Optimized Assay for Thymidine Kinase and Its Application to the Detection of Antibodies Against Herpes Simplex Virus Type 1- and 2-Induced Thymidine Kinase

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An optimized assay for herpes simplex virus type 1- and 2-induced deoxythymidine kinase (dTK) is described which used [125 I]iododeoxyuridine (IUdR) as a substrate. Values for K_m and V_{max} were determined for both viral and cellular dTK, using either deoxythymidine or IUdR as a substrate. A comparison between the two substrates revealed that higher reaction velocities and lower K_m values were obtained with IUdR. A standard assay was designed which uses 10^{-7} M IUdR as a substrate. This assay can detect herpes simplex-induced dTK from as few as two infected cells and is several orders of magnitude more sensitive than conventional dTK assays which use 10^{-5} M dT as a substrate. The assay was also used for detection of antibodies capable of blocking viral dTK. An easily detectable blocking activity, which was shown to be mainly confined to the immunoglobulin G antibody class, was found in most human sera which were positive for complement-fixing antibody against herpes simplex virus.

Since Kit and Dubbs in 1963 (9) reported elevated levels of deoxythymidine kinase (dTK) activity in herpes simplex virus (HSV)-infected cells, the virus induced pyrimidine kinases have been intensively studied. Induction of HSV dTK activity has been made in dTK-deficient mouse fibroblasts and also through transfer of restriction enzyme fragments of HSV deoxyribonucleic acid (16, 23). Cell-free synthesis of HSV-specific dTK has also been achieved (18), and chain termination mutants in the HSV gene have been isolated (21). The genes for HSV type 1 (HSV-1) and HSV-2-induced dTK are thus considered to be located on the viral genome. HSV-induced dTK differs in many properties from host cell dTK; the enzymes are immunologically distinguishable (13) and have different molecular weights (12, 17) as well as different electrophoretic mobilities (16). They also differ in substrate specificity (1, 5, 6, 14), in feedback inhibition by deoxythymidine triphosphate, and in preference for phosphate donors (for a review, see Kit et al. [10]). The presence of HSV-specific dTK activity is now widely used as a marker for HSV infection or transformation. The dTK activities induced by HSV-1 and -2 can be distinguished by their immunological properties, their sedimentation coefficients and their different affinities for certain substrate analogs (1, 12, 22). Several assays for HSV dTK have been reported, most of which use 3 H- or 14 C-labeled deoxythymidine (dT) as a substrate at concentrations of about 10^{-5} M. A different assay was described by Dobersen and Greer (2), using 32 P-

labeled adenosine triphosphate as a phosphate donor instead of radioactively labeled substrate. The assay was designed to study dT analogs and has the disadvantage that it requires a purified enzyme to avoid interference from other phosphotransferases. Another assay, which uses [125 I]iododeoxycytidine as substrate and shows a strong preference for HSV dTK, was recently reported by Summers and Summers (20).

Antisera prepared in animals against HSV-1 and -2 have been reported to contain antibodies against the viral dTK. Rawls et al. (19) screened a large number of sera from patients with cancer in the cervix uteri for antibodies capable of blocking HSV dTK. Although a large fraction of the sera contained complement-fixing (CF) antibodies against HSV, very few were found to be positive for blocking of HSV dTK. This was probably due to the fact that conventional dTK assays used too much enzyme to allow the detection of small amounts of dTK-blocking antibodies in human sera. The current investigation was undertaken to design a more sensitive assay for HSV dTK which also could be used to detect antibodies blocking the viral enzyme activity.

MATERIALS AND METHODS

Cells and media. BHK-21/13S cells were grown in suspension cultures, with density limits of 1×10^5 to 2×10^6 cells per ml. Eagle minimal essential medium was modified by using double concentrations of amino acids and vitamins and by adding 0.1 mg of FeNO_3 per liter. The CaCl_2 was replaced with KCl, and 10% calf serum and 10% tryptose phosphate broth were added

for cell growth. For production of virus stocks and enzyme, only modified minimal essential medium was used.

Sera. Human sera were obtained from the clinical virus diagnostic laboratory in our department. Specific rabbit antisera against HSV dTK were a gift from W. C. Summers, Yale University, New Haven, Conn. The rabbit serum against HSV-1 dTK was prepared against a purified enzyme preparation obtained from cells infected with strain KOS. The HSV-2 rabbit serum was raised against rabbit kidney cells infected with HSV-2 strain 409 X:1. Pooled human immunoglobulins were purchased from Kabi (Stockholm, Sweden).

Virus. The HSV strains were isolated in the clinical virus diagnostic laboratory of our department. They had all been passaged about 10 times. The isolates were typed by Stig Jeansson (Gothenburg) by the immunoelectrophoretic method (7) and by an iododeoxythymidine [IUdR] sensitivity assay (J. S. Gronowitz, manuscript in preparation). In all cases the results were supported by the clinical symptoms of the patients from which the strains were isolated. Most results presented here were obtained with the isolate C42, which is a type 1 strain from a patient with conjunctivitis, and C168, which is a type 2 strain isolated from the vulva. Many experiments were checked with at least three strains of each type.

Enzyme preparation. Spinner cultures were infected with 2 to 50% cytopathic effect (CPE₅₀) per cell, depending on virus strain. The infected cells were harvested 11 to 16 h after infection. Control preparations were made from uninfected BHK cells in the same proliferation stage. The cells were collected by centrifugation, washed with phosphate-buffered saline, and suspended at a density of 5×10^8 cell/ml in stabilizing buffer (Table 1). The cells were then disrupted by sonication for 1 min. Cell debris was removed by centrifugation at 3,600 rpm for 30 min. Finally, the suspension was centrifuged for 2 h at 100,000 $\times g$. The supernatant was collected, diluted, and frozen at -70°C in equal portions. Enzyme quantities given in the text are related to the number of cells from which the enzyme was derived. Dilutions were made in stabilizing buffer and refer to dilutions made from a solution containing 5×10^8 cell equivalents per ml. Protein was assayed according to the method of Lowry et al. (15), and 5×10^8 cells were found to be equivalent to 2 to 5 mg of protein.

Enzyme assay. The assay was performed by mixing 5 μl of the desired enzyme dilution with 35 μl of stabilizing buffer (spacer volume). The mixture was then prewarmed at 37°C for 2 min, and the enzyme reaction was started by addition of 14 μl of assay mixture and 7 μl of substrate solution (see Table 1). Duplicate samples (25 μl) were taken after different incubation times. The enzyme reaction was terminated by spotting the sample on a 1-cm² piece of Whatman DEAE-81 paper kept at 90 to 100°C . The paper was washed four times in 1 mM ammonium formate, once in distilled water, and finally in methanol. [³H]dT-containing samples were dried and counted in Omnifluor (New England Nuclear Corp., Boston, Mass.). Samples containing [¹²⁵I]IUdR were counted in an automatic gamma counter. Isotopes were obtained from New England Nuclear.

Antibody assay. The spacer volume (35 μl) in the enzyme assay was replaced with 25 μl of antibody dilution in stabilizing buffer. Ten microliters of guinea pig serum, diluted 1:5 in stabilizing buffer, was added as a complement source. The mixture of antibody, complement, and appropriate enzyme dilution was incubated for 60 min at 37°C at a final IUdR concentration of 10^{-7} M. The residual enzyme activity was determined after 30 min as described above. Control samples without enzyme or antibody were treated in the same manner.

Polyacrylamide gel electrophoresis. We used the method described by Kit et al. (11) with the following modifications. Thymidine was omitted from all buffers. Electrophoresis was performed in 3-mm-thick slab gels, and the samples were layered directly on top of the gel in a buffer containing 0.06 M Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 6.8, and 15% sucrose. After electrophoresis for 120 min, the gel was divided into two parts with a steel wire.

The positions of the enzymes were determined by the use of 3-mm-wide Whatman DEAE-81 paper strips moistened with reaction mixture. The gels with paper strips were incubated for 10 min at 37°C when [³H]dT or [¹²⁵I]IUdR was used as a substrate and for 20 min when [³H]deoxycytidine (dCyd) was used. Then the strips were washed, dried, cut into 2-mm pieces, and counted as described above. The relative fraction of migration (R_f) value was calculated with bromophenol blue as a reference.

Serum fractionation. Gel filtration was performed in a Sephacryl 300 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) column (2.6 by 45 cm). The column was eluted with a 0.05 M Tris-hydrochloride buffer, pH 8.0, containing 0.5 M NaCl and 0.02% Na₂S₂O₅. The flow rate was 24 ml/h, and 2-ml fractions were collected. Samples of 200 to 500 μl of serum were fractionated with a small amount of ¹²⁵I-labeled sheep anti-rabbit immunoglobulin G (IgG) serum to indicate immunoglobulin-containing fractions. This tracer was shown in a separate experiment to contain mainly material which eluted in the same position as human IgG. The protein content of each fraction was determined spectrophotometrically at 280 nm.

The IgG fraction was adsorbed with protein A-Sepharose (Pharmacia Fine Chemicals). A 215- μl amount of gel suspension containing 2 mg of gel (binding capacity, 1 mg of IgG/mg of gel) was used with 215 μl of serum diluted 1:2. All components were prepared in stabilizing buffer without albumin. The mixture was incubated for 60 min at 37°C ; the sample was then centrifuged at low speed, and the supernatant was collected. The gel was washed three times with a modified stabilizing buffer without albumin which contained 0.05 M Tris-hydrochloride, pH 7.6. To recover bound IgG, the gel was mixed with 215 μl of 0.1 M glycine buffer, pH 2.3, and after 5 min the supernatant was removed and mixed with 40 μl of Tris-hydrochloride buffer, pH 9.3, to restore the pH.

RESULTS

Fractionation of dTK activities by slab gel electrophoresis. Extracts prepared from

uninfected and HSV-infected BHK cells were fractionated by polyacrylamide slab gel electrophoresis as described in Materials and Methods. After separation, the gel was divided in two halves, which were assayed separately with dT or IUdR as substrate. Figure 1A shows the results with C168, an HSV-2 strain. Two peaks

with R_f values of 0.18 and 0.45 were apparent with coinciding IUdR- and dT-phosphorylating activities. The peak with $R_f = 0.18$ was also present in extracts from uninfected BHK cells (Fig. 1B), indicating that this activity was of host cell origin, whereas the peak with $R_f = 0.45$ was exclusively present in extracts from HSV-

TABLE 1. Concentration and composition of buffers used in the dTK assay^a

Component	Stabilizing buffer	Assay mixture	Final concn
Tris-maleate		700 mM	160 mM
Tris-hydrochloride	250 mM		160 mM
MgCl ₂	2 mM	70 mM	17 mM
KCl		88 mM	20 mM
NaF		10 mM	2.3 mM
Adenosine triphosphate	2 mM	35 mM	9.3 mM
Dithiothreitol	3 mM	3 mM	2.7 mM
Glycerol	10% (wt/vol)		6.6% (wt/vol)
Bovine serum albumin	0.5 mg/ml		0.33 mg/ml
pH	7.5	6.5	7.3

^a In the dTK assay, 35 μ l of stabilizing buffer was mixed with 14 μ l of assay mixture and 7 μ l of substrate solution with the appropriate concentration. Five microliters of enzyme solution, diluted in stabilizing buffer, was also added, and the final volume was 61 μ l.

FIG. 1. Electrophoretic separation of dTK activities from uninfected and HSV-2 (strain C168)-infected BHK cells. Extracts were prepared as described in the text and analyzed by polyacrylamide slab gel electrophoresis. The gels were analyzed for dTK activity by using different substrates. (A) Extract from 2×10^5 HSV-2-infected cells. The gel was assayed for dTK activity with [125 I]IUdR (O) or [3 H]dT (X) as substrate. (B) Extract from 10^6 uninfected cells. [125 I]IUdR (O) or [3 H]dT (X) was used as substrate in the dTK assay. (C) Extract from 10^6 HSV-2-infected cells. [125 I]IUdR (O) or [3 H]dCyd (Δ) was used as substrate in the dTK assay. (D) Extract from 5×10^4 HSV-2-infected cells. The gel was assayed with [125 I]IUdR (O) or with mixtures of [125 I]IUdR and unlabeled dT. Mixtures containing 3.1×10^{-7} M IUdR and 6.9×10^{-6} M dT (●) or 3.1×10^{-7} M IUdR and 2.7×10^{-5} M dT ($\blacktriangle$) were used.

infected cells. To confirm the viral origin of the latter peak, we also assayed the gel with dCyd as a substrate (Fig. 1C). This substrate cannot be phosphorylated by dTK from the cytosol of uninfected cells (6, 14). The results revealed a prominent peak of dCyd-phosphorylating activity with $R_f = 0.45$, whereas no activity was demonstrable with $R_f = 0.18$ when this substrate was used. A small peak of dCyd-phosphorylating activity was also detectable at $R_f = 0.75$. This is the expected position of the mitochondrial dTK which has been described by others (11, 14). In the experiments described in Fig. 1A and 1C, no dT- or IUdR-phosphorylating activities were present at $R_f = 0.75$. This was because too little enzyme was used (Fig. 1A) or because the IUdR substrate had too low specific activity (Fig. 1C). Both dT- and IUdR-phosphorylating activities were clearly present at this position under other experimental conditions (data not shown). Experiments similar to those described above were performed with preparations obtained with three HSV-1 strains and two additional HSV-2 strains. In all cases, a prominent activity with an R_f value different from that of host cell dTK was present which could phosphorylate IUdR as well as dT.

To show that the IUdR-phosphorylating activity could be inhibited with dT, we also assayed electrophoretic runs with different mixtures of labeled IUdR and unlabeled dT (Fig. 1D). At a dT concentration of 2.7×10^{-5} M, both the cellular and viral enzyme activities were undetectable (Fig. 1D).

Comparison between dT and IUdR as substrates for viral and cellular dTK. Unfractionated extracts from cells infected with HSV-1 or -2 were used as a source of viral enzyme in these studies. This was possible since the electropherograms showed that the host dTK constitutes a very small fraction of the dTK activity in HSV-infected cells. The reaction velocities for cellular and HSV dTK with dT and IUdR as substrates were determined at seven different substrate concentrations, ranging between 7×10^{-8} and 2×10^{-6} M. Higher concentrations were avoided due to expected substrate inhibition (8). At each concentration, duplicate samples were withdrawn at time zero and at five other times. In assays with IUdR, the viral enzyme preparations were used at a 1:10,000 dilution and the host enzyme was used at a 1:200 dilution. In assays using dT as a substrate, the corresponding dilutions were 1:800 and 1:30. All experimental data were computer processed, with the initial velocities calculated as moles of product formed per minute and enzyme equivalent from 10^3 cells. To obtain Menten plots, the experimental data were ana-

lyzed by using a computer program based on Hanes' method for analysis of enzyme kinetics (Fig. 2). Each curve was computed from two or more experiments. Both HSV-1 dTK and host cell dTK gave higher turnover numbers when IUdR was used as a substrate instead of dT (Fig. 2). Likewise, the HSV-2 dTK gave a higher turnover number with IUdR as a substrate, albeit only at concentrations below 4.5×10^{-7} M. These results indicate that IUdR should be a useful substrate to investigate HSV dTK activities, and it should provide improved sensitivity. The Michaelis constant (K_m) and saturation velocities (V_{sat}) calculated from the data in Fig. 2 are summarized in Table 2. We would like to emphasize that the values obtained with different strains of HSV are not comparable even when the same type is used, since it has been shown that different strains of HSV induce varying amounts of dTK per cell (4). V_{sat} values obtained for different substrates may thus only be compared when preparations from the same strain are used. K_m values similar to those in Table 2 were obtained by using at least two more strains of each serotype (data not shown).

As can be seen from the Menten curves (Fig. 2), an approximately twofold increase in real velocity was obtained when IUdR was used as a substrate instead of dT. The main advantage, however, to using [125 I]IUdR instead of [3 H]dT in the dTK assay is the considerably better counting efficiency that can be obtained with a 125 I-labeled substrate. The counting efficiency was estimated to be 5.3% for [3 H]dT under our experimental conditions and 62% for [125 I]IUdR. Figure 3 shows the difference in sensitivity of the dTK assay when [125 I]IUdR and [3 H]dT were used as substrates. For comparison, the values are calculated for equivalent amounts of enzyme (10^2 cells/sample) and isotope (0.32 μ Ci/sample). The amount of incorporated label increased with diminishing substrate concentrations when IUdR was used. By using equivalent assay conditions with [3 H]dT as a substrate, very small amounts of incorporated label were obtained due to the low counting efficiency of 3 H-labeled substrate. To demonstrate that low substrate concentrations also gave a similar benefit when dT was used as a substrate, Fig. 3 also shows results obtained with 12.5 times more enzyme. Similar results were obtained when HSV-2-infected cells were used as a source of enzyme (data not shown).

To design a reproducible assay capable of detecting small amounts of enzyme, we decided to use a substrate concentration of 10^{-7} M for routine assays (Table 3). Lower substrate concentrations may improve the sensitivity but gave irreproducible results due to the poorly defined

FIG. 2. Menten plots, using IUDR of dT as substrate for the TK isoenzymes. (A) Extract from HSV-1 (strain C42)-infected cells. (B) Extract from HSV-2 (strain C168)-infected cells. (C) BHK cytosol. Velocities are given as moles of product formed per minute and enzyme amount derived from 10^3 cells. Symbols: (○) IUDR; (+) dT.

concentration of commercially available undiluted [125 I]IUDR (about 10^{-8} M). In Fig. 4, the amounts of phosphorylated substrate obtained during a 30-min assay at a substrate concentration of 10^{-7} M, using 0.32 μ Ci of [125 I]IUDR or [3 H]dT per sample, are given as a function of enzyme amount. The nonlinear shape of the

curve at high enzyme concentrations is due to substrate depletion during the assay. The results show that it is possible to measure dTK activity from as few as 10 to 20 HSV-infected cells. In the optimized assay with [3 H]dT as a substrate, approximately 30 times more enzyme was required to obtain equal amounts of incorporated

TABLE 2. K_m values and saturation velocity (V_{sat}) obtained with enzyme preparations from HSV-infected and uninfected cells, using dT and IUdR as substrates

Source of enzyme	K_m ($\times 10^7$ M) ^a		V_{sat} ^b	
	dT	IUdR	dT	IUdR
Uninfected BHK cells	18.9 $\pm$ 2.7	11.3 $\pm$ 1.1	1.62 $\pm$ 0.17	1.69 $\pm$ 0.26
HSV-1 (strain C42)-infected cells	4.6 $\pm$ 0.27	2.8 $\pm$ 0.63	11.1 $\pm$ 0.33	17.6 $\pm$ 2.0
HSV-2 (strain C168)-infected cells	6.6 $\pm$ 0.42	1.9 $\pm$ 0.26	16.4 $\pm$ 0.60	9.5 $\pm$ 0.51

^a Confidence limits are calculated at the 90% level.
^b Expressed as moles $\times 10^{14}$ per minute and amount derived from 10^3 cells.

FIG. 3. Correlation between substrate concentration and incorporation in the dTK assay, using an extract for HSV-1 (strain C42) as the source of enzyme. Each sample contained 0.32 μ Ci of isotope, and the values for incorporation of label refer to a 10-min assay period. Symbols: (O) [125 I]IUdR used as a substrate with enzyme from 10^2 HSV-infected cells. (x) [3 H]dT used as a substrate with enzyme from 10^2 HSV-infected cells. (Δ) [3 H]dT used as a substrate with enzyme from 1.3×10^3 cells.

label. With cells infected with the type 2 strain C168 as a source of enzyme, the difference in enzyme requirement for the two substrates was slightly smaller (data not shown).

Contribution of cellular dTK activity in HSV-1 and -2 dTK preparations. The experimental data also show another advantage of using low concentrations of IUdR as a substrate to assay impure enzyme preparations of HSV-1 and -2 dTK. Table 4 gives a comparison between the reaction velocities at different substrate concentrations when extracts from HSV- and mock-infected cells were used. With decreasing substrate concentrations, the contribution from the cellular enzymes decreased (Table 4). The estimated contribution of host cell dTK activity at different IUdR concentrations was, of course, only valid if cellular dTK was not induced as a result of HSV infection. However, such induction of host dTK did not seem to occur since our electropherograms showed no increase in dTK activity with $R_f = 0.18$ when mock-infected and

TABLE 3. Typical results obtained in an assay of HSV dTK, using a final concentration of 10^{-7} M IUdR^a

Assay time (min)	cpm ^b
0	1,514 $\pm$ 111 ^c
3	4,230 $\pm$ 10
6	6,821 $\pm$ 271
9	9,796 $\pm$ 353
12	14,426 $\pm$ 19
20	19,908 $\pm$ 1,324

^a Extracts from 10^2 cells infected with HSV 2 (strain C168) were used as source of enzyme in each sample.
^b Each sample contained 590,000 cpm of [125 I]IUdR.
^c Duplicate samples were assayed at each time.

HSV-infected preparations were compared. On the contrary, there was an indication that the host dTK decreased as a result of HSV infection (data not shown). Also, from the antibody blocking experiments (see below), there was no indication of an increase in host dTK activity during HSV infection.

Detection of antibodies that block HSV dTK activity. To assay for dTK-blocking antibodies, crude extracts from 10^2 HSV-1 (strain C42)- or -2 (strain C148)-infected cells were used per sample as a source of enzyme. The enzyme preparation was preincubated with different dilutions of antiserum for 1 h before the dTK activity was measured in a 30-min assay, using 0.2 to 0.4 μ Ci of [125 I]IUdR at a final concentration of 10^{-7} M. These experimental conditions were chosen since uninhibited samples gave an incorporation of 15,000 to 30,000 cpm (Fig. 4) with both types of extracts, whereas the back-

ground incorporation in assays without added enzyme was less than 10%. Furthermore, less than 10% of the substrate turned over during the assay, which eliminates the risk of substrate depletion, a factor which otherwise could mask the presence of blocking antibody.

Sera from 76 patients, collected for different diagnostic purposes during a 2-week period, were assayed for dTK-blocking activity and CF titers against HSV. Extracts from both HSV-1- and HSV-2-infected cells were separately used in this assay. Forty-three sera were CF positive, and all of these except five contained dTK-blocking ac-

FIG. 4. Sensitivity of the dTK when [125 I]IUdR and [3 H]dT were used as substrates. Extracts from different amounts of HSV-1 (strain C42)-infected cells were used in a 30-min assay; 10^{-7} M IUdR (O) or dT (x) was used as a substrate, and 0.32 μ Ci of isotope was used in each sample.

TABLE 4. Comparison between IUdR-phosphorylating activity obtained with preparations from uninfected BHK cells and BHK cells infected with HSV-1 (strain C42) and HSV-2 (strain C168)

Type of dTK activity	Turnover ^a at given IUdR concn ($\times 10^7$ M)					
	12.5	6.25	3.125	2.083	1.563	1.042
HSV-1	134	129	92.8	76.2	53.6	41.4
HSV-2	64.4	60.8	46.1	35.4	31.7	24.8
BHK-2	8.91	6.32	3.37	2.46	1.90	1.29
BHK/HSV-1 (%)	6.6	4.9	3.6	3.2	3.6	3.1
BHK/HSV-2 (%)	13.8	10.4	7.3	6.9	6.0	5.2

^a Expressed as moles $\times 10^{15}$ per minute and enzyme amount derived from 10^3 cells.

tivity. (Less than 30% blocking activity by a 1:2 serum dilution was considered negative due to the experimental error of the assay.) The correlation between dTK-blocking activity and serum dilution is shown in Fig. 5. Preliminary results indicate that the antibodies against HSV dTK appear later after infection than the CF antibodies (J. S. Gronowitz and C. F. R. Källander, manuscript in preparation), which could explain the findings of five CF-positive but dTK antibody-negative sera. None of the CF-negative sera was found to have dTK-blocking ability. To demonstrate the specificity of the dTK inhibition assay, some sera that were positive for inhibition of dTK from HSV-infected cells were also assayed against extracts from uninfected BHK cells. None of the tested sera showed detectable inhibition of cellular dTK activity.

To assess the degree of immunological cross-reactivity between dTK from HSV-1 and HSV-2, we also compared the blocking ability of different positive sera against dTK preparations from the two types of HSV. The blocking titer of a serum was defined as the reciprocal dilution that was capable of blocking 30% of the dTK activity when 100 cell equivalents were used per sample (15,000 to 30,000 cpm incorporated in a 30-min assay). The results showed a great variation in the degree of cross-reactivity. Certain human sera which were blocking one type of

dTK activity even at high dilutions were unable to neutralize the heterologous dTK (Fig. 5C, D), whereas other sera showed only a weak preference for one type of dTK activity. The observed cross-reactivity in certain sera could have been due to the fact that many patients were immunized with both HSV-1 and HSV-2. Therefore, we also analyzed two type-specific rabbit antisera. The results showed that the rabbit sera gave a clear cross-reaction, although four to eight times more antibody was required to block the heterologous dTK activity.

To demonstrate that the blocking activity in the positive sera was confined to the immunoglobulin fraction, selected antisera were fractionated before being used in the dTK inhibition assay. Two methods were used for serum fractionation; three sera which were positive in the dTK inhibition assay were fractionated by exclusion chromatography on Sephacryl 300, using ^{125}I -labeled rabbit IgG as a marker. The peak of the dTK-blocking activity coincided with the peak of the IgG fraction, and no peak of dTK-blocking activity was found in other fractions from the column (Fig. 6). Serum fractionation was also carried out by affinity chromatography on protein A-Sepharose. After incubation of positive sera with the protein A-Sepharose, the blocking activity was reduced or eliminated, confirming that the blocking factor was an antibody

FIG. 5. dTK-blocking efficiency of different antisera. Extracts from 10^2 HSV-1 (strain C42) (○)- and HSV-2 (strain C48) (●)-infected cells were used as the source of enzyme. (A) Rabbit antiserum against HSV-1. (B) Rabbit antiserum against HSV-2. (C) Human serum with a CF titer of 1:8 against HSV. (D) Another human serum with a CF titer of 1:8 against HSV.

FIG. 6. Fractionation of an HSV CF-positive serum by exclusion chromatography on Sephacryl 300. Serum (200 μ l) was applied to the column, and the protein contents of individual fractions were determined spectrophotometrically at 280 nm ($\circ$). A small amount of 125 I-labeled IgG was added as a tracer (Δ). Individual fractions were assayed for dTK-blocking activity by mixing 25 μ l with an extract from 10^2 HSV-1 (strain C42)-infected cells. The residual dTK activity was determined in a 30-min assay ($\bullet$).

since the protein A-Sepharose is known to specifically adsorb IgG (Table 5). It was furthermore possible to elute most of the blocking activity from the protein A-Sepharose by acid treatment, which supports the notion that antibodies of the IgG class were responsible for most of the blocking activity in the studied sera. The small amount of residual blocking activity which sometimes remained in the sera after protein A adsorption (Table 5) was probably due to blocking antibodies of types which failed to adsorb to protein A.

DISCUSSION

This study describes a sensitive assay for HSV dTK using [125 I]IuDR as a substrate. IuDR, an analog of dT, has previously been shown to compete with the turnover of dT when used as a substrate for HSV dTK (1). Phosphorylation of IuDR by HSV dTK has also been demonstrated before (3). In our electrophoretic separations of crude extracts from uninfected or HSV-infected cells, we were unable to detect any activity which phosphorylated IuDR but not dT. The peaks of IuDR- and dT-phosphorylating activities coincided, and their electrophoretic mobilities were similar to those described by others for viral and cellular dTK (1, 14, 16). We have also shown that the IuDR-phosphorylating activity can be blocked completely with unlabeled dT. We furthermore found that some HSV CF antisera were able to block more than 90% of the enzyme activity (Fig. 5). The residual activity was of the order expected if cellular dTK is present to the same

TABLE 5. Distribution of dTK-blocking activity in different serum fractions after affinity chromatography with protein A-Sepharose

Serum	Reciprocal blocking titer ^a		
	Unfractionated serum	Absorbed ^b fraction	Supernatant fraction
P 1403	32-64	16	ND ^c
P 1753	256	128	32
P 1769	64-128	32	ND
P 1776	128-256	64-128	32
P 1710 ^d	ND	ND	ND

^a Reciprocal serum dilution that blocks 30% of the dTK activity in a standard dTK assay. The values are calculated from titration curves (see Fig. 4).

^b Eluted from the protein A-Sepharose with acid treatment before titration.

^c No detectable blocking activity.

^d Control serum without enzyme-blocking ability.

extent in infected cultures as in mock-infected cultures. Therefore, on the basis of these results, we believe that the same enzyme activity is measured when IuDR instead of dT is used as a substrate in the dTK assay with crude preparations. If any additional IuDR-phosphorylating activity was present in our unfractionated preparations, it was not detected under the experimental conditions used.

In the HSV dTK antibody assay, we used enzyme amounts equivalent to 10^2 HSV-infected cells per sample (approximately 5×10^{-4} μ g of protein). We used this amount of enzyme in order to maintain the background values below 10% of the counts obtained in a 30-min assay. For significant detection of enzyme, only 10^{-4}

μg of protein is required under our standard assay conditions. For special purposes, it is possible to increase the specific activity of the substrate approximately 10-fold and to prolong the assay time. In this way, the sensitivity can be improved to allow the detection of dTK activity from as little as two infected cells (10^{-5} μg of protein). Conventional methods using 10^{-5} M [^3H]dT require at least 0.5 μg of protein for detection.

The high sensitivity of our dTK assay should be useful to study HSV dTK, which is a widely used marker for HSV transformation (23). The assay combined with other analytical methods such as electrophoresis and antibody inhibition assays should be sensitive enough to easily allow the detection and characterization of small amounts HSV dTK in experimentally transformed cells or possibly in human tumors that might result from HSV infection. By use of the optimized dTK assay, we have been able to design a sensitive antibody assay that detects dTK-blocking antibodies in human sera. By serum fractionation, it was possible to establish that immunoglobulins are responsible for the enzyme-blocking effect. In contrast to earlier reports which have described very few sera with partial blocking capacity, we could detect dTK-blocking activity in many HSV-positive sera and also in commercially available human IgG preparations with titers of up to 1:2,048. The use of the HSV dTK antibody assay for differential diagnosis of HSV-1 and -2 infections as well as the clinical importance of HSV dTK antibodies will be discussed in a separate communication.

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LITERATURE CITED

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