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,
Photodynamic treatment of plasma

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 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, Marberg, 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 tmin, 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.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 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 tmin, uninh. I is the distance on a horizontal line between tmin, uninh. 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)

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
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 the 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>
<thead>
<tr>
<th>Reagent</th>
<th>Sample A not illuminated</th>
<th>Sample B not illuminated</th>
<th>Sample C illuminated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombost</td>
<td>40</td>
<td>117.5</td>
<td>117.6</td>
</tr>
<tr>
<td>Thrombost</td>
<td>40</td>
<td>118.1</td>
<td>118.2</td>
</tr>
<tr>
<td>Recombiplastin</td>
<td>18</td>
<td>39.6</td>
<td>6.2</td>
</tr>
<tr>
<td>Recombiplastin</td>
<td>19</td>
<td>37.7</td>
<td>7.5</td>
</tr>
<tr>
<td>PT-Fib HS</td>
<td>17</td>
<td>30.7</td>
<td>7.9</td>
</tr>
<tr>
<td>PT-Fib HS</td>
<td>18</td>
<td>29.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Thromborel-S</td>
<td>13</td>
<td>38.3</td>
<td>6.1</td>
</tr>
<tr>
<td>Thromborel-S</td>
<td>12</td>
<td>36.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Hepato Quick</td>
<td>15</td>
<td>85.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Hepato Quick</td>
<td>16</td>
<td>85.6</td>
<td>3.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Sample A not illuminated</th>
<th>Sample B not illuminated</th>
<th>Sample C illuminated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombost</td>
<td>41</td>
<td>3.11</td>
<td>4.6</td>
</tr>
<tr>
<td>Thrombost</td>
<td>40</td>
<td>2.87</td>
<td>4.3</td>
</tr>
<tr>
<td>Recombiplastin</td>
<td>18</td>
<td>3.55</td>
<td>8.5</td>
</tr>
<tr>
<td>Recombiplastin</td>
<td>19</td>
<td>3.34</td>
<td>8.0</td>
</tr>
<tr>
<td>PT-Fib HS</td>
<td>17</td>
<td>3.73</td>
<td>12.0</td>
</tr>
<tr>
<td>PT-Fib HS</td>
<td>19</td>
<td>3.57</td>
<td>12.0</td>
</tr>
<tr>
<td>Thromborel-S</td>
<td>13</td>
<td>3.70</td>
<td>4.6</td>
</tr>
<tr>
<td>Thromborel-S</td>
<td>12</td>
<td>3.56</td>
<td>6.8</td>
</tr>
<tr>
<td>Hepato Quick</td>
<td>16</td>
<td>3.10</td>
<td>5.7</td>
</tr>
<tr>
<td>Hepato Quick</td>
<td>16</td>
<td>3.12</td>
<td>5.6</td>
</tr>
</tbody>
</table>

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.

**Discussion**

VSV is a very well characterised lipid envelope 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. This is probably caused by photooxidation of these residues by singlet oxygen, as shown in a study using scavengers of different reactive oxygen species. 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. 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. 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). 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. 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 was 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, 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 Meeswis-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.


---

**PATHOLOGY INTERACTIVE 2000**

**Do you know . . . ?**

*Pathology Interactive* Volume 2 now offers:

Up to three CPD credits per review article

- 1 credit for reading the article and completing the associated questions
- 1 credit for additional reading, following up references
- 1 credit for making notes, identifying learning outcome or identifying further learning needs

CPD credits for working on articles outside your specialty

*Pathology Interactive* Volume 2 Issue 1 includes articles in:

- Microbiology
- Histopathology
- Molecular pathology

**Further issues in 2000 will cover further articles in these specialties, plus:**

- Chemical pathology
- Haematology
- Immunology
  - Including case study and picture quiz formats

*Pathology Interactive* 2000 Volume 2, 4 issues (March, June, September, December) ISSN 1466 5743 Accredited by the Royal College of Pathologists

Subscription rate: £75+VAT personal*, £150 +VAT institutional (multiuser rate on application to the publisher).

Send orders to: BMJ Publishing Group, Journals Marketing Dept. PO Box 299, London WC1H 9TD, UK; fax credit card orders to: +44 (0)20 7383 6402; call subscriptions hotline +44 (0)20 7383 6270; email orders to: subscriptions@bmjgroup.com

*ACP members receive *Pathology Interactive* with their copy of *Journal of Clinical Pathology*, as a membership benefit.
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

J Clin Pathol 2000 53: 470-475
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#ref-list-1

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/
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-emphasised. 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. 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. 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.

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

<table>
<thead>
<tr>
<th>Specimen</th>
<th>M2-AMA</th>
<th>ANCA IIF pattern (titre)</th>
<th>ANCA Combi-kit® ELISA (OD ratio)</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 (2.97)</td>
<td>Not done</td>
</tr>
<tr>
<td>3</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-elastase (1.46)</td>
<td>PBC</td>
</tr>
<tr>
<td>4</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (1.74)</td>
<td>Not done</td>
</tr>
<tr>
<td>5</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.04)</td>
<td>PBC</td>
</tr>
<tr>
<td>6</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (1.24)</td>
<td>PBC</td>
</tr>
<tr>
<td>7</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (1.24)</td>
<td>PBC</td>
</tr>
<tr>
<td>8</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (1.79)</td>
<td>PBC</td>
</tr>
<tr>
<td>9</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (1.27)</td>
<td>Not done</td>
</tr>
<tr>
<td>10</td>
<td>High positive</td>
<td>Negative</td>
<td>Anti-BPI (1.87)</td>
<td>Not done</td>
</tr>
<tr>
<td>11</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (1.02)</td>
<td>PBC</td>
</tr>
<tr>
<td>12</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (2.25)</td>
<td>Not done</td>
</tr>
<tr>
<td>13</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (2.35)</td>
<td>Not done</td>
</tr>
<tr>
<td>14</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (2.46)</td>
<td>Not done</td>
</tr>
<tr>
<td>15</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.16)</td>
<td>Not done</td>
</tr>
<tr>
<td>16</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.26)</td>
<td>Not done</td>
</tr>
<tr>
<td>17</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.36)</td>
<td>Not done</td>
</tr>
<tr>
<td>18</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.46)</td>
<td>Not done</td>
</tr>
<tr>
<td>19</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.56)</td>
<td>Not done</td>
</tr>
<tr>
<td>20</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.66)</td>
<td>Not done</td>
</tr>
<tr>
<td>21</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.76)</td>
<td>Not done</td>
</tr>
<tr>
<td>22</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.86)</td>
<td>Not done</td>
</tr>
<tr>
<td>23</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (3.96)</td>
<td>Not done</td>
</tr>
<tr>
<td>24</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.06)</td>
<td>Not done</td>
</tr>
<tr>
<td>25</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.16)</td>
<td>Not done</td>
</tr>
<tr>
<td>26</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.26)</td>
<td>Not done</td>
</tr>
<tr>
<td>27</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.36)</td>
<td>Not done</td>
</tr>
<tr>
<td>28</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.46)</td>
<td>Not done</td>
</tr>
<tr>
<td>29</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.56)</td>
<td>Not done</td>
</tr>
<tr>
<td>30</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.66)</td>
<td>Not done</td>
</tr>
<tr>
<td>31</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.76)</td>
<td>Not done</td>
</tr>
<tr>
<td>32</td>
<td>High positive</td>
<td>Negative</td>
<td>Anticathepsin G (4.86)</td>
<td>Not done</td>
</tr>
</tbody>
</table>

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

Atypical cytoplasmic ANCA staining (1/40 titre) with homogeneous and speckled patterns was present.

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

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

†Concomitant ANA staining (1/40 titre) with a nuclear membrane pattern was present.


Raised plasma parathyroid hormone related peptide in gastric adenocarcinoma

We report a case of humoral hypercalcaemia 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...
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 \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 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 0.5 mmol/litre, and albumin 26 g/litre. With intravenous hydration and pamidronate (10 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; Nichols Institute) was 6 ng/litre (normal < 1.3). The patient died of progressive liver failure three weeks later.

Hypercalcemia is rarely associated with gastric cancer. In the literature, two cases of gastric adenocarcinoma with hypercalcemia have been reported in which the tumour cells expressed PTHrP. 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 hypercalcemia. PTHrP expression in the tumour tissue was strongly associated with poorly differentiated cancers (34 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.

GEORGE ENGELICH
NIALL SWAN
KEVIN MARTIN
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 results showed that indirect immunofluorescence is a more sensitive technique than antigen specific enzyme linked immunosorbant 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-

Table 1

<table>
<thead>
<tr>
<th>Test</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Positive Predictive Value</th>
<th>Negative Predictive Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>87%</td>
<td>76%</td>
<td>91%</td>
<td>62%</td>
</tr>
<tr>
<td>IF</td>
<td>50%</td>
<td>87%</td>
<td>62%</td>
<td>91%</td>
</tr>
</tbody>
</table>


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.

Savige et al reported their finding of a higher positive predictive value if results of ELISA and immunofluorescence ANCA are combined, but 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 Savige 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" should be revisited.

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 paper and to take the opportunity to add some comments of my own. Following earlier correspondence in the journal, 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. Secretarial or medical staff can enter a short code of up to 35 letters, 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 often measures 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 specimen derivation to the particular clinicians to the responsible consultant.

Nucleotide sequences of primer region

Some problems found in HIV-1 RNA quantification

The polymerase chain reaction (PCR) based assay of human immunodeficiency virus type 1 (HIV-1) RNA quantification is now commercially available and is widely used for the assessment of antiretroviral treatments. The kit is called the Amplicor 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. To surmount this problem, additional gag primers (AG primers) have been pre-empted to allow 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 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 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 altered value.

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 AmplicorTM 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 × 10⁴</td>
<td>2.7 × 10⁴</td>
<td>2.0 × 10⁴</td>
</tr>
<tr>
<td>Case 2</td>
<td>&lt;0.4 × 10¹</td>
<td>3.7 × 10⁴</td>
<td>&lt;0.4 × 10⁴</td>
</tr>
<tr>
<td>Case 3</td>
<td>4.2 × 10⁵</td>
<td>1.2 × 10⁴</td>
<td>9.2 × 10⁴</td>
</tr>
<tr>
<td>Case 4</td>
<td>1.7 × 10⁴</td>
<td>5.0 × 10⁵</td>
<td>3.5 × 10⁵</td>
</tr>
<tr>
<td>Case 5</td>
<td>1.6 × 10⁵</td>
<td>6.1 × 10⁵</td>
<td>3.5 × 10⁵</td>
</tr>
<tr>
<td>Case 6</td>
<td>4.6 × 10⁶</td>
<td>&lt;0.4 × 10⁴</td>
<td>0.5 × 10⁵</td>
</tr>
<tr>
<td>Case 7</td>
<td>3.1 × 10⁶</td>
<td>&lt;0.4 × 10⁴</td>
<td>&lt;0.4 × 10⁴</td>
</tr>
</tbody>
</table>

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 mean 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).
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 difference 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 Quan-tiplex® 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
NORIHARU 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

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 coronal 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 coronal 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 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 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 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.
- 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 coronal 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.

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.


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](https://www.jclinpath.com)

The authors reply

Dr 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 might 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 sensitivity 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.

www.jclinpath.com