A diazophenylthio-ether-IgG probe for analysing fibrin- and fibrinogen-related antigens

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Summary
A technique for identifying and quantitating fibrin- and fibrinogen-related antigens (FRAs) in serum is described. Panspecific antifibrinogen IgG is bound covalently to diazotised aminophenylthio-ether cellulose paper discs. Under conditions of antibody excess, a disc probe extracts 95% of all fibrinogen-related antigen from appropriately diluted serum samples. Bound antigen is then eluted from the washed probe and electrophoresed on a 4 - 11% gradient SDS-polyacrylamide gels. Derivatives of cross-linked fibrin, non-cross-linked fibrin and fibrinogen may thus be identified by their molecular weights, and their relative concentrations determined by densiometric analysis. The total amount of FRA is estimated by a radio-immunoassay which uses labelled D-dimer as the competitive antigen. The probes can extract as little as 0.20 and 0.15 μg/ml of D-dimer and D-monomer, respectively. Specific FRA binding was confirmed with Western blotting. Potential interfering substances do not influence antigen binding to the discs and, since complete elution of all antigens is achieved, discs may be reused. This procedure provides estimates of the serum levels of fragments YY, DY, X, DD, Y and D. The procedure is simple to perform and, as a research tool, may help to delineate some of the features of clotting disturbances in clinical states.

Materials and methods

Purification of antigens
Fibrinogen was prepared by repeated ammonium sulphate precipitation of pooled citrated human plasma. The final precipitate was dissolved in 0.05M trisodium citrate and dialysed exhaustively against 0.05M tris-HCl (pH 7.4) with 0.15M NaCl and 0.02% (w/v) sodium azide (buffer A).

Fibrinogen-derived D-monomer and fragment E were prepared as described previously. Non-cross-linked fibrin monomers were prepared according to the method of Connaghan et al. Fibrin-derived D-dimer was prepared using fast protein liquid chromatography. Purified human fibrinogen was clotted and the resulting cross-linked fibrin digested with plasmin. Calcium chloride, 5 mM, was added to all buffers to prevent destabilisation of the FRAs. The clotting mixture consisted of 100 mg fibrinogen in buffer A, 25 units of human thrombin (Sigma) in 50% (w/v) glycerol and 0.005M t-cysteine (BDH). The clot, formed at 37°C for 24 hours, was homogenised, washed extensively with 0.05M tris-HCl (pH 7.4) in the presence of 150 mM NaCl and 5 mM CaCl₂ and digested with plasmin for 18 hours at 37°C. The digestion mixture consisted of 5.5 mg porcine plasminogen (Sigma), 3000 Ploug units of urokinase (Leo Pharmaceutical), 5 mM CaCl₂, 150 mM NaCl and 0.1M tris (pH 7.4) in 5 ml of this buffer. Digestion was stopped by adding 1 000 kallikrein inactivator units of aprotinin (Novo Industries) and 25 mM aminocaproic acid (Lederle). The mixture was dialysed against 25 mM imidazole/HCl buffer (pH 8.0).

All samples were degassed and filtered through a 0.22 μm filter (Millipore) before chromatofocusing on a Mono P HR 5/20 chromatofocusing column (Pharmacia) pre-equilibrated with 0.025M imidazole/HCl buffer (pH 7.4). A linear descending pH gradient was generated with 200 ml of 1:10 dilution polybuffer 74 (Pharmacia) adjusted to pH 3.5 with 1M HCl, 2 ml of which was applied to the column before sample application. The flow rate was 1 ml/min and aliquots of 2.5 ml were collected. The pH and absorbance (280 nm) of the eluate were continuously monitored.

Aliquots of fractions containing protein were analysed electrophoretically using SDS-polyacrylamide gel electrophoresis. Fractions free of fragment E were pooled, concentrated by dialysis against polyethylene glycol (MW 6 000) and applied to a Superox 6B gel filtration column (100 x 2.5 cm) (Pharmacia) and eluted with 0.1M tris/HCl (pH 7.4) containing 5 mM CaCl₂. Fractions containing D-dimer were pooled, concentrated and stored at -70°C.
Preparation of IgG

Rabbits were immunised with D-dimer, D-monomer, fragment E and human fibrinogen, respectively. Each antisera was assessed by immunoprecipitation and by titration against the various radiolabelled fragments. IgG was purified by ammonium sulphate precipitation and dialysed exhaustively into sodium borate buffer 0.1M (pH 9.5).

Preparation of IgG-diazophenylthio-ether (DPT) cellulose paper discs

APT cellulose paper (Schleicher & Schuell), stored at 4°C in a light- and oxygen-free environment, was activated by diazotisation. All procedures were carried out at 4°C. APT paper was placed in 250 ml of 0.5% NaNO2. Freshly prepared 0.145M NaNO2 was then added, 0.5 ml for each 10 ml HCl. The paper was left in this solution for 30 minutes, after which it was washed twice with distilled water, followed by two washes with 166 mM NaCl; 166 mM acetic acid and 33 mM sodium acetate (pH 4.0). The resulting activated DPT paper was incubated in the freshly prepared IgG solution (50 µg/ml) for 12 hours and gently shaken.

Residual binding capacity was quenched by incubating the paper with 10% ethanolamine in 0.1M tris/HCl (pH 9.0) at 37°C. After 2 hours the paper was washed and stored in 0.1M sodium phosphate buffer with 0.9% NaCl at 4°C. Immediately before use, 6 mm diameter discs were prepared using a cork borer (Gallenkamp).

Assessment of IgG binding to paper discs

In order to quantitate the amount of IgG binding, discs onto which IgG had been bound were incubated in 0.2M sodium borate buffer (pH 9.0) containing 0.1M sodium dithionite to reduce the azo-bonded ligands. After 2 hours of constant shaking, the protein concentration of the buffer was measured.

Assay procedure

Paper discs were incubated with appropriately diluted sera. Degree of dilution was calculated according to the total FRA level, as determined by radio-immunoassay. Incubation was carried out for 2 hours at room temperature with constant shaking with a total volume of 500 µl. After vigorous washing with PBS, elution of bound antigen was achieved by placing the discs in 500 µl of 0.1M tris/HCl (pH 8.8) for 2 hours at room temperature.

SDS-polyacrylamide gel electrophoresis

Electrophoresis was performed according to the method of Laemmli with 4–11% gradient gels cast at room temperature in a SE 200 slab gel electrophoresis unit (Hoeffer Scientific Instruments). Gels were polymerised for 1 hour. Electrophoresis was run for 2 hours at room temperature using constant current (20 mAmp) with a running buffer consisting of 0.1M tris (pH 8.8) with 0.1% SDS and 1.5% glycerol. The gels were fixed and stained in a solution containing 33% methanol, 10% TCA and Coomassie brilliant blue (Sigma) and then de-stained in an aqueous solution of 25% ethanol and 8% acetic acid. Molecular weights were determined by comparison with known standards (Pharmacia).

Within-gel and between-gel variations were assessed by electrophoresing the same sample in each of 10 lanes of a gel and in a single lane in 25 different gels. The densitometric profiles of each of these runs were compared.

Radiolabelling of FRAs

For studies requiring radiolabelled antigens, D-dimer, D-monomer and fibrinogen were labelled with either iodine-125 or iodine-131 by a modification of the iodine monochloride method. Extent of labelling was determined by precipitation of the protein with 20% trichloro-acetic acid; 86.4 – 94.3% of the label was protein-bound in 6 samples (mean 91.2%, SD ± 2.1%).

Radio-immunoassay of FRAs

The radioligand (125I) D-dimer and antifibrinogen antisera were used throughout. D-dimer was labelled by the chloramine-T method of Hunter and Greenwood and the labelled protein was separated from free iodide by Sephadex G-100 gel filtration chromatography. Labelled D-dimer was assessed using immunoprecipitation, chromato-electrophoresis, TCA precipitability and electrophoresis with autoradiography. All samples were assayed in duplicate, and separation of bound and free ligands was achieved by precipitation of the bound fraction with a donkey anti-rabbit serum. Optimal dilutions of the first and second antibodies and for normal rabbit serum were determined with dilution curves and grid precipitation patterns. Logit transform slopes, potencies and affinities of the various fibrin and fibrinogen fragments for antifibrinogen serum were compared. Standard curves were fitted by the four-parameter logistic model of Rodbard and Hutt and the least-squares test was used to determine slopes, intercepts and standard error. Minimum sensitivity of the assay was 120 pg.

Assessment of DPT-IgG discs

Specific FRA binding to the discs was assessed by Western blotting. Rabbit anti-human fibrinogen serum was the first antibody and peroxidase-conjugated goat anti-rabbit serum the second. The detection system included 3,3'-diaminobenzidine tetrahydrochloride (BDH) or 4-chloro-naphthol (Sigma) as the substrate. Serum free of FRAs was used to assess nonspecific binding to the discs. This serum was obtained by passage through a Sepharose 4B column (Pharmacia) onto which antifibrinogen IgG had been covalently bound. The absence of FRAs in the eluate was confirmed on electrophoresis and by obtaining no line of immunoprecipitation against antifibrinogen serum. Binding efficiency of the discs, as well as the influence of other serum proteins and molecules on FRA binding to the discs was assessed. Optimal serum dilutions, paper disc size and incubation/elution conditions were defined. All experiments were performed in duplicate at 4°C, room temperature and at 37°C.

Blood sample collection

Venous blood was obtained from subjects with a 21-gauge needle without the use of a tourniquet. After discarding the initial 3 ml, 10 ml of blood was collected into a clean cold syringe. For preparation of plasma, 3.8% sodium citrate and 25 mM amino-caproic acid were added to 5 ml of blood. After centrifugation at 2000 g for 15 minutes, the supernatant fluid was immediately frozen and stored at –70°C until required. Serum was prepared by clotting citrated plasma on ice with an equal volume of 2 M urea containing 0.1M EACA, 0.025M CaCl₂ and thrombin 2 U/ml. The clot was left undisturbed for 4 hours at 0°C after which the serum was extruded with a wooden applicator stick, centrifuged for 20 minutes at 10000 g and immediately diluted and incubated with the paper discs.

Densitometric analysis

Densitometry was performed on a Berthold Apparatus densitometer which automatically calculated ratios and relative percentages under the curve.

Results

The purity of fibrinogen, D-dimer, D-monomer and fragment E prepared as described above is shown in Fig. 1. Immunoreactivity of antisera raised against these preparations was confirmed using Western blotting and immunodiffusion. Standard curves comparing IgG in solution with IgG covalently linked to the insoluble matrix were parallel and of similar sensitivi-
Fig. 1. 11% gradient SDS-polyacrylamide gel electrophoresis of fibrinogen, D-dimer, D-monomer and fragment E (lane 2). Fibrinogen (lane 3), D-dimer (lane 4), D-monomer (lane 5) and fragment E (lane 6) were electrophoresed separately to demonstrate their purity and relative electrophoretic mobilities. Lane 1 contains a mixture of standard high-molecular-weight markers (Pharmacia). The anode is at the bottom.

Fig. 2. Comparison of the affinity of antifibrinogen IgG for fibrinogen, D-dimer, D-monomer and D-monomer. The antifibrinogen IgG being assessed was in solution (A) or bound covalently to activated APT paper discs (B). The percentage of fibrinogen, D-dimer and D-monomer bound to the IgG is demonstrated on the Y axis and the amount of protein used (in D-monomer equivalents) is on the X axis.

Fig. 3. 11% gradient SDS-polyacrylamide gel electrophoresis of FRAs extracted from normal serum. Antigens are identified according to their molecular weights as determined by their electrophoretic mobilities.
TABLE I. BINDING OF FRAs TO DISCS IN THE PRESENCE OF POTENTIAL INTERFERING SUBSTANCES

<table>
<thead>
<tr>
<th></th>
<th>Control serum</th>
<th>Bilirubin</th>
<th>Cholesterol</th>
<th>Albumin</th>
<th>Haem</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1^251) Fg</td>
<td>95,1 ± 1,7</td>
<td>95,1 ± 0,9</td>
<td>97,2 ± 1,3</td>
<td>96,2 ± 0,8</td>
<td>94,0 ± 0,7</td>
</tr>
<tr>
<td>(1^251) DD</td>
<td>98,0 ± 2,2</td>
<td>96,8 ± 1,2</td>
<td>98,1 ± 1,3</td>
<td>98,0 ± 0,9</td>
<td>98,0 ± 0,9</td>
</tr>
<tr>
<td>(1^251) D</td>
<td>95,5 ± 1,1</td>
<td>94,4 ± 1,0</td>
<td>93,3 ± 1,3</td>
<td>93,3 ± 1,2</td>
<td>95,5 ± 0,8</td>
</tr>
</tbody>
</table>

Radio-labelled fibrinogen, D-dimer or D-monomer was added to a control serum. To this serum was added bilirubin, cholesterol, albumin or haem in quantities greater than their physiological concentrations. The mixture was incubated with the D.FT-ICG disc for 2 hours and then washed vigorously in 0.5M phosphate buffer (pH 7.4). The paper discs and the incubation mixtures were then counted on an auto-gamma scintillation spectrometer (Packard). Data represent mean ± SD for 6 determinations and are corrected for non-protein-bound radioactivity.

Elution of bound radio-labelled antigens was initially rapid, followed by a slow linear release. By 2 hours all of the label was in the elution buffer, with no detectable radioactivity left on the disc.

During preparation of serum samples, the percentage incorporation of radio-labelled fibrinogen, D-dimer and D-monomer into the plasma clot was 95.2 ± 1.2%, 2.6 ± 0.4% and 2.6 ± 0.3% respectively.

Proteins extracted from sera by the probe were shown to react with antifibrinogen IgG by Western blot and to have electrophoretic mobilities corresponding to identifiable fibrinogenetic fragments. Other proteins which could not be identified by their mobility (Fig. 3), were also shown to react with antifibrinogen IgG (Fig. 4). The topic of presumptive FRAs has been reviewed recently.

Albumin is apparently the only quantitatively important protein which binds to the disc probes nonspecifically.

The minimal detectable concentration of D-dimer, D-monomer and fibrinogen on electrophoresis was 0.20, 0.15 and 0.25 µg/ml respectively. Linear dose-response on electrophoresis for D-dimer, D-monomer and fibrinogen was observed between 25-250, 5-250 and 10-200 ng/ml of incubation mixture respectively when purified fragments were tested separately, in mixtures, and when added to serum controls.

When cholesterol (4 mg/ml), bilirubin (120 mg/ml), haemoglobin (2 g/l), or albumin (60 mg/ml) was added to serum, antigen affinity for the discs was not reduced or altered (Table I).

The proportions of D-dimer to D-monomer demonstrated by densitometric analysis of the polyacrylamide gels were similar to those present in the initial incubation mixtures (Table II). In addition, ratios of (1^251) D-dimer to (1^31) D-monomer in the initial incubation mixtures corresponded well with the ratios subsequently observed bound to discs (Table II).

An incubation period of 2 hours was chosen since maximal binding had been achieved by this time. Under the above conditions, the probes bound radio-labelled FRAs as follows: 97.0 ± 2.5% of D-dimer (N = 6), 95.2 ± 1.6% of D-monomer (N = 6) and 95.8 ± 2.3% of fibrinogen (N = 6). After incubation with discs for 2 hours, serum mixtures contained less than 150 pg of residual FRA as measured by radio-immunoassay. This result indicates that recovery of FRA by the probe is greater than 99%. This small difference possibly suggests loss of FRA by adsorption to the assay apparatus.

Protein quantities of 0.15 µg/ml or greater could be detected on densitometry of the polyacrylamide gel. The coefficient of variation between runs on electrophoresis, which included the use of two batches of APT paper as well as IgG prepared from the same rabbit at different times, did not exceed 6.2%, 5.3% and 8.1% for D-dimer, D-monomer and fibrinogen respectively (Table III). Within-run coefficient of variation did not exceed 6.0%, 9.9% and 4.4% for D-dimer, D-monomer and fibrinogen respectively (Table IV).
The normal values for fibrinogen and the principal FDPs in serum are given in Table V. The mean total FRA level was 0.167 ± 0.043 μg/ml. The range was 0.038-0.186 μg/ml. The applicability of this technique to the study of clinically relevant pathological conditions was demonstrated in 20 patients with hepatocellular carcinoma. These patients are known to produce a hepatoma-associated dysfibrinogen and have been shown to have enhanced antifibrinolytic activity in their sera. All patients had elevated total FRA levels (mean 1.23 μg/ml, range 0.41-2.78 μg/ml). In comparison with the electrophoretic patterns obtained from control subjects, the FRA profiles of these patients demonstrated high molecular bands similar to the X-oligomers described by Graeff et al. These bands all reacted with antifibrinogen serum on Western blot. Fig. 5 shows a representative profile of FRAs from one of these patients.

Discussion

A variety of procedures have been described for quantitating and identifying circulating fibrinogen, non-cross-linked- and cross-linked-fibrin derivatives in serum and plasma. Quantitative procedures including radio-immunoassays and enzyme-linked immunoassays provide no comparative analysis of the various circulating fragments, while techniques aimed at extracting various FRAs fail to recover them in proportion to their initial concentrations.

Our procedure for antigen extraction from serum samples is designed to take place under conditions of antibody excess. This allows maximum FRA binding to the discs thereby avoiding the unequal antigen recovery seen with precipitation extraction techniques. The method detects 0.2 and 0.2 μg/ml of D-dimer and D-monomer respectively, comparing...

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### Table III. Between-Run Variation for FRAs in a Serum Sample

<table>
<thead>
<tr>
<th>Fm</th>
<th>DD</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>13.1</td>
<td>28.4</td>
</tr>
<tr>
<td>SD</td>
<td>0.7</td>
<td>1.8</td>
</tr>
<tr>
<td>CV %</td>
<td>5.3</td>
<td>6.2</td>
</tr>
</tbody>
</table>

The FRAs from a serum sample from a healthy adult donor were extracted and electrophoresed. The procedure was repeated 25 times, each time employing a separately prepared polyacrylamide gel.

### Table IV. Within-Run Variation of FRA Proportions in 3 Sera

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fg</td>
<td>DD</td>
<td>D</td>
</tr>
<tr>
<td>Mean %</td>
<td>12.6</td>
<td>28.6</td>
</tr>
<tr>
<td>SD</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>CV %</td>
<td>4.5</td>
<td>6.0</td>
</tr>
</tbody>
</table>

FRAs were extracted from serum and subjected to 4-11% gradient gel electrophoresis. The extracted FRAs were electrophoresed in each of 10 lanes of a gel. This was repeated with 3 different serum samples.

### Table V. FRA Levels in Serum from Healthy Individuals

<table>
<thead>
<tr>
<th>Fm</th>
<th>YY</th>
<th>DY</th>
<th>X</th>
<th>DD</th>
<th>Y</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean %</td>
<td>12.7</td>
<td>5.5</td>
<td>1.6</td>
<td>1.9</td>
<td>29.9</td>
<td>11.3</td>
</tr>
<tr>
<td>SD</td>
<td>1.7</td>
<td>0.7</td>
<td>0.4</td>
<td>0.3</td>
<td>3.1</td>
<td>1.7</td>
</tr>
<tr>
<td>CV %</td>
<td>13.1</td>
<td>12.7</td>
<td>25.2</td>
<td>14.7</td>
<td>10.2</td>
<td>14.9</td>
</tr>
<tr>
<td>Mean ng/ml</td>
<td>21.2</td>
<td>9.7</td>
<td>2.7</td>
<td>3.2</td>
<td>50.0</td>
<td>18.9</td>
</tr>
<tr>
<td>SD</td>
<td>2.6</td>
<td>1.2</td>
<td>0.7</td>
<td>0.5</td>
<td>0.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Range</td>
<td>16.1 - 25.4</td>
<td>7.2 - 10.4</td>
<td>2.2 - 5.2</td>
<td>1.8 - 3.9</td>
<td>41.0 - 60.2</td>
<td>15.1 - 23.3</td>
</tr>
</tbody>
</table>

FRAs were extracted from the sera of 30 healthy adult donors with ages 24-61 years. None was on any medication nor did any have clinical reasons for FRAs to be elevated. The FRAs were subjected to electrophoresis and the FRA proportions were determined by densitometry. Quantitation was achieved by radio-immunoassay.
favourably with the tanned red cell haemagglutination inhibition immunosassay, the most sensitive of the commercial assays available, which detects 0.5 - 2.0 µg/ml and which does not differentiate between the various breakdown fragments.29

APT paper was selected since it allows constant immobilisation of immunoglobulins to its matrix so that these are not lost from the paper during assay washes, probing and elution. Proteins which form labile complexes with nitrocellulose paper form stable linkages with DPT paper by means of covalent linkages to the diazoum group.29 Other support media are limited by adsorption of macromolecules, structural weaknesses and difficulties in binding proteins covalently without altering their function.

The log log transformation slopes comparing antigen affinity for bound antifibrinogen IgG with IgG in solution are similar. Fibrinogen and fragment D-monomer have similar affinities for the antifibrinogen IgG whereas D-dimer has a somewhat higher affinity, as previously described.12

Western blotting confirms the affinity that the immobilised IgG has for circulating FRAs. Only albumin binds non-specifically to the probe in quantities sufficient to be detected on the polyacrylamide gel. Disc incubation in serum free of FRAs resulted in a small amount of albumin in the eluate, and this amount increased when FRAs were added to the incubation mixture. The reasons for this are not apparent. Attempts made to prevent this binding by altering temperature or buffer composition, by adding fatty acids and detergents known to bind to sites on the albumin molecule, or by altering the incubation and washing buffers, failed to reduce albumin affinity for the probe. However, albumin does not interfere with the assay procedure since it was not detected in the radio-immunosassay, did not alter or inhibit FRA binding to the discs and could be excluded from the densitometric profile of the eluted antigens.

Electrophoresis in the presence of SDS disrupts non-covalent aggregates of cross-linked complexes which probably exist in a double-stranded form with a heterogeneous mixture of D and E domains.31 Lysis of fibrin monomer yields fragments that cannot be distinguished from fibrinogenolytic products on electrophoresis. The present system cannot distinguish polymers of fibrinogen from those of non-cross-linked fibrin, their presence reflecting the action of both thrombin and active factor XIII. However, since our intention is to be able to distinguish lysis of cross-linked fibrin from lysis of fibrinogen and non-cross-linked fibrin, this co-electrophoresis does not interfere with our determinations. Electrophoresis demonstrates the presence of a 340 kD band which reacts with antifibrinogen serum. Both fibrinogen and fibrin monomer migrate in this region and, since more than 97% of labelled fibrinogen is incorporated into the clot, a very small proportion of this band is likely to be fibrinogen.

Despite the variation in size between the several circulating FRAs,13,14 their recovery on the probe and their subsequent elution was proportional to their concentration under a variety of circumstances. Recovery studies utilising labelled D-monomer and D-dimer indicates 95 - 98% capture by the probe. Excellent recovery was also documented by measuring (by radio-immunoassay) the difference in FRA levels in the incubation mixtures before and after exposure to the disc probe.

Radiolabelled D-dimer was used as competitive antigen in the assay because this system is least likely to underestimate the amount of fibrin derivatives.12

Gaffney and Perry15 have recently suggested that assays for FRAs should be performed on plasma samples, since current FDP assays used on serum may fail to detect all the relevant FDP present in the originating plasma. They showed that as much as 75% of the native FRAs are incorporated into the clot during serum preparation, contrasting with the 9% detected by Connaughan et al.,20 and the 2 - 3% found by us. These differences may possibly arise from a difference in techniques. Gaffney and Perry15 measure plasma FRAs using the method of Rylatt et al.,32 capturing them with a monoclonal antibody, either pan-specific for most of the FDPs or monospecific for most of the cross-linked fibrin fragments only. The captured antigens are then tagged with a peroxidase conjugated pan-specific antibody. In terms of measuring cross-linked fibrin derivatives, problems may arise where high levels of fibrinogen and its high-molecular-weight derivatives are present, such as in plasma samples, since they may compete for binding with cross-linked fibrin derivatives being measured. In our studies, conditions for blood collection, storage and clotting were chosen according to the recommendations of Merskey et al.,20 whereby the lowest possible amount of in vitro non-cross-linked fibrin and fibrinogen degradation was allowed to take place. Using this blood sample collection technique, only 2 - 3% of D-dimer and D-monomer were incorporated into the clot and under the particular conditions chosen for clotting, more than 97% of the radiolabelled fibrinogen was incorporated. This is clearly an underestimate of native fibrinogen incorporated into the clot because clotting of radiolabelled fibrinogen is always in the presence of a 340 kD band which reacts with antifibrinogen serum. Recovery of Fibrinogen from serum by the disc probe/electrophoresis technique indicates that more than 99.9% of native fibrinogen is incorporated into the clot. A disadvantage of using plasma is that the high concentration of fibrinogen would obscure the presence of degradation fragments of fibrinogen or cross-linked fibrin with similar electrophoretic mobilities as fibrinogen, such as X, YD and YY.14 Analysis of FDPs in serum allows one to identify all non-clotable derivatives but does not, however, detect high molecular weight factor XIII cross-linked polymers that would be removed by clotting.

The method we have described not only provides measurements of the principle terminal cross-linked and non-cross-linked FDPs, but also of earlier high-molecular-weight fragments. We believe this procedure provides a useful research tool with which in vitro fibrin(ogen)olysis can be monitored. Furthermore, it may be clinically useful since it allows one to distinguish intravascular coagulation with lysis of cross-linked fibrin from primary fibrinogenolysis and lysis of non-cross-linked fibrin.

We gratefully acknowledge the support of the South African Medical Research Council and the Cancer Research Trust.

REFERENCES

One of the most useless pieces of advice a doctor can give a patient is to cut down on the number of cigarettes he smokes. On the face of it the advice is logical, since epidemiologists tell us that the risks of lung cancer and coronary heart disease are proportional to daily cigarette consumption. However, there is some evidence that smokers compensate for cutting down on the number they smoke by extracting more tar, nicotine and carbon monoxide from each cigarette.

This has once more been demonstrated in a study of 13 paid volunteers who had smoked an average of 39 cigarettes a day (range 20 - 60) for many years. The research team (Benowitz et al. 1986; 315: 1310) admitted the volunteers to hospital and studied them in four experimental smoking blocks, each of which lasted 3 - 4 days. In the first block they were given their own brand of cigarette *ad lib* while in the next three they were given 15, 10 or 5 of their usual brand to smoke each day. The intake of tar was estimated in terms of mutagenic activity in the urine, while nicotine and carbon monoxide were also measured. In a short article in *The Lancet* (1986; ii: 1386) Severino and Morriati argue that medicine and not politics should maintain control of the diagnostic process and that it is perfectly legitimate to include this entity in the DSM-III.