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MURINE T CELL RESPONSES TO MELITTIN AND ITS ANALOGS[1]

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The 26-residue peptide melittin from bee venom elicits high IgG1 and IgE responses in selected strains of mice. The antibody responses were shown previously to be specific mainly for the region of residue 20-26. The T cell epitope of melittin in H-2^d-restricted mice is now found to be primarily in residue 11-19, corresponding to an α-helical amphiphilic segment of the molecule. Melittin-specific T cell lines have varying responses to different structural analogs of the melittin T cell epitope, and the results indicate that the antigenicity of T cell epitope peptides depend more on their primary structure than on their secondary structure. Melittin-specific T cell clones are found to be CD4$^+$ and secrete IL-4, and are restricted to presentation on I-A^d or I-E^d. The I-A^d- or I-E^d-restricted clones differ in their responses to different analogs of melittin.

Melittin is a 26-residue peptide of honeybee venom, comprising 50% of venom dry weight (1). About one-third of bee-venom allergic individuals have melittin-specific IgE (2, 3). The murine antibody response to melittin is strain specific, indicating genetic control of the T cell response, and IgE and IgG1 responses are obtained irrespective of the adjuvant used (4). Melittin has linear and helical amphiphilicity and it has a strong propensity to assume α-helix structure in association with hydrophobic environments (5). These characteristics were found to be important for antibody responses,[3] and they make melittin a useful model for investigation of the relationship of structure and antigenicity of T cell epitopes. Melittin's known activity as an allergen, albeit a minor one, also makes it a good choice to investigate intrinsic characteristics of an Ag that may enhance allergenicity by selecting for specific T cell responses. The availability of proteolytic and synthetic melittin fragments allow detailed investigation to identify relevant T cell epitope(s) and to determine how responding T cells might influence the course of immunity.

Studies of T cell clones in the murine immune system have led to the identification of two functionally different Th cell phenotypes: Th1 cells secrete IL-2 and IFN-γ, and are implicated in helping cellular immunity; Th2 cells secrete IL-4 and IL-5, and are implicated in helping humoral immunity (6). Further experiments in mouse (7) and rat (8) systems suggests that Th1 cells are primary T cells that mature or differentiate into Th2 cells. IL-4 induces B cell isotype switching to IgE and IgG1 in mice in vitro (9) and in vivo (10) and to IgE and IgG4 in humans in vitro (11).

To characterize the T cell response of H-2^d mice to melittin, we have mapped the T cell epitope using melittin fragments and analogs (Fig. 1) to stimulate primary and long term T cells in vitro. Several peptides were tested in vitro and in vivo for their ability to induce peptide- and melittin-specific T cell responses. Peptide analogs that differ in their primary and secondary structures (Fig. 2) were tested to determine the effect of these changes on antigenicity in vitro. Melittin-specific T cell clones from primary immunized lymph node cells were studied for phenotype to determine if there is a correlation of T cell activity and high IgE response to melittin.

MATERIALS AND METHODS

Peptides. The preparation and characterization of melittin and its analogs are described our earlier paper (4) and in a manuscript submitted for publication.[3] The exceptions are 1-20-G_4Q_2 and 1-20-D_6 which are gifts from Dr. Henning Lowenstein of ALK A/B, Horsholm, Denmark. Peptide 7-19 is prepared by digestion of melittin with α-chymotrypsin for greater than 20 min (4) and 7-19-lac was prepared by conjugation with p-aminophenyl-β-D-lactopyranoside as described (see footnote 4).

Derivatives of peptide 8-21 were prepared by condensation with GlyOMe[4] in the presence of 1-ethyl-3-dimethylaminopropylcarbodiimide-HCl as described for the preparation of peptide-lactoside derivatives (see footnote 4). Products were purified by reverse phase chromatography. Peptide 8-21-NAcUrea represents a side product of the condensation reaction, an O to N shift of the carbodiimide-activated peptide intermediate to a stable NAcUrea.

Several melittin T cell epitope analogs were prepared synthetically (Fig. 2). One series of peptides, representing derivatives of peptides 10-21 and 11-21, were prepared with a free or acetylated N-terminus and a free or amide C-terminus using FMOC chemistry with a RaMPS semiautomatic apparatus (Du Pont, Wilmington, DE). A standard solid phase procedure with symmetric anhydride single coupling to either RapidAmide (C-terminal carboxamide) or Wang (free C-terminus) was used according to the RaMPS manual. Leucine-16 was coupled as the preactivated OPfp ester (Du Pont). Where indicated, peptides were acetylated according to the RaMPS manual.

Peptide 7-21 analogs with single or dual amino acid substitutions at position 12 and 14 were also prepared. These peptides were synthesized using a novel multisynthesis strategy on a derivatized film solid phase support (12). Common steps of deprotection, neutralization, washing, and coupling of identical amino acids were performed simultaneously, whereas the coupling of different amino acids was carried out in separate vessels. A standard solid phase

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[3] **Fehlner, P. F., L. Kochoumian, and T. P. King.** Murine IgE and IgG responses to melittin and its analogs. *Submitted for publication.*

[4] Abbreviations used in this paper: GlyOMe—glycine methyl ester; Ac, acetyl; Am, amide; HFP, 1, 1, 1, 3, 3, 3-hexafluro-2-propanol; lac, *p*-aminophenyl-β-D-lactopyranoside; NAcUrea, N-acyl urea; NMSR, 1% normal mouse serum culture medium; 10R, culture medium with RPMI 1640; RCM, rat conditioned medium.

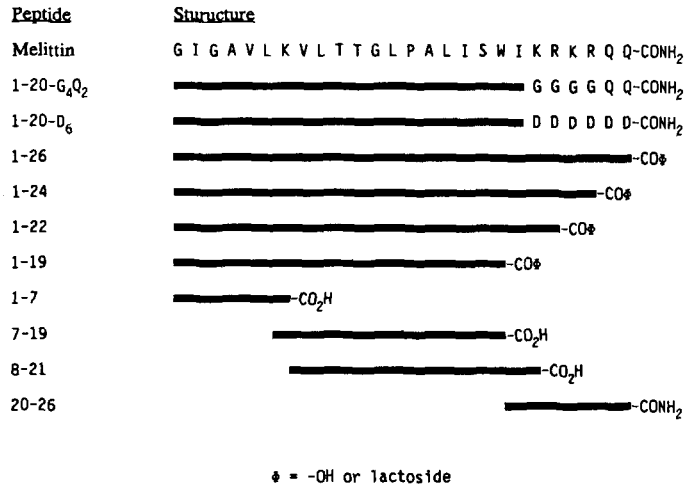

Figure 1. Structures of melittin and its fragments or analogs. The numbers refer to positions in melittin sequence.

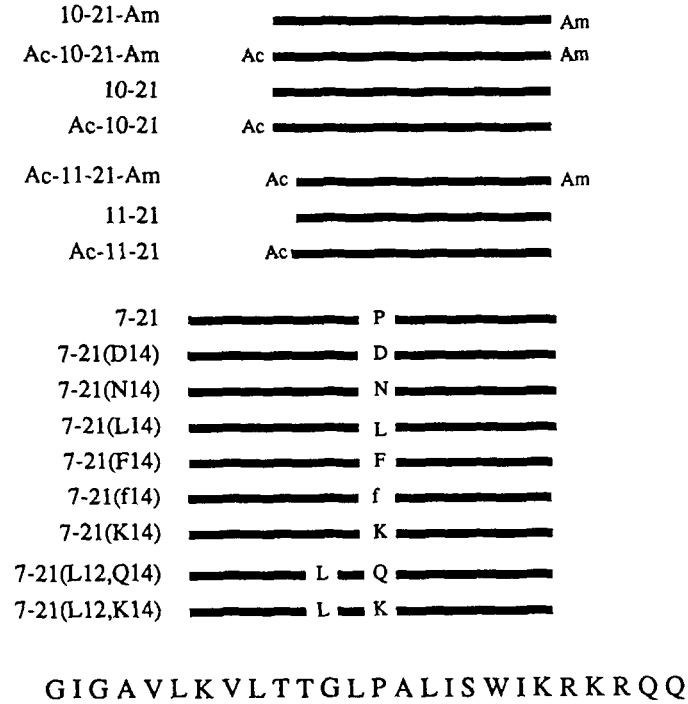

Figure 2. Structures of melittin T cell epitope analogs. The numbers refer to positions in melittin sequence which is shown at *bottom*. *Ac* and *Am* indicate N-acetyl and C-terminal carboxamide, respectively. *f* indicates D-phenylalanine.

procedure, using double dicyclohexylcarbodiimide coupling with 3.5 equivalents, was used, except for leucine-13 and glutamine-14, which were double coupled as symmetric anhydrides.

All the peptides were purified by reverse phase chromatography, and further characterized by HPLC, amino acid analysis and mass spectrometry as described (see footnote 4) and the results are summarized in Table I.

Circular dichroism. Circular dichroism spectra of 10 μM peptides in PBS or 20% HFP (Aldrich, Milwaukee, WI) in 80% PBS were obtained on an AVIV 62 DS spectrometer. Secondary structure was calculated using the "prosec" program provided by AVIV.

Initiation and propagation of melittin-specific T cell lines and clones. The cell lines and clones tested in this report are listed in Table II. Various procedures were adapted from Reference 13. Vaccines consisted of peptide in a 1:1 emulsion of PBS and CFA (Calbiochem-Behring, La Jolla, CA). Female BALB/c mice of 6 to 10 wk of age (The Jackson Laboratory, Bar Harbor, ME or Charles River Breeding Laboratories, Inc., Wilmington, MA) were immunized i.p. with 0.2 ml of 10 μM peptide or s.c. in the base of the tail with 0.05 to 0.1 ml of 25 μM peptide. Spleen cells were obtained from mice immunized i.p. 8 to 10 days earlier and were prepared in a single cell suspension after hemolysis in 0.16 M NH4Cl. Draining lymph node cells (inguinal and periaortic) were obtained from mice immunized s.c. and prepared in a single cell suspension. Primary spleen and lymph node cells were cultured at a density of 5 to 10 $\times$ 10^6 cells/ml and stimulated with 0.3 to 0.8 μM melittin. Culture medium (10R) consisted of RPMI 1640 (GIBCO, Grand Island, NY), 10% FCS (Hazelton Systems, Inc., Aberdeen, MD, endotoxin low), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO), 10 mM HEPES (Sigma Chemical Co., St. Louis, MO), and 54 μM 2-ME (14).

Long term cultures were maintained by repeated restimulation and rest as described (14), though frequently cells were only harvested before restimulation (15). T cells (1 to 2 $\times$ 10^6) were restimulated on 5 to 10 $\times$ 10^7 irradiated splenic feeder cells with 0.4 μM melittin in 10 ml of 10R in a 25-cm^2 culture flask (Corning Glass Works, Corning, NY). Syngeneic feeder cells were freshly irradiated (3300 rad from a ^{135}Cs source) BALB/c spleen cells that were not cleared of red blood cells. Blast cells (T cells 4 days after restimulation) and rested T cells were harvested by centrifugation (600 $\times$ *g* for 30 min) over 2 cm of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), washed extensively and counted in the presence of trypan blue.

We have also prepared irradiated feeder cells from large numbers of spleens and cryopreserved the extra cells (4 $\times$ 10^7 cells in 4.5 ml of 10R containing 7.5% DMSO). Human peripheral blood mononuclear cells are preserved in this way (16). Upon thawing, cell recovery was about 40%, and red blood cells were almost totally absent. These cells worked well as feeder cells for maintaining long term cultures and in proliferation assays.

T cells were cloned from long term cultures by limiting dilution. One day after bulk restimulation, cells were seeded at 0.3 cell/well in a 96-well plate and cultured in 10R with 20% RCM as described (14). Primary lymph node cells from four CAF_1 and two DBA/2 mice were cloned in a limiting dilution analysis 1 day after in vitro stimulation of the primary cells (17). Wells were seeded with 10^5, 10^4, 10^3, and 10^2 lymph node cells per well and an equal volume of 2.5 $\times$ 10^5 irradiated feeder cells in 100 μl of 10R supplemented with 20% RCM and 0.4 μM melittin. After 10 to 16 days, all wells were restimulated with the addition of 5 $\times$ 10^6 irradiated feeder cells in 100 μl supplemented with 10% RCM and 0.4 μM melittin for a final volume of 0.2 ml. After another 10 to 16 days, 100-μl aliquots of cell suspensions were removed from each well and cultured with irradiated feeder cells, melittin (0.4 μM) and RCM (10%) in 200 μl volume for a proliferation assay. This technique has been used successfully with human derived clones to identify wells with responding colonies (18). Positive wells incorporated ^{3}H-thymidine at least two to three times above the baseline level, which was determined from the average of randomly selected samples from the set of wells with a low frequency of positive colonies. Clones were expanded into 24-well plates (Corning) with 5 $\times$ 10^6 feeder cells, 0.4 μM melittin, and 5% RCM in 10R. Once the cells could be counted, cultures were restimulated with 1 to 2 $\times$ 10^5 T cells/ml, 5 to 10 $\times$ 10^6 feeder cells/ml and 0.4 μM melittin in 10R without RCM.

Proliferation assays. Proliferation assays with primary cells were performed as described (13). Primary spleen or lymph node cells were obtained 9 to 11 days after immunization and were cultured in 10R, or 1% normal mouse serum culture medium which differs from 10R in that 1% normal mouse serum is used in place of 10% FCS. Spleen cells were obtained from mice immunized with four or more biweekly injections of peptide in alum as described (see footnote 4), and lymph node cells were obtained from mice immunized s.c. in the base of the tail.

Proliferation assays with long term lines and clones were made 10 to 16 days after restimulation. The cells were cultured at 1 to 2 $\times$ 10^4 cells/well with 2 to 5 $\times$ 10^5 irradiated spleen cells or 1 $\times$ 10^3 A20 cells and Ag in 200 μl of 10R. The A20 feeder cells were treated with 50 μg/ml of mitomycin C (Sigma), an anti-mitotic agent, for 90 min at 37°C. A20 cells, a BALB/c-derived B cell lymphoma line (19), were the gift of Dr. Joshua Metlay of this university. After 3 days, cultures were pulsed with 0.5 or 1.0 μCi of ^{3}H-thymidine, 2 Ci/mmol (Amersham Corp., Arlington Heights, IL), in 25 to 50 μl of RPMI 1640 for 16 to 20 h. DNA was harvested on glass fiber filters using a PHD cell harvester (Cambridge Technology, Watertown, MA) and ^{3}H-thymidine incorporation was determined by scintillation counting in ReadySafe cocktail (Beckman Instruments Inc., Fullerton, CA).

Lymphokine assays. IL-4 activity was determined by the B cell costimulation assay (20). Small resting B cells (10^3/well) were cultured with a-Ig-coated Sepharose and T cell culture fluid supernatant in the presence or absence of the anti-IL-4 mAb 11B11 (21) for 3

TABLE I

Characterization of T cell epitope peptides

Peptide[a]	Reverse Phase Chromatography[b]	Amino Acid Analysis[c]	Mass Spectorometry[d]	
			Expected	Found
8-21	40%[1]	+		ND
8-21-glyOMe	41	+		ND
8-21-NAc-Urea	42	+	1596	1596.8
7-19	39.4%[2]	+		ND
7-19-lac	36.0	+		ND
10-21-Am	43	+		ND
Ac-10-12-Am	44	+		ND
10-21	41	+		ND
Ac-10-21	42	+		ND
Ac-11-21-Am	44	+		ND
11-21	41	+		ND
Ac-11-21	45	+		ND
7-21	21.6 min[3]	+	1640	1640.51
7-21(D-14)	21.9	+	1658	1658
7-21(N-14)	21.4	+	1657	1657
7-21(L-14)	24.0	+	1656	1656.1
7-21(F-14)	24.0	+	1690.1	1690.1
7-21(f-14)	23.8	+	1690.1	1690.1
7-21(K14)	20.1	+	1671.1	1671.2
7-21(L12,K14)	25.9	+	1727.2	1727.3
7-21(L12,Q14)	27.8	+	1727.1	1727.1

[a] See Figure 2 or nomenclature of peptide structures; peptides 7–21(F-14) and (f-14) denote that the residue at position 14 are L-and D-phenylalanine, respectively.

[b] Reverse phase chromatographies were made using 1) Varian MCH-10 column, percent refers to 2-PrOH concentration; 2) Du Pont Protein Plus column, percent refers to CH_3CN concentration; and 3) a Vydac C18 column, time of elution CH_3CN gradient.

[c] +denotes that the peptides show the expected composition.

[d] Molecular weight was determined by fission mas spectrometry; expected values were calculated using the five most abundant isotopes.

TABLE II

Melittin-specific T cell lines and clones[a]

Cell Line: Clone	Mouse Strain	Immunization	Tissue Source
A1:	BALB/c	1-22-lac in CFA, s.c	Lymph nodes
A1:B10, H11	BALB/c	1-22-lac in CFA, s.c.	Lymph nodes
R3:	BALB/c	Melittin in CFA, i.p.	Spleen
R4:	BALB/c	Melittin in CFA, i.p.	Spleen
R4: A2	BALB/c	Melittin in CFA, i.p.	Spleen
L1: B9, B12	CAF_1	Melittin CFA, s.c.	Lymph nodes
L2: A10, A11, B7	CAF_1	Melittin in CFA, s.c.	Lymph nodes
M1: A8, B8	CAF_1	Melittin in CFA, s.c.	Lymph nodes
M2:A9, B4, B5, B8, B12	CFA_1	Melittin in CFA, s.c	Lymph nodes
DBA/2	DBA/2	Melittin in CFA, s.c.	Lymph nodes

[a] All cell lines or clones were maintained on irradiated spleen cells from BALB/c mice with the exception of M2.B8, for which spleen cells from A/J mice were used.

days. The cells were pulsed as in the proliferation assay, and harvested after 20 h.

IL-2 was determined by its growth activity on CTTL (22) cells in the presence or absence of anti-IL-2 mAb S4B6 (23). CTTL cells were used 48 h after re-stimulation and cultured with T cell culture supernatant for 48 h. Cells were then pulsed as in the proliferation assay and harvested 4 h later. The reagents for above assays were gifts of Drs. Marian Birkeland and Jeffery Ming of this university.

Immunofluorescence. CD4 and CD8 were detected by direct immunofluorescence staining. FITC-labeled anti-Lyt-2 (CD8) and phycoerythrin-labeled anti-L3T4 (CD4; both from Becton Dickinson, Mountain View, CA) were used at 10 and 2 μg/ml, respectively, to stain 10^6 T cell blasts in 100 μl of PBS, 1% BSA, 0.1% NaN_3, according to the manufacturer's instructions. CD45 and CD45R were detected by indirect immunofluorescence. Culture fluid from rat-derived hybridomas secreting mAb TIB122 (anti-CD45; American Type Culture Collection, Rockville, MD) and mAb MB23G2 (anti-CD45R) (24), were used at ¼ dilution, and were gifts of Dr. Marian Birkeland. Bound rat mAb was detected with a FITC-mouse-anti-rat reagent at 5 μg/ml (Boehringer Mannheim, Indianapolis, IN). Cells were incubated with antibody for 60 min at 4°C (these conditions were repeated with the FITC-mouse-anti-rat reagent for indirect immunofluorescence), then washed and fixed in 10% formalin. Immunofluorescence data were obtained on a FACscan II (Becton Dickinson).

MHC class II restriction. Restriction of clones and lines to I-A^d and I-E^d was tested in a proliferation assay by incubating H-2a,d-restricted cells (CAF1 cells) with irradiated feeder cells in the presence of mAb B21.2 (anti-I-A^d), 11B.32 (anti-I-E^d), or, as a control for non-specific inhibition of presentation, 10.216 (anti-I-Ak). The mAb were present in culture fluid supernatant, which was used at a concentration of 1.6 to 25%. mAb culture fluids were the generous gift of Dr. Ralph M. Steinman of this university.

RESULTS

Melittin T cell epitope. As listed in Table II, several long term T cell lines were obtained from mice immunized with melittin and one line, A1, was from mice immunized with 1-22-lac. Figure 3 *a*, *b*, and *c* show the results of proliferation assays with polyclonal lines A1, R3, and R4, respectively. All three lines responded to melittin, 1-22, 1-19, and 8-21, but not to peptides 1-7 and 20-26 (data not shown). Thus the T cell epitope of melittin in

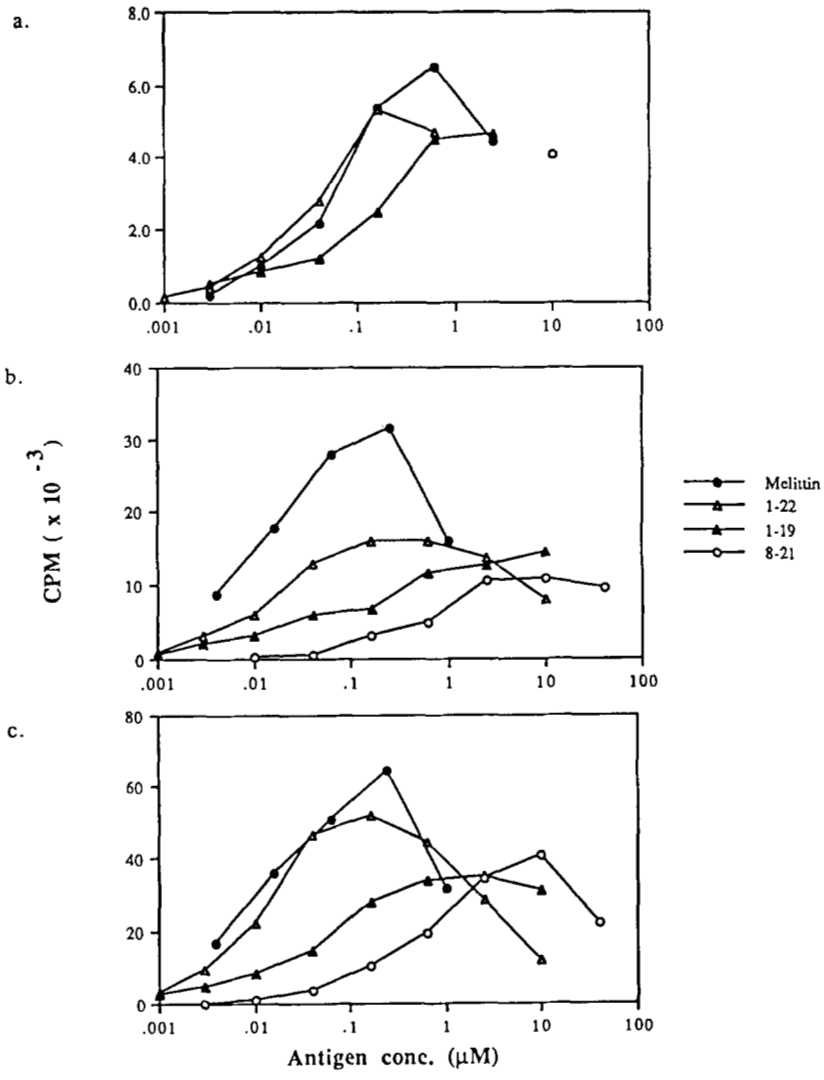

Figure 3. Proliferation of long term melittin specific T cell lines. *a*, Line A1, after four passages in vitro; background 144 cpm. *b*, Line R3 after six passages; background 116 cpm. *c*, Line R4 after six passages; background 150 cpm.

BALB/c mice is located in residue 8-19. Similar results were observed with primary lymph node and spleen cells (data not shown). The proliferation assay also indicated fine specificity of the different lines for structural differences in the peptides. Line A1 and R4 responded equally well to melittin and 1-22, and with reduced efficiency to 1-19 and 8-21. However, line R3 responded poorly to 1-22, and much less efficiently to 1-19 and 8-21 than the other two lines.

Immunogenicity of melittin analogs for T cell response. BALB/c mice were immunized s.c. with peptides 7-19 and 7-19-lac and draining lymph node cells were challenged in vitro with melittin, 1-19 and 7-19-lac. The results of the proliferation assay are shown in Figure 4*a*, and indicate that T cells specific for 7-19 and 7-19-lac can be stimulated in vitro with 7-19-lac, 1-19 or melittin with similar efficiency.

Spleen cells from mice immunized i.p. with melittin, 1-24, 1-20-G_4Q_2, and 1-20-D_6 in alum adjuvant for antibody studies in a separate paper (see footnote 4) were also tested for proliferative responses in vitro to the immunogen and to peptide analogs. The results in Figure 4*b* show that all peptides elicited T cell responses. In vitro responses of cells immunized with 1-20-G_4Q_2 and 1-20-D_6 were weaker against melittin than the immunogen. These peptides have less α-helical character than melittin (see footnote 4). Interestingly, the response of 1-24 immunized cells is enhanced against melittin relative to the immunogen.

Structure of T cell epitope peptides. To study the influence of structural changes on the antigenicity of T cell epitopes, we prepared peptides 10-21 and 11-21 with free or blocked N- and C-terminus (Fig. 2). Data on the antigenicity of these peptides as tested with T cell line A1 are given in Figure 5; the magnitude of proliferations corresponded to their dose-responses (data not shown). Peptides with an acetylated N-terminus were more antigenic than their free N-terminus counterparts. This effect was also seen with blocked C-terminal group by comparison of peptide Ac-10-21-Am vs Ac-10-21 and Ac-11-21-Am vs Ac-11-21. However peptide 10-21-Am was less antigenic than 10-21.

Enhanced activity of C-terminal blocked peptides was also seen with T cell lines R3 and R4. These lines showed better dose-responses to 1-19-lac peptide relative to 1-19 and to a blocked 8-21 peptide relative to 8-21, such that the response to the peptide derivative was comparable to melittin (data not shown).

The most dramatic preference for C-terminal blocked peptides is evident in Figure 6, which shows the proliferation of clone A1.B10 derived from line A1 to 8-21 and two 8-21 derivatives. This clone responded equally well

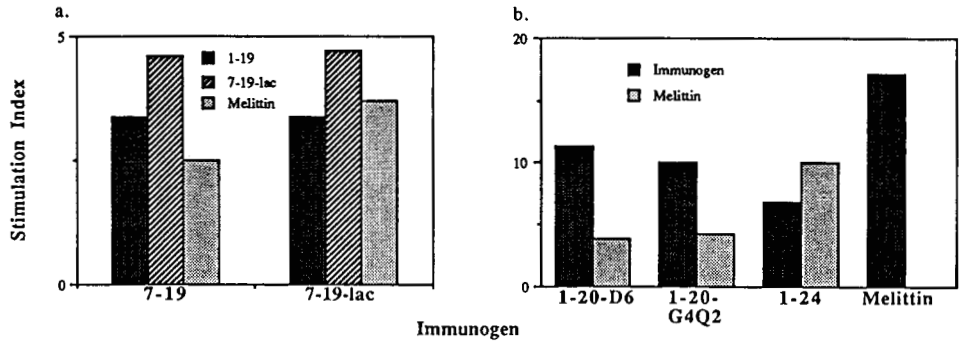

Figure 4. Proliferation of peptide-specific lymphocytes in presence of various melittin analogs. *a*, Lymph node cells from groups of three mice immunized with 7-19 or 7-19-lac were tested in 10R medium. Ag concentration was 0.16 μM for melittin and 0.25 μM for the analogs. Stimulation index represents the radioactivity of sample divided by that of background; data are the mean for three mice, and SD is <20%. *b*, Spleen cells from groups of two to three mice immunized with the indicated immunogens were tested in NMSR medium. Ag concentration was 0.63 μM for all samples. Background incorporation ranged from 880 to 5680 cpm.

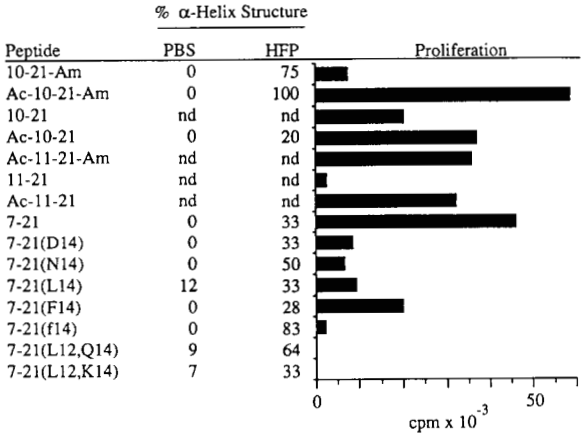

Figure 5. Antigenicity and secondary structure of melittin T cell epitope peptides. Antigenicity was measured by a proliferation assay using melittin-specific cell line A1 and irradiated spleen cells as feeder cells; these cells were recovered from cryopreservation and used immediately. Peptide concentration was 10 μM. Background incorporation was 350 cpm. Secondary structure were determined from circular dichroism spectra of 10 μM peptide in PBS or 20% HFP.

to melittin, 8-21-GlyOMe, and 8-21-NAcUrea, but not at all to 8-21. Peptide 8-21-GlyOMe has a neutral C-terminus with an ester group; 8-21-NAcUrea has a positive charge in the C-terminal group. Despite the chemically dissimilar C-terminal structures of these derivatives, they are nearly indistinguishable antigenically in the proliferation assay. However, they are distinguished by having a blocked C-terminus while 8-21 has a free carboxyl group at the C-terminus.

To test further whether enhanced helicity of a peptide would produce higher antigenicity, a series of 15-mer peptides, corresponding to residue 7-21 of melittin with single amino acid substitutions at position 14 (proline), or dual substitutions at positions 12 (glycine) and 14 were prepared. Proline and glycine are known to be α-helix breakers (25), and their occurrence in the middle of a peptide sequence is likely to influence the helicity. These analogs were tested for their antigenicity with T cell line A1 (Fig. 5). Substitutions with acidic (D), polar (N), or hydrophobic (L or F) amino acid residues showed varying degrees of reduction in antigenicity in vitro. Interestingly, substitution with D-phenylalanine did not destroy activity, although molecular modeling suggested that the resulting structure would be very different from the native peptide. Two peptides with dual substitutions at position 12 (L) and 14 (K or Q) were inactive but a peptide substituted only at position 14 with K was also inactive (data not shown).

Percent α-helix structure of peptides in aqueous and organic (HFP) solvent were obtained from analysis of circular dichroism (Fig. 5). No strong correlation of secondary structural propensity and T cell antigenicity was observed. For examples, peptide 7-21(L14), with detectable helical character in aqueous buffer, was antigenic, but peptides 7-21(L12, Q14) and 7-21(L12, K14), also with α-helix structure in aqueous solution, were not. Similarly, peptides 7-21, 7-21(D14), and 7-21(N14) contained very similar conformational characteristics in PBS and HFP, including β-sheet structure (data not shown), yet only peptide 7-21 was found to be a strong T cell Ag.

Phenotype of T cell clones. CAF_1-derived T cell clones were harvested 4 days after restimulation for direct and indirect immunofluorescence. Expression of CD4 and CD8, the MHC class II (helper) and class I (cytotoxic) T cell restriction markers, and CD45 and CD45R was determined. CD45 is found on all $CD3^+$ T cells, and the isoform CD45R (R for restricted) is expressed on primary T cells (26) and on long term IL-4 secreting T cell clones (24). As shown in Table III, all clones were positive for CD4 and negative for CD8 in flow microfluorimetry analysis. The cells also generally showed medium to high

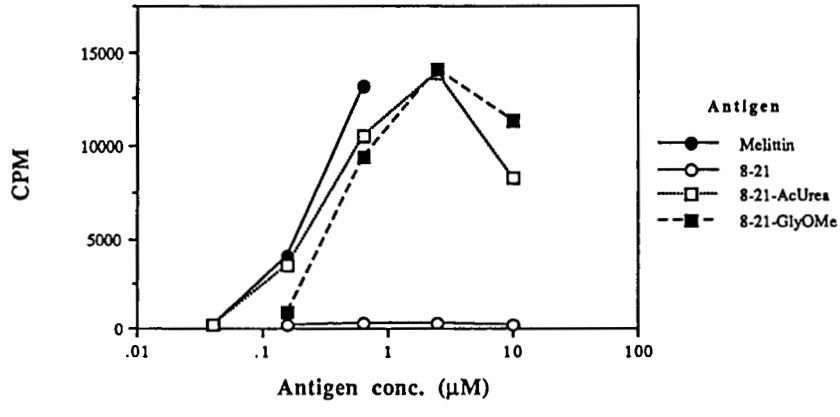

Figure 6. Sensitivity of clone A1.B10 to structural variations of melittin T cell epitope. Cloned T cells from line A1 were cultured at 10^4 cell/well with 10^3 mitocycin-C treated A20 cells in the presence of melittin, 8-21, and derivatives of 8-21 in 10R medium. Background incorporation was 110 cpm.

TABLE III

Phenotypes and IL-4 production

Clone	Mean Fluoresscence Intensity (Arbitary Units)					B Cell Costimulation Assay for IL-4 (cpm)			CTTL Assay for IL-2 (cpm)	
	Control[a]	Direct		Indirect		+a-Ig	+a-Ig +11b11	S.N.[b] only	+S4B6	S.N.[b] only
		CD4	CD8	CD45	CD45R					
A1.H11	8.9	1100	ND	80	90	4185	1349	2254	ND	ND
M2.B8	5.1	74	5.3	960	160	2558	618	1222	1502	4840
L1.B9	2.7	1500	ND	270	260	2197	1398	2208	961	1311
L1.B12	9.2	1400	6.1	960	960	3134	1069	896	688	827
L2.A10	9.4	630	8.5	830	620	1417	778	592	385	569
L2.A11	8.0	1200	6.6	670	710	2975	1938	1696	733	910
L2.B7	9.9	1600	7.9	690	640	2409	1863	1581	1011	1391
M1.A8	7.6	1200	6.2	1100	ND	4112	1263	899	1028	1250
M1.B8	11	1000	9.3	910	750	2505	1691	873	1004	1118
M2.A8	8.1	1200	7.1	750	590	807	759	468	1096	1028
M2.B4	7.8	1100	7.2	730	410	3018	726	1730	962	1451
M2.B5	5.3	170	9.3	310	110	2229	855	1317	1080	1224
M2.B12	7.7	330	10	240	50	1891	835	1626	1230	2345

[a] The negative control was FITC-mouse anti-rat reagent for both direct and indirect labled cells.

[b] S. N., supernatant of T cell clone, and 11B11 and S4B6 are anti-IL-4 and anti-IL-2 mab, respectively. Background incorporations for B cell and CTTL assays were 690±40 cpm.

expression of CD45R relative to CD45, and very high levels of CD45R relative to the control. Similar findings were made with DBA/2 derived T cell clones (data not shown).

After four passages in culture supplemented with RCM and two passages without exogenous growth factors, supernatants from 12 cloned lines harvested after stimulation with Ag on irradiated feeder cells for 4 days were tested by the B cell costimulation assay for IL-4 activity. The results in Table III suggest that eight of these clones produced IL-4 as the B cell costimulatory activities of the supernatants decreased by >twofold in the presence of monoclonal anti-IL-4 antibody, and that four clones possibly produced IL-4 as their cosimulatory activities decreased by about 30% and that one clone M2.A9 was IL-4$^-$ because it showed no decrease. These clones were also tested for IL-2 secretion by CTTL assay. The results in Table III suggest that only one clone M2B8 produced IL-2 as its CTTL activity was suppressed by >threefold by monoclonal anti-IL-2 antibody, and that five clones possibly produced IL-2 as their activities decreased by 30 to 50%, and that six clones did not produce IL-2 because they showed no decrease.

Class II molecule restriction and sensitivity to peptide structure. Clones derived from CAF_1 primary lymphocytes and long term lines were tested for Ia restriction, sensitivity to stimulation by melittin, and epitope recognition, and the results are summarized in Table IV. Ia restriction was determined by inhibition analysis with anti-I-A^d- and anti-I-E^d-specific mAb. About half the cells were found to be restricted to I-A^d and half to I-E^d, with one exception that clone L2.A11 is restricted to both I-A^d and I-E^d. In a concurrent proliferation assay, these cells were also found to have different sensitivities for stimulation by melittin. The I-A^d restricted clones required a lower concentration for ½ maximal melittin stimulation, 0.17 ± 0.10 μM (n = 4), than I-E^d-restricted clones, 0.36 ± 0.13 μM (n = 4). Similar differences in sensitivity to melittin stimulation and Ia restriction were observed with 14 DBA/2-derived clones (data not shown).

The apparent difference in melittin binding affinity for the respective Ia molecules was manifested in the ability of clones to respond to melittin fragment 7-21. Clones restricted to I-A^d responded to fragment 7-21, but clones restricted to I-E^d did not (Table IV). Two representative clones were selected for further tests: L2.B7, restricted to I-A^d and M1.B8, restricted to I-E^d. These clones were >90% inhibited by little as 1.6% of the appropriate Ia-specific mAb culture fluid supernatant, but not by comparable concentrations of the other Ia-specific mAb. Both clones proliferated upon challenge with 1-26, 1-26-lac, 1-24, and 1-24-lac (data not shown), in addition to melittin and 1-22-lac, as presented in Figure 7. The I-A^d restricted clone proliferated in response to all peptides tested; the I-E^d-restricted clone failed to respond to peptides 1-20-G_4Q_2, 1-20-D_6, and 7-21, and responded only weakly to peptide 1-19-lac. These results suggest either that the first three of these analogs can not be presented by I-E^d, or that they are not recognized by the TCR of the clone tested. However, primary spleen cells from BALB/c mice immunized with 1-20-D_6 were inhibited from proliferation by anti-I-A^d but not by anti-I-E^d (data not shown), suggesting that 1-20-D_6 is preferentially presented by I-A^d.

DISCUSSION

Melittin-specific T cells. We have obtained a number of melittin-specific T cell lines or clones from BALB/c, CAF1, and DBA/2 mice. The T cell clones (13 tested) were found to have CD4, CD45, and CD45R cell surface markers, and 12 of these 13 clones secreted IL-4. Moreover the majority of IL-4-secreting clones secreted no IL-2 or at low level. These results suggest that the majority of melittin-specific clones that we studied are of the Th2 type although we did not establish conclusively the absence of Th1 type cells by assaying IFN-γ.

Evidence with these T cell clones indicates that melittin is presented by I-A^d or I-E^d molecules. Interestingly, I-A^d- or I-E^d-restricted clones differ in their proliferative responses to the melittin analogs. The analogs that stimulate only I-A^d-restricted clones were found to be either ineffective or weak immunogens for antibody response (see footnote 4).

Structure of the melittin T cell epitope. The results with different peptides indicate that the dominant T cell epitope of melittin is located predominantly in residue 11-19. Peptides that include this segment stimulate melittin-specific cell lines in vitro, and peptides with melit-

TABLE IV

Ia restriction, melittin sensitivity, and epitope 7-21 recognition of T cell clones

Clone	Precent Inhibition[a] in Presence of Anti-			(Melittin) 1/2 Max. Response (μM)	Response to 7-21[b]
	I-A^d	I-E^d	I-A^k		
L1.B9	11	**100**	37	2.5	ND
L1.B12	40	**100**	36	0.5	−
L2.A10	67	**100**	ND	2.5	ND
L2A11	**96**	**90**	14	0.21	ND
L2.B7	**89**	10	16	0.055	+
M1.B8	0	**100**	5	0.2	−
M2.A9	**100**	53	ND	0.3	+
M2.B4	13	**79**	43	0.35	ND
M2.B5	**100**	29	52	0.4	ND
R4.A2	**100**	57	83	ND	ND
A1.B10	**100**	32	45	ND	ND
A1.H11	**100**	27	34	0.15	+

[a] T cell clones, 2×10^4 cells/well, were stimulated by 0.1 μM melittin and 5×10^5 irradiated CAF_1 spleen cells with or without anti-Ia antibodies. Antibodies were in culture fluid from the appropriate h ybridoma: which were used at 1/4 dilution. Anti-I-A^k is included as a control for non-specific effects of binding Ia molecules to the APC. Percent inhibition is caluclated as follows:

$$\frac{(\text{cpm} + \text{anti-Ia} - \text{cpm background})}{(\text{cpm} - \text{anti-Ia} - \text{cpm background})} \times 100$$

Bold type indicates significant inhibition relative to the anti-I-A control.

[b] Peptide 7–21 was tested at 10 μM.

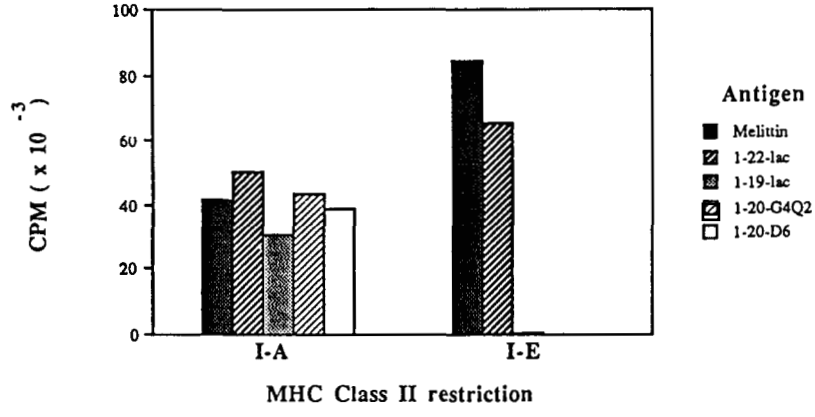

Figure 7. Proliferation of I-A- and I-E-restricted clones to immunogenic peptides. Ag concentrations were 0.32 μM for I-A restricted clone L2.B7 and 1.25 μM for I-E restricted clone M1.B8. Background incorporation was 104 cpm.

tin's 1-20 sequence but different 21-26 sequences elicit similar primary T cell responses in vivo.

Polyclonal melittin specific T cell lines show preference for T cell epitopes with fine structural variations. Our results with N- and/or C-terminal blocked peptides indicates that they show increased antigenicity and helical potential in comparison to the free peptides (Fig. 4). Blocking the terminal functional group of a peptide theoretically increases helical potential since free amino and carboxyl groups have unfavorable charge interactions with the helix dipole (27). These results with blocked peptides would suggest the importance of the secondary structure for T cell epitopes. However, our other findings with peptide analogs which vary by amino acid substitutions (Fig. 5) clearly indicate the predominance of primary structure on antigenicity, because enhanced helical propensity could not overcome the effects of an unsuitable amino acid substitution.

These results with melittin agree in part with the algorithms developed by Berzofsky and Rothbard and coworkers (28, 29) to characterize secondary structure requirements for T cell epitopes based on amphiphilicity and primary structure. However, experiments to identify T cell epitopes of a single Ag in a large number of mouse species indicate that almost the whole sequence of the protein was available to act as the T cell epitope; most of the identified epitopes correlated with one or the other algorithm, and some correlated with neither (30). A recent study found no correlation of experimental helical propensity with antigenicity of epitope peptides from pigeon and moth cytochrome-C (31). In this study, net positive charge of the peptide epitopes correlated with antigenicity in vitro, presumably due to the enhanced attraction of positively charged peptides to the negatively charged cell membrane. Melittin has a strong positive charge character in the C-terminus.

Correlation of structure and antigenicity for peptide epitopes may be important for immunotherapy, especially when therapy is directed to a specific MHC molecule. A peptide analog to the N-terminal fragments epitope of MBP that acted as an heteroclitic antigen for MBP-specific T cells in vitro was found to inhibit induction of experimental autoimmune encephalomyelitis in vivo (32). Thus it may be interesting to explore competition of the

various synthetic analogs against melittin, with the hope that one, perhaps evidencing enhanced structural propensity, will block melittin-specific responses by acting as an inhibitor for presentation on Ia.

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