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Niosomes as a novel peroral vaccine delivery system

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Abstract

The feasibility to develop a peroral vaccine delivery system based on non-ionic surfactant vesicles (niosomes) was evaluated using BALB/c mice. Ovalbumin was encapsulated in various lyophilized niosome preparations consisting of sucrose esters, cholesterol and dicetyl phosphate. Two different formulations were compared in this study. The specific antibody titres within serum, saliva and intestinal washings were monitored by ELISA on days 7, 14, 21 and 28 after intragastric administration. Only encapsulation of ovalbumin into Wasag®7 (70% stearate sucrose ester, 30% palmitate sucrose ester (40% mono-, 60% di/tri-ester)) niosomes resulted in a significant increase in antibody titres. Administration of ovalbumin and empty niosomes did not exert a similar effect, neither did administration of any control formulation. In contrast to ovalbumin loaded Wasag®7 niosomes, application of the more hydrophilic Wasag®15 (30% stearate sucrose ester, 70% palmitate sucrose ester (70% mono-, 30% di/tri-ester)) niosome preparations did not result in an increase in antibody titres.

Keywords: Niosomes; Peroral; Immunization; Ovalbumin; Sucrose ester

1. Introduction

The success of vaccine technology in controlling infectious diseases has made remarkable contributions to public health and quality of life, because it has been proven that vaccines have saved more lives than drugs (Powell, 1996). Presently most vaccines for protection against both superficial and systemic pathogens are administered parenterally in order to avoid antigen degradation by gastric acidity and proteolytic enzymes in the gut. However, the majority of infectious diseases is mediated through pathogen contact with the large area of the body's mucosal surfaces, e. g. the gastro-intestinal tract, respiratory tract and urogenital tract. These surfaces are protected by their own secretory immune system which cannot always be activated efficiently by the parenteral route (Fujii et al., 1993; Mestecky et al., 1996). Oral vaccination, however, activates the gastro-intestinal immune system (Nakamura et al., 1998). Stimulation of specific secretory IgA responses in the intestine by the (per-) oral route can lead, via lymphocyte homing and the mucosal immune system, to the same specific secretory IgA antibodies at extraintestinal mucosal sites. Oral delivery of vaccines is more attractive, since this offers a

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number of inherent advantages over the parenteral route, including ease and convenience of administration, increased patient compliance, reduced costs and minimal side effects and thus a greater possibility of frequent boosting. Easy to use oral vaccine delivery systems can play an important role in immunization programmes against many infections in developing countries. New oral vaccine formulations also offer the possibility to control infectious diseases which cannot be prevented, yet.

Most of the oral vaccines so far are based on live attenuated organisms. Rather new approaches to oral vaccination employ synthetic peptides, proteins and polysaccarides. Due to their susceptibility to degradation during gastric and intestinal passage, these compounds require more sophisticated delivery systems. These systems should protect the incorporated vaccines from degradation, deliver the vaccine specifically to immuno-competent cells in the gut (Peyer's patches) and be biodegradable.

In a series of experiments Eldridge et al. (1990) have shown that the uptake of particles via the Peyer's patches is dependent on their size and hydrophobicity. Particles smaller than 10 μm may be taken up by the Peyer's patches, and it has been suggested, that particles smaller than 5 μm can traffic to other tissues such as the spleen, via the known pattern of lymphoid drainage, whereas particles larger than 5 μm remain in the Peyer's patches. The most hydrophobic particles are absorbed to the greatest extent.

An interesting group of vaccine carrier systems are formulations based on non-ionic surfactant vesicles (niosomes) consisting of one or more lipid bilayers encapsulating an aqueous core. Niosomes themselves are only weakly immunogenic. The vesicles are able to encapsulate both lipophilic and hydrophilic drugs and to protect them against acidic and enzymatic degradation in the gastro-intestinal tract (Yoshida et al., 1992). Tomizawa et al. (1993) have shown that vesicles with a negatively charged surface are preferably taken up via the Peyer's patches. These studies confirm and elucidate the benefit of a negatively charged surface.

Niosomes can be prepared in the same way as documented for liposomes. The sucrose ester surfactants used in our studies are able to form gel state bilayers at 37°C and have been chosen due to their easy biodegradability and very low toxicity. Previous studies (Rentel et al., 1994) have shown that the niosome preparations used for the in vivo studies were stable against bile salts, rat bile and porcine bile in vitro.

2. Materials and methods

The vesicles were prepared from cholesterol (purity > 95%), dicetyl phosphate (both obtained from Sigma, St. Louis, MO, USA) and sucrose ester surfactants (Wasag®7 and Wasag®15, purchased as food additives from Schmidt, Amsterdam, NL) at a molar ratio of 5:1:4. Wasag®7 consists of 70% stearate sucrose ester and 30% palmitate sucrose ester (40% mono-, 60% di/tri-ester) and possesses a HLB value of 7. Wasag®15 consists of 30% stearate sucrose ester and 70% palmitate sucrose ester (70% mono-, 30% di/tri-ester); its HLB value is 15.

All other chemicals were of analytical grade (Merck, Darmstadt, Germany). The water was deionized and sterile filtered (Milli-Q® UF plus system, Millipore, Etten-Leur, NL).

2.1. Sonication method

Stock solutions (150 mM) of the surfactants in a chloroform–methanol mixture (2:1 (v/v)) were vigorously mixed. The organic solvent was removed in a Speed Vac concentrator (Savant Instrument, Farmingdale, NY, USA) for 16 h at − 1.0 bar. Sucrose solution (5.00 ml, 10% (m/v)) was added to the surfactant film and the samples were heated in a water bath (80°C) for 15 min. The samples were sonicated for 60 s using a Branson sonicator 250 (Branson Ultrasonics, Danbury, CT, USA) with a 4.7 mm microtip. The procedure resulted in small unilamellar vesicles which were cooled down to 25°C directly after sonication.

2.2. Dehydration–rehydration method

The model protein ovalbumin (grade V, purity > 99%, Sigma) was encapsulated by using the method described by Kirby and Gregoriadis (1984). Briefly, the samples were flash-frozen as a thin shell by immersing them in liquid nitrogen, and directly lyophilized. The freeze-dried membranes were resuspended with 1.5 ml ovalbumin (10 mg/ml) dissolved in phosphate buffered saline (PBS, PhEurII, pH 7.4).

The vesicle sizes (in all formulations clearly below 0.5 μm) were measured by photon correlation spectroscopy (Malvern 4700 c with a 25 mW He-Ne-laser and Malvern Automeasure V5.22 software; Malvern Instruments, Worcestershire, UK) and the encapsulated amount of ovalbumin (Wasag®7: approximately 18%, Wasag®15: around 8%) was determined by size exclusion chromatography with Sephadex® G100SF (Pharmacia, Woerden, NL) as described earlier by Rentel et al. (1994).

2.3. In vivo antibody production

Groups of female BALB/c mice (age at the beginning of the experiment: 6–8 weeks) were starved over night and fed intragastrically 0.5 ml of one of the following five different formulations on days 0, 2 and 13:

1. Ovalbumin loaded niosomes: The samples were rehydrated as described above. Non-encapsulated ovalbumin was removed by filtration with Centrisart® I filters (Sartorius, Göttingen, Germany) resulting in 100 mM preparations encapsulating approximately 2 mg ovalbumin per ml (Wasag®7) or 1 mg ovalbumin per ml (Wasag®15), respectively. The samples were used immediately.
2. PBS, PhEurII, pH 7.4
3. Ovalbumin in PBS: The comparative amount of ovalbumin (1 or 2 mg/ml) was dissolved in PBS, PhEurII, pH 7.4.
4. Empty niosomes: 100 mM preparations were obtained by rehydration with PBS only.
5. Empty niosomes + ovalbumin: 100 mM preparations of empty niosomes with the comparative amount non-encapsulated ovalbumin were obtained by rehydration of the lyophilizate with PBS and adding ovalbumin solution directly before administration.

Finally, all mice of the groups 1, 3 and 5 received 1 mg ovalbumin per dose (Wasag®7) or 0.5 mg, respectively (Wasag®15).

Blood samples were collected before administration of any formulation and on days 7, 14, 21 and 28 from the tail vein and allowed to clot at 4°C. The serum was separated by centrifugation (14 000 × g for 10 min).

Intestinal washings and saliva samples were collected on the same days as the blood samples. Saliva production and intestinal discharge were stimulated by an i.p. injection of 1 μg pilocarpine/g body weight after feeding 1.0 ml iso-osmotic lavage solution (Elson et al., 1984).

2.4. ELISA procedure

The individual samples from all the mice were assayed separately. The antibody responses were determined by ELISA (Engvall, 1980; Naisbett, 1991). Microtitre plates were loaded with ovalbumin, incubated at 37°C for 2 h and washed in a Titertek® microplate washer (ICN Biomedicals, Irvine, UK) with PBS, PhEurII, pH 7.4 containing 0.05% (v/v) Tween® 20 (ELISA buffer). A special blocking step to prevent non-specific binding to the antibodies seemed not to be necessary (Naisbett, 1991). Mouse serum (50 μl), or 50 μl saliva or intestinal fluid, were incubated with bound antigen and washed with ELISA buffer to remove any unbound or non-specifically bound antigens. The plates were then incubated with the diluted second antibody (biotinylated anti-mouse IgG or IgA, respectively), washed again and incubated with diluted biotinylated streptavidin–peroxidase conjugate (all obtained from Sigma). After an intense final washing step an enzymatic colour reaction substrate consisting of *o*-phenylenediamine and H_2O_2 in phosphate–citrate buffer was used to stain the samples. The samples were allowed to react for 15 min and the enzymatic reaction was stopped by adding H_2SO_4 subsequently. The coloured product was read at 490 nm on a BioRad 3550 UV plate reader (Bio-Rad, Veenendaal, NL).

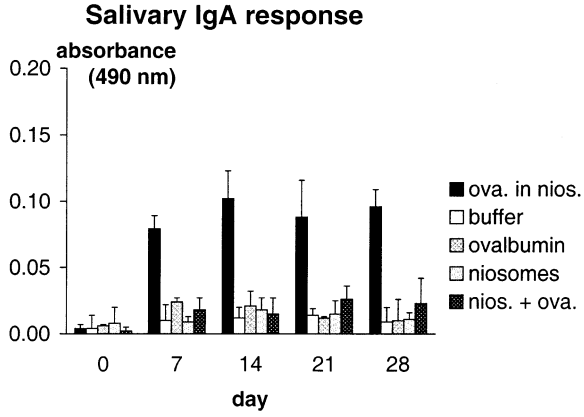

Fig. 1. Wasag®7 niosomes: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. All groups besides the PBS control group ($n = 5$) consist of 10 animals.

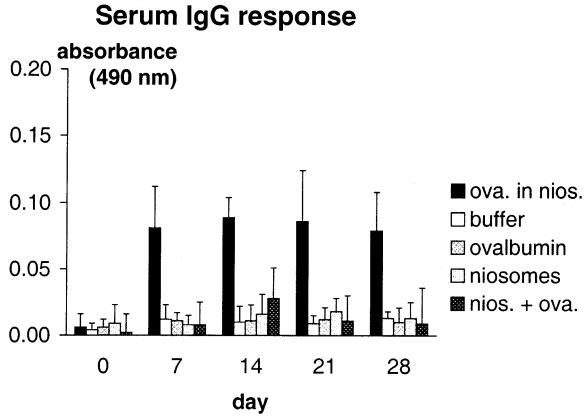

Fig. 3. Wasag®7 niosomes: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. All groups besides the PBS control group ($n = 5$) consist of 10 animals.

3. Results

In the figures the mean values of the absorbance at 490 nm subtracted from the average of the background level are plotted as a function of time. The error bars indicate the S.D.

Only encapsulation of ovalbumin in Wasag®7 niosomes resulted in a significant increase in specific antibodies (Figs. 1–3).

Administration of empty niosomes and ovalbumin solution seems not to exert an immuno response, neither does administration of one of the other control formulations. The more hydrophilic Wasag®15 niosomes failed in raising specific antibodies (Figs. 4–6].

The application of a boosting dose on days 2 and 13 seems not to have any detectable effect.

4. Discussion

Oral immunization provides many advantages over the commonly used parenteral route of vacci-

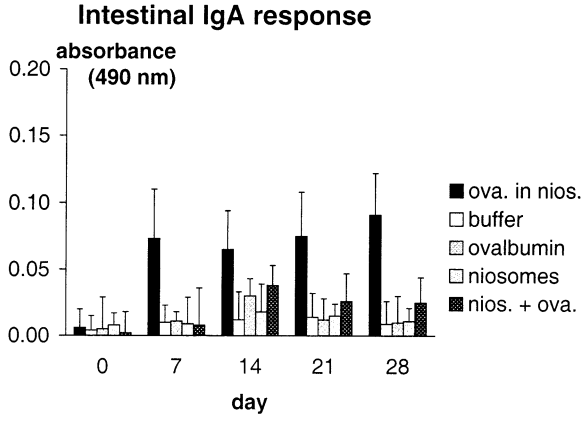

Fig. 2. Wasag®7 niosomes: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. All groups besides the PBS control group ($n = 5$) consist of 10 animals.

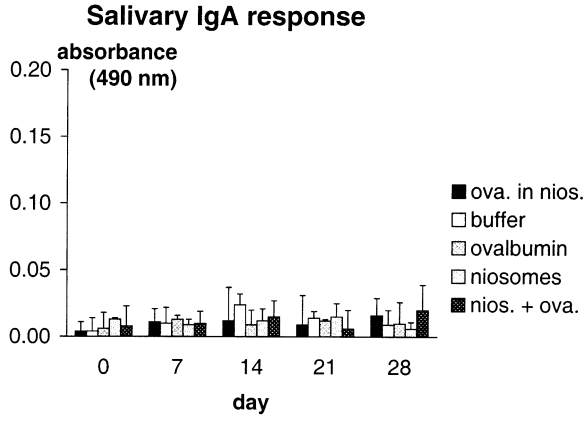

Fig. 4. Wasag®15 niosomes: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. All groups besides the PBS control group ($n = 5$) consist of 10 animals.

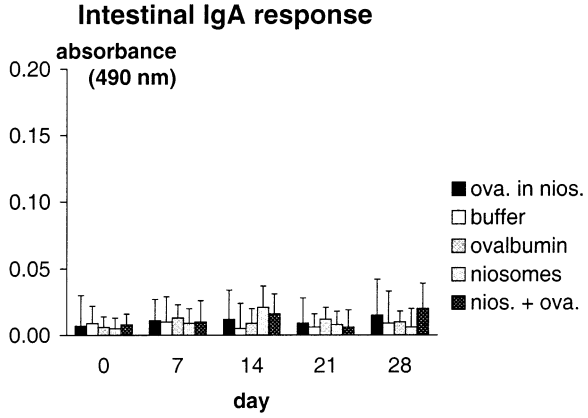

Fig. 5. Wasag®15 niosomes: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. All groups besides the PBS control group ($n = 5$) consist of 10 animals.

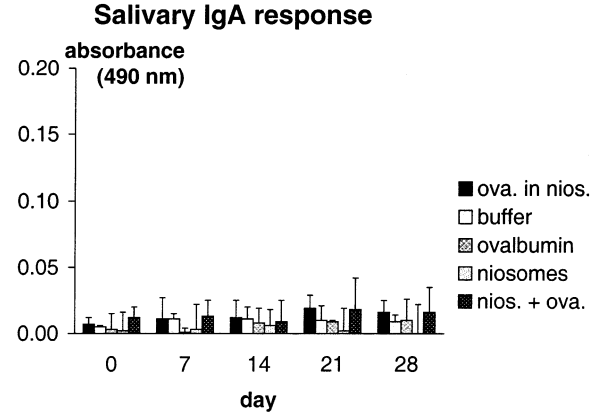

Fig. 7. Wasag®15 niosomes rehydrated with the double amount of ovalbumin: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. The PBS control group and the empty niosome group consist of four animals, all other groups consist of seven animals.

nation. Promising initial results have been obtained with vesicular carrier systems based on non-ionic surfactants like Novasomes® or NISV's (Alexander and Brewer, 1993) and provide a role for these systems in peroral vaccine delivery. Oral delivery of Wasag®7 niosomes showed in comparison to s.c. or i.p. injection (over 10 times higher antibody titres, unpublished data) a weak, but significant increase in antibody titres. Ovalbumin loaded Wasag®15 did not potentiate the immune response. This may be due to the increased hydrophilicity of these vesicles which may prevent them from being taken up by the Peyer's patches. Another reason, the fact that the Wasag®15 niosomes are only entrapping half of the amount the Wasag®7 vesicles do, was investigated in a series of additional experiments. By using a higher concentrated protein solution (20 mg/ml) for the re-

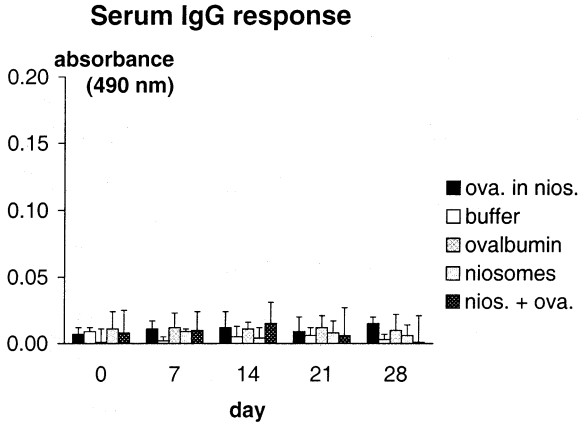

Fig. 6. Wasag®15 niosomes: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. All groups besides the PBS control group ($n = 5$) consist of 10 animals.

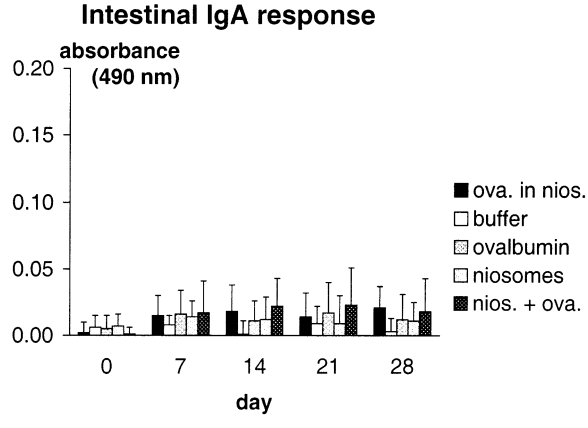

Fig. 8. Wasag®15 niosomes rehydrated with the double amount of ovalbumin: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. The PBS control group and the empty niosome group consist of four animals, all other groups consist of seven animals.

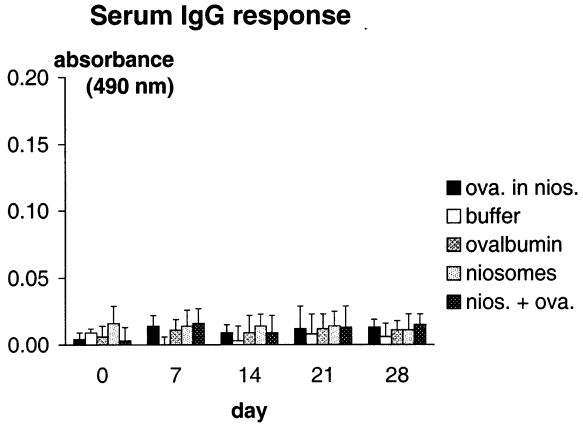

Fig. 9. Wasag®15 niosomes rehydrated with the double amount of ovalbumin: mean values of the absorbance at 490 nm subtracted by the average of the background level. Error bars indicate the S.D. The PBS control group and the empty niosome group consist of four animals, all other groups consist of seven animals.

hydration of the Wasag®15 lyophilizate, the total amount of ovalbumin encapsulated in the Wasag®15 vesicles was similar to that encapsulated in the Wasag®7 vesicles; all mice of groups 1, 3 and 5 received 1 mg ovalbumin per dose. However, the Wasag®15 niosomes failed again in potentiating the immunoresponses as measured in saliva, serum and intestinal washings (Figs. 7–9). Preliminary results using confocal laser scanning microscopy (CLSM) and fluorescent labelling of both ovalbumin (with fluorescein) and membrane components (with Bodipy®665/676 = (*E*,*E*)-3,5-bis-(4-phenyl-1,3-butadienyl)-4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) showed that the labelled membrane components are taken up by the Peyer's patches in easily detectable amounts whereas the ovalbumin fluorescein conjugate could only be detected in small quantities after application of the Wasag®7 niosomes and in even much smaller quantities in the Wasag®15 vesicles (Rentel et al., 1998). These results may explain the weak immunogenic ovalbumin reaction with Wasag®7 niosomes and the failure with Wasag®15 samples. Currently efforts are being made to increase the stability of the niosomes in gastric acid in order to increase the immunogenic potential of these vesicular carriers. The development of peroral vaccine delivery systems based on niosomes seems to be a possibility.

Acknowledgements

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