Sensitive Nonradiometric Method for Determining Thymidine Kinase 1 Activity

ANDERS ÖHRVIK, MARIA LINDH, ROLAND EINARSSON, JACQUES GRASSI, and STAFFAN ERIKSSON

Background: Thymidine kinase 1 (TK1) is a cytoplasmic enzyme, produced only in the S-phase of proliferating cells, that has potential as a tumor marker. Specific determination of TK1 in serum is difficult, in part because of differences in the physical properties of serum TK1 compared with cytoplasmic TK1.

Methods: The first step in the new assay was phosphorylation of 3'-azido-2',3'-deoxythymidine (AZT) to AZT 5'-monophosphate (AZTMP) by TK1 present in patient material. The AZTMP formed was measured in a competitive immunoassay with specific anti-AZTMP antibodies and AZTMP-labeled peroxidase. Results were compared with those of a TK radioenzyme assay (REA) for 78 samples from patients suffering from hematologic diseases.

Results: The detection limit was 78 μIU/L, and within-run CVs <20% were seen for samples with TK1 down to 130 μIU/L. Cross-determination of the mitochondrial isoenzyme TK2 activity was <0.1%. Between-assay imprecision (CV) was 3.5–7.4%, and the within-assay imprecision was 4.1–9.1%. In studies of recovery and linearity on dilution, measured values ranged from 84% to 115% of expected at concentrations of 0.26–10.4 μIU/L. Results of the new assay (μIU/L) = 0.109 × TK REA (U/L) + 0.092. Heterophilic antibodies did not interfere in the assay. The upper 95th percentile, in 100 healthy individuals, was 0.94 μIU/L, and the median value was 0.43 μIU/L.

Conclusion: The TK1 enzyme-labeled immunoassay uses a stable substrate, is precise, appears to be accurate, and is resistant to interferences. It may provide a practical tool in the management of hematologic malignancies.

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Thymidine kinase 1 (TK1;4 ATP:thymidine 5'-phosphotransferase; EC 2.7.1.21) is a cytoplasmic enzyme found only in proliferating cells, with production restricted to the S-phase of the cell cycle (1, 2). This enzyme and its activity have therefore been used as a reliable and sensitive marker for cell proliferation. TK1 transfers a γ-phosphate group from a nucleoside triphosphate to the 5'-hydroxyl group of thymidine or deoxyuridine, and these products are further phosphorylated to DNA precursors. TK1 is a homodimer or homotetramer with a 24-kDa monomer subunit (2–4). TK1 can phosphorylate analogs with modifications at the N-3 or C-5 position of the pyrimidine ring and the 3' position of the ribose (4–8); it also phosphorylates clinically important nucleoside analogs, including 5-fluoro-2'-deoxythymidine and 3'-azido-2',3'-deoxythymidine (AZT) (4–6). TK1 activity begins in late G1 phase; increases during S phase, coinciding with the increase in DNA synthesis; and becomes undetectable (2, 9–11). The role of the TK1 promoter in regulation has been reviewed (11), and the mechanism for cell-cycle-dependent degradation of TK in most G2-M cells has been determined (12).

The mitochondrial thymidine kinase TK2 can also phosphorylate thymidine and deoxyuridine. The concentration of TK2 in tissues is not correlated with proliferation. TK2 plays an essential role in the synthesis of mitochondrial DNA precursors and is involved in certain

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Nonstandard abbreviations: TK1 and TK2, thymidine kinase 1 and 2, respectively; AZT, 3'-azido-2',3'-dideoxythymidine; REA, radioenzyme assay; AZTMP, 3'-azido-2',3'-dideoxythymidine 5'-monophosphate; AZXMP, 3'-azido-2',3'-dideoxy-5'-monophosphate-5-(5-amino-1-pentyl)thymidine; KLH, keyhole limpet hemocyanin; HRP, horseradish peroxidase; BSA, bovine serum albumin; TMB, 3,3',5,5'-tetramethylbenzidine; hrTK1, human recombinant thymidine kinase 1; DPE, dithioerythritol; PBS, phosphate-buffered saline; DSS, disuccinimidyl suberate; and TMPK, thymidylate kinase (thymidine-5'-monophosphate kinase).
forms of mitochondrial diseases but not in diseases related to cell proliferation (13, 14).

The substrate specificity of TK2 is different from that of TK1, e.g., thymidine analogs such as AZT are phosphorylated at much lower efficiency by TK2 than by TK1 (4, 5), a feature that is of particular importance for the assay described here.

Serum TK has been determined by use of a commercially available, highly sensitive 125I-deoxyuridine radioenzyme assay (TK REA). This assay can provide prognostic information in malignancies, and in some cases can aid in the choice of therapy (15–22). The assay has disadvantages in that it uses 125I, is relatively complex, and partly measures the activity of TK2. In most cases the latter is not a major concern because TK1 is the predominant form in serum, but in certain instances, particularly when cellular extracts are assayed, false-positive results may occur.

Although immunochemical methods have been reported (23–29), a highly sensitive but convenient approach for routine measurement of serum TK1 in hematologic malignancies has not been forthcoming. Furthermore, antibody-based assays would most probably provide different clinical information than would activity-based measurements (23).

Here we describe the development and analytical validation of an enzyme-labeled immunoassay for TK1 activity. An abstract of this study, describing some preliminary work, has been published (30).

Materials and Methods

REAGENTS

AZT, AZT-related compounds, 3’-amino-2’,3’-deoxythymidine, and natural nucleosides and nucleotides were all purchased from Sigma-Aldrich. AZT 5’-monophosphate (AZTMP), AZT, and 3’-azido-2’,3’-deoxy-5’-monophosphate-5N-1-(5-aminopentyl)thymidine (AZXMP) were dissolved in deionized water and kept at −78 °C. The concentrations of filtered solutions were checked by absorbance measurements. Keyhole limpet hemocyanin (KLH) was purchased from Pierce.

Horseradish peroxidase (HRP) and rabbit and goat IgG of reagent grade were purchased from Sigma, and bovine IgG and bovine serum albumin (BSA) were purchased from Bayer/Pentex. Concentrations of filtered immunoglobulin solutions were determined by ultraviolet absorbance measurements, and stock solutions were aliquoted and kept at −20 °C. Ready-to-use 3,5,5,5’-tetramethylbenzidine (TMB) and TMB stop solutions were from BioFx. All microtiter plates were from Nunc ProClín 300™ (Rohm and Haas) was purchased from Supelco. Affinity-purified secondary rabbit anti-goat immunoglobulin was purchased from DakoCytomation.

STATISTICAL ANALYSIS

Statistical analyses were performed with StatGraphics plus 4.0. Error limits are based on 95% confidence intervals, and experiments were repeated at least once with two or more replicates per sample.

Purified TK1

The production of human recombinant TK1 (hrTK1) has been described elsewhere (31). Native TK1 was isolated from HeLa cells as follows. Exponentially grown confluent HeLa cells were harvested, by centrifugation, from the growth medium after trypsinization, and the cells were resuspended in lysis buffer containing 50 mmol/L HEPES, 5 mmol/L dithioerythritol (DTE), 2 mmol/L ATP, and 2 mmol/L MgCl2· H2O. The cell suspension was subjected to five freeze-thaw cycles at −70 °C and 25 °C, respectively. The ruptured cells were centrifuged at 48 000g for 1 h at 2 °C to pellet cellular debris, and the supernatant containing TK was tested by electrophoresis as described below.

TK Isoenzyme Purification Control

To check that no TK2 enzyme was present in the crude extract, we performed rod electrophoresis, followed by REA detection with the prolifigen® TK REA assay (DiaSorin AB), which detects both TK1 and TK2 (32). A sample containing 100 µL of supernatant, 40 µL of 500 mL/L glycerol, and 5 µL of 10 g/L bromphenol blue was analyzed on 5% polyacrylamide gel in electrophoresis buffer (pH 8.6) containing 190 mmol/L glycine, 0.2 mmol/L ATP, 25 mmol/L Tris, 1 mmol/L MgCl2, and 3 mmol/L β-mercaptoethanol for 3 h at 100 °C. The gel was then sliced into pieces and mixed with the assay buffer. The activity in each slice was determined and plotted against the relative mobility. The tested samples showed no detectable TK2 activity (data not shown).

Preparation and Purification of Antibodies

Preparation of the Immunogen.

The preparation of polyclonal goat antiserum was done as described above for the preparation of rabbit antiserum and is summarized below (33). An AZTMP derivative, AZXMP, was synthesized as described (33). The purity of AZXMP was >99%, and the identity was confirmed by mass spectrometry, 1H nuclear magnetic resonance, and 31P-nuclear magnetic resonance (data not shown). The hapten molecule AZXMP was coupled to the carrier protein KLH by use of glutaraldehyde at 6 °C to better control the reaction. KLH (198 mg) in 10 mmol/L sodium phosphate–0.15 mol/L NaCl (pH 7.2) was added to AZXMP (13.5 mg) in 80 mmol/L phosphate buffer (pH 7.2) and mixed with 0.21 mol/L glutaraldehyde (final concentration, 23 mmol/L). The reaction mixture was incubated for 23 h under agitation. Subsequently, the solution was dialyzed against 0.1 mol/L phosphate-buffered saline (PBS), and the resulting conjugate was kept at −78 °C until required. The coupling efficiency was checked by the ultraviolet absorbance of free AZXMP in the filtrate after ultrafiltration. A mean (SD) coupling of 0.035 (0.004) g of AZXMP/g of KLH was obtained.
Immunizations and purification of antibodies. Polyclonal antibodies were raised in goats by multiple intradermal injections of 1 mg of AZXMP-KLH emulsified in Freund’s complete adjuvant. Booster doses were given monthly, and after three rounds of immunization, booster intervals were extended to 8-week cycles. Bleedings were done every second week by Agrisera AB (Vännäs, Sweden). Goat antibodies were affinity-purified on AZXMP coupled to cyanogen bromide-activated Sepharose Phast Flow (Amersham Biosciences) with 2.5 μmol of AZXMP per milliliter of gel.

Affinity-purified sera were further purified by gel filtration on a Superdex 200 preparative-grade gel (Amersham Biosciences). The peak corresponding to monomeric IgG was isolated, and ProClin 300 was added as a preservative to a final concentration of 5 mL/L. Purified antibodies were kept at 2–8 °C until further use. The reactivity and specificity of the antibodies were checked in the immunoassay described below. For use in a TK1 ELISA, the antibodies were diluted to 25 μg/L in a diluent consisting of 50 mmol/L phosphate buffer, 1 mL/L ProClin 300, and 1 mL/L Triton X-100 (pH 8.0) supplemented with 100 mg/L bovine IgG, 100 mg/L rabbit IgG, and 0.25 mL/L Tween 20. Blocking was achieved by the addition of 5 mL/L ProClin 300 (pH 7.4) and stored at −80 °C.

Preparation of HRP Conjugate
AZXMP was coupled to HRP by use of the homobifunctional reagent disuccinimidyl carbonate (DSS), which incorporates an 8-carbon chain linker. AZXMP was initially coupled through its primary amino group to one of the two active esters on the DSS molecule, giving AZXMP-DSS. To obtain an equimolar coupling product, an excess of 8 moles of DSS per mole of AZXMP was used, and the reaction was performed at 23 °C. AZXMP (500 μL; 2.0 g/L in 0.1 mol/L sodium borate buffer, pH 8.8) was mixed with DSS (500 μL; 8.4 g/L in acetonitrile), and the coupling reaction was terminated after 60 min by injection on the separation column. Before injection, 1 mL of 20 mmol/L KH₂PO₄ was added, and the pH was adjusted to 4.6 with HCl. The yield of AZXMP-DSS was 60–70% after purification on a C2/C18 pepRPC HR16/10 reversed-phase column (Amersham Biosciences). The product was eluted with a methanol–water (9:1 by volume) gradient. Fractions were pooled, and the product was immediately frozen at −78 °C and subsequently lyophilized to remove the methanol used in the reverse-phase chromatographic step. The product was dissolved in H₂O-acetonitrile (9:1 by volume) and then immediately frozen and kept at −80 °C. The concentration and purity of the final product were determined by reverse-phase chromatographic analysis using AZTMP as an internal standard.

AZXMP-DSS was conjugated to amino groups on HRP, giving AZXMP-DSS-HRP (Fig. 1). For a standard reaction, 15 moles of AZXMP-DSS were added per mole of HRP. The reaction was performed at 18–22 °C for 90 min. A 100-μL portion of 3.4 mmol/L AZXMP-DSS was added to HRP (197 μL; 5.0 g/L) and further supplemented with 170 μL of H₂O and 5 μL of 1 mol/L NaOH, giving a pH of 8.8. The AZXMP-DSS-HRP conjugate was separated from low-molecular-weight material by gel filtration on a Superdex 200 HR 10/30 column (Amersham Biosciences). The fractions with AZXMP-DSS-HRP were pooled and diluted 10-fold in 10 mmol/L PBS containing 25 g/L BSA, 0.1 g/L K₂FeCN₆, 0.1 g/L bromphenol blue, and 5 mL/L ProClin 300 (pH 7.4) and stored at −80 °C.

Coating of Microtiter Plates
The secondary antibody (rabbit anti-goat IgG) was diluted in 0.1 mol/L carbonate buffer (pH 8.5) to a concentration of 5 mg/L, of which 100 μL was used to coat each well of C8 Maxisorbent plates. Microtiter wells were incubated for 20 h at 18–22 °C and subsequently washed three times with 250 μL of 10 mmol/L PBS (pH 7.5) containing 0.5 mL/L Tween 20. Blocking was achieved by the addition of 250 μL/well of a solution of 13 mmol/L PBS (pH 7.5) containing 100 g/L sucrose and 10 g/L BSA, followed by incubation at 18–22 °C for 20 h. Wells were then emptied, dried under low humidity for 20 h at 22 °C, and sealed. Plates were washed three times in wash buffer before use.

Assay Procedures
Principle for the TK1 assay method. In the first step of the assay, TK1 present in the patient sample catalyzes the phosphorylation of a specific substrate (AZT) to the corresponding 5'-monophosphate (AZTMP). In the second step, primary polyclonal goat anti-AZTMP antibodies are added, and this is done in microtiter plates coated with an excess of rabbit anti-goat antibodies together with HRP-labeled AZTMP (Fig. 1).

Competition between the generated AZTMP and the HRP-labeled AZTMP occurs, and bound immune complex is captured by the secondary antibodies. Unbound HRP-labeled AZTMP is washed away, and the bound HRP is detected and related to the AZTMP concentrations, i.e., the TK1 activity present in the original sample.

TK1 assay. A 50-μL portion of serum or calibrator was added to 50 μL of the substrate buffer (170 mmol/L
tracer (450 μg/L HRP) and 50 μL of polyclonal goat anti-AZTMP antibodies (25 μg/L). After incubation for 2 h at 18–25 °C with agitation, the wells were washed three times with 250 μL of 0.1 mol/L phosphate buffer (pH 7.4) containing 1 g/L BSA and 1 mL/L Triton X-100—was added. The incubation, wash, and detection steps were then performed as described for the TK1 assay.

**ANALYTICAL VALIDATION**

**Calibration.** Native purified cytosolic TK1 diluted in buffer [50 mmol/L HEPES (pH 7.4) containing 75 mL/L fetal calf serum, 4 mmol/L ATP, 12.5 g/L polyethylene glycol 8000, and 82 mmol/L mannitol] was used as the calibration material. Assay calibration was initially done against the TK REA assay to give equal U/L values in both assays. Under the conditions described in the REA assay, 1 U of TK REA enzyme activity is equal to 1.2 × 10−12 katal (34). However, TK ELISA results are presented in IU (1 IU = 1 μmol/min = 16.67 nmol/s = 16.67 nkatal). In some cases, the TK ELISA values are presented in REA U/L to compare the assays. This is described in more detail in the Results section.

**Cross-reactivity with nucleotides and nucleosides.** The cross-reactivity with AZT-related compounds, nucleosides, and nucleotides was investigated by establishing the corresponding calibration curves (see the section for the AZTMP assay). Tested compounds were AZTMP, AZXMP, AZT, thymidine, thymidine 5'-monophosphate, thymidine 5'-diphosphate, UMP, and ATP.

**Cross-determination of TK2 activity.** Purified recombinant human TK2 was isolated as described elsewhere (35). The amount of AZTMP generated per mole of TK2 was compared with the AZTMP generated by TK1. The TK1 assay was performed, and the activity was calculated using the TK ELISA IU/L.

**Optimization of the assay to eliminate interference.** The TK ELISA was performed with various concentrations of bovine, rabbit, and goat IgG in the primary antibody solutions. The responses of patient sera with heterophilic antibodies (n = 8) and normal samples covering the range measured were investigated. The immunoglobulin interference could also be verified by use of the TK ELISA assay. High-molecular-weight components were eliminated after the TK reaction step and before the AZTMP detection by filtration through M, 10 000 cutoff filters (Centricon 10; Amicon). Recovery of AZTMP was >99%, and the presence of normal serum did not influence the AZTMP detection assay. A mock ELISA assay was also performed in which the AZT substrate was replaced by the diluent. Performance evaluations were all done with the optimum concentrations of 100 mg/L bovine IgG, 100 mg/L rabbit IgG, and 0.25 mg/L goat IgG.

**Dilution linearity and analytical recovery.** The linearity on dilution was evaluated with six human serum samples (hematologic diseases) containing increased concentrations of TK1 (3.12–11.18 mIU/L). The assay diluent (9 g/L NaCl, 91 g/L glycerol) was used to prepare the dilutions, and every sample was tested undiluted and diluted by factors of 1.25, 1.67, 2.5, 5, and 10.

The recovery was evaluated by adding stock solutions of native TK1 to calibrator diluent to obtain samples with activity values of 8.06–8.71 mIU/L. These samples were then further diluted by factors of 1.25, 1.67, 2.5, 5, and 10 as described above.

Recovery and dilution linearity were also tested by diluting five human sera (1.55–3.50 mIU/L) with sera from five blood donors (0.58–0.81 mIU/L) in the ratios indicated above.

**Detection limit and functional sensitivity.** The lowest detectable TK1 activity was obtained by measuring the inaccuracy of the zero calibrator in 20 replicates and then calculating the dose associated with the mean signal for the zero calibrator + 3 SD. Precision was determined by analyzing >200 serum samples in duplicate.

**Imprecision.** We determined the within-assay, between-assay, and total imprecision according to the variance component model recommended by NCCLS (36). Two operators performed 10 assays on 5 days. Each sample was run in four replicates per assay, giving a total of 40 determinations. The seven samples were native sera from patients with hematologic diseases added to delipidated normal human sera.

**Reference interval.** A total of 100 serum samples from apparently healthy Swedish blood donors were measured in duplicate with the TK ELISA. The samples were

HEPES, 62 mmol/L NaOH, 15.4 mmol/L MgCl2 · 6 H2O, 142 mmol/L mannitol, 7.7 mmol/L ATP, 12 mmol/L DTE, 187 μmol/L AZT, and 1.0 mmol/L UMP) in uncoated flat microtiter wells. The wells were incubated for 60 min at 37 °C, and the reaction was terminated by the addition of 50 μL of 74 mmol/L hydrogen peroxide per well. The plate was placed at room temperature and mixed at 500 rpm for 10 min before 50 μL was transferred to a microtiter plate well coated with rabbit anti-goat antibodies together with 50 μL of the AZXMP-DSS-HRP tracer (450 μg/L HRP) and 50 μL of polyclonal goat anti-AZTMP antibodies (25 μg/L). After incubation for 2 h at 18–25 °C with agitation, the wells were coated flat microtiter wells. The wells were incubated for 60 min at 37 °C and mixed at 500 rpm for 10 min before 50 μL was transferred to a microtiter well coated with rabbit anti-goat antibodies together with 50 μL of the AZXMP-DSS-HRP tracer (450 μg/L HRP) and 50 μL of polyclonal goat anti-AZTMP antibodies (25 μg/L). After incubation for 2 h at 18–25 °C with agitation, the wells were washed three times with 250 μL of 0.1 mol/L phosphate buffer (pH 7.4) containing 1 g/L BSA and 1 mL/L Triton X-100—was added. The incubation, wash, and detection steps were then performed as described for the TK1 assay.

AZTMP assay. For the AZTMP assay, the TK reaction step was omitted, and the AZTMP calibrator was diluted in an appropriate diluent. The total reaction volume was 150 μL, and 50 μL of each component—HRP-DSS-AZXMP tracer (450 μg/L HRP), anti-AZTMP antibody (25 μg/L), and AZTMP calibrator [diluted in 0.1 mol/L phosphate buffer (pH 7.4) containing 1 g/L BSA and 1 mL/L Triton X-100]—was added. The incubation, wash, and detection steps were then performed as described for the TK1 assay.

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Reference interval. A total of 100 serum samples from apparently healthy Swedish blood donors were measured in duplicate with the TK ELISA. The samples were
obtained from the Karolinska Hospital blood bank (Stockholm, Sweden).

Clinical samples in method comparison. We tested 78 serum samples from patients with clinically confirmed hematologic diseases (54 with non-Hodgkin lymphoma, 17 with Hodgkin lymphoma, 2 with follicular non-Hodgkin lymphoma, 4 with chronic lymphocytic leukemia, and 1 with multiple myeloma) in parallel and in duplicate with the TK ELISA and the TK REA. The samples were collected in 2002 and obtained from the National Cancer Institute (Budapest, Hungary).

Measurement of TK activity in breast cancer tumor extracts. The TK activity was measured in 24 cytosols from tumor extracts of patients with metastasis-free breast cancer (34).

TK1 assay calibration

This assay combines an enzyme reaction with a competitive immunoassay for the detection of a specifically generated reaction product. In the first step, the TK1 present in patient material converts AZT to AZTMP. The amount of AZTMP generated is directly proportional to the activity of the enzyme present in the sample (Fig. 2).

If the same type and concentration of calibrator is used in the TK ELISA and the TK REA, the enzyme activities would differ in katal by a factor of ~2. This is explained by the great difference in substrate concentrations between the two assays. The concentration of the iododeoxyuridine in the TK REA is 120 nmol/L, which is 0.050 times its $K_m$ value (32), whereas the AZT substrate concentration in the TK ELISA is 93.5 μmol/L, which is 31 times its $K_m$ value (6). The TK ELISA thus works under saturating kinetic conditions, whereas the conversion rate (because of the low substrate concentration) is lower in the TK REA. This was done to maximize the sensitivity of the assay with radioactive substrate.

Cross-reactivity and evaluation of the anti-AZTMP antibodies

The reactivity and specificity of the goat anti-AZTMP antibodies were improved by purification with affinity chromatography and gel filtration. The purification improved the assay imprecision and allowed better batch-to-batch control. The antibodies specifically recognized AZXMP or AZTMP and gave low cross-reactivity with natural nucleotides or nucleosides. The results obtained were similar to those reported earlier for rabbit antibodies (33). Cross-reactivity with thymidine 5′-monophosphate, UMP, and AZT was <0.1%, 0.001%, and 0.0006%, respectively (data not shown).

Cross-determination of TK2 activity

The cross-determination of human recombinant TK2 activity, with AZT as a substrate, compared with hrTK1 was <0.1% measured at a TK concentration of 7 μg/L. In comparison, the corresponding cross-determination of TK2 activity was 4% in the TK REA. The mean (SD) activity of the hrTK1 preparation was determined by the TK ELISA assay to be 2100 (270) nmol · mg⁻¹ · min⁻¹, which is in the range reported previously (31).

Optimization of the assay to eliminate interference

The assay could be sensitive to antibodies in the sample reacting especially with primary goat antibodies. Apart from human anti-mouse antibodies, circulating antibodies...
against immunoglobulins of other species are also frequently present in blood from patients and may interfere with the assay (37). Bovine, goat, and rabbit IgG concentrations were optimized by use of eight sera containing heterophilic antibodies. Significant interference was observed when no protective IgG was added (Table 1). A cocktail containing bovine, goat, and rabbit IgG was the most efficient formulation. No single IgG alone, even at the highest concentrations, was by itself capable of eliminating interference in all samples tested (data not shown). TK ELISA results could also be obtained by eliminating immunoglobulins before the immunodetection step, and these values were used as reference values.

The TK REA does not suffer from interference from immunoglobulins, and the TK REA results were used for comparison. Only one of the eight serum samples showed a very low but detectable TK activity (92 IU/L; 9262 IU/L) when the AZT substrate was omitted, and this was a false-positive signal, of unknown origin, in the TK ELISA assay.

DILUTIONLINEARITY AND ANALYTIC RECOVERY
The assay was linear on dilution down to 0.26 mIU/L; measured values were 88–115% of expected for the six investigated samples (Fig. 4). Dilution of samples in blood donor sera gave comparable results (84–105%). Recovery of TK1 in calibrator diluent was 97–113%.

DETECTION LIMIT AND FUNCTIONAL SENSITIVITY
The detection limit was 78 µIU/L (0.6 U/L), and the intraassay CV was <20% down to 0.13 mIU/L. Acceptable recoveries and linearity on dilutions were observed even as low as 0.26 mIU/L.

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<th>TK ELISA, mIU/L</th>
<th>TK REA U/L</th>
<th>HWM removal</th>
<th>No substrate</th>
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<td>1.86</td>
<td>0.36 (2.8)</td>
<td>0.27 &lt;0.07</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Effect of blockers on heterophilic antibody interference with TK ELISA results.

The TK reaction was performed with the AZT substrate omitted. Interference-free result should be <0.07 mIU/L (below detection limit).

IMPRECISION
Within-assay CVs were 4.1–9.1%, between-run CVs were 3.5–7.4%, and total pooled CVs were 6.0–11%. At activities of 0.90, 1.52, 1.82, 2.56, 2.80, 3.62, and 4.78 mIU/L, the total CVs were 9.8%, 8.0%, 8.6%, 9.1%, 11%, 6.0%, and 6.0%, respectively. The between-operator imprecision (CV) estimate pooled for all seven samples was 1.8%.

HIGH-DOSEROOK EFFECT
With the TK ELISA, we examined 20 sera having TK REA values in the range of 200–4000 U/L. The samples were tested undiluted and diluted 50-fold. All undiluted samples gave lower absorbances at 450 nm (0.10–0.44 absorbance units) than the highest calibrator (0.488 absorbance units), whereas diluted samples gave the expected high activity: 4.33 mIU/L to 0.274 IU/L. Serial dilutions of 0.1 g/L hrTK1 were also analyzed. No high-dose hook was observed (data not shown).

REFERENCE INTERVAL
The cutoff value estimated as the upper 95th percentile was 0.94 mIU/L (7.2 U/L, TK REA), and the median value was 0.43 mIU/L.

METHOD COMPARISON WITH THE TK REA
We conducted a clinical correlation study by testing the TK REA and TK ELISA in parallel, using sera from 78 patients with hematologic diseases. The simple linear regression analysis of TK REA values ranging from 2 to 130 U/L (Fig. 5) gave the following equation: TK ELISA (mIU/L) = 0.109 × TK REA + 0.092 (r = 0.98). The $S_{y|x}$ was 0.476 mIU/L, and the SE of the slope and intercept...
were 0.002 and 0.061 mIU/L, respectively. The narrow range of 2–10 U/L into which 59 samples fell gave TK ELISA (mIU/L) values of 0.118 ± 0.092 mIU/L; r = 0.98; S_y = 0.476 mIU/L; n = 78. An enlargement of the region from 0 to 10 U/L is shown as a scatter plot in B.

Fig. 5. Comparison of the TK-REA and TK ELISA measurements for 78 sera from patients with hematologic diseases.

(A), the equation for the line (y = 0–130 U/L) is: y = 0.109x + 0.092 mIU/L; r = 0.89. For this subset, use of the more appropriate Deming regression model gave: TK ELISA (U/L) = 0.85 × TK REA + 0.5 U/L (for 2–130 U/L) and TK ELISA = 0.98 × TK REA − 0.1 U/L (for 2–10 U/L). TK REA U/L is given here in both assays for an easier numeric comparison. One TK REA U/L corresponds to 0.13 mIU/L in the ELISA.

MEASUREMENT OF TK ACTIVITY IN BREAST CANCER TUMOR EXTRACTS

Aliquots of the samples were, after thawing, immediately tested in the TK ELISA. The median value was 33.5 mIU/mg of protein (or 250 TK mU/mg), and the range was 9.1–84.1 mIU/mg of protein. Median (range) TK REA values on the same samples were 350 and 47–760 mU/mg of protein, respectively. It is clear from these preliminary experiments that the ELISA can be used to measure cytosolic TK1 activities.

Discussion

The TK ELISA described here was designed to provide a reproducible, enzyme-based, nonradiometric method to specifically determine TK1 activity. This was achieved by designing a two-step strategy. In the first step, TK1 present in the patient sample phosphorylates AZT to the corresponding 5'-monophosphate (AZTMP). In the second step, a competitive ELISA measures the amount of AZTMP by use of specific goat antibodies against AZTMP and HRP-labeled AZTMP. Hydrogen peroxide was introduced to stop the TK enzyme and eliminate DTE. DTE or other sulphydryl reagents are necessary for optimum serum TK activity (38), but they interfere strongly with the peroxidase activity.

Here we describe a convenient and easy method to separate the enzyme substrate and the product formed by use of an antibody specific for the 5'-monophosphate product. A crucial point is the choice of substrate and the pyrimidine 5'-monophosphate to which the antibody is directed. An unnatural substrate is preferred because the assay relies on indirect quantification of a nucleotide. In addition, the antibodies would have to be very specific and selectively bind to the product in the presence of large amounts of substrate without exhibiting significant cross-reactivity to endogenous substances. AZT and its monophosphate derivative, AZTMP, are ideal candidates against which antibodies could be raised. Highly specific antibodies against AZT and AZTMP have been generated previously and used in clinical settings to determine the concentrations of these compounds in serum and extracts from tissues and cells (33, 39). More importantly, the substrate specificity of TK1 differs from that of TK2; AZT is much less efficiently phosphorylated by TK2 than TK1 (4, 5). Furthermore, AZTMP constitutes a stable metabolic product in blood, serum, and cell extracts and is not degraded or further phosphorylated at a significant rate (40–42).

In human cells treated with AZT, activation by TK1 is a necessary step, and there is an accumulation of AZTMP because the next step catalyzed by thymidylate kinase (TMPK) is very inefficient (6, 43). Some viral TKs (e.g., varicella zoster and herpes simplex virus type 2) also possess a TMPK activity as part of the same TK protein or have a TMPK enzyme with broader substrate specificity than that of human TMPK (44, 45). AZTMP generated in this manner can be further phosphorylated to form AZT 5'-diphosphate, which does not significantly bind to the anti-AZTMP antibody (33). Evaluation of patients suffering from viral diseases should be initiated to test this hypothesis.

Our polyclonal goat anti-AZTMP antibody was very specific for AZTMP, comparable to previously described rabbit antibodies (33). The affinity purification was intro-
duced to minimize lot-to-lot variations and to control the amount of specific antibodies in the assay. This procedure also improved the overall precision of the assay. Our estimates of cross-determination of TK2 activity (<0.1%) in the TK ELISA and TK REA assay (<4%) agree well with published data on the enzymatic activities and substrate specificities of these enzymes (46, 47). The cross-determination of TK2 activity in the TK ELISA is low enough, at least for serum measurements, to make the assay TK1 specific in clinical applications.

Because the assay is reagent-limited with regard to primary anti-AZTMP antibody, the assay would be especially sensitive to antibodies present in sera from patients that react with the goat antibodies. A cocktail of bovine, rabbit, and goat IgG was found to be effective to quench this effect in all samples tested. Nevertheless, it is still possible that samples containing human anti-mouse antibodies or heterophilic antibodies may cause interference if the concentrations of the interfering antibodies are high enough or if they have a unique specificity. Such cases can be tested by running the TK ELISA with or without filtration through a molecular weight cutoff filter before the detection of AZTMP. Alternatively, the procedure may be modified by replacing the assay buffer with a sample diluent. In the latter case, the determination should be <0.13 mIU/L if there is no interference.

The reproducibility, linearity on dilution, and recovery of the TK ELISA was satisfactory. The dilution behavior and recoveries of serum TK1 parallel those of calibrator TK1 in diluent prepared from HeLa cells. The detection limit (<130 μIU/L) is low enough to allow measurement of the TK activity present in sera from all individuals. The precision of the assay is also adequate for clinical management. The assay method can be converted to a fully automated system with some modifications (48), thus allowing automated determinations. Contrary to the TK REA assay, the ELISA assay can easily be standardized by calibration against AZTMP and use of a fixed conversion rate between IU/L and AZTMP formed in the assay, which is another major advantage of the new method.

A series of recent publications describe the production and use of antibodies against TK1 (23–29). The authors of these reports have reported several interesting results, but there are some fundamental differences and problems with immunology-based measurements of TK1. One general concern is that the sensitivities of antibody-based assays are usually lower than those of assays based on enzyme activity because there is considerable amplification of the signal during the enzymatic reaction. Therefore, it is probably unlikely that an immunologic assay can accurately determine, by a method suitable for routine use, the low concentrations of serum TK1 protein found in healthy individuals.

A second major problem is that the antibodies may react with both biologically active and inactive or degraded TK1 present in serum. Furthermore, modifications of serum TK1, such as those described earlier, will most likely alter the reactivity with different TK1 antibodies. It is known that TK1 is found in serum in a stable multimeric form (38), probably originating from disrupted proliferating tumor cells. It is possible that several epitopes will be masked in this protein conformation and that determinations using agents that bind to the TK1 protein or peptides derived from TK1 may thus give information other than direct activity measurements (23, 29).

The clinical correlation study in which we tested the TK REA and TK ELISA in parallel with sera from patients suffering from hematologic diseases showed high correlation and good agreement between the methods. Differences between the two assays are expected in certain cases, as discussed above. When we randomly screened 50 sera from patients with unknown diseases in the two assays, 5 samples gave significantly different results between the assays. Three of these samples were above the cutoff in the TK ELISA but were below the cutoff in the TK REA. We confirmed that only enzymatic activity was measured in the TK ELISA for these three samples because the results were below the detection limit in the ELISA when the AZT substrate was omitted. These results warrant further studies to clarify the mechanism(s) responsible for the discrepancy.

This is, to our knowledge, the first nonradiometric assay able to accurately measure serum TK1 activity in healthy individuals and that could be automated for large-scale determinations. This assay may enable full exploitation of serum TK1 as a tumor marker.

In non-Hodgkin lymphoma, serum TK activity correlates with clinical staging and provides important prognostic information on (progression-free) survival. It can also be used to monitor treatment and detect recurrent disease (15). Serum TK determinations in patients with multiple myeloma differentiate between stable or progressive disease and may guide the treatment decision (16, 17). Recent results indicate that serum TK concentrations seem to distinguish between progressive and indolent disease in early-stage chronic lymphocytic leukemia (18, 19). Initial serum TK concentrations reflect the proliferative activity and mass of leukemic cells. Several studies have shown that pretherapeutic serum concentrations predict the probability of achieving complete remission and survival (17).

Measurement of TK activity in breast cancer tumor extracts (cytosols) has been reported for 908 primary breast cancer patients, and it was concluded that high TK values were an independent factor for metastasis-free survival (20). In another recent study with 1692 patients with primary breast cancer, high TK values were shown to be an important risk factor in node-negative patients and seemed to be associated with beneficial effect of adjuvant chemotherapy (21, 22). The development of specific inhibitors of human TK1 activity to be used in combination with inhibitors of de novo pyrimidine synthesis is a major challenge to improve the efficiency of
chemotherapy (49). Measurement of TK1 activity could be a critical issue in this area.

In conclusion, preliminary results show that the TK ELISA can be used for cytosol measurements. A high AZT concentration probably makes the assay robust against interfering factors, and UMP was included to protect AZTMP from enzymatic degradation. Further studies with the ELISA and cytosol preparations may provide results of value in cancer diagnostics. Furthermore, it appears that the TK ELISA is a technically accurate and reliable method to determine TK activity in sera from patients with hematologic malignancies. Therefore, the TK ELISA could be of value in the clinical setting for the management of patients with cancers.

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