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Molecular Parameters in Melittin Immunogenicity

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Abstract: Based on immunogenicity studies, two T-cell epitopes in melittin were found to be functional in guinea pigs, one being centrally located, the other one residing in the C-terminal chain. In Balb/c mice only the central epitope was found to be active. A human T-cell clone was found by T-cell proliferation studies to employ strictly the C-terminal chain. Truncation of melittin peptides at the N-terminus did not markedly affect the capacity of guinea pigs to develop anti-IgG responses towards peptidic epitopes and towards a C-terminally attached haptenic group. Attachment of various substituents inside and outside the T-cell epitopic areas had no marked effect on antibody responses. In contrast, the substituents positioned within a T-cell epitope abolished T-cell proliferation. This difference between whole animal data and cellular *in vitro* responses is presently not understood. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

The hexacosapeptide melittin showing unusually high immunogenicities in several species is a major component of honey-bee venom and an allergen in humans [1, 2]. In dilute aqueous solutions its linear chain does not assume a particular configuration but in membrane-mimicking solvents and in lipid bilayers, in highly concentrated aqueous solution as well as in the crystalline state, it has an α-helical conformation [3–6]. The molecule folds into two helical regions interspaced by a Pro-hinge at position 14 (cf. Table 1 for the melittin sequence), whereas the C-terminal segment 21–26 remains as a random structure. The structure is amphipatic in two ways. First, the N-terminal helix shows a segregated hydrophobic and a hydrophilic side, then, compared with the helical moiety, the C-terminal chain is extremely polar which may be described as primary amphiphilicity [7]. Owing to primary amphiphilicity melittin would insert inself perpendicular to the cell membrane and eventually form bundles with hydrophilic pores [6]. On the other hand the helical (secondary) amphipathy would also be expected to allow molecular positions parallel to the membrane [8, 10].

Melittin, in addition to being haemolytic and toxic to eukaryotic cells, exhibits antibacterial activity [11]. It shares this property with a number of similar small peptides which are collected under the term 'animal (insect) peptide antibiotics' [12]. These peptides, like the cecropins from Hyalophora, Bombyx and other species, are also linear, mostly helical and hinged by Gly/Pro containing helix-disrupting segments.

In the present study we show that melittin in guinea pigs exhibits two T-cell epitopes, one centrally located, the other one at the C-terminal end. The C-terminal epitope was also found to be reactive on a human T-cell clone established from an individual allergic to bee-venom; it was not active

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in Balb/c mice, on the other hand. Other parameters of interest were the effect of large substituents present within T-cell epitopes on immunogenicity and the effect of chain shortening at the N-terminus. As recently published [13] immunogenicities of the peptides were studied by using melittin-derived peptides fitted with a single 2,4-dinitro-6-carboxylphenyl (Dncp) haptenic group at the C-terminal end. The haptenic structure serves as an effective antigenic determinant regularly selected by B-cells for antibody production. It is then sufficient to measure anti-Dncp antibody responses in order to characterize peptide immunogenicity.

MATERIALS AND METHODS

Synthetic peptides

Solid-phase peptide synthesis according to the Fmoc/*tert*-butyl strategy was carried out essentially as described previously [10]. The final products were characterized by electrospray mass spectrometry and these data, together with sequences and abbreviations, are given in Table 1.

ELISA

Polystyrene microtitre plates (Dynatech) were coated with N^1-Dncp–diaminohexane and used as described before [13, 14]. The detecting antibodies goat anti-guinea pig IgG (H + L) alkaline phosphatase and goat anti-mouse IgG (H + L) alkaline phosphatase were obtained from Jackson Immunoresearch Laboratories, West Grove, PA. Titres were expressed as the highest reciprocal dilution (D_r) of the antisera giving an absorbance of 1.0 after 30 min incubation with 4-nitrophenylphosphate (1 mg/ml in 0.05 M carbonate buffer – 1 mM $MgCl_2$, pH 9.8).

Immunodot Assay

This was performed as described previously [10].

Immunization of Animals

GOHI-guinea pigs (out-bred, female, 250 g) from BRL, Ltd, Füllinsdorf, Switzerland, and Balb/c mice (female, 8–12-weeks-old) from IFFA, Credo, Saint-Germain sur l'Arbresle, France, were immunized with Dncp–peptides as described before [13, 10].

T-cell Clone

The melittin-specific human T-cell clone ReT 19 was generated at the Swiss Institute of Allergy and Asthma Research, Davos, from cells of an allergic individual R.H. [15, 16]. The clone was maintained in culture by biweekly restimulation with allogeneic peripheral blood mononuclear cells irradiated with 6000 rad and PHA (Difco Laboratories, Detroit, Michigan). T cells were used 10–14 days after restimulation.

An EBV line from the allergic donor R.H. was as described [17] and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (Life Technologies, Paisley, UK), 25 mM HEPES buffer, 10 μg/ml streptomycin and 100 U/ml penicillin. These cells were used as antigen-presenting cells in the proliferation assays with the melittin-derived peptides.

The complete culture medium consisted of RPMI 1640 supplemented with 10% pooled heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 25 mM HEPES buffer pH7.4, 2 mM L-Glutamine, 25 μg/ml human transferrin (Biotest, Dreieich, Germany), 10 μg/ml streptomycin and 100 U/ml penicillin (Animed, Basel, Switzerland). The medium used to culture the T-cell clone was enriched with 10 U/ml natural and recombinant h IL-2 (a gift from Dr A. Cerny, Inselspital, Bern, Switzerland).

T-cell Proliferation

In proliferation assays 2.5×10^4 per well ReT 19 cells were incubated in 96-well U-bottom tissue culture plates with different concentrations of melittin–peptides and with 5000 autologous EBV-transformed B-cells irradiated with 6000 rad. After two days the cells were pulsed with [^{3}H]-thymidin (Amersham, Little Chalfont, UK) for 12 h. Cells were harvested on glass fibre disks and counted in a microplate beta-counter (Inotech Filter Counting System INB 384, Inotech, Dottikon, Switzerland). Proliferative responses are given as a stimulation index (SI) obtained after the division of cpm from antigen-stimulated cultures by cpm from antigen-free control cultures.

RESULTS

Effect of Chain Shortening at the *N*-terminus on Immunogenicity

A series of melittin Dncp–peptides was tested for Dncp-specific antibodies in guinea pigs. A reference immunization was done with melittin (1–26)K(Dncp)G, **MD**, previously synthesized and

shown to give ELISA titres D_r in guinea pigs of between 10^4 and 10^6 [13]. The data found for **MD** in the present experiment are shown together with those from the truncated peptide **M**(13–26)**D** in Figure. 1. It is evident and originally unexpected that the truncated peptide still elicits a considerable antibody response. The expectation was that in **M**(13–26)**D** a marked decrease in immunogenicity should become noticeable because previous data have shown that chain shortening at the C-terminus down to **M**(1–19)**D** did not remove guinea-pig immunogenicity [13] and that therefore a centrally

Table 1 Synthetic Peptides Derived from Melittin[a,b]

Abbreviations	Sequences	MS-analysis exp./found
Melittin*(1–26)K(ac)G,**M**[c]	G′IGAVLKVLTTGLPALISFIKRKRQQK(ac)G	3063.76 *3063.32 ± 0.36*
M(2–26)**D**	IGAVLKVLTTGLPALISFIKRKRQQK(Dncp)G	3188.76 *3187.83 ± 0.35*
M(4–26)**D**	AVLKVLTTGLPALISFIKRKRQQK(Dncp)G	2976.52 *2975.81 ± 0.23*
M(7–26)**D**	KVLTTGLPALISFIKRKRQQK(Dncp)G	2693.14 *2693.11 ± 0.61*
M(9–26)**D**	LTTGLPALISFIKRKRQQK(Dncp)G	2465.84 *2465.64 ± 0.41*
M(11–26)**D**	TGLPALISFIKRKRQQK(Dncp)G	2251.57 *2250.47 ± 1.1*
M(13–26)**D**	LPALISFIKRKRQQK(Dncp)G	2093.42 *2091.95 ± 0.89*
M(9–19)**D**	LTTGLPALISFK(Dncp)G	1527.69 1526.85
M(13–19)**D**	LPALISFK(Dncp)G	1155.27 *1154.59 ± 0.1*
M(16–26)**D**	LISFIKRKRQQK(Dncp)G	1812.06 *1812.36 ± 0.18*
M(19–26)**D**	FIKRKRQQK(Dncp)G	1498.67 *1498.97 ± 0.44*
MD–3Cap	GIK(Cap)AVLKVLTTGLPALISFIKRKRQQK(Dncp)G	3401.09 *3400.29 ± 0.28*
MD–7Cap	GIGAVLK(Cap)VLTTGLPALISFIKRKRQQK(Dncp)G	3329.97 *3329.14 ± 0.15*
MD–11Cap	GIGAVLKVLTK(Cap)GLPALISFIKRKRQQK(Dncp)G	3357.04 *3356.65 ± 0.29*
MD–15Palm	GIGAVLKVLTTGLPK(Palm)LISFIKRKRQQK(Dncp)G	3499.28 not done
MD–19Palm	GIGAVLKVLTTGLPALISK(Palm)IKRKRQQK(Dncp)G	3423.19 not done
M–3**D**[d]	GIK(Dncp)AVLKVLTTGLPALISFIKRKRQQK(ac)G	3316.71 *3316.22 ± 0.40*
MD–3**D**[d]	GIK(Dncp)AVLKVLTTGLPALISFIKRKRQQK(Dncp)G	3485.00 *3484.17 ± 0.14*

[a]Melittin is a modified sequence containing Phe instead of Trp at position 19 of the original sequence. In melittin*, Lys at position 27 is acetylated (abbreviation: ac).
[b]Peptides were characterized also by amino acid analysis with amino acid ratios within the expected limits (± 10%)
[c]The melittins **MD** (melittin (1–26)K(Dncp)G), **DMD** (Dncp–melittin (1–26)K(Dncp)G) and **DM** (Dncp–melittin 1–26) have been described previously [13]. G′ stands for *N,N*-dimethylglycin.
[d]**M**–3**D** and the homologues **M**–7**D**, **M**–11**D**, **M**–15**D**, **M**–19**D**, **M**–23**D** as well as **MD**–3**D** with its homologues **MD**–7**D**, **MD**–11**D**, **MD**–15**D** and **MD**–19**D** have been described before [10].

located T-cell epitope must be present similar to the epitope 11–19 in H-2^d restricted mice [18]. The entire series of data is summarized in Table 2 which confirms a rather regular and unremarkably diminishing anti-Dncp IgG response of the peptides truncated stepwisw by three amino acids. Some of the antisera were also assessed against **MD** by immunodot assay which primarily measures anti-peptidic responses [10]. Truncation has no marked effect on anti-peptide antibody generation (Figure. 2). It should be noted that in contrast to ELISA where log values are reported, a linear scale is used here, i.e. even the relative OD values of **M**(9–26)**D** are still considerable.

Location of T-cell Epitopes

Immunogenicity data from the truncated peptides and in particular the human T-cell proliferation data (see below) suggested that the C-terminal part of melittin might be involved in T-cell epitopic activity. This was confirmed when peptides from the central and C-terminal parts were studied. Table 3 shows that a segment as small as **M**(19–26)**D** gave antibody titres up to $D_r \times 10^4$ in guinea pigs. Similar responses were observed with segment **M**(13–19)**D**. The central T-cell epitope may start before position 13 but position 9 seems not involved because there is no increase to be seen among the highest titres. It cannot be stated whether the two epitopes are adjacent or overlapping. It would seem probable that at least the flanking proportions are shared (cf. 'Discussion'). There are some animals in two groups which do not respond at all. This is not unusual in outbred guinea pigs and has been discussed before [13].

In Balb/c mice the C-terminal T-cell epitope is not recognized since the segments **M**(16–26)**D** and **M**(19–26)**D** gave no evidence of immunogenicity (Table 4). It may be of interest to note that the reference immunizations with **MD** and Dncp–HSA included in Table 4 show that **MD** has a similar immunogenicity when compared with the protein immunogen. This has also been noted before in guinea-pig immunizations [13].

Effect of Large Substituents on Murine Immunogenicity

Since Balb/c mice, in contrast to guinea pigs, are addressing only one centrally located T-cell epitope,

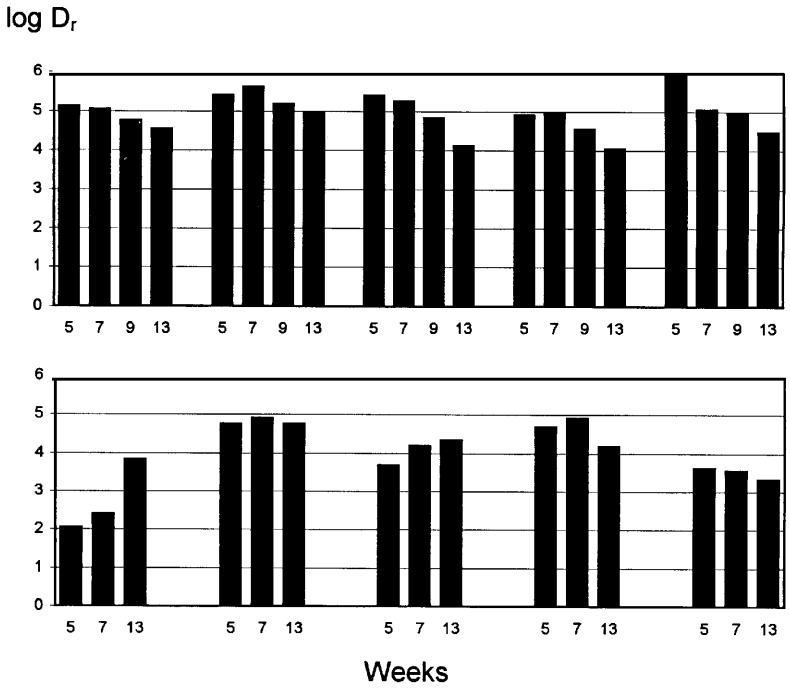

Figure 1 Anti-Dncp IgG-titres from ELISA, 5–13 weeks after priming. Each group of columns represents the response of an individual guinea pig. The immunogen in the upper section was **MD** with boosts after 2, 4, 6, 8 and 12 weeks; in the lower section **M**(13–26)**D** with boosts after 2, 4, 6 and 11 weeks.

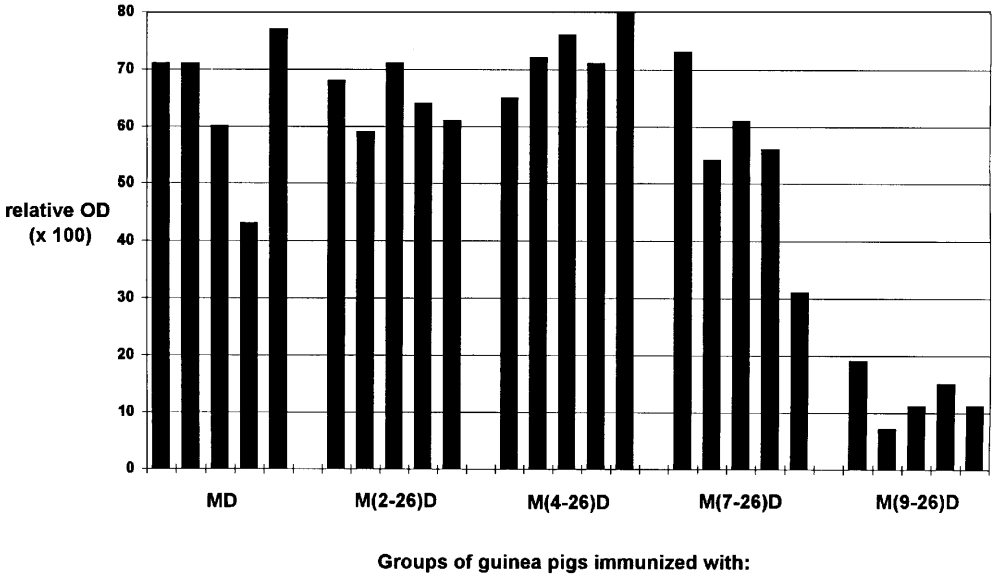

Figure 2 Anti-IgG titres from the immunodot assay of guinea-pig antisera, the nitrocellulose strips being charged with 1 μl dots of **MD** (5 mg/ml). Each individual animal is represented by one column indicating the titre after five weeks. Boosts were applied two and four weeks after priming.

the testing focused on murine immunogenicity. Three Dncp–melittin peptides containing substituents in the region of the central epitope 11–19 were tested and showed no remarkable effect on immunogenicity (Table 5), i.e. the titres were quite within the range obtained with **MD** or Dncp–HSA of Table 4. It is remarkable, in particular, that **MD**-15palm with its centrally located long palmitoyl chain does not give any evidence for interference with MHC or T-cell receptor binding. It cannot be excluded, however, that before cellular processing and before binding of the peptide fragments to MHC molecules, the substituents which are of the acyl-type in Table 5 may be enzymatically split off. We have been unable to find evidence on this point and have therefore included a series of dihaptenated peptides according to Table 6. In this series the second Dncp in the various positions within the chain may be taken as a substituent and not, like the C-terminal Dncp, as an immunochemically relevant hapten involved in recognition at the B-cell level. Table 6 shows that again no marked effect becomes evident. In fact immunogenicities are similar when Dncp-substituents within the T-cell epitopic area and outside (**MD**–3**D**, **MD**–7**D**) are compared. Again the titres were within the range of **MD** and Dncp-HSA of Table 4. In the case of Dncp we deal with a nitrated, rather exotic, aryl substituent which is unlikely to become removed by enzymatic splitting.

T-cell Proliferation Study

The T-cell proliferation pattern of unhaptenated melittin and C- and/or N-terminally haptenated melittins was similar, each one showing a maximum in the same concentration range (Figure 3). The fact that **MD** and **DMD** but not **DM** gave a lower stimulation than **M** indicates that the hapten at the C-terminus interferes to some extent as a constituent of the C-terminal T-cell epitope which is established in a further series (see below).

Evidence that the T-cell epitope is localized in the C-terminal chain came from the observation that truncation of the N-terminus starting with **M**(2–26)**D** up to **M**(16–26)**D** and **M**(19–26)**D** did not abolish proliferation as shown in Table 7. On the other hand **M**(9–19)**D** and **M**(13–19)**D** of Table 7 were negative. It is also of interest that **M**–3**D**, **M**–7**D**, **M**–11**D** and **M**–15**D** were all good stimulators comparable to the SI of **M** (cf. Figure 3), whereas **M**–19**D** and **M**–23**D** were virtually negative as shown in the lower section of Table 7. It seems, therefore, that not only the C-terminal hapten but also the haptenic substituents

Table 2 ELISA Anti-Dncp IgG Titres (log D_r) of Guinea Pigs Immunized with Truncated Melittin Peptides

Animals immunized with	Animal no.	Titres weeks after priming[a]			
		5	7	9	13
MD	70	5.1	5.1	4.8	4.6
	71	5.5	5.7	5.2	5.0
	72	5.4	5.3	4.9	4.1
	73	4.9	5.0	4.6	4.1
	74	6.0	5.1	5.0	4.5
M(2–26)**D**	75	5.5	5.5	5.2	4.4
	76	5.0	5.1	4.5	4.4
	77	5.5	5.6	5.0	4.5
	78	5.6	5.7	5.0	4.7
	79	5.0	5.0	4.8	4.6
M(4–26)**D**	80	5.5	5.6	5.1	4.5
	81	5.5	5.6	5.0	4.5
	82	5.7	5.8	5.3	4.9
	83	6.0	5.7	5.4	4.8
	84	5.7	6.0	5.2	4.8
M(7–26)**D**	3	5.2	5.1	5.1	4.9
	4	5.7	5.6	5.7	5.2
	5	5.0	5.0	5.1	4.5
	6	5.4	5.4	5.3	4.8
	7	4.9	4.7	4.4	4.2
M(9–26)**D**	8	3.7	3.3	3.3	3.4
	9	4.4	4.2	3.9	3.4
	10	4.3	4.1	4.0	3.5
	11	4.2	4.9	4.5	4.2
	12	4.6	4.4	4.4	4.2
M(11–26)**D**	31	0	0		0
	32	3.4	4.1		4.3
	33	4.2	4.2		3.9
	34	0	3.2		4.3
	35	4.2	4.4		4.0
M(13–26)**D**	36	2.1	2.4		4.0
	37	4.8	5.0		4.9
	38	3.8	4.2		4.4
	39	4.8	5.0		4.3
	40	3.6	3.6		3.4

[a]Boosts were given after 2,4,6,8 or 10 and 12 weeks. In the case of **M**(11–26)**D** and **M**(13–26)**D**, the boost after 8 or 10 weeks was omitted.

in positions 19 and 23 interfere with the T-cell epitope.

Figure 4 shows that interference by substituents occurs also with **MD**–19Palm whereas caprylyl and palmitoyl substituents outside the T-cell epitope segment had no marked influence since the stimulations by the corresponding peptides were almost as strong as that of **MD** in Figure 3. Palmitoyl in position 15 may, however, reach the epitopic area and interfere there to some extent.

DISCUSSION

Melittin peptides monohaptenated at the C-terminus have shown high immunogenicity in guinea pigs

Table 3 ELISA Anti-Dncp IgG Titres (log D_r) of Guinea Pigs Immunized with Dncp–Peptide Segments

Animals immunized with	Animal no.	Titres weeks after priming[a]	
		5	7
M(9–19)**D**	41	0	0
	42	0	2.1
	43	3.5	2.5
	44	3.7	0
	45	0	0
M(13–19)**D**	46	3.0	2.2
	47	3.8	4.2
	48	0	0
	49	3.9	3.9
	50	2.2	3.7
M(16–26)**D**	51	0	2.1
	52	4.4	4.4
	53	3.5	3.7
	54	3.5	3.6
	55	4.3	3.7
M(19–19)**D**	56	4.2	3.2
	57	3.8	4.1
	58	4.6	4.9
	59	2.8	2.7
	60	2.4	2.3

[a]Boosts were given after two, four and six weeks.

Table 4 ELISA Anti-Dncp IgG Titres (log D_r) of Balb/c Mice Immunized with C-terminal Dncp–Peptide Segments

Animals immunized with[a]	Animal no.	Titres weeks after priming[b]			
		4	6	8	11
M(16–26)**D**	1	0	0	0	
	2	0	0	2.0	
	3	0	0	0	
	4	0	0	0	
	5	0	0	0	
M(19–26)**D**	6	0	0	0	
	7	0	0	0	
	8	0	0	0	
	9	0	0	2.1	
	10	0	0	0	
MD	15		3.2	0	0
	16		3.8	4.5	4.9
	17		3.7	3.2	4.8
	18		3.3	4.9	4.4
	19		3.3	3.2	4.8
Dncp–HSA	21		4.6	4.8	5.2
	22		4.6	5.2	5.3
	23		4.8	5.0	5.2
	24		4.8	5.1	5.3

[a]Dncp–HSA is a haptenated human serum albumin with 14 Dncp groups [13].
[b]Boosts were given after five and seven weeks; in the case of **MD** and Dncp–HSA also after ten weeks.

comparable to the immunogenicity of protein conjugates [13]. The same result was obtained in the present study in Balb/c mice although the general level of IgG titres was lower than in guinea pigs. We assume that melittin undergoes the same steps during the development of the antibody response as a protein immunogen, i.e. the peptide is taken up by antigen-presenting cells, internalized, fragmented and brought to the cell surface associated with MHC class II glycoproteins. The associated fragments contain the T-cell epitopes interacting and stimulating the helper T cells. It is possible that melittin peptides act in conjunction with large carrier molecules although little direct evidence is available. One report deals with heparin-melittin complexes which give enhanced anti-IgG and IgE responses compared with melittin alone [19]. Heparin is thought to become secreted from mast cells following a bee sting. The amphipathic properties of melittin, however, allow other associations with a variety of carriers which may make melittin and melittin-derived peptides functionally large immunogens.

Truncation of melittin peptides at the N-terminus did not remove the antibody producing capacity in guinea pigs, an observation which is at variance with data from mice where even a deletion of the two N-terminal amino acids resulted in a loss of this capacity [20]. An interpretation explaining the difference is presently not available but since truncation data seem species-dependent, they cannot, in our opinion, be used to explain immunogenicities in general terms.

The present view on peptides which bind to MHC class II molecules is that they have their N- and C-termini protruding out of the MHC groove which strongly binds a nonamer core in the middle of 12–15mer peptides. However, peptides shorter than nonamers may also bind very well to the groove [21,22]. The sequences flanking the core region appear to increase the binding by non-specific interactions with the MHC molecules [23]. It also seems that variations of the flanking chains can influence T-cell recognition [24]. Our finding in

Table 5 ELISA Anti-Dncp IgG Titres (log D_r) of Balb/c Mice Immunized with Substituted Dncp–Melittin Peptides

Animals immunized with	Animal no.	Titres weeks after priming[a]		
		4	6	10
MD–11Cap	81		4.1	4.4
	82		4.2	4.8
	83		4.2	5.0
	84		4.3	4.9
	85		4.3	5.0
MD–15Palm	86	0	3.0	2.9
	87	0	4.2	3.8
	88	0	3.5	3.6
	89	3.4	4.3	4.1
	90	3.2	4.4	4.0
MD–19Palm	91	3.5	5.5	4.7
	92	4.2	5.3	4.8
	93	0	5.1	4.3
	94	2.7	5.6	4.6
	95	3.4	5.1	4.3

[a]Boosts were given after five and nine weeks, in the case of **MD**–11Cap after five and seven weeks.

Table 6 ELISA Anti-Dncp IgG Titres (log D_r) of Balb/c Mice Immunized with Dihaptenated Melittin Peptides

Animals immunized with	Animal no.	Titres weeks after priming[a]			
		4	5	8	11
MD–3**D**	101	3.6	4.7		4.9
	102	2.9	4.2		4.8
	103	2.9	4.2		5.1
	104	3.6	4.2		5.0
	105	3.7	4.9		5.1
MD–7**D**	106	3.5	not done		not done
	107	3.6	5.2		5.1
	108	3.2	4.5		4.4
	109	3.1	5.1		4.9
	110	3.0	5.0		4.9
MD–11**D**	111		5.1	4.9	
	112		4.4	4.4	
	113		4.6	5.1	
	114		5.1	5.1	
	115		4.7	5.0	
MD–15**D**	116	2.9	4.4		4.9
	117	2.5	4.3		4.7
	118	3.7	4.3		4.2
	119	3.0	4.5		4.5
	120	3.0	4.7		4.9
MD–19**D**	121	3.9	5.1		4.8
	122	3.4	5.0		4.9
	123	3.7	5.4		5.0
	124	3.1	5.3		5.2
	125	3.6	4.9		4.9

[a]Boosts were given after four and nine weeks, in the case of **MD**–11**D** after five and seven weeks.

guinea pigs that the decapeptide **M**(19–26)**D** and the tridecapeptide **M**(16–26)**D** showed similar immunogenicities indicates that the core sequence is near the C-terminal end. On the other hand a central core sequence is signalled by the immunogenicity of **M**(13–19)**D**, a nonapeptide.

The two T-cell epitopes in melittin may be used to discuss two earlier points. (a) It was our experience that with complete melittin we rarely found non-responding animals in a group of out-bred guinea pigs during immunogencity tests. With peptides lacking the C-terminal chain, non-responders were more frequent [13]. This can now be attributed to the lack of one of the epitopes in the shorter peptides. The non-responding is primarily due to genetic restriction involving lack of interaction between peptide fragments and MHC class II molecules. (b) An important point has been whether the cytotoxicity and in particular the haemolytic activity of melittin would be involved in providing the high melittin immunogenicity [13]. It was concluded that good, although, in comparison to melittin, lower IgG titres obtained in guinea pigs with non-haemolytic melittin(1–19)-Dah–Dncp removed the possibility of a significant effect of haemolysis. Since melittin in guinea pigs provides two T-cell epitopes whereas the shorter (1–19) peptide has lost the C-terminal epitope, the lower immunogenicity may be expected to derive from this fact.

Of considerable interest is the potential effect of substituents in and outside of T-cell epitopes. Allergic diseases as well as autoimmune processes may involve altered constituents of the organism which are transformed into immunogens. The generation of B-cell epitopes by chemical attachment of low molecular weight chemicals including drugs to proteinaceous carriers is well studied, but the production of functioning T-cell epitopes out of carriers within the body originally not reacting against itself is not sufficiently clear [25]. Melittin peptides with their well-studied structure and relative simplicity may therefore be taken as a good model to address relevant questions in this field.

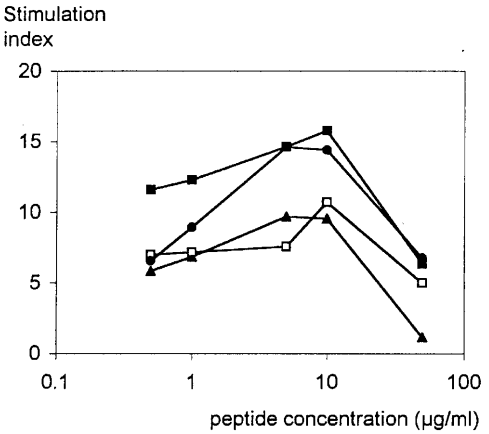

Figure 3 Stimulation of the human T-cell clone ReT19 by **M** (■); **DM** (●); **DMD** (□); **MD** (▲).

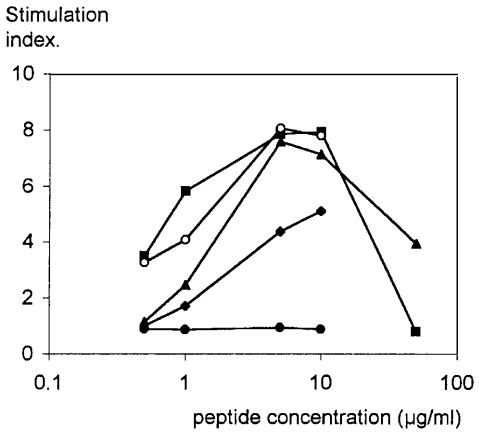

Figure 4 Stimulation of the human T-cell clone ReT19 by **MD**–3Cap (■); **MD**–7Cap (▲); **MD**–11Cap (○); **MD**–15Palm (◆);**MD**–19Palm (●).

The finding that T-cell epitope-connected substituents abolished T-cell proliferation but did not affect whole animal immunizations (Table 7 and Figures 3 and 4 versus Tables 5 and 6) was unexpected. This discrepancy suggests that the substituent-impaired interaction between T-cell epitope and MHC class II glycoprotein or T-cell receptor can be overruled by a mechanism not presently understood. Final assessment has to await direct evidence with regard to the removal or non-removal of substituents before cellular processing, i.e. within the intact animal, but given the expectation that non-splitting is rather probable, the question becomes very important. The finding that substituents outside the T-cell epitopes have no effect on antibody production and T-cell proliferation is also of interest. Concerned here are **M**–3**D** and **M**–7**D** in murine immunization (Table 6) and **M**–3**D** and **M**–7**D** in human T-cell proliferation (Table 7). It is not to be expected that flanking parts obligatorily contribute to the MHC-interactions and the lack of an effect is therefore not surprising. On the other hand in the human proliferation, positions 3 and 7 may be too far removed from the core sequence to have an effect.

Within the field of animal peptide antibiotics, melittin as such is not invoked owing to its considerable haemolytic activity. But derivatives and analogues such as the recently studied cecropinA-melittin hybrids [26] may become interesting. In this connection a detailed understanding and evaluation of the factors involved in the immunogenicities of these amphipatic molecules is important and necessary.

Table 7 Proliferation of the Human T-cell Clone ReT19

Peptide	Stimulation index with peptide concentrations (µg/ml)				
	0.5	1	5	10	50
M(2–26)**D**	3.6	5.6	9.4	11.5	3.5
M(4–26)**D**	2.6	2.8	8.5	10.9	11.3
M(7–26)**D**	2.8	3.6	7.9	10.2	10.5
M(9–26)**D**	3.2	4.1	7.4	8.9	11.2
M(11–26)**D**	2.8	3.4	7.3	8.2	10.6
M(13–26)**D**	1.5	2.4	6.8	10.0	10.4
M(16–26)**D**	1.2	1.7	4.5	8.3	13.4
M(19–26)**D**	1.9	2.5	6.6	8.8	13.2
M(9–19)**D**	0.7	0.9	1.0	0.8	1.1
M(13–19)**D**	0.8	0.9	0.9	0.8	1.1
M–3**D**	10.3	10.3	14.6	12.6	11.5
M–7**D**	13.6	12.1	9.4	10.2	11.8
M–11**D**	12.6	13.4	11.8	11.0	1.0
M–15**D**	13.0	14.7	12.1	10.6	not done
M–19**D**	0.8	0.7	0.8	0.9	not done
M–23**D**	0.7	0.9	2.2	1.2	not done

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REFERENCES

1. T. P. King, A. K. Sobotka, L. Kochoumian and M. Lichtenstein (1976.) Allergens of honey bee venom. *Arch. Biochem. Biophys. 172*, 661–671.
2. D. M. Kemeny, M. G. Harris, L. J. G. Youlten, M. Mackenzie-Mills and M. H. Lessof (1983). Antibodies to purified bee venom proteins and peptides I. Development of a highly specific RAST for bee venom antigens and its application to bee sting allergy. *J. Allergy Clin. Immunol. 71*, 505–514.
3. C. R. Dawson, A. F. Drake, J. Helliwell and R. C. Hider (1978). The interaction of bee melittin with lipid bilayer membranes. *Biochim. Biophys. Acta 510*, 75–86.
4. J. C. Talbot, Dufourcq, J. de Bony, J. F. Fancon and C. Lussan (1979). Conformational change and self association of monomeric melittin. *FEBS Lett. 102*, 191–193.
5. T. C. Terwilliger and D. Eisenberg (1982). The structure of melittin II. Interpretation of the structure. *J. Biol. Chem. 257*, 6016–6022.
6. H. Vogel and F. Jähnig (1986). The structure of melittin in membranes. *Biophys. J. 50*, 573–582.
7. R. Schwyzer (1992). Conformations and orientations of amphiphilic peptides induced by artificial lipid membranes: Correlations with biological activity. *Chemtracts – Biochem. Molec. Biol. 3*, 347–379.
8. T. C. Terwilliger, L. Weissman and D. Eisenberg (1982). The structure of melittin in the form I crystals and its implication for melittin's lytic and surface activities. *Biophys. J. 37*, 353–361.
9. R. Von Grünigen and C. H. Schneider (1989). Antigenic structure of the hexacosapeptide melittin: Evidence for three determinants, one with a helical conformation. *Immunology 66*, 330–342.
10. V. Curcio-Vonlanthen, H. Rolli and C. H. Schneider (1996). Immunogenicity studies with haptenated melittin peptides: Implication for membrane involvement during the recognition step. *J. Peptide Sci. 2*, 251–260.
11. H. G. Boman (1982). Humoral immunity in insects and the counterdefense of some pathogens. *Fortschr. Zool. 27*, 211–222.
12. H. G. Boman (1995). Peptide antibiotics and their role in innate immunity. *Annu. Rev. Immunol. 13*, 61–92.
13. Z. Zhao, H. Rolli and C. H. Schneider (1995). Immunogenicity of dinitrocarboxyphenylated melittin: The influence of C-terminal chain shortening, N-terminal substitution and prolin insertion at positions 5 and 10. *J. Peptide Sci. 1*, 140–148.
14. M. F. Kasper, C. H. Schneider, H. Rolli, B. Angst and A. L. de Weck (1986). Diagnostic reagents in drug allergy: Immunochemical specificity in the 1,2-diphenyl-pyrazolidinedione series. *Immunobiology 173*, 98–109.
15. J. M. Carballido, N. Carballido-Perrig, G. Terres, C. H. Heusser and K. Blaser (1992). Bee-venom phospolipase A2-specific T-cell clones from human allergic and nonallergic individuals: Cytokine patterns change in response to the antigen concentration. *Eur. J. Immunol. 22*, 1357–1363.
16. J. M. Carballido, N. Carballido-Perrig, M. K. Kägi, R. H. Meloen, B. Wüthrich, C. H. Heusser and K. Blaser (1993). T-cell epitope specificity in human allergic and nonallergic subjects to bee-venom phospholipase A2. *J. Immunol. 150*, 3582–3591.
17. T. Wyss-Coray, C. Brander, K. Frutig and W. J. Pichler (1992). Discrimination of human CD4 T-cell clones based on their reactivity with antigen presenting T-cells. *Eur. J. Immunol. 22*, 2295–2302.
18. P. F. Fehlner, R. H. Berg, J. P. Tam and T. P. King (1991). Murine T-cell responses to melittin and its analogs. *J. Immunol. 146*, 799–806.
19. L. S. Kind and E. Allaway (1982). Enhanced IgE and IgG anti-melittin antibody formation induced by heparin–melittin complexes in mice. *Allergy 37*, 225–229.
20. T. P. King, M. R. Coscia and L. Kochoumian (1993). Structure–immunogenicity relationship of melittin and its N-terminal truncated analogs. *Biochemistry 32*, 3506–3510.
21. H.-G. Rammensee in: *Peptides in Immunology*, C. H. Schneider, Ed., p. 39–53, Wiley, Chichester 1996.
22. H.-G. Rammensee (1995). Chemistry of peptides associated with MHC class I and class II molecules. *Curr. Opin. Immunol 7*, 85–96.
23. G. Malcherek, V. Gnau, S. Stevanovic, H.-G. Rammensee, G. Jung and A. Melms (1994). Analysis of allele-specific contact sites of natural HLA-DR17 ligands. *J. Immunol. 153*, 1141–1149.
24. D. A. A. Vignali and J. L. Strominger (1994). Amino acid residues that flank core peptide epitopes and the extracellular domains of CD4 modulate differential signalling through the T-cell receptor. *J. Exp. Med. 179*, 1945–1956.
25. S. Martin and H. U. Weltzien (1994). T-cell recognition of haptens, a molecular view. *Int. Arch. Allergy Immunol. 104*, 10–16.
26. P. Juvvadi, S. Vunman, E. L. Merrifield, H. G. Boman and R. B. Merrifield (1996). Hydrophobic effects on antibacterial and channel-forming properties of cecropin A–melittin hybrids. *J. Peptide Sci. 2*, 223–232.

