

A Three-Layer Immunoradiometric Assay for Determination of IgG Subclass Antibodies in Human Sera ("IgG Subclass RAST")

Validation of the Subclass Specificity, and Establishment of Equipotency

R. DJURUP,¹ I. SØNDERGAARD,¹ C. G. M. MAGNUSSON² and B. WEEKE¹

¹Laboratory of Medical Allergology, Medical Department TA, Rigshospitalet, and University of Copenhagen, Copenhagen, Denmark, and ²Institute of Cellular and Molecular Pathology, Unit of Experimental Medicine, Université Catholique de Louvain, Brussels, Belgium

We report the development of a three-layer immunoradiometric assay (TIRA) for measurement of IgG antibodies of all four subclasses in human sera. The first layer consists of diluted human serum, the second layer is monoclonal mouse antibodies to human IgG subclasses, and the third layer is ¹²⁵I-labelled rabbit anti-mouse IgG. Monoclonal anti-IgG1, anti-IgG3 and anti-IgG4 reacted only with their complementary IgG subclass, whereas the anti-IgG2 showed slight cross-reactivity to immunoglobins of other subclasses and classes and to light chain proteins. The observed cross-reactivity was found to be without importance, when the TIRA was applied to measurement of IgG subclass antibodies. Equipotency was established by use of appropriate dilutions of the monoclonal antibodies, and the assay was calibrated by use of human reference serum. The TIRA therefore permits reliable inter-individual and intra-individual comparisons of the IgG antibody response in all four subclasses. Non-specific binding obtained with pooled normal human serum was below 0.33 %. Inter-assay coefficient of variation was between 18 and 27 %. The TIRA was applied to measurement of IgG subclass antibodies to timothy grass pollen in sera from grass pollen allergics undergoing immunotherapy.

Key words: blocking antibodies; hay fever; IgG antibodies; immunotherapy; monoclonal antibodies; solid-phase radioimmunoassay.

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CLINICAL ASPECTS

IgG antibodies of different subclasses seem to play an important role in the clinical outcome of an allergen encounter. Thus, there is increasing evidence that an IgG4 response may protect the individual against allergy (1, 5, 22) and – as part of a common mucosal system of immunity – against undue immunological reactions in general (17). The role of IgG4 antibodies in immunity is, however, controversial (20, 22, 25, 27), and at least one subtype of IgG4 may function as a short-term anaphylactic antibody (27), giving rise to allergic reactions. Due to lack of suitable methods for measuring IgG antibodies of the other three subclasses, their role in allergy has not been studied in any detail. As IgG antibodies of different subclasses may play different functional and regulatory roles in allergy and during immunotherapy, we consider determination of all four subclasses of IgG antibodies to relevant allergens an important part of the complete work-up of allergic patients, especially when the patients are undergoing immunotherapy. This paper presents an easy and inexpensive method for studying the IgG subclass response to any allergen of interest.

In recent years there has been much interest in the subclass nature of IgG antibodies ("blocking antibodies") formed during immunotherapy of allergic patients 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, 10).

IgG subclass antibodies to allergens have been measured by the red cell linked antigen-antiglobulin reaction (RCLAAR) (6), the defined antigen substrate spheres system (DASS) (10), the radio-allergo-sorbent test (RAST) (10), and the enzyme-linked immunoadsorbent test (ELISA) (9). In all these assays, IgG subclass antibodies were measured by use of extensively absorbed polyclonal antisera to human IgG subclasses. Such specially prepared antisera are not generally available. Moreover, none of these assays showed completely satisfactory performance characteristics as regards non-specific binding, subclass specificity, sensitivity, reproducibility, or convenience. Continued efforts to develop alternative assays with improved performance characteristics therefore seemed warranted.

We here report the development of a triple-layer immunoradiometric assay (TIRA), based on the RAST principle and the use of monoclonal antibodies to human IgG subclasses. The assay shows very low non-specific binding, absolute subclass specificity, good sensitivity and acceptable precision. The assay furthermore displays equipotency and thus permits reliable intra-individual comparison of the IgG subclass response to the allergens in question. All reagents are commercially available and the assay is easy to perform and inexpensive to run.

MATERIAL AND METHODS

Antisera, immunoglobulins and animal sera

Polyclonal sheep antisera (precipitating) to human IgG subclasses were from the Central Laboratory of the Netherlands Red Cross Transfusion Service (CLB), Amsterdam. Affinity-purified goat anti-human IgG was from E-Y laboratories, San Mateo, Ca., USA. All other polyclonal antisera were obtained from

DAKO, Copenhagen, Denmark, as IgG fractions.

Monoclonal antibodies to human IgG subclasses (mAbs) were obtained from Seward Laboratory, London, UK, as unpurified ascitic fluid containing 1 mg/ml azide (code No.: BAM 15 (anti-IgG1), BAM 10 (anti-IgG2), BAM 08 (anti-IgG3) and BAM 11 (anti-IgG4)). All mouse monoclonal antibodies were of the IgG1 subclass. The ascitic fluids were used without further purification. To inhibit proteolysis, aprotinin was added to the concentration 100 kU/ml. The ascitic fluid was divided in aliquots of 100 μ l and stored at -20°C . After thawing, the mAbs were stable for at least 4 weeks at 4°C .

Mouse immunoglobulin (MIgG) was from Sigma, St. Louis, Missouri, USA.

Polyclonal human IgG (HIgG) was isolated from pooled normal human serum by precipitation with 33% saturated ammonium sulphate followed by alternating dialysis against distilled water and acetate buffer, pH 5.0 (to remove lipoproteins (14)), and ion-exchange chromatography in DEAE-Trisacryl (LKB, Bromma, Sweden) using 0.01 M sodium phosphate buffer, pH 6.5, for elution of IgG (21). The IgG subclass distribution of this IgG preparation was 66% IgG1, 24% IgG2, 6.7% IgG3 and 3.0% IgG4 as determined by single radial diffusion (Mancini-technique) against polyclonal subclass specific antisera from CLB.

Purified monoclonal IgM (98% purity) and polyclonal (secretory) human IgA (96% purity) were from Behringwerke AG, Marburg, W. Germany.

Immunochemically pure monoclonal human IgE (IgE-JK) was isolated from a serum with an IgE M component by fractional ammonium sulphate precipitation, gel filtration, chromatofocusing (Pharmacia, Uppsala, Sweden) and subtraction affinity chromatography as described in detail elsewhere (Malling, Djurup, Magnusson et al., manuscript in preparation).

Normal rabbit serum (NRS) was from Gibco Europe Ltd., Paisley, Scotland. Sodium azide (1 mg/ml) was added to prevent bacterial growth. Bovine gammaglobulin was obtained from Sigma.

Allergens

Partially purified, standardized, freeze-dried timothy grass pollen extract (Aquagen SQ 225) was from Allergologisk Laboratorium (ALK), Copenhagen, Denmark.

Buffers:

AB: 0.1 M acetate, 0.5 M NaCl, pH 4.0.

BB: 0.1 M borate, 0.5 M NaCl, pH 8.0.

CB: 12.6 g NaHCO_3 /l, 5.4 g Na_2CO_3 , pH 9.5.

GB: 0.1 M glycine, 0.65 M NaCl, 10 ml/l Tween 20, 15 mM NaN_3 , pH 9.2.

IB: On the assay day the necessary amount of incubation buffer (IB) was prepared by adding 5 g bovine gammaglobulin and 50 ml NRS to 950 ml GB. The buffer is *not* stable and must be freshly prepared each day (7).

PBS: 0.01 M potassium phosphate, 0.15 M NaCl, 1.5 mM NaN_3 , pH 7.4.

PBS⁺: 0.1 M potassium phosphate, 0.15 M NaCl, 1.5 mM NaN_3 , pH 7.5.

WS: 0.65 M NaCl, 1 ml/l Tween 20, 20 mg/l chlorhexidine acetate, in distilled water.

Other reagents

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (CDI) was from Sigma. When not otherwise stated, other chemicals were from Merck, Darmstadt, W. Germany.

Saturated ammonium sulphate (SAS) was prepared by adding 760 g ammonium sulphate to 1 l PBS at room temperature.

Immunoadsorbents

Crude immunoglobulin fractions were prepared from polyclonal antisera to human IgG subclasses (CLB) by precipitation with 33% SAS. These fractions were immobilized to carboxymethyl agarose (BioRad Labs., Richmond, Cal., USA) by carbodiimide (CDI) as recommended by the manufacturer, and used for subtraction affinity chromatography (see below).

Mouse immunoglobulin (2 mg/ml swollen gel) and human immunoglobulin (HIgG) (10 mg/ml swollen gel) were coupled to CNBr-activated Sepharose 4 B (Pharmacia) as described by the manufacturer.

Patients' sera and controls

Sera were obtained from 20 grass (timothy) pollen allergic patients undergoing immunotherapy with alum-adsorbed timothy extract (Alutard SQ 255, ALK). The patients and the therapy procedure have been described elsewhere (28). Samples collected just before beginning of treatment, and after 3 years of immunotherapy, were used in this study.

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

For assessment of non-specific binding we used pooled normal human serum (NHS) obtained from *non-allergic* donors (7).

To estimate the variability in background binding (non-specific and specific) in normal sera, we tested 20 sera obtained from blood donors with the same sex ratio and mean age as the patients.

Purification of human IgG myeloma proteins

IgG myeloma proteins were isolated from sera obtained from patients with multiple myeloma. First, electrophoretic mobility of the myeloma proteins was determined by agarose electrophoresis of the sera in barbitone buffer, pH 8.6. The myeloma proteins were then isolated by chromatofocusing (26), followed by subtraction (negative) affinity chromatography (2, 11):

The chromatofocusing was performed exactly as described by the manufacturer (Pharmacia) using a 1×40 cm (bed dimensions) column of polybuffer exchanger PBE 94 (Pharmacia). Myeloma serum (0.2–0.4 ml) was applied to the column and eluted at flow rate 0.4 ml/min at room temperature. Fractions of 5 ml were collected. For isolation of myeloma proteins with cathodic electrophoretic mobility, the PBE 94 column was equilibrated with 0.025 M ethanolamine- CH_3COOH . The proteins were eluted

over the pH range 9–6 by use of polybuffer 96 (Pharmacia) adjusted to pH 6.0 with CH_3COOH . Proteins with gamma anodic mobility were isolated on a PBE 94 column equilibrated with 0.025 M Tris- CH_3COOH , pH 8.3, and elution was done with a mixture of polybuffer 96 (30% (v/v)) and polybuffer 74 (Pharmacia) (70% (v/v)) adjusted to pH 5.0 with CH_3COOH . For isolation of proteins with fast (beta) anodic mobility, the PBE 94 column was equilibrated with 0.025 M imidazol- CH_3COOH , pH 7.4, and elution was performed with polybuffer 74 adjusted to pH 4.0 with HCl. The eluant was monitored for its absorbance at 280 nm. Peak fractions were pooled. The proteins were precipitated with 50% SAS, dialyzed against PBS for 24 h at 4°C and concentrated to approximately 5 mg/ml by ultrafiltration using immersible CM 30 ultrafiltration units (Millipore, Bedford, Ms., USA). The IgG subclass content of the peak(s) was determined by double immunodiffusion against polyclonal subclass specific antisera. To ensure that the myeloma protein was not contaminated with polyclonal IgG of the other subclasses, each IgG myeloma protein isolated by chromatofocusing was absorbed with antisera to the other three subclasses, insolubilized to carboxymethyl agarose with CDI. To take an example, an IgG1 protein was incubated 16 h with 3 ml (settled volume) of a gel, to which 33% SAS immunoglobulin fractions of 1 ml anti-IgG2, 1 ml anti-IgG3 and 1 ml anti-IgG4 had been insolubilized. Incubation was performed at room temperature with slow end-over-end rotation. The myeloma protein was eluted with PBS^+ , concentrated and stored in small portions at -20°C until use. To inhibit proteolysis of IgG3 (21), epsilon-amino-caproic acid (Sigma) and phenyl-methyl-sulfonylfluoride (Sigma) was added to the concentrations 0.1 M and 2 mM, respectively, immediately after isolation of IgG3 myeloma proteins by chromatofocusing.

Isolation of kappa and lambda light chains

Kappa and lambda light chain proteins were isolated from urines from patients with multiple

myeloma by precipitation with 65% SAS followed by gel-filtration on Sephacryl S-200 (Pharmacia) (16).

Determination of protein concentration

Protein concentration was determined by measurement of the absorbance at 280 nm, using $E_{1\text{ cm}}^{1\%} = 14$ for IgE proteins and $E_{1\text{ cm}}^{1\%} = 11.8$ for light chain proteins.

Coupling of proteins to nylon balls

IgG myeloma proteins of each subclass, IgA, IgM, IgE, kappa and lambda light chain proteins were coupled to HCl-treated 5 mm nylon balls (Aco Plast, Elsinore, Denmark) with CDI as described in (15) with some modifications: The nylon balls were washed in distilled water, incubated for 24 h with 3.5 M HCl, washed with distilled water, reacted with concentrated acetic anhydride for 2 min, washed successively with CB, PBS and distilled water. The balls were then incubated with 1 g CDI in 100 ml distilled water per 1,000 balls for 10 min, washed with PBS and incubated with 2 µg/ball protein in PBS (100 ml PBS per 1,000 balls) for 2 h at room temperature and for 20 h at 4°C. Any residual active groups were then blocked with 0.5 M ethanolamine in PBS for 2 h at room temperature. Non-covalently bound substances were removed by washing alternately with AB and BB (3 times) and finally with PBS, in which the balls were stored at 4°C until use. The immunoreactivity of the immobilized IgG myeloma proteins has not shown any deterioration during 6 months' storage.

Timothy grass pollen extract was coupled to HCl-treated nylon balls with glutaraldehyde as described previously (7). The timothy-coated balls were stable for at least 6 months, when stored in PBS at 4°C.

Affinity-purification and ^{125}I -labelling of rabbit anti-mouse immunoglobulin

To remove any antibodies with reactivity to human IgG, 10 ml rabbit antiserum to mouse IgG were first recirculated for 2 h at 0.5 ml/

min at room temperature over a 5 ml (settled volume) Sepharose column, to which polyclonal human IgG had been immobilized. The anti-serum was then eluted with BB and transferred to another column containing 5 ml Sepharose, to which mouse IgG had been coupled. The mouse IgG-Sepharose column was washed with 100 ml BB to remove unbound antibodies. The rabbit anti-mouse IgG antibody (RaMIgG) was then eluted with 0.1 M glycine buffer, pH 2.35. The eluate was immediately neutralized with 1/5 volume 1.0 M Tris-HCl buffer, pH 8.0, concentrated by precipitation with 50% SAS and transferred to PBS⁺ on a PD 10 column (Pharmacia) as described by the manufacturer. The protein concentration was adjusted to 1 mg/ml by addition of PBS⁺ and the antibody stored in aliquots at -20°C.

Labelling with ¹²⁵I was done with a modification of the chloramine-T method (7, 12): 100 µg (100 µl) RaMIgG was incubated for 1 min with 0.75 mCi ¹²⁵I (supplied as sodium iodide (Radiochemicals Centre, Amersham, UK)) and 100 µg (50 µl) chloramine-T in PBS⁺. The reaction was stopped by addition of 100 µg (50 µl) sodium metabisulphite in PBS⁺. The ¹²⁵I-labelled antibody was separated from low molecular weight substances on a PD 10 column equilibrated with 10% (v/v) NRS in PBS⁺, containing 1 g/l sodium azide, and stored in this buffer at 4°C. Specific activity was approximately 5 mCi/mg and trichloroacetic acid precipitability better than 98%. The labelled antibody can be used for at least 2 weeks without appreciable changes in specific or non-specific binding.

Assessment of equipotency and specificity of the monoclonal antibodies (mAbs) to human IgG subclasses

To determine the equipotent dilution of the monoclonal antibodies for the four human IgG subclasses, balls coated with an IgG myeloma protein of the complementary subclass were incubated with serial two-fold dilutions (1:500 to 1:32,000) of the monoclonal antibody (ascitic fluid) (Fig. 1a). The antibodies were diluted in a special incubation buffer (IB) which minimizes

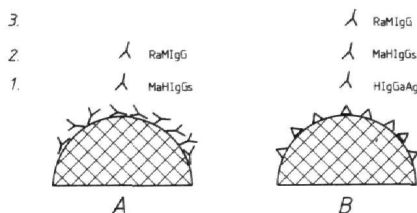


Fig. 1. Principle of the assay: A: Two-layer assay for assessment of equipotency and cross-reactivity. Purified immunoglobulins (IgG1, IgG2, IgG3, IgG4, IgA, IgE or IgM) or light chain proteins are immobilized on the balls. The balls are then incubated with diluted monoclonal mouse IgG (MaHIgGs) to human IgG1, IgG2, IgG3 or IgG4 (first layer), washed, and incubated with ¹²⁵I-labelled rabbit anti-mouse IgG (RaMIgG) (second layer).

B: Three-layer assay for determination of IgG subclass antibodies. Allergen extract is immobilized on the balls. The balls are incubated with diluted human serum (HIgGaAg) (first layer), washed, incubated with equipotent dilutions of monoclonal mouse IgG to human IgG subclasses (second layer), washed, and finally incubated with ¹²⁵I-labelled rabbit anti-mouse IgG (third layer).

non-specific binding (7). Each ball was incubated with 0.2 ml diluted antibody for 2 h at 37°C, washed 6 times with wash solution (WS) and then incubated overnight at room temperature with approximately 40 ng ¹²⁵I-labelled RaMIgG in 1.0 ml IB (7). The balls were washed 6 times with WS and counted for 1 min in a gamma-counter with counting efficiency 0.45. All determinations were done in duplicate.

To detect any cross-reactivity of the monoclonal antibodies, balls coated with IgG myeloma proteins of the four IgG subclasses, with IgA, IgM and IgE, and with kappa and lambda light chain proteins, were incubated with the monoclonal antibodies in equipotent dilutions (see Results) as described above. All IgG myeloma test proteins were of kappa light chain type. IgA was a polyclonal (secretory) protein. IgE was of the lambda light chain type, and IgM of the kappa light chain type.

Determination of IgG subclass specific antibodies by a three-layer immunoradiometric assay (TIRA)

Balls coated with timothy grass pollen extract were incubated for 2 h at 37°C with 0.2 ml serum diluted 1:100 in IB per ball. The balls

were washed 6 times with WS, incubated with 0.2 ml/ball diluted monoclonal anti-IgG1 (1:1,000), anti-IgG2 (1:500), anti-IgG3 (1:24,000) or anti-IgG4 (1:24,000) for 2 h at 37°C. The balls were then washed 6 times with WS, incubated overnight at room temperature with approximately 40 ng ^{125}I -labelled RaMIgG in 1.0 ml IB, washed 6 times with WS, and counted for 1 min in a gamma-counter. All determinations were done in duplicate.

The assay was calibrated by use of dilutions of a human reference serum obtained from a patient (JN) who has been undergoing immunotherapy with a grass pollen mixture for several years. This serum was arbitrarily ascribed 100 U IgG4 antibody to grass pollen/ml undiluted serum. Dilutions of this serum were used to construct a standard curve for the IgG4 system. Antibody responses in the other three subclasses were calibrated by use of the same (IgG4) standard curve, assuming parallel serum dilution curves in all four subclasses.

RESULTS

Coupling of IgG myeloma proteins to nylon balls

To assess whether the coupling procedure actually resulted in binding of identical amounts of IgG myeloma proteins of the different subclasses, balls coated with eight different

myeloma proteins, two of each subclass, were incubated with the same amount (about 80 ng) of ^{125}I -labelled goat anti-human IgG overnight at room temperature. The balls bound from 19.1 to 25.3% of the added radioactivity ($\bar{x}$ = 22.2%; SD = 2.27%; CV = 10.2%).

Titration of the monoclonal mouse antibodies to human IgG subclasses

The reactivity of each of the four monoclonal antibodies against its complementary IgG subclass protein is shown in Fig. 2. The values are corrected for the non-specific binding found with incubation buffer in the first step. Non-specific binding was 0.32%, 0.16%, 0.13% and 0.16% for balls coated with IgG1, IgG2, IgG3 and IgG4 myeloma proteins, respectively.

The reactivity of each monoclonal antibody was measured against two different, purified, IgG myeloma proteins of the complementary subclass. Each of the four monoclonal antibodies reacted identically with the two complementary proteins (results not shown).

The dilution giving about 8% specific binding, corresponding to about 20,000 cpm/ball, was used for further studies and as the equipotent dilutions in the TIRA. The equipotent dilution for anti-IgG3 and anti-IgG4 antibody was determined by more detailed titration, in the region of 1:16,000 to 1:32,000, than shown

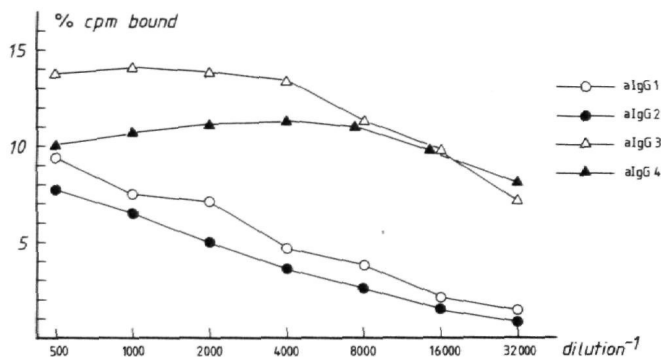


Fig. 2. Titration of mouse monoclonal antibodies to human IgG subclasses. Balls coated with the complementary IgG subclass protein were incubated with two-fold serial dilutions of the monoclonal antibody and then with ^{125}I -labelled RaMIgG in the two-layer assay. Abscissa: reciprocal dilution. Ordinate: binding (corrected for background) in percent of the added radioactivity.

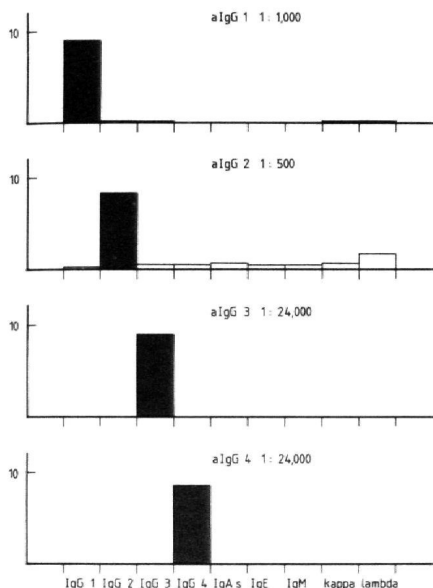


Fig. 3. Reactivity of monoclonal antibodies in the two-layer assay to immunoglobulins of different classes and subclasses, and to light chain proteins. Abscissa: type of test protein. Ordinate: percent specific binding (total binding corrected for non-specific binding, which was in the range 0.14 to 0.33%). Note that the equipotent dilutions selected actually resulted in almost identical specific binding of the four monoclonal antibodies to their complementary IgG proteins (toned columns).

in Fig. 2. The equipotent dilutions were 1:1,000, 1:500, 1:24,000 and 1:24,000 for anti-IgG1, anti-IgG2, anti-IgG3 and anti-IgG4, respectively.

Subclass-specificity of the assay

The reactivity of the four monoclonal antibodies against IgG1, IgG2, IgG3, IgG4, IgA, IgE, IgM, kappa and lambda proteins is shown in Fig. 3.

Binding less than twice the background binding obtained with incubation buffer was considered insignificant.

Anti-IgG1, anti-IgG3 and anti-IgG4 antibodies bound only to their complementary IgG myeloma protein.

Anti-IgG2 showed moderate cross-reactivity to the lambda protein and low, but significant

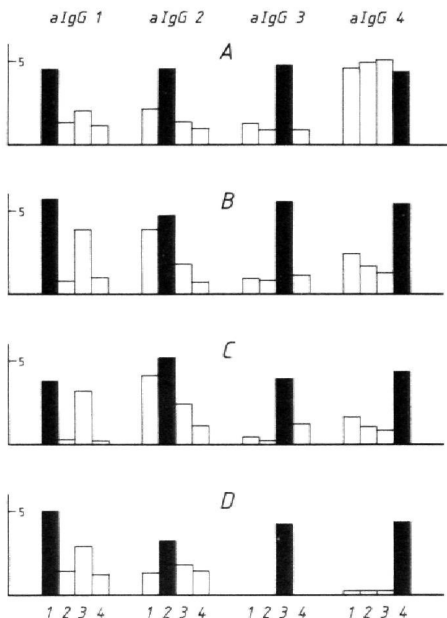


Fig. 4. Reactivity of polyclonal antisera in the two-layer assay to IgG subclasses. Abscissa: type of IgG myeloma subclass. Ordinate: percent specific binding. Antisera "D" were from CLB. The suppliers of the other antisera have not been stated in Material and Methods. None of the antisera were claimed by the producers to be specific for radioimmunoassays or other sensitive techniques.

cross-reactivity to all other test proteins. This finding does not invalidate the subclass-specificity of the three-layer immunoradiometric assay for measurement of IgG subclass antibodies (see Discussion).

For comparison, the reactivity of *polyclonal* sheep antisera to human IgG subclasses, obtained from four different commercial suppliers (A, B, C and D), is shown in Fig. 4. ^{125}I -labelled affinity-purified rabbit anti-sheep IgG was used as detector protein in this system.

The subclass-specificity of the monoclonal antibodies was confirmed by incubation with two different IgG myeloma proteins of each subclass (results not shown).

Binding of ^{125}I -labelled rabbit anti-mouse IgG to eight purified human IgG myeloma proteins, two of each subclass, did not exceed 0.33%. In the presence of mouse antibodies to human IgG

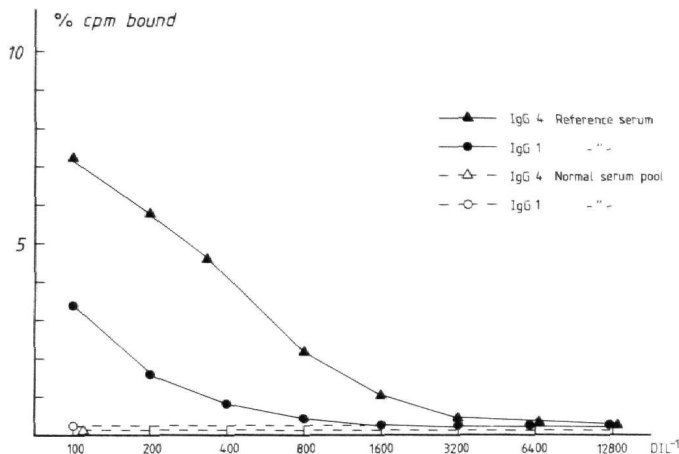


Fig. 5. Titration of the reference serum JN and of pooled normal human serum in the three-layer assay (TIRA). Serial two-fold dilutions of the reference serum JN and of pooled NHS were tested in the TIRA. Abscissa: reciprocal dilution. Ordinate: total binding (not corrected for non-specific binding). Specific IgG2 or IgG3 antibodies were not found in the reference serum.

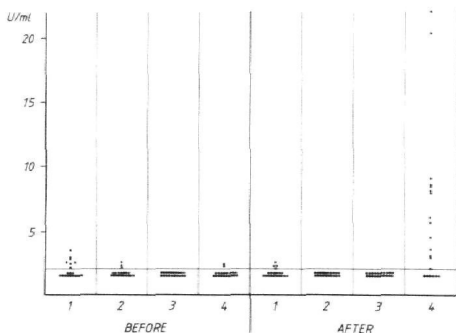


Fig. 6. IgG subclass antibodies to timothy grass pollen in sera from grass allergic patients. Sera obtained before start of immunotherapy and after 3 years of desensitization with timothy extract were tested in the TIRA. Results are expressed as unit IgG subclass antibody per ml undiluted serum. Abscissa: IgG subclass. Ordinate: U antibody/ml serum.

subclasses, binding as high as 20% in the two-layer assay was observed.

Measurement of IgG subclass antibodies to timothy grass pollen extract

Serial two-fold dilutions (1:100 to 1:12,800) of the reference serum JN and of pooled normal human serum (NHS) obtained from non-allergic

donors (7) were run in the TIRA as described in Material and Methods. The results are shown in Fig. 5.

Non-specific binding obtained with NHS did not exceed 0.33% in any of the subclasses, and was independent of serum dilution in the range stated.

Total binding found with JN in the dilution 1:100 was 3.3% and 7.2% in the IgG1 and IgG4 subclasses, respectively. Significant binding was not demonstrable in the IgG2 IgG3 subclasses. The binding ratio (total binding divided by non-specific binding) was 11.8 and 55.4 for the IgG1 and IgG4 subclasses, respectively, at the dilution 1:100 of serum JN.

All patients' sera and controls were tested in duplicate in the dilution 1:100. In each subclass, specific binding was calculated by subtraction of non-specific binding (obtained with NHS in the same dilution) from the total binding observed.

Dilutions of the reference serum JN, corresponding to 1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01 unit IgG4 antibody (see Material and Methods), were used to produce a standard curve.

By use of this IgG4 antibody standard curve, the specific binding observed in each of the

Table 1
Inter-assay precision^a

Sample	IgG subclass	Mean U/ml (% cpm bound)	CV (%) ^b
HD	IgG1	4.30 (0.55)	18 (32)
HD	IgG4	12.00 (1.89)	24 (15)
12/4	IgG1	17.54 (2.77)	26 (22)
12/4	IgG4	3.21 (0.36)	27 (22)

^a Seven determinations in duplicate run on 7 different days within a month. The same batch of timothy-coated balls was used on all days. Significant amounts of IgG2 or IgG3 antibodies were not detected in the two sera on any assay day. The sera were tested in the dilution 1:40.

^b The mean is given as unit antibody per ml serum and as per cent cpm bound (the latter value in brackets). The coefficient of variation (SD/mean) has been calculated for both modes of result expression and is stated in per cent.

four subclasses with a given serum was expressed as units (U) antibody/ml undiluted serum. Values below 2 U/ml were considered insignificant.

Only one of the 20 normal sera contained significant amounts of IgG antibodies to timothy (4.5 U/ml IgG1 and 3.0 U/ml IgG4 antibody).

The amounts of IgG subclass antibodies to grass pollen found in the patients' sera obtained before the start and after 3 years of immunotherapy are shown in Fig. 6.

Detailed results, showing the IgG subclass response during the course of immunization and the correlation between the IgG subclass response and clinical parameters, will be presented elsewhere (8).

The inter-assay coefficient of variation was assessed as part of another study (8), in which the sera were tested in the dilution 1:40. The results are shown in Table 1.

DISCUSSION

Elimination of non-specific binding and selection of serum dilution in the routine TIRA

The major obstacle for successful application of the RAST principle to measurement of IgG and

IgG subclass antibodies in human sera has been unacceptably high non-specific binding (10, 13, 25). We have recently demonstrated (7) that it is possible to eliminate non-specific binding by use of a non-porous sorbent (a plastic ball) and a special incubation buffer (IB) of high ionic strength and high pH (pH 9.2), using bovine gamma globulin and normal rabbit serum as balance proteins. In fact, our IgG RAST (7) shows high binding ratios, even when sera are tested in the dilution 1:10.

In the TIRA presented in this paper we have selected the serum dilution 1:100 for routine measurements for two reasons: First, parallel serum dilution curves were obtained in the IgG RAST at dilutions greater than 1:40 (7). This indicates that the assay is working in antigen excess and validates inter-individual comparison of the IgG response even to complex allergen mixtures (7, 13). Furthermore, great antigen excess reduces or eliminates interference caused by specific antibodies of other classes (e.g. IgE) or subclasses (3, 19). The capacity of the TIRA to detect high levels of both IgG1 and IgG4 in the same (reference) serum demonstrates that the assay is insensitive to competition between antibodies of different subclasses.

Second, non-specific binding in a given subclass remains constant in the range of dilutions from 1:100 to 1:12,800 (Fig. 5). Correction for non-specific binding can therefore be made by use of a single dilution (1:100) of NHS in each subclass. This applies also to the standard curve run, simplifying the assay and reducing practical work and costs.

Specificity of the assay

The antigen specificity of the first (serum) incubation step of the assay was documented during the development of the IgG RAST (7).

In this paper we have validated the subclass specificity of the TIRA: The results presented in Fig. 3 demonstrate that the TIRA specifically measures IgG subclass antibodies of each of the four subclasses without important cross-reactivity to the other three subclasses, to other immunoglobulin classes (IgA, IgE, IgM), or to light chain proteins. In this context we want to

stress the importance of using absolutely pure proteins for testing cross-reactivity in highly sensitive assays. Simple ion-exchange chromatography does not necessarily provide IgG myeloma proteins of sufficient purity for testing cross-reactivity in such assays (3, 11, 18, own unpublished results). In fact, Lowe's group, who developed the monoclonal antibodies used in this study, admitted that they could not decide whether the observed cross-reactivity of the monoclonal antibodies to IgG myeloma of other subclasses was genuine, or due to contamination of the myeloma proteins with polyclonal IgG (18). To solve this very important problem, we isolated two myeloma proteins of each subclass from sera with high concentrations of the protein in question and low concentrations of the other subclasses, by use of an optimized chromatofocusing procedure (taking into consideration the pI of the protein), followed by removal of any contaminating trace amounts of the other subclasses by subtraction affinity chromatography. Evidently (Fig. 3), absolutely pure IgG myeloma proteins were obtained by this procedure.

Assuming that the test proteins were pure, the cross-reactivity of the monoclonal anti-IgG2 (Fig. 3) must therefore be caused by a) non-specific protein-protein interaction, b) presence of a "contaminating" mouse immunoglobulin with broad reactivity against human immunoglobulins (18) in this ascitic fluid, or c) genuine cross-reactivity to a common determinant in all the test proteins. The cross-reactivity against the monoclonal lambda chain protein is probably too high to be explained by (a) or (b), and we consider genuine cross-reactivity the most likely explanation of this observation. Other purified lambda proteins were not available, but it should be noted that the anti-IgG2 showed almost insignificant reactivity to the IgE myeloma protein (Fig. 3) which is of the lambda type.

High IgG2 antibody level was not found in any of the 20 patients sera tested (Fig. 6), nor in the reference serum JN, which contains very high levels of both IgG1 and IgG4 antibody (Fig. 5).

We therefore argue that the observed cross-reactivity of the anti-IgG2 to the monoclonal

lambda test protein does not invalidate the subclass specificity of the TIRA for measurement of a polyclonal IgG subclass response to allergens.

Turning to the specificity of the third incubation step with ^{125}I -labelled rabbit anti-mouse IgG, it suffices to note that the labelled antibody did not bind significantly to any of the eight different human IgG myeloma proteins nor to the other test proteins.

The concept and importance of equipotency

The results presented in Fig. 2 indicate that the order of affinity of the four monoclonal antibodies is IgG3 > IgG4 > IgG1 > IgG2 (23). If these antibodies (or other subclass antibodies with the same rank of affinities) were used in the same dilution (e.g. 1:500) or in the dilution giving the highest specific binding, the amount of IgG3 and IgG4 antibodies would have been significantly overestimated. This fact seems to have been overlooked by several investigators (e.g. (9, 10)), but taken into consideration by others (3, 6).

For intra-individual comparison of the IgG response in different subclasses, it is necessary that the signal (cpm bound) is the same for equivalent amounts of antibody in different subclasses. To ensure such equipotency in the TIRA, we have used exactly the same amount (2 μg /ball) of the different IgG myeloma proteins to coat the balls used to titrate the monoclonal antibodies. By incubation of the different balls with ^{125}I -labelled goat anti-human IgG with reactivity against common determinants we showed that our coupling procedure resulted in binding of the same amount of the different proteins to the balls. The equipotent dilution used in this study therefore should be fully reliable, and a certain amount of allergen-specific antibody should result in the same binding, whichever subclass the antibody belongs to.

Validity of the calibration, sensitivity and precision of the TIRA

In this work, as a preliminary approach, we

have calibrated the antibody response in all four subclasses by use of an IgG4 standard curve, obtained with dilutions of a reference serum. Validity of this approach requires that serum dilution curves are parallel in all four subclasses. Taking into consideration the different total amount of IgG1 and IgG4 antibody in the reference serum (Fig. 5), the IgG1 and IgG4 serum dilution curves are for practical purposes parallel (13). The amount of IgG2 and IgG3 antibodies in the sera tested in this work was too low to permit meaningful studies of serum dilution in these subclasses. A more exact method for calibration, permitting expression of results in absolute amounts of antibody (e.g. $\mu\text{g/ml}$), is under investigation.

Until true standardization has been obtained, the absolute sensitivity of the assay cannot be assessed. However, the amount of IgG antibody to pollen allergens in sera from immunized allergics is low, and lower than the amount of IgG to most other types of allergens (animal dander, mite, insect venom) (7, 24, 25). The amount of IgG to pollen is probably about 1 $\mu\text{g/ml}$ or even less (7, 24). As significant amounts of IgG4 antibodies could be detected in most sera from immunized patients (Fig. 6), we conclude that the sensitivity of the TIRA is sufficient for monitoring the IgG subclass response to most allergens during immunotherapy.

The inter-assay precision of the TIRA (CV 18 to 27%) is somewhat poorer than that found in our two-layer IgG RAST (CV 8 to 20%) (7). We believe that this is due to the extra incubation step included in the TIRA. Thus, a decrease in precision may be the price to pay for the increase in sensitivity obtained with a three-layer assay (see below). However, the precision permits subdivision of positive sera in several classes and is considered sufficient for assessment of the IgG subclass response during immunotherapy.

Practical advantages of the TIRA for IgG subclass antibody determination

We have demonstrated that the TIRA is a reliable and convenient method for determination

of IgG subclass antibodies. Some of the practical advantages provided by a three-layer (or multi-layer) immunoradiometric assay are: 1) Purification of the subclass specific antibodies is not necessary (3, 4). This applies to both polyclonal and monoclonal antibodies. However, it is possible that isolation of the anti-IgG2 from the mouse ascitic fluid might have eliminated the cross-reactivity to some of the test proteins (see above). 2) A certain degree of amplification is obtained for each layer added (3, 19). This increase in sensitivity makes it possible to use high serum dilutions, thereby minimizing non-specific binding and eliminating interference by specific antibodies of other classes and competition between specific antibodies of different subclasses. 3) Only one labelled detector protein is needed. This makes the assay very versatile (4), reduces work with highly radioactive compounds and makes it easier to standardize the assay. 4) Equipotency is easy to assess and to establish. The only disadvantage with a three-layer assay is the extra incubation step and the decrease in assay precision, this may cause.

Finally, we want to draw attention to the fact that not all allergens can be coupled covalently to the nylon balls used in this TIRA. However, such allergens (e.g. wasp venom) are successfully adsorbed to polystyrene balls (Djurup, Malling & Søndergaard, manuscript in preparation).

In conclusion, the TIRA seems to be a useful assay for measurement of IgG subclass antibodies in human sera.

To obtain high and reproducible specific binding, the incubation buffer must be prepared from freshly thawed rabbit serum and supplied with 20 mM EDTA (final concentration).

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R. Djurup is Research Fellow at the University of Copenhagen.

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Address:

René Djurup
Laboratory of Medical Allergology
Rigshospitalet TA 7542
Tagensvej 20
DK-2200 Copenhagen N
Denmark

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