

MELITTIN-SPECIFIC MONOCLONAL AND POLYCLONAL IgE AND IgG₁ ANTIBODIES FROM MICE¹

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Melittin, a bee venom peptide consisting of 26 amino acid residues, elicited high IgG and IgE antibody responses in mice of BALB/c and CAF₁ strains, but not in mice of A/J, AKR, and C57BL/6 strains. Greater than 80% of the melittin-specific antibodies in sera of responder mice were found to bind the hydrophilic carboxyl-terminal heptapeptide of melittin. Three melittin-specific monoclonal antibodies were obtained from responder mice by the hybridoma technique. Two are of the IgG₁ isotype and one is of the IgE isotype. One monoclonal antibody of the IgG₁ isotype binds the carboxyl-terminal heptapeptide of melittin, while the other two monoclonal antibodies do not. However, competitive binding studies suggest that all three monoclonal Ig bind at the same, or adjacent, site of melittin. These findings, together with the known amphiphilic property of melittin, suggest that the immunogenicity of this peptide is a consequence of its binding to cell surface phospholipids.

Honey bee venom consists mainly of peptides and proteins (1). The major component of bee venom is the peptide melittin, which consists of 26 amino acid residues. The primary structure of melittin (1) is shown in Figure 1, and its x-ray crystal structure is also known (2). Melittin has the property of binding to cell membrane phospholipids. Another major venom component is the protein phospholipase A₂, consisting of 128 amino acid residues. These two components account for 50% and 12% of the venom weight, respectively.

Nearly all bee venom-sensitive people have elevated levels of IgE and IgG antibodies specific for phospholipase, while only about one-third of these people have melittin-specific antibodies (3-6). The immunogenicity of melittin, although occurring only in certain individuals, is of interest because peptides are usually poor immunogens. Another bee venom peptide, apamin (consisting of 18 amino acid residues), for example, is nonimmunogenic in venom-sensitive people so far tested (4, 6). Melittin has also been reported to induce antibody response in mice of the Swiss-Webster stock (7).

In this report, we show that melittin is highly immunogenic in certain strains of mice, and that its major, if

not only, antigenic determinant is in the hydrophilic carboxyl-terminal heptapeptide region of the molecule.

MATERIALS AND METHODS

Melittin was isolated from honey bee venom according to the published procedure (4), and was further purified by chromatography on CM-cellulose in pH 4.75 ammonium acetate buffer containing 4 M urea (8). The purity of the samples was established through amino acid analyses and the correct molar extinction coefficient at 280 nm.

Peptic and tryptic fragments of melittin (9) shown in Figure 1 were obtained as follows. Melittin (4 mg per ml) was digested with pepsin (25 µg per ml) in 0.05 M HCl at 37°C overnight; the digest was applied directly to the SE-53 cellulose column for isolation of peptides. Melittin (4 mg per ml) was digested with trypsin (0.2 mg per ml) in 0.05 M NH₄HCO₃ at 25°C overnight; the turbid digest was dissolved in 0.2 N acetic acid and was lyophilized again after 72 hr (see Table I, footnote). Both pepsin and trypsin were crystalline preparations from Worthington Biochemical Corp. (Freehold, NJ).

Peptic and tryptic digests of melittin were separated by ion-exchange chromatography on a 20 x 0.9-cm column of SE-53 cellulose (Whatman, Clifton, NJ) at a flow rate of 60 ml per hr (Fig. 2). The column was first eluted with a linear pH gradient from 3.05 to 5.90, followed with a second pH gradient from 5.90 to 7.90. The compositions of these three buffers were: pH 3.05, 10 mM ammonium formate and 50 mM formic acid; pH 5.90, 95 mM ammonium acetate and 5 mM acetic acid; and pH 7.90, 95 mM ammonium acetate and 1 M NH₄HCO₃.

The indicated fractions of Figure 2 were recovered by lyophilization. They were characterized by amino acid analysis (10) after 20-hr hydrolysis in 6 N HCl at 110°C and 0.1 Torr. The fractions were also examined by high-pressure liquid chromatography on a reverse-phase column (MCH-10; Varian Instrument Co., Palo Alto, CA) by using a linear CH₃CN gradient of 1% per ml in 0.05% trifluoroacetic acid. The analytical results of these samples and their yields are given in Table I.

The mice in this study were females with body weights of 20 to 25 g, obtained from the Jackson Laboratory (Bar Harbor, ME). Freund's complete adjuvant was from Calbiochem (La Jolla, CA), and alum was prepared as described (11). Immunizations were performed by the i.p. route, and sera were collected from mice by retro-orbital plexus puncture. A standard hybridoma procedure (12) with one modification was used to obtain antibody-secreting hybrid cell

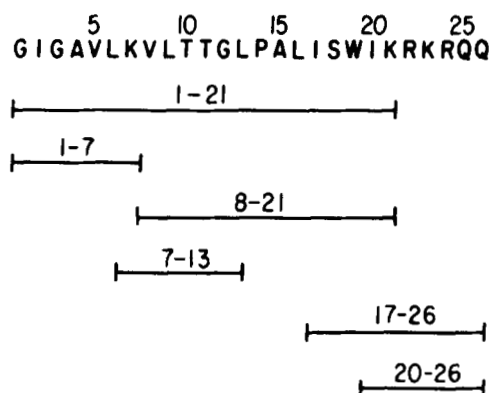


Figure 1. Amino acid sequences of melittin and its peptic or tryptic fragments. The carboxyl-terminal residue of melittin is in the form of amide (1).

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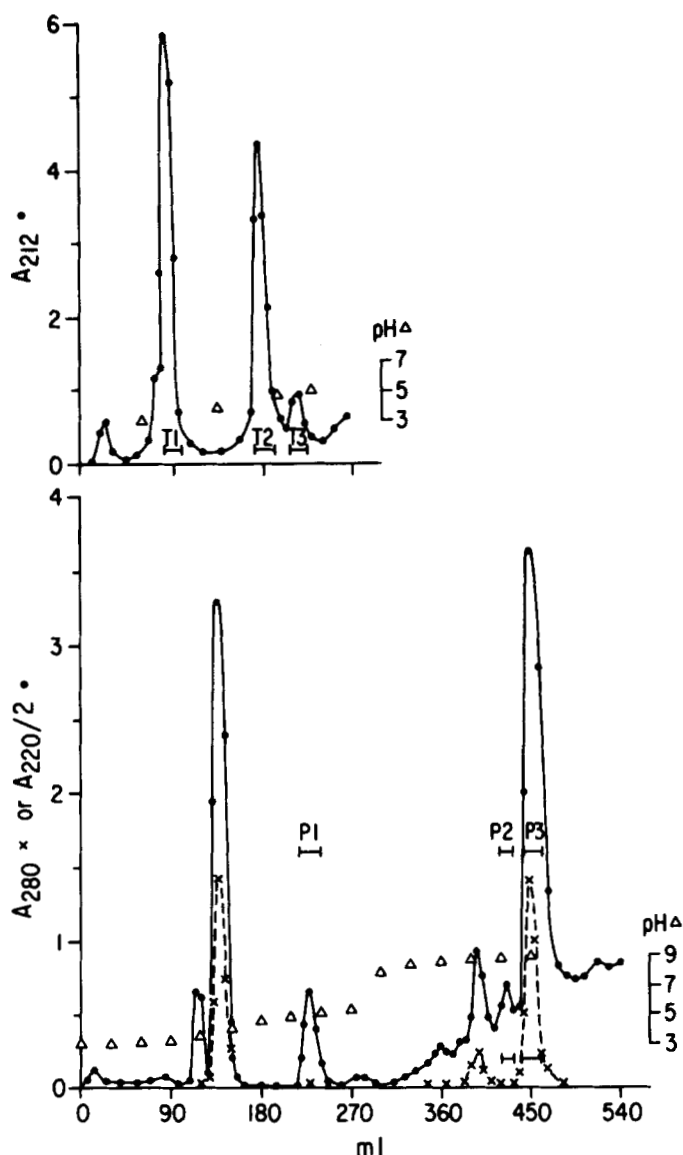


Figure 2. Separation of trypsin and pepsin digests of melittin on SE-53 cellulose. In the upper pattern, 5.2 μ mol of trypsin-digested melittin were used, and in the lower pattern, 11 μ mol of pepsin-digested melittin were used.

lines of spleen cells and plasmacytoma SP 2/0 cells (13). The modification was that the hybrid cells were grown in standard hybridoma medium containing 100 μ M hypoxanthine and 16 mM thymine. Hybrid cells were cloned by limiting dilution to give about 0.3 cell per well.

Melittin-specific monoclonal antibodies were isolated from ascites of pristane-treated mice that had been inoculated with about 0.5×10^6 hybrid cells. The antibodies were precipitated from ascites at 40% $(\text{NH}_4)_2\text{SO}_4$ saturation. After dialysis, they were chromatographed on a 10 x 0.9-cm column of CM-Affi Gel Blue (Bio-Rad Laboratories, Richmond, CA), which is an agarose gel containing blue dye and carboxymethyl groups. The column was equilibrated with a pH 7.95 buffer of 0.05 M Tris-HCl + 0.1 M NaCl. The unbound peak containing antibody was further purified by affinity chromatography on a column of melittin-coupled Sepharose 4B, followed by gel filtration on a 200 x 0.9-cm column of Sephadex G-100. The affinity column was prepared by reaction of 10 mg of melittin with 1 g (dry weight) of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) (14). The purity of antibodies was tested by SDS-gel electrophoresis (15) after reduction and by electrofocusing in agarose gel (Isogel System; FMC Corp., Homer City, PA).

Melittin-specific antibodies were measured by solid-phase enzyme immunoassay (14, 16) with two minor modifications: a) the volumes of reagents were reduced to 20 μ l, and b) the absorbance changes were read directly in wells with a BIOTEK EIA Reader. The solid-phase antigens were plastic wells with noncovalently bound melit-

tin, which were prepared by overnight treatment with a 5- μ g-per-ml melittin solution in 50 mM Tris HCl buffer of pH 7.95. All mouse antibody solutions were made in a pH 7.95 buffer containing 0.05 M Tris-HCl, 0.5 M NaCl, 1 mg per ml bovine plasma albumin, and 0.5 mg per ml Tween 20. The solid-phase bound melittin-specific mouse antibodies were detected by reaction with rabbit anti-mouse Ig (DAKO Immunochemicals, Westbury, NY), followed with sheep anti-rabbit IgG conjugate with horseradish peroxidase.

To determine the isotype of mouse antibodies, rabbit antibodies specific for the individual mouse Ig heavy or light chains were used in place of rabbit anti-mouse Ig. These rabbit antibodies were from a commercial mouse Ig subtype identification kit (Boehringer-Mannheim, New York, NY), with the one exception that rabbit antibodies specific for mouse ϵ -chain were prepared in this laboratory (14) with the use of a monoclonal mouse IgE specific for the 2,4-dinitrophenyl (DNP) group (17) as the immunogen. The specificity of these reagents were established with appropriate mouse immunoglobulins, and the presence of 1% cross-contamination would have been detected under the conditions of the assay.

Melittin-specific mouse IgE was also measured by passive cutaneous anaphylaxis in retired male breeder rats (18). Four hours after sensitization of skin sites with 0.1-ml aliquots of appropriately diluted mouse antibodies, the rat was challenged i.v. with 1 ml of a solution containing 1 mg of melittin and 5 mg of Evan's Blue in phosphate-buffered saline.

Inhibition of binding of melittin-specific antibodies to solid-phase melittin by increasing concentrations of melittin or its fragments in solution-phase was made at ambient temperature in the same pH 7.95 buffer used for enzyme immunoassay. The antibody concentration chosen was at a level at which there was about 30% maximal binding of the antibodies to the solid-phase melittin, and it corresponded to about 150 ng per ml of specific Ig. The data are expressed as percent of maximal absorbance change that reflects the change in antibody concentration in the presence of inhibitor.

Inhibition of binding of biotinylated antibodies to solid-phase melittin by unlabeled antibodies was made under the same conditions described above, with the exception that the biotinylated antibodies bound to solid-phase melittin were detected with horseradish peroxidase-labeled avidin (19). Biotinylation was performed by reaction of 3.2 μ M antibody with 0.91 mM biotin N-hydroxy-succinimide ester (Calbiochem) at pH 7.2 for 1 hr, followed by dialysis. Peroxidase-labeled avidin was prepared by reaction of 14 μ M 4-mercaptobutyrimidyl-avidin and 28 μ M 4-(4'-dithiopyridyl)-butyrimidyl-peroxidase at pH 6.6 and 25°C overnight, followed by chromatography on Sephadex G-100. The product was estimated from its absorbance ratio at 403 and 280 nm to contain 0.7 mol of peroxidase per mol of avidin. Chemical modifications of avidin (Sigma Chemical Co., St. Louis, MO) and peroxidase (Miles Laboratories, Elkhart, IN) were made according to a published procedure (20).

RESULTS

Immunogenicity of melittin in mice. As listed in Table II, mice of five different strains were immunized i.p. with a mixture of melittin and either alum or Freund's complete adjuvant. At specified times, their sera were analyzed for melittin-specific total Ig by solid-phase enzyme immunoassay, and for melittin-specific IgE by passive cutaneous anaphylaxis. In Figure 3 are shown two representative enzyme immunoassays of serum samples which were collected from BALB/c and A/J at week 6 after three immunizations (experiments 5 and 6 of Table II). Also shown in Figure 3 are the assay data for a sample of monoclonal melittin-specific IgG₁, the isolation of which will be given later (fusion 4 in Table III). The concentrations of melittin-specific Ig in the serum samples of Table II were estimated by using this monoclonal IgG₁ as the standard.

The results in Table II show that the immunized BALB/c and CAF₁ mice had high serum levels of melittin-specific Ig (400 to 1100 μ g per ml), the A/J mice had low levels (1 to 2 μ g per ml), and the AKR and C57BL/6 mice had no detectable level (<0.1 μ g per ml). The A/J, BALB/c, and CAF₁ mice had comparable serum levels of melittin-specific IgE with passive cutaneous anaphylaxis ti-

TABLE I
Amino acid compositions and yields of peptic and tryptic fragments of melittin^a

Amino Acid	Tryptic Peptide ^b			Peptic Peptide		
	T1 (1-21)	T2 (8-21)	T3 (1-7)	P1 (7-13)	P2 (20-26)	P3 (17-26)
Thr	1.76 (2)	1.91 (2)	0.15	2.00 (2)	0.06	
Ser	0.96 (1)	1.20 (1)	0.15	0	0.07	0.95 (1)
Glu	0.15	0	0.30	0.02	2.01 (2)	2.05 (2)
Pro	0.92 (1)	0.95 (1)	0	0		0
Gly	3.38 (3)	1.21 (1)	1.93 (2)	1.13 (1)	0.12	0.12
Ala	2.38 (2)	1.00 (1)	0.96 (1)	0.01	0.01	0
Val	2.44 (2)	1.00 (1)	0.98 (1)	0.93	0.02	0
Ileu	2.47 (3)	1.64 (2)	0.99 (1)	0.04	1.03 (1)	1.80 (2)
Leu	4.20 (4)	2.83 (3)	1.10 (1)	2.02 (2)	0	0
Lys	1.77 (2)	1.02 (1)	1.02 (1)	1.00 (1)	2.03 (2)	2.03 (2)
Arg	0				2.12 (2)	2.01 (2)
Trp	ND ^c (1)	ND (1)				ND (1)
Yield	16%	20%	23%	59%	40%	34%

^a The expected values for the fragments listed in Figure 1 are given in parentheses.

^b Some preparations of peptide T2 were contaminated with another tryptic peptide, glutamylglutamine amide. Acetic acid treatment converted the majority of this basic dipeptide to a neutral dipeptide, pyroglutamylglutamine amide, which was eluted at the void volume shown in Figure 2. Peptide T2 free of the basic dipeptide was obtained by rechromatography on SE-53 with a linear pH gradient from 3.30 to 5.90.

^c ND, not determined.

TABLE II
Melittin-specific antibody response in mice

Expt. ^a	Strain ^b	Adjuvant ^c	Ig μ g/ml ^d		IgE titer	
			wk 6	wk 11	wk 6	wk 11
1	AKR	Freund's	<0.1	ND ^e	<5	ND
2	C57BL/6	Freund's	<0.1	ND	<5	ND
3	CAF ₁	Freund's	990	ND	80	ND
4	CAF ₁	Alum	730	400	640	320
5	BALB/c	Alum	420	1100	160	80
6	A/J	Alum	2	1	80	ND

^a Groups of two to four mice were immunized i.p. on wk 0, 3, 5, and 8 with 0.2 ml of melittin (4 μ g) and alum (1 mg) in pH 6.4 phosphate buffer, or with 0.2 ml of melittin (4 μ g) in an emulsion of equal parts of phosphate buffer and Freund's complete adjuvant.

^b CAF₁ is an F₁ hybrid of BALB/c $\times$ A/J.

^c Ascites were formed in mice immunized with melittin and Freund's complete adjuvant, and they had the same antibody levels as the sera.

^d Serum levels of melittin-specific Ig and IgE were measured by enzyme immunoassay and passive cutaneous anaphylaxis, respectively. IgE titer represents the reciprocal of highest dilution to give a positive reaction; the lowest dilution tested is 1/5. Serum pools of each group of mice were analyzed.

^e ND, not determined.

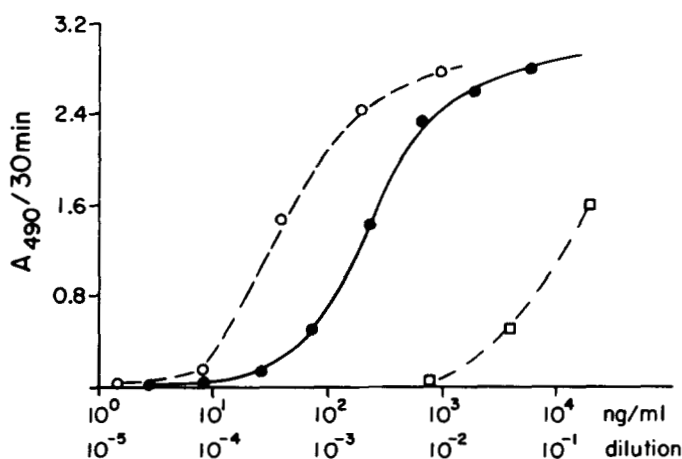


Figure 3. Solid-phase enzyme immunoassay for melittin-specific Ig. $\circ$ and $\square$ are for sera from wk-6 bleedings of BALB/c and A/J mice, respectively; concentrations expressed as serum dilutions. $\bullet$ are for monoclonal IgG₁ from fusion 4; concentrations given in ng per ml.

ters of 80 to 640, even though the A/J had very low levels of specific Ig when compared to the BALB/c and CAF₁ mice. Also as shown in Table II, CAF₁ mice had similar serum levels of specific Ig irrespective of the adjuvant used.

Melittin-specific monoclonal antibodies. By the hybridoma technique, three stable cell lines secreting

melittin-specific Ig were obtained. A total of eight fusion experiments were performed by using the spleen cells from five BALB/c mice, two CAF₁ mice, and one A/J mouse as indicated in Table III. Under similar experimental conditions with spleens from mice immunized with bee venom phospholipase, five to 30 antibody-secreting clones were usually obtained from each fusion (unpublished results).

Melittin-specific monoclonal Ig were isolated from ascites induced with the hybrid cells of Table III, in yields of 1 to 5 mg per ml of ascites. The monoclonals from fusions 2 and 4 were found to be of IgG₁ isotype, whereas that from fusion 6 was found to be of IgE isotype. The light chains of these three Ig were found to be κ -type. The minimal concentration of the melittin-specific monoclonal IgE required to give a passive cutaneous anaphylaxis reaction in rats was found to be about 8 ng per ml as compared to about 1 ng per ml reported for the DNP-specific monoclonal IgE (17). This difference probably reflects a lower affinity of the melittin-specific IgE for the antigen because the IgE sample used is free of other Ig on immunoassay (<1%). On SDS gel electrophoresis of the reduced monoclonal Ig, two predominant bands corresponding to the heavy and light chains were observed for each sample together with several faint, fast-moving bands. On electrofocusing, two closely spaced bands were observed for each of the two monoclonal IgG₁, and three

TABLE III
 Hybridomas secreting melittin-specific antibodies^a

Expt.	Mouse Strain	No. of Weeks After Primary Immunization	No. of Wells with Specific Ab	Ab Isotype
1	BALB/c	3	0	—
2	"	5	1	IgG ₁
3	"	10	0	—
4	"	10	1	IgG ₁
5	"	13	0	—
6	CAF ₁	5	1	IgE
7	"	10	0	—
8	A/J	5	0	—

^a The mice used were from experiments 4 to 6 in Table II. In each experiment, the spleen cells from a single mouse were used. Sera from individual mice used for fusion were found to have antibody levels similar to those of their respective groups in Table II. The fusion mixture was distributed into about 760 wells, each containing 0.2 ml volume. Two to four weeks after fusion, 40 to 200 wells had clones of cells, and these wells were tested for the presence of melittin-specific antibodies.

groups of two to three closely spaced bands were observed for the monoclonal IgE. These electrophoretic data are not known.

Antigenic specificity of the melittin-binding monoclonal and polyclonal Ig. This antigenic specificity was studied by testing six different fragments of melittin (Fig. 1) to inhibit the binding of specific Ig to solid-phase melittin. The inhibition data of Figure 4 are for the polyclonal Ig in sera of BALB/c and CAF₁ mice. As a control, high concentrations of melittin in solution completely inhibited the binding of specific Ig to solid-phase melittin. Of the six fragments tested, only fragments corresponding to residues 17-26 and 20-26 showed inhibition of binding. The data for the fragments that did not show inhibition are not given in the figure, except for fragment 1-21. The maximal extent of inhibition by fragment 17-26 or 20-26 was about 70% for sera of BALB/c mice, and it was nearly 100% for sera of CAF₁ mice. The concentrations of melittin or fragments required for half-maximal inhibition of each serum sample were about the same in the 10⁻⁶ M range, indicating that the specific Ig bound the fragments as tightly as they did the intact melittin. The serum samples used in Figure 4 were collected at wk 6 after primary immunization, but similar findings were obtained at wk 13.

In Figure 5 is shown the inhibition of binding of mon-

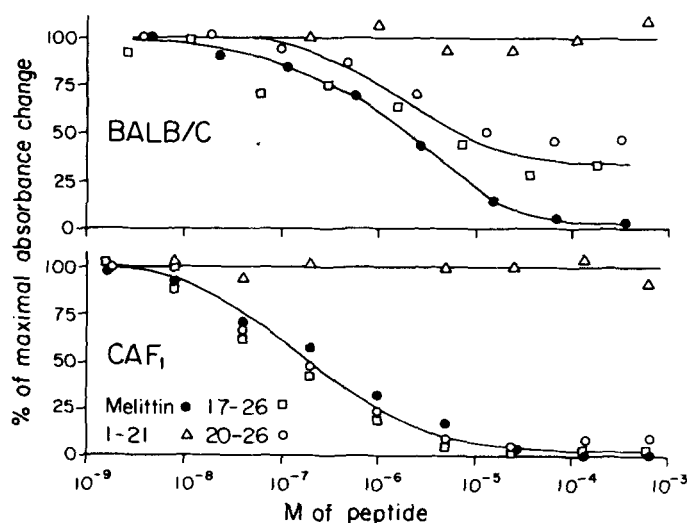


Figure 4. Inhibition of binding of polyclonal melittin-specific Ig to solid-phase melittin by melittin or its fragments. Upper and lower patterns were obtained with sera from wk-6 bleedings of BALB/c and CAF₁ mice, respectively.

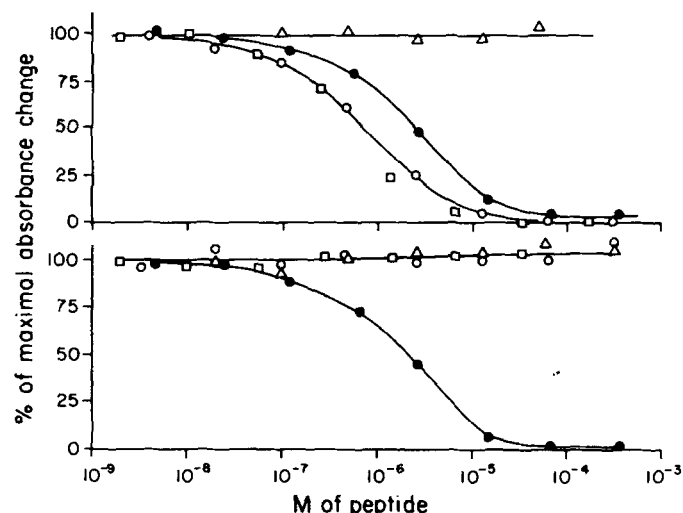


Figure 5. Inhibition of binding of monoclonal melittin-specific Ig to solid-phase melittin by melittin or its fragments. Upper and lower patterns were obtained with monoclonal Ig from fusions 4 and 6, respectively. Symbols have the same designations as those described for Figure 4.

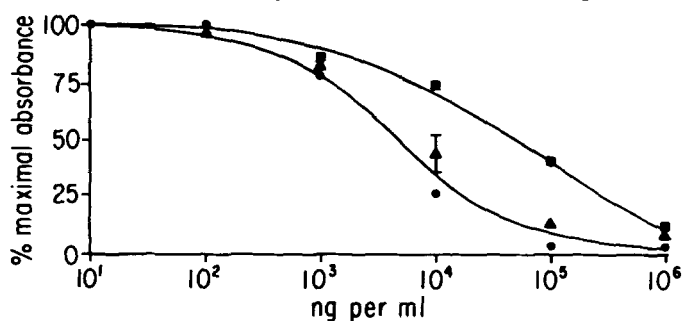


Figure 6. Competition of binding of biotinylated monoclonal IgG1 from fusion 4 to solid-phase melittin by unlabeled monoclonal Ig. ●, ■, and ▲ represent monoclonal Ig from fusions 2 (IgG₁), 4 (IgG₁), and 6 (IgE), respectively.

oclonal Ig from fusions 4 and 6 to solid-phase melittin by melittin and its fragments. The data of monoclonal IgG₁ from fusion 2 are not shown, being nearly identical with those of IgE from fusion 6. All three monoclonal Ig were inhibited by melittin at about the same concentration of 10⁻⁶ M. Of the fragments tested, only fragments 17-26 and 20-26 showed inhibition with monoclonal IgG₁ from fusion 4, but not with the monoclonal Ig from fusions 2 and 6.

The three monoclonal Ig were compared for their inhibition of binding of biotinylated IgG₁ from fusion 4 to solid-phase melittin. The data in Figure 6 show that the

three monoclonal Ig are equally effective in this regard. Accordingly, the results suggest that they bind at the same, or adjacent, regions of the melittin molecule, as does the biotinylated IgG₁.

DISCUSSION

On immunization of five strains of mice with melittin, mice of the BALB/c strain and its F₁ hybrid with the A/J strain produced high serum levels of 400 to 1000 µg per ml of specific Ig, and the other strains did not (Table II). These serum levels of specific Ig are about the same as those of BALB/c mice immunized with the bee venom protein phospholipase (unpublished results). Therefore, melittin can be as highly immunogenic as protein antigens are in selected strains of mice. Our findings suggest that there is genetic control of the melittin-specific immune response. Genetic control of immune response to proteins is usually complex because each protein has multiple antigenic determinants (21). Melittin is a small peptide, and has fewer determinants than large proteins. Our findings from inhibition studies suggest strongly that melittin has only one major antigenic determinant, although the presence of other minor determinants cannot be excluded. Therefore, melittin may be useful for studying the genetic marker of human immune response to a single determinant, just as has been done with a small ragweed antigen, termed Ra5, of 5000 daltons (22).

Inhibition studies with different fragments of melittin indicate that 70 to 100% of the melittin-specific Ig in sera of BALB/c and CAF₁ mice bind only the carboxyl-terminal fragment 20-26 and its larger homolog fragment 17-26 (Figs. 1 and 3). Therefore, the findings indicate that the major antigenic determinant of melittin is located in the region containing residues 20-26, a conclusion supported by data obtained with monoclonal melittin-specific Ig. The monoclonal IgG₁ from fusion 4 (Table III) binds fragment 17-26 or 20-26 even more tightly than it binds melittin, but the other monoclonal IgG₁ from fusion 2 or the IgE from fusion 6 binds only melittin and not its fragments (Fig. 4). Although the three monoclonal Ig do not have the same binding properties for melittin fragments, they are nevertheless equally effective in competing for the binding of melittin (Fig. 5). Owing to the small size of melittin, steric hindrance may prevent the simultaneous binding of two Ig at separate antigenic sites of the molecule. Therefore, the competition data of the three monoclonal Ig do not establish that they bind at the same, or adjacent, sites of melittin. If they do bind at the same site, i.e., fragment 20-26, they may differ in their specificity for the different conformers of this fragment. It is likely that the fragment will have greater conformational freedom than the melittin molecule itself. Also, there remains the possibility that the monoclonal Ig from fusions 2 and 6 bind at site(s) other than that of fragment 20-26. These site(s) may represent minor determinants of melittin. It is possible that, for unknown reasons, the two hybridomas specific for the minor determinants are obtained in equal frequency as that for the major determinant.

Melittin is an amphiphilic molecule (Fig. 1). The region from residue 1 to 20 contains mainly hydrophobic residues, whereas the region from residue 21 to 26 contains only hydrophilic residues. The conformation of melittin crystals is known to be an α -helix with a slight bend at

the midportion (2). The surface of the helix from residue 1 to 20 is also amphiphilic because it can be divided lengthwise into two halves; one half consisting mainly of hydrophobic side chains and the other half of hydrophilic side chains. Because of this amphiphilic character, melittin in solution can associate to form a tetramer, and it can bind to cell surface phospholipid bilayers. A synthetic analog of melittin, with markedly altered amino acid residues 1 to 20 but retaining the amphiphilic character, was found to retain these physicochemical properties of melittin (23).

Physicochemical studies (24, 25) indicate that melittin is monomeric in phospholipid bilayers, and that the hydrophobic portion of the helix is in contact with the phospholipid bilayers while the hydrophilic portion is accessible to aqueous environment. Our finding that the hydrophilic fragment 20-26 is the major antigenic determinant of melittin is in accord with this model of phospholipid-bilayer bound melittin. Furthermore, this model provides a possible explanation of the immunogenicity of this peptide: namely, binding to cell surface phospholipid bilayer changes melittin from being a soluble antigen into a multivalent cellular antigen with its hydrophilic carboxyl-terminal fragment as a hapten group. This property, as well as the tendency of melittin to form tetramers, can enhance the immunogenicity of melittin both by increasing its circulatory life in the host and by altering its processing by macrophages and lymphocytes. This line of reasoning may also explain why the known antigenic determinants of globular proteins usually involve the exposed hydrophilic regions of the molecule (26, 27). These two biochemical properties of melittin also help to explain why melittin can elicit passive cutaneous anaphylaxis in passively sensitized rats, because release of chemical mediators from IgE-sensitized cells is known to require bridging of cell surface IgE antibodies by multivalent antigens (28).

The isolation of a hybrid cell line secreting specific IgE is generally thought to be an event of low frequency. Special techniques with the use of x-ray-irradiated or drug-treated mice are used to decrease suppressor cell populations so as to increase the frequency of isolation of IgE-secreting cell lines (29). In the present studies, standard procedures were used, and one of three cell lines that we obtained secretes IgE. This may be a chance event, or it may be related to the unusual physicochemical properties of melittin.

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