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 μM 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 μM 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.

(J Clin Pathol 2000;53:470–475)

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 treatment. Since 1974, the Federation of the Netherlands Thrombosis Centres has organised a national EQA scheme for its members. 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. 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.

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. 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 μM in plasma. 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,
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 × 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 × 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/m², 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 Claus.8 Factors II, V, VII, and X were determined with coagulation assays using human tissue factor in undiluted plasma samples deficient in the respective coagulation factors. Prothrombin times were determined with Innovin (Dade Behring, Marberg, Germany), Thrombotest (Nycomed), 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 tmin, uninhib 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.7 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 theuffy 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 hematocrit 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 ml 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 tmin uninhib. I is the distance on a horizontal line between tmin uninhib and the intercept of the line for coumarin plasma with that horizontal line.
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. Interlaboratory 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 2 Infectivity of vesicular stomatitis virus (VSV) after treatment with 1 µM methylene blue and incandescent light (700 W/m²). The VSV titer is expressed on a log scale. Two different pooled coumarin plasmas were used in two separate experiments (open squares and circles).

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 3 Fibrinogen activity of pooled coumarin plasma after treatment with 1 µM 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).

Figure 4 Coagulation factor activities of pooled coumarin plasma after treatment with 1 µM methylene blue and incandescent light (700 W/m²). 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.

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/m²). Before illumination, either air (open circle) or nitrogen (open square) was bubbled through the plasma, or no additional treatment was given (cross).
Photodynamic treatment of plasma

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.

Under the conditions of photodynamic treatment used in our study (20 minutes illumination with white light (700 W/m²) in the presence of 1 µM 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.

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 photo-oxidation 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.1 1 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 and inhibitors react differently with bovine tissue factor than with human thromboplastin. The prothrombin times and INRs determined with plain rabbit and human thromboplastin in 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.4 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 test 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 decrease of INR 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,1 1 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.

The authors thank Ms E Witteveen, Ms C van Rijn, Mrs H Schafer-van Mansfeld, and Mrs J Meerwis-Braun for excellent technical assistance. Mr G van de Kamp (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) supervised the preparation of control blood specimens. Financial support was received from the Federation of the Netherlands Thrombosis Services.


Photodynamic treatment of pooled coumarin plasma for external quality assessment of the prothrombin time
A M H P van den Besselaar and A C E Moor

doi: 10.1136/jcp.53.6.470

Updated information and services can be found at:
http://jcp.bmj.com/content/53/6/470

These include:

**References**
This article cites 13 articles, 1 of which you can access for free at:
http://jcp.bmj.com/content/53/6/470#BIBL

**Email alerting service**
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

**Errata**
An erratum has been published regarding this article. Please see next page or:
/content/53/8/646.2.full.pdf

**Topic Collections**
Articles on similar topics can be found in the following collections
- Immunology (including allergy) (1664)

**Notes**

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/
Letters

M2-AMA do not directly produce ANCA indirect immunofluorescence patterns

The importance of distinguishing atypical cytoplasmic indirect immunofluorescence patterns from the “classical,” centrally accentuated cytoplasmic immunofluorescence pattern on ethanol fixed human neutrophils has recently been re-emphasized.11 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 specificity produced positive indirect immunofluorescence patterns on ethanol fixed human neutrophils,11 or whether these sera contain concomitant ANCA. While the atypical cytoplasmic immunofluorescence pattern on ethanol fixed human neutrophils has recently been re-emphasized, the 10 perinuclear immunofluorescence patterns in M2-AMA sera produced positive indirect immunofluorescence 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, proteinase 3, cathepsin G, lysozyme, and bactericidal/permeability increasing protein (BPI).

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

<table>
<thead>
<tr>
<th>Specimen</th>
<th>M2-AMA</th>
<th>ANCA IIF pattern (titre)</th>
<th>ORGenTec Combi-kit® ELISA OD (titre)</th>
<th>Liver biopsy result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.47)</td>
<td>PBC</td>
</tr>
<tr>
<td>2</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>3</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>4</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>5</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>6</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>7</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>8</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>9</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>10</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>11</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>12</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>13</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>14</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>15</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>16</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>17</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>18</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>19</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>20</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>21</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>22</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>23</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>24</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>25</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>26</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>27</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>28</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>29</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>30</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>31</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
<tr>
<td>32</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (2.79)</td>
<td>Not done</td>
</tr>
</tbody>
</table>

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.

www.jclinpath.com
polyloid 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 × 10^9/litre; platelets, 460 × 10^9/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 µmol/litre; lactate dehydrogenase, 1765 units/litre; carcinoembryonic antigen, 327 µg/litre; ferritin, 302 µg/litre; Fe, < 2 µmol/litre; and total iron binding capacity, < 21 µmol/litre. Chest x ray was negative. Two weeks later the patient developed mental confusion and dehydration. Serum calcium was 2.8 mmol/litre, phosphorus 0.96 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 radionucleic bone scan nor magnetic resonance scan of the brain suggested metastases. The intact PTH (IRMA; Nichols 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 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.2 PTHrP expression in the tumour tissue was strongly associated with poorly differentiated cancers (54% of 54) 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 progression or is a byproduct of oncogene activation (for example, ras and src) remains to be determined.

GEORG ENGELICH
NIALL SWAN
KEVAN L MARTSHORN
Boston University School of Medicine and Boston Medical Center, Departments of Medicine and Pathology, 80 East Concord Street, Boston, MA 02118, USA


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)”1 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 number 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 anticardiolipin. We believe that the use of ELISAs alone to diagnose a systemic necrotising vasculitis is analogous to testing for systemic lupus erythematosus with anti-dsDNA antibodies rather than initially screening for antinuclear antibodies by indirect immunofluorescence.

JUDY SAVAGE
Division of Investigative Medicine, Austin and Repatriation Medical Centre

WENDY POLLOCK
Gribble’s Pathology, 14 Yarra Street, Private Bag 1800, South Yarra, Victoria 3141, Australia


The authors reply

Our study was carried out following the preliminary observation that in our laboratory a positive immunofluorescence result for a false positive 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.

Savage 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 assays are used 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 = 0.0564) yet has a signifi-
ently better specificity (92% ± 90%; p = 0.0006) and positive predictive value (73% ± 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. ELISA and immunofluorescence ANCA are considered together.

In fact, combining immunofluorescence and ELISA ANCA resulted in a lower positive predictive value if results of ELISA and immunofluorescence ANCA are combined. This was not found in our study. Combining immunofluorescence and ELISA ANCA are considered together. Combining immunofluorescence and ELISA ANCA are considered together.

Finally, in addition to the ecocervical and endocervical endpoints we routinely comment upon the presence of CIN (cervical intraepithelial neoplasia) at the deep lateral edge. This is the edge that runs between the superior, endocervical edge of the specimen to the lateral, ecocervical 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 replaparisation process, so that it will not be detected on colposcopy or cytopathological surveillance. Residual disease, if undetected, has been suggested as a cause of late invasive cervical carcinoma in patients treated for CIN.

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 blood is now commercially available and is widely used for the assessment of antiretroviral treatments. The kit is called the AmpliCox™ HIV-1 monitor 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 To surmount this problem, additional gag primers (AG primers) have been previously used to address this need (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 basic substitutions. In our laboratory, we have examined over 1500 samples (148 cases) using the Ver. 1.0 kit, and among 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 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 equivocal 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 awaited result.

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).

Table 1 AmpliCox™ HIV-1 monitor kit

<table>
<thead>
<tr>
<th>Case</th>
<th>Ver. 1.0</th>
<th>Ver. 1.0 (+)</th>
<th>Ver. 1.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>3.6 × 10^3</td>
<td>2.7 × 10^3</td>
<td>2.0 × 10^3</td>
</tr>
<tr>
<td>Case 2</td>
<td>&lt;0.4 × 10^1</td>
<td>3.7 × 10^3</td>
<td>&lt;0.4 × 10^1</td>
</tr>
<tr>
<td>Case 3</td>
<td>3.2 × 10^3</td>
<td>1.2 × 10^4</td>
<td>9.2 × 10^3</td>
</tr>
<tr>
<td>Case 4</td>
<td>1.7 × 10^4</td>
<td>5.0 × 10^5</td>
<td>3.5 × 10^4</td>
</tr>
<tr>
<td>Case 5</td>
<td>1.5 × 10^6</td>
<td>6.1 × 10^7</td>
<td>3.5 × 10^6</td>
</tr>
<tr>
<td>Case 6</td>
<td>4.0 × 10^5</td>
<td>&lt;0.4 × 10^1</td>
<td>3.5 × 10^5</td>
</tr>
</tbody>
</table>

Test results of each case are summarised. Measurements with AmpliCox™ HIV-1 monitor test kits Ver. 1.0, 1.0 plus, and 1.5 were performed according to the manufacturer’s protocol. The current coefficient of variation on the same samples tested with the AmpliCox™ 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).
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.lbl.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 region as the other cases. The results of sequence analysis of the primer regions suggests that the minor differen-ces 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 such a common kit 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, suggest-ing that these kits are mutually complemen-tary. 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 Corpora-tion, 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 conclu-sion, 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.

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 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 counsel-lors routinely contact the next of kin in all cases, discussing with them forensic and coro-nial procedures and outlining the counselling service, which includes individual, group and liaison 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 regu-larly 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 to discuss 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 meeting 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 nec-essary 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 aspect of the meeting is most clearly 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 it might be.

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 Hemi-sphere are finally becoming aware of the value of such a system.

Correction


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.