Serological Properties of Thymidine Kinase Produced in Cells Infected with Type 1 or Type 2 Herpes Virus

By M. E. THOULESS

Department of Virology, Medical School, University of Birmingham, B15 2TJ, England

(Accepted 18 August 1972)

SUMMARY

Antisera produced in rabbits against extracts of cells infected with type I or type 2 herpes simplex virus neutralized thymidine kinase activities of six type I and six type 2 herpes virus strains. There was intertypic neutralizing activity, suggesting some common antigenic determinants, but much greater intratypic activity, suggesting that the enzyme bears type-specific determinants.

The effects of some anti-type I sera were complicated since they also stabilized the type 2 enzyme activity.

When anti-type I and anti-type 2 sera were absorbed with extracts of cells infected with thymidine-kinase deficient mutants of homologous type they retained their power to neutralize thymidine kinase activity but completely lost their ability to neutralize infectivity. Each absorbed serum gave a single precipitin line against the parent strain and none against the parent of the other type. It was surmised that these precipitin lines were associated with type-specific thymidine kinase antigens.

INTRODUCTION

Herpes simplex virus type I and type 2 differ in many properties. They differ in their ability to grow in chick embryo fibroblasts (Figueroa & Rawls, 1969), size of pocks produced on the chorioallantoic membrane of eggs (Parker & Banatvala, 1967), permissive temperature for virus growth (Ratcliffe, 1970), antigenic properties and serological response of the host (Plummer, 1964; Nahmias & Dowdle, 1968), and differences in the density of their nucleic acids (Goodheart, Plummer & Waner, 1968). They also differ with respect to the thymidine kinase they induce in infected cells. The type I enzyme is rather stable at 40 °C and the type 2 enzyme is unstable (Thouless & Skinner, 1971; Ogino & Rapp, 1971). The type I enzyme appears to be virus specified (Klemperer et al., 1967). It is inactivated by antiserum to herpes type I infected RK 13 cells but not by antiserum to pseudorabies-infected RK 13 cells and, likewise, pseudorabies thymidine kinase is inactivated by antiserum to pseudorabies-infected cells but not by that to herpes-infected cells (Buchan & Watson, 1969).

It is of interest to investigate antigenic and serological differences between herpes virus type I and 2 induced thymidine kinase. This simple objective has proved technically difficult to attain. The first complication is that the thermolability of the type 2 enzymes has compelled us to modify the neutralization test used by Klemperer et al. (1967). For the same reason we have been forced to modify the preparation of the antigen used for immunizing rabbits. The interpretation of results has been further complicated by the heterogeneous action of different anti-herpes type I sera on the type 2 enzyme; some of which even appear
to stabilize enzyme activity (Thouless & Skinner, 1971). However, by use of selected antisera it is possible to distinguish type 1 and type 2 herpes strains by neutralization of the thymidine kinases that they induce. Evidence for this and for type specific antigenic determinants on thymidine kinase as detected by immunodiffusion are reported in this paper, together with an account of the stabilization phenomenon.

**METHODS**

*Cell lines.* BHK 21 C13 cells, grown in supplemented Eagle’s medium containing 10% tryptose phosphate broth and 10% calf serum (ETC) (Vantsis & Wildy, 1962), were used in the preparation of infected cell extracts for the experiments.

RK 13 (Beale, Christofinis & Furminger, 1963) cells were grown in the same medium with rabbit serum substituted for calf serum for preparation of antigen for immunizing rabbits.

Bromodeoxyuridine-resistant BHK 21 cells were produced in this Department by Dr A. Buchan and Mrs C. Wallis by growing the cells in the presence of increasing concentrations of bromodeoxyuridine (BUdR, Sigma) until they finally withstood 100 μg BUdR/ml of Eagle’s medium with 10% calf serum.

*Virus strains.* The following virus strains were used in this work: herpes virus type 1, strain HFEM, NASH, $2$, $3$, HIL and WAL; herpes virus type 2, strain G-LOV, G-BRY, G-PAR, G-3345, G-2037 and G-2248 (Thouless & Skinner, 1971); herpes virus type 1 thymidine-kinase-deficient mutant, 2006 (Dubbs & Kit, 1964), hereafter referred to as 1-TK-; herpes virus type 2 thymidine-kinase-deficient mutant of G-BRY, hereafter referred to as 2-TK-.

The latter mutant was prepared by the method of Dubbs & Kit (1964), using BUdR-resistant BHK 21 cells. Cells of this type ($3 \times 10^7$) were infected with $2 \times 10^8$ p.f.u. of G-BRY in the presence of 25 μg BUdR/ml medium (Eagle’s medium with 10% calf serum). The yield was $2 \times 10^{-4}$ p.f.u./cell. Plaques were picked from dishes where there was only 1 plaque/dish using a fine-drawn Pasteur pipette. The cells were disrupted in an ultrasonic water bath (Electrosonic Headland, London). The virus yield from each plaque was propagated 5 times in cells in the presence of 25 μg BUdR/ml medium. The isolates were then propagated in BHK cells in ETC medium. The virus derived from plaque 6 was selected for further study because not only did it fail to induce thymidine kinase, but it was particularly effective in depressing host-cell thymidine kinase activity.

*Antisera.* Antiserum 192, 115, 303 and 186 (referred to as anti-type 1 sera) were prepared using freeze-dried antigen prepared from RK 13 cells infected with the type 1 herpes simplex strain HFEM. The antigen + Freund’s incomplete adjuvant was injected intra-muscularly according to the prolonged schedule described by Watson *et al.* (1966). Antiserum 305 was similarly prepared by Dr A. Buchan using the thymidine-kinase-deficient strain 2006.

At first, antisera were prepared by the above method against freeze-dried extracts of RK 13 cells infected with type 2 viruses. When it was discovered that the thymidine-kinase activity of type 2 virus was lost after freeze-drying, this step was omitted and infected RK 13 cells were stored at $-70$ °C until required. Accordingly, antisera were prepared against frozen RK 13 cells infected with G-BRY. Antiserum 409 was prepared using a similar schedule to that of Watson *et al.* (1966). Sera were derived from bleeds taken 10 days after the 7th and 8th injection. Antiserum 367 was prepared using the lymph-node injection technique (Watson & Wildy, 1969). RK 13 cells infected with G-BRY were ultrasonically disrupted in 5 ml antitype 1 serum (192) which stabilized the type-2 thymidine kinase (see Results). The allotypes of rabbits 367 and 192 were the same. The extract was centrifuged at 100,000 g for 30 min
Herpes virus types I and 2 thymidine kinase

Vol. 21, No. 3
309

at 4 °C. The supernatant fluid, concentrated by pressure dialysis, was stored at −70 °C. Volumes of 0.1 ml + 0.1 ml Freund's adjuvant were injected into the popliteal lymph nodes on two occasions at an interval of 6 weeks. This was followed by two subcutaneous injections and later two intramuscular injections of unconcentrated antigen. Antisera 409 and 367 are referred to as anti-type 2 sera.

The pre-immune sera were taken from rabbits 409 and 367.

**Antigen preparation for agar gel diffusion tests.** BHK cells (10⁶) were infected at an input multiplicity of 10 p.f.u./cell. The cells were harvested 12 h after infection, washed in phosphate buffered saline and resuspended in 1 ml of distilled water. The cells were then disrupted, either in an ultrasonic water bath or with an MSE ultrasonic disintegrator.

**Immunodiffusion tests.** These were carried out in 1% agar (Watson et al. 1966). A pattern of six wells, equally spaced around a central well, each 8 mm in diameter and separated by 3 mm was used. Each well was filled to capacity (approximately 0.12 ml) with antigen or antiserum, both undiluted and diluted 1/3 and left at room temperature for 48 h for the lines of precipitation to develop.

**Serum absorption.** This was carried out as described by Buchan, Luff & Wallis (1970). About 10⁶ BHK cells were disrupted 12 h after infection and mixed with 1 ml antiserum. The mixture was shaken at 4 °C overnight. It was centrifuged at 100000 g for 1 h under liquid paraffin and the supernatant fluid was concentrated to 1 ml by pressure dialysis.

**Preparation of herpes infected BHK cell extracts.** BHK cells were infected with herpes simplex virus at an input multiplicity of 10 to 20 p.f.u./cell, incubated at 37 °C for 7 h, washed in PBS and finally resuspended at 10⁸ cells/ml in distilled water. The suspension was ultrasonically disrupted and centrifuged at 100000 g under liquid paraffin for 30 min. The layer between the pellet and the paraffin was taken off and stored in small volumes at −70 °C until required for thymidine kinase assays.

**Determination of thymidine kinase activity.** The thymidine kinase activity was measured as described by Klemperer et al. (1967). We have found, unlike Lowry, Bresnick & Rawls (1971), that the pH optimum for the type 2 enzyme is the same as for the type 1 (just under pH 6). The reaction mixture containing phosphate buffer pH 6 and a final concentration of 5 mM-MgCl₂, 5 mM-ATP and 106 μM-[2-¹⁴C]-thymidine and water, was mixed in bulk and dispensed in 0.21 ml samples in conical tubes. Cell extract (0.04 ml) to be assayed was added suitably diluted in tris+HCl+NaCl. The mixtures were incubated for 10 min at 37 °C, boiled for 2 min and centrifuged at 20000 g for 10 min to remove denatured protein. Supernatant fluids (0.05 ml) were spotted on to DEAE cellulose paper and washed in 1 mM-ammonium formate. The products of the reaction stuck to the paper and the radioactivity was measured in a liquid scintillation counter.

**Thymidine kinase neutralization.** All sera used were dialysed for 2 days against 0.02 M-phosphate buffer pH 6. Samples (0.1 ml) of antiserum were mixed at 0 °C with 0.1 ml of cell extract diluted so that controls gave 17 to 20000 ct/min/assay. The mixture (0.04 ml) was added to the reaction mixture and assayed within 2 min because of the instability of the type 2 enzyme. Neutralization took place extremely rapidly. When the incubation time of the assay at 37 °C was varied from 2.5 to 15 min the level of activity of type 2 thymidine kinase + anti-type 2 serum barely increased, whereas the activity of the enzyme + pre-immune sera increased in a linear fashion with increasing incubation time. In some experiments the enzyme–serum mixture was reassayed after 30 and 60 min at 25 °C.

**Neutralization of infectivity.** The antisera were diluted 1/5 in phosphate buffered saline. They were heated to 56 °C for 30 min to inactivate complement. Samples (0.2 ml) were mixed with 0.2 ml of a virus suspension containing 4 × 10⁴ p.f.u./ml. After 1 h at room
temperature a 1/100 dilution was made. This was assayed by the plaque assay method of Russell (1962) in BHK cells under carboxymethyl cellulose overlay.

RESULTS

Specificity of neutralization of enzyme activity by selected antisera

Thymidine kinase neutralization tests were done on six type 1 and six type 2 herpes virus strains using three selected sera (Table I). The anti-type 1 serum (192) reduced the activities of all type 1 strain preparations by 87 to 95% and those of type 2 strain preparations by 0 to 28%. The anti-type 2 sera (367 and 409) behaved reciprocally, reducing the enzyme activity of type 2 strains by 87 to 96% and those of type 1 strains by 37 to 64%. The results suggested the presence of type-specific antigenic determinants on enzymes from all strains. They also suggested the presence of common antigenic determinants, though this was more evident from the behaviour of the type 1 virus enzymes treated with heterologous antiserum than of those of type 2. The enzyme of strain G-BRY was exceptional since it was neutralized to a greater extent by this heterologous antiserum than were the other type 2 enzymes. This result was typical of numerous other experiments with this particular enzyme and this antiserum. Some other heterologous antisera reduced the activity of this enzyme less, but others reduced it even more (cf. Fig. 3). This is further discussed below.

Table 1. Percentage residual activity of thymidine kinase after mixing with equal volumes of serum at 0°C for 2 min

<table>
<thead>
<tr>
<th>Herpes virus strains</th>
<th>Residual activity* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-type 1 serum no. 192†</td>
</tr>
<tr>
<td>Type 1</td>
<td></td>
</tr>
<tr>
<td>S3</td>
<td>13</td>
</tr>
<tr>
<td>HIL</td>
<td>10</td>
</tr>
<tr>
<td>s2</td>
<td>6</td>
</tr>
<tr>
<td>HFEM</td>
<td>5</td>
</tr>
<tr>
<td>WAL</td>
<td>11</td>
</tr>
<tr>
<td>NASH</td>
<td>9</td>
</tr>
<tr>
<td>Type 2</td>
<td></td>
</tr>
<tr>
<td>G-BRY</td>
<td>72</td>
</tr>
<tr>
<td>G-2248</td>
<td>93</td>
</tr>
<tr>
<td>G-3345</td>
<td>91</td>
</tr>
<tr>
<td>G-LOV</td>
<td>93</td>
</tr>
<tr>
<td>G-PAR</td>
<td>83</td>
</tr>
<tr>
<td>G-2037</td>
<td>100</td>
</tr>
</tbody>
</table>

* Cell extract was used at a dilution which gave 17000 to 20000 cts/min assay when mixed with pre-immune serum or with buffer. Results are corrected for background counts which were always less than 200 cts/min. † Stock booknumber of immunized rabbit.

Experiments with anti-type 1 and 2 serum absorbed with the homologous type of thymidine kinase-deficient mutant

Association of thymidine kinase with a diffusible antigen

Buchan et al. (1970), making use of the 1-TK- mutant, showed that the thymidine kinase activity induced by herpes simplex virus type 1 was associated with a diffusible antigen. We have done similar experiments using the 2-TK- mutant. Anti-type 2 serum (409) gave multiple precipitin lines in immunodiffusion tests when set up against either type 1 or type 2
Herpes virus types I and 2 thymidine kinase

Infected cell extracts. When the antiseraum was absorbed with an excess of extracts of 2-TK⁻ infected cells it gave one precipitin line only against the parent type 2 virus infected cell extract and none against the type 1 or 2-TK⁻. Conversely, anti-type 1 serum (192) gave multiple precipitin lines against type 1- or 2-cell extracts, but when absorbed with excess 1-TK⁻ infected cell extract gave only one precipitin line against type 1 and none against type 2 or 1-TK⁻ infected cell extracts (Fig. 1).

![Fig. 1. Type-specific thymidine kinase precipitin line. 1As/1-TK⁻: anti-type 1 serum (192) absorbed with type 1 thymidine-kinase deficient mutant. 2As/2-TK⁻: anti-type 2 serum (409) absorbed with type 2 thymidine-kinase deficient mutant. 1Ag: herpes type 1 antigen. 2Ag: herpes type 2 antigen. 1-TK⁻Ag: herpes type 1 thymidine-kinase-deficient mutant antigen. 2-TK⁻Ag: herpes type 2 thymidine-kinase-deficient mutant antigen.](image)

Neutralization of thymidine-kinase activity and infectivity by absorbed antisera

Samples of the absorbed antisera (192, 409) used in the previous experiment were tested for their ability to neutralize the thymidine-kinase activities of both types of virus. Here an unexpected difficulty was encountered, since absorption with excess cell extract resulted in the non-specific removal of IgG (kindly determined by Professor P. G. H. Gell, using the immunodiffusion assay). For this reason the experiment was controlled by absorbing samples of serum in parallel with uninfected BHK cells. The results (Table 2) showed that absorption of the antisera with extracts of cells infected with TK⁻ mutants of homologous type did not remove their enzyme-neutralizing properties. At the same time the antisera were tested in plaque reduction tests against the type-2 strain G-BRY; antisera absorbed with extracts of cells infected with homologous type of thymidine kinase-deficient virus had completely lost their ability to neutralize infectivity whereas those absorbed with uninfected cells had not.

Thus, the anti-type 1 and anti-type 2 sera blocked with the homologous type of thymidine kinase-deficient mutant did not neutralize infectivity but did neutralize thymidine kinase activity. This suggested that the sera were specific for thymidine kinase and supports the hypothesis that the type specific precipitin lines produced by the absorbed sera were, in fact, thymidine kinase antigens.
Table 2. Neutralization of herpes virus type 1 and 2 induced thymidine kinase activity by anti-
type 1 and 2 sera absorbed with the homologous type of thymidine kinases mutant infected
BHK cells*

<table>
<thead>
<tr>
<th>Serum</th>
<th>Herpes type 2 G-BRY</th>
<th>Herpes type 1 HFEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thymidine kinase activity ct/min/assay</td>
<td>Residual activity (%)</td>
</tr>
<tr>
<td>Pre-immune</td>
<td>16500</td>
<td>100</td>
</tr>
<tr>
<td>Anti-type 2 serum (409) absorbed with BHK cells</td>
<td>3700</td>
<td>22</td>
</tr>
<tr>
<td>Anti-type 2 serum (409) absorbed with 2-TK^-</td>
<td>2300</td>
<td>14</td>
</tr>
<tr>
<td>Anti-type 1 serum (192) absorbed with BHK cells</td>
<td>9000</td>
<td>55</td>
</tr>
<tr>
<td>Anti-type 1 serum (192) absorbed with 1-TK^-</td>
<td>10300</td>
<td>62</td>
</tr>
</tbody>
</table>

* Enzyme neutralization tests were carried out as described in the Methods section. An equal volume of serum and a suitable dilution of the enzyme preparation were mixed on ice and assayed immediately.

The distinction between neutralizing and stabilizing activities of antisera on
thymidine kinase induced by herpes simplex virus type 2 strains

Stabilization and neutralization could be distinguished by holding enzyme and antiserum
mixtures at 25 °C for varying periods before assay. Under these conditions enzyme prepara-
tions, mixed with pre-immune serum or buffer, lost about 60% of their activity after 30 min
and 75% after 60 min at 25 °C, presumably due to thermal inactivation (Fig. 2). Several
dilutions of sera 409 (anti-type 2) and 192 (anti-type 1) were tested for neutralization and
stabilization of enzyme activity. The log enzyme activities were plotted against time (Fig. 2)
since this allowed ready comparison of their rates of decay during the experiment. Increasing
dilutions of anti-type 2 serum (409) resulted in decreasing neutralizing activity at whatever
time point comparisons were made. Indeed, in Fig. 2a the curves ran parallel, indicating no
stabilization. By contrast, anti-type 1 serum (192) (Fig. 2b) showed at lower dilutions only
slight neutralization followed by substantial stabilization. At higher dilutions this serum
did not influence the enzyme activity.

Heterogeneity of activity of anti-type 1 sera with herpes type 2 G-BRY thymidine kinase

The neutralizing and stabilizing activities of various anti-type 1 sera were compared,
using conditions similar to the previous experiment. The results (Fig. 3) showed that anti-
serum 305, prepared against strain 1-TK^-, neither neutralized nor stabilized type 2 thymidine
kinase but gave values similar to those with pre-immune serum or buffer. Other anti-type 1
sera showed a spectrum of neutralizing activities and stabilized the enzyme at different levels.
Thus, the anti-type 1 sera showed a variety of combinations of neutralizing and stabilizing
effect. A point of some interest (data not given) was that type 2 enzyme stabilized with
anti-type 1 serum (192) could still be neutralized by anti-type 2 serum, albeit at a slower rate.
**Herpes virus types 1 and 2 thymidine kinase**

![Graph](image-url)

**Fig. 2.** The neutralizing and stabilizing effects of dilutions of (a), an anti-type 2 serum (409) and (b), an anti-type 1 serum (192) on herpes type 2 thymidine kinase: ○—○, undiluted; □—□, 1/4; △—△, 1/8; ■—■, 1/16; ◇—◇, 1/32; ●—●, pre-immune.

![Graph](image-url)

**Fig. 3.** Neutralization and stabilization of herpes type 2 thymidine kinase (G-BRY) by various anti-type 1 sera. △—△, tris buffer, pH 7.2; □—□, pre-immune serum; ○—○, 305, antiserum to 1-TK- infected cells; ●—●, 186; ▼—▼, 192; ■—■, 115; △—△, 303.
DISCUSSION

Klemperer et al. (1967) have shown that antiserum to herpes type 1 infected RK cells inhibits the thymidine kinase from type 1 infected cells. Buchan et al. (1970) have shown this inhibition to be specific by absorbing the antiserum with a type 1 thymidine-kinase deficient mutant (1-TK-). The absorbed antiserum still neutralized thymidine-kinase activity although not infectivity, and, further to this, gave only one precipitin line against the type 1 parent antigen. This work has been extended in parallel with comparable work with type 2 serum and a type 2 thymidine-kinase-deficient mutant. It has been shown that the type 2 thymidine kinase was specifically inhibited by type 2 sera prepared from RK antigen with active enzyme at the time of rabbit inoculation. The type 2 thymidine kinases were not greatly inhibited by an anti-type 1 serum (192), which markedly stabilized the enzyme. Anti-type 1 serum (303) neutralized G-BRY thymidine kinase without, apparently, stabilizing it; other anti-type 1 sera showed intermediate behaviour. The results with some of these sera were compatible with the presence of common antigenic determinants. The type 1 thymidine kinases, however, were specifically neutralized by anti-type 1 serum (192) and reduced to half their activity by type 2 sera, clearly suggesting the presence of antigenic determinants common to both types of thymidine kinase. Anti-type 2 serum absorbed with 2-TK- did not neutralize infectivity of G-BRY but inhibited its thymidine kinase activity; in immuno-diffusion tests it gave one precipitin line only against the type 2 (G-BRY) antigen and none against type 1 (HFEM). The anti-type 1 serum blocked with 1-TK- gave one precipitin line against type 1 (HFEM) antigen and none against type 2 (G-BRY), thus giving further evidence for a thymidine kinase type-specific antigen. There was therefore a paradox; common antigenic determinants were revealed by enzyme neutralization but not by the precipitin tests. This is unexplained and requires further investigation.

The mechanism of stabilization of the type 2 enzyme is obscure. It is not even certain that the effect is due to an antibody though we believe this to be so. Unfortunately, it has not been possible to verify this since stocks of pre-immune sera from the rabbits yielding stabilizing antisera are exhausted, but it is hoped to examine the problem with fresh sera. However, if stabilization is due to an antibody, this would suggest that the type 2 enzyme does, indeed, possess antigenic determinants in common with that of type 1. Further, it suggests that these are situated away from the active site of the enzyme since stabilizing antibody seems neither to interfere with enzyme activity nor enzyme neutralization.

Finally, it is clear that if antisera are carefully selected for their effect against known type 1 and 2 thymidine kinase preparations, they may be used to distinguish type 1 from type 2 herpes strains by enzyme neutralization tests.

This work was supported by grants from the United Birmingham Hospital Endowment Fund and the Medical Research Council. Thanks are also due to Shell International Petroleum Company Limited for general support to the Department.

I should like to thank Professor P. Wildy for advice and encouragement throughout this work and Dr A. Buchan and Dr H. S. Bedson for help with the preparation of this paper. I should also like to thank Mrs Joan Edwards for help with rabbit serum preparation.
REFERENCES


(Received 19 May 1972)