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An Immunoblotting Procedure Following Agarose Gel Electrophoresis for Subclass Typing of IgG Paraproteins in Human Sera

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Summary: A method for subclass typing of IgG paraproteins in human sera following agarose gel electrophoresis is presented.

After electrophoretic separation, serum proteins were blotted by capillary diffusion onto nitrocellulose. Mouse anti-human IgG_1–IgG_4 monoclonal antibodies were exposed to bound IgG. Goat anti-mouse IgG alkaline phosphatase conjugate was employed as second antibody.

Thirty six sera were examined, in which the presence of an IgG paraprotein had previously been proved by agarose gel electrophoresis and subsequent immunofixation with anti-IgG antiserum. The subclass frequency distribution was 27 IgG_1, 6 IgG_2, 2 IgG_3 and 1 IgG_4.

By means of IgG subclass typing in 8 out of these 36 sera, a total of 12 additional monoclonal bands (1 to 2 bands per serum) were detected which were not seen after immunofixation electrophoresis with anti-IgG antiserum. Eleven of 12 additional bands belonged to an IgG subclass different from that of the bands already detected by immunofixation electrophoresis. Light chain typing was performed in 9 of 12 bands found additionally. Two of these 9 bands belonged to a light chain class different from that of the bands already detected by immunofixation electrophoresis.

The method described can be employed to further elucidate the possible diagnostic and prognostic significance of the subclass type of an IgG paraprotein.

Introduction

At present, immunofixation following agarose gel electrophoresis is the method of choice for classifying human paraproteins according to their heavy and light chain type (1).

On the basis of structural differences human IgG molecules can be categorized into the four subclasses IgG_1–IgG_4. Application of immunofixation to subclass typing of IgG paraproteins is, however, problematic, because

(a) polyclonal antibodies against human IgG subclasses seldom fulfil the criteria of specificity and high avidity (2), and

(b) monoclonal antibodies against human IgG subclasses are characterized by high affinity (3, 4) but do often not readily precipitate their corresponding antigens because of their restricted specificity; therefore their use in immunofixation is limited (5).

Recently immunoblotting has been proposed as an alternative method for heavy and light chain typing of human paraproteins (6). By this method the protein pattern generated in a gel is transferred to a porous membrane (e. g. nitrocellulose) to produce a replica; the purpose of this transfer is to facilitate the binding of antibodies to their corresponding antigens on the membrane (for review see l. c. (7)).

The present communication describes an immunoblotting procedure following agarose gel electrophoresis for subclass typing of IgG paraproteins in human sera. Parts of this work have been presented in a preliminary form (8).

Materials and Methods

Samples

We examined

(a) 36 sera in which the presence of an IgG paraprotein had previously been proved by agarose gel electrophoresis and subsequent immunofixation with anti-IgG antiserum (Paragon® Immunofixation Electrophoresis Kit, Beckman Instruments GmbH, Munich, Germany, No. 444930) and

(b) 'adult donor serum' (pooled from healthy adult donors) (The Binding Site Ltd., Birmingham, G. B., No. BP050, lot no. A5565).

Electrophoresis

For subclass typing of IgG paraproteins sera were diluted to 0.2 g/l IgG with 'B-2 barbital buffer' pH 8.6 (10 mmol/l 5,5-diethylbarbituric acid; 50 mmol/l 5,5-diethylbarbituric acid sodium salt) (component of the Paragon® Immunofixation Electrophoresis Kit). Diluted sera were then electrophoretically separated in agarose gels for 30 min using the Paragon® system according to the manufacturer's recommendations (Beckman Instructions 015-246513-H). Diluted serum (5 µl, corresponding to 1 µg of IgG) was applied to the gel surface.

Tab. 1. Processing of blotted nitrocellulose membranes. During all assay stages, except for step 3, membrane strips were contained in plastic tubes (1.5 cm × 10 cm) which were rotated by a rolling mixer, 2.5 ml reagent being employed per tube and incubation step. All incubation steps were performed at room temperature.

1. For blocking of free binding sites, place membrane strips for 1 h into Tris · HCl buffer (20 mmol/l, pH 7.5, with 500 mmol/l NaCl) containing 30 g/l gelatine.
2. Wash twice for 5 min each with Tris · HCl buffer containing 0.5 ml/l Tween® 20.
3. Place Parafilm® on a glass plate, put membrane strips onto Parafilm® and cover with mouse anti-human $IgG_1 - IgG_4$ monoclonal antibodies diluted 1 + 1000 with Tris · HCl buffer containing 0.5 ml/l Tween® 20 and 10 g/l gelatine. Apply 500 µl of diluted antibody solution per strip and incubate for 1 h in a humid chamber.
4. Wash twice for 5 min each with Tris · HCl buffer containing 0.5 ml/l Tween® 20.
5. Incubate for 1 h with an alkaline phosphatase conjugate of goat anti-mouse IgG antibodies diluted 1 + 3000 with Tris · HCl buffer containing 0.5 ml/l Tween® 20 and 10 g/l gelatine.
6. Wash twice for 5 min each with Tris · HCl buffer containing 0.5 ml/l Tween® 20 and once for 5 min with Tris · HCl buffer.
7. Incubate membrane strips for 30 min with 'colour development solution'.
8. Wash twice for 5 min each with doubly distilled water. Dry membrane strips by laying them on filter paper.

Transfer of proteins to a blotting membrane

Nitrocellulose membranes (pore size 0.45 µm, 15 cm × 9.2 cm) (Bio-Rad Laboratories GmbH, Munich, Germany, No. 162-0114) cut to the same size as the gel tracks (44 mm × 8 mm) as well as blot absorbent filter paper (thin) (Bio-Rad, No. 162-0118) were dampened with Tris · HCl buffer (20 mmol/l, pH 7.5, with 500 mmol/l NaCl). After electrophoresis the gel tracks were overlaid with nitrocellulose strips, 2 layers of moistened blot absorbent filter paper (thin), 10 layers of dry blot absorbent filter paper (thick) (Bio-Rad, No. 165-0962), a glass plate and a weight of 2 kg. Transfer of proteins to the membrane strips was allowed to proceed for 90 min.

Processing of blotted membrane strips

All reagents necessary for processing of blotted membrane strips are included in an Immun-Blot® Assay Kit (Bio-Rad, No. 170-6461).

'Colour development solution' was prepared by adding 1 ml of 'colour reagent A' (10 g/l nitroblue tetrazolium and 400 mmol/l $MgCl_2$ in 700 ml/l aqueous dimethylformamide) and 1 ml of 'colour reagent B' (5 g/l 5-bromo-4-chloro-3-indolyl phosphate (as *p*-toluidine salt) in dimethylformamide) to 100 ml of Tris · HCl buffer (0.1 mol/l, pH 9.5).

Details of the procedure are listed in table 1. Characteristics of the mouse anti-human $IgG_1 - IgG_4$ monoclonal antibodies used are given in table 2.

Tab. 2. Characteristics of the mouse anti-human $IgG_1 - IgG_4$ monoclonal antibodies used.

Clone	Murine isotype	Specificity for human IgG	Catalogue No.*
HP 6070	IgG_1 κ	IgG_1 (Fc)	MH 1015
HP 6002	IgG_1 κ	IgG_2 (Fc)	MH 1021
HP 6047	IgG_3 κ	IgG_3 (hinge region)	MH 1032
HP 6023	IgG_3 κ	IgG_4 (Fc)	MH 1042

* Medac GmbH, Hamburg, Germany; data are from the manufacturer's specification sheets.

Controls

The following controls were used:

(a) Mouse monoclonal antibodies were substituted for 'mouse IgG' (isolated from pre-immunized mice) (Medac GmbH, Hamburg, Germany, No. 10400, lot No. 0703) diluted 1 + 1000 with Tris · HCl buffer (20 mmol/l, pH 7.5, with 500 mmol/l NaCl) containing 0.5 ml/l Tween® 20 and 10 g/l gelatine (see tab. 1; step 3). The IgG concentration of diluted 'mouse IgG' solution (2 mg/l) was fourfold higher than that of diluted anti-human $IgG_1 - IgG_4$ monoclonal antibodies.

(b) 'Normal serum minus IgG' (free of IgG as proved by immunoelectrophoresis; total protein concentration: 57 g/l) (Sigma Chemical Co., St. Louis, U. S. A., No. S 5143, lot 29F4866) diluted 1 + 50 with 'B-2 barbital buffer' (see above) was used as sample. Diluted 'normal serum minus IgG' (5 µl) (corresponding to 5.6 µg of total protein) was applied to the gel surface (see above).

Light chain typing of IgG paraproteins

For light chain typing of IgG paraproteins a method described by *McLachlan* (9) was modified as follows: Membrane strips were placed on Parafilm® and covered with mouse anti-human IgG_1-IgG_4 monoclonal antibodies diluted 1 + 100 with doubly distilled water and incubated for 1 h in a humid chamber. Free binding sites were then blocked (see tab. 1, step 1) and strips were washed (see tab. 1, step 2). After electrophoresis (see above) proteins were allowed to diffuse into the pretreated membrane strips (see above) but only IgG belonging to one subclass was retained at the membrane strips. After another washing (see tab. 1, step 4) strips were incubated with alkaline phosphatase conjugates of goat anti-human κ and λ light chain antibodies (Medac GmbH, No. H 16108 and H 16208) diluted 1 + 1000 with Tris · HCl buffer (20 mmol/l, pH 7.5, with 500 mmol/l NaCl) containing 0.5 ml/l Tween® 20 and 10 g/l gelatine. After renewed washing (see tab. 1, step 6) colour development was performed according to steps 7 and 8 (s. tab. 1).

Results

After electrophoretic separation the localizations of the four IgG subclasses from 'adult donor serum' differed clearly from each other: IgG_2 was shifted to the anode as compared with IgG_1. IgG_3 was positioned extremely near the cathode whereas IgG_4 had a pronounced anodal mobility (fig. 1).

All the IgG paraproteins (from 36 sera examined), whose presence had previously been proved by immunofixation, showed a specific interaction with just one of the four IgG subclass-specific antibodies without unspecific binding to antibodies against the other three IgG subclasses (fig. 2). Moreover, controls (a) and (b) did not yield any staining.

The subclass frequency distribution of these IgG paraproteins was 27 IgG_1, 6 IgG_2, 2 IgG_3 and 1 IgG_4.

By application of 1 µg of total IgG to the gel surface we were able to classify IgG paraproteins according to their subclass type in 34 of 36 sera examined. In just 2 sera with IgG_2 paraproteins, the monoclonal immunoglobulins appeared as rather broad IgG_2 fractions; by using lower amounts of IgG, however, distinct IgG_2 bands were detectable so that subclass typing was possible (fig. 3).

By subclass typing in 8 of these 36 sera, a total of 12 additional monoclonal bands (1 to 2 bands per serum) was detected, which were not seen after immunofixation electrophoresis. The subclass frequency distribution of the additional bands was 4 IgG_1, 6 IgG_2 and 2 IgG_3. Eleven of these 12 bands belonged to an IgG subclass different from that of the bands already detected by immunofixation electrophoresis.

Light chain typing was performed in 9 of the additional 12 monoclonal bands (fig. 4). Two of these 9 bands belonged to a light chain class different from that of the bands already detected by immunofixation electrophoresis. Further details are given in table 3.

Discussion

A routine clinical laboratory technique for subclass typing of IgG paraproteins in human sera following agarose gel electrophoresis has not hitherto been reported (5).

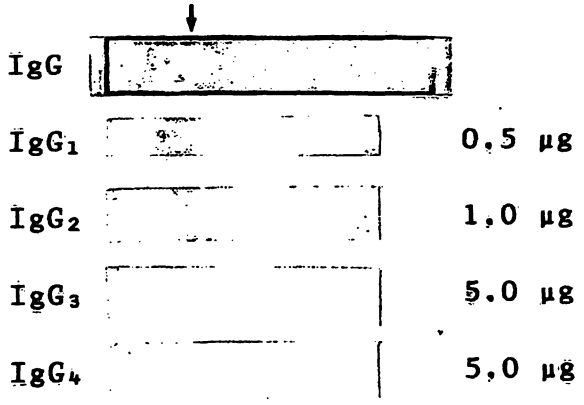

Fig. 1. Electrophoretic mobility of IgG_1-IgG_4 subclasses in 'adult donor serum'
The cathode is on the left, the anode on the right. The point of sample application is indicated by an arrow. The lane at the top shows the detection of total IgG from 'adult donor serum' (pooled from healthy adult donors) by agarose gel electrophoresis and subsequent immunofixation with anti-IgG antiserum. Beneath: nitrocellulose strips revealing the electrophoretic mobility of IgG_1-IgG_4 subclasses. The amount of total IgG applied is noted on the right.

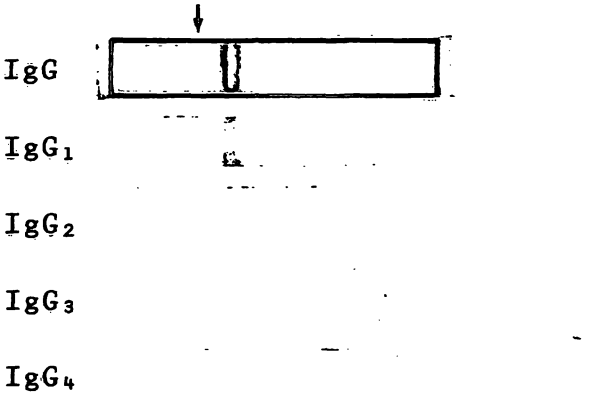

Fig. 2. Subclass typing of an IgG_1 paraprotein.
The cathode is on the left, the anode on the right. The point of sample application is indicated by an arrow. The lane at the top shows the detection of an IgG paraprotein by agarose gel electrophoresis and subsequent immunofixation with anti-IgG antiserum. Beneath: nitrocellulose strips which were exposed to antibodies against the four IgG subclasses.

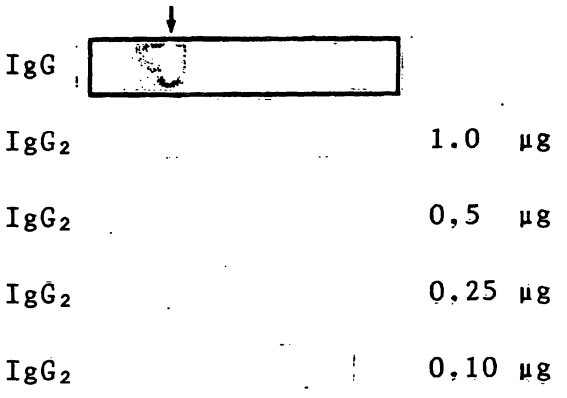

Fig. 3. Subclass typing of an IgG_2 paraprotein yielding equivocal results when 1 µg of total IgG is applied to the gel surface.
The cathode is on the left, the anode on the right. The point of sample application is indicated by an arrow. The lane at the top shows the detection of an IgG paraprotein by agarose gel electrophoresis and subsequent immunofixation with anti-IgG antiserum. Beneath: nitrocellulose strips which were exposed to antibodies against IgG_2; different amounts of total IgG were applied as indicated on the right.
Note that only by application of 0.1 µg of total IgG, does the IgG paraprotein appear as a distinct band.

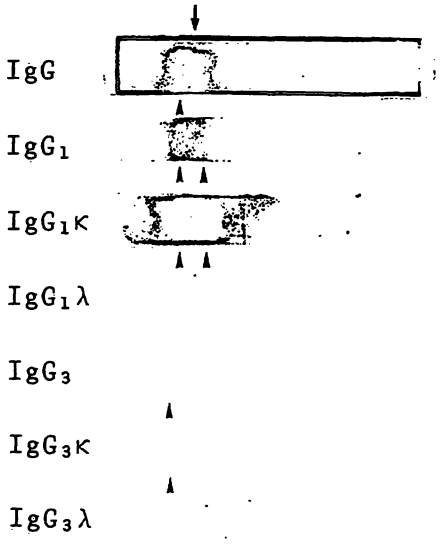

Fig. 4. Subclass and light chain typing of IgG paraproteins additionally found (as compared with immunofixation with anti-IgG antiserum).
The cathode is on the left, the anode on the right. The point of sample application is indicated by an arrow. The lane at the top shows the detection of an IgG paraprotein by agarose gel electrophoresis and subsequent immunofixation with anti-IgG antiserum. For subclass typing, nitrocellulose strips were exposed to anti-IgG_1 and anti-IgG_3 antibodies (denoted by 'IgG_1' and 'IgG_3').
For light chain typing (as described under Materials and Methods) nitrocellulose strips were pretreated with anti-IgG_1 and anti-IgG_3 antibodies and exposed to anti-κ light chain and anti-λ light chain antibodies (designated by '$IgG_1\kappa$', '$IgG_1\lambda$', '$IgG_3\kappa$' and '$IgG_3\lambda$').
The monoclonal bands which were detected are indicated by arrow-heads.

Tab. 3. Subclass and light chain type of monoclonal bands not detected by immunofixation with anti-IgG antiserum.

Number of serum	IgG paraproteins detected by immunofixation with anti-IgG antiserum	IgG paraproteins found only by immunoblotting with IgG subclass-specific antibodies
1	$IgG_1\kappa$	$IgG_1\kappa$, $IgG_3\kappa$
2	$IgG_1\kappa$	$IgG_2\kappa$, $IgG_2\kappa$
3	$IgG_1\kappa$	$IgG_2\kappa$, $IgG_2\kappa$
4	$IgG_1\kappa$	$IgG_2\lambda$
5	$IgG_1\lambda$	$IgG_2\kappa$
6	$IgG_2\kappa$	IgG_1*
7	$IgG_2\lambda$	$IgG_3\lambda$
8	$IgG_3\lambda$	IgG_1*, IgG_1*

* Light chain typing not performed.

The method for subclass typing of IgG paraproteins presented in this report additionally increases the diagnostic sensitivity for the detection of monoclonal gammopathies; this can be explained as follows:

(a) Detection of monoclonal bands at low concentrations is often hampered by high concentrations of polyclonal immunoglobulins. Application of IgG subclass-specific antibodies enables a partial reduction of that 'background' which consists of polyclonal IgG;

(b) For immunofixation, a precipitation of immune complexes is required, so that repeated dilutions of samples must often be performed to detect monoclonal bands at low concentrations. By application of immunoblotting, however, monoclonal bands differing widely in concentration can be seen by using one combination of sample and antibody dilution (6).

Since the monoclonal bands found additionally (as compared with immunofixation) had a low concentration, light chain typing of these bands was hampered by the obscuring 'umbrella effect' (10) of the light chains of polyclonal immunoglobulins. To overcome this problem, IgG molecules belonging to a specified subclass were selectively transferred to nitrocellulose strips coated with antibodies against this subclass; IgG subclass-specific light chains were then subsequently detected (9). Comparing the method for subclass typing presented in this paper with those published previously, we feel that our procedure has the following advantages.

(a) *Fasullo* et al. (5) used peroxidase-conjugated monoclonal antibodies of the same clonal origin as those employed by us for an immunoblotting procedure after agarose gel electrophoresis; by this method, in sera containing IgG paraproteins, all four IgG subclasses appeared as thick bands despite the predominance of one IgG subclass.

We preferred to employ unconjugated monoclonal antibodies and alkaline phosphatase-conjugated secondary antibodies to achieve unequivocal subclass typing of IgG paraproteins.

(b) *Radl* et al. (13) has already shown that the diagnostic sensitivity for the detection of monoclonal gammopathies can be increased (as compared with immunofixation) by immunoblotting and application of IgG subclass-specific antibodies. However, light chain typing of additional bands is often difficult (see above). Therefore we applied 'affinity blotting' (9) to overcome this problem.

(c) *Magnusson* et al. (12) quantitatively determined the concentrations of the four IgG subclasses in sera from patients with known IgG paraproteinaemia. In this way IgG subclass typing is only possible if the concentration of that IgG subclass to which the paraprotein belongs clearly exceeds the upper reference limit.

(d) *Schur* et al. (11) studied the subclass frequency distribution of IgG paraproteins by immunoelectrophoresis using polyclonal antibodies against the four IgG subclasses, but employed antisera against IgG_2, IgG_3 and IgG_4 only when there was no reaction to antiserum against IgG_1. Since polyclonal antibodies often do not fulfil the criterion of specificity (2), an unspecific reaction cannot be excluded by such a procedure.

The subclass frequency distribution of IgG paraproteins in human sera found by us is concordant with that reported earlier (11); the same applies to the electrophoretic mobility of polyclonal $IgG_1 - IgG_4$ (12).

The method described here may be employed to further elucidate the possible diagnostic and prognostic significance of the subclass type of an IgG paraprotein (11, 13).

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