

Characterization of IgG-Subclass Antibodies to Individual Allergens by Crossed Radioimmuno-electrophoresis

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A crossed radioimmuno-electrophoretic (CRIE) method for detection of human IgG subclass specificities against individual antigens is described. After crossed immunoelectrophoresis (CIE) of the antigens the immunoplates are incubated with serum, washed and incubated with mouse monoclonal anti-human IgG-subclass antibodies. After washing, the plates are finally incubated with the ^{125}I -labelled detector protein, rabbit anti-mouse IgG, and washed. The plates are then placed on an X-ray film for autoradiography. The specificity of the method was tested by inhibition with antigens in the first layer and by inhibition with myeloma sera containing only one IgG subclass protein in the second layer. The specificity of the third layer was assured by affinity purification of the rabbit anti-mouse IgG antibodies. The method was shown to be sensitive and specific. The test systems were timothy (*Phleum pratense*) pollen antigens and house dust mite (*Dermatophagoides pteronyssinus*) antigens.

Key words: IgG-subclass antibodies; crossed radioimmuno-electrophoresis; immunotherapy.

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CLINICAL ASPECTS

Measurement of specific IgG antibodies and the whole concept of blocking antibodies, mainly of the IgG class, is now being replaced by a more varied view on the different functional and regulatory roles that the different IgG-subclasses may play in allergy and particularly during immunotherapy. Evidence that IgG₄ antibodies may play an important role in the protection of an individual against allergy is now emerging (1, 5, 15). However, the subject is controversial (13, 15, 17, 18) as IgG₄ may function as short-term anaphylactic antibody (18). The role of the other subclasses of IgG in allergy has not yet been studied in detail. Therefore a detailed study of the IgG subclass antibody response in allergic patients before and during immunotherapy, together with clinical parameters, might throw further light on the regulatory and functional aspects of these antibodies. In addition, it is now possible to compare the IgE response with the IgG-subclass response to individual allergens during immunotherapy, with the possible result that, in the future, the therapy outcome can be predicted at an earlier stage.

Detailed study of the immune response during immunotherapy is very important as it will lead to better understanding of the protective mechanisms involved. As a consequence, this might result in methods which would give a better prediction of the outcome of immunotherapy in a given patient at an early stage of

the treatment. Furthermore, this understanding could possibly lead to more efficient forms of immunotherapy.

In recent years great interest has been given to measurement of IgG and IgG-subclasses in conjunction with immunotherapy, and it has been suggested that clinical improvement may

be correlated to the antibody response in one or more of the four IgG subclasses (6, 9).

Recently, we have described methods for measurement of IgG and IgG-subclasses by a RAST technique, and a method for determination of the IgG_{1,2,4} specificity to individual antigens by CRIE technique (7, 8, 19). The present paper describes a CRIE method for determination of IgG_{1,2,3,4}-subclass specificity to timothy pollen and house dust mite allergens. It is a three-layer method, thus only a single radio-labelled detector-protein is necessary for all four subclasses, and has the additional advantage of enhancing the detection of the subclass IgG. The use of monoclonal antibodies to human IgG subclass antibodies gives a low background in this CRIE technique.

MATERIAL AND METHODS

Antigens

Timothy (*Phleum pratense*) pollen antigens were timothy alpha fraction freeze dried bulk from Miles Laboratories, 5,720 PNU/mg (batch No. TI-241). House dust mite (*Dermatophagoides pteronyssinus*) and birch pollen (*Betula verrucosa*) antigens were partially purified extracts (SQ 503 and SQ 108) from Allergologisk Laboratorium (ALK), Copenhagen, Denmark.

Antisera

Antibodies against timothy (*Phleum pratense*) pollen and house dust mite (*Dermatophagoides pteronyssinus*) were raised in rabbits using established procedures, and the immunoglobulin fraction was isolated as described (11).

Monoclonal antibodies to human IgG-subclasses were purchased from Seward Laboratory, London, U. K., as ascitic fluid containing 1 mg/ml sodium azide (code Nos. BAM 15 (anti-IgG₁), BAM 10 (anti-IgG₂), BAM 08 (anti-IgG₃) and BAM 11 (anti-IgG₄)). Except for the addition of aprotinin (100 KU/ml) to inhibit proteolysis, the ascitic fluids were used as re-

ceived. The ascitic fluids were stored at -20°C in aliquots of 100 µl and once thawed were kept at 4°C at which temperature they were stable for at least 3 weeks.

Normal rabbit serum (NRS) (Gibco Europe Ltd., Paisley, Scotland) was heat-inactivated at 56°C for 30 min and sodium azide (1 mg/ml) was added. Bovine gammaglobulin was from Sigma, St. Louis, U.S.A.

Polyclonal sheep antisera (precipitating) to human IgG-subclasses were from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands. Rabbit anti-mouse IgG was obtained from DAKO, Copenhagen, Denmark. (see also section concerning labelling procedure).

Sera

Sera from patients allergic to timothy pollen antigens and house dust mite allergens were collected at the Dept. of Allergology, Rigshospital. The patients had received immunotherapy for at least 1 year. Normal human sera (NHS) were supplied by the blood bank at the Rigshospital. No history concerning the donors could be obtained.

Sera from patients with clinical myelomatosis were used for specificity testing. The sera contained an M-component of IgG type as found by agarose electrophoresis, and type and amount of the IgG subclass were determined by single radial immunodiffusion (Mancini technique) against polyclonal subclass specific antisera from CLB. Sera containing only one of the four subclasses were selected for inhibition studies.

The IgG₁ myeloma serum used contained 85.5 g/l IgG₁ and the IgG₄ myeloma serum contained 52.7 g/l.

Incubation buffer and washing solution

A glycine buffer (GB) containing 0.1 M glycine, 0.65 M NaCl, 10 ml/l Tween 20, 15 mM NaN₃, pH 9.2 is used as basis for the incubation buffer. On the assay day the necessary amount of incubation buffer (IB) was prepared by adding 5 g

bovine gammaglobulin and 100 ml NRS to 900 ml GB. The incubation buffer is not stable and must be freshly prepared (8).

The washing solution contains 0.65 M NaCl, 1 ml/l Tween 20 and 20 mg/l chlorohexidine acetate in distilled water.

¹²⁵I-labelled rabbit anti-mouse immunoglobulin

The rabbit anti-mouse immunoglobulin was purified by immunosorption against sepharose coupled polyclonal human IgG and by affinity chromatography on sepharose coupled mouse IgG as described previously (7). Labelling with ¹²⁵I was done using a modification of the chloramine-T method (8, 10). The specific activity of the labelled antibody was approximately 4 mCi/mg.

Crossed immunoelectrophoresis (CIE)

CIE was performed as previously described (20) using 1% agarose (HSA, Litex, Denmark) in a buffer containing 0.024 M sodium barbital, 0.073 M Tris and 0.01 M NaN₃, pH 8.6.

In the first dimension 10 µl of the timothy pollen extract or 30 µl of the *Dermatophagoides pteronyssinus* extract was run for 30 min at 10 V/cm. The second dimension electrophoresis was run at 2 V/cm for 18 h using either 12 µl/cm² gel or 3 µl/cm² gel of the respective antibodies. The immunoplates were washed, pressed and dried in cold air as described previously (20).

CRIE procedure

The dried immunoplates were placed in plastic lids and incubated for 3 h with 360 µl serum in 7 ml incubation buffer. The plates were washed 4 times with washing solution and incubated for 3 h with 7 ml of a solution containing the monoclonal mouse anti-human IgG-subclass antibody diluted 1+10,000. Again the plates were washed 4 times with washing solution and incubated overnight with 7 ml incubation buffer containing ¹²⁵I-rabbit anti-mouse IgG (4 × 10⁵ cpm). Finally the plates were washed 4 times with washing solution, dried in hot air

and placed in an X-ray cassette for autoradiography in 3 h, 6 h, 24 h, 3 days and 14 days.

Specificity testing

The specificity of the reactions in each layer was tested in the following way. All possible combinations of NHS or patient serum or buffer in the first layer, monoclonal antibody solution or buffer in the second layer, and ¹²⁵I-labelled anti-mouse IgG in the third layer were tested. Inhibition with free antigen was tested by incubation of 360 µl serum with 100 µl allergen extract for 3 h before use in CRIE. In a similar manner inhibition with IgG₁ and IgG₄ myeloma sera of the reaction in the second layer was tested by incubation of 7 ml of the monoclonal mouse anti-human IgG-subclass solution with 2.5 µl IgG₁ containing myeloma serum, or with 4 µl IgG₄ containing myeloma serum, for 24 h before use in CRIE. An additional experiment was performed using serum heated to 56°C for 4 h in order to exclude that the reactions seen were due to IgE.

RESULTS

Fig. 1 shows detection of IgG-subclass antibodies to timothy pollen antigens by CRIE as well as the control experiments.

Fig 1A and B show the detection of IgG 1 and IgG 4 respectively against timothy pollen antigens in a patient serum and Fig. 1C and 1D show the result of a similar experiment using NHS.

Other experiments (not shown) showed that no IgG₂ and IgG₃ antibodies against timothy pollen antigens could be detected in any of the patient sera. In order to determine the specificity of the reaction in the first layer the patient serum was incubated with antigen for 3 h before incubation with the immunoplate. The result of this experiment is shown in Fig. 1E where an almost total inhibition of the reaction can be seen.

The next step in the specificity testing was to determine the specificity of the mouse anti-human IgG-subclass monoclonal antibodies by

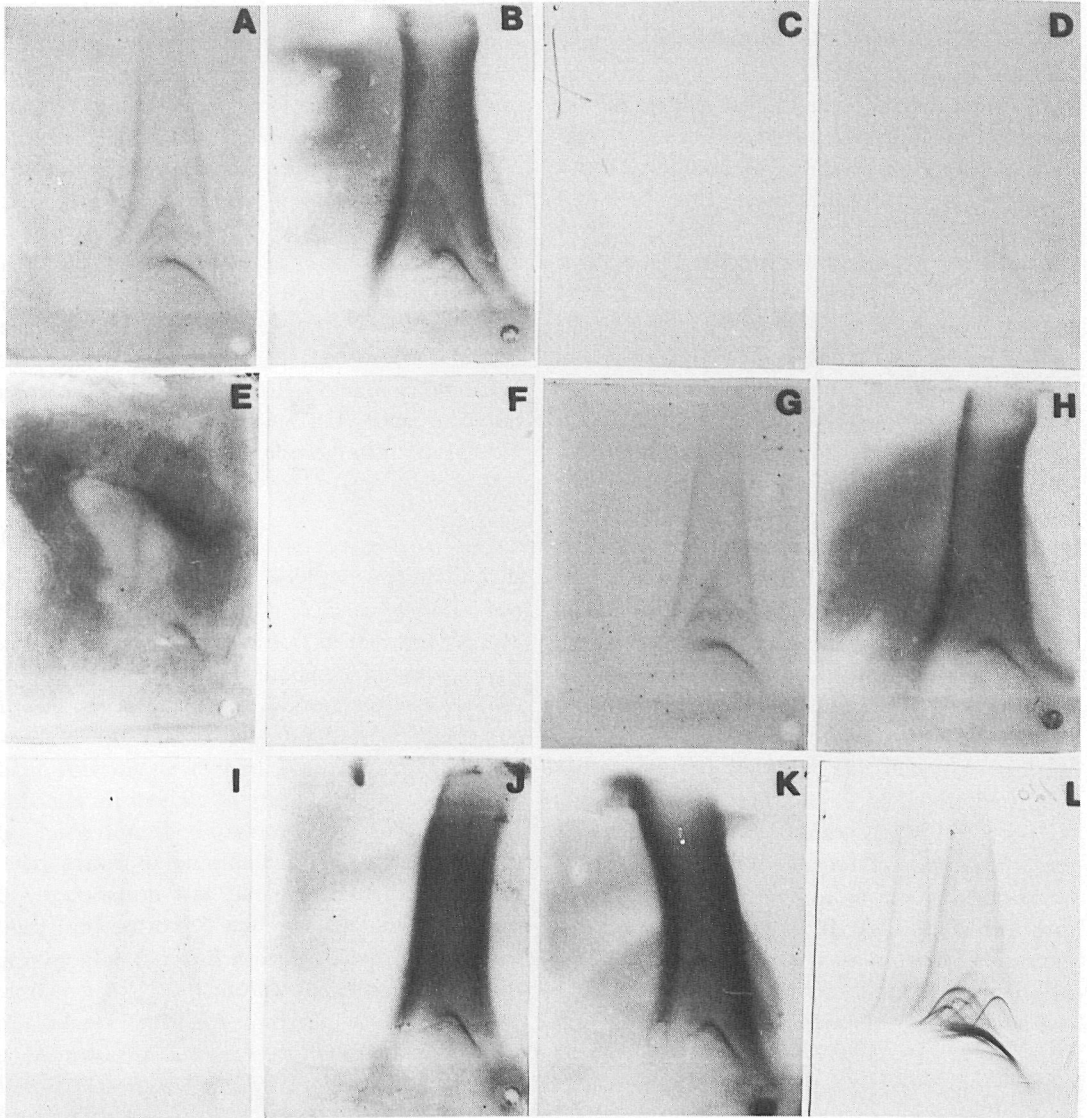


Fig. 1. Photographs of X-ray films of IgG-subclass CRIE experiments on antigens from timothy (*Phleum pratense*). A: Patient serum and detection with anti-IgG₁. B: Patient serum and detection with anti-IgG₄. C: NHS and detection with anti-IgG₁. D: NHS and detection with anti-IgG₄. E: Patient serum inhibited with free antigen and detection with anti-IgG₄. F: Patient serum inhibited with IgG₁ and detection with anti-IgG₁. G: Patient serum inhibited with IgG₄ and detection with anti-IgG₁. H: Patient serum inhibited with IgG₁ and detection with anti-IgG₄. I: Patient serum inhibited with IgG₄ and detection with anti-IgG₄. J: Patient serum inhibited with birch pollen antigen and detection with anti-IgG₄. K: Patient serum heat inactivated at 56°C for 4 h and detection with anti-IgG₄. L: CIE plate stained with Coomassie Brilliant Blue.

incubation with human myeloma sera in the second layer of this CRIE procedure. In Fig. 1F it can be seen that anti-IgG₁ is inhibited by a myeloma serum containing IgG₁ and that anti-IgG₄ is likewise inhibited by a myeloma serum

containing IgG₄. As can be seen from the figure, anti-IgG₁ is not inhibited by IgG₄ and anti-IgG₄ is not inhibited by IgG₁.

Fig. 1J shows the CRIE result with patient serum inhibited with birch pollen extract, and

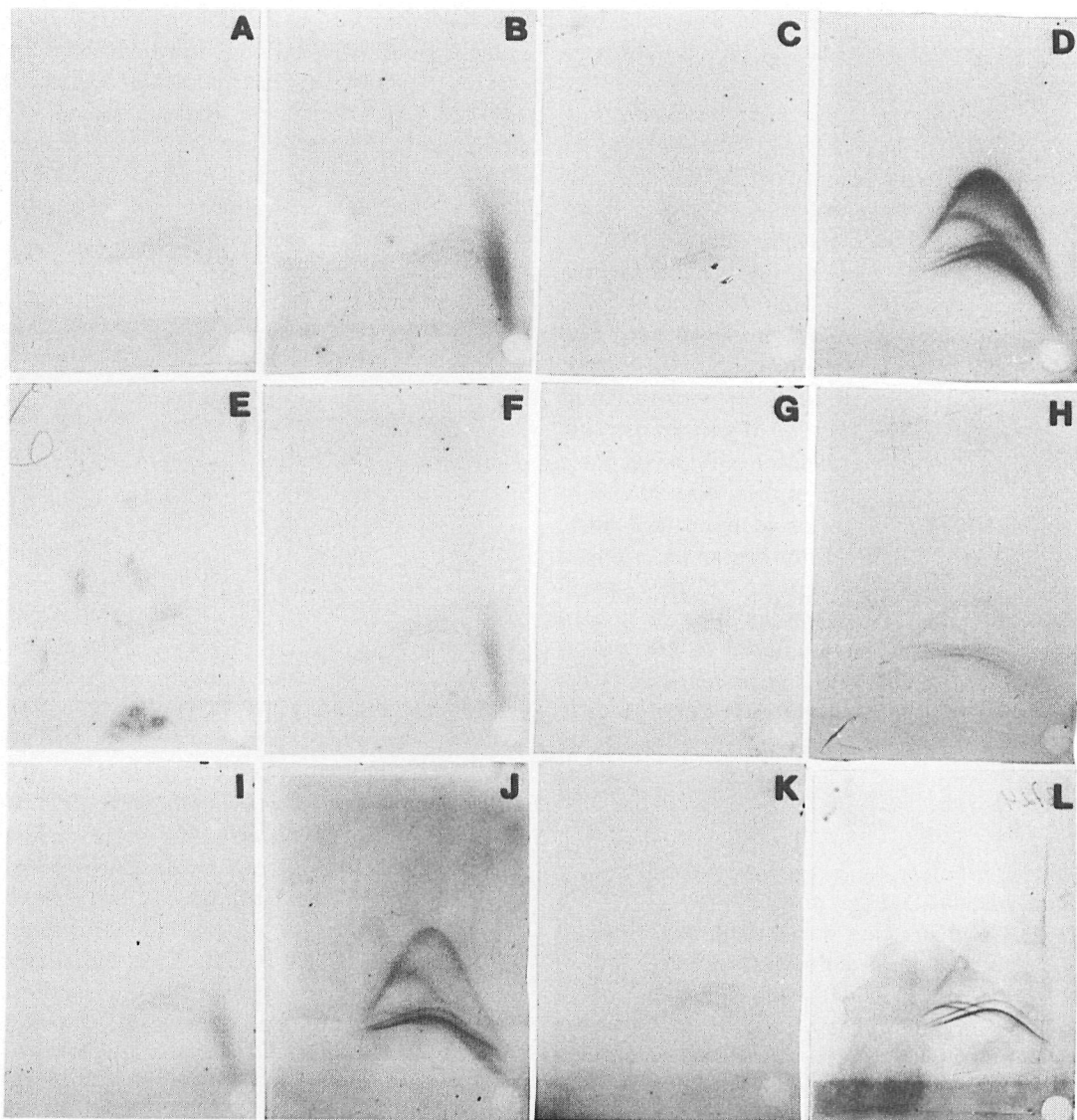


Fig. 2. Photographs of X-ray films of IgG-subclass CRIE experiments on antigens from house dust mite (*Dermatophagoides pteronyssinus*). A: Patient serum and detection with anti-IgG₁. B: Patient serum and detection with anti-IgG₂. C: Patient serum and detection with anti-IgG₃. D: Patient serum and detection with anti-IgG₄. E: NHS and detection with anti-IgG₁. F: NHS and detection with anti-IgG₂. G: NHS and detection with anti-IgG₃. H: NHS and detection with anti-IgG₄. I: Patient serum inhibited with free antigen and detection with anti-IgG₄. J: Patient serum inhibited with free antigen and detection with anti-IgG₂. K: NHS inhibited with free antigen and detection with anti-IgG₄. L: CIE plate stained with Coomassie Brilliant Blue.

Fig. 1K the result with heat inactivated serum using detection with anti-IgG₄ in both cases. Finally, Fig. 1L shows a CIE plate stained with Coomassie Brilliant Blue to show the total amount of antigens in this system.

Fig. 2 shows the results for IgG CRIE with

house-dust mite antigens. Fig. 2A-D show the detection of IgG₁, IgG₂, IgG₃ and IgG₄ antibodies against house dust mite antigens using patient serum. The same experiment but using NHS is shown in Fig. E-H. inhibition of patient serum with antigen and detection of IgG₂ and

IgG₄ antibodies are shown in Fig. 2I-J and inhibition of NHS with antigen and detection of IgG₄ antibodies in Fig. 2K. Other experiments (not shown) were performed demonstrating that the reaction could be inhibited in the other subclasses as well. The inhibition experiments show that the faint reaction seen with NHS could be inhibited and this was due to IgG-subclass antibodies present in this serum pool. It should be noted that photodocumentation of CRIE results is very difficult. However, the results mentioned in the text are in full accordance with the results obtained by direct visual inspection of the CRIE films and less clearly visualised in the photograph.

DISCUSSION

Determination of IgE specificity against individual allergens in allergen extracts by CRIE has been a widely used method in the past decade (2, 12, 21, 22). CRIE methods for analysis of IgG antibodies against honey bee venom (14) and against horse hair and dander (19) have been recently developed. Determination of IgG-subclass antibodies by the CRIE technique has not been described previously.

In order to eliminate non-specific binding, the incubation was performed in buffer with high ionic strength and pH containing bovine gammaglobulin and normal rabbit serum. Previously it has been shown that this combination of balance proteins and slightly dissociating condition improves the signal-to-noise ratio in RAST based techniques for determination of IgG and IgG-subclass antibodies (7, 8). Although the solid phase antigen containing matrix is different in the two cases this buffer was shown to work very well also in the CRIE technique by suppressing non-specific binding.

The specificity of the reactions involved in each layer was confirmed by inhibition experiments. In the first layer it was shown that human IgG-subclass antibody binding to antigens precipitated in the gel could be inhibited by incubation of a serum sample with antigens in free form. Only the relevant antigen could inhibit the reaction, e.g. birch pollen antigens did not inhibit the reaction against timothy

pollen antigens. The reaction between the bound IgG-subclass antibodies in the first layer and the mouse anti-human IgG-subclass antibodies could be inhibited by the complementary myeloma IgG-subclass immunoglobulins. Only IgG₁ and IgG₄ subclass containing myeloma sera were used in the CRIE experiments, but specificity data for all four mouse anti-human IgG-subclass antibodies have been obtained by the IgG-subclass RAST published previously (7). The specificity of the ¹²⁵I-labelled rabbit anti-mouse immunoglobulin was assured by immunosorption against human IgG with a representative subclass distribution and, finally, by affinity chromatography on sepharose coupled mouse immunoglobulin. As a further control of the specificity of the detection protein the experiments were performed where all possible combinations of the two first layers with and without the appropriate antibodies were tried. In these experiments, and also in experiments using normal control sera, only very weak non-specific binding to the precipitates could be recognized, probably due to cross reaction between the detection protein and rabbit IgG or due to hydrophobic interactions in the precipitates.

Previous studies by ourselves and others (1, 5, 6, 7, 9, 16, 17) have shown that significant amounts of pollen specific IgG antibodies cannot be detected in sera from normals. Hyposensitization results in substantial increase in the IgG₄ level in most pollen allergic patients, but also a rise in the IgG₁ level is seen in the early phases of immunization. A detailed study of the IgG-subclass and IgE response to individual allergens before and during immunotherapy and their relation to clinical parameters will be described elsewhere (Søndergaard et al., in preparation).

The well-known IgE CRIE method is a two layer assay where anti-human IgE is radiolabelled. The use of a three layer method in the present study has some advantages:

- 1) The subclass specific antibodies need not be purified as would be the case if they were to be radiolabelled (3, 4). This is because the radiolabelled detector protein rabbit anti-mouse IgG is highly purified.

2) The assay uses only one detector protein, thus reducing the work with highly radioactive materials. The favourable reaction kinetics reduce the reaction time in each step which, despite the extra incubation step, makes the total time used for this three layer assay comparable to the time used in the two layer IgE CRIE. In fact work in our laboratory is in progress to use the same detector protein and a monoclonal mouse anti-human IgE antibody for IgE CRIE, thus further reducing the number of radio-labelled antibodies needed when both the IgE and IgG subclass antibody responses are to be evaluated.

In conclusion the IgG-subclass CRIE seems to be well suited for detailed study of the antibody response to individual allergens, for instance, during immunotherapy.

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