

Crossed Radioimmuno-electrophoretic Studies of Bee Venom Allergens

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Crossed immunoelectrophoresis (CIE) glass slides of honey bee venom showing 18 immunoprecipitates were used in crossed radioimmuno-electrophoresis (CRIE) experiments with sera from 25 patients allergic to honey bee venoms. Phospholipase A, acid phosphatase, hyaluronidase and melittin were demonstrated in the immunoprecipitates using zymographic techniques and direct hemolysis of sheep erythrocytes. The CRIE experiments verified the allergenicity of these proteins. In addition, another antigen (Ag-I) with allergenic activity was identified. This allergen is probably identical to the previously described allergen C in honey bee venom. The presence and complexity of multiple forms of the known allergenic proteins in honey bee venom, was well illustrated in the CIE/CRIE system employed.

Key words: allergens; bee venom; CRIE.

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Crossed radioimmuno-electrophoresis (CRIE) (2, 17, 19, 20) is probably the best method available today for the identification of distinct allergens in complex solutions (4, 17). The technique is based on incubation of relevant crossed immunoelectrophoresis (CIE)-plates with serum samples taken from patients with IgE mediated allergy. IgE-binding to immunoprecipitates of the allergens is visualized by incubation with ¹²⁵I-anti-IgE and subsequent autoradiography. The amount of specific IgE binding to the precipitates (or the degree of radiostaining) may be semi-quantified using a CRIE reference system (2). According to this system, CRIE classes from A (dense radiostaining) to G (weak radiostaining) are assigned to the precipitates. Definitions of major, intermediate and minor allergens have been based on CRIE (3, 17). CRIE has successfully been used to identify allergens in a number of allergen sources (17, 20).

Recently, allergens from insect venoms have been subjected to intensive research (9, 12, 14, 15). In bee venom, phospholipase A, acid phosphatase, hyaluronidase, melittin and a protein called allergen C have been identified as allergens (11, 13, 14, 16). The success of CRIE depends heavily upon the quality of the CIE-plates employed; the antiserum employed in CIE must be capable of forming immunoprecipitates with all allergens applied.

In a paper by Einarsson et al. (7) the preparation of high resolution bee venom CIE-plates is described, showing 17 immunoprecipitates detectable with Coomassie Brilliant Blue staining (7).

The purpose of the present study was to identify the allergens in the CIE pattern of unfractionated bee venom preparations directly by means of CRIE.

MATERIAL AND METHODS

Honey bee venom

Honey bee venom, which had been obtained by electrical stimulation, was obtained from Mr. C. Mraz, Champlain Valley, Middlebury, Vermont (Apis Mellifera Lots 91232 and 00500).

Antisera

Antisera to bee venom protein were raised in rabbits by multiple bi-weekly intracutaneous immunizations with 300 µg bee venom in Freund's complete adjuvant, essentially as described by Harboe & Ingild (10).

Patient sera

Sera were obtained from 25 individuals sensitive to bee venom according to criteria described by Valentine et al. (18). The patients had not been subjected to immunotherapy.

IgE

Specific and total IgE in the patient sera were determined using Phadebas RAST® and Phadebas PRIST® kits (Pharmacia Diagnostics, Uppsala, Sweden), respectively, according to the manufacturer's instructions.

Crossed immunoelectrophoresis (CIE)

CIE (5) was carried out on 8 × 8 cm glass plates, essentially in accordance with the previously described procedure (7), using a Tris/Barbital buffer (pH 8.6) in 1% agarose (Agarose A, Pharmacia Fine Chemicals, Uppsala, Sweden). Samples of 5 µl bee venom (15 g/l) were run in the first dimension electrophoresis for 60 min. The second dimension of electrophoresis was performed for 20 h with 10 V/cm at 15°C, using 13 µl antiserum per cm² gel.

Crossed radioimmuno-electrophoresis (CRIE)

The washed CIE-plates were incubated with 0.7 ml of the patient's serum diluted to 12 ml with

the CRIE incubation buffer (0.5 mol/l phosphate buffer, pH 7.4, containing 0.9% sodium chloride and 0.3% dextran 10) for 24 h at 25°C in suitable plastic boxes. The plates were washed as previously described, and incubated with approximately 250,000 cpm ¹²⁵I-anti-IgE (Phadebas RAST Isotope Reagent) in 12 ml total volume for another 24 h. The plates were washed, dried and placed on X-ray photographic film for autoradiographic exposure. All plates were exposed several times, using time periods varying from a few hours to several weeks, so that each exposure corresponded to one particular CRIE class, using a CRIE reference system (2). The exposure periods were increased in a two-fold manner; the shortest period denoted CRIE class A and the longest exposure period denoted CRIE class G. Each precipitate giving rise to radiostaining, was said to be in a CRIE class corresponding to the shortest exposure period necessary to give visible radiostaining.

Identification of known bee venom components in the CIE pattern

In the accompanying paper by Einarsson et al. (7), a more detailed description is given of the identification of known bee venom components in the CIE pattern. The various bee venom enzymes were identified by zymographic techniques. Additional agarose gels, containing various substrates, were molded on top of the CIE plates. Acid phosphatase activity was assayed by hydrolysis of the chromogenic substrate *p*-nitrophenyl-phosphate. Phospholipase A activity was assayed by using lecithin as substrate. Hyaluronic acid was used as substrate for hyaluronidase. Melittin was measured by its direct hemolytic activity, using washed sheep erythrocytes as substrate. In general, hydrolysis was visualized as stained or cleared regions. The identifications based on zymography, were verified in CIE modifications by combining isolated bee venom components with the crude bee venom preparation (7).

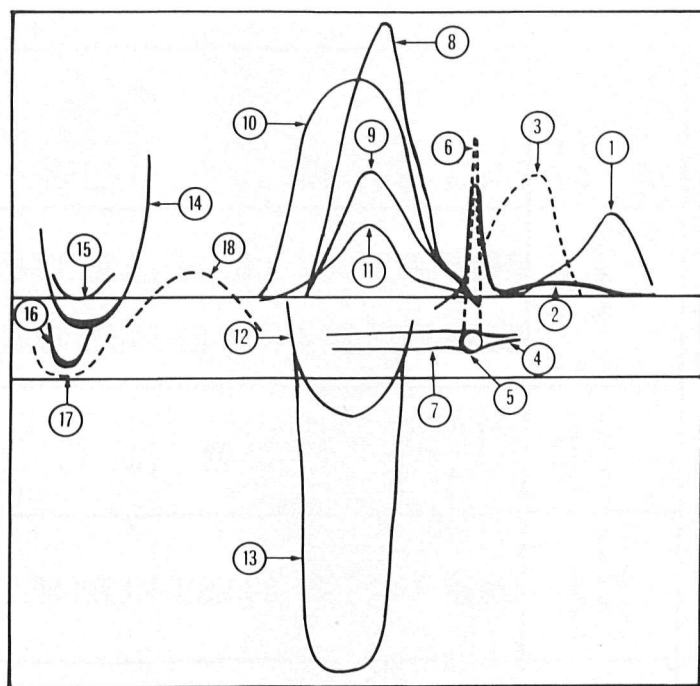


Fig. 1. CIE reference pattern of bee venom with arbitrary numbers assigned to the precipitates (7).

RESULTS

The CIE reference pattern of bee venom is demonstrated in Fig. 1 with arbitrarily numbered immunoprecipitates. The various enzyme activities associated with the immunoprecipitates are shown in Table 1. CRIE was performed with 25 individual sera prior to protein staining of the CIE plates. Fig. 2 shows examples of CRIE-films thus obtained. The CRIE results are summarized in Table 1.

Precipitates 8 and 9 (representing hyaluronidase) were recorded together. The phospholipase A-containing precipitates Nos. 12 and 13 showed partial identity and were recorded together. Another molecular form of phospholipase A, antigen No. 11, was not recorded, due to its position within the hyaluronidase precipitates. The melittin-containing precipitates Nos. 14, 15, 16 and 17 were recorded as a group.

The antigen in precipitate No. 2 (representing acid phosphatase) did not appear to be

homogeneous. In the CIE pattern it consisted of a peak connected to a curved line. In CRIE, some patients showed specific IgE-binding mainly to the peak, others mainly to the linear part of the precipitate.

Interpretation of radiostaining corresponding to precipitates 4, 5, 6 and 7, was difficult. These precipitates were connected to the application well. In spite of this, clearly different and characteristic radiostaining patterns appeared corresponding to these precipitates (Fig. 2).

When one patient serum was used (A.M.), radiostaining was observed corresponding to a precipitate (Ag-18) which was not visible following Coomassie Brilliant Blue staining.

No definite pattern could be established when trying to relate the CRIE pattern for individual patients to the total IgE-levels, RAST results or clinical picture.

As controls, CIE plates were incubated with saline instead of the patient serum. No radiostaining at all was seen corresponding to the control plates, even in CRIE class G.

Table 1

Total IgE and specific IgE against bee venom, and CRIE radiostaining of the different precipitates in a CIE reference pattern of bee venom. The intensity of the radiostaining is shown as hatched squares symbolizing the CRIE classes (A-G)

Patient	IgE U/ml	Antigen No RAST PRU/ml	Allergen C 1 3	Acid phosphatase 2 4 6	Acid phos- phatase and mellitin 5	Phospholi- pase A and mellitin 7	Hyaluro- nidase 8/9	Phospholipase A 10 (11) 12/13	Mellitin 14/15/16/17	18
I.L.	50	53								
K.N.	450	30								
M.G.	780	14.5								
E.B.G.	10	6.6								
J.N.	50	6.4								
J.M.	40	6.1								
S.S.	70	5.5								
J.E.T.	180	5.2								
B.A.L.	350	4.6								
S.E.H.	50	4.4								
P.O.B.	2080	4.0								
B.B.	40	4.0								
M.W.	80	3.9								
E.O.	30	2.7								
L.J.	30	2.7								
O.R.	50	2.6								
S.C.	250	2.5								
G.H.A.	30	2.5								
S.G.	460	2.3								
A.M.	170	1.9								
I.M.H.	30	1.6								
G.B.P.	125	1.0								
A.S.	170	0.5								
T.D.	370	0.5								
B.J.	7.	0.3								

CRIE CLASSES



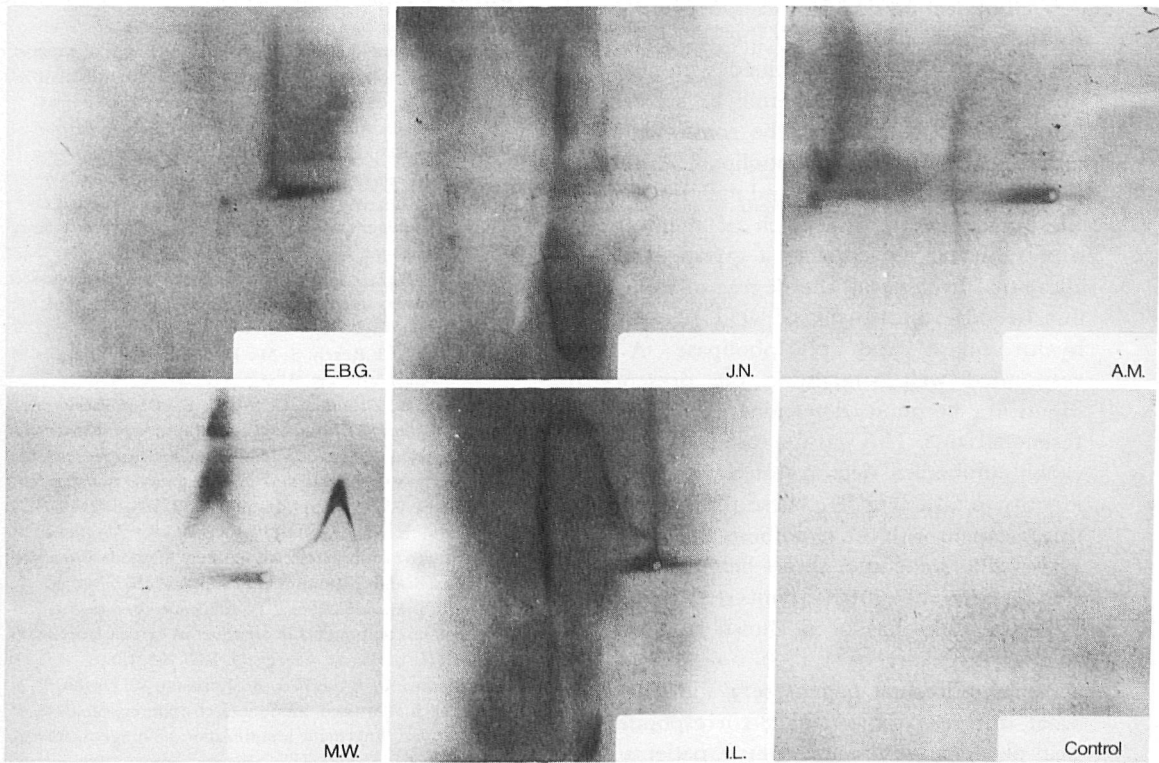


Fig. 2. X-ray films from CRIE experiments with five sera and a control.

DISCUSSION

The CIE pattern employed seemed to have the necessary resolution and to contain immunoprecipitates of all known important allergenic components in honey bee venom. Although Coomassie Brilliant Blue staining revealed 17 precipitates, the final number of precipitates in the reference pattern (Fig. 1) is 18, since an additional precipitate was demonstrated by CRIE. Although the pattern contained 18 precipitates, several of them represented the same enzymatic activity (7).

The most important known allergens in honey bee venom; phospholipase A, acid phosphatase, hyaluronidase and melittin all gave rise to radiostaining in CRIE, thus showing good agreement between previous findings and the present study. In addition to the enzymes

described, seven of the 25 sera were shown to have specific IgE against antigen No. 1 (Ag-1). It is suggested that Ag-1 is identical to allergen C, described by Hoffman et al. (13). One precipitate (Ag-18) was detected only in CRIE, using a particular patient serum (A.M.). Ag-18 may be a variant of melittin (complexed with another protein) or an unidentified protein/peptide component that is present in concentrations too low to be detected with protein stain (Coomassie Brilliant Blue). Only one immunoprecipitate (Ag-3) did not bind IgE from any of the sera used, and must be regarded as a non-allergenic antigen in bee venom.

Radiostaining corresponding to precipitates 4, 5, 6 and 7, was difficult to interpret because these precipitates were fuzzy, and tailed back to the application well. Possibly these precipitates contained partially denatured material.

A complicating factor was the presence of multiple forms of several of the allergens. The present CRIE study, combined with the previous zymographic and hemolytic studies (7), demonstrated that it may be somewhat oversimplified to regard phospholipase A, hyaluronidase, acid phosphatase and melittin as single allergens (11, 14, 16). Each of them should preferably be regarded as a group of similar allergens. In general, the degree of radiostaining to different forms of acid phosphatase, hyaluronidase and phospholipase A corresponded well, suggesting the presence of identical allergenic determinants on the different variants. This was the case even when the rabbit antibodies demonstrated partial or no identity in CIE (Fig. 1). These results would be in agreement with the hypothesis that the rabbit IgG-system sometimes shows better discrimination between molecular variants than the human IgE-system, as has been shown for cod and hazel pollen allergens (1, 6, 8).

Some individual patient sera showed somewhat different radiostaining corresponding to acid phosphatase precipitates (i.e. patients K.N., S.E.H. and B.B.). This may suggest minor differences in allergenic determinants on the various molecular forms. Alternatively, the differences may be caused by different parts of the molecules being available for IgE binding in the various precipitates.

CIE/CRIE has allowed a study of the individual bee venom components with respect to allergenicity without purification of the components. It is interesting to note that if CRIE had been the sole technique used to study allergens in honey bee venom, the present study would have indicated that the number of distinct allergens present in the venom was considerably higher than the five allergens previously described (11, 13, 14, 16). This would have been misleading, and the combination of CRIE and the accompanying zymographic/hemolytic study (7), however, has given detailed, and probably more correct, information regarding allergens in honey bee venom. This illustrates that it may be advantageous to combine different techniques in analyzing CIE patterns.

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