of fusion was calculated based on the decrease in pyrene excimer fluorescence at 480 nm, taking the excimer fluorescence of unfused virosomes as the 0% fusion level and the fluorescence after addition of $C_{12}E_8$ as the 100% fusion level. Virosomes were fusion inactivated by an incubation at pH 5.0, 37 °C for 20 min. This pH was achieved by adding a small pre-titrated volume of 0.1 M MES, 0.1 M HAc to the virosome suspension. After fusion-inactivation, the pH of the virosome solution was adjusted to pH 7.4 with a pre-titrated volume of 0.2 M Tris buffer (pH 8.5).

2.7. Antigen presentation assay

DCs (2×10^4) were plated in duplicate wells of 96-well flat-bottom tissue culture plates in 100 μl supplemented RPMI. Free OVA, virosome-encapsulated OVA or liposome-encapsulated OVA was added overnight, at the indicated concentrations. In the case of liposome-encapsulated OVA, the incubation was performed in the presence or absence of targeting (anti-DNP) or control Ab (5 μg/ml). DCs were then washed before the addition of 1×10^4 OT4H.1D5 T cell hybridoma cells or 2×10^4 OT-1 transgenic T cells. After 48 h of incubation in supplemented RPMI the undiluted supernatant fluids were harvested and frozen. IL-2 content in the supernatants was measured by adding 1×10^4 CTLL cells overnight, followed by a pulse of [^{3}H] thymidine (1 μCi per well) for an additional 6 h and measurement of the [^{3}H] thymidine incorporation by CTLL cells. IL-2 values were derived from a standard curve using CTLL in the presence of recombinant mouse IL-2 (Roche).

CD8+ T cell cytotoxicity was evaluated by the JAM test [25]. Five thousand RMA cells were [^{3}H] thymidine-labeled (0.25 μCi/ml) and OVA peptide SIINFEKL-pulsed (1 μM) overnight. Then, the RMA cells were washed and added to wells containing T cells, which had been incubated under various experimental conditions for 5 days. After 5 h incubation, cells were harvested and radioactivity in DNA was counted by scintillation. Under these conditions, 5000 RMA cells incorporated 2000–10,000 cpm and spontaneous lysis in the presence of DCs incubated without antigen was indistinguishable from that of RMA cells incubated alone. This value was taken as 100% viable cells. Maximum lysis using these cells was about 70% of incorporated [^{3}H] thymidine, obtained by incubation of cells in Triton X-100 and DNase. Under our experimental conditions, OT-I but not 1D5 cells were cytotoxic (data not shown).

2.8. FACS analysis and confocal microscopy

Binding of virosomes and liposomes to DCs was analyzed by incubation of DCs with 10 nM of OVA–FITC in virosomes or liposomes for 1 h at 37 °C. Fusion-active or fusion-inactive virosomes were used. The liposomes were incubated with the DCs in the presence or absence of 5 μg/ml anti-DNP Ab. Then, cells were washed, fixed in 2% formaldehyde and analyzed in a FACScan® cytofluorimeter (Becton Dickinson, Franklin Lakes, NJ).

The expression of cellular markers on DCs was determined after incubation of DCs with 10 nM of OVA in fusion-active virosomes, in fusion-inactive virosomes, in non-targeted liposomes or in FcγR-targeted liposomes for 24 h. As a control, DCs were incubated in medium or LPS (5 μg/ml) for the same amount of time. After the 24 h incubation the DCs were washed and stained or incubated for an additional 24 h in supplemented RPMI. Cell surface

staining was performed using the following antibodies: anti-MHC class I (FITC-labeled mouse IgG2b mAb 5F1, anti-H-2^b), anti MHC class II (FITC-labeled mouse IgG2a mAb 10.2.16, anti-I-A^k), anti-CD40 (FITC-labeled rat IgG2a mAb FgK45), anti-ICAM-1, anti-B7.1 and anti-B7.2 (FITC conjugated mAbs from Pharmingen). The control antibody used was anti-CD69. After 1 h at 4 °C, cells were washed, fixed with 2% *para*-formaldehyde and analyzed in a FACScan® cytofluorimeter. The results were analyzed using CELLQuest™ software. The gate was placed on cells expressing the FcγR and this gate was used for all analysis. Of the DC population used in these studies (7-day culture protocol) about 80% of the living cells were in this gate.

For confocal analysis, DCs were attached to glass coverslips coated with poly-L-lysine (Sigma) (0.01% (w/v) in distilled water) for 20 min in medium without FCS at room temperature, followed by 15 min incubation in complete medium. After washing, DCs were incubated with 1 nM of OVA–FITC in fusion-active or fusion-inactive virosomes for 4 h at 37 °C. The cells were washed again and fixed in 4% *para*-formaldehyde for 15 min. After washing, confocal laser scanning microscopy was performed on the cells as previously described using a Leica TCS 4D instrument (Leica, Heidelberg, Germany) [21].

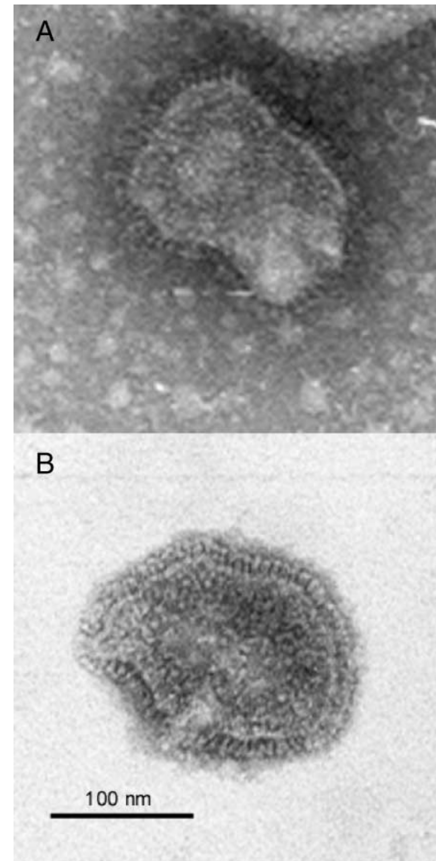

Fig. 1. Electron microscopy of influenza virosomes. The morphology of OVA virosomes (A) is similar to that of empty virosomes (B).

3. Results

3.1. Characterization of the virosomes

The morphology of influenza virosomes was similar to that of native virus as determined by transmission electron microscopy (Fig. 1). The images clearly demonstrate the hemagglutinin and neuraminidase spikes on the virosomes. No morphological difference could be seen between the empty virosomes and the OVA virosomes. The mean diameter of the virosomes was about 200 nm, comparable to that of the liposomes we used.

The pH-dependent fusion activity of virosomes was determined using a lipid mixing assay with erythrocyte ghosts as target membranes. Previous studies have shown that empty virosomes reconstituted from influenza virus have the same pH-dependent fusion characteristics as the native influenza virus [26–28]. The optimal pH for fusion of A/Johannesburg influenza virus is pH 5.5. Virosomes containing OVA and empty virosomes displayed a similar fusion activity at this optimal pH, indicating that encapsulation of OVA has no effect on the fusion activity of the virosomes (Fig. 2). Virosomes were fusion-inactivated by a pre-incubation at low pH to determine the role of the fusion activity of virosomes for the delivery of encapsulated protein to the cytoplasm of DCs. After this treatment all of the fusion activity of the virosomes was eliminated (Fig. 2, Curve C).

3.2. Fusion-active virosomes, fusion-inactive virosomes and FcγR-targeted liposomes bind to DCs

Binding of the virosomes and FcγR-targeted liposomes to DCs was determined to ensure that fusion-active

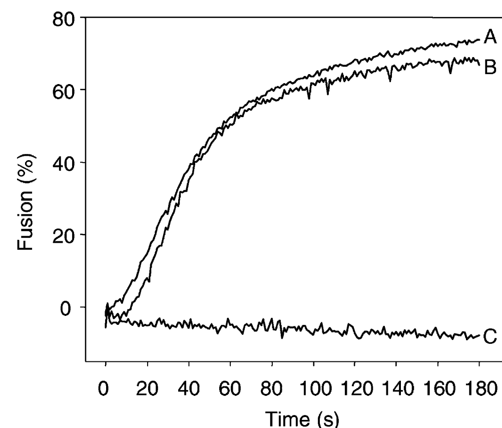

Fig. 2. Fusion activity of virosomes determined by a fluorescence excimer quenching assay. Fusion activity of empty virosomes (A) and OVA virosomes (B) are similar at pH 5.5. Virosomes were fusion-inactivated by a pre-incubation at pH 5.1 (C).

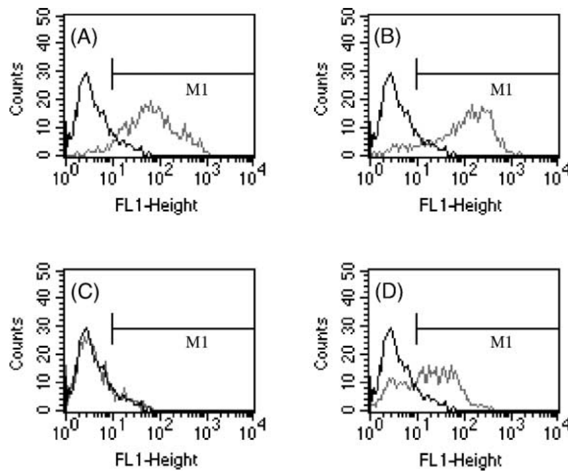

Fig. 3. Binding of liposomes and virosomes to DCs. Black lines represent DCs without OVA–FITC. Gray lines represent DCs incubated for 1 h at 37 °C with 10 nM of OVA–FITC in fusion-active virosomes (A), fusion-inactive virosomes (B), DNP-liposomes in the absence of anti-DNP Ab (C) FcγR-targeted liposomes (DNP-liposomes in the presence of anti-DNP Ab) (D).

virosomes, fusion-inactive virosomes and FcγR-targeted liposomes bind to DCs to similar extents. An equal amount of OVA–FITC in fusion-active virosomes, fusion-inactive virosomes, liposomes or FcγR-targeted liposomes was incubated with DCs at 37 °C for 1 h. After washing, the FITC fluorescence associated with DCs was measured by FACS analysis (Fig. 3). The amount of OVA–FITC associated with DCs was similar for fusion-active and fusion-inactive virosomes (97 and 94% binding). Irreversible denaturation of the influenza virus hemagglutinin, therefore, does not affect its capacity to bind to cell surface proteins containing sialic acid residues. Binding of FcγR-targeted liposomes was slightly less (64%). As previously reported [19], non-targeted liposomes did not bind to DCs in the absence of the opsonizing anti-DNP. Any major differences in antigen presentation by DCs upon incubation with OVA in virosomes or FcγR-targeted liposomes can therefore not be ascribed to differences in the level of binding to DCs.

3.3. Incubation of DCs with fusion-active OVA virosomes, fusion-inactive OVA virosomes or FcγR-targeted OVA liposomes results in upregulation of expression of cellular markers

The expression of different cellular markers was determined after incubation of DCs with either fusion-active OVA virosomes, fusion-inactive OVA virosomes, OVA liposomes or FcγR-targeted OVA liposomes. As a positive control, DCs were incubated with LPS. There was no difference in expression levels of MHC class I and II between the DCs incubated with fusion-active virosomes or fusion-inactive virosomes (Fig. 4). Also, no differences were observed between the expression of MHC class I and II after incubation with the FcγR-targeted liposomes or either of the virosome preparations. Incubation with LPS resulted in higher expression levels of MHC class I and II as compared to incubation with the virosome and liposome preparations, especially at the 24 h time-point. The expression of the other cellular markers tested (CD40, ICAM-1, B7.1 and B7.2) was increased to the same extent upon incubation with virosomes or FcγR-targeted liposomes. Upon LPS incubation the DCs upregulated CD40 and ICAM-1 at both time-points tested and B7.1 and B7.2 only at the 48 h time-point. Incubation of DCs with non-targeted OVA liposomes did not result in upregulation of any of the cellular markers tested. Thus, the expression of all of the tested maturation markers on DCs was increased to the same extent upon incubation with OVA virosomes, either fusion-active or fusion-inactive, as with FcγR-targeted OVA liposomes.

3.4. Fusion-active, but not fusion-inactive virosomes can deliver their contents into the cytoplasm of DCs

DCs were incubated with fusion-active and fusion-inactive OVA–FITC virosomes for 4 h to investigate if the delivery of OVA–FITC to the cytoplasm of DCs was dependent on the fusion activity of the virosomes. DCs incubated with fusion-active OVA–FITC virosomes showed diffuse cytoplasmic fluorescence (Fig. 5A). The nuclei of two of these cells can be clearly distinguished, demonstrating that the plane in which these cells were photographed contains the nucleus. The FITC staining observed is therefore in the cytosol of these cells and not on their membrane. In contrast, DCs incubated with fusion-inactive OVA–FITC virosomes showed primarily vesicular fluorescence, indicative of endosomal localization of the marker (Fig. 5B). As a control, DCs were incubated with FcγR-targeted OVA–FITC liposomes. Similar to the results observed with fusion-inactive OVA–FITC virosomes fluorescence was primarily vesicular upon incubation of DCs with FcγR-targeted OVA–FITC liposomes (data not shown).

3.5. Fusion activity of virosomes is required for efficient MHC class I presentation of encapsulated antigen

DCs were incubated with fusion-active OVA virosomes or fusion-inactive OVA virosomes and subsequently co-cultured with the H-2K^b plus SIINFEKL-specific T cells from OT-1 mice to investigate differences in MHC class I presentation. As a control, DCs were incubated with FcγR-targeted OVA liposomes, OVA liposomes, free OVA and empty virosomes. Incubation of DCs with fusion-active virosomes resulted in MHC class I presentation at picomolar concentrations of OVA (Fig. 6A). Upon incubation of DCs with free OVA, fusion-inactive OVA virosomes or FcγR-targeted OVA liposomes, no stimulation of OT-1 cells was detected at the concentrations tested. As expected, empty virosomes did not induce MHC class I presentation of OVA. These responses were observed by analysis of secretion of IL-2, measured at 48 h.

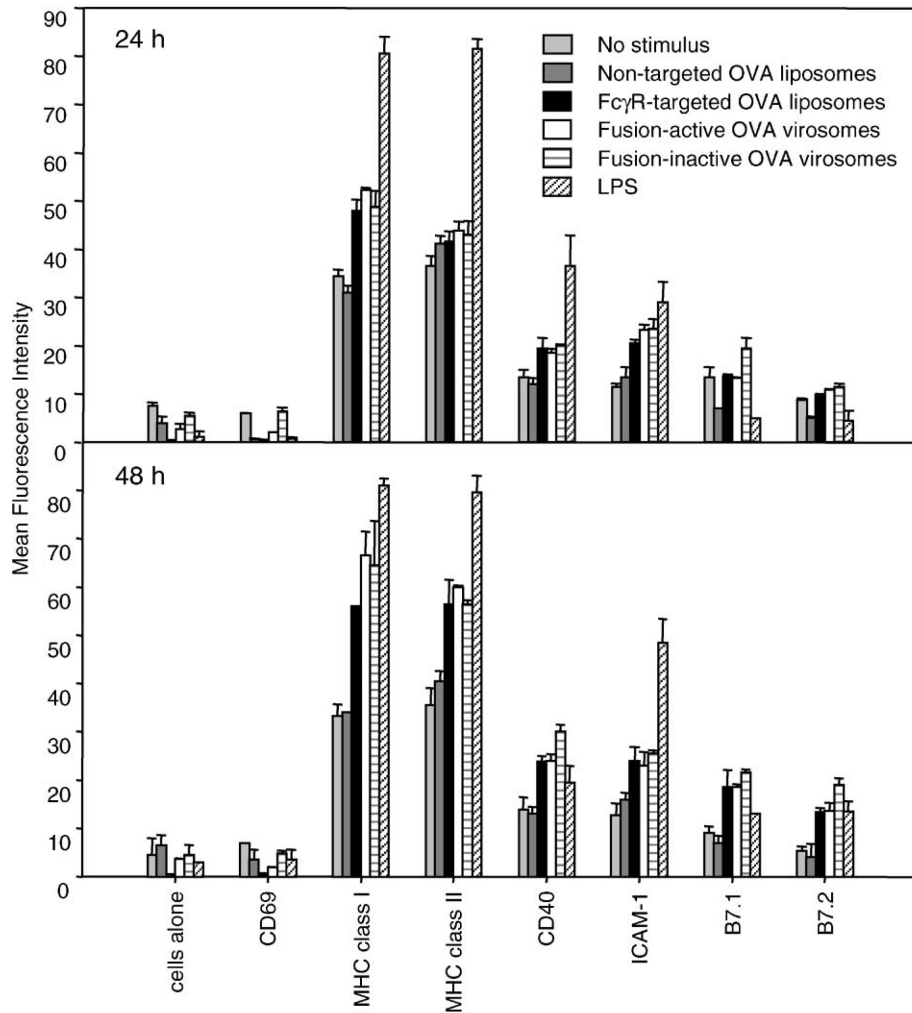

Fig. 4. Fusion-active, fusion-inactive virosomes and FcγR-targeted liposomes stimulate expression of MHC class I and II, CD40, ICAM-1, B7.1 and B7.2 on DCs. DCs were incubated for 24 h with 10 nM of OVA in fusion-active virosomes, fusion-inactive virosomes, non-targeted liposomes or FcγR-targeted liposomes. Cells were incubated with 5 μg/ml LPS as a positive control and with supplemented RPMI as a negative control. After incubation, DCs were washed and used for immunofluorescence or cultured for an additional 24 h in supplemented RPMI before use in immunofluorescence. Immunofluorescence staining was performed as described in Section 2.

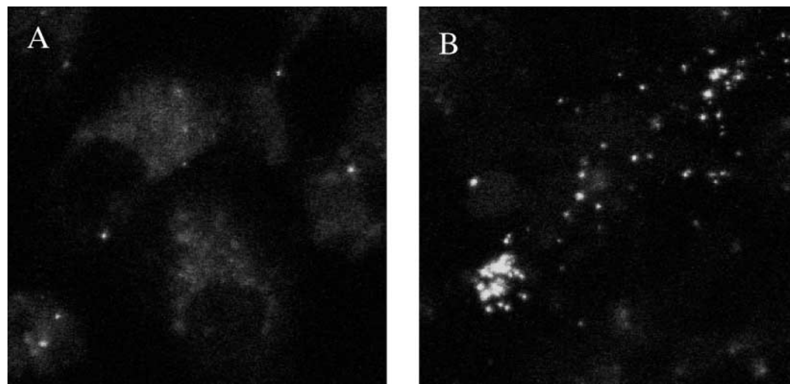

Fig. 5. Delivery of OVA to DCs. CBA/B6 DCs were incubated with fusion-active (A) or fusion-inactive (B) OVA virosomes for 4 h at 37 °C and prepared for confocal microscopy as described in Section 2.

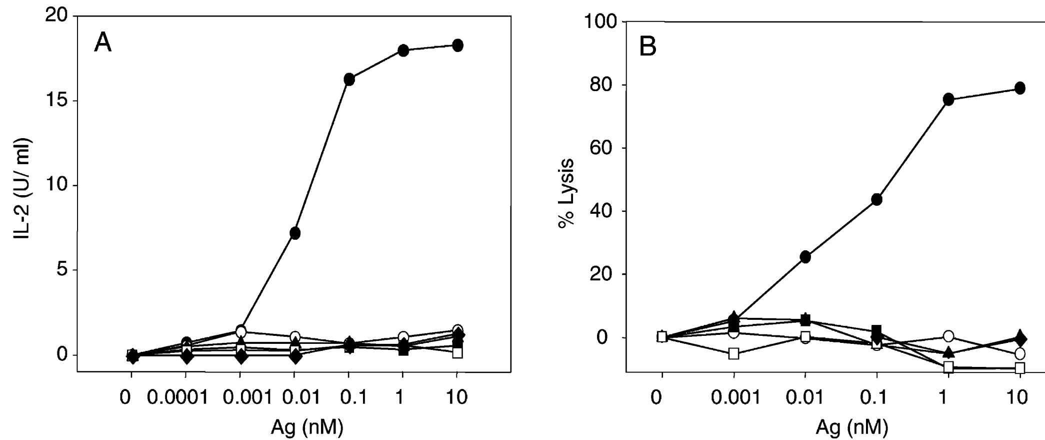

Fig. 6. Fusion activity of virosomes is required for efficient MHC class I presentation of encapsulated antigen. DCs were incubated with free OVA (◆), fusion-active OVA virosomes (●), fusion-inactive OVA virosomes (○), OVA liposomes (□), FcγR-targeted OVA liposomes (■) or empty virosomes (▲) and subsequently co-cultured with the H-2K^b plus SIINFEKL-specific T cells from OT-1 mice to investigate differences in MHC class I presentation. MHC class I presentation was evaluated by production of IL-2 by the OT-1 cells (A) and by induction of cytotoxicity against SIINFEKL-pulsed RMA cells (B). Results shown are representative of four experiments for IL-2 production and representative of two experiments for the cytotoxicity assay.

Subsequent induction of cytotoxicity of OT-1 T cells against SIINFEKL-pulsed RMA cells was measured at 5 days by a JAM test. Here, only OT-1 cells co-cultured with DCs that were incubated with fusion-active OVA virosomes showed cytotoxicity against the target cells (Fig. 6B). Similar to the production of IL-2, a response was already observed if the OT-1 cells were stimulated by DCs that were incubated with picomolar concentrations of OVA in fusion-active virosomes.

3.6. *No fusion activity is needed for efficient MHC class II presentation of encapsulated antigen*

DCs were incubated with OVA-containing fusion-active or fusion-inactive virosomes. As a control, the DCs were incubated with OVA in FcγR-targeted or non-targeted liposomes. Subsequently, DCs were co-cultured with the T cell hybridoma 1D5 to detect MHC class II presentation of OVA peptide. Efficient MHC class II presentation of OVA peptide was observed at picomolar concentrations of OVA for the FcγR-targeted liposomes, the fusion-active virosomes and the fusion-inactive virosomes (Fig. 7). Again, no response was observed with free OVA, non-targeted OVA liposomes or empty virosomes at the concentrations tested. The failure of empty virosomes to generate MHC class I or II presentation of OVA peptides indicates that virosomes do not contain chicken OVA from the eggs on which the native influenza virus was cultured. Western blot analysis of empty virosomes confirmed this observation (data not shown). Furthermore, it shows that in vitro proliferation of T cells in response to virosomes does not occur under these experimental conditions.

Thus, fusion activity is not important for efficient MHC class II presentation of an antigen by DCs, provided that the antigen binds to cells and is endocytosed.

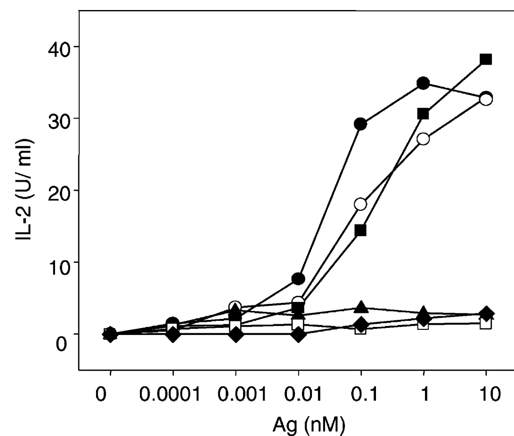

Fig. 7. Efficient MHC class II presentation of encapsulated antigen by DCs. DCs were incubated with free OVA (◆), fusion-active OVA virosomes (●), fusion-inactive OVA virosomes (○), OVA liposomes (□), FcγR-targeted OVA liposomes (■) or empty virosomes (▲) and subsequently co-cultured with the T cell hybridoma 1D5 to investigate differences in MHC class II presentation. Results shown are representative of four experiments.

4. Discussion

The experiments described in this paper show that fusion-active virosomes are highly effective in their delivery of encapsulated protein antigen for MHC class I presentation by bone marrow-derived DCs. DCs cultured in the presence of picomolar concentrations of OVA encapsulated in fusion-active virosomes are able to stimulate specific CD8+ T cells. Moreover, these CD8+ T cells are primed to become CTLs, as shown by killing of SIINFEKL-loaded target cells. MHC class I presentation depends on the fusion activity of the virosomes. In this study, fusion-inactive virosomes or FcγR-targeted liposomes were not able to

generate MHC class I presentation of OVA peptide by DCs in vitro at the antigen concentrations tested (up to 10 nM). We previously demonstrated that higher antigen concentrations of free OVA and liposomal OVA are required for efficient MHC class I presentation by DCs [19]. Since the fusion-active and fusion-inactive virosomes bind to the same extent to DCs, the capacity to fuse is responsible for the effective MHC class I presentation of antigen delivered by virosomes. After binding to sialic acid residues on the surface of cells, influenza virosomes, as the influenza virus, are taken up via receptor-mediated endocytosis. The low pH inside the endosomes triggers conformational changes in the influenza virus hemagglutinin [12]. These changes expose the fusion peptide, which mediates the fusion of the virosomal membrane with the endosomal membrane. Fusion of these membranes causes the release of the contents of the virosome into the cytoplasm of the cell, thereby permitting the entry of protein encapsulated in the virosome into the classical MHC class I processing pathway.

Strong CTL responses and the maintenance of T cell memory require not only presentation of the antigen in MHC class I but also CD4+ T helper cell activity and therefore presentation of antigen peptides in the context of MHC class II [29]. MHC class II presentation of the model antigen OVA is as efficient in fusion-inactive virosomes as in fusion-active virosomes or FcγR-targeted liposomes, all of which are taken up by receptor-mediated endocytosis. This indicates that fusion, as expected, is not needed for this process. As shown previously, untargeted OVA liposomes can induce MHC class II presentation of OVA peptide at higher antigen concentrations [19].

Since maturation and expression of co-stimulatory molecules of DCs are necessary for effective MHC class I and II presentation of antigen to naïve T cells, we also determined the level of expression of maturation markers on DCs after incubation with virosomes or FcγR-targeted liposomes. Our results show that fusion-active virosomes, fusion-inactive virosomes and FcγR-targeted liposomes do not differ in their capacity to bind to DCs and induce expression of MHC class I, MHC class II, CD40, ICAM-1, B7.1 or B7.2 on DCs. Thus, the more effective MHC class I presentation of OVA peptide by DCs in response to fusion-active OVA virosomes as compared to fusion-inactive OVA virosomes or OVA in FcγR-targeted liposomes is not due to differences in binding or upregulation of maturation markers.

Although in our present study FcγR-targeted liposomes were unable to deliver OVA for efficient MHC class I presentation, in DCs cultured in the presence of GM-CSF for longer periods than 7 days, the delivery of OVA by FcγR-targeted liposomes approached the efficiency of delivery by fusion-active virosomes reported here [19]. A possible mechanism for such an increase in delivery efficiency with cell maturation is the transport pathway that has been described in DCs which allows antigens to escape from the endosome into the cytosol [19,30]. This pathway, the mechanism of which is as yet undefined, could be responsible for the processing of OVA for MHC class I presentation upon uptake of FcγR-targeted OVA liposomes by DCs that have been cultured for longer periods of time (12 days). Fusion-active virosomes do not need this special pathway. Virosomes are able to introduce their contents into the cytosol of DCs and thus into the conventional MHC class I pathway at earlier stages of DC development.

Several studies on cells other than DCs have used carriers that can deliver their contents into the cytoplasm. Nakanishi et al. described the in vitro delivery of OVA in fusogenic liposomes to EL-4 cells to sensitize these cells as targets for MHC class I-restricted killing by OVA-specific CTL [31]. The fusogenic liposomes were prepared by the incubation of liposomes with Sendai virus envelopes [32]. These liposomes therefore contain genetic material of Sendai virus. The viral RNA is inactivated by UV irradiation before use of the fusogenic liposomes. Furthermore, compared to our fusion-active virosomes, relatively high concentrations (nM) of OVA were needed for MHC class I presentation in these experiments. Also, pH-sensitive liposomes have been used to deliver antigen for MHC class I presentation in vitro and in vivo. These liposomes destabilize upon protonation and release their contents into the cytosol. Sensitization of EL-4 cells for CTL recognition has been observed with pH-sensitive liposomes at micromolar concentrations, which is much less efficient than the MHC class I presentation induced with fusion-active virosomes [33,34]. Using macrophages as an APC, pH-sensitive liposomes could deliver OVA for MHC class I and II presentation to T cell hybridomas at nM concentrations in vitro, whereas pH-insensitive liposomes could only deliver their contents for MHC class II presentation [35]. Therefore, both fusogenic virosomes and pH-sensitive liposomes can deliver their contents to cells for MHC class I presentation. However, fusogenic virosomes are more efficient in delivering their contents to cells for MHC class I presentation. The ability of virosomes to bind to sialic acid residues on cells and to be taken up by receptor-mediated endocytosis probably accounts for their superior delivery capacity compared to conventional liposomes.

Previously, we have shown the efficiency of fusion-active virosomes in delivering peptide antigen in vivo [36]. Immunization of mice with a peptide of the influenza virus nucleoprotein (NP) encapsulated in fusion-active virosomes resulted in the induction of a potent CTL response. In contrast, fusion-inactive virosomes containing NP peptide or empty virosomes admixed with NP peptide failed to induce a CTL response. Thus, fusion activity of the virosomes is essential, both for delivery of a peptide antigen in vivo for induction of a CTL response and for induction of MHC class I presentation of a whole protein antigen in vitro. In this paper we have shown efficient delivery of a whole protein antigen by virosomes for presentation in MHC class I and II in vitro. The fact that virosomes can efficiently deliver their contents into the MHC class I and II route of DCs makes

them excellent candidates for use as a vaccine delivery system.

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