

A Modified Crossed Radioimmuno-electrophoretic Method for Determination of Allergen-Specific IgG Antibodies

Elimination of Non-Specific Binding by use of F(ab')₂-Fragments of Rabbit Antibodies and ¹²⁵I-Labelled Protein A

IB SØNDERGAARD, RENÉ DJURUP and BENT WEEKE

Medical Dept. TA, Division of Allergology and Laboratory for Medical Allergology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

A crossed radioimmuno-electrophoretic (CRIE) method for detection of specific IgG antibodies in patients' sera against horse hair and dander was developed. The unacceptably high non-specific binding encountered when substituting ¹²⁵I-labelled antihuman IgG for ¹²⁵I-labelled antihuman IgE in an ordinary CRIE was eliminated by the combined use of ¹²⁵I-labelled Protein A as detector, and F(ab')₂-fragments of the allergen-specific rabbit antibodies. The low background binding thus obtained makes the method useful for detection of specific IgG in sera where the ratio between specific and non-specific IgG is low. Therefore the method should also be applicable to other antigen/allergen systems.

Key words: IgG antibodies; immunotherapy.

Accepted for publication 8 September 1983

CLINICAL ASPECTS

A generally applicable IgG-CRIE method would be of interest for monitoring the specific IgG response during immunotherapy, especially in the evaluation of new hyposensitization regimens (e.g. with chemically or otherwise modified allergens). In this context it would be of interest to compare the IgG and the IgE response, thus enabling prediction of the therapeutic response at an earlier phase of the immunotherapeutic regimen. Investigations along these lines are now possible.

Crossed radioimmuno-electrophoresis (CRIE) has been used for several years to determine patients' IgE specificities and to identify allergens (1, 8, 16, 17). Determination of IgG against allergens ("blocking antibodies") has been accomplished by several methods (2, 3, 5, 9, 11, 12, 13, 18), but no general methods for detailed studies on IgG specificities are yet available although some attempts have been made (10, 14). The present paper describes a general method for studying IgG specificities by using CRIE, thus enabling the study of similarities and differences in the IgE and the IgG reactivity in allergic patients. This may lead to a better

understanding of the role of IgG during immunotherapy, and may predict the success or failure of a given immunotherapeutic regimen at an earlier stage of the treatment. In the present study extract of horse hair and dander was used as test system.

MATERIAL AND METHODS

Antigen

A standardized extract of horse hair and dander (SQ 552) was purchased from Allergo-

logisk Laboratorium (ALK), Copenhagen, Denmark. The extract contains all the major allergens as measured by IgE-CRIE.

Sera

Serum from a patient allergic to horse hair and dander and undergoing immunotherapy with an extract of horse hair and dander was used in the experiments.

Normal human sera (NHS) were supplied by the hospital's blood bank. No history concerning the donors could be obtained.

Antiserum, immunoglobulins and immunosorbent

Antibodies against horse hair and dander were raised in rabbits using established procedures (4). The immunoglobulin fraction was isolated as described (4) and the $F(ab')_2$ -fragments of the IgG were prepared as follows:

To 20 ml antibody solution containing approximately 8 mg/ml IgG is added 7.7 g $(NH_4)_2SO_4$ (resulting in 50% saturated ammonium sulphate) under stirring. The solution is transferred to centrifugal tubes and after 1 h at room temperature the precipitate is isolated by centrifugation for 20 min at 2000 *g*. The precipitate is dissolved in 5 ml 0.1 M acetate buffer pH 4.5 and the solution dialysed overnight at 4°C against the same buffer. To this solution is then added 3.2 mg pepsin (pepsin: IgG ratio = 1:50 (w/w)) obtained from Sigma (P7012) St. Louis, USA, and the digestion proceeds at 37°C for 24 h. Digestion is stopped by adding 3 ml 1.0 M Tris-HCl containing 0.003% chlorhexidine. From the now clear solution the $F(ab')_2$ -fragments are isolated by gel filtration on a sephacryl S-200 (Pharmacia, Uppsala, Sweden) column 2.5 × 100 cm at a flow of 0.25 ml/min. The elution buffer is 0.1 M Tris-HCl, 0.2 M NaCl pH 7.7 containing 0.002% chlorhexidine. The isolated $F(ab')_2$ -fragments are concentrated by adding 0.58 g of $(NH_4)_2SO_4$ /ml (resulting in a 75% saturated ammonium sulphate solution) under stirring. After 1 h at room temperature and centrifugation for 20 min at 2000 *g* the precipitate is dissolved in 10 ml 0.9% NaCl containing 1.5 mM NaN_3 and finally dialysed

against the same solution at 4°C overnight. The purified $F(ab')_2$ fragments are kept frozen until use.

Protein A was obtained from Pharmacia (Uppsala, Sweden). Sepharose coupled anti-human IgE was obtained from the IgE test kit (Pharmacia, Uppsala, Sweden). Affinity purified goat antihuman IgG was purchased from E-Y Laboratories (San Mateo, Ca., USA, cat. No. AF-001).

Crossed immunoelectrophoresis (CIE)

CIE was performed as previously described (15) using 1% agarose (LSA, Litex, Denmark) in a buffer containing 0.024 M sodium barbital, 0.073 M Tris and 0.01 M NaN_3 , pH 8.6.

In the first dimension 1 µl of the standardized extract was run for 30 min at 10 V/cm. The second dimension electrophoresis was run at 2 V/cm for 18 h using 10 µl of the isolated $F(ab')_2$ fragments/cm² gel.

The immunoplates were washed, pressed and dried in cold air as described previously (15).

¹²⁵I-labelled protein A and ¹²⁵I-labelled antihuman IgG

Protein A was labelled with ¹²⁵I by use of the Boulton-Hunter reagent with a modification of a method described previously (7). The solution of ¹²⁵I-labelled Boulton-Hunter reagent (Amersham, UK) in benzene (0.75 mCi) was evaporated to dryness under nitrogen. A solution of protein A (375 µl of a solution containing 0.5 mg/ml) in 0.1 M borate buffer, pH 8.5, was added and incubated for 15 min with occasional shaking. Then a solution of glycine (375 µl of a solution containing 1 mg/ml) in 0.1 M borate buffer pH 8.5 was added. After 30 min the labelled protein A was isolated on a PD 10 column (Pharmacia, Uppsala, Sweden) previously washed with 50 mM phosphate buffer pH 7.5 containing 0.25% gelatin and 400 mg/l NaN_3 .

¹²⁵I-labelled antihuman IgG was prepared by the chloramine-T method exactly as described elsewhere (3).

CRIE procedure

The dried immunoplates were placed in plastic lids and incubated for 3 h with 200 μ l serum in 7 ml 0.07 M phosphate buffer pH 7.0. The plates were washed 10 times in 0.15 M NaCl and incubated for 3 h with ^{125} I-labelled protein A or anti-IgG (8×10^5 cpm) or with ^{125} I-labelled anti-IgE (4×10^5 cpm) in 7 ml of phosphate buffer 0.05 M, pH 7.5 containing 0.3% bovine serum albumin, 0.15 M NaCl, 15 mM NaN_3 and 2.7 mM $\text{Na}_2\text{-EDTA}$. Finally, the plates were washed 10 times with 0.15 M NaCl, dried in hot air and placed in an X-ray cassette for autoradiography. The specificity of the reaction was tested in several ways. Inhibition with free antigen was tested by incubation of 200 μ l serum with 50 μ l horse extract. The influence of IgE on the detection of IgG was studied by CRIE experiments before and after removal of IgE by incubation of 1 ml serum for 16 h with 1 ml sedimented sepharose coupled antihuman IgE.

RESULTS

Fig. 1 shows the detection of IgG antibodies against horse hair and dander antigens by CRIE, as well as the control experiments. Fig. 1A and B shows the binding when patient serum is used compared with serum from a 1000 donor pool. The inhibition of the reaction between IgG and the different horse antigens in the gel by free antigen is shown in Fig. 1C.

In order to study the influence of IgE on the detection of IgG, CIE plates were incubated with serum before and after removal of about 90% of the total IgE by sepharose coupled antihuman IgE (350 KU/l before, 42 KU/l after) (Fig. 1D–F). It can be seen that removal of IgE has no influence on the detection of IgG by ^{125} I-labelled protein A. The use of ^{125} I-labelled antihuman IgE as detector shows that IgE has in fact been removed (Fig. 1E) as compared with the IgE CRIE in Fig. 1J. When the differences in binding between patient serum and the 1000 donor pool, using the three different detector proteins (protein A, anti-IgG and anti-IgE), are evaluated it is clear that only protein A

and anti-IgE can differentiate between the sera (Fig. 1A and 1B; 1G and 1H; 1J and 1K). Furthermore, it can be seen that this patient reacts with IgE and IgG antibodies against the same antigens.

^{125} I-labelled protein A gives a background radiostaining on the gels but there is no problem of unspecific binding to the precipitates in the gel.

DISCUSSION

Detection of IgE antibodies to the different antigens in complex mixtures has been possible for many years, but the application of this technique to the detection of IgG antibodies has only been successful when detecting antibodies to honey bee venom (10). This has been due to the high non-specific background binding of the antihuman IgG used. The reason for the success in detecting IgG to honey bee venom is the favourable ratio between specific and non-specific IgG in this system permitting extreme dilution of the serum.

In the present study it was shown that ^{125} I-labelled protein A is superior to affinity purified antihuman IgG. The binding of protein A to the rabbit antibodies in the gel could easily be prevented by pepsin digestion of the rabbit antibodies and isolation of the F(ab')_2 -fragments.

The specificity of the detection of IgG antibodies against the different antigens precipitated in the gel has been established for different reactions involved. First, the inhibition of the binding of IgG to the precipitates by free antigens together with the fact that normal human serum IgG does not bind to the precipitates, proves the specificity of the IgG binding. Second, the fact that removal of IgE from the patient's serum does not change the radiostaining when ^{125} I-labelled protein A is used as detector protein clearly indicates the usefulness of this detector in IgG-CRIE. However, the use of ^{125} I-labelled affinity purified antihuman IgG as detector protein failed to differentiate between patient serum and NHS. This might be due to reactivity of this antibody against determinants on the rabbit F(ab')_2 -fragments.

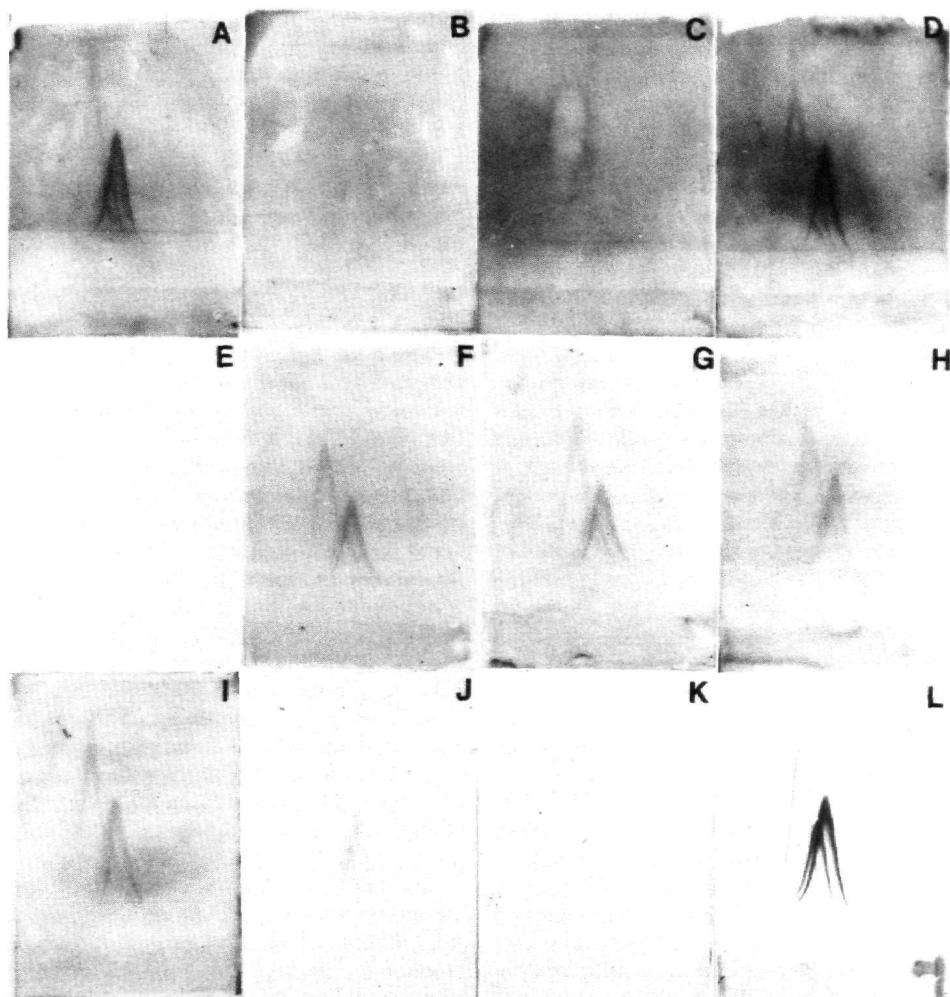


Fig. 1. Photographs of X-ray films of IgE- and IgG CRIE experiments on antigens from horse hair and dander.

Fig. 1A: Patient serum and ^{125}I -labelled protein A as detector.

B: NHS pool and ^{125}I -labelled protein A as detector.

C: Patient serum inhibited with free antigens and ^{125}I -labelled protein A as detector.

D: Patient serum absorbed with anti-IgE and ^{125}I -labelled protein A as detector.

E: Patient serum absorbed with anti-IgE and ^{125}I -labelled anti-IgE as detector.

F: Patient serum absorbed with anti-IgE and ^{125}I -labelled anti-IgG as detector.

G: Patient serum and ^{125}I -labelled anti-IgG as detector.

H: NHS pool and ^{125}I -labelled anti-IgG as detector.

I: Patient serum inhibited with free antigens and ^{125}I -labelled anti-IgG as detector.

J: Patient serum and ^{125}I -labelled anti-IgE as detector.

K: NHS pool and ^{125}I -labelled anti-IgE as detector.

L: CIE-plate stained with Coomassie Brilliant Blue.

For details of the CIE and the IgG-CRIE see text.

In the preparation of rabbit IgG F(ab')₂-fragments only established laboratory procedures are used, thus little extra work is involved in the standard CRIE procedure. Other methods for detection of IgE and IgG antibodies to the same antigens have been described previously. These include a method for electrophoretic transfer from polyacrylamide gels onto a nitrocellulose membrane and subsequent incubation with serum and ¹²⁵I-labelled antihuman IgE or protein A (14). Another means of detecting antibody binding zones in gels is to fix the proteins before reaction with antibodies, using glutaraldehyde (6). The main advantages of the present method are that detection of IgE with CRIE is such a well known procedure that the results from the two systems can be compared and, furthermore, both the identity and cross-reactivity of antigens can be analysed by established procedures. In isoelectric focusing cross-reacting proteins are sometimes difficult to detect. Many years of work with the CRIE method has shown that the risk of not detecting important allergens in antigen extracts is very small because careful preparation of rabbit antibodies will give antibodies against almost every antigen. However, with this technique, low molecular weight antigens might be too small to be precipitated and thus go undetected. However, as these antigens will be detected by RAST analysis with dialysed and non-dialysed material and a pool of patient sera, this one point of criticism against the CRIE technique is not valid.

The technique will be useful for studies on IgG/IgE response to different allergens during immunotherapy and might give an early prediction of the outcome of hyposensitization.

ACKNOWLEDGEMENTS

We wish to thank Lisbeth Abrahamsen for excellent technical assistance. The Danish Medical Research Council and the University of Copenhagen are thanked for financial support.

REFERENCES

1. Aukrust, L.: Crossed radioimmuno-electrophoretic studies of distinct allergen in two extracts of *Cladosporium herbarum*. *Int. Arch. Allergy Appl. Immunol.* 58, 371-386. 1979.

2. Djurup, R., Magnusson, C. G. M., Minuva, U., Søndergaard, I., Østerballe, O. & Weeke, B.: An automated particle counting immunoassay (PACIA) for determination of blocking antibodies against timothy grass pollen in sera from desensitized allergics. *Allergy* 38, 173-182, 1983.
3. Djurup, R., Søndergaard, I., Minuva, U. & Weeke, B.: A nylonball solid-phase radioimmunoassay for specific antibodies in human sera. Application to measurement of IgG antibodies to pollen allergens. *J. Immunol. Methods* 62, 283-296, 1983.
4. Harboe, N. & Ingild, A.: Immunization, isolation of immunoglobulins, estimation of antibody titre. *Scand. J. Immunol.* 2, Suppl. 1, 161-164, 1973.
5. Johansson, S. G. O., Foucard, T., Hjelm, T. & Sjøkvist, J.: Determination of specific IgG antibody to soluble antigens by absorption on Protein A-sepharose. *Scand. J. Immunol.* 3, 881, 1973.
6. Keck, K., Grossberg, A. L. & Pressman, D.: Specific characterization of isoelectrofocussed immunoglobulins in polyacrylamide gel by reaction with ¹²⁵I-labeled protein antigens or antibodies. *Eur. J. Immunol.* 3, 99-102, 1973.
7. Langone, J. J.: ¹²⁵I-labeled Protein A: Reactivity with IgG and use as a tracer in radioimmunoassay. In van Vunakis, H. & Langone, J. J. (eds.): *Methods Enzymol.* 70A, 356, 1980.
8. Löwenstein, H., Markussen, B. & Weeke, B.: Identification of allergens in extract of horse hair and dandruff by means of crossed radioimmuno-electrophoresis. *Int. Arch. Allergy Appl. Immunol.* 51, 38-47, 1976.
9. Müller, U., Johansson, S. G. O. & Streit, G.: Hymenoptera hypersensitivity: IgE, IgG and haemagglutinating antibodies to bee venom constituents in relation to exposure and clinical reaction to bee stings. *Clin. Allergy* 8, 267-272, 1978.
10. Nordvall, S. L., Uhlin, T. & Einarsson, R.: Determination of specific IgG antibody by crossed radioimmuno-electrophoresis. *Allergy* 38, 183-189, 1983.
11. Prahl, P., Skov, P., Minuva, U. & Weeke, B.: Measurement of IgG antibodies against timothy pollen allergens. *Allergy* 36, 123-127, 1981.
12. Shimizu, M., Wicher, K., Reisman, R. E. & Arbesman, C. E.: Measurement of IgG antibodies to bee venom by radioallergosorbent test (RAST). *J. Allergy Clin. Immunol.* 57, 211, 1976.
13. Sobotka, A. K., Valentine, M. D., Ischizaka, K. & Lichtenstein, L. M.: Measurement of IgG-blocking antibodies: Development and application of radioimmunoassay. *J. Immunol.* 117, 84-89, 1976.
14. Sutton, R., Wrigley, C. W. & Baldo, B. A.: Detection of IgE- and IgG binding proteins after electrophoretic transfer from polyacrylamide gels. *J. Immunol. Methods* 52, 183-194, 1982.
15. Weeke, B.: Crossed immunoelectrophoresis. *Scand. J. Immunol.* 2, Suppl. 1, 47-56, 1973.
16. Weeke, B. & Löwenstein, H.: Allergens identified in crossed immunoelectrophoresis. *Scand. J. Immunol.* 2, Suppl. 1, 149-153, 1973.

17. Weeke, B., Søndergaard, I., Lind, P., Aukrust, L. & Løwenstein, H.: Crossed radioimmuno-electrophoresis (CRIE) for identification of allergens and determination of the antigenic specificities of patients IgE. *Scand. J. Immunol.* *17*, Suppl. 10, 265–272, 1983.
18. Wide, L.: A RAST neutralization test for determination of blocking antibodies after hyposensitization. *Int. Arch. Allergy Appl. Immunol.* *52*, 219–226, 1976.

Address:

Ib Søndergaard

Laboratory for Medical Allergology, TA 7542

Rigshospitalet

20 Tagensvej

DK-2200 Copenhagen N

Denmark

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.