

Allergenic and biological activities of melittin from honey bee venom

B. F. MACKLER,* A. S. RUSSELL† and G. KREIL‡

* *Division of Immunology, Kennedy Institute of Rheumatology, London W6 7DW, England*

† *Rheumatology Division, University of Edmonton Medical School, Edmonton, Alberta, Canada*

‡ *Department of Chemistry, Institute for Molecular Biology of the Austrian Academy of Science, Vienna, Austria*

Summary

Patients sensitive to bee stings had immediate weal-and-flare (WF) and painful late reactions when skin tested with purified melittin, the main toxin of bee venom. The WF reactions were immunologically non-specific, as melittin is a potent histamine-releasing agent. The late reactions which persisted after 4-5 hr appeared to result from immunological hypersensitivity to bee stings since normal patients had no late reactions. Reaginic and precipitable antibodies against melittin were not found in the sera from any of the bee sting sensitive patients. Cellular hypersensitivity against melittin was not detected by the leucocyte migration inhibition test.

The haemolytic and leucocyte migration inhibition activities of melittin were associated with the intact molecule and not the carboxy-terminal heptapeptide.

Introduction

Allergy against bee venom is a serious medical problem in sensitive individuals; a single sting may cause serious consequences with occasional anaphylactic reactions. Patients with anaphylactic-type responses suffer respiratory obstruction, vascular collapse and haemorrhagic reactions. Non-fatal reactions tend to be immediate and localized; however, symptoms may become more severe and generalized with time. The characteristics of painful reactions in non-sensitive individuals suggested that the pharmacological activity of bee venom may contribute to, and actually accentuate, the diversity of clinical symptoms seen in sensitive patients (Barr, 1971).

In the venom of the honey bee, *Apis mellifica*, several substances have been identified which induce severe local inflammatory reactions and also show other biological effects. This has in particular been demonstrated for the main toxin, a peptide called melittin which amounts to 50% by weight of the dried venom (Habermann, 1968a, b). The structure of this peptide, which contains twenty-six amino acids, has been determined (Habermann & Jentsch, 1967). Studies on the pharmacological

properties of melittin show that it decreases blood pressure, causes muscle contraction and readily lyses red blood cells (Habermann, 1968a, b). Although melittin has been described as a poor immunogen, it may be allergenic. Habermann (1968b) has reported that his own allergy acquired during the handling of dried venom is directed against purified melittin and a similar case has occurred in the laboratory of one of the authors (G.K.). It seems possible, therefore, that at least in some bee venom allergies melittin both sensitized the person and elicited the reaction.

The present study sought to define further the possible role of melittin in naturally occurring bee venom allergy. The purified intact toxin and a fragment derived from melittin by enzymic cleavage were assayed for their haemolytic and leucocyte migration inhibition activities on the human peripheral blood cells from allergic and normal individuals. The skin reactivity of these patients to melittin was assessed by the prick test, then by intradermal injection. The sera of allergic patients were tested by passive transfer (P.K.) tests on rhesus monkeys for reaginic antibodies.

Methods and materials

Melittin

A highly purified preparation of melittin was obtained from bee venom (Firma Mack, Illertissen, Germany) by published procedures (Kreil & Kreil-Kiss, 1967; Kreil & Bachmayer, 1971). Purity was checked by the absence of aspartic acid in total hydrolysates of melittin, an amino acid present in all other components of bee venom.

Melittin fragments

Melittin (75 mg) dissolved in 3 ml water (pH 8.5) was digested with 0.4 mg chymotrypsin (SIGMA, St Louis, U.S.A.) and 0.08 mg trypsin inhibitor (SIGMA). This solution was incubated at 28°C for 15 min, frozen and thawed, then finally centrifuged (1000 g) at 4°C for 20 min. The supernatant was rich in fragment 20-26AA, the C-terminal heptapeptide. This solution is extracted three times with an equal volume of n-butanol and dried. The heptapeptide was further purified by high voltage paper electrophoresis at pH 4.8 (1% pyridine acetate buffer, Whatman 3MM paper, 50 V/cm, 90 min); under these conditions the peptide migrated just ahead of free lysine.

Haemolysis

The haemolytic activity of melittin and its fragment were tested on fresh human erythrocytes. Cells were obtained in citrate, then washed five times in isotonic saline and resuspended to a 1% solution. The tests were done in plates with a volume of 0.2 ml melittin and 0.2 ml 1% human RBC per well. The plates were incubated at 37°C for 1 hr, then visually read for their 100% haemolytic end points.

Inflammation

Patients with a clinical history of bee sting allergy and normal non-reactive individuals were skin tested with purified melittin. All allergic patients had previously undergone severe localized or generalized reactions after bee stings on at least one occasion within the last 2 years. These reactions were either immediate or delayed. A preliminary series of prick tests were made with increasing melittin concentrations (0.0000001–100 µg); followed by intracutaneous injection (0.1 ml volumes) of these dilutions. Skin reactions were examined at 15 min and 24 hr for inflammation.

Human leucocyte migration inhibition

The migration inhibitory activity of melittin and its carboxy-terminal (20-26AA) heptapeptide were assayed on leucocytes separated from the blood of allergic and normal individuals. Leucocytes were washed twice in Eagle's Minimum Essential Medium (MEM) supplement with 10% horse sera, then resuspended to the desired cell concentration. The microcapillary technique described by Federlin *et al.* (1971) was used with two 10 (volume) capillaries per chamber. After incubation at 37°C for 18 hr, the areas of migration were measured by planimetry and the percentage inhibition calculated from the sixteen replicate capillaries for each dilution, obtained from four experiments.

Reaginic antibodies

The presence of human reaginic antibodies in the sera of bee venom sensitive patients was assessed by passive transfer into rhesus monkeys, an animal in which human reagins will fix to cells (Layton, Yamanka & Denko, 1962). Sites were sensitized by intracutaneous injections of sera, then challenged 48 hr later with melittin and reactions read after 30 min. Two melittin challenge concentrations (1 and 10 µg) were used against 0.1 ml doses of sera; these melittin concentrations induced a small weal-and-flare in the rhesus monkey. Since melittin itself has an inflammatory effect, suggestive evidence of reaginic antibodies could be obtained only when the immediate reactions at the sensitized sites were greater than the non-specific reactions induced by equivalent amounts of melittin.

Results*Haemolytic activity*

Melittin caused 100% human erythrocyte lysis at concentrations of 25 µg/ml or 0.0075 M. The 20-26AA heptapeptide was found not to be haemolytic at the range of concentrations tested (0.061–0.001 M). The structural features responsible for the haemolytic activity of melittin were thus not present in the carboxy terminal heptapeptide.

Intracutaneous skin tests of allergic and normal patients

Patients with clinical histories of bee sting allergy were skin tested for their reactivity to purified melittin (Table 1). Skin reactions were observed at 15 min (reaginic) and 24 hr (delayed); few measurements were made at 5 hr because patients were unable to spend the entire day under observation. The immediate skin responses were a weal-and-flare (WF) type which became quite oedemous by 24 hr. Several patients (Nos. 1, 2 and 5) had swelling, pain and irritation at 24 hr quite similar to that experienced after bee stings. The other two patients (Nos. 3 and 4) who normally suffered anaphylactic reactions had milder 24 hr reactions. In contrast, eight normal individuals gave only immediate WF reaction which quickly disappeared, with no reactions evident by 5 hr. The immediate WF reactions were probably induced by the non-allergic inflammatory activity of melittin because these occurred in similar intensities in both normal and allergic patients. The later 24 hr responses evident in allergic patients only appeared to be an immunological manifestation. No biopsies were performed to determine the cellular nature of these reactions.

Table 1. Intracutaneous skin tests of allergic and normal patients with melittin. Patients were intracutaneously skin tested with 0.1 ml volume of various melittin dilutions and the reactions read at 15 min, 5 hr and 24 hr. Reactions were quantitated by size (diam. in mm), visual morphology: weal-and-flare (WF), and oedema (E). Notation: ND, not done.

Melittin conc. per site (μ g)	Normal patients (average of eight individuals)						Allergic patients											
	15 min		5 hr	24 hr		1	2		3		4		5		Average			
	min	hr	min	hr	15 min		24 hr	15 min	24 hr	15 min	24 hr	15 min	24 hr	min	hr			
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
0.001	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
0.10	0	0	0	0	WF	0	WF	0	0	0	0	0	0	0	WF	0		
					11		10								4			
0.1	WF	0	0	0	WF	0	WF	E	5	0	7	E	WF	0	WF	E		
	12				10		20	10				10	4		9	4		
1.0	WF	0	0	0	WF	E	WF	E	WF	WF	WF	E	WF	E	WF	E		
	17				30	20	22	20	12	10	10	15	8	10	16.5	13		
10.0	WF	0	0	0	WF	E	ND	ND	WF	E	WF	E	WF	E	WF	E		
	38				43	33			31	15	40	15	18	17	33	18		
15.0	WF	0	0	0	ND	ND	WF	E	WF	E	WF	E	WF	E	WF	E		
	43						35	30	45	40	39	25	49	35	42	32.5		

Human leucocyte migration

Melittin inhibited the migration of peripheral leucocytes from allergic and normal individuals (Fig. 1). Leucocytes from four allergic and five normal patients were equally sensitive to melittin; the standard deviations for points on the two curves overlapped. Trypan blue dye exclusion showed most of the cells inhibited by high melittin concentrations (0.0055–0.015 M) had died while leucocytes exposed to lower concentrations still lived. The lytic effect of high melittin concentrations may have masked any antigen mediated inhibitory responses in the allergic patients.

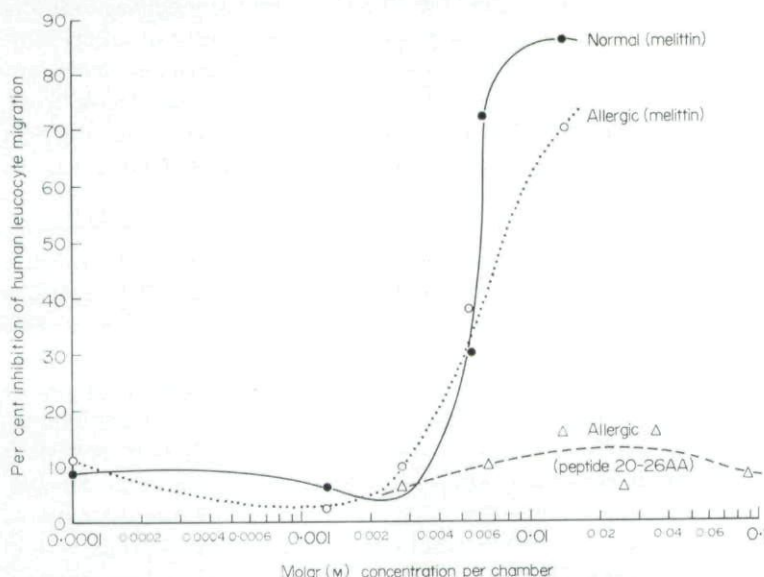


Fig. 1. Melittin inhibition of leucocytes from bee sting allergic and normal individuals. Each point on the melittin curves represents the mean inhibition obtained from sixteen capillaries per dilution in four experiments: four allergic patients (Nos. 1, 2, 3 and 5) and four normal individuals. Two experiments were done utilizing the 20-26AA heptapeptide on the leucocytes from patients 1 and 3.

The inability of even high concentrations of the non-haemolytic 20-26AA heptapeptide (0.109 M) to inhibit allergic leucocyte migration suggested that this amino acid sequence did not contain the antigenic determinant of melittin.

Passive transfer (PK-like) experiments

In passive transfer experiments into rhesus monkeys, allergic sera did not alter the intensity of the immediate inflammatory reactions induced by the challenge dose of melittin, and did not differ from reactions at sites injected with normal sera. Reaginic antibodies against melittin were not detectable by these passive type transfer experiments.

Discussion

Although melittin has been described as a poor immunogen (Habermann & El Karemi, 1956), patients sensitive to bee stings had significant inflammatory reactions 24 hr after challenge with melittin. These reactions were probably a consequence of an immunological hypersensitivity since similar late reaction did not occur in any of the six normal patients. Arthus-like (Insect Allergy Committee, 1965) and delayed-type (Bernard, 1966) reactions at the site of bee sting have been described. We were unable by either

the passive transfer (reaginic) or leucocyte migration inhibition (cellular) experiments to identify the specific nature of the immunological hypersensitivity in our five allergic patients. However, the onset of symptoms after skin testing and the experiences of laboratory workers in handling melittin were consistent with the description of reactions after insect stings (Bernard, 1966; Mackler & Kreil, 1972). Melittin therefore was an allergen in sensitive people.

Purified melittin lysed human erythrocytes and leucocytes. The 20-26AA heptapeptide did not lyse these human cells. In tests on guinea-pig (Mackler & Kreil, 1972) and rabbit (Habermann, 1968) erythrocytes, the 20-26AA heptapeptide was found to have low haemolytic activity. This activity was probably attributable to its highly basic (hydrophilic) nature. An 18-26AA nonapeptide of melittin completely lacks any haemolytic activity on both human and guinea-pig erythrocytes (Mackler & Kreil, 1972), indicating that the more proximal amino acids (Nos. 18 and 19) did not augment the haemolytic activity of the heptapeptide. Apparently only intact melittin has the ability to disrupt cells as well as synthetic membranes (Sessa *et al.*, 1966).

The absence of late melittin induced inflammation reactions in normal persons contrasts with their presence in normal guinea-pigs (Mackler & Kreil, 1972). Intracutaneous injections of melittin in guinea-pigs induced immediate-type WF reactions whose intensities increased until they reached their maximum levels at 24 hr. Frequently these inflamed sites later became necrotic. This later inflammation probably represented a greater susceptibility of guinea-pigs to the pharmacological activity of melittin since massive polymorph cell infiltration occurred without evidence of circulating antibody (Mackler & Kreil, 1972). In guinea-pigs, melittin may persist at the injection site; whereas it is rapidly removed or inactivated in normal persons. This ability to limit the effects of melittin may explain why few allergic patients (<2.8% of 2606 cases) underwent severe reactions of the central nervous system (Insect Allergy Committee, 1965; Bernard, 1966). However, in animals an injection of bee venom frequently induces neurologic changes similar to those induced in this low percentage of humans (Schneegans, 1961).

The demonstration of reaginic antibodies *in vitro* was complicated by the non-specific ability of melittin to release histamine (Jasani *et al.*, 1972a; Higginbotham & Karnella, 1971) and *in vivo* by its non-specific inflammatory activity. Our passive transfer experiments could therefore be taken as only suggestive evidence of the lack of reaginic antibody activity. Antibodies to melittin were moreover not detectable in sera from these five sensitive patients when tested by the ability of the sera to block melittin-induced histamine release from rat mast cells (Jasani *et al.*, 1972b) and by passive transfer into guinea-pig skin (Mackler & Kreil, 1972). The failure to find anti-melittin antibodies in allergic sera is consistent with the observation that antibodies to melittin have not been found in sera from bee keepers who formed antibodies against two high molecular weight constituents of bee venom—phospholipase A and hyaluronidase (Habermann, 1968; Habermann & El Karemi, 1956). It is possible that because of the peculiarities of melittin that antibodies to it may have been present in such studies (Langlois, Shulman & Arbesmann, 1965; Shulman, Langlois & Arbesmann, 1966) but were not detected by the conventional antibody assay methods.

Acknowledgments

We wish to thank the physicians and their patients for cooperation in this study and the Arthritis and Rheumatism Council for general support.

Rhesus monkeys (*Macaca mulatta*) used were generously provided by Mr Erneas Hartley, National Institute for Medical Research, Mill Hill. The technical assistance of Mr David Page and Miss Wendy Partridge is gratefully acknowledged.

References

- BARR, S.E. (1971) Allergy to hymenoptera stings. Review of the literature, 1953-70. *Annals of Allergy*, **29**, 49.
- BERNARD, J.H. (1966) Severe hidden delayed reactions from insect stings. *New York Journal of Medicine*, **66**, 1206.
- FEDERLIN, K., MAINI, R.N., RUSSELL, A.S. & DUMONDE, D.C. (1971) A micro-method for peripheral leukocyte migration in tuberculin sensitivity. *Journal of Clinical Pathology*, **24**, 533.
- HABERMANN, E. (1968a) Pharmakologie und Toxikologie von Hymenopterengiften. *Reviews of Physiology, Biochemistry and Experimental Pharmacology*, **60**, 220.
- HABERMANN, E. (1968b) *Traité de Biologie de l'Abeille* (Ed. by R. Chauvin), Vol. 3, p. 384.
- HABERMANN, E. & EL KAREMI, M. (1956) Antibody formation by protein components of bee venom. *Nature*, **178**, 1349.
- HABERMANN, E. & JENTSCH, J. (1967) Sequenzanalyse des Melittins aus den tryptischen und peptischen Spaltstücken. *Hoppe-Seyler's Zeitschrift für Physiologische Chemie*, **348**, 37.
- HIGGINBOTHAM, R.D. & KARNELLA, S. (1971) The significance of the mast cell response to bee venom. *Journal of Immunology*, **106**, 233.
- INSECT ALLERGY COMMITTEE (1965) American Academy of Allergy: insect sting allergy. *Journal of the American Medical Association*, **193**, 115.
- JASANI, B., MACKLER, B.F., KREIL, G. & STANWORTH, D.R. (1972a) (In preparation).
- JASANI, B., STANWORTH, D.R., MACKLER, B.F. & KREIL, B. (1972b) A study of the structural basis of the histamine releasing activity of certain basic polypeptides. Abst. Chem. Soc. (Brit.), Swansea, 7 April 1972.
- KREIL, G. & BACHMAYER, H. (1971) Biosynthesis of melittin, a toxic peptide from bee venom. *European Journal of Biochemistry*, **20**, 344.
- KREIL, G. & KREIL-KISS, G. (1967) The isolation of N-formylglycine from a polypeptide present in bee venom. *Biochemical and Biophysical Research Communications*, **27**, 275.
- LANGLOIS, C., SHULMAN, S. & ARBESMANN, C.E. (1965) The allergic response to stinging insects. II. Immunologic studies of human sera from allergic individuals. *Journal of Allergy*, **36**, 12.
- LAYTON, L.L., YAMANKA, E. & DENKO, C.W. (1962) Demonstration of human reagins to foods, cat dander, and insect, and ragweed and grass pollens. *Journal of Allergy*, **33**, 271.
- MACKLER, B.F. & KREIL, G. (1972) (In preparation).
- SCHNEEGANS, E. (1961) Mort néo-natale par malformation multiples graves à la suite de l'action du poison d'Abeilles. *Strasbourg Médecine*, **12**, 698.
- SESSA, G., FREER, J.H., COLACICCO, G. & WEISSMAN, G. (1966) Interaction of lytic polypeptide, melittin, with lipid membrane systems. *Journal of Biological Chemistry*, **244**, 3575.
- SHULMAN, S., LANGLOIS, C. & ARBESMANN, C.E. (1966) The allergic response to stinging insects. V. Fractionation of whole body and venom sac extracts of bee. *Journal of Allergy*, **35**, 350.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.