Photodynamic treatment of pooled coumarin plasma for external quality assessment of the prothrombin time

A M H P van den Besselaar, A C E Moor

Abstract

Aims—To determine the conditions of photodynamic inactivation of vesicular stomatitis virus (VSV) added to pooled coumarin plasma and the effects of the photodynamic treatment on the prothrombin times and international normalised ratio (INR) in a Netherlands national external quality assessment scheme.

Methods—Pooled coumarin plasma samples were illuminated with visible light in the presence of 1 μ M methylene blue. Inactivation conditions for VSV in pooled coumarin plasma were determined using an end point dilution assay. Plasma illuminated for 20 minutes was mixed with red blood cells and mailed to participants of the Netherlands external quality assessment (EQA) scheme. Prothrombin times and INRs were determined with various thromboplastin reagents.

Results—Photodynamic treatment using 1 μ M methylene blue and 700 W/m² caused 4.7 log inactivation of VSV in pooled coumarin plasma. Fibrinogen and coagulation factors II, V, VII, and X were decreased slightly by the treatment. These conditions caused prolongation of the prothrombin time in EQA surveys. The magnitude of the effect was different for various thromboplastin reagents. The increase of the INR was negligible when measured with the Thrombotest reagent. With other reagents, an approximately 5–16% increase of the INR was observed. Interlaboratory variation of the INR was not affected by photodynamic treatment.

Conclusions—Photodynamic treatment of pooled coumarin plasma is very effective for the inactivation of some enveloped viruses such as VSV, but has only a limited effect on the prothrombin time and INR. Photodynamic treatment can be used to improve the viral safety of coumarin plasma for EQA of the prothrombin time and INR.

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Keywords: prothrombin time; international normalised ratio; external quality assessment; photodynamic treatment

External quality assessment (EQA) of the prothrombin time and international normalised ratio (INR) is an important component of quality assurance of oral anticoagulant treat-

ment. Since 1974, the Federation of the Netherlands Thrombosis Centres has organised a national EQA scheme for its members.¹ Each year, 10 surveys are performed with five liquid control blood samples in each survey. Many Dutch centres are using the Thrombotest reagent (Nycomed, Oslo, Norway), which is a modification of the prothrombin time test.² It is used with citrated venous blood. As far as possible, survey materials should resemble test specimens, and should include a range of coagulation activities to be measured, especially at critical levels for decision making. The survey materials in the Netherlands scheme are prepared by mixing washed human red blood cells with various pooled plasma samples obtained from normal individuals and patients treated with oral anticoagulant drugs.³

There is a risk of contamination with blood borne viruses when a large number of patients' specimens are used for the preparation of pooled plasmas. Although the pooled plasmas are tested for hepatitis antigen and human immunodeficiency virus (HIV) antibodies, the risk of infection of laboratory workers cannot be eliminated completely. Leakage of survey material from the primary container may occur during transportation and postal delivery. EQA organisers might be held responsible for injury caused by survey materials. To improve the safety of the survey materials, we evaluated photochemical methods for the inactivation of viruses in pooled plasma samples.

In transfusion medicine, photodynamic treatment has been studied for the inactivation of blood borne viruses in blood components. A photodynamic virus inactivation method using methylene blue is being applied routinely in the production of virus inactivated plasma.⁴ Methylene blue was selected because it is used clinically and because of its known toxicological properties. The standard procedure for photodynamic treatment involves illumination with visible light at a methylene blue concentration of 1 μ M in plasma.⁵ Upon illumination, methylene blue becomes excited, and energy transfer from the excited state to molecular oxygen dissolved in the plasma can cause the formation of singlet oxygen. This very reactive oxygen species can damage lipids, proteins, and viral nucleic acids. Photodynamic treatment with methylene blue effectively inactivates various enveloped viruses, including HIV. Polymerase chain reaction analysis revealed that hepatitis B virus,

Haemostasis and Thrombosis Research Centre, Leiden University Medical Centre, 2300 RC Leiden, The Netherlands
A M H P van den Besselaar

Department of Molecular Cell Biology, University of Leiden, 2300 RA Leiden, the Netherlands
A C E Moor

Correspondence to:
Dr van den Besselaar,
Haemostasis and Thrombosis Research Centre, Department of Haematology, C2-R, Leiden University Medical Centre, PO Box 9600, 2300 RC Leiden, The Netherlands
email: Abesselaar@hematology.azl.nl

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hepatitis C virus, HIV-1, and probably also the non-enveloped parvovirus B19 are sensitive to methylene blue/light treatment.⁶

The purpose of our study was to determine the conditions for effective photodynamic inactivation of vesicular stomatitis virus (VSV) added to pooled coumarin plasma, and to study the effect of photodynamic treatment on the prothrombin time and INR of the plasma to be used for preparation of survey samples in the Netherlands EQA scheme. We report the results of two surveys in which photodynamic treatment was applied.

Materials and methods

PREPARATION OF POOLED PLASMAS

Venous blood was collected from coumarin treated patients using evacuated tubes containing 0.105 M buffered sodium citrate (Becton Dickinson Vacutainer® Systems; Becton Dickinson, Franklin Lakes, New Jersey, USA). Plasma was obtained by centrifugation at 2000 $\times g$ for 10 minutes. Samples with INRs in the range 1.5 to 3.0 or 2.5 to 4.0 were pooled and centrifuged for a second time, but now at 29 000 $\times g$ for 30 minutes. After careful decantation the pooled plasmas were frozen in closed plastic containers at -70°C . After thawing in a waterbath at 37°C for 15 minutes, the pooled plasmas were mixed with HEPES buffer (final concentration in plasma, 0.05 M; pH 7.3). In some experiments, the oxygen dissolved in the plasma was removed by slowly bubbling nitrogen through the plasma before photodynamic treatment.

PHOTODYNAMIC TREATMENT

Methylene blue was obtained from Sigma Chemical Co (St Louis, Missouri, USA). A stock solution (1 mM in water) was prepared. Each plasma sample was mixed with methylene blue (1 μM final concentration). The plasma was then illuminated at room temperature, in 50 ml polystyrene tissue culture flasks, using a 500 W halogen lamp. The plasma was stirred during the illumination. A large glass vessel with running tap water was placed between the lamp and the flask to absorb the heat generated by the lamp. The distance between the lamp and flask was 12 cm. Irradiance at the site of the flask was 700 W/m^2 , as measured with a Gentec TPM-310 photometer.

VSV INFECTIVITY ASSAY

Measurement of the infectivity of VSV by an endpoint dilution assay was performed as described previously.⁷ Results were expressed as percentage of the control (no illumination).

COAGULATION ASSAYS

The following assays were performed in the authors' laboratory. Fibrinogen was determined according to the method of Clauss.⁸ Factors II, V, VII, and X were determined with coagulation assays using human tissue factor and plasma samples deficient in the respective coagulation factors. Prothrombin times were determined with Innovin (Dade Behring, Marburg, Germany), Thrombotest (Ny-

comed), and PT-Fib HS (Instrumentation Laboratory, Breda, The Netherlands). Instrument specific international sensitivity index values were used to calculate INR values. Thrombotest dilution plots were made essentially as described by Hemker and co-workers.⁹ In these plots, Thrombotest clotting times t of diluted plasma samples are plotted against the dilution factor D . According to these authors,⁹ the quantity of a competitive inhibitor present in undiluted plasma can be estimated from the distance I on a horizontal line between $t_{\min, \text{uninh}}$ and the intercept of the t - D line of the experimental plasma with that horizontal line (fig 1).

PREPARATION OF SURVEY SAMPLES

The preparation of survey samples was modified from the original procedure.³ Red blood cells of blood group O were obtained as packed cells in CPDA-1 (citrate phosphate dextrose adenine) from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. After washing of the red blood cells with phosphate buffered saline and removal of the buffy coat, the cells were incubated with a solution containing sodium chloride (0.051 M), trisodium citrate (0.017 M), and sodium HEPES (0.1 M), pH 8.6. After two hours incubation at room temperature, the cells were centrifuged and the supernatant was discarded. The cells were then mixed with the pooled coumarin plasma samples, aiming at a haematocrit value of 0.4–0.45. A mixture of penicillin and streptomycin was added to prevent microbial growth. The pH of the artificial blood was approximately 7.5. The blood was distributed in 1.0 aliquots in capped polypropylene tubes. The blood samples were prepared on Monday and mailed to the participants of the EQA scheme on Tuesday. Participants were instructed to store the samples at room temperature until analysis on Friday—four days after preparation. Participants used their routine method for prothrombin time and INR determination.

Figure 1 Thrombotest dilution plots of normal plasma (cross), pooled coumarin plasma before illumination (open circle) and after illumination with methylene blue for 20 minutes (open square). The regression equations for the latter two plots are $y = 47.3 + 27.6x$ and $y = 48.0 + 30.1x$, respectively. The intercept of the line for normal plasma with the Y axis is named $t_{\min, \text{uninh}}$. I is the distance on a horizontal line between $t_{\min, \text{uninh}}$ and the intercept of the line for coumarin plasma with that horizontal line.

Figure 2 Infectivity of vesicular stomatitis virus (VSV) after treatment with 1 μM methylene blue and incandescent light (700 W/m^2). The VSV titer is expressed on a log scale. Two different pooled coumarin plasmas were used in two separate experiments (open squares and circles).

The homogeneity of the samples was determined in the authors' laboratory by prothrombin time testing of 10 samples, using Simplastin Excel-S as thromboplastin reagent and a Coagamate-MTX as coagulometer.

STATISTICAL EVALUATION OF EQA RESULTS

Prothrombin times and INRs reported by the participants of the Netherlands EQA scheme were evaluated for each group using the same brand of thromboplastin reagent, irrespective of the type of instrument (coagulometer) used. When a participant's result was greater than two times the mean value of the group using the same reagent, it was regarded as an outlier and excluded from the final calculations. Inter-laboratory variation was expressed as coefficient of variation (CV) in per cent.

Results

VSV INFECTIVITY

The inactivation of VSV in pooled coumarin plasma was determined using an infectivity assay, based on the cytopathological effect of VSV on A549 cells, as scored by light microscopy after 72 hours. As shown in fig 2, complete inactivation of added VSV (4.7 log)

Figure 3 Fibrinogen activity of pooled coumarin plasma after treatment with 1 μM methylene blue and incandescent light (700 W/m^2). Before illumination, either air (open circle) or nitrogen (open square) was bubbled through the plasma, or no additional treatment was given (cross).

Figure 4 Coagulation factor activities of pooled coumarin plasma after treatment with 1 μM methylene blue and incandescent light (700 W/m^2). Factor V activity was measured in plasma treated with air (open circle), or nitrogen (open square). In plasma without preceding air or nitrogen treatment, factor V (cross), factor II (closed circle), factor VII (closed triangle), and factor X (closed square) were measured.

could be obtained by treatment with 1 μM methylene blue and 20 minutes illumination with white light (700 W/m^2). These conditions were used for the preparation of EQA samples.

COAGULATION ACTIVITY

The inactivation of fibrinogen and factor V in pooled coumarin plasma was determined as a function of the illumination time. As shown in fig 3, a biphasic decrease of the fibrinogen concentration occurred in the presence of air. Under a nitrogen atmosphere, there was practically no decrease of the fibrinogen concentration. Factor V activity decreased approximately 10% in the presence of air, but no change was seen under nitrogen (fig 4). Similarly, there was

Figure 5 International normalised ratio (INR) of pooled coumarin plasma determined with recombinant human thromboplastin (Innovin) after treatment with 1 μM methylene blue and incandescent light (700 W/m^2). Before illumination, either air (open circle) or nitrogen (open square) was bubbled through the plasma, or no additional treatment was given (cross).

Figure 6 International normalised ratio (INR) of pooled coumarin plasma determined with bovine thromboplastin (Thrombotest) after treatment with 1 μ M methylene blue and incandescent light (700 W/m²). Before illumination, either air (open circle) or nitrogen (open square) was bubbled through the plasma, or no additional treatment was given (cross).

a slight decrease of factor II and factor X activities in the first 15 minutes of the illumination. In the following hour, no measurable change was seen. Factor VII activity remained constant in the first hour of the illumination. INR values measured with Innovin (recombinant human thromboplastin) increased rapidly in the first 15 minutes (with air), followed

Table 1 Mean prothrombin times (seconds) and interlaboratory variation (CV) in two separate external quality assessment surveys

Reagent	n	Sample A not illuminated		Sample B not illuminated		Sample C illuminated	
		Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
Thrombotest	40	117.5	5.4	117.6	5.4	118.2	5.3
Thrombotest	40	108.1	5.6	109.1	5.9	110.6	6.1
Recombiplastin	18	39.8	6.2	39.3	6.0	42.1	5.8
Recombiplastin	19	37.7	5.7	38.2	4.4	41.6	5.3
PT-Fib HS	17	30.7	7.0	31.0	7.2	33.7	8.3
PT-Fib HS	18	29.8	7.4	30.2	6.2	33.6	6.8
Thromborel-S	13	38.3	6.1	38.1	6.3	40.1	7.1
Thromborel-S	12	36.8	6.7	37.5	7.2	39.9	7.8
Hepato Quick	15	85.1	4.1	84.7	3.9	91.5	4.2
Hepato Quick	16	85.7	3.6	87.0	3.6	92.4	3.3

The artificial blood samples were prepared separately for each survey. Artificial blood sample A was prepared from pooled coumarin plasma without addition of methylene blue and without illumination. Blood sample B was prepared from the same pooled coumarin plasma but with addition of methylene blue and without illumination. Blood sample C was prepared from the same pooled coumarin plasma but with the addition of methylene blue and with illumination. n, number of laboratories.

Table 2 Mean international normalised ratio (INR) and interlaboratory variation (CV) in two separate external quality assessment surveys. INR values were calculated and reported by each participating laboratory

Reagent	n	Sample A not illuminated		Sample B not illuminated		Sample C illuminated	
		Mean	CV (%)	Mean	CV (%)	Mean	CV (%)
Thrombotest	41	3.11	4.6	3.11	4.9	3.13	4.5
Thrombotest	40	2.87	4.3	2.89	4.3	2.93	4.3
Recombiplastin	18	3.55	8.5	3.52	8.0	3.75	8.4
Recombiplastin	19	3.34	8.0	3.38	7.0	3.71	7.5
PT-Fib HS	17	3.73	12.0	3.79	11.9	4.24	13.9
PT-Fib HS	19	3.57	12.0	3.64	10.9	4.16	14.0
Thromborel-S	13	3.70	4.6	3.69	5.6	3.92	5.7
Thromborel-S	12	3.56	6.8	3.62	7.1	3.90	7.6
Hepato Quick	16	3.10	5.7	3.10	6.3	3.35	7.1
Hepato Quick	16	3.12	5.6	3.15	5.2	3.36	5.1

The artificial blood samples were prepared separately for each survey. Artificial blood sample A was prepared from pooled coumarin plasma without addition of methylene blue and without illumination. Blood sample B was prepared from the same pooled coumarin plasma but with addition of methylene blue and without illumination. Blood sample C was prepared from the same pooled coumarin plasma but with the addition of methylene blue and with illumination. n, number of laboratories.

by a phase of slow increase (fig 5). Similar results were obtained with PT-Fib HS, a rabbit tissue thromboplastin (not shown). INR values measured with Thrombotest were only slightly increased (fig 6). In Thrombotest dilution plots (fig 1), the slope of the line obtained with illuminated plasma was greater than that with non-illuminated plasma. Furthermore, the distance *I* was slightly decreased after illumination.

EXTERNAL QUALITY ASSESSMENT OF PROTHROMBIN TIME AND INR

Two EQA surveys were performed in November and December 1998. In each survey, one sample was illuminated as described above, a second sample was prepared from the same coumarin pool with methylene blue but not illuminated, and a third sample was prepared from the same coumarin pool without methylene blue and not illuminated. The homogeneity of the samples was good (CV < 0.9%). The mean prothrombin times and interlaboratory CV are shown in table 1, and the mean INR values in table 2. The mean prothrombin times of the samples prepared from the same coumarin pool not illuminated with and without methylene blue were almost the same; the differences were not greater than 2%. The mean clotting times and INRs reported by participants using the Thrombotest reagent were almost the same before and after illumination of the pooled coumarin plasmas in the presence of methylene blue. In contrast, there was a substantial increase in clotting time and INR with the other reagents; the increase ranged from 5% to 16%. The interlaboratory variation of the prothrombin times and INRs was not different before and after illumination.

Discussion

VSV is a very well characterised lipid enveloped virus that is used as a model for the study of photodynamic virus inactivation. The virus inactivating properties of methylene blue and other phenothiazine dyes in combination with visible light have been known for many years. Viruses vary in their sensitivity to methylene blue, but the susceptibility of HIV-1 to photoinactivation was similar to that of VSV.⁵

Under the conditions of photodynamic treatment used in our study (20 minutes illumination with white light (700 W/m²) in the presence of 1 μ M methylene blue) there was complete inactivation of all VSV added to pooled coumarin plasma buffered with HEPES (fig 2). Our results are in agreement with those of Lambrecht *et al* who used fresh frozen plasma isolated from blood donations.⁵

Photodynamic treatment of pooled coumarin plasma resulted in a substantial decrease of the functional fibrinogen concentration as measured with the Clauss method. The photodynamic inactivation of fibrinogen was, as expected, dependent on the presence of oxygen in the solution (fig 3). Lowering the oxygen concentration by bubbling with

nitrogen resulted in the complete absence of photodynamic damage. Previously, it has been shown that illumination in the presence of methylene blue resulted in a substantial reduction of histidine and tryptophan residues.¹⁰ This is probably caused by photooxidation of these residues by singlet oxygen, as shown in a study using scavengers of different reactive oxygen species.¹¹ The relative decrease of factor II, V, VII, and X activities (fig 4) was smaller than that of fibrinogen, which is in agreement with the observations by other investigators.^{5 11 12} Furthermore, the activities of factors II, V, VII, and X remained practically constant after 15 minutes. The decrease in fibrinogen was biphasic and continued after 15 minutes. The biphasic kinetics might be the result of oxygen depletion occurring under our experimental conditions, which causes a slower rate of photodynamic damage in the second part of the illumination period. It seems that air bubbling before illumination cannot prevent the biphasic kinetics of activity loss. An alternative explanation for the observed effect might be that the different amino acid residues in the proteins undergo photooxidation at different rates. The partly oxidised proteins are apparently still able to function to some extent.

The photodynamically induced increase of the prothrombin time and INR is probably caused by inactivation of fibrinogen, and coagulation factors II, V, VII, and X.^{5 11 12} In the absence of oxygen the prothrombin time remained almost constant. Therefore, the increase of the prothrombin time in the presence of oxygen can be attributed to photooxidation of the proteins involved. The relative increase was not the same with the various thromboplastin reagents. The prothrombin times and INRs determined with plain rabbit and human thromboplastin increased relatively more than those determined with Thrombotest, a bovine thromboplastin combined with adsorbed bovine plasma. These results suggest that photooxidised coagulation factors and inhibitors react differently with bovine tissue factor than with human and rabbit tissue factor. In a Thrombotest dilution plot (fig 1) the slope of the pooled coumarin plasma was increased by photodynamic treatment and the distance *I* was decreased. This could be interpreted as a decrease of factor X activity and of competitive inhibitor protein induced by vitamin K antagonists (PIVKA).⁹ Because several coagulation factors are affected by the photodynamic treatment, it is not a simple task to determine which factors are responsible for the prolongation of the prothrombin time. Further experiments are needed to resolve this question.

Previous studies have shown that the liquid artificial blood samples used in the Netherlands EQA scheme are not stable.³ During storage at room temperature, a slow increase of the prothrombin time was seen. All participants were requested to perform the tests on the same day, so that the effect of

deterioration was approximately the same in all samples. Differences in ambient temperature between the various parts of the Netherlands are small and the transit time is usually not greater than 24 hours. Furthermore, the homogeneity of the samples on the day of testing was very good (CV < 1%). The interlaboratory variation of the samples treated with methylene blue/light was not substantially different from the variation of the control samples when the variation was calculated for each reagent group separately (tables 1 and 2). In most of the reagent groups, an increase of the mean prothrombin time and INR was seen after photodynamic treatment of the pooled coumarin plasma. In contrast, the mean clotting time and INR reported by the users of the Thrombotest reagent did not change greatly after photodynamic treatment. As a result, the differences between the reagent group mean values increased after photodynamic treatment. It should be noted that in the control samples that were not illuminated, there were also important differences in the INR between the reagent groups (table 2). For example, the mean INR reported by users of PT-Fib HS was 20% greater than the mean INR reported by Thrombotest and Hepato Quick users. After illumination, the difference increased to 35%. The differences in INR are the result of deterioration of the samples and a different response of the various reagents to deterioration. Therefore, the performance of each participant of the EQA scheme must be assessed by comparison with results from other laboratories using the same reagent. In agreement with previous reports,¹³ it is not possible to assign a single INR value to these EQA samples that would be valid for all reagents.

In conclusion, our results show that photodynamic treatment of pooled coumarin plasma is effective for viral decontamination, without important impairment of its usefulness for EQA of the prothrombin time and INR.

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Letters

M2-AMA do not directly produce ANCA indirect immunofluorescence patterns

The importance of distinguishing atypical cytoplasmic indirect immunofluorescence patterns from the "classic", centrally accentuated cytoplasmic immunofluorescence pattern on ethanol fixed human neutrophils has recently been re-emphasised.¹⁻³ Autoantibodies to other cytoplasmic autoantigens such as antimitochondrial antibodies (AMA), antismooth muscle antibodies, and antiribosomal-P antibodies have also recently been reported to produce atypical cytoplasmic immunofluorescence patterns on ethanol fixed human neutrophils.¹ However, an alternative explanation is that the atypical cytoplasmic immunofluorescence patterns might be produced by concomitant antineutrophil cytoplasmic antibodies (ANCA) in these sera, especially in cases of autoimmune liver disease. Therefore, we investigated: (1) whether sera containing AMA with confirmed M2 specificity⁴ produced positive indirect immunofluorescence patterns on ethanol fixed human neu-

trophils, and (2) the precise neutrophil antigen specificity of such sera.

Thirty two sera from different patients with confirmed M2-AMA specificity (using the Inova Diagnostics (San Diego, California, USA) M2-AMA enzyme linked immunosorbent assay (ELISA)), were tested by indirect immunofluorescence on in house, ethanol fixed, normal human neutrophils and commercial HEP-2 cells (Sanofi Diagnostics Pasteur Chaska, Minnesota, USA). Twelve of the 32 patients had liver biopsies, which showed primary biliary cirrhosis in nine cases and autoimmune chronic active hepatitis in three. All 32 sera were also tested on the ORGenTec (Mainz, Germany) Combi-kit® ELISA against the following neutrophil antigens: proteinase 3, myeloperoxidase, lactoferrin, elastase, cathepsin G, lysozyme, and bactericidal/permeability increasing protein (BPI).

Table 1 gives the results. Thirteen of the 32 M2-AMA sera produced positive indirect immunofluorescent staining on the ethanol fixed neutrophils: ten had a perinuclear pattern (two of which might have been produced by concomitant ANA), and three had an atypical cytoplasmic pattern. The ORGenTec Combi-kit ELISA revealed that 22 (comprising all 13 sera that were immunofluorescence positive, and nine that were negative) of the 32 sera contained ANCA directed specifically against the following

neutrophil antigens: BPI (n = 13), cathepsin G (n = 8), lactoferrin (n = 7), elastase (n = 2), proteinase 3 (n = 2), and lysozyme (n = 1). Five of these 22 sera contained ANCA against two neutrophil antigens, one against three neutrophil antigens, and one against five neutrophil antigens.

The immunofluorescence staining pattern did not appear to predict antigen specificity because the three atypical cytoplasmic immunofluorescence positive sera were associated with either antikathepsin G antibodies (n = 2) or anti-BPI antibodies (n = 1). Furthermore, the 10 perinuclear immunofluorescence positive sera were associated with either anti-BPI antibodies alone (n = 2), antikathepsin G antibodies alone (n = 1), antilactoferrin antibodies alone (n = 1), antilysozyme antibodies alone (n = 1), or a combination of two or more antibodies (n = 5). Finally, 10 of the 32 sera were negative for ANCA by both immunofluorescence and ELISA.

Therefore, we conclude that M2-AMA do not directly produce positive indirect immunofluorescence patterns on ethanol fixed human neutrophils. Rather, a positive indirect immunofluorescence pattern in M2-AMA positive sera appears to be produced by concomitant ANCA directed against a variety of neutrophil antigens such as BPI, cathepsin G, and lactoferrin. Further studies are necessary to determine whether autoantibodies to other cytoplasmic autoantigens directly produce positive immunofluorescence patterns on ethanol fixed human neutrophils,¹ or whether these sera contain concomitant ANCA.

Table 1 Results of M2-AMA ELISA, ANCA IIF and ANCA Combi-kit® ELISA

Specimen	M2-AMA ELISA	ANCA IIF pattern (titre)	ANCA Combi-kit® ELISA (OD ratio)	Liver biopsy result
1	High positive	Negative	Anti-BPI (2.47)	PBC
2	High positive	Negative	Negative	PBC
3	High positive	Negative	Negative	PBC
4	High positive	Atypical cytoplasmic (1/40)	Antikathepsin G (1.31)	PBC
5	High positive	Negative	Antilactoferrin (1.08)	PBC
6	High positive	Negative	Anti-BPI (1.24)	PBC
			Antiproteinase 3 (1.13)	
7	High positive	Negative	Anti-BPI (1.79)	PBC
8	High positive	Negative	Anti-BPI (2.79)	Not done
9	High positive	Negative	Negative	Not done
10	High positive	Negative	Negative	Not done
11	High positive	Perinuclear (1/160)	Anti-BPI (1.60)	Not done
			Antikathepsin G (2.02)	
12	High positive	Negative	Anti-BPI (1.02)	Not done
			Antilactoferrin (2.35)	
13	High positive	Perinuclear (1/160)	Anti-BPI (3.04)	Not done
			Antikathepsin G (2.14)	
			Antilactoferrin (1.6)	
14	High positive	Perinuclear (1/160)	Anti-BPI (1.17)	Not done
15	High positive	Perinuclear (1/640)	Antilactoferrin (4.37)	Not done
16	High positive	Perinuclear/nuclear (1/160)†	Anti-BPI (1.30)	Not done
17	High positive	Negative	Antilactoferrin (1.07)	Not done
18	High positive	Negative	Antilactoferrin (1.03)	Not done
19	Mod positive	Perinuclear (1/40)	Antilysozyme (1.41)	PBC
20	Mod positive	Negative	Negative	AICAH
21	Mod positive	Perinuclear/nuclear (1/40)#	Antikathepsin G (1.02)	AICAH
22	Mod positive	Negative	Antikathepsin G (4.24)	Not done
23	Mod positive	Negative	Negative	Not done
24	Mod positive	Perinuclear (1/160)	Anti-BPI (4.01)	Not done
			Antikathepsin G (1.74)	
			Antielastase (1.11)	
			Antilactoferrin (2.12)	
			Antiproteinase-3 (1.05)	
25	Mod positive	Negative	Negative	Not done
26	Low positive	Atypical cytoplasmic (1/40)	Antikathepsin G (1.00)	PBC
27	Low positive	Perinuclear (1/160)	Anti-BPI (1.49)	AICAH
			Anti-elastase (1.46)	
28	Low positive	Negative	Negative	Not done
29	Low positive	Atypical cytoplasmic (1/40)	Anti-BPI (1.35)	Not done
30	Low positive	Negative	Negative	Not done
31	Low positive	Perinuclear	Anti-BPI (1.74)	Not done
			Antikathepsin G (1.03)	
32	Low positive	Negative	Negative	Not done

ORGenTec ANCA Combi-kit® ELISA OD (optical density) ratio: positive ≥ 1 , negative < 1 .

†Concomitant ANA staining (1/640 titre) with a nuclear membrane pattern was present.

#Concomitant ANA staining (1/40 titre) with homogeneous and speckled patterns was present.

AICAH, autoimmune chronic active hepatitis; AMA, antimitochondrial antibodies; ANCA, antineutrophil cytoplasmic antibodies; BPI, bactericidal/permeability increasing protein; ELISA, enzyme linked immunosorbent assay; IIF, indirect immunofluorescence; PBC, primary biliary cirrhosis.

R C W WONG
R WILSON
J NEIL
Division of Immunology,
Queensland Health Pathology Services,
Royal Brisbane Hospital,
Herston 4029, Brisbane, Australia

S ADELSTEIN
Department of Clinical Immunology,
Royal Prince Alfred Hospital, Australia

R A SILVESTRINI
E M BENSON
Department of Immunopathology, ICPMR,
Westmead Hospital, Australia

L POWELL
Queensland Institute of Medical Research, Australia

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Raised plasma parathyroid hormone related peptide in gastric adenocarcinoma

We report a case of humoral hypercalcemia associated with a rapidly growing gastric carcinoma. To our knowledge, this is the first such case of gastric carcinoma reported with raised plasma parathyroid hormone related peptide (PTHrP) and absent bone metastases.

A 69 year old woman presented with fatigue and intermittent sharp epigastric pain for one week. Upper gastrointestinal radiographs and endoscopy demonstrated a necrotic, friable mass in the mid stomach. Biopsy of the mass and a surrounding satellite

Figure 1 The figure shows representative tissue from multiple gastric biopsies in which the expression of parathyroid hormone related peptide (PTHrP) was investigated using a horseradish peroxidase labelled polyclonal rabbit antibody directed against amino acids 1–34 of human PTHrP: (A) Gastric adenocarcinoma showing expression of PTHrP and (B) gastric adenoma showing no expression of PTHrP.

polypoid lesion showed poorly differentiated adenocarcinoma and gastric adenoma with high grade dysplasia, respectively (fig 1). Four weeks after the first symptoms had arisen the liver edge was palpable 3 cm below the costal margin. Computed tomographic scan of the abdomen demonstrated several hypodense lesions in the liver and aortic adenopathy compatible with metastases. The following results were found: haematocrit, 0.24; white blood cell count, $12.7 \times 10^9/\text{litre}$; platelets, $460 \times 10^9/\text{litre}$; calcium, 2.54 mmol/litre; albumin, 31 g/litre; international normalised ratio, 1.2; alkaline phosphatase, 1075 units/litre; alanine amino transferase, 16 units/litre; total bilirubin, 10.26 $\mu\text{mol/litre}$; lactate dehydrogenase, 1765 units/litre; carcinoembryonic antigen, 327 $\mu\text{g/litre}$; ferritin, 302 $\mu\text{g/litre}$; Fe, < 2 $\mu\text{mol/litre}$; and total iron binding capacity, < 21 $\mu\text{mol/litre}$. Chest x ray was negative. Two weeks later the patient developed mental confusion and dehydration. Serum calcium was 2.8 mmol/litre, phosphorus 0.5 mmol/litre, and albumin 26 g/litre. With intravenous hydration and pamidronate (90 mg), serum calcium rapidly became normal and the patient's mental status returned to baseline. Neither radionuclide bone scan nor magnetic resonance scan of the brain suggested metastases. The intact PTH (IRMA; Nichol's Institute) was 6 ng/litre (normal, 10–65). However, PTHrP (1–40 IRMA; Nichols Institute) was 5.4 pmol/litre (normal < 1.3). The patient died of progressive liver failure three weeks later.

Hypercalcaemia is rarely associated with gastric cancer. In the literature, two cases of gastric adenocarcinoma with hypercalcaemia have been reported in which the tumour cells expressed PTHrP.^{1,2} In both cases, bone metastases were present and plasma PTHrP values were not reported. Alipov *et al* found that PTHrP was expressed on tumour cells in 71 of 92 patients with gastric adenocarcinoma, none of whom had humoral hypercalcaemia.³ PTHrP expression in the tumour tissue was strongly associated with poorly differentiated cancers (34 of 34) as opposed to well differentiated ones (10 of 23). Normal gastric mucosa

and adenomas did not express PTHrP. Our case is consistent with the findings of Alipov *et al* because cancer cells expressed PTHrP, whereas adenoma did not (fig 1). Our case is notable for rapid clinical deterioration coupled with raised tissue and plasma PTHrP. These results suggest that PTHrP expression is associated with poor prognosis in gastric cancer. Whether PTHrP plays a direct role in cancer progression⁴ or is a byproduct of oncogene activation (for example, ras and src⁵) remains to be determined.

GEORG ENGELICH

NIAL SWAN

KEVAN L HARTSHORN

Boston University School of Medicine and Boston Medical Center, Departments of Medicine and Pathology, 80 East Concord Street, Boston, MA 02118, USA

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ELISA is the superior method for detecting antineutrophil cytoplasmic antibodies in the diagnosis of systemic necrotising vasculitis

Dr Harris's results¹ showed that indirect immunofluorescence is a more sensitive technique than antigen specific enzyme linked immunosorbent assay (ELISA) for the diagnosis of systemic necrotising vasculitis (70% *v* 50%) but that ELISAs have a higher positive predictive value (87% *v* 76%). It was the greater sensitivity of indirect immunofluorescence that prompted the "Inter-

national consensus statement on testing and reporting antineutrophil cytoplasmic antibodies (ANCA)"² to require all laboratories to screen for ANCA by indirect immunofluorescence, but to confirm the specificity of fluorescent sera by ELISA. In our hands, adherence to the minimum requirements of the consensus statement results in a higher positive predictive value than either indirect immunofluorescence or ELISA alone (62% compared with 44% and 50%, respectively). Screening by indirect immunofluorescence has the additional advantages of being a quicker and cheaper technique than using the two commercial antigen specific ELISAs that are usually required and, furthermore, indirect immunofluorescence might demonstrate coincidental but unsuspected autoantibodies such as antinuclear antibodies. We believe that the use of ELISAs alone to diagnose a systemic necrotising vasculitis is analogous to testing for systemic lupus erythematosus with anti-double stranded DNA antibodies rather than initially screening for antinuclear antibodies by indirect immunofluorescence.

JUDY SAVIGE

University Department of Medicine,
The University of Melbourne,
Austin and Repatriation Medical Centre,
Heidelberg, Victoria 3084, Australia

PAUL NEESON

MICHELE TREVISIN

PETER GAMBEL

Division of Investigational Medicine,
Austin and Repatriation Medical Centre

WENDY POLLOCK

Gribble's Pathology, 14 Yarra Street,
Private Bag 1800, South Yarra,
Victoria 3141, Australia

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The authors reply

Our study was carried out following the preliminary observation that in our laboratory a positive immunofluorescence antineutrophil cytoplasmic antibody (ANCA) result was less frequently associated with a diagnosis of systemic necrotising vasculitis than a positive enzyme linked immunosorbent assay result (PR3 or myeloperoxidase). Our clinicians frequently did not alter management based on the immunofluorescence ANCA result. In fact, our renal physicians invariably proceeded with renal biopsy, regardless of immunofluorescence results, to confirm or refute the diagnosis of systemic necrotising vasculitis. The ELISA result frequently came back too late to alter clinical management, the ELISA test being done when immunofluorescence was positive. Our study has shown that for active systemic necrotising vasculitis, ELISA has a superior positive predictive value and specificity, and comparable sensitivity to the immunofluorescence technique.

Savige *et al* quoted our sensitivity results with respect to "all cases" of systemic necrotising vasculitis, active and inactive combined, which are largely irrelevant parameters in clinical practice because the need is to identify active cases. The principal result of the study was the finding that in active, biopsy confirmed cases of systemic necrotising vasculitis, ELISA ANCA is just as sensitive (85% *v* 88%; *p* = 0.056) yet has a signifi-

cantly better specificity (97% *v* 90%; $p = 0.0006$) and positive predictive value (73% *v* 50%; $p = 0.0013$) compared with immunofluorescence ANCA.

The fact that the sensitivity of ELISA ANCA falls as inactive cases are added to active cases implies that ELISA more quickly becomes negative as active disease settles, whereas immunofluorescence remains positive. This observation suggests that ELISA is also a better test in following disease activity after diagnosis and initiation of treatment.

Savidge *et al* reported their finding of a higher positive predictive value if results of ELISA and immunofluorescence ANCA are combined. This was not found in our study. In fact, combining immunofluorescence and ELISA ANCA resulted in a lower positive predictive value than ELISA ANCA alone. It would be of interest to review the data Savidge *et al* have used.

Based on our results, we conclude that ELISA ANCA is the principal serological test for the diagnosis of systemic necrotising vasculitis. Immunofluorescence ANCA should be avoided because its inferior specificity and poor positive predictive value open the way to incorrect or delayed diagnosis and treatment. We would like to restate the importance of recognising the different clinical syndromes caused by systemic necrotising vasculitis and of appropriate histological testing even with a positive or negative ELISA ANCA result.

We have not investigated the value of ANCA with respect to the diagnosis of other conditions, such as inflammatory bowel disease, and so we do not recommend which tests should be used in a particular laboratory. We have only compared ELISA and immunofluorescence ANCA in a particular disease (systemic necrotising vasculitis) and found ELISA to be superior by all criteria. Our results indicate that the 'International consensus statement on the testing and reporting of ANCA'¹ should be revisited.

ANNE HARRIS
DAVID GILLIS
MATTHEW VADAS
GRACE CHANG

Division of Investigational Medicine,
Austin and Repatriation Medical Centre, Heidelberg,
Victoria 3084, Australia

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The effect of using templates on the information included in histology reports on specimens of uterine cervix taken by loop excision of transformation zone (LETZ)

I should again like to congratulate Dr Al-Nafussi and her colleagues for providing us with an interesting and stimulating paper¹ and to take the opportunity to add some comments of my own. Following earlier correspondence in the journal,^{2,3} I have sought to develop a system of standardised phrases that are used in reporting the features listed in the paper by Reid *et al*.¹ Secretarial or medical staff can enter a short code of up to 35 letters, which is expanded electronically to produce a phrase or sentence in coherent English. In this department, we use the Telepath system, which allows more than one such code to be used in any given report. Snomed codes are linked to the codes and automatically included in the departmental database. Furthermore, it is possible to recover reports in which a given standardised phrase or sentence has

been used. This allows us to identify the proportion of cases with specific findings such as involved specimen edges, traumatised squamocolumnar junctions, or the presence of endocervical epithelium or squamous epithelium at the end of the endocervical canal. Because these are quality features that are to some extent under the control of the colposcopist or surgeon, it is envisaged that we can then provide feedback on the adequacy of specimens deriving from particular clinics to the responsible consultants.

Finally, in addition to the ectocervical and endocervical edges we routinely comment upon the presence of CIN (cervical intra-epithelial abnormality) at the deep lateral edge. This is the edge that runs between the superior, endocervical edge of the specimen to the lateral, ectocervical edge of the specimen. Although this is composed of cervical stroma with variable degrees of cautery artefact, we regard this involvement as being important because there is the potential of residual disease being covered in the re-epithelialisation process, so that it will not be detected on colposcopy or cytological surveillance. Residual disease, if undetected, has been suggested as a cause of later invasive cervical carcinoma in patients treated for CIN.^{4,5}

S BRADBURN
MK HEATLEY

Department of Pathology, 5th Floor, Duncan Building,
The Royal Liverpool University Hospital,
Prescot Street, Liverpool L7 8XP UK

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Some problems found in HIV-1 RNA quantification

The polymerase chain reaction (PCR) based assay of human immunodeficiency virus type 1 (HIV-1) RNA in plasma is now commercially available and is used widely for the assessment of antiretroviral treatments. The kit is called the AmplicorTM HIV-1 monitor test kit version 1.0 from Roche Diagnostics (Tokyo, Japan). However, this system is not sensitive enough for the accurate measurement of genetic subtypes A and E, and it gives falsely low titres for these virus subtypes.^{1,2} To surmount this problem, additional gag primers (AG primers) have been provided by Roche for research use (Ver. 1.0 plus). Furthermore, a new improved version (Ver. 1.5) was developed recently, which is said to yield accurate results not only on subtype B but on subtypes A and E. With the Ver. 1.0 plus kit, adding the AG primer set from the Ver. 1.5 kit to the PCR master mixture containing the Ver. 1.0 primer set makes it possible to amplify even subtype A and E viruses. In the Ver. 1.5 kit, the downstream primer is 12 bases downstream from the Ver. 1.0 primer position, whereas the upstream primer position is unchanged but the primer has two base substitutions. In our laboratory, we have examined over 1500 samples (148 cases) using the Ver. 1.0 kit, and among

Table 1 AmplicorTM HIV-1 monitor kit

Case	Ver. 1.0	Ver. 1.0 (+)	Ver. 1.5
Case 1	3.6×10^4	2.7×10^4	2.0×10^4
Case 2	$<0.4 \times 10^3$ $<0.4 \times 10^3$	3.7×10^4 2.1×10^4	$<0.4 \times 10^3$
Case 3	4.2×10^3	1.2×10^4	9.2×10^3
Case 4	1.7×10^3	5.0×10^4	3.5×10^4
Case 5	1.6×10^3	6.1×10^4	3.5×10^4
Case 6	4.6×10^4 3.1×10^4	$<0.4 \times 10^3$ $<0.4 \times 10^3$	0.5×10^3

Test results of each case are summarised.

Measurements with Amplicor HIV-1 monitor test kits Ver. 1.0, 1.0 plus, and 1.5 were performed according to the manufacturer's protocol.

The per cent coefficient of variation on the same samples tested with the Amplicor HIV-1 monitor test kits Ver. 1.0 and 1.0 plus in our laboratory were 19.9% ($n = 17$) and 31.9% ($n = 12$), respectively. In cases 2 and 6 the viral load was confirmed with another independent measurement (second result).

them, 150 samples (65 cases) were also measured with the Ver. 1.0 plus kit. As expected, most cases with the HIV-1 subtypes A and E, which could not be measured with the Ver. 1.0 kit, could be measured with the Ver. 1.0 plus kit. However, we were particularly interested in a few cases that gave unexpected results. Clinically and epidemiologically, these patients are not different from our other patients infected with HIV-1 subtypes A or E. As shown in table 1, case 1 gave equivalent results with all of the kits but in cases 2 to 5 higher results were obtained with the Ver. 1.0 plus kit than with the Ver. 1.0 kit. We measured these specimens with the Ver. 1.5 kit (kindly provided by Roche Diagnostics, Japan). In these five cases, all but case 2 revealed equivalent values with both the Ver. 1.0 plus and Ver. 1.5 kits. Unexpectedly, in case 2, only the Ver. 1.0 plus kit yielded the accurate results.

On the other hand, in case 6, a higher result was obtained with the Ver. 1.0 kit but not with the Ver. 1.0 plus or Ver. 1.5 kits. An additional two cases showed a similar trend, although the degree of discrepancy was less severe (data not shown).

Nucleotide sequences of primer region

Upstream primer region

SK462 primer AGTTGGAGGACATCAAGCAGCCATGCAAT

Consensus B ---G---G-----
Consensus A ---G---G---C---G---T---
Consensus E ---G---G---C---G---A-----

Case 1 ---G---G---C---G---A-----
Case 2 ---G---G---C---G---A-----
Case 3 ---G---G---C---G---A-----
Case 4 ---G---G---C---G---A-----
Case 5 ---G---G---C---G---A-----
Case 6 ---G---G---C---G---A-----

Downstream primer region

SK431 primer AGAGAACCAAGGGGAAGTACATAGCA

Consensus B -----G-----
Consensus A -----G-----
Consensus E --G-----G-----

Case 1 --G-----G-----
Case 2 --G-----G-----
Case 3 --G-----G-----
Case 4 --G-----G-----
Case 5 --G-----G-----
Case 6 --G-----G-----

Figure 1 The alignment of each primer region. The SK462 and SK431 primers were used in the AmplicorTM HIV-1 monitor test Ver. 1.0 kit. The sequences of these primers were obtained from Roche Diagnostics. Consensus sequences were reproduced from the HIV Sequence Database WWW home page (see text). The sequences shown in this table are from the sense strand.

To clarify the cause of these discrepant results, sequence analysis of the amplified regions of these cases was performed. The nucleotide sequences of these cases were found to be homologous to subtype A or E virus when they were aligned with the consensus sequences of HIV-1 subtypes A, B, and E obtained from the HIV Sequence Database WWW home page (Sequences. (Online.) <http://hiv-web.lbnl.gov>. 30 October 1999, last date accessed.). Surprisingly, the sequences of the primer regions of all cases were completely identical (fig 1). In conclusion, cases 2 and 6 are measurable by one of these kits, Ver. 1.0 plus and Ver. 1.0, respectively; and case 1 is measurable by all kits despite having the same nucleotide sequence in the primer regions as the other cases. The results of sequence analysis of the primer regions suggests that the minor differences in sequence between the virus and the primers does not always affect amplification efficiency in these kits. Although such cases might form a minor population among HIV-1 infections, these results indicate that some cases could not be measured by a single kit. As far as we have experienced, even if one kit fails to measure virus, the other will yield the expected viral load, as judged by disease history, CD4 count, treatment, and so on, suggesting that these kits are mutually complementary. If other methods, such as nucleic acid sequence based amplification or branched DNA (bDNA) systems, are available, it would be useful to test with them. We have found some cases of subtype A or E that have shown a higher viral load with bDNA (Chiron Quantiplex™ HIV RNA 2.0 assay Chiron Corporation, Emeryville, California, USA) than the Amplicor HIV-1 monitor test kit (data not shown). In patients infected with subtype B virus, the correlation between the Amplicor HIV-1 monitor test kit and the bDNA method was excellent ($r = 0.904$; $n = 21$). In conclusion, because the major difference in the three versions of the Amplicor HIV-1 monitor test kit is the primer set, we emphasise that for accurate quantitative measurement using this kit various additional primer sets that can amplify similar regions are needed.

SATORU YOSHIDA
NOZOMI YUSA
NORI HARU SATO

Department of Laboratory Medicine, Institute of
Medical Science, University of Tokyo, 4-6-1,
Shirokanedai, Minato-ku, Tokyo, 108-8639, Japan

MIEKO GOTO
AIKICHI IWAMOTO

Department of Infectious Diseases and Applied
Immunology, Institute of Medical Science, University of
Tokyo

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Next of kin clinics

We read with interest the considered views of Professor Vanezis and Dr Leadbeatter regarding the role of forensic pathologists dealing with the next of kin of those people whose deaths are subject to medicolegal investigation.¹ It might be of interest to your readers to know that the issues raised by the authors have been dealt with at the department of forensic medicine, at Westmead in Sydney for the past decade.

The department offers a specialist grief counselling service, targeting the needs of

families attempting to come to terms with the complications of a coronial investigation at a time of crisis and great distress. The counsellors routinely contact the next of kin in all cases, discussing with them forensic and coronial procedures and outlining the counselling service, which includes individual sessions, group debriefing, and court support. The service operates with the full support of the New South Wales State Coroner, but is funded solely by the department of forensic medicine.

Professor Vanezis and Dr Leadbeatter propose the provision of "next of kin clinics", conducted by the pathologist, to discuss postmortem findings. Such a service is regularly provided at Westmead as part of the counselling unit's brief. Although thoroughly endorsing the authors' remarks on the need for accurate and timely information, we have found that several alterations need to be made to the model broadly outlined by the authors for the families to gain maximum benefit from the information sessions.

These include:

- Ongoing contact between the family and the counsellor from the time of necropsy to the receipt of its results, to ensure that the family is confident that forensic staff will be both frank and reliable in the delivery of information and support.
- A preliminary session between the family and the counsellor, to ensure that all the family's concerns are identified, thereby ensuring that the meeting with the pathologist is as comprehensive as possible.
- The presence of the counsellor at the meeting as mediator and support person for the family. Such a mediation role involves ensuring that all the family's issues are adequately considered, that the tendency some pathologists have to use jargon is kept under control, and that clarification is sought where necessary.

We have found that these alterations are necessary to ensure that the emotional needs of the family and the occasionally unavoidable power imbalance between grieving relatives and a medical specialist are properly dealt with. This latter concern can most clearly be seen in the reluctance of non-medically trained relatives to seek clarification and to admit to doubts and concerns while speaking with a representative of "the system", however well intentioned.

We have been pleased with the success of the service over many years, and self reported feedback from clients suggests both a sense of confidence in the forensic system and in coronial findings as a result. We are delighted that our colleagues in the Northern Hemisphere are finally becoming aware of the value of such a system.

JOHN DRAYTON
PETER S J ELLIS
TONY PURCELL

Department of Forensic Medicine, Institute of Clinical
Pathology and Medical Research, Westmead Hospital,
Westmead NSW 2145, Australia

1 Vanezis P, Leadbeatter S. Next of kin clinics: a new role for the pathologist. *J Clin Pathol* 1999;52:723-4.

The authors reply

Drs Drayton *et al* are to be congratulated on setting up what appears to be a splendid service for relatives, and we note with interest their comments, particularly on how we could modify our work practice in the UK.

We have always been conscious of the fact that when running such clinics we have had to tread very carefully, bearing in mind the sen-

sitivities of relatives, as well as our medicolegal obligations to the investigating authorities. The system we have adopted is designed to provide information to the next of kin regarding the necropsy performed on their loved one, and to answer any concerns they might have regarding our findings. It is not designed to be a specialist grief counselling service. We have intentionally not attempted to take this approach because as pathologists we are not grief counsellors. Nevertheless, it would be extremely useful for all doctors to have some formal training in dealing with the bereaved. As you can appreciate, we have given some thought to the structure we have adopted, and what our role would be in such clinics in relation to our own situation in the west of Scotland, and obviously we would like to see the service that we provide extended to other areas in the UK. As we have stated in our paper, the service we offer at the present time, albeit limited when compared with the one offered in Australia, is nevertheless very welcome and we believe of some benefit to the next of kin.

PETER VANEZIS

Department of Forensic Medicine and Science, The
University of Glasgow, Glasgow G12 8QQ, UK

STEPHEN LEADBEATTER

Wales Institute of Forensic Medicine,
The Royal Infirmary, Cardiff CF2 1SZ, UK

Correction

Photodynamic treatment of pooled coumarin plasma for external quality assessment of the prothrombin time. Van den Besselaar AMHP, Moor ACE. *J Clin Pathol* 2000;53:470-5.

Because of an error in the publishing process, the wrong figure was published as fig 6 in this paper. The following figure is the correct one. The journal apologises for this error.

Figure 6 International normalised ratio (INR) of pooled coumarin plasma determined with bovine thromboplastin (Thrombotest) after treatment with 1 μ M methylene blue and incandescent light (700 W/m²). Before illumination, either air (open circle) or nitrogen (open square) was bubbled through the plasma, or no additional treatment was given (cross).