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Effectiveness of liposomes as adjuvants of orally and nasally administered tetanus toxoid

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Summary

Tetanus toxoid was incorporated into liposomes composed of equimolar concentrations of distearoyl phosphadylcholine (DSPC) and cholesterol. We investigated the non-parenteral delivery of free or liposome entrapped tetanus toxoid to guinea pigs and measured the subsequent IgG anti-tetanus-antibody response, using an ELISA. Liposome formulation significantly improved the immune response when compared to the free antigen when delivered via the nasal, oral and i.m. routes. However, 10 × concentration of tetanus toxoid entrapped in DSPC introduced mucosally (nasally and orally) was necessary to produce an IgG antibody titre similar to those obtained via the i.m. delivery. This study suggests that liposomes, administered through the oral and nasal routes, have considerable potential as mucosal adjuvants and warrant further investigation.

Introduction

Vaccination programmes against tetanus, which use formaldehyde-inactivated tetanus toxin purified from *Clostridium tetani*, are highly effective in the prevention of tetanus in western countries (Bizzini, 1984). However, some countries are unable to fully implement these programmes, resulting in significant annual mortality from neonatal tetanus (Stanfield and Galazka, 1984). It is possible that the use of an effective oral tetanus vaccine may help to alleviate some of the problems associated with widespread vaccination in developing countries.

Liposomes are microscopic phospholipid vesicles composed of one or more concentric phospholipid bilayers, and were first described for their function as adjuvants for protein antigens by Allison and Gregoriadis (1974). The physicochemical properties of liposomes can influence their utility as vaccine adjuvants. Variables considered, include the liquid-crystalline phase-transition temperature (T_c) of the lipids, the charge of

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the lipids, inclusion of cholesterol, ratio of protein to lipid, and of the liposomes in the preparation (Eppstein et al., 1990).

Liposomes act as immunological adjuvants for diphtheria toxoid (Allison and Gregoriadis, 1974) and cholera toxin (Pierce and Sacci, 1984). In addition, studies with hepatitis B surface antigens have shown that the immune response can also be cell-mediated (Manesis et al., 1979), an event that plays a major role in protection against most viral infections.

Liposomes have been delivered by various routes (Patel and Ryman, 1981). For example, subcutaneously delivered liposomes of large size disintegrate locally, probably upon attack by infiltrating macrophages. In contrast, smaller vesicles enter the lymphatic system and localise avidly into the lymph nodes (Ryman and Tyrrell, 1980, Gregoriadis, 1981). This has stimulated research using liposomes either as reservoirs for sustained release of drugs, or as vaccines (Gregoriadis, 1981).

The use of liposomes as an oral dosage form has been considered, since they may either protect the entrapped drugs from digestive degradation or increase absorption of poorly absorbed drugs from the gastrointestinal tract (Sessa and Weissmann, 1970). The oral route of administration using liposomes has been employed in a limited number of studies performed in animal models (Patel and Ryman, 1981; Patel et al., 1982). The stability of liposomes in the gastrointestinal tract is essential to their success (Chiang and Weiner, 1987a;b).

This study was aimed at investigating the potential adjuvant effect of liposomes on tetanus toxoid, when delivered via the nasal, oral and i.m. routes compared to delivery in simple solution and to develop a non-parenteral immunization procedure which stimulates a strong systemic immunity.

Materials and Methods

Liposome preparation

Multilamellar dehydration-rehydration (DRV) vesicles composed of equimolar phospholipid (66 μmol) and cholesterol were prepared as described previously (Kirby and Gregoriadis, 1984). Distearoylphosphatidylcholine (DSPC) (Lipid Products, S. Nutfield, Surrey, U.K.) and cholesterol (Sigma, Poole, U.K.) were dissolved in 10 ml of chloroform and evaporated to dryness under reduced pressure at 58°C. The lipid film was dried under nitrogen for 10 min and then dissolved in 2 ml distilled water at 58°C. The mixture was sonicated at 58°C resulting in the formation of single unilamellar vesicles (SUV). The liposomes were centrifuged at $2500 \times g$ for 15 min to remove larger lipid aggregates and titanium particles released from the sonicator probe. 2 ml of SUV were mixed with 2 ml distilled water containing 1000 μg of tetanus toxoid vaccine, flash-frozen as a thin shell and freeze-dried overnight. The preparation was rehydrated with distilled water, using a volume equivalent to one-tenth of the total volume of SUV used (i.e., 0.2 ml in the present case). The rehydration procedure was aided by light vortexing the preparation then left to stand for 30 min at 58°C. Isotonic phosphate-buffered saline (PBS), pH 7.4, equivalent to one-tenth of the total volume of SUV used was added to the preparation, which was vortexed lightly and left to stand for 30 min at 58°C. A further volume of PBS equivalent to eight-tenths of the total volume of SUV used was added, the mixtures vortexed and allowed to stand for 30 min at 58°C.

The liposomes were separated from non-entrapped material by diluting with PBS to 20 ml followed by centrifugation at $10\,000 \times g$ for 30 min. The pellet was finally resuspended in 1 ml PBS and the supernatants were used indirectly to estimate the percentage entrapment of tetanus toxoid in the liposomes using the protein assay of Lowry et al. (1951), by determining ^{125}I radioactivity and with a high pressure liquid chromatography (HPLC) method.

In vitro stability assay of liposomes

Tetanus toxoid was radiolabelled with ^{125}I (Na^+ salt) (Amersham International, U.K.) by the chloramine-T method based on that of Hunter and Greenwood (1962). Briefly, 10 μl of 2 mg/ml protein PBS was mixed with 15 μl PBS and 10 μl

(1 mCi) ^{125}I. To this was added 25 μl chloramine-T solution (4 mg/ml PBS), 100 μl sodium metabisulphite (2.4 mg/ml PBS) and 20 μl sodium iodide (10 mg/ml PBS). Radiolabelled protein was separated from free ^{125}I salt on a Sephadex G-25 column (Pharmacia) and the activity assessed in a 1282 Compugamma counter (LKB Instruments Ltd, Croydon, U.K.).

Liposomes containing tetanus toxoid (TT) together with a trace of ^{125}I-iodinated TT were prepared and diluted with PBS to 3 mg/ml TT. Percentage entrapment was measured by assaying ^{125}I radioactivity associated with liposomes. Aliquots (2.5 ml) of liposome suspension were mixed with 0.1 M PBS pH 7.4, or glycine/HCl, pH 2.2. The mixtures were incubated at 37 or 4°C. At the start (t_0) and at intervals throughout the 2 h incubation, 400 μl aliquots were taken from each mixture. A portion (100 μl) was retained and the remainder was centrifuged to separate free and liposome-entrapped TT. The volumes of the supernates and pellets were measured and the TT content of each of the fractions was assessed for gamma emission.

Assessment of protein integrity

The TT is a very large protein (150 kDa), with one major component. The integrity and antigenicity of TT were checked by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques.

The assessment of TT stability was investigated using SDS-PAGE electrophoresis, protein was analysed before encapsulation, after encapsulation from supernatants and after release from the liposomes using a 12% gel and the Mini-Protean system. The immunogenic properties of the molecule after release were also assessed in vitro by immunoblotting (Western blotting). Blots were visualised using 4-chloro-1-naphthol.

Liposome uptake by the gastrointestinal tract

Liposomes containing tetanus toxoid were prepared as described above and dosed orally to rats. Tissue sections were taken 1 h later from the Payer's patches regions of the ileum and examined by transmission electron microscope (TEM).

Immunization schedule

Pigmented guinea pigs ($n = 5$) were immunized via the i.m., oral and nasal routes. A vaccine dose of 30 μg and 60 μg tetanus toxoid was given nasally (0.2 ml) and orally (0.5 ml) on weeks 1, 2 and 4 in phosphate-buffered saline (PBS) or 30 μg when entrapped in DSPC liposomes. In addition, 6 μg of free tetanus toxoid vaccine in PBS and 3 μg of encapsulated toxoid were given i.m. (0.2 ml) on weeks 1 and 4.

A further two groups of guinea pigs ($n = 5$ each group) were also immunized via the nasal and oral routes using commercially available alum adsorbed tetanus toxoid (Mérieux Tetavax, Mérieux U.K. Ltd, Maidenhead) with 60 μg toxoid/animal/dose. Another group was also dosed following the same protocol but administering cimetidine (Tagamet, Smith Kline and French Laboratories) 2 h before giving the tetanus toxoid solution orally at a dose of 50 μg/kg body weight.

In vivo tissue distribution of nasally administered liposomal tetanus toxoid

Dunkin Hartley guinea pigs, weighing 300 g, were divided into three groups ($n = 3$). Each guinea pig was dosed nasally (i.n.) with liposome entrapped ^{125}I-labelled tetanus toxoid 0.2 ml PBS containing 0.017 μCi ^{125}I-TT in 30 μg TT on days 1–3.

A group of guinea pigs was killed 24 h and another 48 h after administration of vaccine and a post mortem carried out. The lungs, liver and spleen were removed washed in PBS and homogenised by grinding in Griffiths tubes. The aliquots of tissues were weighed and the ^{125}I radioactivity were measured. Blood was also collected, initially hourly (up to 4 h) and then at 24 h and 48 h intervals from all the animals by cardiac puncture, until they were killed and blood samples analysed for radioactivity content. The third group of animals were kept until the end of the immunization studies (11 weeks). The amount of radioactivity recovered in the organs was then calculated and expressed as a percentage of the total inoculated radioactivity.

Enzyme linked immunosorbent assay (ELISA)

Antibody responses to the tetanus toxoid in immunized guinea-pigs were monitored by a mi-

croplate ELISA (Davis and Gregoriadis, 1989). Tetanus toxoid (5 μg/ml) in 0.05 M Na^+ carbonate-bicarbonate buffer (pH 9.6) was added to Dynatech microelisa plates and incubated overnight at 4°C. The wells were washed three times with 9.5 mM sodium phosphate buffer containing 0.8% NaCl and 0.05% Tween 20 (PBST), pH 7.4.

Dilutions of guinea-pig serum in PBST + 0.1% bovine serum albumin (Sigma, Poole, U.K.) were incubated in the plates for 2 h at room temperature. The total amount of guinea pig bound IgG was estimated by the addition of rabbit anti-guinea-pig IgG-horseradish peroxidase conjugate (ICN, Pharmaceuticals, U.K.).

TABLE 1

Antibody titres to tetanus toxoid determined by ELISA in the sera of guinea-pigs vaccinated on weeks 1, 2 and 4 (oral and nasal routes of delivery) or weeks 1 and 4 (i.m delivery) (n = 5)

Route and formulation	protein (μg)	Pre-bleed	Titre of serum IgG antibody (± SD)		
			Week 3	Week 6	Week 11
Oral					
DSPC [a] liposomes	30	< 6.40 (0 13)	< 6 40 (0.13)	10.10 (1.06)	10.53 (0 95)
PBS [b]	30	< 6 30 (0.42)	< 6.20 (0.64)	< 6.40 (0 50)	< 6.40 (0.40)
PBS	60	< 6 30 (0 55)	< 6 30 (0.55)	7.80 (1.58)	< 7.30 (0 63)
Alum adsorbed	60	< 6.82 (0.47)	< 6.82 (0.47)	< 7.62 (2.01)	< 7 28 (1 24)
PBS.cim [c]	60	< 6.20 (0.12)	< 6 40 (0.90)	< 7 80 (1 43)	< 7.00 (1 34)
Nasal					
DSPC liposomes	30	< 6.30 (0.27)	< 6 30 (0.27)	10.56 (0 60)	10.20 (0.58)
PBS	30	< 6.30 (0 25)	< 6.40 (0 60)	7 90 (0.65)	7.20 (0.85)
PBS	60	< 6.10 (0.61)	< 6.20 (0 42)	9.15 (0 97)	8.30 (0 95)
Alum adsorbed	60	< 6.60 (0.15)	< 7.10 (0.56)	9 37 (0.71)	7.37 (0.97)
Intramuscular					
DSPC liposomes	3	< 6.30 (0.13)	< 6.50 (0.53)	9.37 (0.81)	10 70 (0.66)
PBS	6	< 6.30 (0.18)	< 6.70 (0.41)	10.40 (0.78)	11 53 (1.05)
Control	0	< 6.30 (0.40)	< 6 30 (0.62)	< 6.60 (0.67)	< 6.40 (0.36)

[a] Distearoylphosphatidylcholine.
[b] Phosphate buffered saline
[c] Cimetidine.
Titre is expressed as $\log_2 \pm$ SD of the inverse of the maximum dilution of serum.

Anti-tetanus immunoglobulin (The Wellcome Foundation Ltd, London) was used as a standard serially diluted from 1 : 100 on each plate. Protein A peroxidase (concentration 0.5 μg/ml) was used to estimate the total amount of IgG bound to the tetanus toxoid.

After 1 h incubation at room temperature the enzyme substrate 3,3′,5,5′-tetramethylbenzidine (TMB, Miles, U.K.) was added (0.15 mM TMB in 0.1 M sodium acetate, pH 6.8, containing 0.003% (v/v) H_2O_2) and the reaction allowed to proceed for 10 min. The enzyme reaction was stopped by the addition of 2 M H_2SO_4 and the absorbance at 450 nm measured (Titertek Multiscan, Flow Labs, U.K.). The endpoint of each titration was defined as the dilution at which the absorbance (450 nm) was 0.2 (instrument zeroed on the reagent blank). This dilution was expressed as a titre after correction by reference to a standard on each plate.

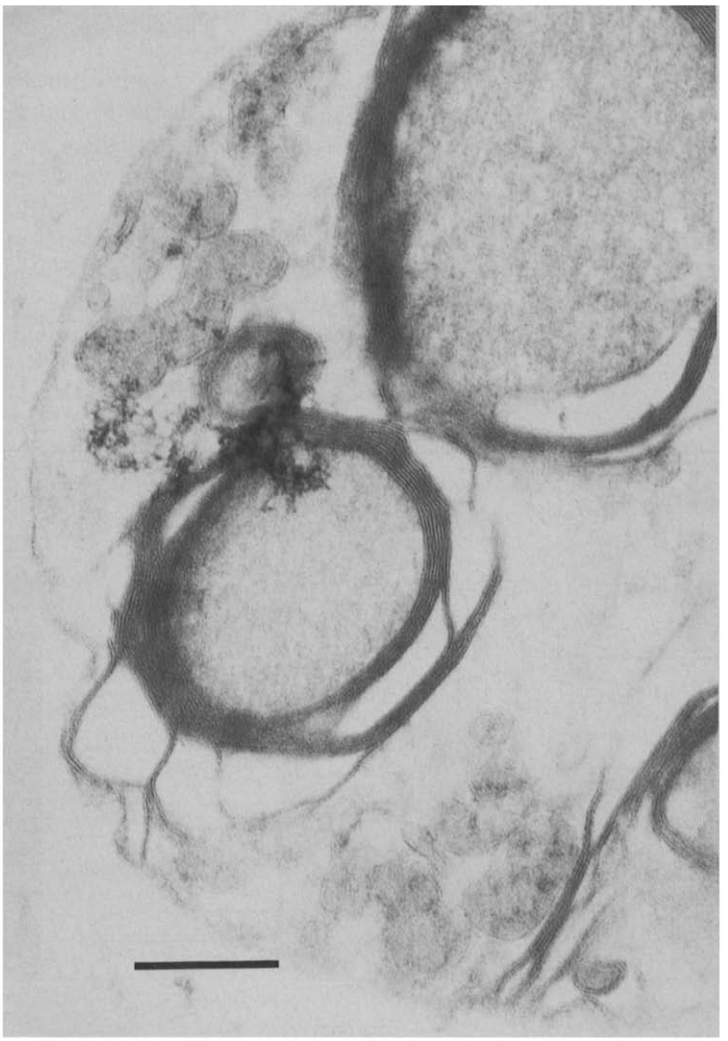

Fig. 1. Electron microscope photographs of rat gut tissue sections taken after oral delivery of tetanus toxoid entrapped in DSPC liposomes (bar = 200 nm).

Statistical methods

Comparisons between the i.m. vaccination group and other groups, using ELISA data, were analysed for significance using Student's unpaired *t*-test. Differences with $P < 0.05$ were considered to be significant.

Results

Immune responses

The anti-toxoid IgG antibody titres prior to and after vaccination of guinea pigs via the i.m., nasal and oral routes using different formulations of tetanus toxoid are shown in Table 1. Significantly, positive serum IgG responses were seen after 6 weeks for animals immunized with DSPC liposome encapsulated TT though both nasal and oral routes ($P < 0.05$) when compared to control group (Table 1).

In the case of oral delivery, low dose free TT (30 μg) did not result in any significant serum anti-body titre after immunization. When a higher dose (60 μg) was delivered, a significant response (compared to pre-immunization titres ($P < 0.05$)) was observed at week 6, but by week 11 titres were back to pre-immunization levels. For the groups of animals immunized with high dose TT in adsorbed form and after the delivery of cimetidine, IgG-anti-tetanus antibody titres did not increase whereas delivery of 30 μg TT associated with DRV DSPC liposomes, after boostering, gave rise to much higher and long lasting antibody titres (weeks 6 and 11). The antibody titres obtained with liposomal toxoid were significantly higher even when compared to high dose free toxoid ($P < 0.05$).

For nasal delivery, although both low and high dose toxoid gave rise to an increase in anti-toxoid-IgG response, these responses were only short-lived (week 6) and decreased by week 11. The liposomal antigen on the other hand produced a significantly high serum immune responses, when compared to the same quantity of free protein. At week 11 antibody titres obtained following liposomal delivery of toxoid were still significantly higher than with both free forms ($P < 0.05$). Alum adsorbed TT delivered nasally

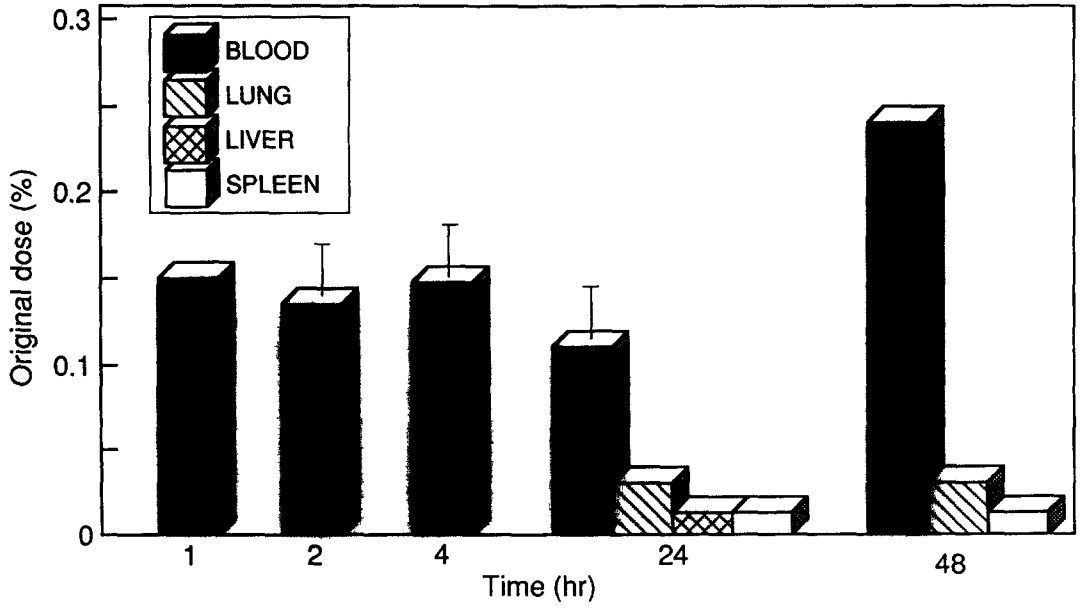

Fig. 2. Fate of liposome-entrapped ^{125}I-labelled tetanus toxoid following i.n. inoculation Results are expressed as per cent of original dose (per ml for blood, and per g for the tissue).

gave results similar to those with free PBS delivery.

Comparable (high and long-lasting) immune responses to that achieved by the conventional i.m. route were obtained using the oral and nasal route of delivery of tetanus toxoid entrapped in DSPC liposomes. Half the amount of toxoid entrapped in DSPC liposomes resulted in an equivalent titre (no significant difference) with free TT when administered i.m. (Table 1).

Protein stability

Results of gel electrophoresis and immunoblotting showed that the use of DRV liposome technology ensured the avoidance of damaging conditions during entrapment of antigen, since no differences were observed in the number of bands and their locations on SDS gels and also protein was recognised by antibodies raised against toxoid (Western blotting – results not shown).

Tissue distribution of nasally delivered liposomal tetanus toxoid

The distribution of radioactivity in each tissue at different time intervals after i.n. liposomal TT delivery is shown in Fig. 2. Highest concentrations were maintained in the lung during an 48 h experiment period. Liver and spleen showed only barely detectable amounts of antigen (0.02% original dose/g tissue) during this time. The radioactivity measured in blood samples was much higher than that of the other tissues although initial levels of circulating radiolabel were lower than that at 48 h.

Liposome characteristics

The entrapment mean values (% ± S.D. of toxoid used) for the DSPC liposomes used in this study prepared using the dehydration-rehydration procedure (Kirby and Gregoriadis, 1984), was 68% ± 8.37 ($n = 6$). This value is lower than that obtained by Davis et al. (1986/1987) which was 82.3 ± 3.4 ($n = 8$). The phospholipid to antigen mass ratio for these liposomes was 80:1. The liposome in vitro stability assay involved the measurement of the gamma emission from the fractions of the aliquots taken at timed intervals during the incubations and this allowed the percentage of tetanus toxoid released from the DSPC liposomes to be calculated. The TT was slowly released from the DSPC liposomes at pH 7.4, until by the end of the 2 h period about 10% had been released at 4°C and 15% at 37°C. Incubation in the presence of glycine/HCl, pH 2.2, resulted in an increased release of TT from the DSPC liposomes compared to the more alkaline medium until at the end of the 2 h incubation 20% at both and 37°C had been released. Therefore, the results suggest that the DSPC liposomes are stable in the acid environment of the gastrointestinal tract as was suggested by other authors (Rowland and Woodley, 1980, 1981). In addition, after oral dosing of DSPC liposomes containing tetanus toxoid to rats, subsequent electron microscope examination of gut tissue sections showed liposome structures which appeared to be intact (Fig. 1).

Discussion

The experiments described demonstrate the efficacy of liposomes in delivering a model antigen (TT) through mucosal surfaces.

Oral immunization may result in the stimulation of local and systemic antibody-mediated and cell-mediated responses and may, under certain circumstances, result in the suppression of these same responses. This process known as 'oral tolerance induction' has been demonstrated against protein antigens (Thomas and Parrot, 1974). It has been proposed that this process protects against dietary hypersensitivities by suppressing harmful hypersensitivity responses to food antigens (Wold et al., 1989).

Oral delivery of tetanus toxoid vaccine entrapped in DSPC liposomes resulted in comparable post vaccination IgG antibody levels to the i.m. delivery (Table 1). When antigen was administered in free form (at high dose) only short-lived antibody response was detected: with low dose no response was seen. It has been shown to be generally more difficult to induce oral tolerance to a particulate antigen than to a soluble antigen (Challacombe and Tomasi, 1980). The encapsulation of toxoid into liposomes renders this soluble

antigen into a particulate one. The poor antibody response to oral immunization of free tetanus toxoid could be due to inactivation and limited ability of toxoid to traverse the gut epithelia in free form. On the other hand, the ability of cholera toxin and its β subunit to prime the immune system upon oral feeding may be due to binding to or cross-linking of G_{m1} ganglioside molecules on the surface membrane of lymphoid cells in the GALT (Holmgren et al., 1973). In addition, DeAizpura and Russell-Jones (1988) demonstrated that certain antigens, such as K99 and 987P pili (two bacterial adhesins of enterotoxigens *E. coli*) and lectins of various binding specificities induced systemic and intestinal immune responses. Many molecules that have the capacity to bind to the intestinal epithelium can also be transported across the epithelial barrier, to enter the circulation and elicit an immune response.

Oral delivery of tetanus toxoid, entrapped in DSPC liposomes, improved the immune response when compared to free antigen, even when the free antigen was at two-fold higher dose, possibly because liposomal TT was more stable in the harsh environment of the gastrointestinal tract. In fact, after oral dosing of DSPC liposomes containing tetanus toxoid to rats, subsequent electron microscope examination of gut tissue sections showed that liposome structures appeared to be intact (Fig. 1). Recently, another group (Childers et al., 1990) has also shown endocytic vesicles containing liposomes and electron dense particles in M-cells. Furthermore, the stability studies presented suggest the liposomes would be stable in the acidic environment of the gastrointestinal tract agreeing with the previous studies (Rowland and Woodley, 1980, 1981), where only less than 20% of toxoid was released after 2 h. The selective localisation of the liposome-associated antigen (TT) may partly explain its better efficacy in provoking higher and longer lasting antibody response (memory formation). The free antigen even after being taken up may channel through the closely associated lymphoid cells and lymph nodes, more rapidly resulting in less antigen retention around these areas. Compared to antigens alone, *Streptoccus mutans* cell wall antigen in liposomes have been shown to induce greater mucosal and systemic immune responses in rats when given by gastric intubation (Wachsmann et al., 1986). In fact, Wachsmann et al. (1986) were able to show the presence of antigens up to 17 days in Peyer's patches when delivered with liposomes.

Also, cimetidine administration at quantities which have been shown to prevent gastric secretions (Brimblecombe et al., 1978) carried out prior to TT delivery to reduce the acidity of the gut and so improve the immune response. This would result in a higher IgG post-vaccination level when the tetanus toxoid was orally delivered. However, cimetidine did not appear to have any affect. Several approaches have been used to protect antigens from acid pH and proteolysis by gastric enzymes, for example orally ingested antigens have been given with a large amount of sodium bicarbonate to neutralise hydrochloric acid in the stomach (Black et al., 1983).

Nasal immunization may not represent a purely local response because part of the antigenic dosage may be swallowed or inhaled and lead to the stimulation of a more general immune response (Mestecky, 1987). This has been demonstrated in tissue distribution studies, which showed higher deposition in lungs, together with sustained levels in the blood, although a much smaller amount was seen in both liver and spleen. In nasal delivery the use of tetanus toxoid entrapped in DSPC liposomes also improved the response compared to delivery of free antigen (Table 1). The results presented suggest tetanus toxoid entrapped in DSPC liposomes is stable and taken up intact in the gut. The conditions in the nasal cavity are less harsh, therefore they would probably be even more stable. Furthermore, if the liposomes were taken up intact, the subepithelial layer of the nose is highly vascularised and therefore a quantity of liposomes will pass directly into the systemic circulation resulting in a systemic immune response and some liposomes would probably be taken up and delivered to underlying lymphoid cells of nasal associated lymphoid tissue (NALT). However, liposome properties such as permeability, size and surface charge can be altered rapidly when they enter the

general circulation and they can be broken down by lipoproteins, circulating phospholipases or complement (Patel and Ryman, 1981). Nevertheless, the present results indicate that at least some immunologically active tetanus toxoid must have reached immuno-compotent cells which have given rise to systemic immune responses.

Tetanus toxoid has been used for immunization by several investigators. For example, human volunteers were immunized with soluble tetanus toxoid given as an aerosol (particle size 1 μm) which resulted in an increase in serum antibody titre comparable to that resulting from conventional s.c. administration. No antibody titres were found in nasal secretions probably because the particles were not large enough to lodge in the nasal passages. Lower respiratory tract antibody production did occur (Wigley et al., 1970); probably because of the deep penetration of the small toxoid particles into the respiratory tract.

The results using tetanus toxoid, either free or entrapped in DSPC liposomes to immunize guinea-pigs via the i.m. route, show that liposomes containing only half the amount of antigen resulted in the same extent of immune response when compared to free toxoid (Table 1). Davis and Gregoriadis (1987) demonstrated strong responses using liposomes containing tetanus toxoid with a low T_c phospholipid, e.g., phosphatidylcholine, whereas responses were negligible when the phospholipid was substituted with the high T_c phospholipid DSPC. Soluble antigens are processed by antigen presenting cells (APCs) prior to exposure on their surface (Allison and Byars, 1986). This processing can be interfered with by high melting point phospholipids such as DSPC (Davis and Gregoriadis, 1987) at any of the stages between liposome internalisation by cells and peptide migration to their membrane.

The ratio of protein to lipid can also significantly influence the immunogenicity of the liposomal-protein preparation (Davis and Gregoriadis, 1987, 1989). Davis and Gregoriadis (1987) also showed an equally improved response to toxoid for DSPC liposomes with similarly high ratios (for example, 2×10^3). It was tentatively proposed (Davis and Gregoriadis, 1987) that, because of the relatively large amount of lipid involved, disintegration of DSPC liposomes at the site of injection (and antigen release) would be slower, leading to a more efficient presentation of antigen to APC. This could override any inhibitory effect of DSPC on the immunogenicity of antigen taken up by the cells in the liposome form. The observed immune response in the present investigation could be explained by the differences in the composition of DSPC liposomes and use of different animal species compared to studies of Davis et al. (1987). Probably, in the present study the amount of DSPC did not interfere with antigen processing but rather functions as a slow release system.

These findings support the potential usefulness of liposomes in oral and nasal vaccine development.

In conclusion, we have shown that the immunogenicity of TT can be improved when delivered mucosally (orally and nasally) associated with liposomes. Through adjusting the dose and immunization protocol, it is possible to achieve long-lasting systemic humoral responses comparable to those of conventional i.m. delivery. However, further investigations are necessary to clarify the effects of physicochemical characteristics of liposomes on the mucosal adjuvanticity of this system.

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