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Molecular Cloning and Characterization of the Yeast Gene Encoding Fibrillarin, a Major Nucleolar Protein

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THE RELATIONSHIP OF STRUCTURE AND IMMUNOGENICITY OF THE BEE-VENOM PEPTIDE MELITTIN

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DEDICATION

To my parents, Francis and Mary Fehlner.

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Table of Contents

List of Figures

List of Tables

ABBREVIATIONS

1%NMSR--1% normal mouse serum culture medium.

10CM--culture medium with 1:1 RPMI-1640 and Eagle-Hank's medium.

10R--culture medium with RPMI-1640.

Ac--acetyl.

CD--circular dichroism.

CFA--complete Freund's adjuvant.

CH_3CN--acetonitrile.

ConA--concanavlin-A.

DCC--dicyclohexylcarbodiimide.

DCM--dichloromethane.

DMF--dimethylformamide.

DMSO--dimethylsulfoxide.

EAE--experimental autoimmune (or allergic) encephalomyelitis.

EDC--1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride.

FITC--fluorescene isothiocyanate.

GlyOMe--glycine methyl ester.

GPC--gel permeation chromatography.

HF--hydrofluoric acid.

HFP--1,1,1,3,3,3-hexafluoro-2-propanol.

HRP--horseradish peroxidase.

i.p.--intraperitoneal.

iPrOH--isopropanol

lac, lactoside--*p*-aminophenyl-β,D-lactopyranoside.

MAb--monoclonal antibody.

MBP--myelin basic protein.

MHC--major histocompatibility complex.

NAcUrea--N-acyl urea.

NH_4Ac--ammonium acetate.

PBS--phosphate buffered saline.

PLA_2--phospholipase A_2.

RAMG--rabbit-anti-mouse-IgG.

RBC--red blood cell.

RCM--rat conditioned medium.

s.c.--subcutaneous.

SARG--sheep-anti-rabbit-IgG.

TcR--T cell receptor.

TFA--trifluoroacetic acid.

ABSTRACT

Melittin, a bee-venom peptide of 26 amino acids, has well known amphiphilic properties. These properties result in a number of interesting biophysical behaviors and defined quaternary structures; knowledge of these activities has been exploited to understand the relationship of structure and immunogenicity of melittin and melittin analogs. From this analysis, a hypothesis for melittin's *in vivo* immunogenic structure, predicated on binding to cell membranes or other lipophilic environments, is suggested.

Immunogenicity for antibody responses depends on primary, secondary and quaternary structure. The distribution of hydrophobic and hydrophilic amino acids determines "peptide-detergent" properties such as self association and the ability to bind and oligomerize in cell membranes, and only peptides of a minimum size of 24 amino acids are immunogenic. Analogs of melittin that retain these properties elicit strong antibody responses; analogs that lack these properties are weak immunogens for antibody responses. As a result of these structural propensities, melittin-specific antibodies primarily react with the C-terminal epitope of the peptide.

The T-cell epitope of melittin in H-2^d restricted mice is located primarily in residue 7-19. Melittin-specific T cell clones are $CD4^+$, showed high expression of a CD45R isoform associated with T_H2 phenotype, and most appeared to secrete IL-4. Melittin-specific T-cell clones are restricted to both alleles of H-2^d class II molecules, I-A and I-E. I-A restricted clones respond to lower concentrations of melittin and a wide variety of melittin analogs; I-E restricted clones require higher melittin concentrations and fail to recognize some melittin analogs. Differential Ia restriction results in different peptide-specific Ig isotype distribution. I-A only restriction is characterized by increased levels of specific IgG_{2a} and decreased levels of IgG_1 and IgE.

Chapter I.

Preface

Honey bees cause the most common type of insect sting allergy, followed by hornets, wasps and yellowjackets (vespids). Generally allergic symptoms resulting from a sting are mediated by venom specific IgE (1). Atopic or type I allergy was first identified with the activity of a serum factor, called reagin, almost 70 years ago. This agent was characterized as a new class of immunoglobulin, IgE, in the late 1960's (2). The atopic individual develops a sustained specific IgE response, which is a T cell dependent event (3). Although the clinical condition of allergy is usually fairly unpleasant for the sufferer, characterized by symptoms ranging from nasal congestion to anaphylactic shock, IgE antibodies may have some beneficial affects as well, particularly for immunity in response to parasite infections. In schistosomiasis, IgE has been implicated in adherence of macrophages to schistosomules; the identification of $Fc_{\epsilon}R$ on the surface of eosinophils suggested that eosinophil-mediated anti-helminth activity may also involve IgE (2).

IgE binds to the high affinity $Fc_{\epsilon}R$ on the surface of mast cells and basophils (2). When bound specific IgE is exposed to multivalent antigen, cross linking of the $Fc_{\epsilon}R$ occurs and causes the release of histamine and other mediators of anaphylaxis. These mediators cause the symptoms characteristic of an allergy.

Immune responses characterized by high levels of IgE have a genetic association with HLA in humans (4). Specific association of HLA and response to allergens from ragweed (5) have been characterized. Familial, probably genetic, factors have also been associated with sensitivity to hymenoptera venom (6).

While genetic predisposition to atopic disease has been extensively studied, the intrinsic characteristics of antigens that make them allergens are not well identified. This is relevant because allergens distinguish themselves from the usual lot of proteins one is exposed to every day: not all antigens are allergenic; some proteins are dominant allergens; and only minute quantities of allergen are required to sensitize a susceptible individual.

One possible intrinsic characteristic of allergens, currently under investigation by T.P. King and his colleagues, is antigenic cross reactivity. Sensitization to many vespid venoms can follow one sting because of cross reactions with homologous venom proteins from other vespid species. IgG antibodies from mice immunized with one venom protein were found to cross react with homologous venom proteins from other vespids; venom allergic patients' IgG and IgE antibodies also showed a high degree of cross reactivity with homologous venom proteins (7,8). Antigenic cross reactivity is found with non-homologous proteins as well. A white face hornet antigen 5 (*Dol m V*) and tobacco leaf pathogenesis-related protein (prp) share sequence similarity and limited antigenic cross-reactivity as demonstrated with rabbit antibodies to prp (9) and antibodies from *Dol m V* sensitive humans (1). Thus allergens may have cross reactive epitopes in common with homologous proteins or ubiquitous environmental proteins, or both.

In order to study possible intrinsic allergenic characteristics further we chose as a model melittin. Melittin is a 26-residue peptide that constitutes 50% of honey-bee venom dry weight (10). While melittin is an allergen for about 30% of bee venom sensitive people (11-13), phospholipase A_2, acid phosphatase and hyaluronidase constitute the most important venom allergens (11,13). Nevertheless, melittin's small size makes it attractive to dissect the relevant B- and T-cell epitopes involved in its immunogenicity and allergenicity. Another bee venom peptide of 18 amino acids, apamin, is not known to induce an antibody response in humans (11,13), and thus was rejected as our model.

Despite the simplification of size in studying melittin, the peptide poses another problem: to determine just why it is so immunogenic. Usually peptides are poor immunogens for antibody responses. Melittin on the other hand was found to be highly immunogenic in mice of the H-2^d haplotype for IgG and IgE responses (14). Clues to this unusual property of strong immunogenicity are found in the

peptide's structure. The N-terminal amino acids are primarily hydrophobic, and the C-terminal amino acids are all hydrophilic, including 4 basic residues (10). The secondary and tertiary structures of melittin are well known from crystallography (15), nuclear magnetic resonance (NMR) spectrometry (16,17), circular dichroism (CD) spectroscopy (18), and Raman spectroscopy (19). Melittin is known to mediate a number of biophysical activities (see Chapter II) resulting from its linear and α-helical amphiphilic character.

Given the well established structure of melittin, the primary focus of this thesis is to demonstrate the relationship of structure and immunogenicity of melittin at the B cell and T cell level. To do this we have prepared a series of melittin analogs with varied C-terminal composition and a series of T-cell epitope analogs with different N-terminal and C-terminal composition or single amino acid substitutions. Chapter II describes the preparation and biochemical and biophysical characterization of melittin and some derivatives of interest. The importance of the N-terminal and C-terminal residues of melittin for many of its biophysical activities and the effect of C-terminal substitutions is determined. Chapter III considers the immunogenicity of the peptides studied in Chapter II, and firmly correlates structure with immunogenicity for antibody responses. We find that a combination of sufficient length and amphiphilic character determines the strength of the antibody response to these peptides. The observation that antibodies to melittin and its immunogenic derivatives are specific for C-terminal epitopes is explained in terms of melittin's molecular structure. Chapter IV presents the mapping of melittin's T-cell epitope, including the effect of peptide structural propensity on antigenicity as determined by *in vitro* assays. Melittin- and peptide-specific T cells are characterized for phenotype, epitope recognition and MHC class II restriction. Melittin-specific helper T cells, which appear to be predominantly of the T_H2 phenotype, are found restricted to both I-A^d and I-E^d of MHC class II. There is a

clear correlation between class II restriction and *in vitro* dose response to melittin, and structural effects on restriction of antigenic peptide derivatives.

Peptide-structure effects on both B- and T-cell immune response suggest a model for the immunogenicity of melittin and for allergenicity of antigens in general, and this is discussed in Chapter V. Although the derived model is speculative, it suggests a course of investigation to further establish a role for intrinsic features of antigens that make them allergens.

Chapter II.

Structure and biophysical properties of melittin and some analogs.

INTRODUCTION

Melittin has well known linear amphiphilic structure, with predominantly hydrophobic residues in the N-terminal 20 amino acids and a concentration of 6 hydrophilic, including 4 cationic, amino acids at the C-terminus. It also has α-helical amphiphilic structure in the 20 residue N-terminus as evident from examination of the helical wheel diagram (Figure 2.1), in which putative polar side chains Lys7, Thr10, Thr11 and Ser18 are segregated on one surface of the helix. Consequently, melittin demonstrates marked biophysical activity, including self association as a tetramer (18), hemolysis (20), and insertion in bilayer lipid membranes to form voltage-gated channels (21). It is under conditions involving hydrophobic interaction that melittin assumes predominantly α-helical structure (discussed in Chapter I). At very dilute concentration, melittin structure is primarily random coil (17,18).

A series of melittin derivatives varied in length and composition at the C-terminus (Table 2.1) were prepared and characterized with regard to changes in biophysical activity resulting from the changes in structure. Structure-activity relationships determined for these peptides are correlated with immunogenicity for antibody responses in Chapter III.

MATERIALS AND METHODS

Melittin. Melittin was isolated from bee venom (Sigma) and was purified on CM-52 cation exchange resin by elution with a linear gradient of 0.02 M to 0.3 M ammonium acetate-acetic acid (NH_4Ac buffer) in 4 M urea as described (14). This chromatography allowed the resolution of the acyl-Nα and the free forms of melittin. Previous workers had identified the acyl-melittin variant, comprising about 5-25% of total melittin, as formyl-Nα melittin (22). However, mass spectrometry proves that our variant is acetylated, not formylated. The molecular ion masses of

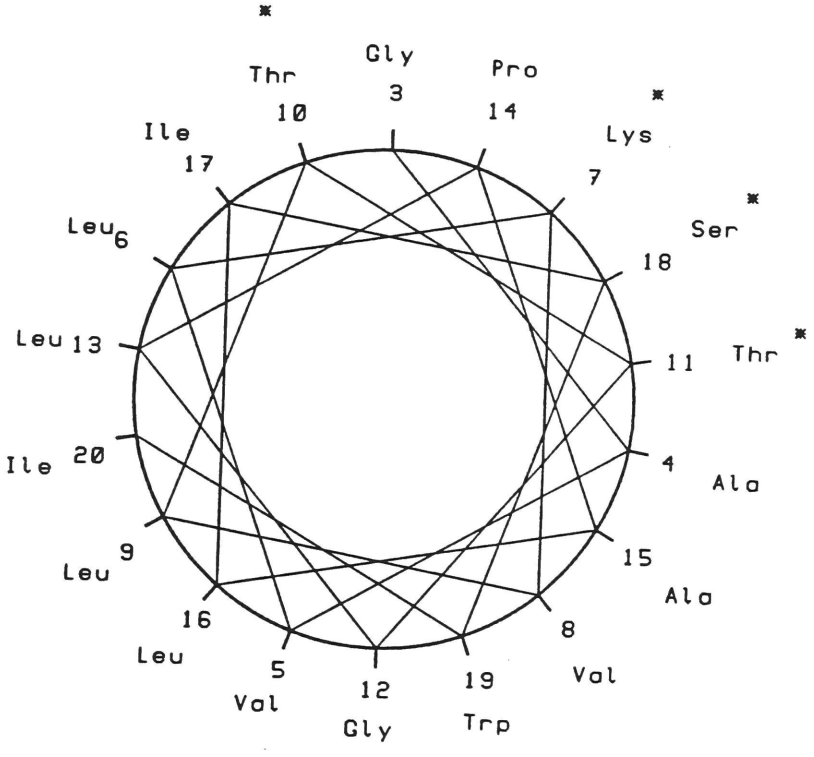


Figure 2.1. Edmundson helical wheel diagram of melittin. Melittin segment 3-20 was drawn using a program created by The Rockefeller University Computing Services. Hydrophilic amino acids Lys7, Thr10, Thr11 and Ser 18 are marked with an asterisk (*). Note that these amino acids, along with glycine-3 (no definite hydrophobic/hydrophilic preference) and proline-14 (hydrophobic but a high propensity to be found on the surface of proteins in solvent contact) constitute the putative hydrophilic face of the α-helix.

free and acyl 1-22 melittin (Ac-1-22, see below for clostripain digestion) were 2307.1 (calculated 2306.9) and 2349.5 (calculated 2348.9), respectively, with a mass difference of 42.4 (CH_3CO- = 43).

Proteolytic digestion products. Fragment 1-22 was obtained from digestion of melittin (8 mg per ml) with clostripain (0.045 mg per ml; Boehringer Mannheim Biochemicals) in 0.05 M Tris-HCl, pH 7.95 containing 2.5 mM dithiothreitol, 2 mM $CaCl_2$ and 2 M urea for 2 hours at ambient temperature. Fragments 1-19, 7-19 and 20-26 were obtained from digestion of melittin (10 mg per ml) with α-chymotrypsin (0.03 mg per ml; Worthington Biochemical Corp.) in 0.05 M Tris-HCl, pH 7.95, for 10 min. Longer reaction time (30 min to 1 hr) increased the yield of 7-19 peptide relative to 1-19. Clostripain and α-chymotrypsin digests were diluted 3-fold with 0.02 M NH_4Ac in 4 M urea and chromatographed on a 20 x 0.9 cm column of CM-52 cation exchange resin (Whatman). The column was washed with 40 ml of 0.02 M NH_4Ac, 4 M urea, and eluted with a linear 400 ml gradient of 0.02 to 0.3 M NH_4Ac, 4 M urea. Fractions of 4 ml volume were collected and peptide detected by absorbance at 280 nm. Peptides 1-19 and 7-19 eluted in 40 ml volume; peptide 1-22 elutes in 122 ml volume; for comparison, melittin elutes in 220 ml volume.

Fractions containing peptides from CM-52 chromatography were pooled, made 0.1% in TFA, and applied to a 6 x 0.9-cm (2 g) column of Polygosil C-18 (Rainin) silica for reverse phase chromatography. The peptides were eluted in a linear gradient of 0 to 60% isopropanol, 0.1% TFA. Peptide 7-19 is eluted in 27% iPrOH; 1-19 in 39% iPrOH; 1-22 in 35% iPrOH;

Synthetic peptides. Melittin 1-26 and 1-24 were prepared by The Rockefeller University Sequencing Facility with an Applied Biosystems 430A using the ABI STD1-R protocol with t-Boc/DMF chemistry and double coupling of symmetric anhydrides on PAM resin (to yield free carboxyl groups). The tryptophan residue was deprotected with 20% piperidine in DMF for 1.5 hours prior to HF cleavage.

Facility quality control was checked by amino acid analysis and analytical HPLC on the cleaved peptide. All reagents were from Applied Biosystems except DMF and DCM, from Burdick & Jackson. We further purified the peptides by ion exchange chromatography on CM-52 cellulose and reverse phase chromatography on Polygosil C-18 as described above. Peptides 1-20-G_4Q_2 and 1-20-D_6 were obtained pure as gifts from Dr. Henning Lowenstein of ALK A/B, Horsholm, Denmark.

Conjugation with lactoside. Peptides (0.6 mM in 2 x 10^{-4} M HCl, 4 M urea) were conjugated at the C-terminal α-carboxyl position by condensation with 48 mM *p*-aminophenyl-β,D-lactopyranoside (lactoside, lac; Sigma) in the presence of 45 mM 1-ethyl-3-dimethylaminopropylcarbodiimide hydrochloride (EDC; Sigma). The pH of the solution was adjusted to be about 4.8 with the dropwise addition of 1 N HCl. After 2 hours, the reaction was stopped by addition of TFA to 0.1% - 1%, and the conjugate product was purified by Polyglosil C-18 reverse phase chromatography. Peptide-lactoside conjugates were eluted at a lower percentage of iPrOH than the respective peptides.

Characterization. All peptides and peptide-lactoside conjugates were characterized as follows (Table 2.2): HPLC on a 25 x 0.46-cm DuPont ProteinPLUS reverse phase column eluted with a linear gradient of CH_3CN in 0.1% TFA; ultraviolet spectroscopy on a Cary 219 spectrophotometer; amino acid analysis. Further characterization of select peptides and conjugates was made by fast ion bombardment or fission fragment mass spectrometry, which was performed by the Rockefeller University Mass Spectrometry Service. Lactoside content of peptide-lactoside conjugates was determined by the reducing sugar, i.e. Prussian Blue, assay (23). Synthetic peptide structures were confirmed by proteolytic cleavage as described above for melittin.

Biophysical assays. Gel permeation chromatography (GPC) was performed on a 50 x 0.9-cm column of Sephadex G50 (Pharmacia, 18). The following buffers

Table 2.1. Melittin and its derivatives.

Peptide	Preparation	Structure
Melittin	purified from bee venom	G I G A V L K V L T T G L P A L I S W I K R K R Q Q-$CONH_2$
Ac-Nα-Mel	purified from bee venom	Ac- ——— $CONH_2$
1-20-G_4Q_2	synthesis (ALK A/B)	——— G G G G Q Q-$CONH_2$
1-20-D_6	synthesis (ALK A/B)	——— D D D D D D–$CONH_2$
1-26	synthesis	——— -COΦ
1-24	synthesis	——— -COΦ
1-22	clostripain digestion	——— -COΦ
1-19	α-chymotrypsin digestion	——— -COΦ
7-19	α-chymotrypsin digestion	——— -COΦ
20-26	α-chymotrypsin digestion	——— $CONH_2$
8-21	trypsin digestion	——— -CO_2H
1-7	trypsin digestion	——— -CO_2H

Ac = acetylated

Φ = -OH or lactoside

Single letter amino acid abbreviations: A, alanine; D, aspartic acid; G, glycine; I, isoleucine;K, lysine; L, leucine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine;V, valine; W, tryptophan.

Table 2.2. Peptide characterization.

Peptide[1]	HPLC[2] (% CH_3CN)	Amino Acid Analysis[3]	Mass Spectrometry[4] Expected	Found	Lactoside content[5] (mol/mol peptide)
Melittin	45	+		nd	na
1-26	45	+	2847.4	2847.8	na
1-26-lac	44	+	3263.9	3263.5	1.13±.32
1-20-G_4Q_2	48	nd		nd	na
1-20-D_6	nd	nd		nd	na
1-24	46	+	2591.2	2591.8	na
1-24-lac	44.5	+	3007.5	3007.1	1.21±.29
1-22	48.5	+	2306.9	2307.1	na
1-22-lac	45.5	+	2722.6	2722.3	1.15±.49 (n = 2)
1-19	45	+	1909.6	1909.3	na
1-19-lac	43.5	+	2325.0	2325.8	0.97±.21
7-19	39.4	+		nd	na
7-19-lac	36	+		nd	0.98 (n = 1)

1. All peptides had the correct ultraviolet spectra.

2. HPLC was performed on a DuPont ProteinPLUS reverse-phase column, 25 x 0.46 cm, in a linear gradient, 1% per minute, of acetonitrile (CH_3CN) in 0.1% TFA, at a flow rate of 1 ml per minute. Detection was by UV absorption at 280 or 220 nm. Purity is >95-99% for all peptides.

3. Peptides had the expected amino acid composition ±5%.

4. Molecular weight was determined by fission fragment mass spectrometry. Molecular weight was calculated using the five most common isotopes.

5. The reducing sugar assay or Prussian Blue assay was used to quantify lactose content of peptide-lactoside derivatives. Lactose to peptide molar ratios were calculated from three experiments unless indicated, and are reported as an average ± S.Dev. Peptide was quantified by amino acid analysis.

were used: (1) 0.15 M NaCl, 0.05 M Tris-HCl, pH 7.95; (2) 0.15 M NaCl, 0.05 M NH_4Ac, pH 4.75; (3) 1.5 M NaCl, 0.05 M Tris-HCl; (4) 1.0 M NaCl, 0.05 M NH_4Ac. Molecular weight standards were bovine plasma albumin (Armour Pharmaceuticals) of MW 65,000, horse cytochrome-C (Aldrich) of MW 13,400, oxidized insulin β-chain of MW 3500, and a synthetic octapeptide of MW 980.

Fluorescence spectra of the tryptophan side chain of 50 μM peptide in 0.05 M NH_4Ac, pH 4.75 or 0.05 M Tris-HCl, pH 7.95 were obtained on a Perkin-Elmer 650-40 spectrofluorimeter in a 1 cm quartz cuvette with excitation at 284 nm. The tryptophan fluorescence maximum was monitored as a function of NaCl concentration as described (18). Melittin fluorescence shift was not affected by the choice of either 0.05 M Tris-HCl, pH 8.0 or 0.05 M NH_4Ac, pH 4.7 as the buffer.

The hemolysis assay was adapted from (20). Briefly, 50 μl of murine red blood cell (RBC) suspension at a density of 7.2 to 8.0 x 10^8 cells per ml in phosphate buffered saline (PBS) were added to 50 μl of serially diluted peptide and incubated for 30 min at 37 C, 5% CO_2, humid atmosphere. The RBC's were pelleted and the supernatant diluted by 51-fold in 0.05 M Tris-HCl, pH 7.95 for hemoglobin determination by absorbance at 415 nm. A 100% hemolysis sample was prepared by diluting the RBC suspension in 0.05 M Tris-HCl. RBC's were incubated without peptide for baseline hemolysis.

Voltage gated channel studies were made with 1,2-diphytanoyl-3-SN-phosphatidyl choline (DPPC)-decane lipid bilayer membranes as described (24). A cell with two 1.5 ml buffer reservoirs separated by a Teflon septum was used. The bilayer was formed in a pretreated 1 mm diameter hole in the Teflon septum by direct application of lipid in decane after addition of the electrolyte buffer, 0.1 M NaCl in 10 mM Hepes, pH 7.1. Bilayer formation was followed as capacitance increased to a maximum value. Peptide was added asymmetrically (cis) to one reservoir and a trans negative voltage applied through KCl saturated calomel

electrodes. Current was measured as voltage on a Teledyne-Philbrick 1025FET operational amplifier with a feedback resistance of 10^8 Ω and a feedback capacitance of 10-30 pF. Output was digitized and recorded on a personal computer.

Circular dichroism (CD) studies of peptides in PBS were obtained on an AVIV 62DS spectrometer. PBS buffer was filtered through a 0.45 μ filter (Nalge) to remove any suspended material and peptides were dissolved at 200 μM. Cells were round or square quartz of 1 mm path length. Secondary structure was calculated using the "prosec" program provided by AVIV.

Molecular modelling of melittin (Brookhaven data bank, 1MLT) was done on a Stellar mini-supercomputer using CHARMm modelling programs (Polygen).

RESULTS

Melittin and its derivatives. The following melittin derivative peptides (Table 2.1) were prepared as described: 1-26, 1-26-lac, 1-24, 1-24-lac, 1-22-lac, 1-19-lac, 1-20-G_4Q_2, and 1-20-D_6. Melittin and its derivatives were characterized biochemically by a number of criteria as summarized in Table 2.2. These peptides were found to be of high purity (>95 - 99%), of the expected amino acid composition, and correct MW where tested. Peptide-lactoside conjugates were found with peptide : lactoside ratios ranging from 1.21 to 0.97. A rough measure of hydrophobicity of the peptides is evident from the elution position in HPLC. Peptides with greater hydrophobicity elute in a higher percentage of acetonitrile. Melittin and the 1-26 derivatives elute earlier (in 45% CH_3CN), whereas 1-24, 1-22 and 1-19 elute later (>48% CH_3CN). Conjugation with the lactoside group appears to increase hydrophilic character, such that 1-24-lac, 1-22-lac and 1-19-lac elute at lower CH_3CN concentration than their respective peptides.

Peptide amphiphilic character. A number of activities previously applied to study the biophysical activity of melittin, including the propensity to self associate as reflected by GPC and fluorescence maximum shift (18), hemolytic activity (20), and circular dichroism (18) have been applied to elucidate structure and activity relationships of the melittin analogs of interest to us. The results of these studies are summarized in Table 2.3.

Peptide oligomerization. Peptide self association is determined by two methods: relative molecular weight in GPC; and fluorescence shift of tryptophan. GPC of standard molecular weight proteins and peptides on G50 Sephadex in different buffer systems is shown in Figure 2.2. The choice of buffer conditions does not significantly effect elution volumes of the standards. GPC of the peptides at low (0.15 M NaCl) and high (1.0 or 1.5 M NaCl) salt in two buffer systems, 0.05 M Tris-HCl, pH 8.0 and 0.05 M NH_4Ac, pH 4.75, are summarized in Table 2.4. Values for MW determined graphically are estimated to be correct to within 50% and are therefore considered reliable indicators of oligomerization. The results suggest that melittin, 1-26 and 1-24-lac have a high propensity to self associate, since these peptides eluted in low salt buffer with molecular weights consistent with dimer (melittin and 1-26) or trimer (1-24-lac) structures. In low salt conditions, 1-26-lac, 1-24 and 1-22-lac eluted as monomers. In high salt, pH 4.75 buffer, melittin, 1-26 and 1-26-lac all eluted as tetramers. Possibly melittin has a higher order of oligomerization at pH 8.0 in 1.5 M NaCl, i.e. octamer structure. Ac-melittin elutes with an apparent molecular weight greater than melittin in low salt buffer. This behavior is particularly apparent at pH 4.75, where melittin has an apparent MW of 4200 and Ac-melittin has an apparent MW of 7400. This behavior has been observed previously (25).

Fluorescence blue shift of tryptophan maximum emission occurs as the indole group moves from a polar to an apolar environment. Data from GPC and

Table 2.3. Summary of biophysical activity and structure.

Peptide	Self association Fluorescence shift[1] [NaCl]-50 (M)	GPC [2] 0.15 M NaCl	1.0 M NaCl	Membrane activity[3] HD-50 (μM)	Secondary structure[4] % α-helix
Melittin	0.44	dimer	tetramer	4.7	31
1-26	0.79	dimer	tetramer	6.0	2.8
1-26-lac	0.81	monomer	tetramer	3.9	24
1-24	0.85	nd	nd	6.6	2.9
1-24-lac	0.59	trimer	nd	5.0	12
1-22-lac	2.3	monomer	insoluble	5.2	18
1-19-lac	∞	nd	nd	32	0
1-20-G_4Q_2	1.2	nd	nd	42	0
1-20-D_6	2.3	nd	nd	∞	11

1. Fluorescence shift studies were done in 0.05 M ammonium acetate, pH 4.75, 0.45 μ filtered, on a Perkin-Elmer 650-40 spectrometer in 1 cm quartz cuvette. Samples (50 μM) were scanned from 2290-400 nm, excitation λ was 284 nm, slits were 5 nm.

2. Gel permeation chromatography on Sephadex G50 in 0.15 M NaCl or 1.0 M NaCl in 0.05 M NH4Ac. Using the data in Table 2.4 for apparent MW, relative state of association was determined.

3. HD-50 is the concentration of peptide required to cause 50% hemolysis of a suspension of 4 x 10 murine red blood cells in PBS. RBC's were incubated for 30 min. at 37 C with serially diluted peptide. The HD-50 value was determined graphically from figure 2.4.

4. Secondary structural propensity for peptides at 200 μM in PBS was determined from CD spectra using the prosec program supplied by AVIV. Spectra were obtained on an AVIV 62DS in 1 mm quartz cuvettes. Peptide concentration was determined by ultraviolet absorbance.

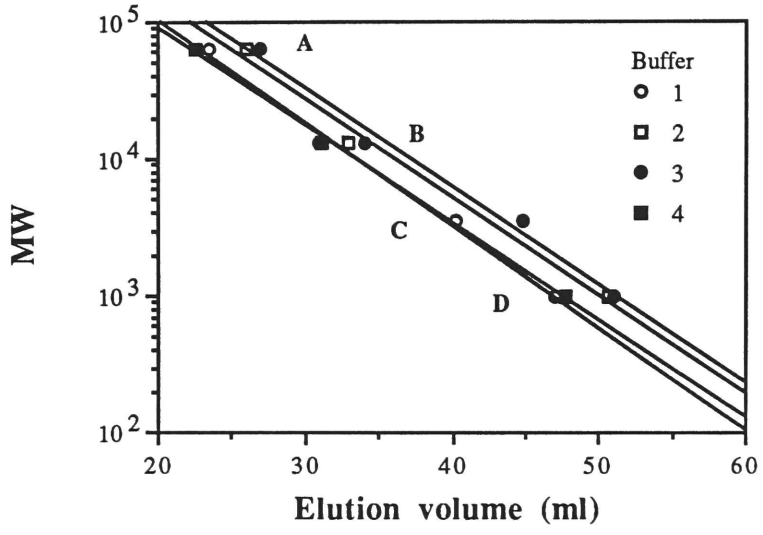


Figure 2.2. Gel permeation chromatography on Sephadex G50. Molecular weight standards and peptides were applied in 0.5 ml volumes to a 50 x 0.9 cm G50 column and eluted at a flow rate of 12 ml per hour. MW standards were: A, bovine plasma albumin (65,000); B, horse cytochrome-C (13,400); C, oxidized insulin β–chain; D, a synthetic octapeptide. Buffers were: 1) 0.15 M NaCl, 0.05 M Tris-HCl, pH 8.0; 2) 0.15 M NaCl, 0.05 M NH_4Ac, pH 4.8; 3) 1.5 M NaCl, 0.05 M Tris-HCl, pH 8.0; 4) 1.0 M NaCl in 0.05 M NH_4Ac.

Table 2.4. Apparent MW of peptides from gel permeation chromatography (GPC).

	Estimated MW Under Various Buffer Conditions			
	1	2	3	4
Peptide	0.15 M Nacl pH 8	0.15 M NaCl pH 4.8	1.5 M NaCl pH 8	1.0 M NaCl pH 4.8
Melittin	5000	4200	25,500	13,700
Ac-melittin	5200	7400	24,000	nd
1-26	5400	nd	nd	14,000
1-26-lac	3400	nd	nd	12,400
1-24	nd	4000	nd	nd
1-24-lac	nd	7500	nd	nd
1-22	2400	nd	insoluble	insoluble
1-22-lac	2800	3600	insoluble	insoluble

Relative MW was determined by extrapolation from the appropriate curve in figure 2.2. Buffer conditions are described in figure 2.2; pH 8 buffer is 0.05 M Tris-HCl; pH 4.8 buffer is 0.05 M NH_4Ac.

The error of estimated MW is probably 50%.

Elution volumes were determined by collecting fractions of 20 drops and weighing the tared collection tubes. Melittin and its analogs caused decreased drop volumebecause of surfactant activity.

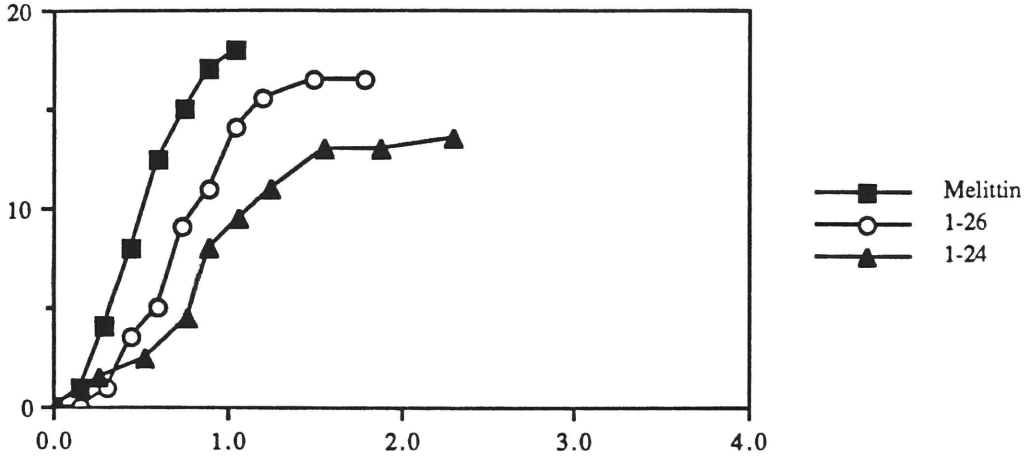
GPC was carried out by applying 0.5 ml of 400 μM peptide to a 50 x 0.9 cm column of Sephadex G50. The flow rate was 12 ml per hour.

previous studies with melittin (18) lead us to conclude that fluorescence shift occurs as peptides self associate. The association curves with increasing NaCl concentration for these peptides are summarized in Figure 2.3. Melittin, which requires the lowest concentration of NaCl for 1/2-maximal shift, shows the highest propensity to self associate. The C-terminal truncated peptides (Figure 2.3a) and peptide-lactoside conjugates (Figure 2.3b) do not associate as readily as melittin. Peptide 1-24-lac follows closely, then 1-26, 1-26-lac, 1-24, and 1-22-lac. This last peptide began precipitating before achieving 1/2-maximal shift, which was estimated by extrapolation of the association curve. Peptide 1-19-lac failed to self associate; the initial shift of 1 nm may be attributed to an intramolecular conformational change. Peptides with C-terminal substitutions do not fit in the same scheme as truncated and lactoside-conjugated melittin (Figure 2.3c). Peptide 1-20-G_4Q_2 undergoes an initial shift with a plateau; as with 1-19-lac, this may be due to an intramolecular conformational change. At high salt concentration a dramatic shift occurs, indicating self association. At this point, however, the peptide begins to precipitate. The anionic peptide 1-20-D_6 requires very high NaCl concentrations to initiate the fluorescence shift, and the slope of the fluorescence maximum shift vs. [NaCl] curve is less than for all the peptides but 1-22-lac and 1-19-lac.

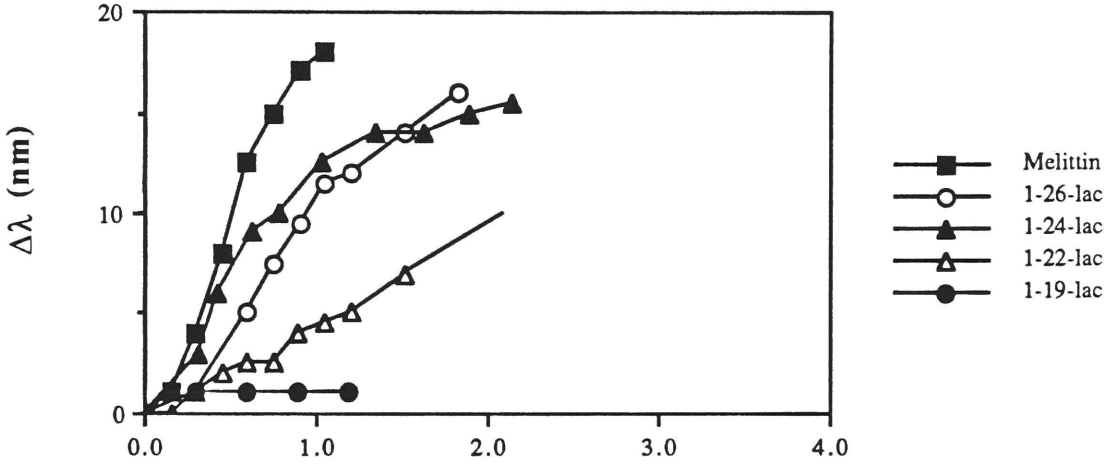
Figure 2.3 indicates that the maximum shift for some peptide derivatives is less than for melittin. This is especially clear for peptides 1-26, 1-24 and 1-20-D_6, all of which reach an asymptote at a lower value of $\Delta\lambda$ than melittin. Possibly the tryptophan residue of these derivatives is in a more polar environment, i.e. more accessible to solvent, than is the case with melittin. Thus these peptides may have slightly different tetramer conformations than melittin.

Figure 2.3. Fluorescence shift of melittin and its analogs versus NaCl concentration. The wavelength of tryptophan fluorescence maximum shifts with increasing salt concentration. Peptide concentration was 50 μM in 0.05 M NH_4Ac, pH 4.8. NaCl concentration was increased by dissolving weighed amounts in the peptide solution. An acidic buffer was used as melittin fragments shorter than 1-24 were insoluble in high NaCl concentration at neutral pH. Individual peptides are indicated to the right of each graph. The results with melittin were repeated three times.

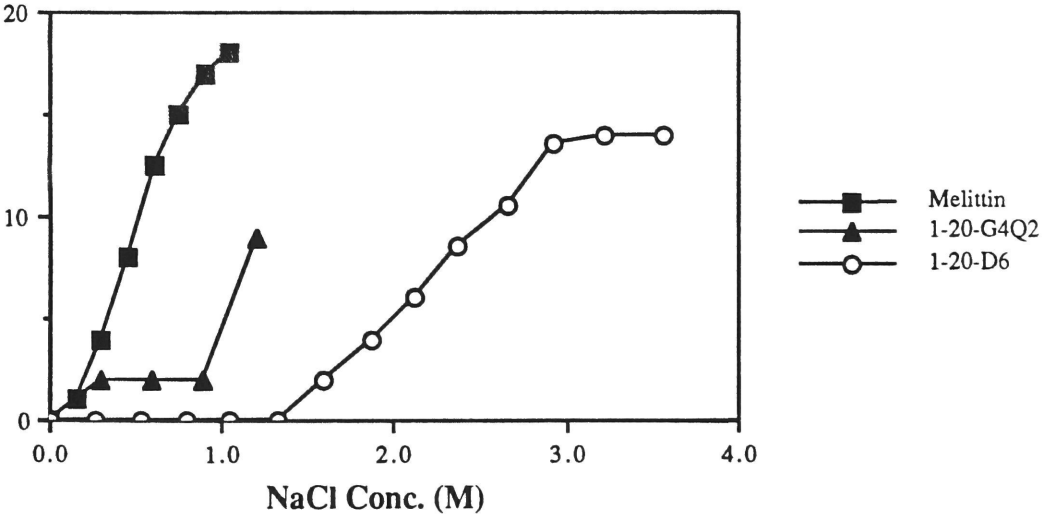
a.


20
10
0
0.0
1.0
2.0
3.0
4.0
Melittin
1-26
1-24

b.


Δλ (nm)
20
10
0
0.0
1.0
2.0
3.0
4.0
Melittin
1-26-lac
1-24-lac
1-22-lac
1-19-lac

c.


20
10
0
0.0
1.0
2.0
3.0
4.0
NaCl Conc. (M)
Melittin
1-20-G4Q2
1-20-D6

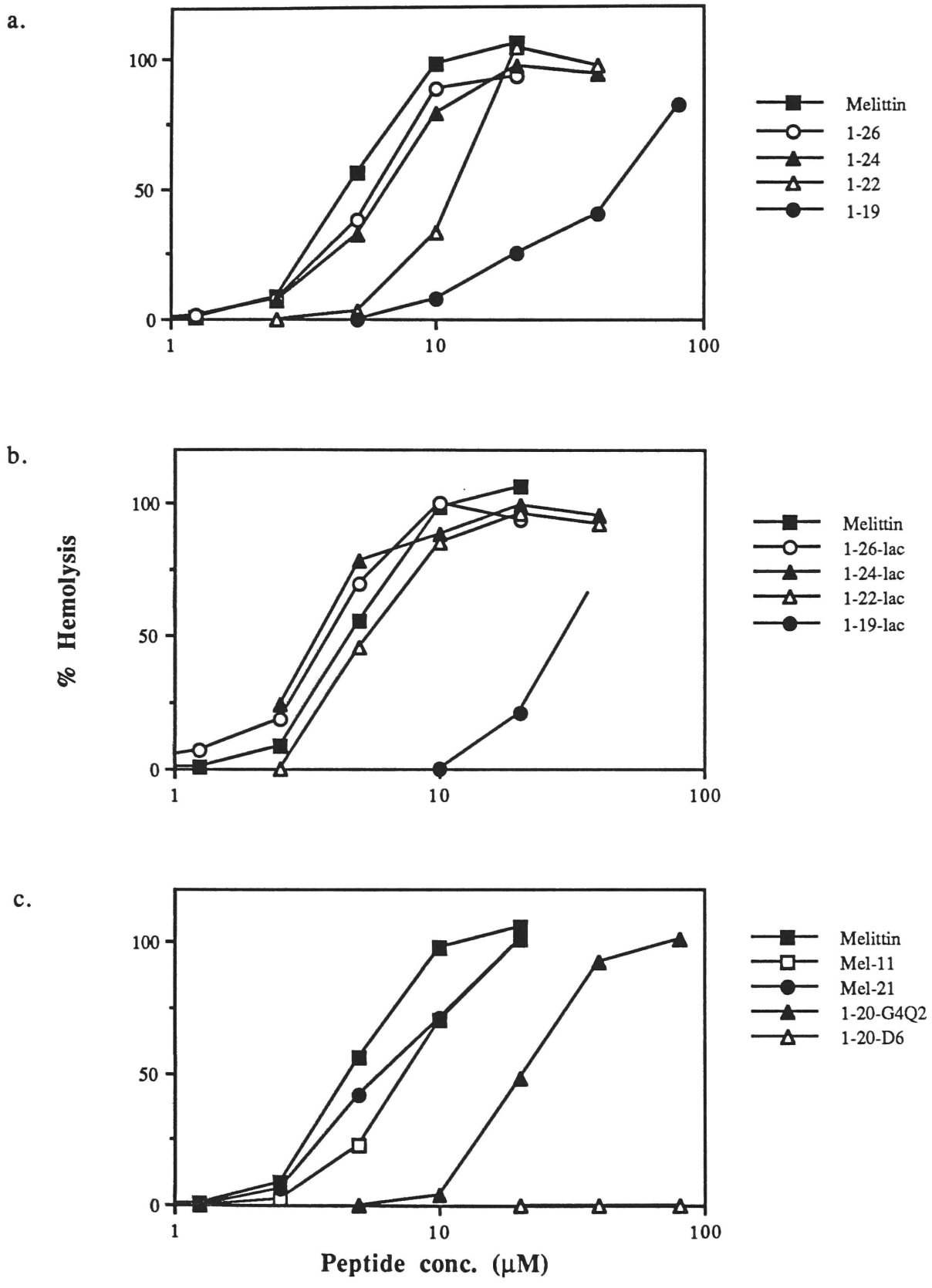
Peptide mediated hemolysis. Red blood cells provide a convenient target for bilayer membrane activity since released hemoglobin is easily monitored by absorbance at 415 nm, and this assay system has been used to study the hemolytic potential of melittin (20,25) and other surface active peptides (26-28). Among the peptides tested, those with C-terminal positive charge and relatively high hydrophilic character in HPLC were found to have greater lytic potential (Figure 2.4). Melittin, 1-26, 1-26-lac, 1-24-lac and 1-22-lac, which are eluted early from HPLC (Table 2.2) and have 2-4 cationic charged groups, have comparable hemolytic activity with fine differences (Figure 2.4a,b). Peptide 1-24, with same charge as melittin but with greater hydrophobic character in HPLC, is less hemolytic. Peptide 1-22, with two positive-charged residues, exhibits lower lytic potential and less hydrophilic character in HPLC than 1-22-lac. A peptide with greater hydrophilic character but lacking C-terminal positive charge, 1-19-lac, is a poor lytic agent. Nevertheless, 1-19-lac was more hydrophilic and hemolytic than 1-19. Peptide 1-20-G_4Q_2 also lacks C-terminal positive charge, and this peptide is poorly lytic (Figure 2.4c). The anionic C-terminal analog, 1-20-D_6, does not show any hemolytic activity whatsoever.

Voltage-gated channel studies. Peptides that include as a minimum segment 1-19 and chimeric melittin-cecropin analogs that include melittin 1-13 form channels in bilayer lipid membranes with comparable voltage gates but different current versus voltage values (C.M. Drain, unpublished results). While channel forming properties reside in the N-terminal residue, the current at a given applied voltage depends on C-terminal structure. Much more current moves through 1-26-lac channels than through melittin channels at the same concentration and voltage, and melittin channels pass more current than 1-22-lac channels, shown in Figure 2.5.

Figure 2.4. Peptide mediated hemolysis of murine red blood cells. Supernatant from a suspension of 3.6 - 4 x 10^8 RBCs per ml incubated for 30 min with peptide at 37°C were assayed for hemoglobin by A_{415}. % Hemolysis is determined by the following equation:

$$\frac{(A_{415\ \text{supernatant}} - A_{415\ \text{baseline}})}{(A_{415\ 100\%\ \text{control}} - A_{415\ \text{baseline}})} \times 100$$

Panels a and b are from one experiment, with the exception of peptides 1-24 and 1-24-lac. Data for these peptides and in panel c are from a different experiment. In panel c, Mel-11 is Ac-melittin; Mel-21 is melittin. The slight increase in melittin concentration requiredfor hemolysis is due to higher RBC density in the second experiment. Replicate assays with melittin, and also with 1-26, 1-26-lac and 1-22-lac, indicate that the error range for this assay is <5%.


a.
100
50
0
1
10
100
Melittin
1-26
1-24
1-22
1-19
b.
% Hemolysis
100
50
0
1
10
100
Melittin
1-26-lac
1-24-lac
1-22-lac
1-19-lac
c.
100
50
0
1
10
100
Melittin
Mel-11
Mel-21
1-20-G4Q2
1-20-D6
Peptide conc. (μM)

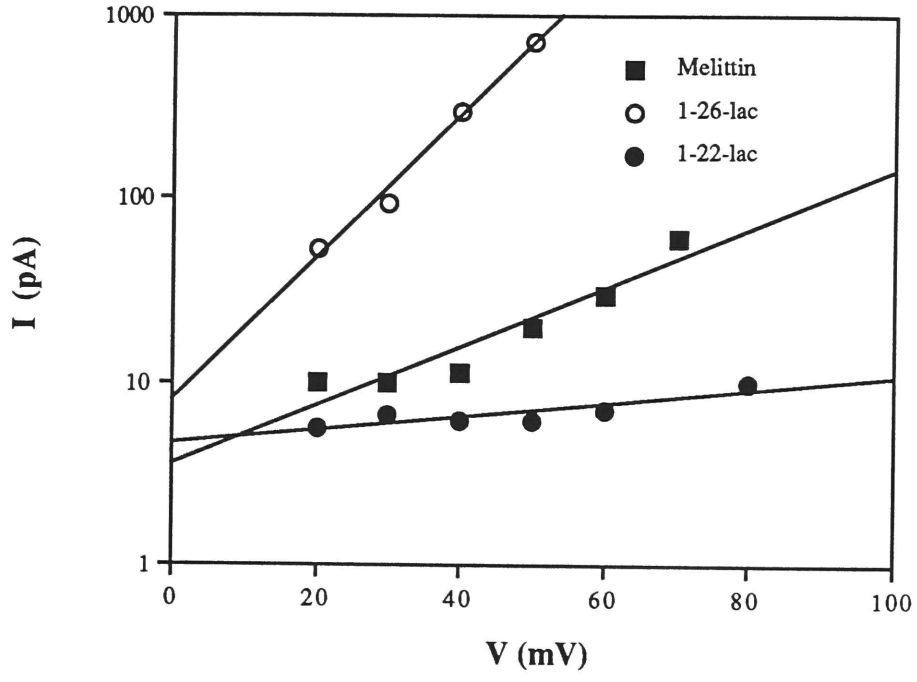


Figure 2.5. Current versus voltage curves for peptide channels. Channels were made asymmetrically in the membrane by adding 30 nM peptide to the cis reservoir of the cell. A trans negative voltage was applied and current measured as described in Materials and Methods.

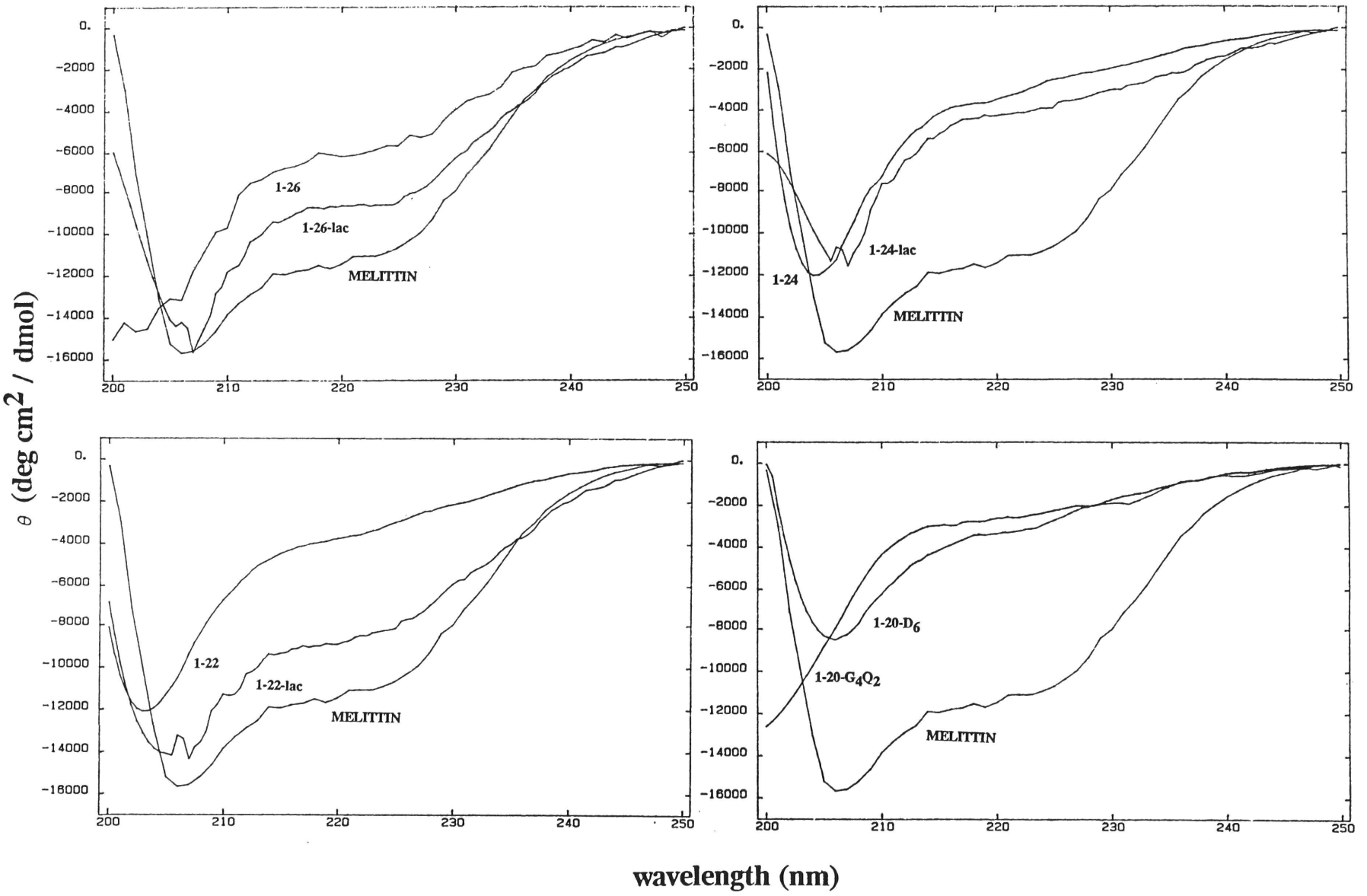
Circular dichroism. Peptides at 200 μM in PBS, conditions which favor self association (26), were analyzed for secondary structure by circular dichroism (CD). Qualitative examination of the CD spectra in Figure 2.6 indicates that melittin has the greatest α-helical content, followed by 1-26-lac, 1-22-lac and 1-20-D_6. Unconjugated derivatives 1-26, 1-24 and 1-22, as well as 1-20-G_4Q_2, have very little helical propensity. Peptide 1-19-lac was not helical under the conditions tested (not shown).

DISCUSSION

Melittin and certain of the derivatives studied demonstrate biophysical properties of self association, membrane association resulting in hemolysis and formation of voltage-gated channels, and propensity for α-helical conformation. These properties are somewhat modified in the melittin derivatives relative to melittin.

Detergent-like membrane activity of these peptides depends equally on the hydrophobic N-terminus and the cationic hydrophilic C-terminus. Previous studies on melittin derivatives have shown that nullification of the C-terminal charge results in loss of hemolytic activity (20,25). Enhancement of PLA_2 activity by melittin also requires the C-terminal charged residue (29). Presence of the lactoside group, itself fairly hydrophilic, does not compensate for lack of positive charge, as shown by the low activity of 1-19-lac in these assays. The failure of 1-20-D_6 to cause hemolysis is not surprising. Cell membranes have an overall net negative charge as a result of the phosphate groups, and the negative charge on the acidic 1-20-D_6 peptide would lead to repulsion from membranes. Strong C-terminal charge repulsion in this peptide also manifests itself in the requirement of extremely high NaCl concentration for self association.

Figure 2.6. Circular dichroism spectra. Spectra of peptides at 200 μM in PBS (except for 1-26, which was 100 μM) were obtained at ambient temperature in a 1 mm path length quartz cell on an AVIV 62DS spectrometer. Raw data were corrected by baseline subtraction prior to further mathematical manipulation. Except for the melittin spectrum, all spectra have been smoothed to correct for noise.


θ (deg cm2 / dmol)
wavelength (nm)
1-26
1-26-lac
MELITTIN
1-24
1-24-lac
MELITTIN
1-22
1-22-lac
MELITTIN
1-20-D6
1-20-G4Q2
MELITTIN

Presence of the C-terminal region is not essential for interaction with lipid bilayers, as shown by the behavior of peptide 1-19 in voltage-gated channel studies. Studies on melittin-cecropin synthetic chimeras indicate that presence of Lys7 in the hydrophobic segment of melittin is essential for forming voltage-gated channels (C.M. Drain, et al, unpublished). Melittin derivatives or fragments lacking C-terminal charge also show high levels of surface activity at the air-water interface (10,25). Melittin and 1-19 demonstrate parallel ability to affect bilayer lipid membrane melting and the amount of lipid-bound water (30). Clearly melittin's N-terminal segment provides the hydrophobic scaffold for melittin's other biophysical activities.

Characterization of structure by circular dichroism and analysis by molecular modelling indicates that C-terminal amides, represented by melittin or by peptide-lactoside conjugates, have more stable structures with increased propensity for α-helix conformation. C-terminal amide hydrogens can form the α-helix H-bond with the α-carboxyl oxygen of Lys23. A negatively charged free carboxylate at the C-terminus also interacts unfavorably with the helical dipole that results from the alignment of carboxyl and amide groups in the helix, whereas carboxamides have a neutral charge and a favorable interaction with the dipole. Concentration of positive charge in the C-terminus also would stabilize the helical dipole (31). Thus, in addition to the electrostatic surfactant activity in membranes resulting from the cationic C-terminus, this region stabilizes helical structure, which has been implicated as essential for the hydrophobic interactions that stabilize peptide oligomerization and lipid association. A synthetic melittin analog with enhanced α-helical conformation and amphiphilic helix character in the N-terminus demonstrated increased self association and hemolytic activity, relative to melittin, amply demonstrating a role for α-helix conformation in these behaviors (26).

Therefore, the hydrophobic activity of melittin and its derivatives depends on the N-terminal 20 residues. Self association and lytic activity, which I will term "peptide-detergent" activity, requires in addition the cationic, hydrophilic C-terminus, with a minimum requirement of the 1-22 sequence. Helical secondary structure is favored in peptides with neutral C-terminal carboxamide groups, and may also be important for activity of the peptides.

Chapter III.

The influence of peptide structure on immunogenicity for antibody responses.

INTRODUCTION

Melittin is highly immunogenic in mice of the H-2^d haplotype, eliciting IgG antibody responses on the order of 400-700 μg/ml (14); in comparison, apamin, the bee venom neurotoxin peptide for which no specific human IgE activity has been found, elicits a maximal antibody response of about 60 μg/ml in the strongest murine responder strain tested (32). Antibodies to melittin are restricted primarily or exclusively to the C-terminal region of the peptide (14). Rabbit antibodies recognize epitopes on the whole peptide surface, but the dominant epitope is the C-terminal segment (33). Specificity of human antibodies to defined epitopes of melittin has not been reported but is an area of interest.

In order to test the relationship of peptide structure and immunogenicity, we immunized mice with the melittin derivatives described in Chapter II of this thesis to test for antibody responses. The peptides have varying immunogenic potencies; in general immunogenicity correlates with size and amphiphilicity. Antibodies specific for highly immunogenic peptides preferentially recognized the C-terminal epitope. Some of the poorly immunogenic peptides were tested for their ability to induce primary lymphocyte proliferation *in vitro* after immunization *in vivo* (Chapter IV). Low immunogenicity does not appear to be due to lack of T-cell activity.

MATERIALS AND METHODS

Peptides. Preparation and characterization of the peptides is described in Chapter II. The following peptides were used in this study: melittin, 1-26, 1-26-lac, 1-24, 1-24-lac, 1-22-lac, 1-19-lac, 1-20-G_4Q_2 and 1-20-D_6 (Table 2.1).

Purified C-terminal peptide-lactoside fragments were obtained by digestion with the appropriate proteolytic enzyme. Digestion of 1-26-lac with clostripain yielded 25-26-lac and 23-26-lac, and digestion with α-chymostrypsin yielded 20-26-lac. Digestion of 1-24-lac with α-chymotrypsin yielded 20-24-lac. The

proteolytic reactions were performed and fragment peptides were purified by reverse phase chromatography on Polygosil C-18 as described above (Chapter II) . C-terminal fragments were characterized by amino acid analysis, the Prussian blue assay, and ultraviolet spectroscopy for structure and purity, as summarized in Table 3.1.

p-Aminophenyl-β,D-lactopyranoside (0.5 mM) was acetylated by reaction with 50 mM acetic anhydride in 0.2 M Tris-HCl, pH 8.0 at ambient temperature, and characterized by ultraviolet spectroscopy and chemical assays (not shown).

Animals. Female BALB/c and DBA/2 mice were obtained from Jackson Laboratories (Bar Harbor, ME) or Charles River Laboratories (Kingston, NY). Mice weighing 20-25 g or 8-10 weeks old at the start of experiments were used. Retired male breeder rats for use in passive cutaneous anaphylaxis assays were obtained from any available specific pathogen free source. Animals were maintained at The Rockefeller University Laboratory Animal Research Center on rodent chow and tap water, and were handled using humane procedures.

Immunization and bleeding. Antigen vaccines consisted of 10 nmol per ml of peptide antigen and 5 mg per ml of alum in 0.05 M phosphate buffer, pH 6.0. Groups of 4 mice were immunized i.p. with 0.2 ml of vaccine (2 nmol of peptide; about 6 μg) on weeks 0, 2, 4 and 6, and were bled by retro-orbital plexus puncture one or two weeks after immunization. Sera from each group of animals were pooled and stored at -20°C. Melittin-specific sera obtained from BALB/c, CAF_1 and A/J mice, which were reported previously (14), were also used.

Immunoassays. Sera were tested for specific IgG by solid phase immunoassay (ELISA) with modifications, as described (34). Briefly, 20 μl of 5 μM (10 μM for 1-22-lac) peptide were adsorbed to wells of a flexible vinyl assay plate (Microtest III, Falcon) by overnight incubation at ambient temperature. Unoccupied protein binding sites were blocked with 2 g/L fish gelatin (Norland

Table 3.1. Characterization of peptide-lactoside fragments.

Peptide-lactoside	Prepared on digestion[1] of melittin analogs (protease/ peptide)	Reverse-phase chromat.[2]	Amino acid analysis[3]	Lactoside content[4]	ε[5] 246 nm
20-26-lac	chymotrypsin/ 1-26-lac	10%	+	1.3	nd
23-26-lac	clostripain/ 1-26-lac	9	+	1.3	15,800
25-26-lac	clostripain/ 1-26-lac	4	+	1.2	15,700
20-24-lac	chymotrypsin/ 1-24-lac	5	+	0.9	16,100

1. Conditions for digestion were the same as are described in Part II.

2. Products were eluted at the indicated isopropanol concentration on reverse phase chromatography on Polygosil as described in Part II.

3. Amino acid analysis was in agreement with expected values ±5%.

4. Ratio of moles lactose per mole of peptide from the reducing sugar assay (See Table 2.2).

5. UV abosrption band is due to the presence of the acyl-*p* -aminophenoxy chromophore in the lactoside group.

Products Inc.) or 0.1 g/L bovine plasma albumin (BPA; Armour Pharmaceutical Co.). Mouse IgG binding to antigen was detected by sequential addition and one hour incubation of 10 μg/ml rabbit-anti-mouse-IgG (RAMG) and 2 μg/ml sheep-anti-rabbit-IgG (SARG) conjugated to horseradish peroxidase (HRP). Antibody isotype was determined by incubation with 10 μg/ml of polyclonal rabbit-anti-mouse-IgG_1, IgG_{2a}, IgG_{2b} (Litton Bionetics), and IgG_3 (Boehringer Mannheim), followed by SARG-HRP. Enzyme activity was detected by addition of 50 μl of substrate solution, consisting of 0.25 mg/ml aminoantipyrene (Kodak), 8 mg/ml phenol, and 0.005% H_2O_2 in 0.1 M phosphate buffer, pH 7.0, for 15 or 30 minutes, and absorbance measured at 490 nm with a plate reader (Bio-Tek Instruments).

Antibody activity is reported as the serum titer, which is the reciprocal of the dilution for 1/3 maximum enzyme activity. A serum titer of less than 5 reciprocal dilutions was considered insignificant. Generally the 1/3 maximum titer is an order of magnitude less than the detectable end point titer, i.e. absorbance >2-fold above background. For melittin, the reciprocal dilution multiplied with 0.1 μg/ml, as determined from the activity of purified monoclonal antibody (14), gives a good quantitative estimate of the antibody concentration in serum.

Antibody specificity and relative binding affinity for fragment epitopes were determined by inhibition assay. Antiserum was diluted to 1/2 or 1/3 maximal activity in the presence of serially diluted soluble antigen and incubated with solid phase antigen. Bound antibody was detected as described above.

Total serum IgE levels were determined by a sandwich ELISA. Assay plates were coated with 10 μg/ml of monoclonal anti-mouse-IgE 6HD5 (35) and unoccupied protein binding sites were blocked with BPA. Hybridoma 6HD5 was the generous gift of Dr. Zoltan Ovary. Wells were incubated with serially diluted sera and bound IgE was detected by 10 μg/ml biotinylated rabbit-anti-mouse-IgE (RAME,33). RAME was biotinylated on reaction of 5.9 μM antibodies with 1.8 mM

of the N-hydroxysuccinimide ester of biotin in 0.1 M phosphate buffer, pH 7.2, for 30 min at ambient temperature. Biotinylated RAME was detected by 2 μg/ml of avidin-HRP (Sigma), and enzyme activity was determined as described above. Specific IgE was detected by passive cutaneous anaphylaxis in rat skin (36).

RESULTS

Kinetics of the antibody response. Sera from groups of four BALB/c mice immunized with melittin, 1-26, 1-26-lac, 1-24, 1-24-lac, 1-22-lac and 1-19-lac were collected one weak after each immunization, pooled and assayed for immunogen specific IgG activity by ELISA (Figure 3.1). No peptide specific activity was detected at week 0 (preimmune sera) or week 1 (after one immunization). Anti-peptide activity was observed in the week 3 sera of mice immunized with melittin, 1-26-lac, 1-20-G_4Q_2, 1-26, and 1-24-lac, with relative activity decreasing in that order. Anti-1-22-lac activity was detected at week 4, anti-1-20-D_6 activity at week 5, and anti-1-24 activity at week 7. No activity was detected in sera from mice immunized with 1-19-lac. Antibody activity was maximal at week 7 with a few exceptions, such as melittin immunized DBA/2 mice, in which week 5 sera showed maximum activity (data not shown).

The rate of increase in serum titer against these peptides corresponds to maximum activity. Melittin and 1-26-lac have the highest early activity and highest titer at week 7. Peptides having delayed onset of the antibody response do not induce high antiserum titers like those against melittin or 1-26-lac. The emphasized diagonal in Table 3.2 compares maximum serum titers for all of the peptide immunogens. In general maximum activity decreases along the diagonal from melittin, with the exception of anti-1-20-G_4Q_2 sera. Thus peptides of similar structure have different potencies for antibody responses.

Figure 3.1. Kinetics of antibody responses to peptide immunogens. BALB/c mice were immunized on weeks 0, 2, 4 and 6 (arrows) and bled one week later, except for 1-22-lac immunized mice, which were bled two weeks later, immediately prior to immunization. Immunogen specific antibody titers were determined by ELISA.

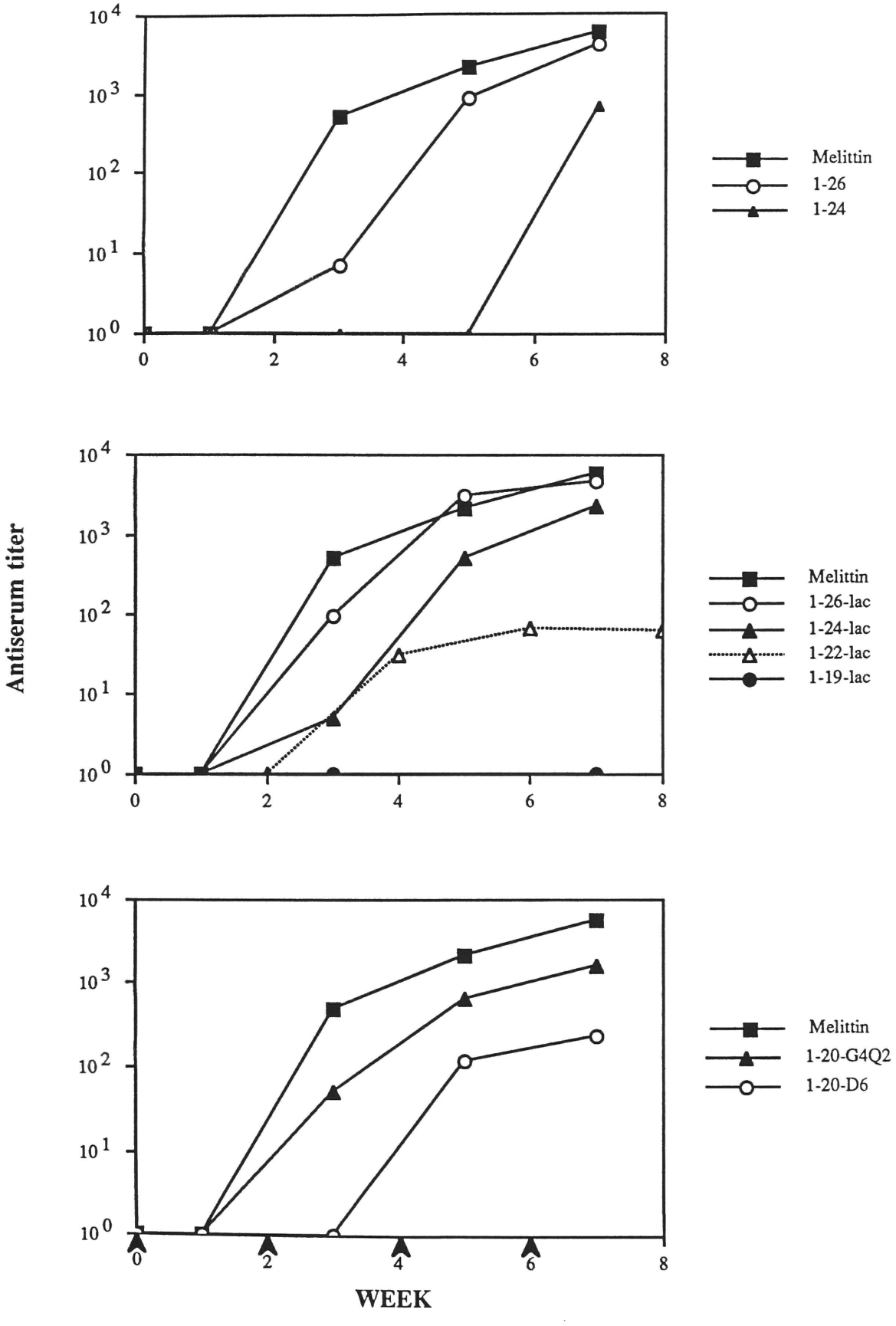

Antiserum titer
10^4
10^3
10^2
10^1
10^0
0
2
4
6
8
Melittin
1-26
1-24
Melittin
1-26-lac
1-24-lac
1-22-lac
1-19-lac
Melittin
1-20-G4Q2
1-20-D6
WEEK

Table 3.2. IgG responses of BALB/c mice to peptide immunogens at week 7.

Immunogen	IgG Titer Against							
	Melittin	1-26	1-26-lac	1-24	1-24-lac	1-22-lac	1-20-G_4Q_2	1-20-D_6
Melittin	**6500**	210	435	0	0	333	300	0
1-26	*4000*	**4000**		310	100			
1-26-lac	300	10	**4000**	0	100	<5		
1-24	0		0	**800**	0			
1-24-lac	0		90	0	**1100**	110		
1-22-lac	10		*90*	0	0	**100**		
1-19-lac	6		5		0	<5		
1-20-G_4Q_2	*420*						**1600**	
1-20-D_6	0							**240**

Peptides were used to coat wells of a 96-well plate at 5 μM, except for 1-22-lac, which was used at 10 μM. Sera were serially diluted in diluent buffer. Results were plotted as A_{490} vs. serum dilution, and the serum titer determined graphically at 1/3 maximum A_{490}.

Boldface type indicates immunogen-specific activity of sera; italic type indicates a high level of cross reactivity with a another analog.

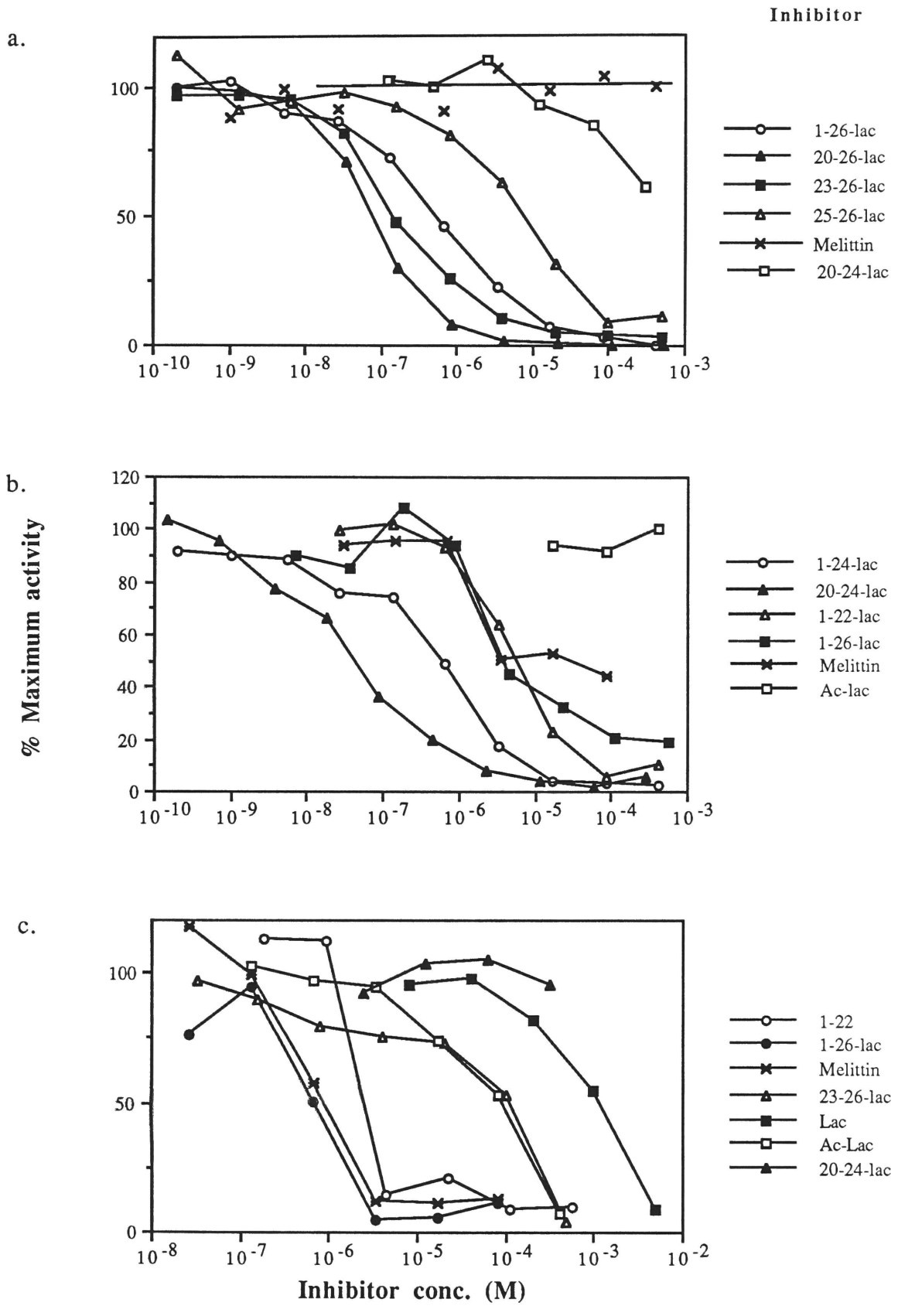
Antibody epitope specificity. Antisera to the peptide immunogens generally showed very low levels of cross reactivity with peptide analogs in ELISA (Table 3.2). For example, melittin specific sera are about 15-fold less active against 1-26-lac, 20-fold less active against 1-22-lac, 30-fold less active against 1-26, and show no activity against 1-24 or 1-24-lac. Antibodies specific for 1-26-lac, 1-24, and 1-24-lac have at least 10-fold lower activity against analog peptides, and in some cases, such as 1-24-lac specific sera, have no activity at all against melittin. Antisera obtained from melittin immunized CAF_1 and A/J mice (14) had reduced activity against 1-26 by 11 to 40-fold and against 1-26-lac by greater than 100-fold (data not shown).

Antibodies to 1-26 do cross react with solid-phase melittin (italicized in Table 3.2). These have comparable activity against melittin and the immunogen itself. In other experiments with DBA/2 mice and BALB/c mice, the melittin-specific activity of 1-26-specific sera was about 3 to 5-fold lower than against 1-26 (data not shown); this still represents higher cross reactivity than the other antisera demonstrate. Antibodies to 1-22-lac cross react poorly with 1-24-lac, despite the shared C-terminal sequence of "KR-lac" in both peptides, but show high cross reactivity with 1-26-lac.

The low level of cross reactivity suggests antibody specificity for C-terminal epitopes since these peptides are identical in the N-terminal 20 residues. Inhibition analysis of antibodies against 1-26-lac and 1-24-lac demonstrate nearly absolute restriction for C-terminal epitopes (Figure 3.2). Affinity constants of the inhibitors relative to the immunogen are apparent from inhibition assays of this type (37).

Inhibition analysis of antibodies against 1-26-lac are presented in Figure 3.2a. Peptide fragments 20-26-lac and 23-26-lac completely inhibit binding of 1-26-lac-specific sera to solid phase 1-26-lac and show higher affinity than 1-26-lac itself. Fragment 25-26-lac binds with somewhat lower affinity than 1-26-lac itself, and inhibits about 95% of the antibodies. Fragment 20-24-lac, the C-terminal

Figure 3.2. Inhibition of binding of immunogen-specific IgG to solid phase immunogen by peptide fragments. All sera are from week 7 bleeding. a. Inhibition of 1-26-lac specific IgG. Sera were diluted 1/2000 and tested against the fragments shown to the right. b. Inhibition of 1-24-lac specific IgG at 1/800 dilution. c. Inhibition of 1-22-lac specific IgG at 1/70 dilution. In another experiment, inhibition of these sera with 1-22-lac was comparable to that of 1-22 (not shown).


Inhibitor
a.
100
50
0
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
10 -4
10 -3
1-26-lac
20-26-lac
23-26-lac
25-26-lac
Melittin
20-24-lac
b.
% Maximum activity
120
100
80
60
40
20
0
10 -10
10 -9
10 -8
10 -7
10 -6
10 -5
10 -4
10 -3
1-24-lac
20-24-lac
1-22-lac
1-26-lac
Melittin
Ac-lac
c.
100
50
0
10 -8
10 -7
10 -6
10 -5
10 -4
10 -3
10 -2
Inhibitor conc. (M)
1-22
1-26-lac
Melittin
23-26-lac
Lac
Ac-Lac
20-24-lac

epitope of 1-24-lac, inhibits incompletely with low affinity. Free lactoside inhibited incompletely at concentrations in excess of 10^{-3} M (data not shown); Ac-lactoside did not inhibit up to that concentration. Although these sera cross-react with solid phase melittin in ELISA, melittin fails to inhibit binding of antibodies to solid phase 1-26-lac. This anomaly presumably results from differences in binding to solid-phase and soluble antigen.

Similar data are observed for 1-24-lac specific sera in Figure 3.2b. The C-terminal fragment of 1-24-lac, 20-24-lac, completely inhibits antibody binding with 10-fold higher affinity than 1-24-lac itself. Antibodies from a bleeding at week 10 after a week 9 boost showed greater affinity for 1-24-lac by a factor of about 4-fold and affinity for 20-24-lac was increased another 10- to 40-fold above that (data not shown). Peptide-lactoside analog 1-22-lac, which has the sequence "KR" rather than "KRKR" preceding the lactoside group, inhibits more than 95% of the activity with reduced affinity; 1-26-lac inhibits about 80% of the activity. Sera specific for 1-24-lac did not cross-react at all with solid phase melittin, yet melittin inhibits about 50% of the antibody activity in this assay, the reverse of the dichotomy presented by the interaction of 1-26-lac-specific sera and melittin. Fragment 25-26-lac (from 1-26-lac), Ac-lactoside and free lactoside failed to inhibit binding at all.

The data suggest that a minimum antibody epitope for the strongly immunogenic peptides 1-26-lac and 1-24-lac is X-X-lac, with X standing for an amino acid. Peptides or fragments with this structure, e.g. QQ-lac for 1-26-lac and 1-22-lac for 1-24-lac, inhibit 95% of peptide-specific antibody activity, albeit with somewhat reduced affinity constants. Antibodies specific for 1-22-lac do not demonstrate this specificity (Figure 3.2c). These antibodies are inhibited by 1-22, 1-26-lac, melittin, 23-26-lac, Ac-lactoside, and free lactoside, though not by 20-24-lac.

IgE responses to peptide immunogens. Specific IgE responses could be determined by passive cutaneous anaphylaxis (PCA) in rat skin. This procedure requires large amounts of antigen for the challenge and was therefore not feasible for some of the immunogens tested*. Increases in specific IgE could be correlated with total IgE in our system (R^2 value of 0.899, R is the correlation coefficient), as shown in Figure 3.3, since a specific IgE responses represents considerable increase over normal IgE levels of < 1 μg/ml. While total IgE to a given immunogen correlates with specific IgE, the total IgE response may have a significant non-specific component due to general effects of the adjuvant (38). Therefore the value for total IgE in Table 3.3 should be considered as a qualitative measure, just as PCA titer is a qualitative value.

Results for total and specific IgE from BALB/c or DBA/2 mice are reported in Table 3.3. These results represent sera with highest activity in PCA. In other experiments, specific and total IgE levels in melittin immunized mice were comparable to those reported for 1-26 and 1-26-lac in this table. Specific IgE response parallels IgG activity, with decreased PCA titer of sera specific for shorter peptides and cross reactivity lower than immunogen specific activity. The high level of cross reactivity of 1-26-lac specific IgE with 1-26 represents an exception to this trend. Also, sera specific for 1-22-lac had high cross reactivity with 1-26-lac and 1-24-lac. Cross reactivity with the lactoside epitope is easy to reconcile, especially with the KR-lac C-terminal sequence of 1-24-lac; the high IgE response, represented by PCA activity of these sera, is unexpected given the low IgG activity of these sera.

Immunization resulted in increased total IgE. Total IgE levels correspond poorly to IgG levels, despite their good correlation with PCA titer. For example, sera against 1-26-lac has much less total IgE than sera against melittin, even though

* Attempts were made without success to develop an ELISA based antigen-specific IgE assay for this study.

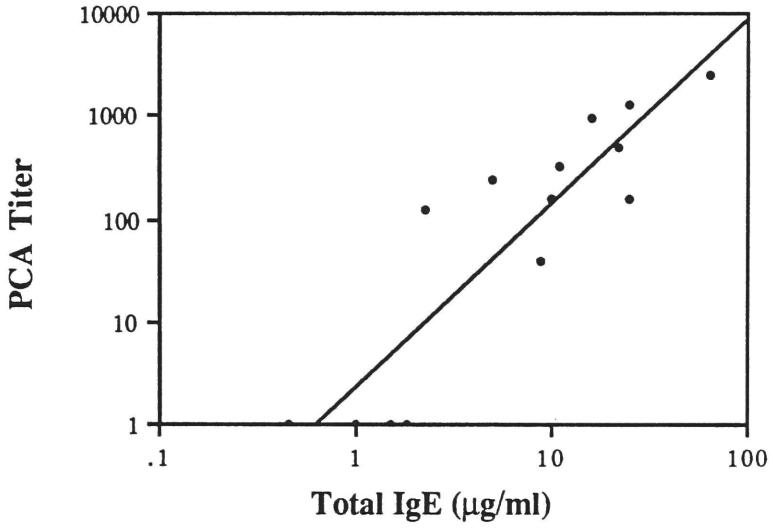


Figure 3.3. Correlation of specific IgE (by PCA) with total serum IgE (by ELISA). Sera were tested for immunogen specific IgE by PCA (see Table 3.3) and for total IgE by a sandwich ELISA. Data are fit by a least squares linear regression analysis (Cricketgraph 1.2), and a correlation coefficient, R^2 of 0.899, is found.

Table 3.3. IgE responses of BALB/c and DBA/2 mice to peptide immunogens at week 7.

Immunogen [1]	Total IgE [2] (μg/ml)	Specific IgE Titer Against [3]			
		Melittin	1-26	1-26-lac	1-24-lac
Melittin (D)	74.0	**2560**	320	160	0
1-26 (B)	8.5	80	**320**	40	80
1-26-lac (D)	5.0	40	320	**320**	80
1-24-lac (B)	11.0				**160**
1-22-lac (D)	14.0			*160*	*640*
1-20-G_4Q_2(B)	4.3				
1-20-D_6(B)	2.5				
normal	<1.0				

1. Strain indicated by (D) is DBA/2, (B) is BALB/c.

2. Total IgE was determined by sandwich ELISA of serially dilute sera. A monoclonal IgE served as the IgE standard. Values represent the mean of three assays.

3. Specific IgE activity was determined by passive cutaneous anaphylaxis (PCA) with serially diluted mouse sera. Rats were challenged i.v. with 300 nmol of peptide in 2 ml of PBS, 5 mg/ml Evan's blue dye. PCA titer, the lowest dilution to give a positive spot, can vary within one dilution.

IgG responses are comparable. Sera from mice immunized with 1-22-lac show high levels of total IgE, despite the low level of IgG detected in these sera. These high levels of total IgE are consistent, however, with the high PCA titers of these sera.

Other immunoglobulin isotypes. Sera against melittin, 1-26-lac, 1-24-lac, 1-24, 1-20-G_4Q_2 and 1-20-D_6 were tested for different Ig isotypes (Table 3.4). IgG_1 comprised 90-99% of the total Ig activity in sera against melittin, 1-26-lac, 1-26-lac and 1-24 (7 sera were tested). Melittin-specific sera reported earlier (14) and from this report had very low levels of IgG_{2a}, ranging from 0-4%, and IgG_{2b}, ranging from 0-6%, and no detectable IgG_3. Similarly, no IgG_3 activity was detected against peptides 1-20-G_4Q_2 or 1-20-D_6. However, 1-20-G_4Q_2- and 1-20-D_6-specific IgG_1 activity was significantly reduced, with a range of 78-92%, and IgG_{2a} activity increased, ranging from 4-11%, relative to melittin and the other peptides. IgG_{2b} activity was slightly increased, with a range of 4-10%. These results were determined from isotype specific titers by ELISA, and so may not represent the quantity of antigen-specific Ig of the particular isotype. However, the same isotype determination system was used for all the sera, so relative data are valid. With this caveat in mind, the bottom line observation is that IgG_{2a} activity increases and IgG_1 activity decreases in response to 1-20-G_4Q_2 and 1-20-D_6 relative to melittin and the truncated melittin peptides. Increased IgG_{2a} activity specific for these antigens correlates with decreased total IgE activity (Table 3.3).

Table 3.4. IgG subclass distribution of peptide immunogen-specific sera.

Immunogen [1]	% Total Ig Activity in [2]			Number of sera	Assays per sera
	IgG1	IgG2a	IgG2b		
Melittin	96±3.8	1.4±1.5	2.4±2.5	7	1
1-20-G_4Q_2	92±0	4±1	4±1	1	3
1-20-D_6	77±7.8	12±4.4	11±3.5	1	3

1. Melittin-specific sera are from 2 BALB/c groups, one CAF1 group, and one DBA/2 group. They also include sera from groups of BALB/c mice immunized with 1-26-lac, 1-24-lac and 1-24 peptides. Sera from the week 7 bleeding were assayed once in each case. Values are average ± S.Dev. for all sera tested or for replicate assays.

2. Immunogen-specific Ig activity was determined from the ELISA titer using isotype-specific rabbit polyclonal antibodies. Total Ig activity was the sum of all Ig-isotype titers (IgG1, IgG2a and IgG2b; no IgG3 activity was detected); % activity is specific titer / total activity x 100.

DISCUSSION

Melittin and its analogs show varying degrees of immunogenicity for antibody responses. These peptides include residue 7-19, which is found to be an immunodominant epitope in H-2^d mice. Melittin, 1-22-lac, 1-20-G_4Q_2, 1-20-D_6, 7-19 and 7-19-lac all elicit primary T cell responses *in vivo* and stimulate melittin specific T cells *in vitro* (Chapter IV), so the different degrees of antibody response do not appear to result from lack of appropriate T cell help. (This conclusion must be weighed in terms of peptide structural effects on class II molecule restriction, which are reported in Chapter IV. The general implications of these observations are considered fully in Chapter V.) The ability of various melittin analogs to elicit IgE as well as IgG antibodies, and the overwhelming preponderance of IgG_1 isotype in the IgG responses, further suggests appropriate helper-T cell activity in of the immune response to these antigens since B cell switching to IgE secretion is an IL-4 mediated (39), therefore T cell controlled, event. The role of T cells in immune response to melittin is reported fully in Chapter IV. Dissociation of IgG and IgE responses to 1-22-lac suggests an interesting mechanism requiring further study.

Two structural elements of these peptides determine immunogenicity for antibody responses. The most obvious, evident from examination of the diagonal of Table 3.2, is length. Size and antibody titer decrease coordinately; sequentially truncated peptide and peptide-lactoside conjugates have clearly diminished potency for antibody responses. Melittin and 1-26, the longest peptides, are immunogenically comparable, and elicit 6 to 9-fold greater antibody activity than 1-24. The shortest peptide tested, 1-22, was not immunogenic (data not shown). Similarly, 1-26-lac is about 4-fold more potent than 1-24-lac, and 40-fold more potent than 1-22-lac; 1-19-lac was not immunogenic at all. Comparison of the response to 1-24 and 1-24-lac, which differ by the presence of the lactoside group, further demonstrates the role of size. First, the antibody response to 1-24-lac comes

up earlier than that to 1-24; second, 1-24-lac-specific activity is 2-fold greater than 1-24. Similar, though not as dramatic results, are evident in comparison of 1-26 and 1-26-lac.

The second structural property that determines immunogenicity is amphiphilicity, as shown qualitatively in Table 3.5. Peptides that were found to demonstrate "peptide-detergent" behavior in Chapter II are more potent immunogens for antibody responses. A peptide with weak amphiphilic behavior, 1-20-D_6, is a poor immunogen, despite the fact that it contains the N-terminal residue implicated in hydrophobic interactions, the 7-19 T cell epitope, the same number of amino acids as melittin, and has definite linear amphiphilic structure from the primary sequence. Peptide 1-20-G_4Q_2, of the same size as melittin, demonstrates less amphiphilic activity than melittin and more than 1-20-D_6, especially with regard to detergent-like behavior, and is moderately immunogenic. The ordering of immunogenicity for antibody responses--melittin, 1-26-lac, 1-26, 1-24-lac, 1-20-G_4Q_2, 1-24, 1-20-D_6, 1-22-lac, 1-19-lac--corresponds roughly to biophysical activity evident from Table 2.3.

More generally, these results suggest that peptides engineered with melittin-like amphiphilic behavior, i.e. a predominantly hydrophobic segment and a contiguous cationic segment, and with an appropriate T-cell epitope, may be useful carrier molecules for chemically defined carrier-hapten constructs and possibly for artificial vaccines.

Table 3.5. Comparison of immunogenicity and amphiphilicity.

Peptide	Immunogenicity[1]	Amphiphilicity[2]		
		Assoc.[3]	Hemol[4]	αHX[5]
Melittin	++++	+	++	+
1-26	+++	+	++	
1-26-lac	++++	+	++	+
1-24	++	+	++	
1-24-lac	+++	+	++	+
1-22-lac	+		++	+
1-19-lac	-		+	
1-20-G4Q2	+++		+	
1-20-D6	++			+

1. Plus signs represent the order of magnitude of the IgG response, as determined from the antibody titer by ELISA.

2. Qualitative values of amphiphilic behavior were determined from experiments reported in Part II. For the sake of comparison with immunogenicity, amphiphilic behavior should be considered as the sum of all + signs.

2. Self association was scored as positive if the concentration of NaCl for 50% fluorescence shift was <1.0 M.

3. Peptides scored ++ if the hemolysis HD-50 value was <10 μM, and + if the HD-50 value was
<100 μM.

4. α-Helix propensity was scored positive if peptide had >10% helix structure at 200 μM as calculated by the "prosec" program.

Chapter IV.

Melittin-specific T cell responses:

Epitope mapping and structural determination;

T cell phenotype, frequency and MHC restriction.

INTRODUCTION

Melittin's well characterized structural propensities make it an interesting model to study how structure influences immunogenicity and antigenicity for T cell responses. One model to predict T-cell epitopes based on identification of primary sequences with amphiphilic α-helical conformational propensity has been proposed (40), yet this model fails to predict all known epitopes. Secondary structural propensities of melittin under a wide variety of conditions are well known, and structural evidence is available from circular dichroism spectra of melittin fragments under normal aqueous and idealized hydrophobic conditions, i.e. in 20% hexafluoroisopropanol, so this analysis potentially sheds light on T-cell epitope secondary structures.

Melittin's known activity as an allergen, albeit a minor one, also makes it a good choice to investigate intrinsic characteristics that may enhance allergenicity by selecting for specific T cell responses. The availability of melittin fragments and the size of the peptide itself 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 helper-T cell phenotypes: T_H1 cells secrete IL-2 and γIFN, and are implicated in helping cellular immunity; T_H2 cells secrete IL-4 and IL-5, and are implicated in helping humoral immunity (41,42). Further experiments in mouse (43) and rat (44) systems suggests that T_H1 (IL-2 secreting) cells are primary T cells that mature or differentiate into T_H2 (IL-4 secreting) cells. There is also evidence for differentiation of helper T cell phenotypes in man based primarily on expression of specific T-cell antigens (45). IL-4 induces B-cell isotype switching to IgE and IgG_1 in mice, shown *in vitro* (39) and *in vivo* (46), and to IgE and IgG_4 in humans, shown *in vitro* (47). Melittin and its derivatives elicit IgE as well as IgG antibodies, and since selection of these

isotypes is under T cell control *in vitro* and *in vivo*, thus melittin-specific T-cells are expected to express the T_H2 phenotype. It is possible than an allergen, e.g. melittin, through some intrinsic property, specific MHC restriction or TcR "selection" promotes T_H2 phenotype. It is possible that an allergen like melittin has some intrinsic charactersitic of sequence or structure that promotes T_H2 phenotype selection, perhaps through a particular MHC restriction or by TcR selection.

T cell activation requires the presentation of processed antigen in the context of self major histocompatability (MHC) antigens. For $CD4^+$ lymphocytes, usually associated with helper responses, antigen presentation is restricted to the MHC class II (Ia) molecules. Selection of antigen by molecules of the MHC represents one level of genetic control of immune response (reviewed in 48). Genetic control of immune response to melittin was observed previously in the strain specificity of the antibody response to melittin: mice of the $H\text{-}2^d$ haplotype respond to melittin, whereas $H\text{-}2^a$ mice are weak responders, and $H\text{-}2^b$ and $H\text{-}2^k$ mice do not respond at all (14). An MHC class II dissociation has been found for melittin sensitivity of English bee keepers and their families (49).

To fully characterize the T-cell response to melittin, we mapped the T-cell epitope using synthetic and proteolytic melittin fragments (Table 2.1, Table 4.1) to stimulate primary and long term T cells *in vitro*. Melittin and its peptide analogs appear to require minimal or no processing at least no C-terminal processing. T-cell phenotype was characterized in clones derived from limiting dilution of immunized cells: melittin-specific T clones are all $CD4^+$ and show high expression CD45R, but have somewhat ambiguous cytokine production profiles. Analysis of MHC class II (Ia) molecule restriction of clones and primary lymphocytes indicates restriction to both $I\text{-}A^d$ and $I\text{-}E^d$ for melittin, but only to $I\text{-}A^d$ for some analogs. These findings have implications for the structure-activity relationship of T-cell epitopes, and how specific T cell responses influence IgE levels.

MATERIALS AND METHODS

Peptides. The preparation and characterization of melittin, 1-26, 1-26-lac, 1-24, 1-24-lac, 1-22, 1-22-lac, 1-19, 1-19-lac, 7-19-lac, 8-21, 1-7, 1-20-G_4Q_2 and 1-20-D_6 is described in Chapter II.

Fragments 1-7 and 8-21 (Table 2.1) were prepared as described (14) by digestion of melittin (4 mg per ml) with trypsin-TPCK (0.2 mg per ml; Millipore Corp.) in 0.05 M NH_4HCO_2 at 25°C for 2.5 hours. Tryptic fragments were separated by chromatography on a 20 x 0.9 cm column of SE-53 cellulose (Whatman) eluted with a linear pH gradient from 3.05 (0.05 M CHO_2H, 0.01 M NH_4HCO_2) to 6.08 (0.005 M HOAc, 0.095 M NH_4Ac) in 4 M urea. Derivatives of peptide 8-21 were prepared by condensation with glycine methyl ester (GlyOMe) in the presence of EDC as described for the preparation of peptide-lactoside derivatives (Chapter II). 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 activated EDC peptide transient forms a stable N-acyl urea (NAcUrea) derivative at the C-terminus. The structure of this product was proven by mass spectrometry (Table 4.2).

Melittin T-cell epitope analogs were prepared synthetically (Table 4.1). One series of peptides, representing N- and C-terminal derivatives of fragments 10-21 and 11-21, were prepared with free or acetylated N-termini and free or amide C-termini using FMOC chemistry with a RaMPS semiautomatic apparatus (DuPont). A standard solid phase procedure with symmetric anhydride single coupling to either RapidAmide (C-terminal carboxamide) or Wang (free C-terminus) was followed. Coupling with 2.5 equivalents of activated amino acid was successful using an HOBt boost, otherwise 5 equivalents were used to couple. The HOBt boost consisted of adding 1.5 equivalents of HOBt and carbodiimide midway through the synthesis. Leu16 was coupled as the preactived OPfp ester

Table 4.1. Melittin T-cell epitope synthetic analogs.

Peptide		Synthesis	Structure
			K V L T T G L P A L I S W I K
10-21	a	semi-automatic, FMOC (RaMPS)	$CONH_2$
	b		Ac $CONH_2$
	c	(PFF)	
	d		Ac
11-21	a		Ac $CONH_2$
	b		
	c		Ac
7-21	1	multiple synthesis, plastic film technique	P
	2		D
	3		N
	4		L
	5		F
	6		(D) F
	7		L Q
	8		L K

All peptides have free N- and C-termini unless otherwise indicated.

Ac = acetylated; $CONH_2$ = carboxamide; (D) = D-amino acid.

Peptides 10-21 and 11-21 were prepared on a DuPont RaMPS semiautomatic apparatus using FMOC chemistry; the 7-21 analogs were prepared by a novel multiple synthesis technique (50) by Dr. Rolf Berg.

(DuPont). Where indicated, peptides were acetylated with excess acetic anhydride and carbodiimide in DMF. The completeness of coupling was tested by the ninhydrin test, and for coupling to proline, the Isatin test (RaMPS manual). Synthesis, cleavage and work up were performed as directed by the RaMPS manual.

Fragment 7-21 analogs with a single amino acid substitution at position 14 (proline in melittin), or positions 12 (glycine in melittin) and 14 were also prepared. Substitutions were chosen to represent the widest range of structural and chemical differences from the native fragment. These peptides were synthesized by Dr. Rolf Berg using a novel multisynthesis strategy on a derivatized film solid phase support of his design (50). Common steps of deprotection, neutralization, washing and coupling of identical amino acids were performed simultaneously, while the coupling of different amino acids was carried out in separate vessels. A standard solid phase procedure, using double DCC coupling with 3.5 equivalents in 30% DMF/DCM was employed, except for Leu13 and Gln14, which were double coupled as symmetric anhydrides in 20% DMF/DCM.

All the peptides were purified by reverse phase chromatography, and further characterized by HPLC, ultraviolet spectroscopy and amino acid analysis as described in Chapter II and shown in Table 4.2.

Circular dichroism. Circular dichroism spectra of 10 μM peptides in PBS or 20% 1,1,1,3,3,3,-hexafluoro-2-propanol (HFP, Aldrich) in 80% PBS were obtained and analyzed as described above (Chapter II).

Mice. BALB/c, DBA/2, A/J and CAF_1 mice were obtained from Jackson Labs (Bar Harbor, ME) or Charles River (Kingston, NY) weighing 20-25 g or at 6-12 weeks of age and maintained as described above (Chapter III).

Table 4.2. Characterization of T-cell epitope peptides. 1. Reverse phase chromatography: a) percent isopropanol (iPrOH) for elution from a Varian MCH-10 (20 x 0.5 cm) reverse phase column in a 1% per min iPrOH gradient in 0.1% TFA at a flow rate of 1 ml/min; b) percent CH_3CN for elution from a DuPont ProteinPLUS column as described (Table 2.2), doublet indicates closely spaced peaks of equal size that could not be resolved; c) time of elution from a Vydac C-18 column in a 1.5% per min CH_3CN gradient in 0.04% TFA at a flow rate of 1.5 ml/min. 2. Amino acid analysis showed the expected values ±5%.

Table 4.2. Characterization of T-cell epitope peptides.

Peptide	HPLC [1]	Amino acid analysis [2]	Mass spectrometry expected	found
8-21	40% [a]	+		
8-21-GlyOMe	41	+		
8-21-NAcUrea	42	+	1596	1596.8
PFF 10-21 a	43% [b]	+		
b	44	+		
c	41	+		
d	42	+		
11-21 a	44	+		
b	41	+		
c	45	+		
RB 7-21 1	21.6 min [c]	+	1640	1640.5
2	21.9	+	1658	1658
3	21.4	+	1657	1657
4	24.0	+	1656	1656.1
5	24.0	+	1690.1	1690.1
6	23.8	+	1690.1	1690.1
7	27.8	+	1727.1	1727.4
8	25.9	+	1727.2	1727.3

Tissue culture. Culture medium (10CM) consisted of 1:1 RPMI-1640 (GIBCO) and Eagle Medium with Hank's Salts (GIBCO), 10% fetal bovine serum (Hazelton, endotoxin low), 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO), 10 mM Hepes (Sigma), and 54 μM 2-mercaptoethanol (51). Medium prepared using only RPMI-1640 (10R) worked just as well.

Syngeneic feeder cells were fresh irradiated (3300 Rads from a ^{135}Cs source) spleen cells that were not cleared of red blood cells. BALB/c spleens were used with H-2^{d} restricted cells; A/J spleen with H-2^{a} restricted cells. Frequently feeder cells were prepared at a density of one spleen, i.e. about 10^8 cells, per 5 ml of culture medium and not counted further. This approximation did not appear to have a significant effect on proliferation assay results when compared with assays using counted splenic feeder cells (data not shown). We have also prepared irradiated feeder cells from large numbers of spleens and cryopreserved the extra cells (4 x 10^7 cells in 4.5 ml of 10R containing 7.5% DMSO). Human peripheral blood mononuclear cells are preserved in this way (52). Upon thawing, cell recovery was about 60%, and red blood cells were almost totally absent. These cells worked well as feeder cells for maintaining long term cultures and in proliferation assays.

Rat conditioned medium (RCM) was prepared essentially as described (53), and ConA activity was neutralized by addition of α-methylmannoside (Sigma) to a concentration of 0.1 M, followed by sterile filtration through a 0.45 μ filter (Nalge).

Initiation and propagation of melittin-specific T cells. These procedures were adapted from (54). Mice were immunized i.p. with 0.2 ml of 10 μM melittin or s.c. in the base of the tail with 0.05-0.1 ml of 25 μM melittin in complete Freund's adjuvant (CFA, CalBiochem). The emulsion was prepared with equal volumes of melittin (at 2X concentration) in PBS and CFA. Spleen cells were obtained from mice immunized i.p. and were prepared in a single cell suspension after hemolysis in 0.16 M NH_4Cl. 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 2.5 x 10^6 cells per ml and stimulated with 0.3-0.8 μM melittin.

Long term cultures were maintained by repeated restimulation and rest as described (51), although frequently cells were only harvested prior to restimulation (53). Blast cells (cultures four days after restimulation) and rested T cells were harvested by centrifugation (600 x *g* for 30 min) over 2 cm of Ficoll-Paque (Pharmacia), washed extensively and counted in the presence of trypan blue.

Cell lines R3 and R4 were derived from spleen cells of BALB/c mice immunized i.p. with melittin; cell line A1 was derived from draining lymph node cells from a BALB/c mouse immunized s.c. with 1-22-lac (Table 4.3).

T cell clones. T cells were cloned from long term cultures A1 and R4 by limiting dilution (Table 4.3). One day after bulk restimulation, cells were seeded at 0.3 cells per well in 48 or 96 wells (Corning 96-well plate) and cultured in 10R with 20% RCM as described (51). Primary lymph node cells from four CAF_1 and two DBA/2 mice (Table 4.3) were cloned in a limiting dilution analysis one day after *in vitro* stimulation of the primary cells (55). Wells were seeded with different numbers of lymph node cells (see Results) and an equal volume of 2.5 x 10^5 irradiated feeder cells pulsed with melittin for a final volume of 100 μl in 10R, supplemented with 20% RCM and 0.4 μM melittin. After 10-16 days, all wells were restimulated with the addition of 5 x 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. Positive wells could be identified visually; however, after another 10-16 days, 100 μl aliquots of cells suspension 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 (56). Positive wells incorporated

Table 4.3. Melittin-specific T cell lines and clones.

Cell line/ clone	Mouse strain	Immunization	Tissue source	*In vitro* conditions
A1 (line)	BALB/c	1-22-lac in CFA, s.c. in the base of the tail	Inguinal and periaortic nodes	Maintained with melittin stimulation.
A1.B10/H11	"			Cloned by limiting dilution from line A1; maintained with melittin stimulation.
R3 (line)	BALB/c	Melittin in CFA, i.p.	Spleen	Melittin stimulation.
R4 (line)	BALB/c	Melittin in CFA, i.p.	Spleen	Melittin stimulation.
R4.A2	"			Cloned by limiting dilution from line R4.
M2.B8	CAF1	Melittin in CFA, s.c. in the base of the tail	Inguinal and periaortic nodes	Cloned from primary cells and maintained on A/J splenic feeder cells.
L1.B9/B12	"	"	"	Cloned from primary cells and maintained on BALB/c feeders.
L2.A10/A11/B7	"	"	"	"
M1.A8/B8	"	"	"	"
M2.B4/B5/B12	"	"	"	"
DBA/2	DBA/2	Melittin in CFA, s.c. in the base of the tail	Inguinal and periarotic nodes	Cloned from primary cells and maintained on BALB/c feeder cells.

^{3}H-thymidine at least 2-3 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 x 10^6 feeder cells, 0.4 μM melittin, and 5% RCM in 10R. Once the cells could be counted, cultures were restimulated with 1-2 x 10^5 T cells per ml, 5-10 x 10^6 feeder cells per ml and 0.4 μM melittin per ml in 10R without RCM.

Proliferation assays. Proliferation assays with primary cells were performed as described (54), with 2.5-5 x 10^5 cells and antigen in 200 μl of 10R in 96-well culture plates. Spleen cells from BALB/c mice immunized i.p. with melittin in CFA were cultured in 10CM; spleen cells from BALB/c mice immunized with melittin + 1-19, 1-24, 1-20-G_4Q_2, or 1-20-D_6 in alum (see Chapter III) were cultured in 1% normal mouse serum supplemented medium (1%NMSR). Lymph node cells from BALB/c mice immunized with peptides 7-19 and 7-19-lac by the s.c. route were cultured in 10R. Primary cells were obtained 9-11 days after immunization. Long term lines and clones harvested 10-16 days after restimulation were cultured at 1-2 x 10^4 cells per well with 2-5 x 10^5 feeder cells and antigen in 200 μl of 10R in 96-well plates. After 3 days, cultures were pulsed with 0.5 or 1.0 μCi of ^{3}H-thymidine, 2 Ci/mmole (Amersham), in 25 - 50 μl of RPMI-1640 for 16-20 hours. DNA was harvested on glass fiber filters using a PHD cell harvester (Cambridge Technology) and ^{3}H-thymidine incorporation was determined by scintillation counting in ReadySafe cocktail (Beckman).

Preparation of irradiated splenic feeder cells has been described above. Glutaraldehyde fixed A20 cells were prepared as described (57) by treating A20 cells with 0.05% glutaraldehyde for 5 min, followed by sterile filtered 1 M lysine and washing. A20 cells were also 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 (58), were the gift of Dr. Joshua Metlay.

Anti-melittin MAb 4, which is specific for the C-terminal epitope of melittin, has been described previously (14).

Lymphokine assays. Culture fluid supernatant from 4-day stimulated T cell cultures was filtered to remove cellular debris (0.45 μ, Schleicher and Schull) and tested for growth activity on CTLL cells in the presence and absence of anti-IL-2 MAb S4B6 (59) (gift of Dr. Marian Birkeland) or anti-IL-4 MAb 11B11 (60) (NCI Biological Response Modifiers Program). IL-2 and IL-4 activity in culture fluid supernatant were compared to results with recombinant murine IL-2 and IL-4 (Genzyme). CTLL cells (61) were used 48 hours after restimulation and cultured at 5×10^3 cells per well with culture fluid supernatant for 18 hours. Cells were pulsed with 1 μCi of ^{3}H-thymidine and harvested 4 hours later. The CTLL line was a gift from Dr. Jeffrey Ming.

IL-4 activity was also determined by the B cell costimulation assay (62). Small resting B cells (10^3 per well) are cultured with α-Ig-coated Sepharose and T cell culture fluid supernatant in the presence or absence of anti-Il-4 (11B11) for three days. The cells were pulsed as in the proliferation assay, and harvested after 20 hours.

Immunofluorescence. For direct immunofluorescence staining, fluorescene isothiocyanate (FITC) labelled anti-Lyt2 and phycoerythrin (PE) labelled anti-L3T4 (Beckton Dickinson) were used at 10 μg/ml 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. For indirect immunofluorescence, culture fluid from hybridomas secreting MAb TIB122 (anti-T200; ATCC) and MAb MB23G2 (anti-CD45R, 63), both rat derived hybridomas, were gifts of Dr. Marian Birkeland, and were used at 1/4 dilution. Bound rat MAb was detected with a FITC-mouse-anti-rat (FITC-MAR) reagent at 5 μg/ml (Boehringer-Mannheim). Cells were incubated with antibody for 60 min at 4°C (these conditions were repeated with the FITC-MAR

reagent for indirect immunofluorescence), then washed and fixed in 10% formalin. Immunofluorescence data were obtained on a FACscan II (Beckton Dickinson).

MHC class II restriction. Restriction of clones and lines to I-A^d and I-E^d was tested by incubating Ia^d restricted cells with irradiated feeder cells in the presence of MAb's B21.2 (anti-I-A^d), 11B.32 (anti-I-E^d), or, as a control for non-specific inhibition of presentation on CAF_1 feeder cells, 10.216 (anti-I-A^k), in a proliferation assay. The MAb's were present in culture fluid supernatant, used at a concentration of 1.6% to 25% (see results). MAb culture fluids were the generous gift of Dr. Ralph M. Steinman.

RESULTS

Melittin T-cell epitope. Figure 4.1 shows the *in vitro* response of spleen cells from a BALB/c mouse immunized i.p. with melittin in CFA. The cells are most responsive to melittin, followed by 1-22, 1-19 and 8-21. The last two peptides are less active, 8-21 by about 100-fold on a molar basis, than melittin. These peptides seem to include all or most of the relevant epitope.

Analysis with long term derived lines confirms the presence of the T cell epitope in peptides 1-19 and 8-21. Figure 4.2 shows the results of a proliferation assay with polyclonal lines A1 (Figure 4.2a), R3 (Figure 4.2b), and R4 (Figure 4.2c). All three lines respond to melittin, 1-22, 1-19 and 8-21 (Figure 4.2), but not to peptides 1-7 and 20-26 (data not shown). Thus it appears that the T cell epitope of melittin in BALB/c mice is substantially located in residue 8-19. The proliferation assay also shows some fine specificity of the different lines. The 1-22-lac immunized line A1 (Figure 4.2a) responded equally well to melittin and 1-22, and with reduced efficiency to 1-19 and 8-21, which was also the case for melittin specific line R4 in Figure 4.2c. However melittin, specific line R3 in Figure 4.2b responds poorly to 1-22, and much less efficiently to 1-19 and 8-21.

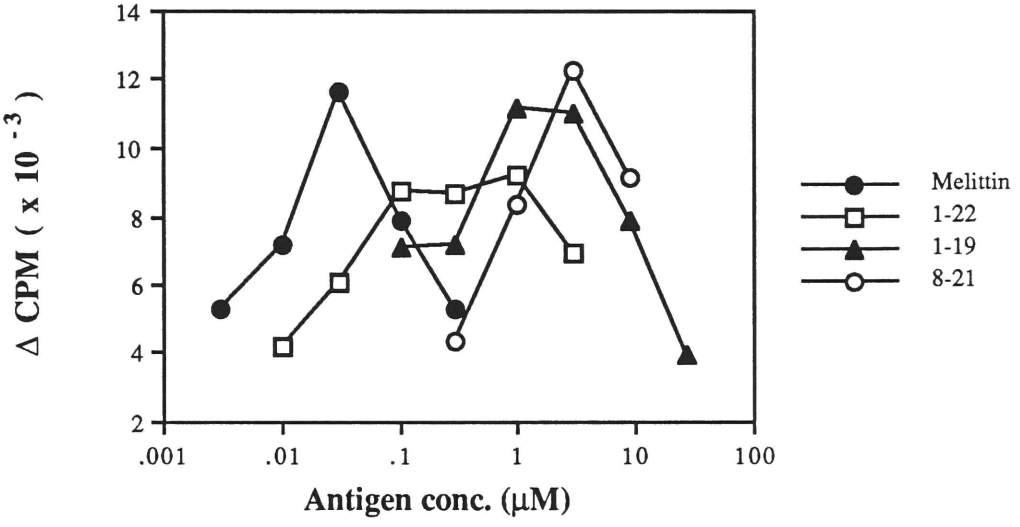
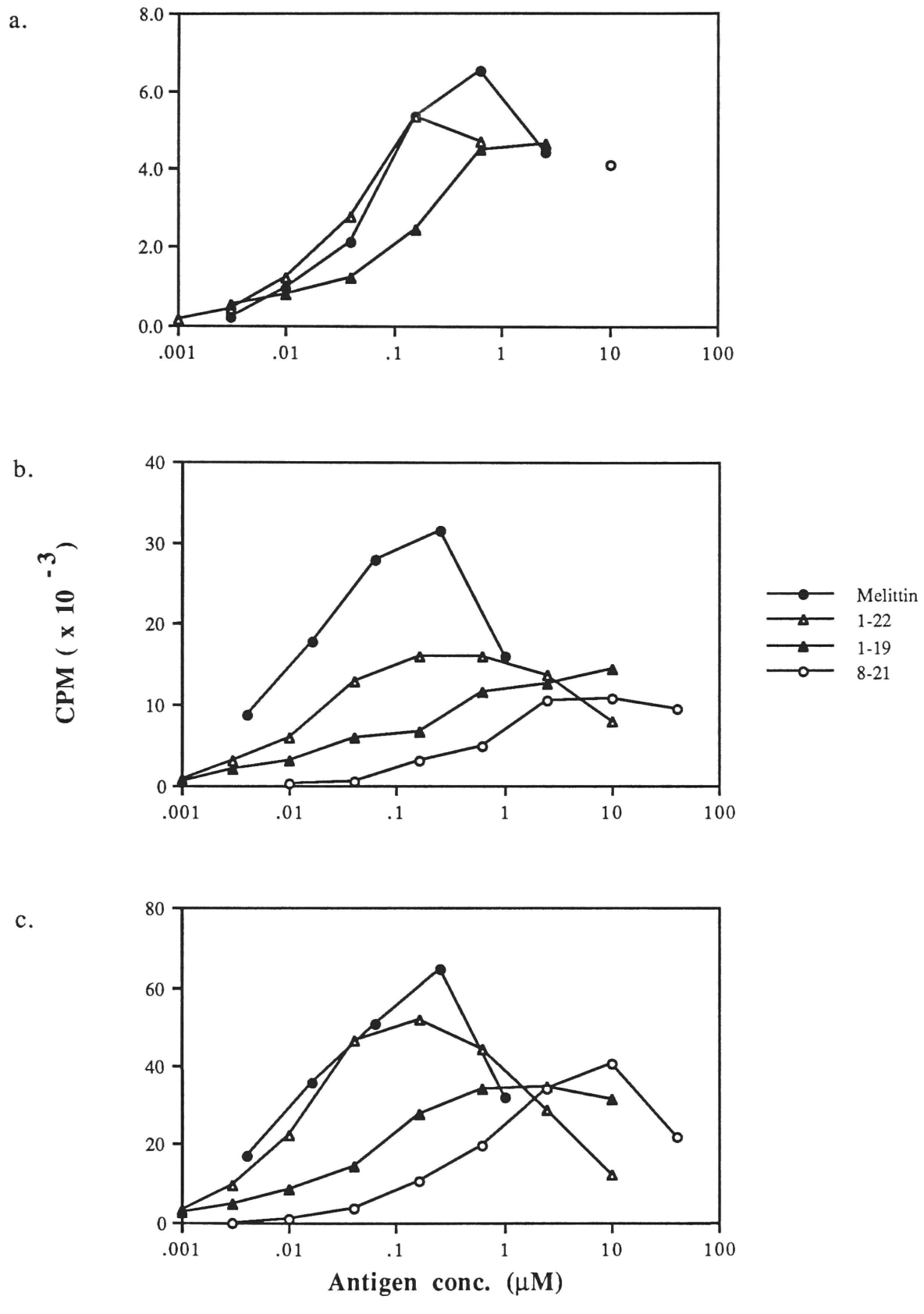


Figure 4.1. Proliferation of melittin immunized spleen cells. Melittin immunization was i.p. in CFA; the spleen was obtained 10 days later. Cultures consist of 5 x 10^5 spleen cells in 200 μl of 10CM. Background incorporation was 6900 cpm.

Figure 4.2. Proliferation of long-term T cell lines. Cell lines were maintained in culture by repeated antigen stimulation and rest. All proliferation assays used 2.5 x 10^5 fresh irradiated spleen cells in 10CM. a) Line A1, 10^4 T cells per well after four passages *in vitro*. Background = 144 cpm. b) Line R3 after 6 passages, 2 x 10^4 T cells per well. Background = 116 cpm. c) Line R4 after 6 passages, 2 x 10^4 T cells per well. Background = 150 cpm.


a.
8.0
6.0
4.0
2.0
0.0
.001
.01
.1
1
10
100
b.
CPM (x 10 -3)
40
30
20
10
0
.001
.01
.1
1
10
100
Melittin
1-22
1-19
8-21
c.
80
60
40
20
0
.001
.01
.1
1
10
100
Antigen conc. (μM)

Immunogenicity of analog peptides. BALB/c mice were immunized s.c. with peptide fragments 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 4.3, and indicate that T cells specific for fragments 7-19 and 7-19-lac can be stimulated *in vitro* with the immunogen, 1-19 or melittin with similar efficiency.

Spleen cells from mice immunized i.p. with melittin + 1-19, 1-24, 1-20-G_4Q_2 and 1-20-D_6 in alum adjuvant for the antibody studies (Chapter III) were also tested for proliferative responses *in vitro* to the immunogen and to peptide analogs. The results in Figure 4.4 show that all the peptides elicited T cell responses. *In vitro* responses of cells immunized with 1-20-G_4Q_2 and 1-20-D_6 are reduced against melittin (4.4a), indicating fine specificity for structure, since these peptides have different structural propensities from melittin. Interestingly, the response of 1-24 immunized cells is enhanced against melittin relative to the immunogen (4.4b).

Structure of T-cell epitope peptides. The results of proliferation with free and derivatized peptides 1-19 and 8-21, shown in Figure 4.5, demonstrate a significant role for peptide structure in antigenicity. The three panels represent lines A1 (a), R3 (b) and R4 (c), as in Figure 4.2. All three lines show significantly enhanced proliferation to 1-19-lac peptide relative to 1-19 and to 8-21-NAcUrea relative to 8-21, such that the response to the derivative peptide is comparable to melittin.

The most dramatic preference for derivatized peptides is evident in Figure 4.6, which shows the proliferation of a clone derived from line A1 (A1.B10) to 8-21 and two 8-21 derivatives. This clone responded equally well 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 positive charge in the derivative group. Despite the chemically dissimilar C-terminal structures of these

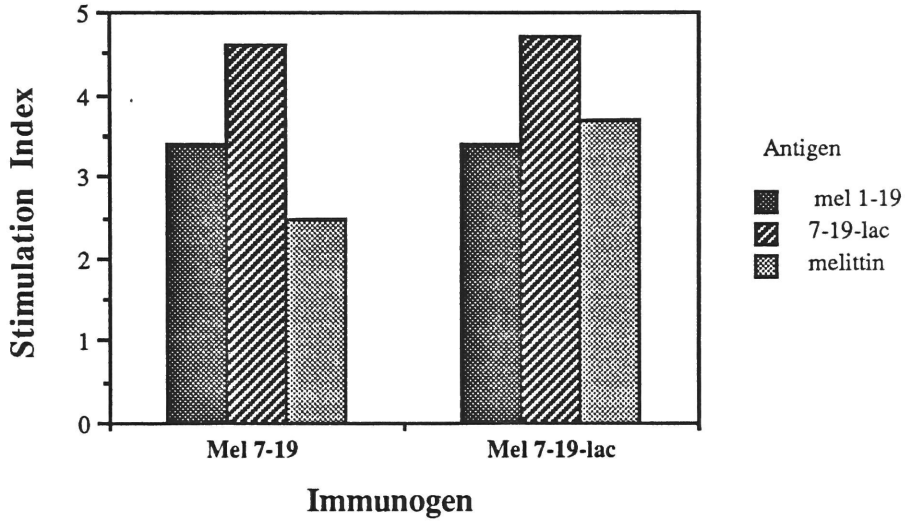
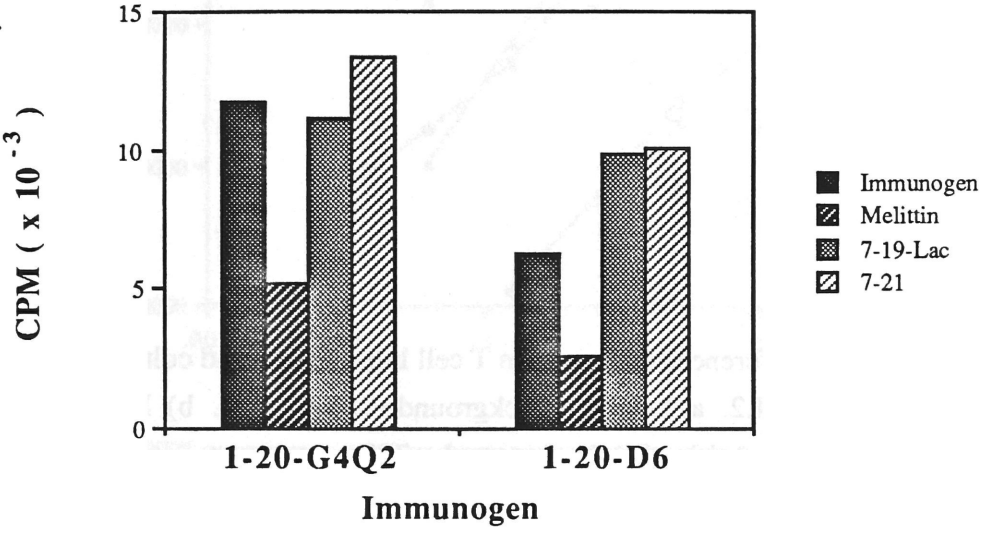


Figure 4.3. Proliferation of fragment immunized lymph node cells *in vitro*. Lymph node cells from groups of three mice immunized with 7-19 or 7-19-lac were tested for proliferation to 1-19, 7-19-lac and melittin. Cells were cultured at 5 x 10^5 cells per well in 10CM. Antigen concentration was 2.5 μM for 1-19 and 7-19-lac and 0.16 μM for melittin. These choices were made for maximum stimulation, although the low melittin dose is necessary to avoid cytolysis by the peptide. Dose response for antigens 1-19 and 7-19-lac were not tested, so no conclusions about relative antigenicity *in vitro* can be made from these data. Stimulation index = cpm with antigen / cpm without antigen; the latter varied from 1600 to 4600 cpm for the cells in this assay. Data reported are the mean SI values; S.Dev. is <20%.

Figure 4.4. Proliferation of spleen cells from peptide-immunogen immunized mice. Spleen cells were cultured at 5 x 10^5 cells per well in 1%NMSR (fetal bovine serum replaced with 1% normal mouse serum). a) Cells from mice immunized four times at two week intervals with 2 nmol of peptide in 1 mg of alum, 0.2 ml 0.05 M phosphate, pH 6.0 (see Part III, immunization procedures). Spleens were recovered 10 days after the last immunization. Antigen concentrations were 2 μM for 1-20-G_4Q_2, 0.1 μM for 1-20-D_6, 0.1 μM for melittin, and 1 μM for 7-21 and 1-19-lac. Background was about 1200 cpm. Data are representative from groups of two mice each. b) Cells from mice immunized at weeks 0, 2, 4, 6, 8 and 16 as detailed in (a) above (mice immunized with 1-20-D_6 were the other pair from the group in (a) above). Spleens were recovered 10 days after the last immunization. Data are presented as the mean stimulation index from groups of mice tested: 1-20-D6, 2 mice; 1-24, 3 mice; melittin + 1-19-lac, 2 mice. Standard deviation of the stimulation index average was <20%. Antigen concentration was 0.63 μM for all peptides tested. Background incorporation was 1350 and 2270 cpm for 1-20D6 immunized mice; 880, 5680 and 2500 cpm for 1-24 immunized mice; and 1560 and 2040 cpm for melittin + 1-19-lac immunized mice. Stimulation index was used instead of cpm because of the heterogenous values of cpm without antigen, i.e. baseline incorporation, and was determined as in Figure 4.3.

a.


15
10
5
0
CPM (x 10 -3)
1-20-G4Q2
1-20-D6
Immunogen
Immunogen
Melittin
7-19-Lac
7-21

b.

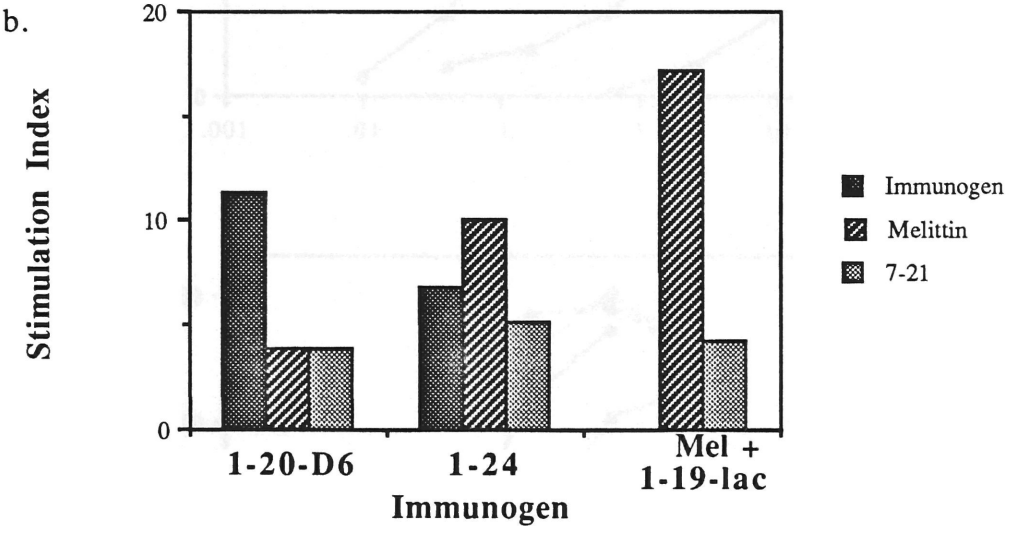
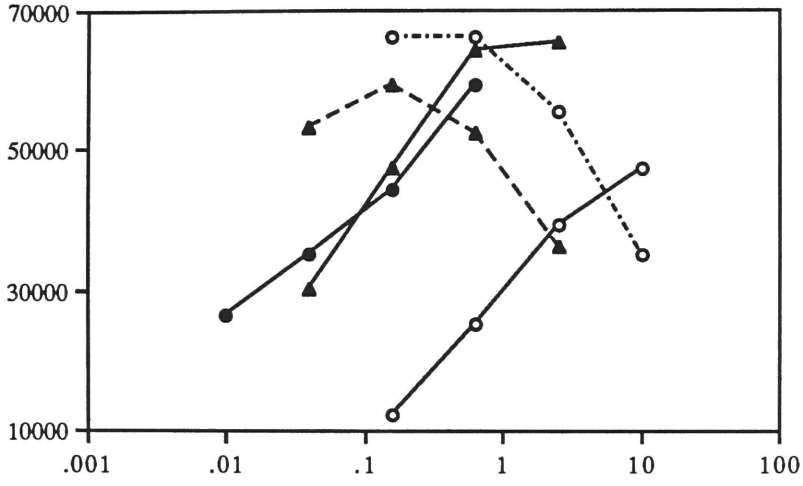
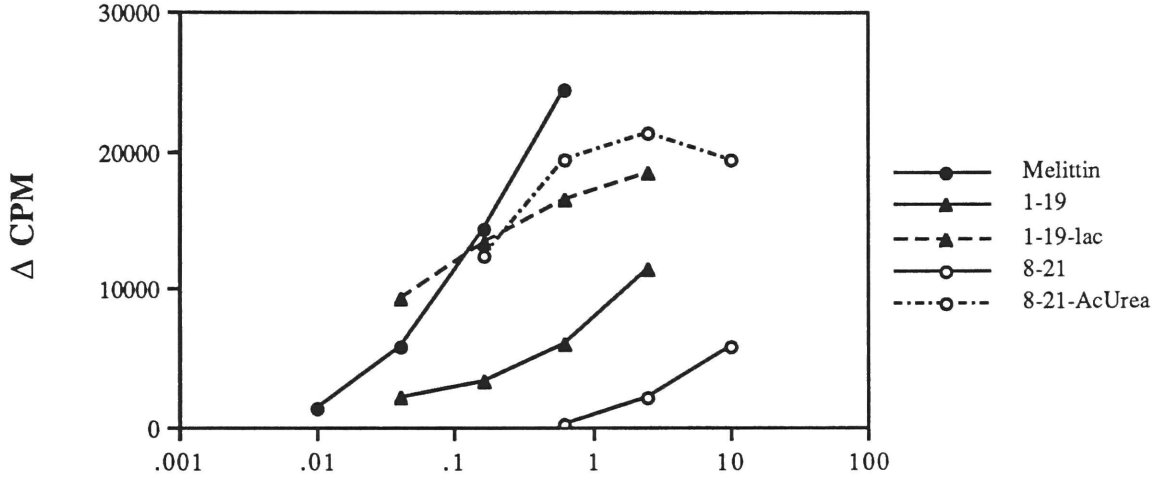

20
10
0
Stimulation Index
1-20-D6
1-24
Mel +
1-19-lac
Immunogen
Immunogen
Melittin
7-21

Figure 4.5. Structural preferences of long term T cell lines. Lines and culture conditions are the same as in Figure 4.2. a) Line A1, background = 18000 cpm. b) Line R3, background = 1640 cpm. c) Line R4, background = 700 cpm.

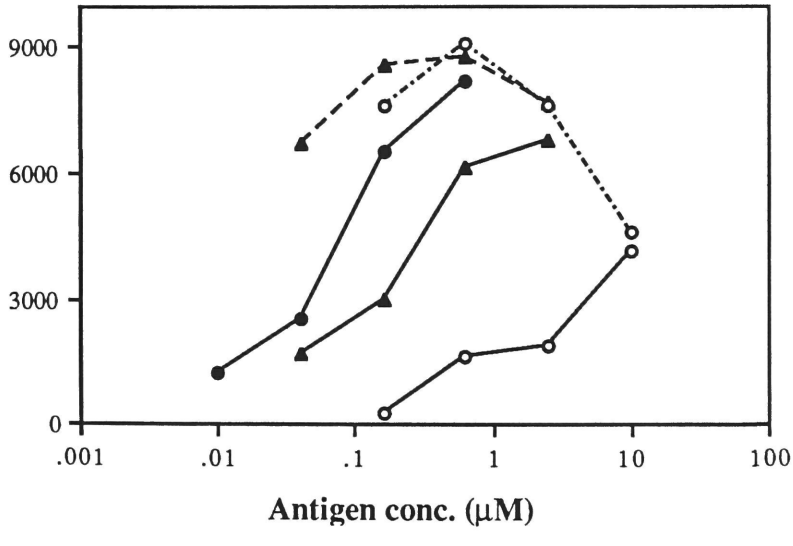
a.


70000
50000
30000
10000
.001
.01
.1
1
10
100

b.


Δ CPM
30000
20000
10000
0
.001
.01
.1
1
10
100
Melittin
1-19
1-19-lac
8-21
8-21-AcUrea

c.


9000
6000
3000
0
.001
.01
.1
1
10
100
Antigen conc. (μM)

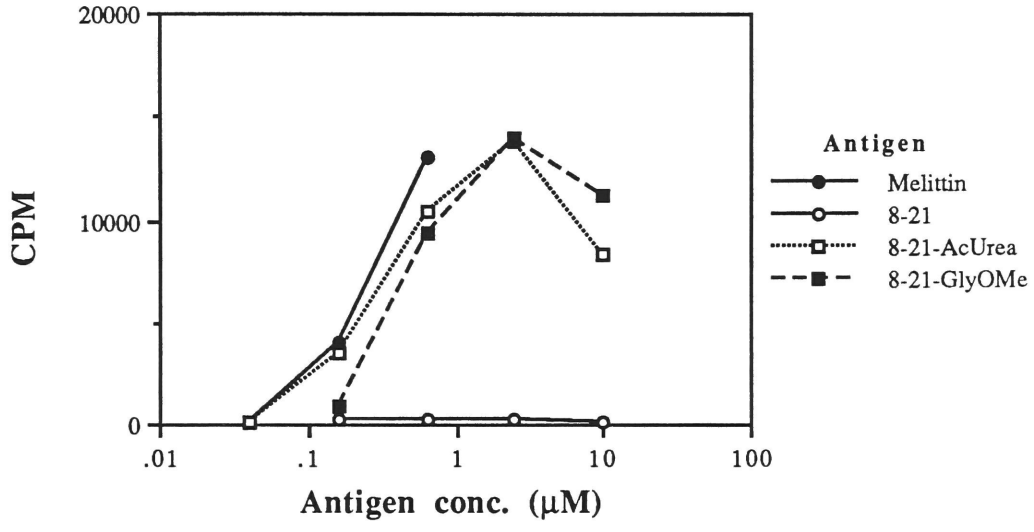


Figure 4.6. Proliferation of clone A1.B10--structure specificity. Cloned T cells from line A1 were cultured at 10^4 cells per well on 10^3 mitomycin-C treated A20 cells in the presence of melittin, 8-21 and derivatives of 8-21 (shown in key at the side of the graph) in 10R medium. Blank incorporation (1 μCi pulse) was 110 cpm.

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.

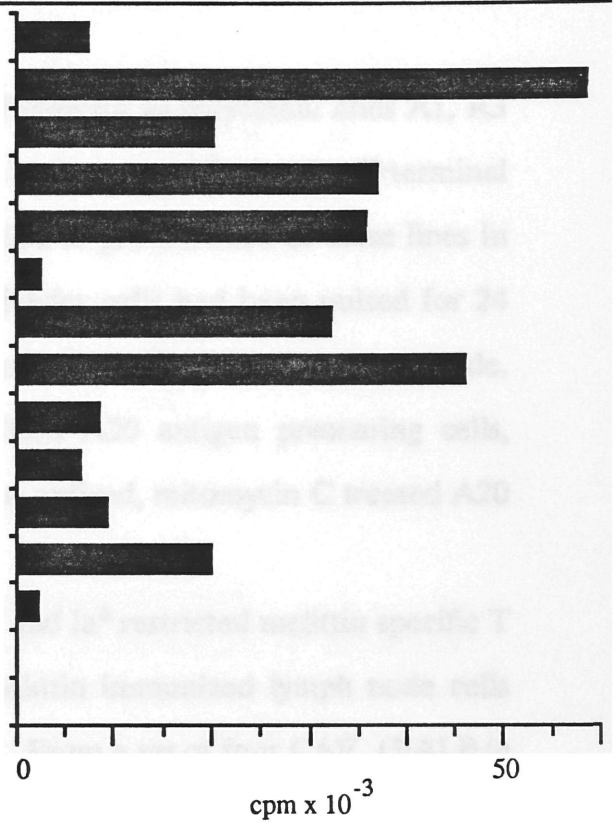
Structural changes in antigenicity of peptides are also evident in the response of T cell line A1 to the peptides represented in Table 4.1. Figure 4.7 shows the results with peptides terminating in an acetylated-Nα or carboxamide functional group. Peptides with an acetylated-N-terminus are more antigenic than their free counterparts; a similar, weaker effect is seen with blocked C-terminal groups with these peptides.

A series of peptides with single amino acid substitutions at Pro14 were found to have varying degrees of antigenicity *in vitro*, again shown in Figure 4.7. These substitutions were made for maximum changes to peptide primary and secondary structure; all substitutions significantly decreased peptide antigenicity *in vitro*. Substitution with lysine, a basic amino acid, in peptide 8 (and another peptide with only position 14 substituted, not shown) abolished antigenicity, but peptide 2, with aspartic acid, an acidic amino acid, and peptide 3 with a polar asparagine were antigenic. Hydrophobic substitution, such as with leucine (peptide 4) and the aromatic amino acid phenylalanine (peptide 5), retained antigenicity. Interestingly, substitution with D-phenylalanine (peptide 6) did not destroy activity, although molecular modelling suggested that the resulting structure would be very different from the native peptide (R. Berg, personal communication). Finally, peptide 7, substituted with leucine at position 12 and glutamine at 14, is predicted and found to have enhanced α-helical propensity, but was not antigenic.

Percent α-helix structure of some peptides in dilute aqueous and organic (HFP) solvent were obtained from analysis of CD spectra (Figure 4.7). No strong correlation of secondary structural propensity and T-cell antigenicity is observed. Peptide 4, with α-helical character in aqueous buffer, was antigenic, but peptides 7

Figure 4.7. Structural influence on peptide immunogenicity for T cell responses *in vitro*.

Peptide	% α-Helix Structure PBS	HFP	Proliferation
10-21-CONH	0	75	
Ac-10-21-CONH	0	100	
10-21	nd	nd	
Ac-10-21	0	20	
Ac-11-21-CONH	nd	nd	
11-21	nd	nd	
Ac-11-21	nd	nd	
1 (7-21)	0	33	
2 (D-14)	0	33	
3 (N-14)	0	50	
4 (L-14)	12	33	
5 (F-14)	0	28	
6 ([D]-F-14)	0	83	
7 (L-12, Q-14)	9	64	
8 (L-12, K-14)	7	33	



A1 cells were cultured at 2 x 10^4 per well with 2.5 x 10^5 irradiated spleen cells in 10R. Both A1 cells and irradiated spleen cells were recovered from cryopreservation for use in this assay. Peptide concentration is 10 μM. Cells were pulsed with 1 μCi of ^{3}H-thymidine; background incorporation was 350 cpm. Secondary structure was determined from analysis of CD spectra of 10 μM peptide in PBS or 20% HFP, 80% PBS by the "prosec" program (AVIV 62DS).

and 8, also with α-helix structure in aqueous solution, were not. Primary structure considerations are more important, as shown by different antigenicities of peptides 1, 2 and 3. These have very similar conformational propensities in PBS and HFP, including β-sheet structure (data not shown), yet only peptide 1 is a strong T cell antigen.

Processing of melittin. Presentation of melittin to polyclonal lines A1, R3 and R4 could be inhibited in the presence of MAb 4, specific for the C-terminal region of melittin. Figure 4.8 shows the inhibition of proliferation of these lines in various concentrations of MAb 4. Irradiated feeder cells had been pulsed for 24 hours with melittin, and were washed to remove any remaining soluble peptide. Melittin was also presented to line A1 on fixed A20 antigen presenting cells, although with decreased effectiveness relative to unfixed, mitomycin C treated A20 cells, shown in Figure 4.9.

T cell frequency. The frequencies of Ia^d and Ia^a restricted melittin specific T cell clones derived from primary culture of melittin immunized lymph node cells were approximated by limiting dilution analysis. From a set of four CAF_1 (BALB/c x A/J F_1) mice, 6.5±1.3 out of 24 wells were positive (frequency = 0.27) when seeded with 10^4 lymphocytes per well on BALB/c ($H\text{-}2^d$) feeder cells. By application of Poisson statistics (as in 55), one gets an approximate value of 1 responding cell per 3×10^4 lymph node cells. Only 1.5±1.7 out of 24 wells seeded with 10^4 cells per well were positive (frequency = 0.063) when cells were stimulated on A/J ($H\text{-}2^a$) feeder cells. This corresponds to 1 responding T cell per 1.5×10^5 lymph node cells.

Limiting dilution analysis of DBA/2 immunized lymph node cells gave similar results for the frequency of Ia^d restricted T cells: 1 per 5×10^4 lymph node cells. This somewhat more precise value was determined graphically from a least squares fit (Figure 4.10). Frequency of responding cells was calculated by

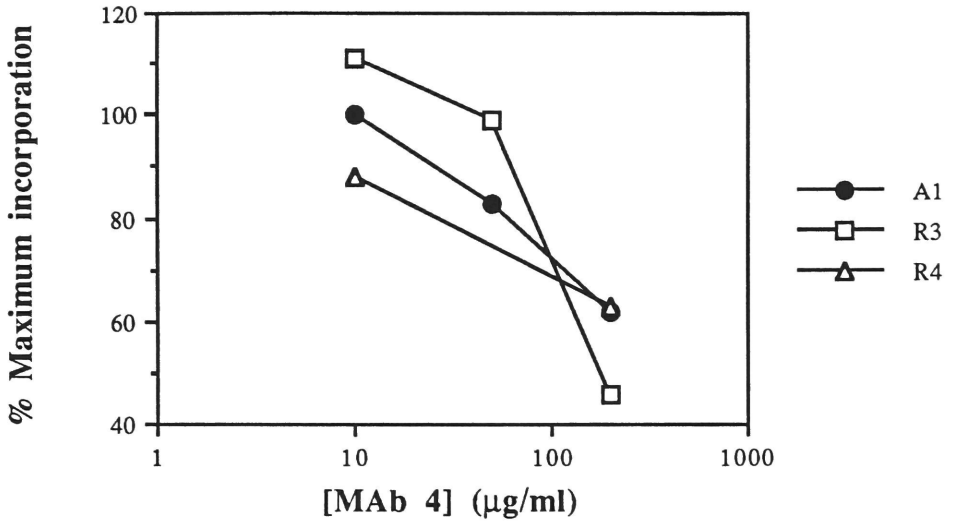


Figure 4.8. MAb 4 specific for melittin inhibits proliferation of melittin-specific T cell lines. Long term melittin specific T cells were cultured at 10^4 per well on 2.5 x 10^5 irradiated spleen cells (BALB/c). The spleen cells had been pulsed for 24 hr with 0.1 μM melittin and washed twice. 100% incorporation was 52,500 cpm for line A1, 9300 cpm for line R3, and 4000 cpm for line R4. Background incorporation was 3600 cpm, 600 cpm and 390 cpm, respectively.

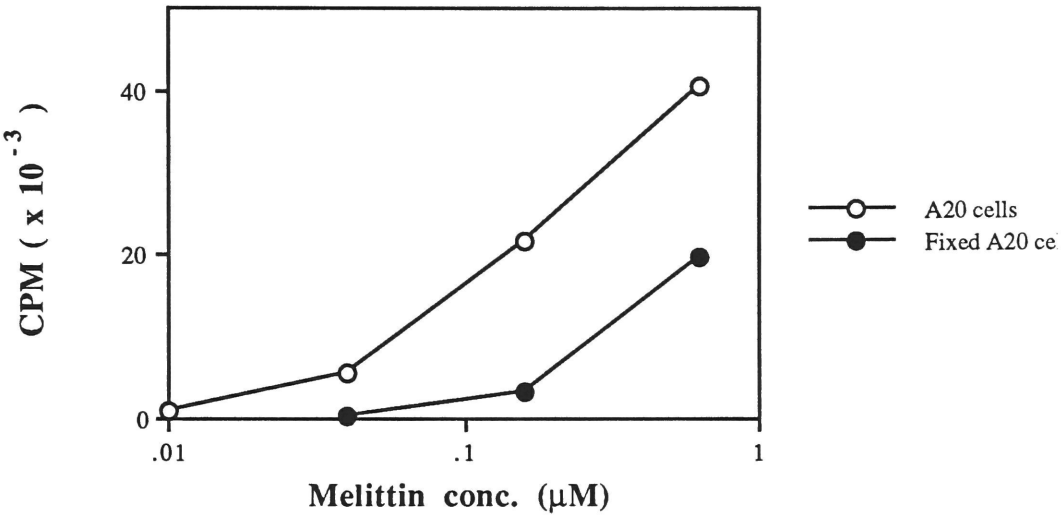


Figure 4.9. Proliferation on fixed APC. A20 cells, 10^3 per well, were glutaraldehyde fixed (closed symbol) or mitomycin-C treated (open symbol). A1 cells, 2 x 10^4 cell per well, were added along with melittin. Background count was 300 cpm. Cells were cultured in 10R medium and pulsed with 1 μCi of ^{3}H-thymidine.

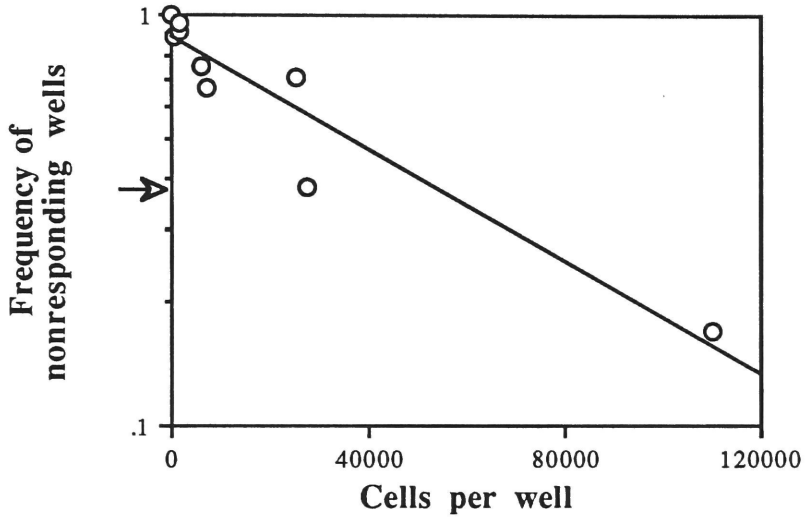


Figure 4.10. Limiting dilution analysis for melittin-specific T cell frequency. Two DBA/2 mice were immunized s.c. in the base of the tail, and draining lymph node cells were cloned under limiting dilution conditions. The frequency of nonresponding wells was plotted as a function of total cell density per well, and a best fit made by least squares analysis. From Poisson statistics, a nonresponding frequency of 0.37 (arrow) corresponds to one cell per well. The frequency of responding T cells was determined graphically at this value.

extrapolation at the nonresponding fraction of 0.37, which according to Poisson statistics corresponds to one responding cell per well.

Phenotype of T cell clones. CAF_1 derived T cell clones were harvested four days after restimulation on Ficoll-Paque for direct and indirect immunofluorescence. Expression of CD4 (L3T4) and CD8 (Lyt2), the MHC class II (helper) and class I (cytotoxic) T cell markers, respectively, and CD45 and CD45R was assayed. TIB122 recognizes CD45 (T200), found on all $CD3^+$ T cells, and MB23G2 recognizes a CD45R (R for restricted) isoform that is expressed on primary T cells (64) and on long term IL-4 secreting T cell clones (63). As shown in Table 4.4, all clones were positive for CD4 and negative for CD8 in FACS analysis. The cells also generally showed medium to high expression of CD45R relative to CD45, and very high levels of CD45R relative to the control. The DBA/2 derived T cell clones tested (6/14; the remainder were lost for unknown reasons) were positive for CD4 and not CD8, and showed high levels of expression of CD45 and CD45R (data not shown).

After four passages in culture supplemented with RCM and two passages without exogenous growth factors, supernatants from 11 cloned lines stimulated with antigen on irradiated feeder cells for four days were tested by the B cell costimulation assay for IL-4 activity, and for IL-4 and IL-2 activity by assaying for growth of CTLL cells in the presence and absence of MAb's 11B11 (anti-IL-4) and S4B6 (anti-IL-2). The results in Table 4.5 are not clear-cut with regard to lymphokine secretion. The B cell costimulation assay indicates that all the clones but M2.A9 and R4.A2 have activity that is inhibited by 11B11. Yet many of the T cell supernatants also have activity that is inhibited by S4B6, or both S4B6 and 11B11, in the CTLL assay. Clearly line R4.A2 is making IL-2 and has no IL-4 activity. Clones M2.B8 ($H\text{-}2^a$ restricted), M2.B4 and M2.B12 show very high levels of IL-2 activity in the CTLL assay, but also have some IL-4 activity in the B cell

Table 4.4. T-cell clone phenotyping by FACS.

	Mean Fluorescence Intensity (arbitrary units)				
		direct [2]		indirect [3]	
Clone	control [1]	L3T4	Lyt2	CD45	CD45R [4]
a.M2.B8	5.1	74	5.3	960	160
d.L1.B9	2.7	1500	nd	270	260
B12	9.2	1400	6.1	960	960
d.L2.A10	9.4	630	8.5	830	620
A11	8.0	1200	6.6	670	710
B7	9.9	1600	7.9	690	640
d.M1.A8	7.6	1200	6.2	1100	nd
B8	11	1000	9.3	910	750
d.M2.A9	8.1	1200	7.1	750	590
B4	7.8	1100	7.2	730	410
B5	5.3	170	9.3	310	110
B12	7.7	330	10	240	50
R4.A2	14	563	8.5	58	39
A1.H11	8.9	1100	nd	80	90

1. The negative control was FITC-MAR; the results are the same with unlabelled cells.

2. L3T4 (= CD4) was labelled with PE, so results are for channel 2 fluorescence intensity; Lyt2 (= CD8) was labelled with FITC, so results are for channel 1 fluorescence.

3. 100% of gated cells were CD4+.

4. Expression of CD45R (MB23G2) increased with stimulation of this cell line. Mean fluorescence intensity of resting T cells before stimulation was about 30 units. No other lines were tested for changes is the level of CD45R expression.

Table 4.5. Lymphokine production of melittin-specific T cell clones.

	Antigen stimulated day 4 supernatants						
	B cell costimulation assay (IL-4)[1]			CTLL assay (Il-2 and IL-4)[2]			
Clone	+ α-Ig	+ α-Ig + 11b11	S.N. only	S.N. only	+ S4B6	+11B11	+ S4B6 + 11B11
M2.B8	2558	618	1222	4840	1502	5000	1182
L1.B9	2197	1398	2208	1311	961	814	722
L1.B12	3134	1069	896	827	688	503	420
L2.A10	1417	778	592	569	385	554	335
L2.A11	2975	1938	1696	910	733	851	576
L2.B7	2409	1863	1581	1391	1011	1392	994
M1.A8	4112	1263	899	1250	1028	922	615
M1.B8	2505	1691	873	1118	1004	1238	850
M2.A9	807	759	468	1028	1096	1068	815
M2.B4	3018	726	1730	1451	962	1376	827
M2.B5	2229	855	1317	1224	1080	1008	820
M2.B12	1891	835	1626	2345	1230	2306	1115
R4.A2	2240	1885	1975	10963	6166	10450	7666
A1.B10	110002	39364	28780	11427	9809	11985	10841
A1.H11	4185	1349	2254	s.n. had no activity			
rMu-IL-2 (5 U/ml)				10439	8257	11148	9751
rMu-IL-4 (250 U/ml)				1859	1881	627	633

1. The B cell costimulation assay consisted of 10^3 small resting B cells per well with 25% T cell supernatant; α-Ig is anti-Ig-coated Sepharose; 11B11 is the anti-IL-4 MAb; S.N. denotes supernatant. B cells incorporated 20,000 cpm in a 1.5% EL-4 supernatant; background incorporation was 650 cpm.

2. The CTLL assay used 5 x 10^3 CTLL cells stimulated 48 prior to use and washed once with 10R; S4B6 is the anti-IL-2 MAb. Background incorporation was 730 cpm.

Both assays were run in 10R medium, and cells were pulsed with 1 μCi of ^{3}H-thymidine.

assay. The other clones were either very weak or showed low levels of both IL-2 and IL-4 activity.

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 4.6. Ia restriction was determined by inhibition analysis with anti-I-A^d and anti-I-E^d specific MAb's. About half the cells are found to be restricted to I-A and half to I-E. In a concurrent proliferation assay, these cells were also found to have different sensitivities for stimulation by melittin. The I-A^d restricted clones require a lower concentration for 1/2 maximal melittin stimulation, 0.17±0.10 μM (n = 4), than I-E^d restricted clones, 0.36±0.13 μM (n = 4). These differences were more dramatic in other proliferation assays (see Figure 4.11; other data not shown). This apparent difference in melittin binding affinity for the respective Ia molecules was also 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.

Similar differences in sensitivity to melittin stimulation in a proliferation assay were observed with DBA/2 derived clones. Proliferation at low melittin concentration is nearly maximal for the more sensitive clones but is reduced for the less sensitive clones. As with the CAF_1 mice, these clones divided evenly for sensitivity to melittin concentration, with 7/14 responding maximally to low concentrations of melittin and 7/14 responding maximally to high concentration. This suggests a similar distribution of Ia restriction to I-A^d (response to low concentration) and I-E^d (high concentration) in DBA/2 mice.

Two representative clones were selected from the CAF_1 experiment: one, L2.B7, restricted to I-A^d and one, M1.B8, restricted to I-E^d. These clones were sensitive to inhibition by as little as 1.56% of the appropriate Ia-specific MAb

Table 4.6. Ia restriction, melittin sensitivity and epitope 7-21 recognition of T cell clones.

Clone	% inhibition in the presence[1] of anti- I-A^d	I-E^d	I-A^{k}[2]	[Melittin] 1/2 max. response (μM)	Response to 7-21[3]
L1.B9	11	**100**	37	2.5	nd
L1.B12	40	**100**	36	0.5	-
L2.A10	67	**100**	nd	2.5	nd
L2.A11	**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	0.18	+
M2.B12	42	**100**	52	0.40	nd
R4.A2	**100**	57	83	nd	nd
A1.B10	**100**	32	45	nd	nd
A1.H11	**100**	27	34	0.15	+

T cell clones, 2 x 10^4 cells per well, were stimulated on 5 x 10^5 irradiated CAF_1 spleen cells in the presence of 0.1μM melittin with or without anti-Ia antibodies. Antibodies were in culture fluid from the appropriate hybridoma: B21.2 fo anti-I-A^d; HB.32 for anti-I-E^d; and 10.216 for anti--I-A^k; the culture fluid concentration was 25%.

1. Percent inhibition is calculated as follows: $\frac{(CPM_{+anti\text{-}Ia} - CPM_{background})}{(CPM_{Melitttin} - CPM_{background})} \times 100$

Bold type indicates significant inhibition relative to the anti-I-A control, i.e. Ia restriction.

2. Anti-I-A^k is included as a control for non-specific effects of binding Ia molecules to the antigen presenting cells.

3. Peptide 7-21 was prepared synthetically by R.B.

culture fluid supernatant but not to 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 4.11. Note that much higher antigen concentrations were required for the same dose-response of clone M1.B8. Clone L2.B7 proliferated in response to all peptides tested, which were the peptides used in the immunogenicity study in Chapter III. Clone M1.B8, however, 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, indicating that I-E^d cannot present peptides 1-20-G_4Q_2 and 1-20-D_6, and has a poor interaction with 1-19-lac.

Class II restriction of peptide immunized spleen cells. To further test the apparent selectivity for peptide structure exhibited by I-E^d, spleen cells from BALB/c mice immunized with melittin + 1-19-lac (essentially melittin immunized), 1-24 and 1-20-D_6 were tested for Ia restriction *in vitro*. The results of this assay are presented in Table 4.7. Cells from mice immunized with 1-20-D_6 did not proliferate as strongly in the presence of melittin and 7-21 (see Figure 4.4a); these cells were inhibited from proliferation by anti-I-A^d but not by anti-I-E^d. Spleen cells specific for 1-24 had a heteroclitic response to melittin and also proliferated well in the presence of 7-21 (see Figure 4.4b). These cells were also preferentially inhibited by anti-I-A^d. The spleen cells immunized with melittin + 1-19-lac responded strongly to melittin, but only weakly to 7-21 (see Figure 4.4c; in contrast to the results with the long term line A1, Figure 4.7). These cells were inhibited more strongly by anti-I-E^d than by anti-I-A^d. However, when challenged only with peptide 7-21, the responding cells were inhibited from proliferation primarily by anti-I-A^d. Thus the response to 7-21 by melittin immunized cells is limited to those T cells restricted to I-A^d.

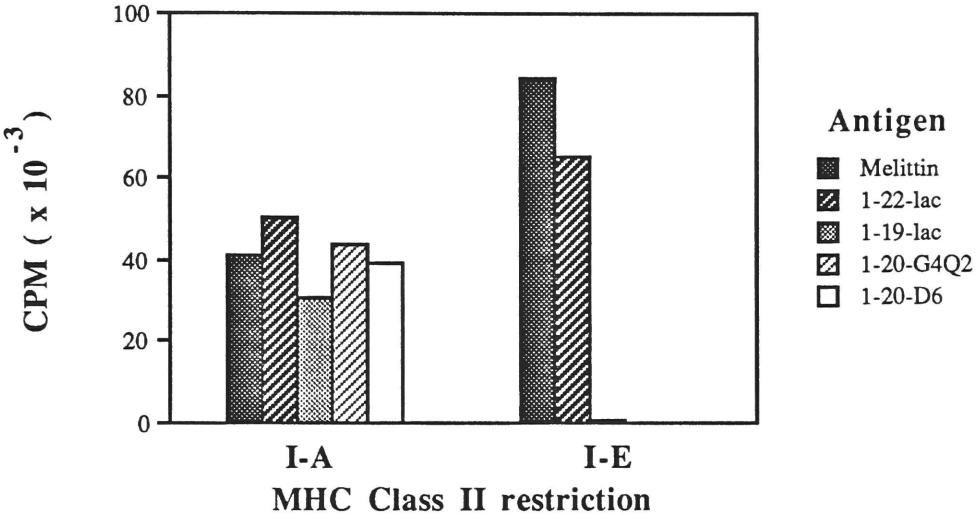


Figure 4.11. Proliferation of I-A and I-E restricted clones to immunogenic peptides. Proliferation of 2 x 10^4 T cells on 5 x 10^5 fresh irradiated BALB/c spleen cells in 10 R medium, 0.5 μCi pulse. Antigen concentration was 0.32 μM for L2.B7 (I-A restricted clone), and 1.25 μCi for M1.B8 (I-E restricted clone); background was 105 and 103 cpm, respectively.

Table 4.7. Primary immunized spleen cell Ia restriction.

Immunogen	No. of mice	Antigen (*in vitro*)	% inhibition in the presence of anti- I-A^d	I-E^d
Melittin + 1-19-lac	2	Melittin	16.5	**46.5**
		7-21	**89**	4
1-24	3	1-24	**71**	2.3
1-20-D6	2	1-20-D6	**96.5**	14.5

Spleen cells from mice immunized 6 times (weeks 0, 2, 4, 6, 8 and 16) with the immunogen in alum were tested for Ia restriction. Cells were cultured at 5 x 10^5 cells per well in 1%NMSR. Inhibition data are for 25% hybridoma culture fluid supernatants of B21.2 (anti-I-A^d) and HB.32 (anti-I-E^d). Antigen concentration was 0.16 μM.

DISCUSSION

Structure of the T-cell epitope. Polyclonal melittin specific T cell lines show preference for T-cell epitopes with fine structural variations. In general, these consisted of peptides with blocked C- or N-terminal groups. Blocking the terminal functional group of a peptide theoretically increases helical potential since free carboxyl and amino groups have unfavorable charge interactions with the helix dipole (31). This structural correlate is best observed experimentally in comparison of CD spectra of peptides and peptide-lactoside derivatives (Figure 2.6). Correlation of structure and antigenicity of the synthetic peptide epitope analogs in Figure 4.7 clearly indicates a role for primary sequence, since enhanced helical propensity could not overcome the effects of an unsuitable amino acid.

Overall preference for epitope fragments with amphiphilic α-helical propensity supports the model for T-cell epitopes advanced by Berzofsky and his colleagues (65). Helical propensity seems to be especially important for restriction to I-E^d, as shown by the inability of peptides with low α-helical structure or propensity, such as 1-19-lac, 1-20-G_4Q_2 and 1-20-D_6 (Figure 2.6), to be presented *in vivo* or *in vitro* on the I-E^d molecule. This Ia molecule in general appears to recognize a limited structural repertoire since a higher concentration of antigen is required for T cells restricted by I-E^d than by I-A^d.

Efforts by Berzofsky and his colleagues to characterize general secondary structure requirements for T-cell epitopes based on amphiphilic considerations have partially succeeded, so that frequently T-cell epitopes predicted by these methods prove to be antigenic (40), as was the case for Pf155-RESA from merozoite stage *Plasmodium falciparum* (66). Rothbard and his colleagues also defined a 4-5 amino acid T-cell epitope consensus sequence with this pattern: charged amino acid or glycine in the 4 amino acid motif, two hydrophobic amino acids, then polar or glycine in the 4 amino acid motif, or hydrophobic or proline and polar or glycine in

the 5 amino acid motif (67). Using this pattern, which was developed by analysis of a number of known T-cell epitopes, a series of epitopes were accurately predicted (68). Many epitopes fit both algorithms, including melittin, which has amphiphilic α-helical structure and the putative consensus of charged (Lys7), two hydrophobic (Val8, Leu9), and one polar (Thr10) amino acids.

Algorithms fail to predict all possible T-cell epitopes, however (40,68). Experiments to identify T-cell epitopes of a single antigen 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, and though most of the identified epitopes correlated with one or the other algorithm, some correlated with neither (69). A recent study found no correlation of experimental helical propensity with antigenicity of epitope peptides from pigeon and moth cytochrome-C (70). 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.

The efforts reported here to correlate secondary structure and antigenicity for specific T cells demonstrate the importance of primary structure, and only partial importance of secondary structure. Nevertheless, 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 1-10 fragments epitope of myelin basic protein (MBP) that acted as an heteroclitic antigen for MBP-specific T cells *in vitro* was found to inhibit induction of experimental autoimmune encephalomyelitis (EAE) *in vivo* (71). Thus it will be useful 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.

Similarity to other I-A^d restricted epitopes. Melittin shares some structural similarity with other I-A^d restricted epitopes: lambda repressor (λ cI) 12-24, *Staphylococcus aureus* nuclease (Nase) 61-78, and chicken ovalbumin (cOVA) 324-336 (72). The sequences of these peptides are shown in Table 4.8. Bold-type residues represent amino acids in contact with the TcR, as shown by the ability of Nase and cOVA analogs substituted in other positions with the appropriate amino acids from the λ cI sequence to stimulate λ cI-specific T cells (72). Presumably other amino acids are involved in binding to the I-A^d molecule.

Two possible alignments of melittin suggest themselves to maximize similarity to the other I-A^d restricted fragments (boxed amino acids). Residue 2-15 of melittin would include Lys7 at a position where a conserved cationic residue is found in the other three peptides. Also Leu6 and Leu9 are consistent with hydrocarbon side chains at those positions in the other epitopes. Ile2 and Val5 are similar to amino acids in cOVA at those positions. However, fragments 7-19-lac and 7-21 represent the I-A^d restricted T-cell epitope of melittin. The best line-up for this segment is with melittin 9-21, thereby matching semi-conserved amino acids Leu9, Thr10, Thr11, Ala15, Ile17, and aromatic Trp19. Val8 of melittin and Ile323 of cOVA are also conserved. This proposed melittin epitope lacks a basic amino acid at the comparable position from the other epitopes, which is interesting because a melittin analog with lysine in proximity to that putative basic position was not antigenic *in vitro*, but an analog with aspartic acid in that position was antigenic (Figure 4.7).

Melittin processing requirements. Two experiments were attempted to determine the extent and necessity of melittin processing. Inhibition of proliferation by MAb 4, specific for the C-terminal segment of melittin, indicates that a significant amount of presented melittin retains the C-terminal region . Previous studies have shown that peptide epitopes are accessible to monoclonal and

Table 4.8. I-A^d restricted peptides.

Antigen source		Epitope sequence
λ cI	12-26	L E D A R R L **K** A **I** Y E **K**
S. aureous nuclease	66-80	V E N A K K I **E** V **E** F D **K**
cOVA	324-336	S Q A V H A A **H** A **E** I N **E**
Melittin	3-15	G A V L K V L T T G L P A
	9-21	L T T G L P A L I S W I K

Boldface type indicates putative TcR contact amino acids; residues inside boxes represent conservative amino acid substitutions, possibly indicative of Ia contacts involving general hydrophobic, hydrophilic or ionic interactions. Sequences of I-A^d restricted T3-cell epitopes and TcR contact residues taken from 72.

polyclonal antibodies (73-75) and other macromolecules (76), and that binding can block 90-100% of proliferation, presumably by interfering with antigen presentation at the level of MHC presentation to the TcR. As with general hydrophobic interactions, melittin's N-terminal segment appears to bind to MHC while the C-terminus is free. Binding of MAb 4 to melittin would interfere with recognition of antigen by the TcR due to steric hindrance. Since the amount of MAb for roughly 60% inhibition represented a 10-fold higher concentration than that of melittin, presumably either binding of antibody to MHC-associated melittin is not favored or melittin is partially processed intracellularly, so that some melittin T-cell epitopes do not include the MAb epitope. Partial processing may depend on Ia restriction. The results with fixed antigen presenting cells also suggest that processing is not required given that these cells act to stimulate T cell proliferation. Decreased dose response and maximum stimulation on fixed cells may be due to an effect of fixation.

Both of these assays, while providing supporting data, suffer from a lack of appropriate controls. The effect of MAb 4 on presentation of other Ia^d restricted epitopes to antigen specific T cells was not determined, so the MAb may inhibit non-specifically. For presentation on fixed cells, the extent of fixation was not determined by some independent means, although the lack of proliferation by the cells, which were not otherwise treated with a mitotic inhibitor, suggests that fixation occurred.

Perhaps the best evidence for non-C-terminal processing of melittin comes from analysis of the *in vitro* antigenicity of melittin analogs that are varied at the C-terminus. For C-terminal structural differences to play a role in the observed antigenicity of these analogs, which presumably occurs at the level of recognition by MHC or the TcR after processing, the C-terminal composition must remain different from melittin i.e. the C-terminus of analog peptides must be intact. These

results do not preclude cleavage in the N-terminus of the peptide, i.e. positions 1-6, since peptides like 7-21 can have comparable activity to melittin. However, cleavage after Lys7 is not indicated since we observed that fragment 7-21 was more antigenic than 8-21.

It is well established that in the absence of cell processing, chemically denatured or proteolytically cleaved cOVA peptides are presented to cOVA-specific T-cell hybridomas (57,77). As a relatively small peptide, melittin may represent essentially a processed epitope so further processing may not be required.

T cell frequency and phenotype. A frequency of 1 in 3-5 x 10^4 lymphocytes specific for melittin may be low. In a recent study, the frequency of responding cells in an immunodominant response was 1 in 475 (55). However, these T cells were enriched by passage through nylon wool and stimulated for one passage in a bulk culture prior to restimulation and cloning. Thus, it is reasonable that under those conditions the frequency of responding cells increased by at least 20 to 50-fold. Adjusting for this possibility, a frequency of one responder per 3-5 x 10^5 lymphocytes reported here would correspond to 1 antigen-specific T cell per 1000 lymphocytes, well within the order of magnitude of the earlier study. The low frequency of Ia^a restricted T cells indicates that A/J mice are poor melittin-specific responders because the T-cell response is limited.

High expression of CD45R as detected by MB23G2 was found to correlate with T_H2 phenotype in a series of clones (63), and further studies with a very large number of tested clones confirm this observation (Marian Birkeland, personal communication). Later studies suggested that expression of this T cell antigen is high in a primary immune response, and decreases with antigen stimulation (64). Thus MB23G2 may identify an antigen more akin to OX-22, the rat T cell antigen associated with primary responses. Expression of OX-22 is diminished in T cells that help B cells and are active in secondary immune responses (78). These

conflicting observations regarding MB23G2 expression on T cell clones and primary polyclonal populations imply that high levels of expression of CD45R as detected by MB23G2 do not definitively establish phenotype *in vivo*.

Analysis of lymphokine profiles fails to shed much more light on the question of T_H1 versus T_H2 phenotype. CTLL cells have a stronger proliferative response to IL-2 than to IL-4, so weak stimulation does not preclude IL-4 activity in the supernatant. However, frequently this activity was poorly inhibited by 11B11, which completely inhibited 250 U/ml of rMu-IL-4 (Table 4.5). No cell line is specific for only IL-4 secretion in the CTLL assay. Most supernatants show very low levels of lymphokine, possibly due to autologous use of the products as growth factors, since these clones were proliferating without exogenous lymphokines. Nevertheless, clones M2.B8 (Ia^a restricted), M2.B4, M2.B12 and A1.B10 appear to make both lymphokines, since they show IL-4 activity in the B cell costimulation assay and high IL-2 activity in the CTLL assay. Cell lines L1.B9, L1.B12, L2.A11, M1.A8, M1.B8, and M2.B5 appear to make both lymphokines because both S4B6, anti-IL-2, and 11B11, anti-IL-4, inhibit partially alone and additively in the CTLL assay. As stated above, R4.A2 clearly produces IL-2; line A1.H11 may produce only IL-4.

One possible explanation for dual lymphokine production would be incomplete cloning, and these lines were not sub-cloned after the limiting dilution cloning from primary culture. However, in other regards, such as Ia restriction and epitope specificity (Table 4.6), these clones behave clonally. Previous workers have identified a $CD4^+$ subset with an unrestricted cytokine profile (79), so the observation of this activity is not without precedent.

Correlation of Ia restriction and immune course. These results will be discussed in Chapter V.

Chapter V.

Honeybee-venom melittin:

a model immunogenic peptide and allergen.

Peptide immunogens.

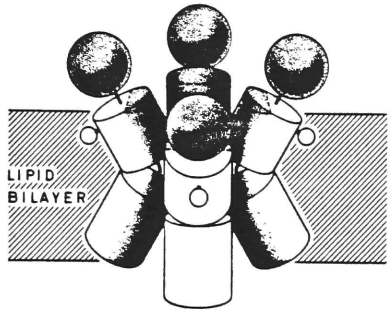
The following characteristics of melittin may apply to peptide immunogens in general: linear amphiphilicity; an N- or C-terminal positive charge; sufficient size (40 Angstroms) to interact with lipid bilayers (about 30 Angstroms) and bind antibody (about 9 Angstroms); an appropriate T-cell epitope. The properties of melittin resulting in its unique structure-activity relationship and its high immunogenicity could conceivably be incorporated as properties of synthetic peptide immunogens. Amphiphilic properties related to the immunogenicity of melittin in this thesis have been applied generally to the design of peptides with predictable behavior (26,80).

It is important to stress that the antibody response to melittin as tested in the murine system is comparable to the response to protein immunogens such as bee-venom phospholipase-A_2 (T.P. King, personal communication) and not to the weak response frequently seen to peptides (for example, apamin in 32). Melittin-specific antibody titers are also an order of magnitude greater than those obtained against foot-and-mouth peptide immunogens prepared by joining the foot-and-mouth B-cell epitope to a foreign (i.e. non-viral) T-cell epitope (81). Melittin immunization also results in high antibody responses despite the use of alum instead of CFA as the adjuvant, whereas other peptide-specific antibody responses are found with immunization in CFA 32, (81,82). Melittin-specific antibody responses can result from immunization with syngeneic, melittin pulsed spleen cells (T.P. King, personal communication). Also, different hapten molecules, analogous to the lactoside group, may be conjugated to immunogenic melittin peptides to create a chemically well defined hapten-conjugate system. This is in contrast to the poorly defined cross linking to carrier proteins such as KLH, in which the number and identity of conjugation points are not known with certainty. Therefore, melittin-like immunogens would offer all the advantages of synthetic vaccines--defined epitopes,

no adverse effects of reactive components of whole organism vaccines, and no contamination with blood products or incompletely attenuated targets--with the added advantage of small size and simple requirements for the delivery vehicle. Given the observation that random peptide-carrier conjugates have a high success rate for eliciting protein-specific antibodies (83), it is possible that the well defined system identified in melittin may provide similar success in eliciting specific reagents.

The structure-activity relationships reported in Chapter II and the dual structural role of length and amphiphilicity developed in Chapter III suggest a model for the *in vitro* structure of the immunogenic peptides. Melittin's structure in crystals (15), and in solution as a tetramer (18), or associated with a detergent micelle (16) is α-helical in residue 1-20 and flexible in residue 21-26. Raman spectroscopy of melittin in lipid bilayer membranes also indicates bent α-helical structure in the N-terminus and flexible structure in the C-terminus (19). Proposed models for melittin's tetramer structure in membranes and in aqueous solution, which may be relevant for its *in vivo* immunogenic structure, are presented in Figure 5.1. Evidence from voltage-gated channel studies indicates that in membranes (Figure 5.1a) melittin forms a tetramer with parallel, asymmetric orientation (21). Tryptophan fluorescence energy transfer studies also indicate a tetramer structure, and fluorescence quenching indicates that tryptophan side chains are located in proximity to one side of the membrane (19). Melittin associates as a pair of antiparallel dimers in the aqueous tetramer structure (Figure 5.1b) due to the charge repulsion of the basic C-terminus. In membranes the charge repulsion effects are diminished because C-terminal groups bend away from each other and the net negative charge of the phosphate head groups of the phospholipids screens the positive charge of melittin.

a.



b.

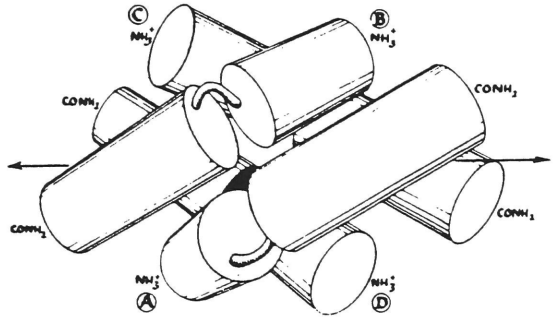


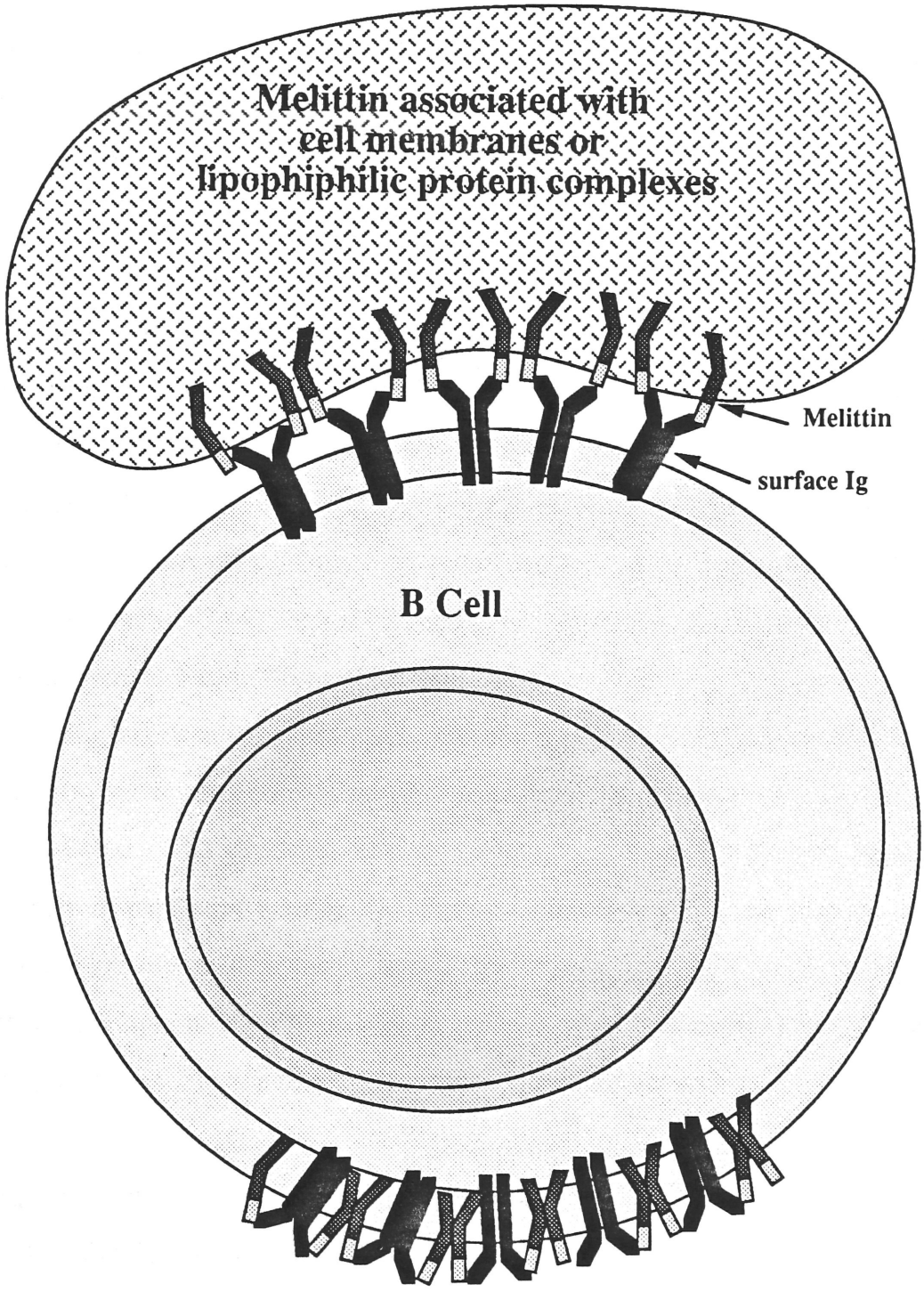
Figure 5.1. Proposed melittin tetramer structures. a. Membrane bound melittin associates in parallel form with the C-terminal groups on the cis membrane surface, i.e. the side to which peptide is added. The hydrophobic surface of the α-helix interacts with the hydrocarbon core of the membrane and the polar side chains provide an ion permeable channel. b. Aqueous melittin tetramer interpreted from the crystal structure is found as a pair of antiparallel dimers with the hydrophobic face of the helix in the center of tetramer. Note in both structures that C-terminal epitopes are accessible for binding to antibody. These structures are from 19 (b is originally from 15).

The aqueous tetramer structure (Figure 5.1b) is interpreted from the crystal structure, and provides the strongest direct evidence for different binding activities of the N-terminal segment and the C-terminal segment. Conclusions drawn from the crystal structure seem to apply to hydrophobic interactions in general. The N-terminal 20 amino acid residue mediates hydrophobic interactions with peptide (self association) and lipid (detergent and bilayer membrane association) and is presumably unavailable for other interactions. The C-terminus has flexible structure and the charge repulsion of the concentrated basic residues insures that this region is free from inter-peptide associations. Thus the restriction of melittin's antibody epitopes to the C-terminus is explained by the availability of this region. The degree of accessibility is apparently represented by such behaviors as self association and hemolysis; clearly the size of the peptide extending beyond the N-terminal scaffold also determines accessibility for recognition by antibody.

This analysis suggests that in its immunogenic form, melittin is bound to cell membranes with the C-terminus accessible at the membrane surface (Figure 5.2). Strong evidence for this structure is found in the activity of 1-20-D_6, which demonstrates poor membrane affinity by failing to lyse red blood cells and was poorly immunogenic, especially relative to 1-20-G_4Q_2, a peptide of comparable size but higher affinity for membrane binding. Estimating 10^{10} total blood cells, 2 nmol of melittin would correspond to 10^5 melittin molecules per cell, a high enough number for specific and non specific membrane binding to play a role in its immunogenicity.

Association with cell membranes would serve to enhance melittin's immunogenicity by two means. First, interaction with membranes or other lipophilic environments, such as lipoproteins, would result in a multivalent antigen structure. Multivalence of melittin, indicated by its apparent propensity to oligomerize either in solution or in membranes, provides a mechanism for surface-

Figure 5.2. Proposed immunogenic structure of melittin. Melittin's hydrophobic N-terminus, indicated by dark shading, inserts in cell membranes or other lipid environments, while the C-terminus is accessible for binding to surface-Ig of the melittin-specific B cell. Cross linking of surface-Ig can occur by interaction with this functionally multivalent melittin. Melittin membrane-associated tetramers are represented by dimers in this figure.



Melittin oligomers in cell membrane crosslinking surface Ig.

Ig cross linking either with melittin located extracellularly in other cell membranes or lipid environments, or located in the responding cell membrane. Cross linking of surface-Ig on resting B cells is an important initial, T cell independent step in B cell activation and humoral immune response (62). The ability of melittin specific IgE, including a monoclonal IgE, to mediate positive PCA responses in rat skin provides direct evidence for multivalence and cross linking ability of melittin *in vivo*. It is useful to note that activation of B cells may play a role in T cell phenotype determination. Antigen presentation by B cells is extremely efficient because the surface-Ig of the B cell acts as an antigen receptor (84). Activation of B cells by cross linking surface Ig increases expression of Ia molecules, further enhancing APC function of these cells (62). Antigen presentation to T_H2 cells proceeds efficiently on activated B cells (43,44), thus B cell activation may select for T_H2 phenotype or may actively induce T cell differentiation to T_H2.

Binding to cell membranes would also serve to maintain relatively high levels of peptide in the system, increasing the opportunity for immune surveillance and response. Other small peptides, such as hormones, are rapidly cleared from the blood stream by metabolic activity. Quick clearance would not be expected to occur for a peptide located predominantly in cell membranes, but might occur for peptide interacting with lipophilic proteins such as albumin or with other lipid environments such as lipoproteins that are themselves subject to metabolic activity.

T-cell epitopes and antibody isotype composition.

Identification of the T cell epitope of melittin restricted to the murine I-A^d MHC class II molecule as segment 7-19 may be useful for similar identification in humans since this indicates a region for consideration. In general the structural propensities identified with melittin are consistent with those expected of T-cell epitopes (48,67).

The predominant T_H2 phenotype of melittin-specific T-cell clones corresponds to the high levels of IgE and IgG_1 though, the lymphokine assays are not conclusive. T_H2 activity may be important for immunogens that elicit high levels of IgE and IgG_1 in mice, especially for IgE. Injection of high levels of 11B11 anti-IL-4 inhibits normal IgE responses in mice (46). Due regard must be given, however, to the fact that T-cell phenotype as characterized by lymphokine production is not assayed until after four to six passages *in vitro*, the first three in the presence of high levels of growth factors in the form of ConA stimulated rat spleen conditioned medium. Thus the culture conditions may themselves play a significant role in selecting the T-cell phenotype. These results call for *in vivo* or earlier *in vitro* studies of T cell phenotype. Messenger RNA amplification (85), *in situ* hybridization (86) or ELISPOT (87), once suitable reagents are available, are all likely techniques to investigate this question more fully.

Differential class II restriction of melittin to both $I\text{-}A^d$ and $I\text{-}E^d$ and of peptides 1-20-G_4Q_2 and 1-20-D_6 to only $I\text{-}A^d$ suggests an interesting mechanism for isotype selection. Ia restriction is coincident with different antigen-specific antibody isotype compositions, summarized in Table 5.1. These results suggest that high IgE and IgG_1 responses are controlled by restriction to $I\text{-}E^d$, and that higher IgG_{2a} responses result from restriction to only $I\text{-}A^d$. Thus one is led to conclude that immunogens that elicit high levels of IgE in $H\text{-}2^d$ restricted mice are restricted or co-restricted to presentation by $I\text{-}E^d$. No correlation is observed between Ia restriction and lymphokine production. Of $I\text{-}E^d$ restricted T cells, 2/5 tested are IL-4 producers, 2/5 produce IL-2, and 1/5 makes both lymphokines. Similarly, $I\text{-}A^d$ restricted clones show IL-2 and IL-4 activity, or show very weak activity overall (see Tables 4.5 and 4.6).

Table 5.1. Ia restriction and Ig isotype composition for peptide-specific responses.

Immunogen [1]	Ia Restriction [2]	% IgG composition [3]			Total IgE [4] (μg/ml)
		IgG1	IgG2a	IgG2b	
"Melittin"	I-A , I-E	96	1.4	2.4	>9.5
1-20-G4Q2	I-A	92	4	4	4.5
1-20-D6	I-A	77	12	11	2.5

1. Immunogens are melittin (in four groups, three strains), 1-26-lac, 1-24-lac and 1-24 in the melittin category, as detailed in Table 3.4

2. Ia activity data are reported in Table 4.7 and figure 4.11.

3. Ig isotype composition is reported in Table 3.4.

4. Total serum IgE in immunogen specific sera are reported in Table 3.3.

Class II allele expression and melittin-specific IgG and IgE responses in BALB/c, CAF, and A/J mice are summarized in Table 5.2. The Ia molecule is made up of an α and β chain (88). For I-E^d, the E_β chain allele is d; the E_α chain allele is called 7. Both polypeptide chains of I-A^d are expressed by the respective d alleles. By comparison, H-2^a mice, e.g. A/J mice, express k alleles of A_α and A_β and E_β. This strain also expresses the E_α 7 allele. Thus H-2^d and H-2^a, i.e. BALB/c and A/J, mice express the same α chain allele in I-E. Moreover, A/J mice, while poor IgG responders to melittin, have high IgE responses, with a PCA titers of 80 (14). It appears that restriction to I-E, and specifically to the E_α 7 allele product, correlates with high levels of IgE to melittin.

Simulteneous antigen restriction to both Ia molecules has been observed before. Both I-A and I-E products were found to contribute to T cell activation in EAE in a rat model, but specific T cell activities, e.g. infiltration and demyelination or delayed type hypersensitivity, were found to correlate with restriction to I-A and I-E, respectively, possibly through separate activation mechanisms (89). How the effects of specific Ia restriction are mediated remain a mystery, but may involve selection of particular TcR genes or gene families.

Table 5.2. H-2 type, class II alleles and melittin-specific IgG and IgE responses of responding mouse strains.

Strain	H-2 [1]	MHC class II alleles [1]				Melittin-specific Ig [2]	
		A_β	A_α	E_β	E_α	IgG (μg/ml)	IgE (PCA)
BALB/c	d	d	d	d	7	400	160
DBA/2	d	d	d	d	7	600	2560
CAF_1	a,d	k,d	k,d	k,d	7	700	640
A/J	a	k	k	k	7	2	80

1. From reference 88.
2. From this thesis and reference 14.

Summary

Melittin provides an interesting model for peptide immunogens with defined structure-activity-immunogenicity relationships. Incorporation of the biophysical properties of melittin in other peptide immunogens, or conjugation of a hapten of interest on a melittin derivative, may provide a new class of easily prepared, immunogenic peptides for vaccine and diagnostic use.

Identification of the immunodominant T-cell epitope in residue 7-19 of melittin provides an good starting point to identify the epitope in humans, particularly since the relevant peptide fragments are easily obtained by proteolysis. Further study of melittin-specific T cell responses are required to firmly correlate Ia restriction and antibody isotype and subclass but the preliminary evidence indicates such a correlation. This correlation, if it holds out under further investigation and proves to be relevant in human immunology, may be very important for the development of immunotherapeutic agents in the treatment of allergy, either by producing agents to enhance restriction to the putative "non-IgE" MHC loci and increase levels of specific IgG or to block activity at the "IgE" MHC locus.

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