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Immunogenicity of Dinitrocarboxyphenylated Melittin: The Influence of C-terminal Chain Shortening, N-terminal Substitution and Prolin Insertion at Positions 5 and 10

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Abstract: Peptides derived from the bee-venom melittin were fitted with the haptenic group dinitrocarboxyphenyl (Dncp) and tested in out-bred guinea pigs for immunogenicity by measuring the IgG anti-Dncp antibody response by ELISA. Dncp-conjugates comprising virtually the entire melittin proved to be strong immunogens producing antibody responses comparable to those of proteins. Weak responses were obtained with considerably shortened sequences. Conjugates with N-terminal Dncp gave markedly reduced antibody responses compared to peptides with C-terminal Dncp. An N-terminal biotinyl substituent abolished the immune response whereas N-terminal lauryl and caprylyl had little effect. Insertion of L-proline into a hexadecapeptide conjugate abolishing the possibility of helix formation gave an immunogen to which individual animals clearly responded on a low level. Oligomerisation, but not the cytolytic activity of melittin peptides, may contribute to the immunogenicities observed.

Keywords: Melittin immunogenicity; T-cell epitopic secondary structure; melittin toxicity; substituent effects

INTRODUCTION

The linear hexacosapeptide melittin is a major component of honeybee venom to which about one-third of individuals allergic to bee-venom develop melittin-specific IgE antibody [1, 2]. Melittin is also a good immunogen in rabbits [3] and in selected strains of mice [4]. We have shown in preliminary reports [5, 6[that melittin-derived peptides fitted with a C-terminal 2,4-dinitro-6-carboxyphenyl (Dncp) haptenic group produce in out-bred guinea pigs anti-Dncp IgG titres approaching titre values obtainable with Dncp–protein. Such a pronounced immunogenicity is unusual for a small peptide and special parameters must therefore be involved. Of course effective T-cell epitopic moieties are a prerequisite, but in addition some of the special properties of the melittin molecule will play a role.

In dilute aqueous solution melittin occurs as random coil but in lipid bilayers and other hydrophobic environments it assumes an essentially helical conformation with two helical moieties comprising positions 1–11 and 12–21 respectively which are bent by 60° relative to each other. The C-terminal segment 22–26 seems non-helical. Oligomerization to tetrameric melittin may form bilayer-spanning polar pores allowing ionic permeability [7–9]. Helix 1–11 is definitely amphipatic and upon immunization in complete Freund's adjuvant distinct antibodies against the polar and non-polar sides of the helix were found [3].

Melittin has detergent-like properties, is strongly haemolytic and generally cytotoxic. Haemolytic capacity seems to depend on the C-terminal basic

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chain since after its removal the remaining segment 1–20 is not lytic although it will still bind to erythrocytes [10, 11]. Relationships between membrane configurations of melittin and its biological effects were reviewed repeatedly [12, 13].

During immunization of guinea pigs, unusual effects related to melittin toxicity were not observed. This may be due to the fact that the peptides are only gradually released from the Freund's adjuvant emulsions used. The observed lack of immune reactivity in several individual animals is not a toxic effect but is related predominantly to a lack of interaction of the melittin epitopes with the genetically specified forms of the MHC-class II molecules in these animals as discussed in later paragraphs.

In the present report we use melittin-derived peptides fitted with a single Dncp haptenic group which is expected to serve as an effective antigenic determinant unfailingly selected by B-cells for antibody production. Immunogenicity of such conjugates is then studied by assessing the anti-Dncp antibody response in out-bred guinea pigs. Since Trp and Phe are known to be immunochemically equivalent, all sequences with Trp at position 19 were synthesized with Phe instead of Trp. The main issues studied were the influence upon immunogenicity of shortening the melittin chain at the C-terminus, the effect of inserting additional Pro into the sequence expected to inhibit helix formation and the role of N-terminal substituents.

MATERIALS AND METHODS

Dncp–Peptides

Peptides were prepared by solid-phase synthesis using the Fmoc/*tert* butyl strategy with the C-terminal Fmoc-amino acid linked to *p*-alkoxybenzyl-alcohol resin [14]. The reaction solvent was *N,N*-dimethylformamide (DMF), the activation usually with HOBt/DCC. Removal of the peptides from the resin was 82.5% trifluoroacetic acid (TFA); 5% phenol; 5% water; 5% Thioanisole; 2.5% ethanedithiol (reagent K). Trifunctional protected amino acids were Fmoc-Gln(Dod), Fmoc-Arg(Mtr), Fmoc-Lys(Boc) and Fmoc-Ser(*t*Bu). Resin and amino acid derivatives were obtained from Bachem, Bubendorf. The Dncp group, 2,4-dinitro-6-carboxyphenyl, was introduced by means of 2-chloro-3,5-dinitrobenzoic acid (*o*-Dncp-Cl) [15]. For C-terminal Dncp, 1,6-diaminohexane (Dah) or Lys was used as an attachment point. For Dah attachments the peptides were fitted at the free C-terminus with N^1-Boc-Dah (Fluka, Buchs) using HOBt/DCC in DMF. The free peptide at this stage had undergone Sephadex gel filtration but not yet final purification. The reaction was sluggish and difficult to complete (ninhydrin test negative in thin-layer chromatography; TLC). After Boc removal by TFA, the reagent was evaporated and the residue taken up in water and lyophilized. A threefold molar excess of *o*-Dncp-Cl was added to the lyophilizate in 1-methyl-2-pyrrolidone and the solution was kept at pH 9 with triethylamine. This reaction took several days for completion. Remaining Dncp-Cl was removed by Sephadex gel filtration.

It was slightly better to start the peptide synthesis with Lys as the first amino acid. This approach could be used with sequences which had, after splitting from the resin and gel filtration, a single free ε-amino group of the C-terminal Lys as the only function reacting with *o*-Dncp-Cl. A satisfactory introduction of C-terminal Dncp is by starting with a Dncp containing unit in the peptide synthesis: Fmoc-Lys was reacted with *tert*-butyl-2-chloro-3,5-dinitrobenzoate [16] and the resulting Fmoc-Lys(Dncp-OtBu) was attached to a Gly-resin as the first step. The final peptide will then have a C-terminus Lys(Dncp)-Gly-OH. N-terminal Dncp was introduced by reacting the resin-bound peptides with the deprotected N-terminal amino group for 5 h with a five fold excess of *o*-Dncp-Cl in DMF while the pH was kept between 8 and 9 with 4-methylmorpholine. Alternatively, Dncp-Gly was used in the last coupling step of the peptide synthesis. Dncp-Gly was prepared as described for N^6-Dncp-aminohexanoic acid [15].

The Dncp–peptides were purified by precipitations as well as Sephadex and Silica gel chromatography until the preparations were homogeneous in at least two different solvents in TLC and upon high-voltage thin-layer electrophoresis (HVTLE). They were further tested by amino acid analysis. After hydrolysis, amino acids carrying the Dncp substituent do not show up at the positions of the free amino acids in the chromatograms.

Dncp–HSA

Human serum albumin (HSA) conjugates with 14 Dncp groups and with 3 groups per molecule were obtained according to previously reported procedures [15].

Immunization of Animals

GOHI-guinea pigs (out-bred, female, 250 g) were obtained from BRL Ltd., Füllinsdorf, Switzerland.

Each animal received subcutaneously 0.05 mg Dncp-peptide or 1.0 mg Dncp–HSA in 1 ml Complete Freund's adjuvant 0.01 M phosphate-buffered saline pH 7.4 1:1. Boosts were made every two weeks with 5 μg Dncp–peptide (100 μg Dncp–protein) given intradermally in 0.1 ml phosphate-buffered saline.

Bleedings were taken one week after a boost. Antisera were stored at −70°C.

ELISA

Polystyrene microtitre plates (Dynatech) were coated with N^1-Dncp–diaminohexane [17] and used as described previously [18]. The detecting antibody was a goat anti-guinea pig IgG-alkaline phosphatase conjugate (Jackson Immunoresearch Laboratories, West Grove, PA). Titres are expressed as the highest reciprocal dilution (*D*) of the antisera giving an absorbance of 1.0.

Toxicity

Assessments by haemoglobin release were made essentially according to DeGrado *et al.* [11] using heparinized human blood as a source for erythrocytes. Cutaneous toxicity was measured on the shaven flanks of white guinea pigs by intradermally injecting 10 μg of the peptide in 0.1 ml of 0.01 M phosphate-buffered saline pH 7.4. Immediately afterwards 1 mg Evans blue in 1 ml phosphate-buffered saline was injected intravenously. After 20 min two perpendicular diameters of the blue skin spots were measured. This assessment is also used for quantifying passive cutaneous anaphylaxis [17].

RESULTS

Characterization of Dncp–peptides

The Dncp–peptides with their abbreviations and some analytical data are shown in Tables I and II. Table II also contains the data of haemoglobin release capacity. The peptide derivatives comprising the full (modified) sequence of melittin show lytic activities similar to melittin. Melittin (1–21)-Dncp and all other derivatives of this or smaller size are virtually negative. On the other hand melittin*(1–19)Dah-Dncp and melittin*(1–16)-Lys(Dncp) showed a cutaneous toxicity regarded as still substantial although significantly lower ($p < 0.05$) than the toxicities of the full-length derivatives as shown in Figure 1.

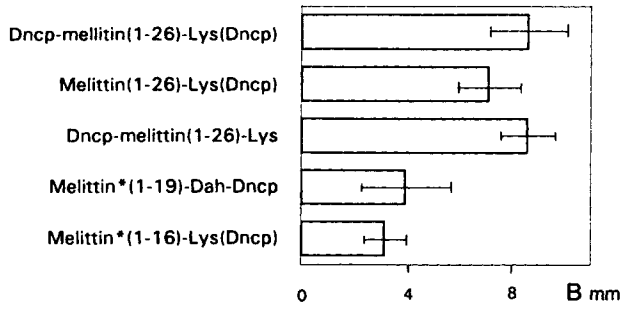

Figure 1 Cutaneous toxicity of Dncp-peptides in guinea pigs. B: mean diameter of blue spot.

Characterization of Immunogenicity

The anti-Dncp response obtained with melittin (1–26)-Lys(Dncp)-Gly and Dncp-melittin(1–26)-Lys(Dncp)-Gly is shown in Figure 2 and compared with the response against $Dncp_{14}$–HSA. In our experience the haptenic response against this protein is among the highest we have ever observed, taking into account not only HSA but BGG and KLH conjugates. Furthermore, when $Dncp_3$-HSA was used as immunogen, titres on ELISA plates coated with 10^5M HSA were around 10^6, i.e. the anti-protein responses were in the same range as the anti-hapten values (data not shown). We therefore conclude that the anti-hapten response of the melittin conjugates is in the same range as that of haptenic proteins.

With melittin conjugates involving shorter sequences, the responses remain high in individual animals. They may be absent in others, however (Figure 3). This phenomenon is not unexpected and is thought to be related to the small (probably one or two) number of T-cell epitope sequences present in the melittin peptides as further elaborated in the Discussion. On the other hand such an effect is rarely seen in hapten–protein conjugates since a multitude of T-cell epitopes are usually available there. The shortest peptides studied thus far are melittin(1–16)-Dah-Dncp and melittin*(1–16)-Lys(Dncp). Their responses are mostly orders of magnitude lower but not strictly negative in any single animal. Little influence is noted when the boosting schedule is altered using longer intervals (Figure 4).

Dncp at the N-terminus Results in Low Immunogenicity

When the haptenic group is positioned at the N-terminus such as in Dncp-Melittin(1–26), a

Table I. Synthetic peptides derived from Melittin[a]

Peptide abbreviation	Sequence	Amino acid molar ratios (expected *found*)											
		Gly	Thr	Ala	Ser	Arg	Pro	Val	Ile	Leu	Phe	Lys	Glu
Melittin*(1–16)-Lys(Dncp)	G'IGAVLRVLTTGLPALK	2	2	2		1	1	2	1	4			
		1.92	*1.95*	*2.09*		*1.03*	*1.09*	*1.86*	*1.05*	*4.15*			
Dncp-Melittin*(1–16)-Lys	GIGAVLRVLTTGLPALK	2	2	2		1	1	2	1	4		1	
		2.30	*1.77*	*1.97*		*0.99*	*1.02*	*1.73*	*0.96*	*3.92*		*1.34*	
Melittin*(1–16,^{5,10}Pro$_2$)-Lys(Dncp)	G'IGAPVLRVLPTTGLPALK	2	2	2		1	3	2	1	4			
		1.96	*1.78*	*2.00*		*1.09*	*3.11*	*1.84*	*1.02*	*3.98*			
Melittin*(1–19)-Dah-Dncp	G'IGAVLRVLTTGLPALISF	2	2	2	1	1	1	2	2	4	1		
		2.01	*1.93*	*1.93*	*0.93*	*1.11*	*1.12*	*1.87*	*2.01*	*4.00*	*1.02*		
Dncp-Melittin*(1–19)-Lys	GIGAVLRVLTTGLPALISFK	2	2	2	1	1	1	2	2	4	1	1	
		2.07	*1.94*	*2.08*	*1.05*	*1.14*	*1.09*	*1.95*	*1.85*	*3.63*	*1.10*	*1.10*	
Melittin*(1–21)(Dncp)	G'IGAVLRVLTTGLPALISFIK	2	2	2	1	1	1	2	3	4	1		
		1.98	*1.87*	*2.02*	*0.96*	*1.09*	*1.03*	*1.99*	*3.11*	*3.97*	*0.98*		
Biotinyl-Melittin*(1–21)(Dncp)	GIGAVLRVLTTGLPALISFIK	3	2	2	1	1	1	2	3	4	1		
		2.70	*2.00*	*2.10*	*1.00*	*1.00*	*1.00*	*1.90*	*3.00*	*4.10*	*1.00*		
Lauryl-Melittin*(1–21)(Dncp)	GIGAVLRVLTTGLPALISFIK	3	2	2	1	1	1	2	3	4	1		
		2.89	*1.93*	*2.09*	*0.98*	*1.09*	*1.02*	*1.97*	*3.06*	*3.99*	*0.98*		
Caprylyl-Melittin*(1–21)(Dncp)	GIGAVLRVLTTGLPALISFIK	3	2	2	1	1	1	2	3	4	1		
		2.93	*1.95*	*2.17*	*1.06*	*1.08*	*1.07*	*1.84*	*2.94*	*3.95*	*1.00*		
Dncp-Melittin(1–26),	GIGAVLKVLTTGLPALISFIKRKRQQ	2	2	2	1	2	1	2	3	4	1	3	2
		2.33	*1.71*	*1.93*	*0.89*	*2.21*	*0.96*	*1.96*	*2.96*	*3.87*	*1.05*	*3.16*	*2.11*
Melittin(1–26)-Lys(Dncp-Gly	G'IGAVLKVLTTGLPALISFIKRKRQQKG	3	2	2	1	2	1	2	3	4	1	3	2
		2.88	*1.83*	*1.99*	*0.92*	*2.10*	*0.97*	*1.89*	*2.97*	*4.05*	*1.01*	*3.25*	*2.05*
Dncp-Melittin(1–26)-Lys(Dncp)-Gly	GIGAVLKVLTTGLPALISFIKRKRQQKG	3	2	2	1	2	1	2	3	4	1	3	2
		2.91	*1.95*	*2.07*	*0.94*	*2.18*	*1.01*	*1.87*	*2.73*	*3.86*	*1.04*	*3.27*	*2.16*

[a] Melittin is a modified sequence containing Phe instead of Trp at position 19 of the original sequence. Gly at the unsubstituted N-terminus is replaced by *N,N*-dimethylglycin (G'). In melittin*, Lys of position 7 is replaced by Arg.
Dah: 1,6-diaminohexane. Amino acid analyses are based on the mean of all residues.

Table II. Some Properties of Melittin-related Peptide Derivatives

Peptide[a]	TLC[b] RF values			HVTLE[b] s_{kat} (mm)	Haemoglobin release[c] Absorbance of haemoglobin released by peptides at	
	A	B	C		5×10^{-7} M	10^{-5} M
Melittin*(1–16)-Lys(Dncp)	0.40	0.17		53	0.013	0.019
Dncp-Melittin*(1–16)-Lys	0.44	0.40		51	0.017	0.029
Melittin*(1–16,^{5,10}Pro$_2$)-Lys(Dncp)	0.38	0.15		47		0.028
Melittin*(1–19)-Dah-Dncp	0.43	0.20		52	0.014	0.029
Dncp-Melittin*(1–19)-Lys	0.49		0.90	51	0.019	0.029
Melittin*(1–21)(Dncp)	0.41	0.17		52	0.015	0.032
Biotinyl-Melittin*(1–21)(Dncp)	0.44		0.71	52	0.012	0.020
Lauryl-Melittin*(1–21)(Dncp)	0.75	0.60	0.89	52	0.015	0.025
Caprylyl-Melittin*(1–21)(Dncp)	0.71	0.59	0.88	55	0.025	0.033
Dncp-Melittin(1–26)	0.28	0.00		48	1.00	1.10
Melittin(1–26)-Lys(Dncp)-Gly	0.00		0.75	49	0.682	1.00
Dncp-Melittin(1–26)-Lys(Dncp)-Gly	0.00		0.77	47	0.742	1.09
Melittin[d]					1.08	1.08
Buffer					0.021	0.021

[a] Cf. Table 1.
[b] TLC: (A) EtOAc:HOAc:H_2O (3:1:1); (B)BuOH:HOAc:$H_2$9 (4:1:1); (C)BuOH:Pyr:HOAc:H_2O (10:9:10) HVTLE: Pyridine:HOAc:H_2O (1:9:90), 5% SDS, pH 3.6 on cellulose F, 28 Vcm^{-1}, 60 min.
[c] An erythrocyte suspension (6×10^{10} cells/l) in 0.3 M sucrose and 0.01 M phosphate buffer pH 7.3 was incubated with the peptides at 22° for 10 min and centrifuged. The absorbances at 578 nm were measured.
[d] Synthetic reference obtained from Bachem, Bubendorf.

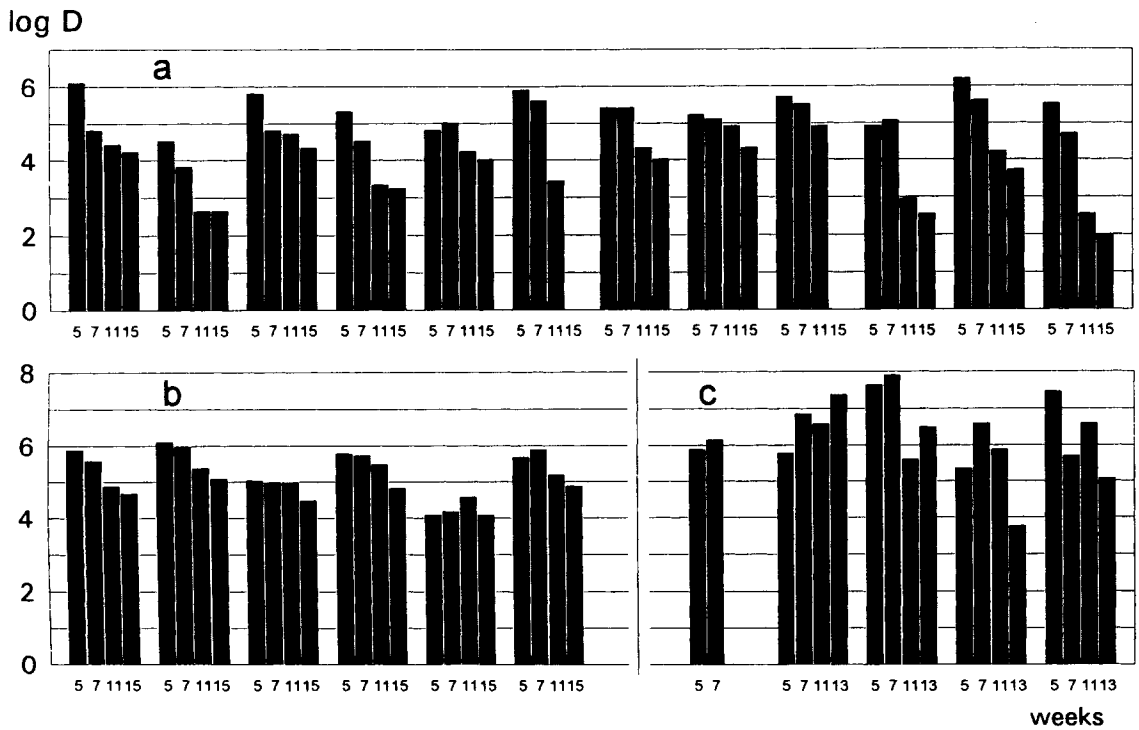

Figure 2 Anti-Dncp titres from ELISA (D) from 5 to 15 weeks after priming. Each group of columns represents the response of an individual guinea pig. The immunogen was in (a): Dncp-melittin (1–26)-Lys(Dncp)-Gly; (b): melittin (1–26)-Lys(Dncp)-Gly; (c): $Dncp_{14}$-HSA.

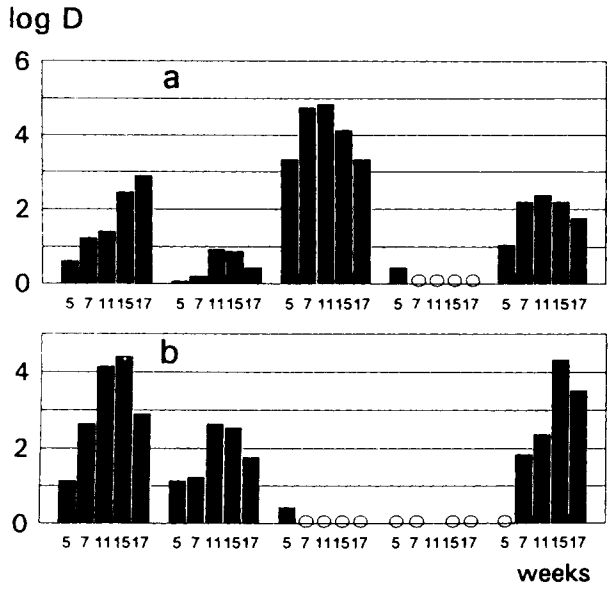

Figure 3 Outline as in Figure 1. The immunogen was in (a): melittin*(1–19)-Dah-Dncp; (b): melittin*(1–21)(Dncp).

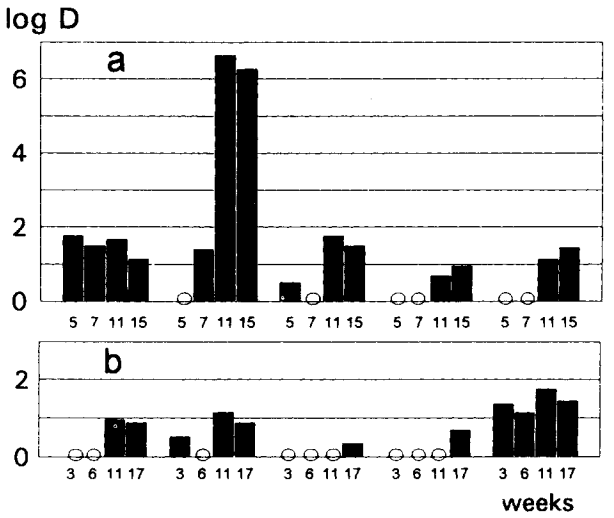

Figure 4 Outline as in Figure 1. The immunogen was melittin*(1–16)-Lys(Dncp). In (a): usual schedule of immunization as indicated under methods; (b): longer intervals between boosts of three weeks after the first boost and later of five and six weeks.

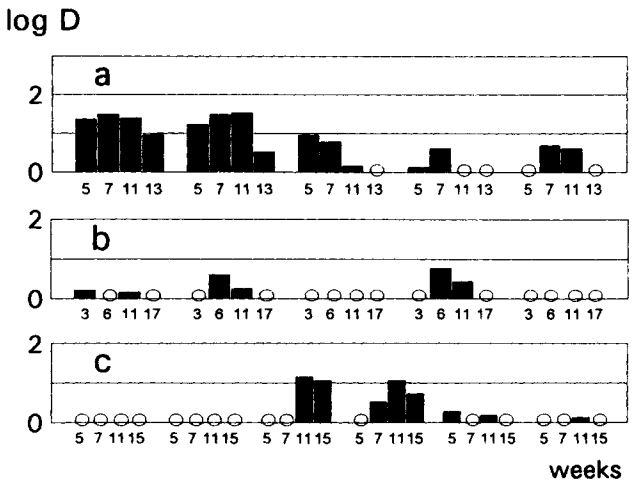

Figure 5 Outline as in Figure 1. The immunogen was in (a): Dncp-melittin(1–26); (b): Dncp-melittin*(1–16)-Lys; (c): Dncp-Melittin*(1–19)-Lys.

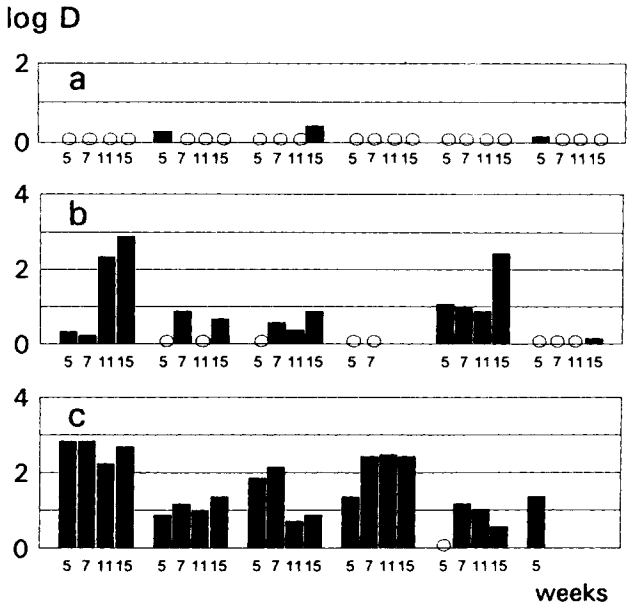

Figure 6 Outline as in Figure 1. The immunogen was in (a): biotinyl-melittin*(1–21)-(Dncp); (b): caprylyl-melittin*(1–21)(Dncp); (c): lauryl-melittin*(1–21)(Dncp).

drastically reduced immunogenicity is seen in Figure 5(a) compared with the effects of Dncp-melittin(1–26)-Lys(Dncp)-Gly and melittin(1–26)-Lys(Dncp)-Gly in Figure 2. Very weak immunogenicities are also noted with Dncp-melittin*(1–16)-Lys and Dncp-melittin*(1–19)-Lys but it is interesting that the responses are not strictly negative in some of the animals (Figure 5(b) and (c)).

Effects of Substituents at the N-terminus

When the N-terminus of melittin*(1–21)(Dncp) was biotinylated, the anti-Dncp was virtually abolished. In contrast, when lauric acid or caprylic acid was attached to the N-terminus, no marked effects on immunogenicity were seen (Figure 6). It should be noted that N-terminal Dncp in Dncp-melittin(1–26)-

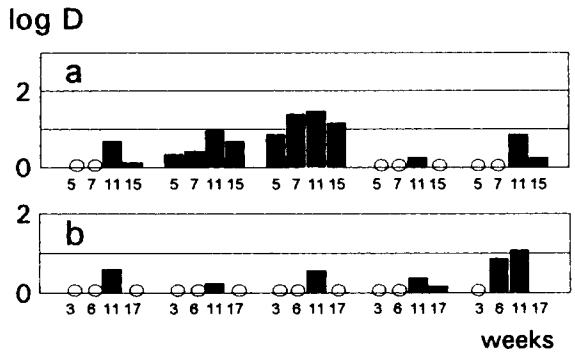

Figure 7 Outline as in Figure 1. The immunogen was melittin*(1–16,^{5,10}Pro$_2$)-Lys(Dncp). In (a): usual schedule of immunization; (b): schedule with longer intervals, cf. legend to Figure 4.

Lys(Dncp)-Gly did not alter the immunogenicity of melittin(1–26)-Lys(Dncp)-Gly although in this situation an effect of two versus one haptenic group on the same molecule may come into play.

Insertion of L-Proline into the Sequence

The insertion of Pro residues at positions 5 and 10 in melittin*(1–16,^{5,10}-Pro$_2$)-Lys(Dncp) is expected to abolish the capacity of the sequence to assume a helical configuration at the cell membrane or in hydrophobic solutions. Furthermore the insertions alter the structure of the T-cell epitopic moiety. It is therefore of interest that the immunogenicity of melittin*(1–16,^{5,10}-Pro$_2$) Lys(Dncp) shown in Figure 7 is not entirely negative and that at least one animal gave a response similar to responses obtained in several animals with melittin*(1–16)-Lys(Dncp) (Figure 4).

DISCUSSION

The Dncp group which is similar to DNP may be introduced by standard methods of peptide chemistry. Although it was originally used for enhancing the water solubility of small conjugates [15] the effect of its carboxylate is of course insufficient in larger hydrophobic peptides. The fact that immunogenicity testing does not obligatorily require water-soluble antigens remains therefore an asset of the present procedure.

It is remarkable that a peptide as small as melittin is able to evoke IgG anti-Dncp antibody responses in the range of protein responses when fitted with a single Dncp-haptenic group. It should therefore be of interest to define the parameters involved in this effect. Obviously, the peptides must contain appropriate T-cell epitopes which can be recognized and contacted by T-cell receptors and MHC-class II proteins. The T-cell epitopic moieties effective in guinea pigs have not been established, but in mice the sequence 11–19 seems primarily involved [19]. The fact that melittin*(1–16)-Lys(Dncp) and melittin*(1–16,^{5,10}-Pro$_2$)-Lys(Dncp) are immunogenic shows that in the sequences 1–16 sufficient T-cell epitopic structure is available. That even the Pro$_2$-inserted sequence 1–16 is immunogenic shows that a helical conformation is not an obligatory property of a T-cell epitope. That the primary structure rather than a secondary configuration is important for melittin-T-cell epitopes has also been concluded from studies of melittin peptides in mice [19], thus de-emphasizing the importance of Berzofsky's algorithm [20] which postulates a helical conformation of T-cell epitopes.

The low antibody responses obtained with the short peptides 1–16 is not unusual but to be expected with peptides of this size. What remains to be explained is rather the strong immunogenicity of the longer melittin peptides. We do not believe that the presentation of eventually more complete T-cell epitopes in the longer peptides has a marked effect. It is more probable that special properties of melittin play a role.

We can exclude haemolytic activity as one of those properties since a number of non-haemolytic peptides gave good antibody responses. On the other hand the non-haemolytic melittin*(1–19)-Dah-Dncp showed cutaneous toxicity which could indicate that non-cytolytic, unphysiological effects play a role. Since also the weakly immunogenic melittin*(1–16)-Lys(Dncp) was toxic, these unphysiological effects are probably not very important. The toxicity test used here is based on permeability increases of the vascular system allowing Evan's blue-stained plasma protein to become dispersed in the surrounding tissue. The effect is mediated by histamine liberated from tissue mast cells. Mast cells are known to dissipate significant amounts of histamine after unphysiological stimulation by various agents [21]. The test is quite sensitive.

Polymerized antigen and also antigen associates frequently show enhanced immunogenicity. Melittin with its tendency to form associates in the cell membrane may, in addition to the channel-forming tetramers [9], establish other oligomeric units which

could be of immunological significance. This has recently been suggested [22] but our data do not allow conclusions in this respect.

From a practical standpoint, the strong immunogenicity of melittin peptides is welcome. Where immunogens with difficult haptens are to be prepared, it may not be feasible to analyse the haptenic moieties actually obtained if a protein is used as carrier. With small peptide carriers, however, such problems are mitigated or may not occur altogether. Work along these lines is presently in progress.

One motivation for initiating the present project is the study of substituent effects. It may be rewarding to learn at what positions small and large substituents can be introduced without impairing or on the other hand resulting in enhancing immunogenicity. Expectedly, N-terminal laurylyl-and caprylyl substituents had no marked effect on immunogenicity since the N-terminal end of melitten is most probably far removed from T-cell epitopic moieties. On the other hand, virtual elimination of immunogenicity with an N-terminal biotinyl residue is interesting. The effect could be due to a substantial change of the distribution pattern of the antigen after injection, i.e. the antigen could be trapped following release from the adjuvant emulsion and thus made ineffective. This notion will have to be evaluated *inter alia* by *in vitro* immunogenicity testing. The finding that Dncp-melittin (1–26) and other peptides with N-terminally positioned Dncp is likewise of interest although not open to a clear interpretation.

The lack of IgG responses in several individuals of the out-bred guinea pigs is interpreted as a lack of T-cell help which is a prerequisite for immune responsiveness to protein and peptide antigens. T-cell help requires *inter alia* the binding of T-cells via T-cell receptors to antigen segments presented in conjunction with MHC-II proteins on antigen presenting cells. MHC-II proteins are highly polymorphic with their structure being genetically controlled. Lack of interaction between peptide and MHC-II molecules has been estimated to be a major reason for immune non-responsiveness. To some extent also a lack of T-cell repertoire must be envisaged [23, 24]. A lack of B-cell response hardly plays a role since anti-Dncp IgG responses are very regularly obtained if reliable T-cell help is provided by a protein carrier (unpublished observations). It is also unlikely that the Dncp group is cleaved off or transferred to other compounds. We have studied this problem with less stable haptenic conjugates and found no evidence for this, as published in part years ago [25].

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