A Simple Method of Screening for Multiple Myeloma of the IgG-3 Type

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SUMMARY
A simple method for distinguishing IgG-3 myelomas from IgG myelomas of the other subclasses is described. The method relies on the increased molecular size of the IgG-3 molecule, and is far quicker than previously described chemical methods of subclass typing.


Human IgG consists of 4 subclasses determined by the γ heavy chain, of which there are 4 variants (γa, γb, γc, and γd). The subclasses are known to differ in their antigenic, structural and biological properties. The IgG-3 molecule differs in size from those of the other IgG subclasses since it has a larger heavy chain of 58-60,000 compared with 50,000 daltons. The γ heavy chain has an extended hinge region consisting of unequal duplications of cysteine-containing peptides, and as such bears more resemblance to α heavy chains than to those of the other γ subclasses.

The IgG-3 molecule has a marked concentration and temperature-dependent aggregation, and of patients with IgG multiple myeloma, those with the IgG-3 subclass have an increased tendency to develop the hyperviscosity syndrome (serum viscosity >4.2 relative to water at 27°C) at much lower myeloma protein concentration — 40 g/l compared with 140 g/l for myelomas of the other IgG subclasses.

The hyperviscosity syndrome presents with the following symptoms: bleeding from mucous membranes, retinopathy with distended veins and haemorrhages, neurological disturbances and congestive heart failure. It is therefore of value to identify the subclass in patients with IgG myelomas, since plasmapheresis, which has been successfully used to combat hyperviscosity, can be initiated selectively in IgG 3-type myeloma patients at a lower myeloma protein concentration.

IgG subclass-specific antisera are difficult to obtain and cross-react with other IgG subclasses. A physicochemical typing method has been described. This method, however, is extremely laborious and time-consuming, since two proteolytic digestion steps on the isolated myeloma protein are required, followed by electrophoresis and autoradiography.

In this report, we describe a simple method for the detection of IgG-3 myelomas. Polyacrylamide gel electrophoresis is used in the presence of SDS (SDS-PAGE), and relies on the increased molecular size of IgG 3 relative to the other IgG subclasses.

MATERIALS AND METHODS
Serum was taken from 8 patients with multiple myeloma of the IgG type. All these patients exhibited clinical features of the disease and had a monoclonal peak on cellulose acetate electrophoresis which was found by radial immunodiffusion to be IgG. Serum was also taken for comparison from 2 patients with a polyclonal increase in γ-globulins, as determined by cellulose acetate electrophoresis.

Polyacrylamide gel electrophoresis, in the presence of 0.1% w/v SDS (SDS-PAGE), was carried out essentially as described by Laemmli. Flat bed gels, 3-20% gradient, size 100 × 160 × 1.5 mm, were used. The running gel contained 0.375M tris-HCl, pH 8.8, and 0.1% SDS. The electrode buffer was 0.025M tris-HCl, pH 8.3, containing 0.2M glycine and 0.1% SDS. Samples containing approximately 10 μg protein were applied in 15 times diluted gel buffer containing 10% glycerol. In some instances β-mercapto-ethanol (5%) was added to diluted samples before application to reduce disulphide bonds. Electrophoresis was carried out for 16 hours at 150 V, after which the gel was fixed in 12.5% trichloracetic acid and stained with Coomassie Blue (Sigma).

IgG was isolated from sera by coupled Sephadex G25/DEAE 52 cellulose chromatography. Gel filtration reduced the ionic strength of the serum, thus allowing serum proteins to bind to the ion exchange resin. Two millilitres bed volume of DEAE 52 cellulose (Whatman) in 5 mM phosphate buffer, pH 7.4, were poured into a column 0.7 × 30 cm in size. After the cellulose had settled, the column was topped with 8 ml Sephadex G25 in the same buffer. Serum 0.5 ml was applied to each column and eluted with the same buffer. Only proteins migrating in the γ region on cellulose acetate electrophoresis were not bound to the DEAE 52 cellulose. All the patients chosen had γ migrating myeloma proteins. The protein eluted from the column was dialysed against 0.06M NH₄HCO₃ and lyophilized.

Papain (BDH) digestion of the isolated myeloma protein was carried out as described by Porter. Fifty micrograms of the isolated IgG was digested for 16 hours at 37°C by 4 μg of papain in 0.1M phosphate buffer pH 7.0 containing 2 mM EDTA. The reaction was stopped by the addition of SDS to 0.1% and by heating for 1 minute at 90°C. Aliquots were then immediately electrophoresed by SDS-PAGE in the presence and absence of β-mercapto-ethanol.

Ouchterlony double radial immunodiffusion on 1% agarose plates containing barbiturate buffer pH 8.6, ionic strength 0.075 μ, was used to type the IgG-3 subclasses. Antisera were obtained from the Central Laboratory of the Netherlands. Ten microlitres each of sample and anti-
serum were applied to the wells. Doubling dilutions were done in 40 mM phosphate buffer, pH 7.4, containing 0.5% BSA, 150 mM NaCl and 10 mM EDTA. The plates were left for 72 hours to develop precipitin lines before being washed with isotonic saline and stained with naphthalene black.

**RESULTS**

SDS-PAGE of whole sera from 8 patients with myeloma and 2 patients with a polyclonal increase in \( \gamma \)-globulins is shown in Fig. 1. Coupled Sephadex G25/DEAE 52 chromatography resulted in the removal of all non-\( \gamma \) migrating proteins from the sera, as determined by cellulose acetate electrophoresis. SDS-PAGE of the eluate (Fig. 2) confirmed the existence of only a single species. Serum from patient 7, who had a polyclonal increase in \( \gamma \)-globulins, was omitted as a control. The myeloma protein from patient 4 (lane 4) has been partly degraded to polypeptides of lower molecular weight by storage of the serum, since an earlier preparation contained only the single high molecular weight species. It can be seen that the myeloma proteins from patients 4 and 5 are of larger molecular size. Their molecular weight was found, by comparative electrophoresis on SDS-PAGE with marker proteins of known molecular weight, to be 158 - 160,000, compared with 150,000 for the other myeloma proteins. The lack of increased staining of the electrophoretogram with the periodic acid-Schiff reagent (PAS) showed that the increased molecular size was not due to increased carbohydrate content. SDS-PAGE of the \( \beta \)-mercapto-ethanol-reduced myeloma proteins showed that the increased molecular weight could be accounted for by an increased size of the heavy chains.

**Table I. Subclass Antiserum Titres of the 8 Myeloma Patients and 2 Patients with a Polyclonal Increase in \( \gamma \)-globulins**

<table>
<thead>
<tr>
<th>Patient</th>
<th>( \gamma_1 )</th>
<th>( \gamma_2 )</th>
<th>( \gamma_3 )</th>
<th>( \gamma_4 )</th>
<th>Remarks</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>&gt;1024</td>
<td>0</td>
<td>8</td>
<td>2</td>
<td>( \gamma_1 )</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3</td>
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<td>0</td>
<td>32</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>&gt;2048</td>
<td>0</td>
<td>( \gamma_3 )</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>64</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>&gt;32</td>
<td>0</td>
<td>32</td>
<td>4</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>7</td>
<td>&gt;32</td>
<td>0</td>
<td>32</td>
<td>0</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>8</td>
<td>&gt;1024</td>
<td>0</td>
<td>&gt;32</td>
<td>2</td>
<td>( \gamma_1 )</td>
</tr>
<tr>
<td>9</td>
<td>&gt;1024</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>( \gamma_1 )</td>
</tr>
<tr>
<td>10</td>
<td>&gt;1024</td>
<td>0</td>
<td>32</td>
<td>2</td>
<td>( \gamma_1 )</td>
</tr>
</tbody>
</table>

Immunological subclass typing revealed that the subclasses of only 5 of the 8 myeloma proteins could be identified using positive precipitin formation at high (>1000) antigen dilution as the criteria for definitive sub-

**Fig. 1. SDS-PAGE of whole sera from 8 myeloma patients (patients 1 - 5 and 8 - 10 in lanes 1 - 5 and 8 - 10), and 2 patients with a polyclonal increase in \( \gamma \)-globulins (patients 6 and 7 in lanes 6 and 7). Lane 1 is on the left. The positions of the IgG and albumin(ALB) are shown.**

**Fig. 2. SDS-PAGE of the isolated IgGs. Patient and lane orientation are the same as for Fig. 1.**

**Fig. 3. SDS-PAGE of papain-digested isolated IgGs. Patient and lane orientation are the same as for Fig. 1. The positions of the Fc and Fd fragments as well as the light chains (L) and peptides (P) resulting from the digestion are shown.**
The isolated proteins were digested with papain in the absence of any added sulphydryl group reagent. The digests were then electrophoresed on SDS-PAGE. It can be seen (Fig. 3) that papain digestion resulted in a heterogeneous collection of peptide fragments.

**DISCUSSION**

In this report we have described a simple method for the detection of IgG myelomas of the γ3 subclass. This diagnosis is important, in view of the known association of the hyperviscosity syndrome with IgG-3 myeloma due to the temperature- and concentration-dependent aggregation of IgG-3.

The described method relies on the increased molecular size of IgG-3, relative to that of the other IgG subclasses. Flat-bed gradient polyacrylamide gels were used, thus allowing immediate comparison of the size of the isolated myeloma proteins. This method has a number of advantages over previously described chemical and immunological methods for IgG subclass typing. The chemical method of Kochwa et al. is extremely laborious and time-consuming (approximately 1 week), whereas IgG-3 determination by our method can be carried out in 24 hours.

Immunological methods have a number of disadvantages. Antisera have restricted availability and are not subclass-specific. Cross-reactivity of all the myeloma proteins with anti-IgG-3 was detected (Table I). Only 5 of the 8 myeloma proteins described here could be definitely subclass-typed using as the criteria positive precipitin formation at high antigen dilution. It is recommended (directions for use of anti-human IgG subclass antisera; Central Laboratory of the Netherlands) that the Ouchterlony plates are left for 72 hours to allow precipitin formation to develop (compare 24 hours by our method). No precipitin formation was obtained with anti-IgG-2 antiserum. This could be explained either by an absence of an IgG-2 myeloma in this study coupled with high antiserum specificity, or by inactive antiserum. We favour this latter explanation, since no cross-reactivity was seen between anti-IgG 2 and the sera of the 2 patients with a polyclonal increase in γ-globulins.

Patient 5 most probably had a myeloma of the γ3 subclass. Not only was the isolated myeloma protein of the same molecular size as that of patient 4, who was typed as IgG 3, but the isolated protein was also papain-digestible. IgG molecules of the γ3 and γ1 subclasses are known to be resistant to papain cleavage (compare with patient 3) (see Fig. 3). No cross-reactivity with anti-IgG 1 or anti-IgG 4 was obtained. Both antisera were active (compare with patient 6).

SDS-PAGE of whole sera (see Fig. 1) resulted in a heterogeneous appearance of IgG molecules. The reason for this phenomenon is not understood, but it may be due to binding of the immunoglobulins to low molecular weight serum components, since ion exchange chromatography removed much of the heterogeneity (Fig. 2). For this reason SDS-PAGE for γ3 subclass determination should be carried out on DEAE 52-treated material.

**REFERENCES**