Chapter 5

Virosome-mediated delivery of protein antigens in vivo: efficient induction of class I MHC-restricted cytotoxic T lymphocyte activity

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Vaccine, in press
Abstract

Induction of CTL responses against protein antigens is an important aim in vaccine development. In this paper we present fusion-active virosomes as a vaccine delivery system capable of efficient induction of CTL responses in vivo. Virosomes are reconstituted viral membranes which do not contain the genetic material of the virus they are derived from. Foreign macromolecules, including protein antigens, can be encapsulated in virosomes during the reconstitution process. Functionally reconstituted virosomes retain the cell binding and fusion characteristics of the native virus. Thus, upon uptake by cells through receptor-mediated endocytosis, virosomes will deliver their content to the cell cytosol. In a previous study, we demonstrated that protein antigens delivered in this manner to dendritic cells are efficiently processed for both MHC class I and II presentation. Here, we studied in vivo induction of cellular immune responses against viroside-encapsulated ovalbumin (OVA) in mice. As little as 0.75 µg OVA delivered by fusion-active virosomes was sufficient to induce a powerful class I MHC-restricted CTL response. All immunization routes that were used (i.m., i.p. and s.c.) resulted in efficient induction of CTL activity. The CTL induced were cytotoxic in a standard 51Cr-release assay and produced IFNγ in response to OVA peptide. Thus, virosomes represent an ideal antigen delivery system for induction of cellular immunity against encapsulated protein antigens.
Introduction

Cellular immunity, cytotoxic T lymphocyte (CTL) activity in particular, plays a crucial role in the clearance of viral infections and the control of tumor development. Therefore, induction of CTL activity is a major goal of vaccine development. Yet, priming of CTL has been difficult to achieve, particularly with classical inactivated vaccine formulations, whereas such inactivated vaccines generally do represent excellent preparations for induction of antibody responses [recently reviewed in Seder 2000]. For many years, this lack of efficient induction of CTL with inactivated vaccines has been thought to be due to their inability to replicate. Indeed, replicating virus was considered to be essential for generating antigen within the cytosol of virus-infected antigen-presenting cells (APC), such that efficient processing of antigen-derived peptides in the context of MHC class I molecules could occur [Braciale 1978, Heemels 1995]. Later it was demonstrated that antigen from inactivated virus that does not replicate but is capable of entering the cytosol can also access the MHC class I presentation route [Yewdell 1988, Hosaka 1985]. In fact, Bender and colleagues showed that dendritic cells (DC), which are considered to be the most important cell type in CTL priming, present antigen from live and inactivated virus with equal efficiency [Banchereau 1998, Théry 2001, Bender 1995]. Recent views on antigen processing emphasize the ability of APC, DC in particular, to acquire and process exogenous protein antigens for MHC class I presentation in a process referred to as cross-presentation [Carbone 1998, Larsson 2003]. These observations indicate that the introduction of antigen into the cytosol of APC suffices for presentation of antigen in the context of MHC class I molecules and priming of CTL.

Within the context of these novel views on antigen processing and presentation, the question emerges again why it has been so difficult to raise CTL responses against inactivated antigen formulations. The answer is probably related to the issue of delivery of the antigen. In order to elicit a potent immune response antigen should be targeted to DC and be delivered efficiently to the DC cytosol in order to access the MHC class I presentation route. Here, we present reconstituted influenza virus envelopes, so-called virosomes, as antigen delivery vehicles that, by virtue of their membrane fusion activity, are capable of introduction of encapsulated protein antigens into the cytosol of DC, thus efficiently priming a class I MHC-restricted CTL response in vivo.

Influenza A virus is an enveloped negative-strand RNA virus belonging to the family Orthomyxoviridae. Entry of the virus into its host cells and delivery of the viral genome to the cell cytosol is mediated by hemagglutinin (HA), the major viral envelope glycoprotein [Matlin 1981, Skehel 2000]. The penetration process involves initial binding of HA to sialic acid residues on the target cell surface. Subsequently, the virus is internalized by receptor-mediated endocytosis, and routed to the endosomal cell compartment. The mildly acidic pH in the lumen of the endosomes triggers a
conformational change in HA such that it achieves its fusion-active state [Skehel 2000]. Fusion of the viral envelope with the endosomal membrane then results in delivery of the viral nucleocapsid to the target cell cytosol.

Virosomes, which have been described first by Almeida et al. in 1975, are reconstituted viral envelopes, which lack the genetic material of the virus but do contain the glycoproteins of the virus they are derived from [Almeida 1975]. We have developed a protocol for the functional reconstitution of virosomes from influenza virus [Stegmann 1987]. This protocol relies on detergent solubilization of the viral envelope, removal of the nucleocapsid, and subsequent reconstitution of the viral membrane proteins, particularly HA, by specific adsorption of the detergent to a polystyrene resin [Stegmann 1993, Bron 1993]. During this reconstitution process foreign molecules, including protein antigens, may be encapsulated within the virosomal lumen. The virosomes, thus produced, retain the receptor-binding and membrane fusion activity of the native virus, due to preservation of the conformational integrity of the viral HA. Accordingly, functionally reconstituted virosomes have the capacity to deliver encapsulated compounds to the cytosol of target cells [Schoen 1993, Bron 1994].

In earlier experiments we have shown that, in vitro, virosomes efficiently deliver their contents to DC for MHC class I and II presentation [Bungener 2002a]. In these studies, active introduction of encapsulated antigen into the DC cytosol, as achieved with fusion-active virosomes, was found to be essential for presentation in the context of MHC class I. In contrast, fusion-active and fusion-inactivated virosomes were found to be equally efficient in delivery of antigen for presentation in the context of MHC class II molecules which involves antigen processing within the endosome itself. In addition to introduction of antigen into the MHC class I and II presentation pathway virosomes were also shown to induce maturation of DC as measured by upregulation of CD40, ICAM-1, B7.1 and B7.2 in these in vitro studies [Bungener 2002a]. In earlier immunization experiments, we have demonstrated that virosomes are suitable carriers for the delivery of antigenic peptides to MHC class I molecules not only in vitro but also in vivo. Immunization of mice with virosomes containing a peptide derived from the influenza nucleoprotein (NP) resulted in successful priming of NP-specific CTL activity [Arkema 2000]. As for the induction of MHC class I presentation of ovalbumin (OVA) by DC in vitro, the induction of these NP-specific CTL was dependent on the ability of the virosomes to fuse with the endosomal membrane.

In the present study, we investigated the ability of influenza virosomes to deliver an intact protein antigen, OVA, for CTL induction in vivo. It is demonstrated that mice immunized with OVA-containing virosomes mount a strong OVA-specific MHC class I-restricted CTL response, as assessed in cytotoxicity and ELISPOT assays as well as by MHC tetramer staining of CD8+ T cells. Fusion activity of the virosomes, although not essential for induction of a response, was clearly beneficial for optimal CTL priming. In addition, virosomes appear to have strong adjuvant activity. Our observations
indicate that virosomes are promising candidates for CTL-inducing vaccines due to their ability to deliver encapsulated antigens to the cytosol of APC in vivo.

**Material and Methods**

**Virosomes**

Purified influenza virus of the H3N2 subtype (A/Johannesburg/33/94 or A/Panama/2007/99) was kindly provided by Solvay Pharmaceuticals (Weesp, The Netherlands). Where indicated, virosomes were prepared from virus inactivated by treatment with $\beta$-propiolactone (BPL). For this inactivation, virus equivalent to 1.5 $\mu$mol of viral phospholipid was incubated with BPL at a final concentration of 0.1% v/v. Inactivation was carried out for 24 h at room temperature under continuous stirring, maintaining a neutral pH during the entire incubation. Virus inactivation was confirmed by Solvay Pharmaceuticals by standard titration of the virus preparation in embryonated chicken eggs. Virus inactivation with BPL according to this protocol results in virus still capable of fusion.

Virosomes were prepared essentially as described previously [Stegmann 1987, Bron 1993, Bungener 2002a, Huckriede 2003]. In short, virus (1.5 $\mu$mol of viral membrane phospholipid) was solubilized in 100 mM octa(ethyleneglycol)-n-dodecyl monoether (C$_{12}$E$_8$), (Calbiochem, San Diego, CA) in buffer containing 5 mM Hepes, 150 mM NaCl and 0.1 mM EDTA (HNE buffer). 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 Chemical Co, St. Louis, MO) to achieve a final concentration of 3 mg/ml. This concentration of OVA corresponds to 2.1 mg/ 1.5 $\mu$mol of viral membrane phospholipid. Subsequently, the detergent C$_{12}$E$_8$ was extracted from the supernatant with BioBeads SM2 (Bio-Rad, Hercules, CA) resulting in the formation of OVA-containing virosomes. The virosomes were separated from non-encapsulated OVA by a discontinuous sucrose density gradient and an OptiPrep® flotation gradient. Finally, the virosomes were dialyzed against HNE buffer and sterilized by filtration through a 0.45 $\mu$m filter.

Complete removal of non-encapsulated OVA was verified by analyzing OVA-containing virosomes on an analytical continuous sucrose density gradient (10-60%, w/v, sucrose in HNE, run for 60 h in a Beckman SW50 rotor); followed by determination of the phospholipid, total protein and OVA contents in all fractions of the gradient. This analysis showed the virosomal phospholipid, protein and OVA migrating in a single band in the gradient (results not shown), indicating that all OVA in the final sample was physically associated with the virosomes.

Virosomal phospholipid content was determined by phosphate analysis [Böttcher 1961]. Virosomal protein (mainly HA) was determined according to Lowry [Peterson
The amount of OVA in the virosome preparations was determined by encapsulating FITC-labeled OVA (Molecular Probes, Leiden, The Netherlands) and comparing the fluorescence intensity in the virosome sample with that of a reference series of OVA-FITC ranging from 0 to 10 µg/ml. The fluorescence intensities of virosomes and OVA-FITC calibration samples were determined, in triplicate, in an FL 500 Microplate Fluorescence Reader (Bio-Tek Instruments, Winooski, VT) at excitation and emission wavelengths of 485 and 530 nm, respectively. In the calibration curve, the fluorescence intensity of OVA-FITC increased linearly with the concentration (correlation coefficient >0.99). Measurements were done both in the absence or presence of 100 mM C_{12}E_{8}, the latter added to ensure complete solubilization of the virosomes and liberation of the encapsulated OVA-FITC. However, the C_{12}E_{8} did not have any significant effect on the fluorescence intensity.

Fusion assay and fusion-inactivation of virosomes
Virosome fusion with erythrocyte ghosts was measured using a lipid mixing assay based on pyrene excimer fluorescence [Stegmann 1993]. Pyrene-PC labeling and fusion monitoring was performed as described earlier [Bungener 2002a]. Virosomes were fusion-inactivated by incubation at pH 5.0, 37°C for 20 minutes in the absence of target membranes. The conformation of the influenza virus hemagglutinin of the H3 subtype is irreversibly changed during this incubation, resulting in fusion-inactivation [Korte 1999]. The pH of 5.0 was achieved by adding a small pre-titrated volume of 0.1 M morpholinoethanesulfonic acid-0.1 M acetic acid 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). Fusion-inactivation of the virosomes was confirmed by the fusion assay.

Animals and immunizations
Specified-pathogen-free female C57BL/6 mice were purchased from Harlan CPB (Zeist, The Netherlands) and used at 8 to 10 weeks of age. The protocol for the animal experiments described in this paper was approved by the Animal Experimentation Ethical Committee of the University of Groningen. Mice were immunized intraperitoneally (i.p.), subcutaneously (s.c.) or intramuscularly (i.m.) with fusion-active or fusion-inactivated OVA virosomes using different doses of viral phospholipids and boosted once two weeks later. As a comparison, mice were immunized with 100 µg of heat-aggregated OVA [Speidel 1997] in the presence or absence of incomplete Freund's adjuvant (IFA). Fusion-active empty virosomes admixed with 10 µg of heat-denatured OVA were used in another comparison experiment. In every experiment mice injected with buffer were used as a negative control. One week after the booster immunization, mice were bled under anesthesia, sacrificed and spleens were
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harvested. Spleen cells were isolated and used in the ELISPot, tetramer and CTL assays.

Cell lines
EL-4 is a C57BL/6 mouse lymphoma cell line (H-2b). EG7OVA is a stable transfectant of EL-4, expressing the cDNA of chicken OVA [Moore 1988]. Both cell lines were cultured in IMDM Glutamax medium (Life Technologies, Paisley, UK) supplemented with 10% FCS (PAA laboratories, Linz, Austria), 100 U/ml penicillin (Life Technologies), 100 µg/ml streptomycin (Life Technologies) and 50 mM β-mercaptoethanol.

CTL assay
Spleen cells were stimulated for 6-7 days with irradiated (100 Gy) EG7OVA cells at an effector-to-stimulator ratio of 25:1 in 25 cm² culture flasks, placed upright. Two days before the ⁵¹Cr release assay, 4 U/ml of recombinant human IL-2 (Strathmann Biotech, Hamburg, Germany) was added to the cultures. CTL activity was determined in a standard 4 h ⁵¹Cr release assay using EG7OVA cells or SIINFEKL-loaded EL-4 cells as targets. EL-4 cells without peptide were used as negative control target cells. ⁵¹Cr release of EL-4 cells without peptide was never above 15%. The target cells were labeled for 1 h with 3.7 MBq ⁵¹Cr per 1x10⁶ cells in 100 µl of medium using ⁵¹Cr-labeled NaCrO₄ (ICN, Costa Mesa, CA). SIINFEKL-loaded EL-4 cells were obtained by adding SIINFEKL at a final concentration of 30 µg/ml during the ⁵¹Cr labeling period. Specific lysis was calculated according to the following formula: % specific lysis = (experimental release – spontaneous release)/ (maximal release – spontaneous release) x 100%. Spontaneous release was determined from target cells incubated without effector cells and maximal release was determined from target cells incubated with medium containing 0.5% Triton X-100. All measurements were performed in triplicate. Spontaneous ⁵¹Cr release was always <15% and the standard errors of the means of the triplicate determinations were <10% of the value of the mean.

CD8⁺ or CD4⁺ cells were depleted from the effector cells by adding 1.5 µg of rat IgG2a antibody specific for mouse CD8 or mouse CD4 (both Pharmingen) to each well of effector cells 3 h before the addition of ⁵¹Cr labeled target cells.

IFNγ ELISpot assay
ELISpot analyses were performed according to a protocol adapted from the method described by Miyahira [Miyahira 1995]. ELISA plates (Greiner, Alphen a/d Rijn, The Netherlands) were coated with purified anti-mouse IFNγ (rat IgG1) (Pharmingen, San Diego, CA) for at least 1 h at 37°C. Plates were washed three times with sterile PBS-Tween (PBS + 0.02% Tween 20) and incubated with blocking buffer (PBS containing 4% RIA Grade BSA) for 1 h. Spleen cells were plated in different quantities in medium containing 5% FCS and incubated overnight with or without 100 ng/ml of the OVA
peptide 257-264 (SIINFEKL, produced by H. Hilkmann, Netherlands Cancer Institute, Amsterdam, The Netherlands). Subsequently, cells were lysed by a 10-min incubation in water and plates were washed five times with PBS-Tween. IFNγ was detected using biotinylated anti-mouse IFNγ antibody and streptavidin-alkaline phosphatase (Pharmingen). The substrate for the alkaline phosphatase was 1 mg/ml 5-bromo-4-chloro-3-indolylphosphate in water containing 6 mg/ml agarose (Sigma), 9.2 mg/ml 2-amino-2-methyl-1-propanol (Sigma) and 0.08 µl/ml Triton X-405. Spots were developed for 3 h at 37°C and counted using a dissection microscope. Background (spleen cells incubated without the SIINFEKL peptide) was less than 5 spots per 10⁶ cells plated. This background was subtracted from the number of spots observed in wells containing spleen cells incubated with peptide to obtain the number of IFNγ-secreting cells.

**Tetramer analysis**
To analyze the number of CD8⁺ T cells specific for the OVA peptide SIINFEKL we used Kb-SIINFEKL tetramers produced in the laboratory of Dr. T. Schumacher (Netherlands Cancer Institute, Amsterdam, The Netherlands). Spleen cells were washed with FACS buffer (PBS containing 0.5% BSA and 0.02% sodium azide) and stained with FITC-conjugated anti-CD8a (Pharmingen) together with PE-conjugated Kb-SIINFEKL tetramers for 20 minutes at 4°C. Spleen cells were washed three times and analyzed by flow cytometry (ELITE, Coulter). Live cells were selected based on propidium iodide exclusion.

**Statistical analysis**
The unpaired Student’s t-test was used to determine if the difference in specific lysis observed between groups of mice was significant. A value of p <0.05 was considered significant.

**Results**

**Induction of CTL activity by immunization of mice with OVA-containing virosomes**
In a previous study, we have demonstrated that influenza-derived virosomes have the capacity to deliver encapsulated OVA to the cytosol of cultured murine DC, as evidenced by the efficient processing and presentation of the OVA-derived peptide epitope SIINFEKL in the context of MHC class I molecules on the DC surface [Bungener 2002a]. In the present study, we immunized mice with OVA-containing virosomes to determine whether virosomes are capable of priming class I MHC-restricted CTL activity *in vivo.*
OVA-containing virosomes were prepared using a 3 mg/ml concentration of OVA during the encapsulation protocol. Under these conditions the typical encapsulation efficiency was 50 µg OVA/µmol phospholipid (or 0.04 µg OVA/µg of virosomal protein), as assessed by encapsulation of FITC-labeled OVA and subsequent determination of the OVA-FITC fluorescence intensity of the virosomes as described in Materials and Methods. Based on the approximation that on average a viroside has a diameter of 200 nm with 100,000 phospholipid molecules per particle, the encapsulated amount of OVA corresponds to approximately 225 OVA molecules per virosome. Theoretically, the volume per µmol of phospholipid of membrane vesicles with a mean diameter of 200 nm is approximately 10 µl [Wilschut 1982]. This would result in an encapsulation of 30 µg of OVA per µmol of phospholipid based on the concentration of 3 mg/ml OVA used during the encapsulation. In the OVA-FITC assay a concentration of 50 µg OVA per µmol of phospholipids was found, suggesting that a fraction of up to 40% of the virosome-encapsulated OVA was associated with the virosomal membrane. It is possible that a minor fraction of this membrane-associated OVA was bound to the external surface of the virosomes. However, if so, this does not affect the membrane fusion activity of the virosomes (membrane fusion activity of OVA-containing virosomes is presented in Figure 1 of [Bungener 2002a]).

Mice were immunized twice, i.p., with 2.5 µg of OVA encapsulated in virosomes. A control received 100 µg of free OVA in the presence of incomplete Freund’s adjuvant (IFA), while also a buffer control was included. Induction of CTL activity was evaluated in a standard 51Cr-release assay using EL-4 cells loaded with the OVA peptide SIINFEKL as targets. EL-4 cells without peptide were used as a negative control in the assay. Figure 1A shows that immunization of mice with OVA-containing virosomes resulted in efficient induction of OVA-specific CTL activity. On the other hand, immunization with a 40-fold higher dose of free heat-aggregated OVA in the presence of IFA was ineffective. Similarly low responses were observed after immunization with 100 µg of heat-aggregated free OVA in the absence of IFA (results not shown). These results indicate that virosomes have the capacity to prime cytolytic activity against an encapsulated protein antigen in vivo.

Although the protocol for virosome preparation, involving disruption of the viral envelope with an excess of detergent, ensures that no active virus is retained in the virosome preparation, future clinical virosomal formulations will likely be prepared from inactivated influenza virus. We therefore evaluated whether virosomes prepared from virus inactivated by treatment with β-propiolactone (BPL) are also capable of inducing CTL responses against encapsulated OVA. As shown in Figure 1B, immunization with virosomes prepared from BPL-inactivated virus resulted in the same strong induction of OVA-specific CTL as immunization with virosomes from active virus. This result indicates that the BPL inactivation protocol has no effect on the ability of virosomes to deliver encapsulated antigen for MHC class I presentation in vivo.
Figure 1. Induction of CTL activity by immunization of mice with OVA-containing virosomes from active or BPL-inactivated influenza virus. Mice were immunized and boosted, i.p., with 2.5 µg of OVA encapsulated in virosomes prepared from active virus (squares in panel A, n=3) or in virosomes prepared from BPL-inactivated virus (squares in panel B, n=3). This dose of OVA in virosomes corresponds to 62.5 µg of viral protein. As a control, a mouse was immunized and boosted with 100 µg of heat-denatured OVA admixed with IFA (circles in panel A, n=1). The booster was given two weeks after the primary immunization. One week after the booster, mice were sacrificed and splenocytes were restimulated in vitro for 6 days with irradiated EG7OVA cells. After restimulation, effector cells were harvested and incubated for 4 h with ⁵¹Cr-labeled EL-4 cells with (closed symbols) or without (open symbols) the OVA peptide SIINFEKL. Data represent mean percentages of specific lysis in each group.

Characterization of the effector cell population
Using MHC tetramer staining, we further characterized the effector cell population activated by immunization of mice with OVA-containing virosomes (2.5 µg of OVA). Spleen cells of mice were stained with PE-labeled MHC tetramers containing the SIINFEKL peptide and with FITC-labeled anti-CD8 antibody directly after harvest (day 0) or after one week of in vitro stimulation with OVA-expressing EG7OVA cells (day 7) (Figure 2A). The tetramers used caused some background staining resulting in a small population of PE⁺/FITC⁻ cells in buffer control mice (mean frequency 1.6 +/- 0.2% of CD8⁺ cells in freshly isolated cells and 1.7 +/- 0.4% in restimulated cells). This background staining has also been observed by others and is due to aspecific binding of the tetramers (T. Schumacher, personal communication). In mice immunized with OVA-virosomes the population of PE⁺/FITC⁻ spleen cells was much larger than in buffer-injected mice. In freshly isolated spleen cells from these mice the frequency of OVA-specific CTL precursors was 3.5 +/- 1.0%. This population was considerably expanded during in vitro restimulation resulting in 28.1 +/- 8.5% of the restimulated CTL reacting with the tetramers.
Figure 2. Characterization of the effector cell population from mice immunized with OVA-containing virosomes by MHC tetramer staining (A), ELISPOT assay (B) and treatment with anti-CD8 prior to and during $^{51}$Cr-release assay (C). Mice were immunized and boosted as described in Figure 1.

For MHC tetramer staining (panel A), spleen cells of immunized mice were incubated with K$^b$-SIINFEKL tetramers directly after isolation (day 0) or after standard restimulation with irradiated EG7-OVA cells (day 7). Results shown are of mice representative for each group.

For the ELISPOT assay (panel B), freshly isolated splenocytes were incubated overnight with or without 100 ng/ml of the OVA peptide SIINFEKL and the numbers of IFN-$\gamma$-secreting spleen cells were calculated by subtracting the number of spots obtained without peptide stimulation (background, always below 5) from the number of spots obtained with peptide stimulation. The number of IFN-$\gamma$-producing cells in splenocytes from mice immunized with 2.5 µg OVA in virosomes (black bars) was compared with the corresponding number in mice immunized with 100 µg of heat-denatured OVA (white bars). All values are averages of triplicate determinations.

In the experiment shown in panel C, restimulated spleen cells from mice immunized with OVA-containing virosomes were treated with 1.5 µg of rat IgG2a antibody specific for mouse CD8 (grey bars) or mouse CD4 (white bars) added to the wells 3 h before the addition of $^{51}$Cr-labeled target cells. As a control, cells were incubated without blocking antibody (black bars). Shown are the mean percentages of specific lysis of SIINFEKL-loaded EL-4 cells at an effector:target ratio of 30:1 of mice injected twice with OVA virosomes ($n=7$). The percentages of specific lysis without antibody or in the presence of anti-CD4 were significantly different from the specific lysis obtained in the presence of anti-CD8 ($p < 0.001$). CTL activity was determined as described in the legend to Figure 1.
We further evaluated the effector cell population(s) activated by viroside-mediated immunization by determining the frequency of IFN$\gamma$-producing cells in the spleens of mice immunized with OVA-containing virosomes (2.5 µg OVA) or 100 µg of heat-aggregated free OVA in an ELISPOT assay. This assay involves stimulation of spleen cell populations with SIINFEKL peptide and subsequent determination of the number of IFN$\gamma$-producing cells using a biotinylated anti-IFN$\gamma$ antibody and streptavidin-labeled alkaline phosphatase. The results of this ELISPOT assay correlated well with the results of the tetramer staining and the $^{51}$Cr release assay. While somewhat variable within each experimental group, the frequency of IFN$\gamma$-producing cells in spleen cells of mice immunized with OVA-containing virosomes was much higher than the corresponding frequency observed in mice immunized with 100 µg of heat-denatured OVA (Figure 2B). No IFN$\gamma$-secreting cells were detected when mice were injected with buffer only.

Finally, we determined whether lysis of target cells, as measured by the $^{51}$Cr release assay is mediated by CD8$^+$ T cells. For this purpose, the effector cells in the $^{51}$Cr-release assay were pre-incubated with an antibody against CD8, and an antibody against CD4, respectively. Specific lysis of OVA-expressing target cells was inhibited significantly (p <0.001) upon pretreatment of the effector cells with the CD8 antibody (Figure 2C). No decrease in cytotoxicity was observed when the effector cells were incubated with anti-CD4.

Taken together, these results indicate that immunization of mice with virosomes containing OVA efficiently primes and activates CD8$^+$ T cells that are directed against the well-described K$^b$-restricted OVA epitope SIINFEKL. These cells are capable of producing IFN$\gamma$ and of lysing target cells presenting the OVA peptide.

**Effects of route of immunization and antigen dose**

In human vaccination protocols the preferred route of immunization is i.m. injection. Accordingly, in our previous study involving immunization of mice with influenza NP peptide, the virosomes were administered i.m. [Arkema 2000]. To determine if this is the most effective route of immunization for protein-containing virosomes, mice were immunized i.p., i.m. or s.c. with OVA-containing virosomes, and the CTL response was evaluated in the $^{51}$Cr-release assay, as described above. Immunization by all three routes resulted in efficient priming of CTL responses against OVA (Figure 3A). I.m. and i.p. immunization were equally effective, while the s.c. route was slightly less effective.

In order to gain more insight in the efficiency of CTL induction by virosomes, we immunized mice with different doses of OVA (0.25 µg, 0.75 µg, 1.25 µg or 2.5 µg) encapsulated in virosomes and determined the minimal dose required for effective CTL priming. At the lowest dose of antigen used (0.25 µg), an modest CTL induction was observed. All of the higher doses of antigen induced strong CTL responses that were not significantly different (Figure 3B). Furthermore, ELISPOT assays revealed that the
number of spleen cells producing IFNγ upon stimulation with the OVA peptide was similar for all of these mice, indicating that the CTL precursor frequency had reached an optimum at a dose of 0.75 µg of OVA per injection (results not shown).

**Mechanism of virosome-mediated priming of CTL activity.**

We hypothesize that virosome-mediated CTL induction relies on delivery of virosome-encapsulated antigen to the cytosol of APC. In order to obtain support for this hypothesis, we immunized mice with free heat-denatured OVA alone or with heat-denatured OVA mixed with empty virosomes. While free OVA, even at amounts of 100 µg, did not induce any significant CTL activity (Figure 2B), the presence of virosomes in the immunization mixture significantly stimulated the induction of OVA-specific CTL. In fact, 10 µg of heat-denatured OVA mixed with virosomes resulted in percentages of specific lysis of target cells of up to 57% at an effector:target ratio of 30:1 (Figure 4A). This result indicates that virosomes have a significant adjuvant effect on admixed antigen. On the other hand, mice immunized in the same experiment with only 1.5 µg of OVA encapsulated in virosomes showed higher specific lysis, while in addition the OVA encapsulated in the virosomes was not heat-denatured.
Figure 4. Mechanism of virosome-mediated priming of CTL activity. For the $^{51}$Cr-release experiment of panel A, mice were immunized and boosted with 1.5 µg of virosoe-encapsulated OVA (n=6; black squares) or with 10 µg of heat-denatured OVA admixed with virosomes (n=2; open circles). Also a buffer control was included (n=1; open squares). In the experiment of panels B and C, mice were immunized and boosted i.p. with 3.5 µg of OVA in fusion-active (n=3, black bars) or fusion-inactivated (n=3, white bars) virosomes. Immunization with fusion-active OVA virosomes resulted in significantly higher specific lysis (B, p <0.03) and higher amounts of IFNγ-secreting spleen cells (C) than immunization with fusion-inactivated OVA virosomes.

In our earlier in vitro study, we demonstrated that the membrane fusion activity of virosomes plays a key role in delivery of virosoe-encapsulated OVA to the cytosol of cultured murine DC and the subsequent presentation of OVA-derived peptides in the context of MHC class I molecules on the DC surface [Bungener 2002a]. Furthermore, in a previous in vivo study involving immunization of mice with virosomes containing an influenza NP peptide, fusion activity of the virosomes was found to be important for the induction of a CTL response against influenza-infected target cells [Arkema 2000]. Therefore, it was of interest to investigate the role of the membrane fusion activity of virosomes in the priming of a CTL response against virosoe-encapsulated OVA.

To this end, an OVA-virosome preparation was split and half of the preparation was fusion-inactivated by preincubation at low pH, as described in Materials and Methods. This low-pH preincubation completely abolished the membrane activity of the virosomes, as determined in the pyrene-based fusion assay (results not shown). Earlier observations have shown that virosomes, fusion-inactivated in this manner, fully retain receptor-binding activity and, thus, are taken up efficiently by APC [Bungener 2002a]. Mice were immunized i.p. with fusion-active or fusion-inactivated virosomes, both containing OVA, and the induction of OVA-specific CTL activity was evaluated using both the $^{51}$Cr-release and the ELISPOT assay. Figure 4B and C summarize the results. Clearly, in the $^{51}$Cr-release assay (panel A) as well as the ELISPOT assay (panel B), responses were substantially lower in the mice immunized with the fusion-inactivated virosomes than in the animals that received fusion-active virosomes. In the CTL assay, fusion-inactivation of the virosomes resulted in a statistically significant reduction of 48% in the percentage of specific lysis. In the ELISPOT assay, the reduction was similar, although in this case, due to larger variations between individual animals, the
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The difference between fusion-active and fusion-inactivated virosomes was not statistically significant. Taken together, the results indicate that membrane fusion activity of the virosomes is important for optimal priming of CTL activity. However, it is noteworthy that fusion activity was not essential, as fusion-inactivated OVA-virosomes induced significant CTL responses, that were higher than CTL responses induced by free OVA at much higher doses.

Discussion

In this paper we demonstrate that immunization of mice with influenza virosomes containing OVA results in efficient induction of a class I MHC-restricted CTL response against the OVA antigen. Staining of spleen cells from immunized mice with K\(^{\text{b}}\)-SIINFEKL tetramers demonstrated a high frequency of precursor T cells specific for the OVA-derived SIINFEKL-epitope. Incubation of the CTL precursors with SIINFEKL peptide resulted in efficient production of IFN\(\gamma\). Moreover, CTL induced by immunization with OVA-containing virosomes were cytotoxic, as demonstrated in a standard \(^{51}\)Cr-release assay. All immunization routes tested (i.m., i.p. and s.c.) resulted in induction of OVA-specific CTL activity, i.m. or i.p. immunization being slightly superior over the s.c. route.

Induction of CTL responses by OVA-containing influenza virosomes was found to be extremely efficient. The minimal dose of antigen needed to induce a strong CTL response was 0.75 µg, while even mice immunized with as little as 0.25 µg of viroso-encapsulated OVA mounted a CTL response. In contrast, with other antigen delivery systems, such as conventional or pH-sensitive liposomes, usually a dose of 25-100 µg of OVA is required to achieve similar responses [Reddy 1991, 1992, Nair 1992, Collins 1992, Zhou 1992, 1994, Wijburg 1998, reviewed in Bungener 2002b]. Even more sophisticated antigen delivery systems like liposomes enriched with monophosphoryl lipid A or liposomes containing membrane proteins from Sendai virus, although reducing the minimally required antigen doses by a factor of 3-5, are still much less effective than the fusion-active influenza-derived virosomes presented in this study [Zhou 1993, Nakanishi 2000].

How can the extraordinary efficiency of virosomes to induce CTL responses be explained? Four unique properties of virosomes might contribute to this phenomenon.

First, the presence of influenza HA in the virosomal membrane facilitates efficient binding and delivery of virosomes, including viroso-encapsulated antigen to APC. Influenza HA binds to cells via sialic acid residues which are found on a wide variety of cells including DC. In the context of vaccination, it is very important that DC, the primary APC for CTL induction, internalize influenza virus as well as influenza-derived

Second, due to the presence of HA the viroosome membrane fuses with the endosome membrane in a pH-dependent fashion, thereby delivering encapsulated antigen directly into the cytosol of target cells. Indeed, fusion activity was found to be indispensable for virosome-mediated delivery of antigen to the cytosol of DC in vitro [Bungener 2002a]. In vivo, the requirement for fusion activity appears to be less stringent since fusion-inactivated virosomes were still capable of inducing CTL responses although to a significantly lower extent than fusion-active virosomes. Most of the current antigen delivery systems facilitate antigen uptake into endosomes or phagosomes of APC [Watts 1997]. However, generally these systems lack the capacity to actively mediate escape of the antigen from the endosomal/phagosomal system to the cytosol, thus relying on the intrinsic capacity of APC to release antigen to the cell cytosol for processing in the class I presentation pathway. The process of antigen release to the cytosol of APC, which is also involved in cross-priming, is still poorly understood. Recent evidence indicates that it might involve transient fusion of phagosomes with the ER after which ER membrane components, notably the protein-translocation channel Sec61, would mediate protein escape to the cytosol [Guermonprez 2003, Houde 2003]. While such a process, at least in DC, might enable the routing of exogenous antigen into the MHC class I processing pathway, it would appear that it is much less efficient than fusion-mediated delivery of protein antigens directly to the cytosol of APC.

Third, virosomes activate not only CD8+ T cells but also CD4+ T helper cells. Help from CD4+ T cells is crucial for the generation of long-lasting antibody responses but also for the priming of effective CTL activity and the generation of memory CTL [Keene 1982, Bennett 1998, McNeela 2001]. As we have shown in an earlier in vitro study, virosome-encapsulated OVA is efficiently processed and presented to class II MHC-restricted T helper cells [Bungener 2002a]. In contrast to class I MHC-restricted antigen presentation, fusion activity was not required for presentation in the context of class II. This observation is in line with the view that class II presentation involves processing of the antigen in the endosomal/lysosomal compartment. In addition to OVA-specific T helper cells HA-specific T helper cells might further contribute to immune stimulation. Immunization with virosomes induces HA-specific T helper cells and incubation of spleen cells from influenza-infected mice with virosomes stimulates T cell proliferation and production of cytokines in vitro (A. Huckriede, unpublished observations). Moreover, influenza-derived virosomes have been shown to promote proliferation of human influenza matrix protein-specific CD8+ T cells when human peripheral blood mononuclear cells were stimulated with the matrix protein epitope and virosomes simultaneously [Schumacher 2004].
Finally, virosomes induce activation/maturation of DC as indicated by upregulation of MHC class I, MHC class II, CD40, ICAM-1, B7-1 and B7-2 [19]. Immature DC which exhibit high phagocytic activity do not activate T cells and may in fact even induce tolerance. In contrast, mature DC are highly potent inducers of T cell responses [Mellman 2001, Jonuleit 2000, Dhodapkar 2001].

In summary, fusion-active influenza-derived virosomes represent a very efficient system for induction of class I MHC-restricted CTL activity against virosome-encapsulated protein antigens. The capacity of virosomes to prime CTL activity is most likely due to their ability to mediate efficient binding of virosomes to APC and to actively deliver virosome-encapsulated antigens to the APC cytosol through fusion of the virosoine membrane with the membrane of endosomes or phagosomes. Through this fusion process virosome-encapsulated protein antigens gain direct access to the MHC class I presentation pathway [Bungener 2002a]. In addition, virosomes induce maturation of DC and activation of T helper cells [Bungener 2002a].

Virosomes have been shown to represent an excellent vaccine formulation for immunization against influenza [Conne 1997]. In these formulations, the virosomal HA primarily functions as an antigen for induction of virus-neutralizing antibodies. In addition, HA ensures binding of the virosomes to APC and acts as a powerful T helper antigen. While it is possible that the virosomes used in current influenza vaccine formulations do express membrane fusion activity, it would appear that such fusion activity is not required for efficient induction of HA-specific antibody responses since there is no need for delivery of the antigen to the cytosol of APC. Influenza-derived virosomes may also serve as a platform for delivery of other antigens bound to the virosoine surface, such as inactivated hepatitis A virions, with the aim to induce a strong antibody response against the virosome-associated antigen [Gluck 1992]. In this case, HA mediates binding and delivery of the virosoine and its foreign antigen to APC and functions as a helper antigen. Again, fusion activity of HA is unlikely to play an important role under these conditions, since an antigen bound to the surface of the virosomes will not be delivered to the cytosol of APC, while also, as argued above, there is no need for escape of the antigen to the APC cytosol for induction of antibody responses. Our present study exploits the entire spectrum of delivery and immunologic capacities of influenza HA, in particular its membrane fusion activity. Thus, virosome-encapsulated foreign protein antigens, including viral as well as tumor antigens, may be directed into the MHC class I presentation pathway for induction of a CD8+ T cell response, the virosomal HA mediating binding and delivery of the encapsulated protein to the cytosol of APC and acting as a powerful T helper antigen.
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The picture on the first page of this chapter is a negative stain electronmicroscopy picture of an influenza virosome containing OVA.
References
