

Adjuvant Action of Melittin Following Intranasal Immunisation with Tetanus and Diphtheria Toxoids

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Melittin, a 26-amino acid peptide and the major active component of the venom of the honey bee—*Apis mellifera*—has recently been shown to have absorption enhancing properties in Caco-2 cells at levels well below the level required for the generation of cytotoxicity. Given the potential of absorption enhancing agents to act as adjuvants when administered nasally [Alpar, H.O., Eyles, J.E., Williamson, E.D. and Somavarapu, S. (2001) “Intranasal vaccination against plague, tetanus and diphtheria”, *Adv. Drug Delivery Rev.* **51**, 173–201] we hypothesized that melittin may have potential as a mucosal adjuvant. Following our initial studies reported here, it was found that the co-administration of 4 µg of melittin in conjunction with tetanus toxoid in BALB/c mice was effective in eliciting markedly enhanced antibody titres in comparison to control groups and groups receiving free antigen administered intranasally. Lower concentrations of melittin were less effective and mice receiving 4 µg of melittin plus antigen exhibited antibody titres significantly higher (i.e. $P < 0.05$) than any of the other groups tested. The observed IgG2a titres were shown to be dependent upon the dose of melittin co-administered with the immunising antigen in a similar fashion to the observed total IgG responses. In summary, melittin has been shown here to have potential as a novel mucosal adjuvant for antigens administered *via* the nasal route.

Keywords: Melittin; Mucosal Adjuvant; Nasal; Vaccine; Absorption enhancer

INTRODUCTION

Current tetanus vaccines are usually combined with diphtheria, acellular pertussis and *Haemophilus influenzae* type b and consist of formaldehyde treated (toxoided) toxins of molecular weight 150 kDa. Protection from tetanus intoxication is mediated by appropriate humoral responses that must be of sufficient specificity and magnitude to facilitate neutralisation (Ourth, 1974). Despite the existence of these vaccines, tetanus still constitutes a significant public health problem in many areas of the world. The requirement for multiple injections of the currently licensed vaccines dictates that there is often comparatively poor coverage in countries where economic or logistical factors make fulfilment of the complete immunisation schedule difficult. For this reason, the World Health Organization (WHO) has indicated that the development of improved immunization strategies for tetanus as well as diphtheria is a priority.

As with tetanus, the diphtheria component of these combined vaccine formulations consists of formaldehyde

treated (toxoided) toxins. The diphtheria toxoid has a molecular weight of 65 kDa. Similarly, protection from diphtheria is mediated by humoral responses. As with tetanus, diphtheria still constitutes a major public health problem in many areas of the world. In the case of diphtheria, this has been given added impetus following recent epidemics in Eastern Europe. The epidemic of diphtheria in the Newly Independent States of the former Soviet Union is the largest outbreak in the developed world in recent years and was considered by the WHO as an international public health emergency (Galazka, 2000), which threatened to spread unchecked, exploiting non-comprehensive vaccine coverage.

The development of new and effective vaccine formulations, in particular easily administered mucosally active vaccines, may improve significantly the potential of such vaccine formulations to ameliorate the effects of diseases such as those caused by *Corynebacterium diphtheriae* and *Clostridium tetani*. It is likely that the discovery and use of a safe and effective mucosal adjuvant would have much interest for many potential applications of vaccine technology.

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Whilst lipophilic agents, such as propanolol, progesterone, pentazocine and fentanyl, administered nasally demonstrate rapid and efficient absorption (Illum, 2002), water soluble drugs or other agents, especially higher molecular weight proteins such as diphtheria or tetanus toxoids, are poorly absorbed by this route when given alone. Delivery systems for drugs or subunit vaccines may overcome poor membrane permeability by enhancing delivery *via* transcellular or paracellular routes. Some liposomal formulations, for example, have been promising enough to make it into human trials (e.g. for insulin delivery, Hilsted *et al.*, 1995) and liposome formulation has also been found to significantly enhance immune responses in comparison to free antigen nasally (Alpar *et al.*, 1992). It is generally thought that these agents enhance transcellular absorption and may assist in uptake of antigen *via* nasal associated lymphoid tissue (NALT) (Almeida and Alpar, 1996).

One alternative approach to the use of a delivery system for mucosally administered drugs or protein antigens is the use of absorption enhancers and surface active agents.

The pseudostratified columnar epithelium of the nasal mucosa is composed of ciliated cells and mucous-secreting goblet cells. Cilia are present at the apical surface and mucous adorns the apices of the flask-shaped goblet cells. The epithelium rests on a lamina propria of loose connective tissue, containing mucous glands. Whilst tight junctions seal intercellular pathways, agents which disrupt these may facilitate transport of molecules through to the underlying connective tissue. The pseudostratified columnar epithelium is found throughout the nasal cavity, trachea, bronchi and bronchioles.

Widely used experimental mucosal adjuvants include cholera toxin (CT) and heat labile enterotoxin (LT). CT and LT are AB5 enterotoxins produced by *Vibrio cholerae* and enteropathic *Escherichia coli*, the primary causative agents of cholera and traveler's diarrhea, respectively. However, the potency of these agents as toxins dictates that they are unsuitable for use in humans in their natural state. Modified or mutant versions of these toxins have attracted much attention as potential mucosal adjuvants although the observed adjuvant action has often been less than that observed for the unmodified toxins. Studies involving such mutant toxins have identified the immunomodulatory action of these agents as being related to cellular effects such as induction of transcription factors by the AB complex and enhanced intracellular cAMP engendered by the enzyme activity of the native toxin (LT) and the residual enzyme activity of a mutant toxin A domain (Ryan *et al.*, 2000).

The mechanisms of action of candidate mucosal adjuvants are potentially multifarious and diverse and the search for non-toxic adjuvants concomitantly comprises a wide variety of agents and strategies. Previous research from our laboratories has identified several potentially effective alternatives to the above described mucosal adjuvants (Alpar *et al.*, 2001). Absorption enhancers used experimentally in nasal drug delivery include non-ionic surfactants, bile salts, fatty acids,

salicylates and chelating agents. However, enhancers such as surfactants, bile salts and fatty acids may exert their effect by disrupting the phospholipid bilayer of epithelial cells, even stripping the outer layer of the mucosa and thus increasing the observed transcellular transport of drugs. For such agents, there may be a direct correlation between membrane damage and observed bioavailability (Illum, 1998). It is well known that sodium-EDTA and other Ca^{2+} chelators may act as absorption enhancers by complexing Ca^{2+} ions, which are important in the functional integrity of the intercellular spaces (Artursson and Magnusson, 1990; Tomita *et al.*, 1988). It is by this mechanism that Ca^{2+} chelators are thought to influence the paracellular transport of molecules such as peptides and proteins that are normally excluded from this pathway. Ca^{2+} chelating agents themselves may also cause problems such as local intolerance due to membrane damage. If such agents penetrate cells, then the interaction with intracellular Ca^{2+} can likely cause damage or cell death. Novel absorption enhancers with potential for drug delivery and hence also as mucosal vaccine adjuvants are of great interest and their suitability for use in the nasal mucosa may ultimately depend upon their mechanism of action and their effects on the epithelial cell membrane.

Melittin is the major active component of the venom of the honey bee—*Apis mellifera*—comprising 50% of bee venom dry weight. A 26-amino acid peptide with no disulfide bridge, the NH_2 terminal part of the molecule is predominantly hydrophobic and the C terminal part is hydrophilic and strongly basic. Melittin can be a monomer, a dimer or a tetramer, although in solutions with physiological pH monomeric protein can be found in roughly equal proportions with tetrameric protein. Melittin is known to act as an inhibitor of protein-kinases and a cell membrane lytic factor. The fact that melittin can act as a lytic agent is thought to be partly due to its detergent-like sequence, which starts with 20 apolar residues and finishes with 4 charged and 2 polar residues. This peptide has attracted some attention as a possible absorption enhancing agent (Liu *et al.*, 1999). It was hypothesised that the absorption enhancing properties of melittin may facilitate its use as a mucosal adjuvant.

MATERIALS AND METHODS

Immunisation Schedules

Adult BALB/c mice received two intranasal doses of 10 μg tetanus or diphtheria toxoids at days 0 and 13 in the presence or absence of 1, 2 or 4 μg of admixed synthetic melittin in a volume of 20 μl . Serum samples were analysed for their antigen specific antibody titres 5 days after the second immunisation at day 18 and at the subsequent timepoints of 90 and 140 days. Intranasal dosing was carried out as described previously (Eyles *et al.*, 2001). Tetanus and diphtheria toxoids were kindly supplied by the Serum Institute, India.

Enzyme Linked Immunosorbent Assays

The ELISA estimation of anti-diphtheria, anti-tetanus and anti-melittin specific antibodies was carried out as outlined using standard ELISA techniques. Briefly, antigen (tetanus, diphtheria or melittin) was added at 100 μ l antigen *per* well (5 μ g/ml) in PBS. Plates were left at 4°C overnight, washed once with PBST, blocked with 50 μ l of 4% bovine serum albumin (fraction V, Sigma, Poole, Dorset, UK) and incubated for one hour at 37°C. After washing three times with PBST, 50 μ l of serum, serially diluted in PBS, was added to the wells and plates were then incubated for one hour at 37°C. Plates were washed again three times with PBST and 50 μ l of conjugate (horseradish peroxidase conjugated anti-mouse isotype specific immunoglobulin; anti-IgG2a, (Harlan sera-lab, Loughborough, UK) or anti-total IgG (Sigma, Poole, Dorset, UK) in PBS, at the manufacturers' recommended dilution for this application, was added to each well. This was then incubated for one hour at 37°C. Colouring agent (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) (Sigma, Poole, Dorset, UK) in citrate buffer incorporating hydrogen peroxide (Sigma, Poole, Dorset, UK) was added to wells after washing plates three times with PBST. The plates were then incubated for half an hour at 37°C and absorbance at 405 nm was then measured using a Dynax MRX microplate reader (Dynax technologies, Billingshurst, W. Sussex, UK).

Cell Viability (MTT Assay)

Cell viability and cytokine production was assessed using splenocyte cultures. Briefly, groups of 4 BALB/c mice were humanely culled and their spleens and/or specified lymph nodes aseptically removed and placed into ice-cold sterile PBS. A crude suspension of spleen cells [in 10 ml working media RPMI 1640 (Life technologies) supplemented (to a resultant concentration of 10%, v/v) with Foetal bovine serum (Life technologies), 20 mM L-glutamine (Sigma, Poole, Dorset, UK), 10^5 U of penicillin litre⁻¹, and 100 mg of streptomycin litre⁻¹ (Sigma, Poole, Dorset, UK)] was prepared by gently grinding the spleen on a fine wire screen. The cell suspension was allowed to settle for approximately 5 min. The liquid was transferred to sterile 20 ml volume "Falcon" tubes, being careful not to disturb the cellular debris at the bottom. The cell suspension was spun at 1200 rpm (200 relative centrifugal force) for 10 min. After centrifugation the supernatant was removed, the cell pellet resuspended in 10 ml fresh working media and the centrifugation procedure was repeated. Following this final centrifugation the cell pellet was resuspended in 5 ml fresh working media, and the cell concentration adjusted to 1×10^7 cells ml⁻¹. Using 96 well tissue culture plates (Fisher, Leics, UK), 100 μ l volumes of viable splenocytes were seeded onto 100 μ l volumes of sterile media or antigen in sterile media

(at the concentrations stated). Covered plates were incubated in a humid (5% CO₂) environment at 37°C for 24 h, after which supernatants were removed and stored at minus 70°C for analysis later. Cytokine levels in the culture supernatants were quantified using DuoSet® capture ELISA (R&D Systems Europe Ltd, Oxfordshire, UK) according to the manufacturers instructions.

Cell viability was evaluated using the MTT assay, which measures the ability of the mitochondrial dehydrogenase enzymes of living cells to reduce the soluble yellow MTT salt [3-(4,5-di-methylthiazol-2-yl)2,5-diphenyl tetrazolium bromide] (MTT, Sigma, Poole, Dorset, UK) into an insoluble purple formazan salt. For this assay, 100 mg of MTT was dissolved in 10 ml PBS and pushed through a 0.2 μ m filter. The MTT solution (30 μ l) was added to the wells for 4.5 h or until the generation of a visible blue precipitate. The culture medium was removed and the precipitate was dissolved in 50 μ l DMSO (dimethyl sulfoxide, Sigma, Poole, Dorset, UK). Absorbance was read at 570 nm and taken as a measurement of cell viability.

RESULTS AND DISCUSSION

Adjuvant Effect of Melittin

It was found that the co-administration of 4 μ g of melittin in conjunction with tetanus toxoid was effective in eliciting markedly enhanced antibody titres in comparison to the control. Lower concentrations of melittin were less effective and mice receiving 4 μ g of melittin plus antigen exhibited antibody titres significantly higher (i.e. $P < 0.05$) than any of the other groups tested (Fig. 1).

The enhanced antibody titres were maintained throughout the duration of the experiment. IgG2a subclass titres are shown in Fig. 2. The observed IgG2a titres were shown to be dependent upon the dose of melittin co-administered with the immunising antigen in a similar fashion to the observed total IgG responses. This was thought to be indicative of a mixed Th1/Th2 type response as although IgG2a is clearly present, the ratio of total IgG to IgG2a is quite high. The presence of antigen specific IgG2a is encouraging for the presence of a well defined Th1 element.

Melittin was also shown to have potential as an adjuvant for diphtheria toxoid (Fig. 3), although the concentrations of melittin used were less than the most effective concentration for tetanus toxoid and the observed titres were correspondingly lower. In both cases the melittin appeared to facilitate the longevity of the observed immune response in comparison to immunisation with antigen alone (Figs. 1 and 3). From the cytokines which were assayed, TNF- α , IL-2, IL-6, IL-1 β and interferon- γ were detected in the supernatants of antigen stimulated spleen cells. Although cytokine production was variable, production

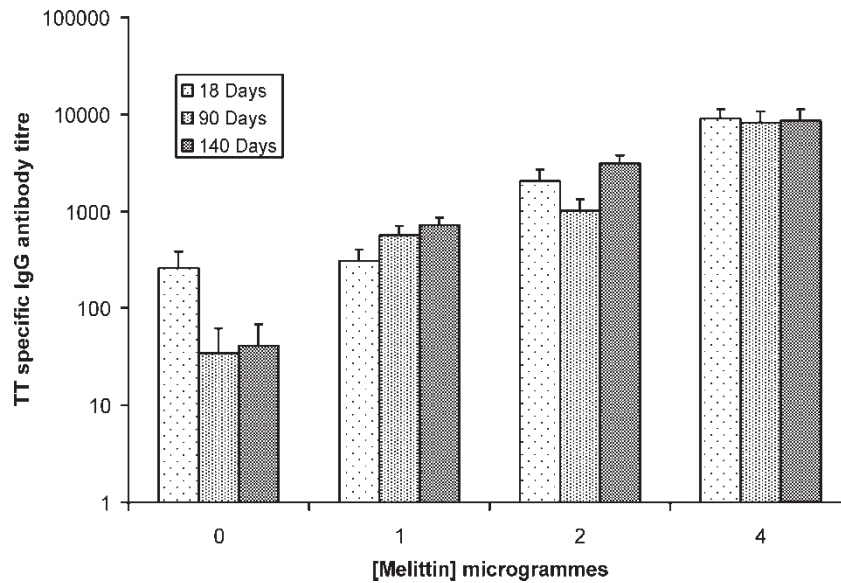


FIGURE 1 *Tetanus toxoid (TT) specific total IgG titres with and without melittin.* The tetanus toxoid specific antibody titre is shown for 18 days (5 days after the booster dose), 90 days and 140 days following intranasal dosing in the presence or absence of melittin as indicated. Values represent the total IgG titre. Error bars represent standard error, $n = 5$.

of these cytokines in the antigen stimulated splenocytes was higher than that shown for the melittin stimulated cells (data not shown).

Cytotoxicity and Safety of Melittin

Using the MTT assay it was found that at 20 $\mu\text{g/ml}$ melittin concentration splenocyte cells were no longer viable. No significant lysis of cells was detected at 2 $\mu\text{g/ml}$ melittin. These results are shown in Fig. 4. However, there was also no observable increase in cell numbers detected by the MTT assay following re-stimulation of splenocytes obtained from pre-immunised mice with either antigen or low concentrations of melittin. In addition, no anti-melittin IgG was detected in the serum of immunised mice.

Synthetic melittin used as an adjuvant for intranasally administered antigen was found to be well tolerated *via* this route at all of the concentrations used and the efficacy of the induction of antigen specific antibody production was increased at higher doses of melittin. In addition, the fact that we were unable to detect anti-melittin antibodies (IgG) in the serum of immunised mice here is encouraging. However, it was thought that the detergent like sequence of melittin may make this molecule unsuitable for some ELISA assays where antigen is adsorbed onto plates. Therefore, it is not possible to say with absolute certainty that just because anti-melittin antibodies were not detected by the ELISA method in the serum of immunised mice, that they were not present.

Melittin is a surface active potent antibacterial agent which binds to both electrically neutral and negatively charged lipid

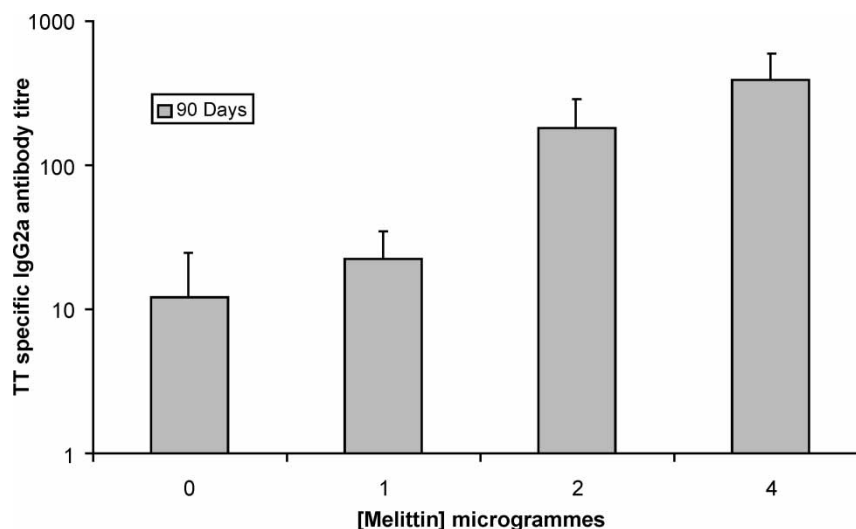


FIGURE 2 *Tetanus toxoid (TT) specific IgG2a subclass titres with and without melittin.* The tetanus toxoid specific IgG2a antibody titre is shown for 90 days following intranasal dosing in the presence or absence of melittin as indicated. Error bars represent standard error, $n = 5$.

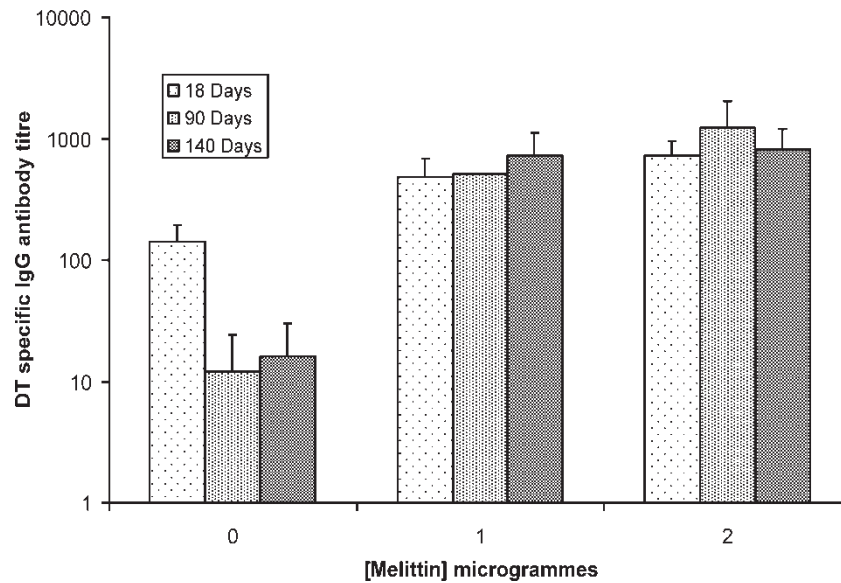


FIGURE 3 *Diphtheria toxoid (DT) specific total IgG titres with and without melittin.* The diphtheria toxoid specific antibody titre is shown for 18 days (5 days after the booster dose), 90 days and 140 days following intranasal dosing in the presence or absence of melittin as indicated. Values represent the total IgG titre. Error bars represent standard error, $n = 5$.

bilayers. Largely unstructured in solution as a monomer, melittin is believed to form an amphipathic α -helical formation when partitioned into membranes, inducing channel formation in bilayers (Allende and McIntosh, 2003). It is thought that melittin is able to preferentially lyse bacterial cells (in particular Gram-positive bacteria) due to the lipid composition of eukaryotic plasma membranes in that the presence of cholesterol reduces bilayer binding efficiency and leakage caused by melittin (Allende and McIntosh, 2003). However, the cytolytic action of melittin has been shown in many eukaryotic cell lines

(Saini *et al.*, 1999). The perceptions of melittin as a cytolytic factor are so well known that it has even attracted attention as a possible immunotoxin (Dunn *et al.*, 1996). Despite these perceived powerful actions on eukaryotic cells, recent research (Liu *et al.*, 1999) has shown that melittin has absorption enhancing properties in Caco-2 cells at levels well below the level required for the generation of cytotoxicity. In this study, the cytotoxic effects of melittin were clearly shown at $20 \mu\text{g ml}^{-1}$ (Fig. 4) but no cytotoxicity was shown at $2 \mu\text{g ml}^{-1}$. However, the lowest concentration of melittin used in the immunisation study was $1 \mu\text{g per dose}$, which is

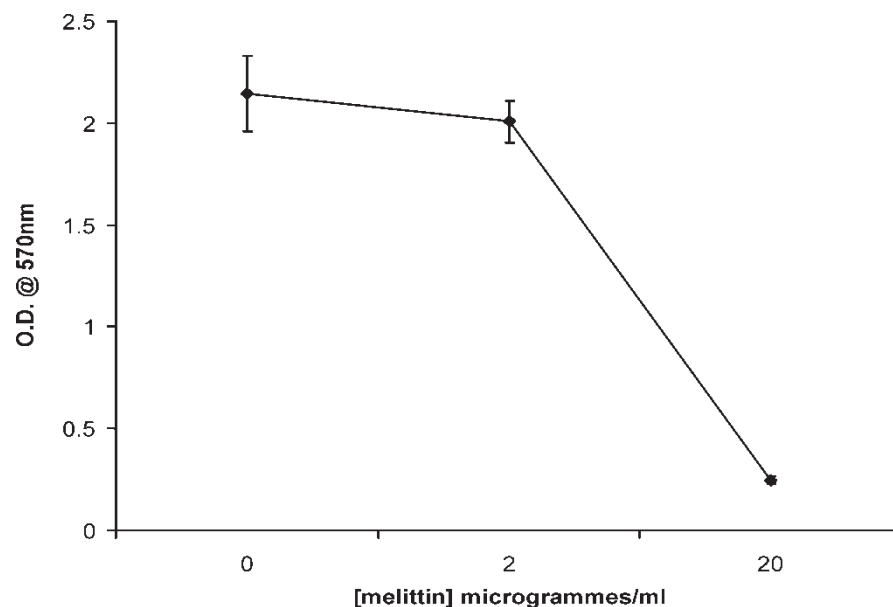


FIGURE 4 *Cell viability following exposure to melittin.* Cell viability is shown as determined by the reduction of MTT measured by optical density at 570 nm. Error bars represent standard deviation, the average of 7 measurements is shown. Spleen cells were stimulated with 0, 2 or $20 \mu\text{g per ml}$ concentration of melittin as shown on the graph.

the equivalent of $50 \mu\text{g ml}^{-1}$ in the dose itself. The melittin concentration may be high enough to cause cytotoxicity and potentially facilitate uptake through membrane damage at the concentration used. However, this does not take account of the dilution effect of nasal secretions and the fact that the vaccine dose may be present at the mucosal surface only for a short period of time, both of which may have the effect of enhancing cell integrity in comparison to the *in vitro* model. In addition, the epithelial cells of the mouse nasal cavity may not be as susceptible to the cytotoxic effects of melittin as the cultured mouse splenocytes used in the cytotoxicity assays.

Melittin in Perspective

Recently, melittin has attracted attention as a possible anti-cancer agent and scientists are working towards the development of a modified melittin molecule in order to reduce the possibility of allergic reactions or anaphylactic shock (Kortt, 1999). Such derivatives may also be of interest as mucosal adjuvants. Whilst it can be speculated that the adjuvant effect observed here is at least in part due to the cell membrane integrity altering properties of melittin, the fact that melittin was found to be well tolerated *via* this route at all of the concentrations used and the findings that melittin can alter the permeability of Caco-2 cell monolayers, apparently by the paracellular route (Liu *et al.*, 1999) indicates that melittin may have a number of actions in addition to surface active properties. There is evidence of diverse actions such as transient activation of phospholipase D in melittin mediated membrane disruption/cytolysis by an uncharacterised signal transduction mechanism (Saini *et al.*, 1999) which indicates that there may be more to melittin than α -helical formation. In addition to the evidence of non-cytolytic absorption enhancement, melittin may be shown to have a diversity of actions as has been shown for more well known mucosal adjuvants such as CT and LT. Melittin certainly warrants further investigation as a potential adjuvant. To our knowledge, this is the first time that this agent has been shown to function as an efficient adjuvant *via* any route. The fact that melittin has been shown here to act as an efficient adjuvant for mucosal immunisation may be of particular interest.

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