Herpesvirus Type 2-induced Thymidine Kinase and Carcinoma of the Cervix

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SUMMARY

Sera from patients with cervical cancer, matched control women, patients with cancers of other sites, and a group of laboratory personnel were examined for neutralizing activity of thymidine kinase induced by herpesvirus type 2. Inhibition of enzyme activity was found with some of the sera; however, it appeared to be more related to the patients’ past experience with herpesvirus type 2 than with the presence or absence of cervical cancer. Thymidine kinase extracted from cervical cancer cells was found to migrate on polyacrylamide gel electrophoresis in a pattern similar to those of the enzymes induced in cells by herpesvirus types 1 or 2. The enzyme of the cancer tissue, however, did not appear to be antigenically related to the virus-induced enzymes.

INTRODUCTION

An etiological relation between herpesvirus type 2 and carcinoma of the cervix has been proposed. Seroepidemiological studies have suggested a greater occurrence of past infections with the virus among cancer patients than among controls (24), and women with laboratory-proven herpes genitalis appear at greater risk of developing cervical neoplastic disease than women without known genital infection with the virus (20). Cells in vitro have been transformed by herpesvirus type 2 (23). In addition, evidence indicating the presence of herpesvirus type 2 DNA and proteins in the human cancer cells has been reported (8, 13, 27).

Recently, antibodies to herpesvirus non-virion antigens have been found in sera of patients with cervical cancer (4, 12, 28) as well as in patients with cancers at certain other sites (12, 28). The functions of the antigens to which these antibodies react have not been determined. One protein induced in cells infected with the herpesviruses is TdR kinase (14, 16). The TdR kinase induced by herpesvirus type 2 is specific for the virus (31). The present study was undertaken to evaluate the possibility that herpesvirus type 2 TdR kinase was present in cervical cancer cells and that patients with cervical cancer had antibodies to the enzyme.

MATERIALS AND METHODS

Sera. Sera were obtained from 30 women with invasive carcinoma of the cervix and from 27 matched control women. These samples were obtained from women of the lower socioeconomic level of Houston, Texas; the criteria for selecting matched controls have been previously described (1, 2). Sera from 18 patients with cancers of sites other than the genital tract (collected by Dr. A. Lipton) and 57 sera from laboratory personnel in the Department of Microbiology were collected at the Milton S. Hershey Medical Center, Hershey, Pa. The latter 2 groups were primarily Caucasian and of the middle socioeconomic level. Antiserum to TdR kinase induced by herpesvirus type 1 or herpesvirus type 2 were provided through courtesy of Dr. M. E. Thouless, Dr. D. H. Watson, and Dr. P. Wildy (16, 31, 34).

Cancer Tissue. Punch biopsies were obtained from cervical lesions of 9 patients and were kindly provided through the efforts of Dr. Alan Kaplan. All specimens were histologically proven invasive squamous cell carcinoma, and none of the patients had received therapy prior to obtaining the specimens.

Enzyme Preparation. The enzyme was prepared from cervical biopsies by homogenizing the tissue in 5 volumes of 0.05 M Tris-HCl (pH 7.5), 0.001 M EDTA, and 0.005 M β-mercaptoethanol in a ground-glass tissue grinder. The homogenate was centrifuged at 15,000 rpm for 1 hr in a Beckman Model 50 rotor. The supernatant was removed and mixed with an equal volume of 0.25 M sucrose with 0.005 M β-mercaptoethanol and stored at -70°.

For assessment of inhibition of type 2 TdR kinase by human sera, enzyme induced by strain 316 D of herpesvirus type 2 was used. Infection and cell extract preparation were the same as previously described (21). Crude extracts were used as the source of TdR kinase. Protein was determined by the method of Lowry et al. (17).

TdR kinase for gel electrophoresis and for neutralization with rabbit antiserum was prepared using strains of type 1 and type 2 viruses previously described (18). In these experiments, infected cells were incubated for 18 hr at 37°,
and the enzyme was prepared by scraping infected cells into the medium. The cells were washed twice in Medium 199 and resuspended in 6 volumes of 0.25 m sucrose with 0.005 m β-mercaptoethanol. The cell suspension was sonically disrupted in a Raytheon sonicator in an ice bath 3 times for 1 min at maximum setting. This material was centrifuged at 15,000 rpm for 1 hr in a Beckman No. 50 rotor. The supernatant was removed and brought to 40% saturation with neutral ammonium sulfate. The material was centrifuged at 3000 rpm in a Sorvall centrifuge at 4°, and the resulting precipitate was resuspended to one-half the original volume of cell suspension, in a buffer consisting of 0.05 m Tris-HCl (pH 7.5), 0.001 m EDTA, and 0.005 m β-mercaptoethanol. This was mixed with an equal volume of 0.25 m sucrose and 0.005 m β-mercaptoethanol and stored at -70°.

**TdR Kinase Assay.** The TdR kinase activity was measured as described previously (21). The reaction mixture consists of: TdR-methyl-14C, 0.1 μCi (35 mCi/mmmole); cold TdR, 8 μmole; ATP, 5 mm; MgCl2, 5 mm; enzyme extract; 0.05 m Tris buffer (pH 9.0), up to 0.25 ml. Serum was involved in the reaction mixture for the experiment examining the effect of serum on TdR kinase activity. The reaction mixture was incubated for 15 min at 38°, boiled for 2 min, and centrifuged to remove denatured protein. The amount of TdR nucleotides in a 25-μl aliquot was measured by the DEAE-cellulose disc method.

In other experiments, reaction mixture consisted of 0.25 mm MgCl2, 5 mM ATP, 0.05 m Tris-HCl (pH 8.0), TdR, 14C to give in each determination 10.2 nmoles and 0.5 μCi of TdR and 50 to 100 μg of prepared enzyme protein. Total reaction volume was 250 μl. The mixture was incubated at 37° for 20 min after which the reaction was stopped by plunging the tubes into an ice bath. Twenty-five μl of the mixture were spotted in DEAE-cellulose papers. After the papers were dry, they were washed by suspension in 0.001 m ammonium formate solution for four 15-min periods. The papers were then washed in ethanol briefly and dried. The papers were then counted in Aquasol (New England Nuclear, Boston, Mass.) scintillation cocktail.

**Gel Electrophoresis.** Polyacrylamide gel electrophoresis was performed according to the method of Davis (7). Approximately 200 μg of protein enzyme preparation were applied to gels in 5- x 90-mm columns. The stacking gel contained 2.5% acrylamide while the separating gel contained 9% acrylamide with a bis:acrylamide ratio of 1:38. Electrophoresis was carried out in Tris-glycine buffer at 4 ma/gel for 2 hr. The gels were sliced into 3-mm slices. The slices were assayed for TdR kinase in the reaction mixture described above. The incubation period was prolonged to 2 hr at 37°. The counting of radioactivity was carried out as described above.

**Effect of Serum on TdR Kinase Activity.** Fifty μl of enzyme extract (herpesvirus type 2-induced TdR kinase, 61.5 μg of protein/50 μl) were mixed in an ice bath with 50 μl of human serum (1:2 or 1:4 dilution) and incubated for 60 min in the ice bath. Then other components described above were added, and the mixture was assayed for TdR kinase activity. The results are expressed in residual TdR kinase activity retained in the presence of the serum when compared to buffer.

In order to determine whether TdR kinase activity from cervical carcinoma was antigenerically related to herpesvirus type 2-induced TdR kinase, neutralization was attempted with antisera possessing neutralizing activity specific for TdR kinase induced by herpesvirus type 1 or type 2. In the neutralization test, the antisera were diluted 1:4 in 0.05 m Tris-HCl, pH 8.0. The diluted antiserum was mixed with an equal volume of enzyme and held at 4° for 5 min. The TdR kinase reaction mixture was then added, and the assay for enzyme activity was conducted in the usual manner. The results of the neutralization test are expressed in residual TdR kinase activity retained in the presence of the antiserum when compared to serum from a nonimmune rabbit.

**RESULTS**

**Inhibition of Type 2 TdR Kinase.** Sera diluted 1:2 or 1:4 were examined for their ability to reduce the TdR kinase activity which was induced by herpesvirus type 2. A 94% reduction in activity was observed with 1 serum sample. However, with most sera the reduction in activity was less than 40%. The results, expressed as percentage of residual activity, are summarized in Table 1. The greatest reduction was observed among Negro women with cervical cancer and matched controls.

<table>
<thead>
<tr>
<th>Study group</th>
<th>Race</th>
<th>No. tested</th>
<th>TdR kinase (% residual activity)</th>
<th>Cervical cancer (p)</th>
<th>Cervical cancer and matched controls (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cervical cancer</td>
<td>Negro</td>
<td>14</td>
<td>79.5 ± 12.7*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>16</td>
<td>81.5 ± 12.5</td>
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<tr>
<td>Matched controls</td>
<td>Negro</td>
<td>14</td>
<td>78.5 ± 26.5</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>13</td>
<td>86.6 ± 10.9</td>
<td>&gt;0.05</td>
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<tr>
<td>Other cancers</td>
<td>Caucasian</td>
<td>18</td>
<td>97.0 ± 7.1</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Laboratory personnel</td>
<td>Caucasian</td>
<td>57</td>
<td>95.9 ± 5.4</td>
<td>&lt;0.01</td>
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</tr>
</tbody>
</table>

* Mean ± S.D.
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their matched controls; mean residual activities of 79.5 and 78.5%, which represented reductions of 20.5 and 21.5%, respectively, for the 2 groups were observed. Comparable reduction in activity was observed among Caucasians with cervical cancer and controls, with mean residual activities of 81.5 and 86.6%, respectively. The degree of reduction of enzyme activity was not significantly different between the cases and controls or between Negro and Caucasian women.

Residual enzyme activity of 85% or less was observed in only 1 of 18 patients with cancer of nongenital sites and in only 3 of 57 laboratory personnel. As can be seen in Table 1, the mean activities of TdR kinase in the presence of sera from nongenital cancer cases and laboratory personnel were similar but were significantly different from the cervical cancer cases and their matched controls.

The inhibition of enzyme activity was similar for sera obtained from women with active cancerous lesions of the cervix and women who had been treated. For women with cancer and their matched controls, correlation coefficients of $-0.3$ to $-0.5$ were obtained when the residual enzyme activity in the presence of sera was compared with the neutralizing antibody titers to herpesvirus types 1 and 2. The relation between neutralizing antibody titers to herpesvirus type 2 and inhibition of TdR kinase activity is shown in Table 2. Residual TdR kinase activity of 80% or less was not observed among 11 sera with neutralizing titers of 1:40 or less, while residual activity of 80% or less was observed among 9 of 17 sera with neutralizing titers greater than 1:201. Thus, inhibition of enzyme activity tended to occur in sera with high titers of neutralizing activity to the virus.

**Migration of TdR Kinase in Polyacrylamide Gels.** Attempts were made to identify the TdR kinase of cervical cancer cells by the migration characteristics in polyacrylamide gel electrophoresis. The migration of TdR kinase induced by 4 strains of type 2 virus in 3 cell types was examined. The results obtained with 1 strain are shown in Chart 1 and are representative of those obtained with all strains of virus tested. The enzymes migrated with $R_F$ values of 0.25 to 0.28.

The patterns of migration of TdR kinase activity was determined for enzyme preparations from 5 cervical carcinomas. Representative results are shown in Chart 2. The major peak of activity was observed at $R_F$ values of 0.22 to 0.28. In 3 of 5 preparations, a 2nd peak of enzyme activity with $R_F$ values of 0.44 to 0.50 was observed. The migration characteristics of the major component of TdR kinase in cervical cancer tissue was similar to that induced by herpesvirus type 2 in rabbit kidney, rabbit embryo, or Vero cells.

**Inhibition of Enzyme Activity with Anti-TdR Kinase Sera.** Antisera to type I and type 2 TdR kinase were examined for their ability to inhibit the activity of the enzyme induced by the virus. The enzymes induced by type 2 virus were unstable, and the optimum time of incubation with the antisera was found to be 5 min. The specificity of the antisera to enzymes is shown in Table 3. Under the conditions of the experiment, 34% of residual activity of type 1 TdR kinase was observed in the presence of antiserum to type 1 virus, and 6% residual activity was observed when type 2 enzyme was mixed with antiserum to type 2 virus. The activities of the enzymes were not inhibited by the heterologous antisera.

The TdR kinase activity obtained from cervical cancer

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**Table 2**

<table>
<thead>
<tr>
<th>Neutralizing activity to herpesvirus type 2</th>
<th>TdR kinase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100-81 persons tested</td>
</tr>
<tr>
<td>≤40</td>
<td>11</td>
</tr>
<tr>
<td>41-200</td>
<td>19</td>
</tr>
<tr>
<td>≥201</td>
<td>8</td>
</tr>
</tbody>
</table>

*Antibody titer expressed as reciprocal of serum dilution producing 50% neutralization.*

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**Chart 1.** Polyacrylamide gel electrophoresis patterns of TdR kinase activity induced by herpesvirus type 2 (strain 333) in (O) rabbit kidney cells, (x) rabbit embryo cells, and (•) Vero cells. The $R_F$ values of the enzyme in uninfected cells were 0.24 and 0.44 for primary rabbit kidney cells; 0.15, 0.47, and 0.54 for rabbit embryo cells; and 0.28 for Vero cells.

**Chart 2.** Polyacrylamide gel electrophoresis patterns of TdR kinase activity extracted from cervical cancer tissue. Samples from 3 different patients are represented.
tissue was not inhibited by the antisera to the virus-induced enzymes. The enzyme was extracted from cancer biopsies of 5 patients and tested with antisera to both virus-induced enzymes. The enzyme activity of 1 sample was too low to evaluate. Of the other 4 samples, there was no significant inhibition of cervical cancer TdR kinase by antiserum to either type 1 or 2 herpesvirus enzymes.

**DISCUSSION**

TdR kinase has been considered a “salvage” enzyme since DNA replication can occur in cells deficient in the enzyme. Elevated levels of TdR kinase activity are associated with rapid proliferation of cells; this has been demonstrated for cells obtained from bone marrow, regenerating liver, and mucosal crypt cells of the gut, as well as malignant tumors (11, 33). It has been suggested that, while not required for replication of normal cells, TdR kinase activity may be rate-limiting in the tumor growth (25, 26). This suggestion is based on the correlation between enzyme levels and the relative growth rate of transplantable mouse hepatomas (5) and the reduced tumorigenicity of SV40 virus-transformed cells in which the levels of TdR kinase were reduced by culturing the cells in the presence of bromodeoxyuridine (3, 25, 26).

Increased activity of TdR kinase is observed in cells infected with DNA viruses (14). The increase occurs not only in cells that are lytically infected but also in cells that undergo malignant transformation (6, 9). The enzyme activity found in SV40 virus-transformed cells appears to be similar to that observed in lytically infected cells, and sera from tumor-bearing animals will neutralize the enzyme activity obtained from either transformed or lytically infected cells (6). If TdR kinase does influence growth potential of tumors as suggested by Rothschild and Black (25, 26) viral-induced enzymes in transformed cells might function in this role.

The enzymes induced by herpesvirus types 1 and 2 are biologically and antigenically distinct from each other and from the cell enzymes (18, 21, 31, 32). In vitro studies have demonstrated a “biochemical” transformation of cells with herpesvirus. TdR kinase with characteristics of the virus enzyme was produced by cells that lacked the capacity to synthesize their own TdR kinase. This was accomplished by infecting the TdR kinase-deficient cells with UV-irradiated virus; the viral enzyme was continually produced through a number of cell divisions (19). From these observations, transformation of cervical epithelial cells by herpesvirus type 2 with continued expression of the viral TdR kinase would appear plausible.

The results of our study suggest that the “non-virion” antigens to which antibodies are made in cervical cancer patients are not TdR kinase. While inhibition of enzyme activity was found with some human sera, it appeared to be more related to the patients’ past experience with herpesvirus type 2 than with the presence or absence of cervical cancer. TdR kinase extracted from cervical cancer tissue had migration characteristics on polyacrylamide gel electrophoresis that were similar to those of virus-induced TdR kinase; however, the enzyme of the cancer tissue did not appear to be antigenically related to the virus-induced enzyme. This study does not rule out the possibility of transformation of cervical epithelial cells by herpesvirus type 2. The virus-induced enzyme, if present, may be labile (21, 32) and not detected by the techniques used. It is also possible that only a small percentage of the cells, if any, express the virus genome, a situation analogous to that observed for expression of antigens in herpesvirus type 2-transformed hamster cells (23).

At least 2 enzymes capable of phosphorylating TdR have been shown to exist in mammalian cells (11, 15, 22, 30). The relative proportions of the enzymes vary during development of the fetus. The enzyme associated with early stages of development, “fetal enzyme,” has been found in malignant tumors (10, 29). The migration characteristics of the TdR kinase of cervical cancer tissue was found to be similar to the fetal enzymes reported for cancers of other sites. The antigenic relatedness of the enzymes found in cancers from different sites would be of value in supporting the concept that they represent expressions of inherited rather than acquired genetic information.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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