

Determination of Specific IgG Antibody by Crossed Radioimmuno-electrophoresis

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A crossed radioimmuno-electrophoretic method was developed for detection of honey bee venom specific IgG antibodies in patient sera. At the serum concentration 1/200 the contrast between specific binding and background was the most favourable. The detection limit was fairly low, approximately 30 kU/l (IgG RAST units). A reference system based on the reference kits in Phadebas IgG-RAST[®] was elaborated.

Key words: antibody; crossed radioimmuno-electrophoresis; IgG.

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CLINICAL ASPECTS

The IgG-CRIE method would enable detailed information of the IgG-antibody response to the various components in a complex allergen extract, e.g. during hyposensitization treatment.

The appearance of "blocking" IgG-antibodies during hyposensitization therapy is regarded as a sign of effective therapy (12). The outcome of the treatment seems to be related to the IgG antibody response even though the relationship is not necessarily causal (11). Various techniques have been developed for measurement of IgG allergen-specific antibodies such as IgG-RAST (13), enzyme-linked immunosorbent assay (ELISA) (15), Sepharose protein-A assay (7, 9) and haemagglutinating techniques (3, 10). All these methods allow detection of the total amounts of allergen specific IgG antibodies. However, to obtain detailed information about the IgG antibody response towards the individual components of a complex allergen extract, the use of these methods requires separation and purification of the individual allergens.

Crossed immunoelectrophoresis (CIE) and crossed radioimmuno-electrophoresis (CRIE) permit direct identification of individual antigens and allergens respectively in complex allergen extracts without previous separation of the individual components (16). The use of a

CRIE-reference system enables semiquantification of the amount of specific IgE-binding to the various immunoprecipitates in the CIE-plate (1). A corresponding technique for detection of allergen-specific antibodies would probably prove valuable, e.g. when monitoring the antibody response during hyposensitization therapy. We have developed an IgG-CRIE procedure permitting identification of individual IgG-binding antigens in honey bee venom.

MATERIAL AND METHODS

Allergen

Honey bee venom (*Apis mellifera*, lot 00500), obtained by electrical stimulation was bought from Mr. C. Mraz, Champlain Valley, Middlebury, Vermont.

Antisera

Antisera to bee venom proteins were raised in rabbits (New Zealand White) by biweekly subcutaneous injections of 300 µg bee venom in

Freund's complete adjuvant, essentially according to Harboe & Ingild (8).

Crossed immunoelectrophoresis (CIE)

CIE was performed essentially as previously described (6), using a 0.025 M TRIS/Tricine buffer (Bio-Rad), pH 8.6 in 1% Agarose (Agarose A, Pharmacia Fine Chemicals, Uppsala). Samples of 5 μ l bee venom (15 g/l) were run in the first dimension electrophoresis at 10 V/cm for 55 min. The second dimension electrophoresis was performed at 2 V/cm for 18 h using 14 μ l rabbit antiserum per cm^2 gel.

Patient sera

Serum from a patient subjected to hyposensitization treatment with honey bee venom was used for the methodological work. Examination of the serum using Phadebas IgG-RAST[®], revealed a content of approximately 480 kU/l, expressed in relation to the Phadebas IgG-RAST reference serum, arbitrarily defined to contain 1,000 kU/l. As the negative control a pool of non-treated patient sera, which in IgG-RAST gave only background values (< 20 kU/l), was used. This serum pool is referred to as the control serum.

In order to obtain sera with known amounts of honey bee venom specific IgG antibodies, keeping other serum protein concentrations at a relatively constant level, serial dilutions of the patient serum were performed in control serum (1:1, 1:2, 1:4, 1:8, 1:16, 1:32). These sera were further diluted in buffer solution to an appropriate concentration for the CRIE assay.

¹²⁵I-anti-IgG antibodies

In all assays the ¹²⁵I-anti-IgG-antibody developed for Phadebas IgG-RAST was used (5).

Crossed radioimmuno-electrophoresis (CRIE)

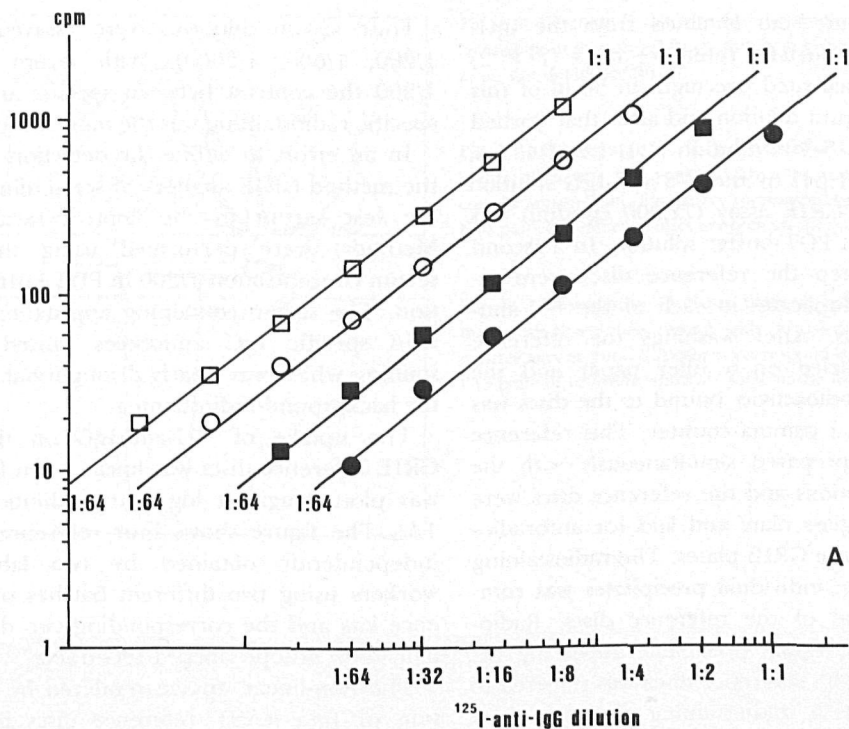
IgG-CRIE was performed essentially as described for IgE-CRIE (2). The washed CIE plates were incubated in plastic boxes overnight in 10 ml diluted patient serum. As the negative

control, control serum was used diluted to the same degree. Four different serum concentrations, 1/20, 1/200, 1/600 and 1/2000, of both the test serum and the control serum were tested in this incubation as well as only buffer solution. The sera were diluted in a phosphate/dextran/Tris (PDT) buffer solution (0.05 mol/l phosphate buffer, pH 7.4, with 0.15 mol/l sodium chloride, 0.3% Dextran 10) and 0.3% Tween. The use of this buffer in the experiments was compared to that of a protein containing buffer solution (0.05 mol/l phosphate buffer pH 7.4, containing 0.15 mol/l sodium chloride, 1 g/l albumin, 170 mg/l rabbit- γ -globulin and 0.3% Tween). After the serum incubation the plates were washed for 4 $\times$ 30 min using 10% PDT buffer solution in 0.15 mol/l sodium chloride. The plates were re-incubated overnight with 10 ml ($\sim$ 72,000 cpm/ml) ¹²⁵I-anti-IgG diluted in one of the buffer solutions. The plates were washed 4 $\times$ 30 min, the last time with distilled water, dried and placed on X-ray photographic film. Autoradiography was performed at -70°C using Du Pont Cronex-4 X-ray films in Kodak Cassettes with Du Pont Lighting Plus Intensifying Screen (14). Sequential exposures were performed and the radiostaining of the different precipitates was compared to that of the discs of a reference system.

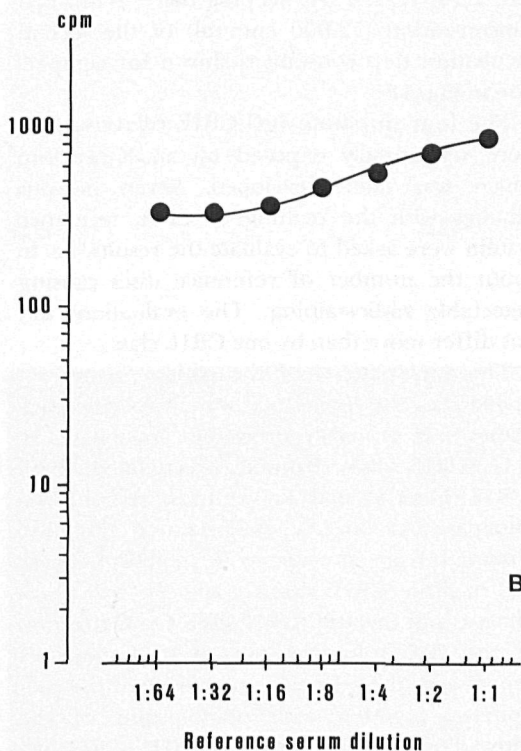
CRIE-reference system

Commercially available Phadebas IgG-RAST reference kits (Pharmacia Diagnostics AB, Uppsala, Sweden) were used in order to establish an IgG-CRIE reference system similar to that described in IgE-CRIE (1). In the IgG-RAST system a standardized set of honey bee venom discs and a pool of sera containing honey bee venom specific IgG antibodies are used as a reference. This reference system is analogous to that used in Phadebas RAST (Pharmacia Diagnostics AB, Uppsala, Sweden) and in IgE-CRIE (1) in which birch pollen discs and a pool of sera from birch pollen allergic patients are used.

For the IgG-CRIE reference system 500 μ l of the reference serum pool was diluted in 750 μ l



A



B

Fig. 1. Radioactive uptake of ^{125}I -anti-IgG on the Phadebas reference discs, means of duplicates.

A) Phadebas reference serum concentration was kept constant while the isotope concentration was varied. Results produced by two laboratory workers (circles and quadrants), using two different batches of Phadebas IgG RAST® reference system (full and open symbols).

B) Effect of varying the reference serum at constant amount of isotope concentration ($\sim 72,000$ cpm/ml).

of the diluent, both obtained from the IgG-RAST kits. Fourteen reference discs (7×2) were each incubated overnight in 50 μ l of this reference serum dilution and after that washed 3×10 min. Serial dilution (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64) of the ^{125}I -anti-IgG solution used in the CRIE assay (72,000 cpm/ml) was performed in PDT-buffer solution. In a second incubation step the reference discs were incubated in duplicates in each of the ^{125}I -anti-IgG dilutions. After washings the reference discs were dried on a filter paper and the amount of radioactivity bound to the discs was measured in a gamma counter. This reference system was prepared simultaneously with the CRIE incubations and the reference discs were taped on a glass plate and laid for autoradiography with the CRIE plates. The radiostaining caused by the individual precipitates was compared to that of the reference discs. Radiostaining of a certain precipitate appearing before that of the reference discs was referred to as CRIE-class A, radiostaining of a precipitate appearing when one pair of the duplicate reference discs, the one incubated with the strongest isotope dilution (1:1), was visible, was referred to as CRIE class B and so forth, analogous to that described for IgE-CRIE (1). In this way a complete CRIE classification of the patient serum was performed.

RESULTS

Patient as well as the pooled control serum gave a faint diffuse radiostaining of the agarose gel of the entire CIE plate. This background radiostaining decreased markedly with the serum dilution and was practically absent when only buffer solution was used. Thus, the observed background radiostaining was caused by unspecific binding of IgG to the agarose gel. A weak background radiostaining from the honey bee venom precipitates 7 and 10 (11) was also observed after extended exposures (CRIE class F). The buffer solution containing rabbit gammaglobulin and albumin gave the same degree of background binding to both the gel and the precipitates as did the PDT buffer solution.

Four serum dilutions were assayed (1/20, 1/200, 1/600, 1/2000). With serum diluted 1/200 the contrast between specific and non-specific radiostaining was the most favourable.

In an effort to define the detection limit of the method CRIE analyses of serial dilutions of the test serum in the control serum (see Methods) were performed using the total serum concentration 1/200 in PDT buffer solution. The serum containing approximately 30 kU/l specific IgG antibodies caused radiostaining which was clearly distinguishable from the background radiostaining.

The uptake of ^{125}I -anti-IgG on the IgG CRIE reference discs was linear when log cpm was plotted against log isotope dilution (Fig. 1A). The figure shows four reference curves independently obtained by two laboratory workers using two different batches of reference kits and the corresponding two different batches of isotope labelled IgG-tracer.

The non-linear uptake rendered by incubation of IgG RAST reference discs in serial dilutions of the reference serum (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64) keeping the ^{125}I -anti-IgG concentration (72,000 cpm/ml) of the second incubation step constant is shown for comparison in Fig. 1B.

The four duplicate IgG-CRIE reference kits were sequentially exposed on an X-ray film which was later developed. Seven persons familiar with the reading of CRIE reference system were asked to evaluate the results, i.e. to count the number of reference discs causing detectable radiostaining. The evaluations did not differ more than by one CRIE class.

The IgG pattern of the patient serum revealed that the honey bee venom specific antibodies were primarily directed to precipitates 10 (11) (CRIE class B) and precipitates 12/13 (CRIE class C) – both known to represent phospholipase A. Specific radiostaining was also observed from precipitate 7 – phospholipase and melittin (CRIE class C), and 5 – acid phosphatase and melittin (CRIE class C). A diagram of the CIE reference picture of honey bee venom with the arbitrary numbering of the precipitates, together with photographs of the autoradiographic patterns in CRIE of the pa-

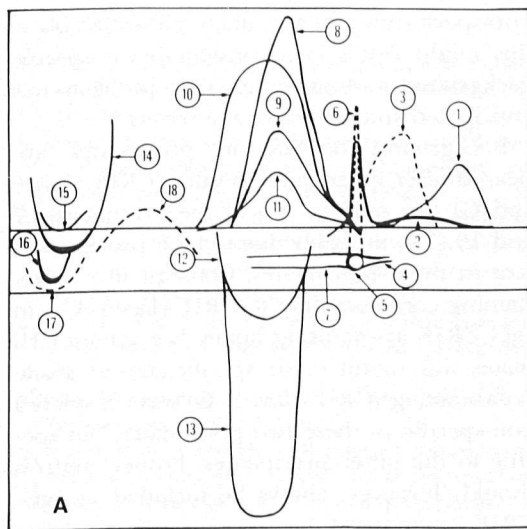
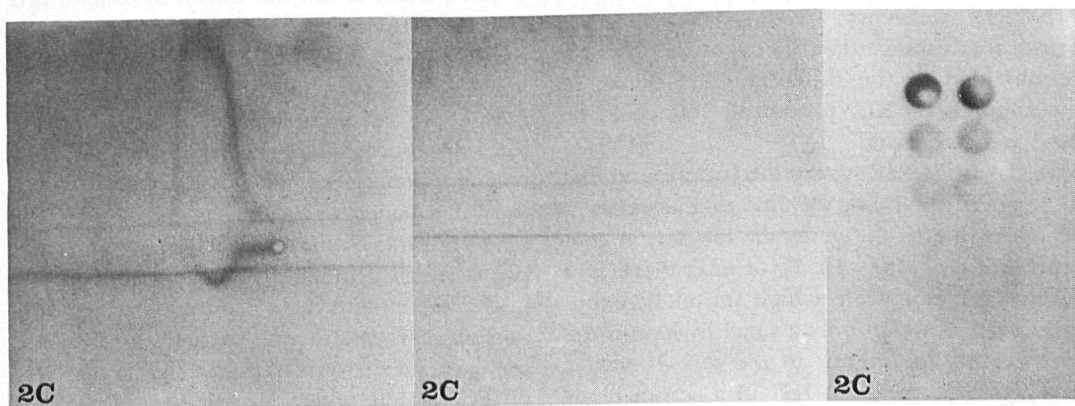
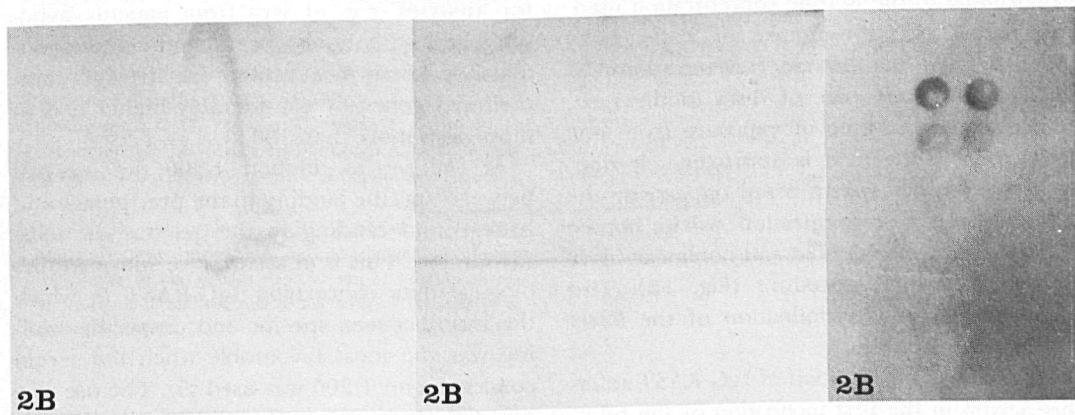


Fig. 2A. Diagram of the crossed immunoelectrophoresis reference pattern with arbitrary numbers given to the precipitates. For details see (6).

Fig. 2B. Photographs of X-ray films of IgG-CRIE experiments of honey bee venom with a patient serum and a control serum pool. Exposures corresponding to CRIE class E (4 pairs of reference discs are seen on the X-ray film).

Fig. 2C. Photographs of X-ray films of IgG-CRIE experiments of honey bee venom with a patient serum and a control serum pool. Exposures corresponding to CRIE class F (5 pairs of reference discs are seen on the X-ray film).



tient serum, the control serum and the corresponding reference discs are shown in Fig. 2.

DISCUSSION

The IgG-CRIE method allows detection and semiquantification of serum IgG-antibodies to the individual components of honey bee venom. This is possible without prior isolation and purification of the various components of the allergen extract. If comparisons between assays performed on different occasions and at different laboratories are required, the use of a reference system is mandatory. This is due to the instability of the radiolabelled tracer system. The described reference system is analogous to that used in IgE-CRIE (1). The uptake of ^{125}I -anti-IgG on honey bee venom reference discs preincubated with a reference serum dilution was proportional to the isotope concentration used. This means that the uptake of radioactivity doubled when the isotope concentration was doubled from one pair of discs to the next. Consequently, the time of exposure from one CRIE class to the next is approximately doubled. A reference system based on varying the reference serum concentration would not be feasible due to the very flat and nonlinear curve rendered by this procedure (Fig. 1B). This would not make discrimination of the lower CRIE classes possible.

We chose a concentration of IgG RAST reference serum in the first incubation of the reference discs, which gave approximately the same binding of radioactivity to the reference discs in the IgG-CRIE reference system as that in the IgE-CRIE reference system. This gave the practical advantage that the exposure times recommended in the different CRIE-classes in the rapid IgE-CRIE procedure (14) could be used as a guideline.

In the IgE-CRIE system the background due to unspecific binding of IgE to the plates is very low due to the generally low serum concentrations of IgE. In IgG-CRIE there is a reverse situation with a high serum IgG concentration of which only a small fraction is the specific IgG of interest in the test. If only a small part of the total IgG in a serum binds

non-specifically when incubating the CIE plate, this might result in a considerable unspecific background radiostaining. This problem was anticipated and proven by the results.

Background binding to precipitates appeared after extended exposure (CRIE class F and G) and only to two of the precipitates, 7 and 10 (11), probably due to high protein content in these precipitates. Consequently radiostaining corresponding to CRIE-classes A–E in IgG CRIE assays using honey bee venom CIE plates was found to be specific. Lower grade radiostaining (CRIE class F, G) was considered non-specific to these two precipitates, but specific to the other precipitates. Proper controls should, however, always be included in IgG-CRIE experiments.

The detection of approximately 30 kU/l for specific radiostaining appears to be satisfactory for analyses, e.g. of sera from patients hypersensitized with honey bee venom. Their contents of honey bee venom specific IgG antibodies is generally considerably higher and in most cases more than 100 kU/l (4, 17).

At the serum dilution 1/200 the contrast between specific binding to the precipitates and background binding to the gel was the most favourable. This is in accordance with methodological data concerning IgG-RAST in which the ratio between specific and unspecific binding was the most favourable when the serum concentration 1/200 was used (5). The use of a protein containing buffer did not appreciably decrease the unspecific binding of IgG to gel or precipitates.

The patient serum was shown to contain IgG antibodies to several bee venom precipitates in the CIE plate, all known to contain phospholipase-A. This substance is also known to be the most important allergen of bee venom (2).

Comparison of the IgE and the IgG antibody response patterns to honey bee venom in hypersensitized patients as well as in bee keepers are made possible by the IgG-CRIE method. It may prove valuable in allergy research and possibly in therapy monitoring of patients during hypersensitization treatment. Further studies are, however, needed to investigate the applicability of IgG-CRIE in other allergens.

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