Tumour Production by HSV-2 Transformed Lines in Rats and the Varying Response to Immunosuppression

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SUMMARY

Rat embryo cells transformed by two ts mutants of HSV-2 strain HG 52 and also by u.v.-irradiated HSV-2 strain 333 were inoculated into highly inbred host rats, either newborn or which had undergone various immunosuppressive treatments. The latent period before a palpable tumour (a fibrosarcoma) was detected varied directly with the degree of immunosuppression of the host. Transformed cells could form tumours after a latent period of nearly 2 years. All tumours were invasive and in some cases metastatic. The continuing expression of HSV information in 10 tumour cell lines was demonstrated by perinuclear and cytoplasmic staining in immunofluorescence studies using a rat antiserum directed against the early polypeptides of HSV-2 HG 52 infection and a rabbit serum prepared against a 24 h cell infection with HSV-2 HG 52 ts 1. Sera from tumour-bearing rats fluoresced the surface of unfixed human or rat embryo cells 4 to 5 h after infection with HSV-2 HG 52. In addition the rabbit antiserum (4740 or 4741) fluoresced the surface of 80% of the tumour cells in culture. Transplanted tumours after 20 passages in vitro and taking up to a year to again form a tumour in a host rat also showed specific HSV cytoplasmic and perinuclear fluorescence in tests with the rat antiserum directed against early polypeptides of HSV-2 lytic infection.

INTRODUCTION

Oncogenic transformation of cultured rodent embryo cells has been described in hamsters by Duff & Rapp (1971, 1973), Kutinova et al. (1973), Kimura et al. (1975); in rats by Macnab (1974, 1975, 1976), Darai & Munk (1976), Darai et al. (1977), Kucera et al. (1977); and in mice by Boyd et al. (1975) and Boyd & Orme (1975). Expression of HSV information has been shown by means of immunofluorescence studies, and neutralizing antibody has been detected in sera from some tumour-bearing animals (Duff & Rapp, 1971, 1973; Boyd et al. 1975; Kimura et al. 1975; Macnab 1976).

Immunosuppression by anti-lymphocyte serum has been shown to increase the onco

This report describes the formation of tumours (fibrosarcomas) in newborn rats and rats subjected to differing degrees of immunosuppression, arising as a result of injection of
cells transformed by ts mutants of HSV-2 and u.v. irradiated HSV-2, and the relationship between the time of expression of the tumour in vivo and the degree of immunosuppression of the rat host. Metastases were observed in lungs of 50% of non-immunosuppressed rats. Continuing expression of HSV genetic information in the tumours was detected by cytoplasmic and perinuclear fluorescence with two different HSV antisera, one a rat serum directed against the early proteins of HSV-2 infection. The surface of transformed cells also showed HSV-specific fluorescence. Sera from tumour-bearing animals showed surface fluorescence with human embryo lung and rat embryo cells infected with HSV-2 HG 52 4-5 h after release from a cycloheximide block.

METHODS

Rats. Hooded Lister rats have been maintained in our laboratory in excess of 11 years as a closed colony. The colony is propagated by mating siblings of the same litter. No spontaneous tumours have been recorded during the period of this study. The breeding stock is confined, however, to young animals.

Cells

Hood cells. A series of rat embryo cell lines produced by passage of different rat embryo primary cultures in Eagle's medium supplemented with 10% (v/v) tryptose phosphate broth and 10% (v/v) calf serum. These cells were used as control cells in tumour experiments and immunofluorescence tests.

Transformed cells. These were described by Macnab (1974, 1975, 1976). (1) Rat embryo primary cells transformed by u.v.-inactivated HSV-2 strain 333 and called RE-HSV-2-333. (2) Rat embryo primary cells transformed by HSV-2 strain HG 52 ts 1 and called RE 1. (3) Rat embryo primary cells transformed by HSV-2 strain HG 52 ts 7 and called RE 7.

The earliest passage at which sufficient cells were available to begin animal experiments was passage 17. The experiments described here were done with passages 17 to 23 except where otherwise stated.

Media. Eagle's growth medium (Glasgow modification) supplemented by 10% (v/v) calf serum, 10% tryptose phosphate broth (E10CT). Eagle's medium supplemented by 5% calf serum (E5C) was used to set up cells for immunofluorescence; 100 µg/ml streptomycin, 100 units/ml penicillin and 0.2 mg/ml of the antimycotic agent n-butyl p-hydroxy benzoate were added to Eagle's growth medium. Phosphate buffered saline (PBS; Dulbecco & Vogt, 1954) was used as diluent.

Preparation of HSV-2 antisera

Rat antisera. An antiserum directed principally against the early proteins synthesized by HSV-2 (Rakusanova et al. 1971; Honess & Roizman, 1974; Ben-Porat et al. 1975) was prepared by infecting Hooded Lister rat embryo primary cells at 37 °C with 10 p.f.u./cell HSV-2 strain HG 52 in the presence of 50 µg/ml cycloheximide. After absorption for 1 h, virus was removed and the cells washed three times in Eagle's medium before a further 7 h incubation in the presence of cycloheximide. Cycloheximide was removed by washing three times in Eagle's medium and the infected cells were then incubated for another 3 to 4.5 h. The cells were again washed three times, harvested and disrupted by sonication. It should be stressed that this antiserum was prepared in cells from the same inbred colony of rats as was used in cell transformation and tumour studies, so eliminating, as far as possible, cross-reacting or non-specific fluorescence.

An emulsion of cells in Freund's complete adjuvant was injected into Hooded Lister rats
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(one half intradermally and one half intramuscularly). Three identical boosts were given at 10-day intervals and the animals bled out after a further 10 days.

Four different batches of this antiserum were made and the specificity of each was determined by indirect immunofluorescence tests on rat embryo cells infected with HSV-2, HG 52 (early and late after infection).

Rabbit antisera. Antisera 4740 and 4741 were made by Dr M. C. Timbury of this Institute. RK cells were infected with HSV-2 HG 52 is 1 (1 p.f.u./cell) at 38 °C for 24 h. The cell extracts were injected intramuscularly in Freund's complete adjuvant. Animals were given three booster injections at weekly intervals. Two intravenous injections of emulsified cells were then given at weekly intervals and the animals bled. The specificities of these antisera were determined by indirect immunofluorescence tests on BHK C13 cells infected with HSV-2 HG 52 (early and late after infection).

Both HSV and control antisera were absorbed with \(1 \times 10^8\) fixed rat embryo and BHK C13 cells per 1 ml of sera before use. If necessary, absorption was repeated to remove non-specific staining. Fluorescein conjugate was absorbed in the same way.

Fluorescein conjugated anti-rat immunoglobulin made in goats was obtained from Nordic Immunological Laboratories, Maidenhead, Berkshire, England. Fluorescein conjugated anti-rabbit immunoglobulin made in sheep was obtained from Wellcome Research Laboratories, Beckenham, Kent, England.

Immunofluorescence test. Approx. \(1 \times 10^6\) cells were seeded on to coverslips in Eagle's medium supplemented with 10% calf serum and incubated at 37 °C overnight to give well spread cells at about 50% confluence. The cells were washed three times in Eagle's medium without calf serum, rinsed, fixed for 4 min in 1:1 acetone:methanol at -20 °C and air dried. Cells not used immediately were stored desiccated at -20 °C or -70 °C until use. At testing, cells were rinsed briefly in PBS and incubated with 1/5 to 1/10 dilution of rat antiserum or a 1/5 to 1/10 dilution of rabbit antiserum for 30 to 60 min at 37 °C and 30 to 60 min at 4 °C. Cells were then washed with agitation for 30 to 60 min in two to three changes of PBS. Fluorescein conjugate at a dilution of 1/10 was then applied for 1 h at 37 °C or overnight at 4 °C. Cells were washed as before and mounted in PBS/glycerol. Control rat embryo cells were prepared in exactly the same way. Rat embryo cells showing early proteins were produced by infecting rat embryo cells with 2 p.f.u./cell HSV-2 HG 52 in the presence of 50 µg/ml cycloheximide. After 7 h the cells were washed with Eagle's medium three times and incubated for a further 3 to 4.5 h before washing and fixing exactly as has been described above for the transformed cells. In some tests 5% calf serum was added to the PBS wash to remove any non-specific staining due to the serum in the growth medium. Tests were performed in which control, transformed and infected cells were each reacted with test serum and control serum (from a non-injected animal or where possible from a pre-bleed).

Rat anti-lymphocyte serum (ALS) was purchased from Microbiological Associates, Bethesda, Maryland, who claim minimum skin allograft survival of 20 days and a cytotoxicity titre of 1/6000.

Rat anti-thymocyte serum (ATS) was produced in New Zealand rabbits by intravenous injections of \(10^9\) fresh rat thymocytes (prepared as a single cell suspension in PBS by dissecting fresh rat thymus tissue mechanically). A booster dose was given after 14 days and the rabbits bled out at 21 days. The serum was inactivated at 56 °C for 30 min and was
absorbed with a suspension of rat and mouse red blood cells. The method was an adaptation of Nehlsen (1971). The cytolysis titre of this serum was 50% at a dilution of 1/3000.

Immunosuppression regime of newborn rats by both ALS and ATS consisted of three injections of 0.1 ml each, given intraperitoneally (i.p.) at 2-day intervals; the experimental cells were injected 2 days later.

Both ALS and ATS behaved similarly in their ability to immunosuppress rats as measured by the length of time taken for a tumour to grow in the host rat. This was the only criterion under test in these experiments with ALS and ATS and no experiments were carried out to compare the relative efficiencies of the two sera with respect to graft rejection studies – a field outside this study.

Thymectomy. Rats were thymectomized at about 21 days old, i.e. when ready to be weaned. Seven days after thymectomy the rats were subjected to further immunosuppression by X-irradiation and/or ALS and ATS (as described in Tables 1 to 3) and injected with experimental cells.

Tumour production, transplantation, examination for metastases and formation of tumour cell lines. Rats were inoculated either subcutaneously (s.c.) into the back of the neck or intraperitoneally (i.p.) with suspensions of transformed cells in Eagle’s medium or PBS. The inoculation used was 5 × 10^6 or 1 × 10^6 cells. The time after inoculation before tumours first appeared was termed the latent period (LP). In three cases discrete tumours were removed (tumour resection) under ether anaesthesia and the incision closed using Martin Michel clips. Otherwise the tumours were removed at post mortem when the lungs, liver, spleen and lymph nodes were examined for metastases. The animals were bled by heart puncture prior to post mortem and sera were stored separately at −20 °C. Tumours were cultured to form tumour cell lines by dissociation of tissue in 0.25% trypsin and subsequent cultivation in EioCT. Specimens of tumour tissue and of metastases were fixed in formal saline for histology and in glutaraldehyde for electron microscopy.

Tumours were transplanted by inoculating newborn to 7 day old rats with tumour cells, either (a) by inoculating 5 × 10^6 or 1 × 10^6 freshly trypsinized tumour cells, or (b) by inoculating 5 × 10^5 or 1 × 10^6 cells of a tumour cell line passaged for varying passages in vitro. Tumours were also serially passaged in animals for three or four generations.

RESULTS

Oncogenicity

Initially, 125 newborn rats were injected with the three different types of transformed cell lines, i.e. REI, RE7 and RE HSV-2-333. No tumours developed within 12 to 24 weeks, which was the time taken for a hamster tumour to appear after injection of transformed cells (Macnab, 1974). Thymectomy of newborn animals was not successful as mothers frequently ate the offspring. X-irradiation of newborn – up to 48 h-old rats was also tried, with a poor success rate as many baby rats died due to radiation. Thymectomy of weanling rats was not suitable as an immunosuppressive measure as has previously been found by Arnason et al. (1962). The two immunosuppressive treatments, i.e. Table 1: a high degree of immunosuppression by thymectomy combined with ALS and/or ATS and sometimes accompanied by X-irradiation, and Table 2: a medium degree of immunosuppression by ALS or ATS, were then implemented, together with Table 3: injection of transformed cells into newborn animals. Animals were observed for 2 to 2.5 years until a tumour appeared or until death.

Table 1 demonstrates that all the three HSV-2 transformed rat embryo cell lines RE HSV-
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Fig. 1. Diagrammatic representation of the time of appearance of tumours in animals inoculated with HSV-2-transformed rat embryo cells, expressed as a function of time in weeks and degree of immunosuppression. Classification on y axis: 1, rats injected as newborns, corresponding to Table 3; 2, rats immunosuppressed by ALS or ATS before injection, corresponding to Table 2; 3, rats highly immunosuppressed by X-irradiation and/or thymectomy together with ALS and/or ATS, corresponding to Table 1. Bar on x axis indicates weeks during which the tumours arose.

2-333, RE1 and RE7 formed tumours 12 to 16 weeks after injection into inbred rats which had been subjected to a high degree of immunosuppression by thymectomy accompanied by either ALS or ATS or both, and in some cases X-irradiation. The cells in all cases were injected subcutaneously (s.c.) into the back of the neck and the tumours arose at the site of inoculation.

Table 2 records the results of experiments performed on animals subjected to a medium degree of immunosuppression which consisted of treatment as newborns by ALS or ATS. Cells in this case were injected in different experiments either s.c. into the back of the neck or i.p. The latent period before tumours arose was 36 to 40 weeks after injection.

Table 3 shows the results of injecting transformed cells into animals from birth to 48 h old. Cells here were injected in different experiments both s.c. and i.p. Tumours arose 57 to 102 weeks after injection of the transformed cells. RE1 cells at passage 17 gave rise to tumours about 80 weeks after injection, while RE1 cells at passage 23 gave rise to tumours 57 weeks after injection. These results might suggest that continuous growth of the transformed cells in vitro before injection may select for cells which have a higher oncogenic potential. However, it is also difficult to inject all animals at exactly the same age in hours so some variation may be due to differences in immunocompetence after birth. Fig. 1 shows, by diagrammatic representation, the relative times of appearance of tumours in the host rat which had undergone different types of immunosuppression. RE7 cells occasionally caused the animals to die, even 1 year after injection and no tumours were seen. No specific cause of death was detected.

Most tumour experiments were done by injecting the transformed cells s.c. into the back
Table I. Oncogenicity of HSV transformed and control rat embryo cell lines into rats which had received a high degree of immunosuppression, i.e. thymectomy* together with ALS or ATS in some cases in combination with X-irradiation

<table>
<thead>
<tr>
<th>Immunosuppressive treatment</th>
<th>Age of rat on inoculation of cells (in days)</th>
<th>Cell type injected†</th>
<th>Passage no. of cell line injected</th>
<th>No. of cells injected</th>
<th>Route of inoculation</th>
<th>No. of tumours observed</th>
<th>LP‡ (in weeks) for particular type of immuno-suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymectomy + ALS + ATS§</td>
<td>40</td>
<td>RE1</td>
<td>18</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE1</td>
<td>21</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>15, 14, 3</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE1</td>
<td>23</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE7</td>
<td>17</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE7</td>
<td>20</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>14, 14, 0</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE7</td>
<td>23</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>12</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE HSV-2-333</td>
<td>19</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE HSV-2-333</td>
<td>20</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>14, 14, 7</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE HSV-2-333</td>
<td>23</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>Hood control</td>
<td>20</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td>Thymectomy + ALS + ATS</td>
<td>40</td>
<td>RE control</td>
<td>2</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

* Thymectomy as weanlings, i.e. 20 days old.
† RE1, rat embryo cells transformed by HSV-2 HG 52 ts 1 at NPT; RE7, transformed by HSV-2 HG 52 ts 7 at NPT; RE-HSV-2-333, transformed by u.v.-irradiated HSV-2-333; Hood control, rat embryo control cells serially passaged to form a control cell line; RE control, rat embryo primary or secondary control cells.
‡ LP, latent period between time of injection of transformed cells and appearance of tumour.
§ ALS, anti-lymphocyte sera, administered in three 0·1 ml doses at intervals of 2 days; ATS, anti-thymocytic sera administered in the same way as ALS.
Table 2. Oncogenicity of HSV transformed and control rat embryo cell lines into rats which had received a medium degree of immunosuppression by treatment of 24 h old rats with either ALS or ATS*

<table>
<thead>
<tr>
<th>Immunosuppressive treatment</th>
<th>Age of rat on inoculation of cells (in days)</th>
<th>Cell type injected†</th>
<th>Passage no. of cell line injected</th>
<th>No. of cells injected</th>
<th>Route of inoculation</th>
<th>No. of tumours observed</th>
<th>No. of rats inoculated</th>
<th>LP for tumour (in weeks)</th>
<th>Mean LP (in weeks) for particular type of immunosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td>7</td>
<td>RE1</td>
<td>17</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_1$</td>
<td>36-40</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>RE1</td>
<td>21</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_1$</td>
<td>36-40</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>RE1</td>
<td>22</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_1$</td>
<td>36-39</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>RE1</td>
<td>23</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_1$</td>
<td>36-38</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>RE7</td>
<td>18</td>
<td>$1 \times 10^6$</td>
<td>i.p.</td>
<td>$\text{T}_1$</td>
<td>36-45</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>RE7</td>
<td>21</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_1$</td>
<td>36-45</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>RE-HSV-2-333</td>
<td>17</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_0$</td>
<td>36-40</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>ATS</td>
<td>7</td>
<td>RE-HSV-2-333</td>
<td>21</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_0$</td>
<td>36-38</td>
<td>37</td>
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</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>Hood control</td>
<td>17</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_0$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>ATS</td>
<td>7</td>
<td>Hood control</td>
<td>20</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>$\text{T}_0$</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
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</table>

* In addition 14 rats treated with ALS or ATS or a combination of both were observed for 2 to 2½ years and no tumours were detected. Abbreviations as in footnote to Table 1.
† Rats injected with all these cell types were killed at 30 weeks after injection and examined for tumours. Although no tumours were found their littermates developed tumours at later dates.
Table 3. Oncogenicity of HSV transformed and control rat embryo cell lines in rats which had received no immunosuppression, i.e. cells were injected up to 24 h after birth*

<table>
<thead>
<tr>
<th>Immunosuppressive treatment</th>
<th>Age of rat on inoculation of cells (in days)</th>
<th>Tumour cell type injected†</th>
<th>Passage no. of cell line injected</th>
<th>No. of cells injected</th>
<th>Route of inoculation</th>
<th>No. of rats inoculated</th>
<th>No. of tumours observed</th>
<th>LP for tumour (in weeks)</th>
<th>Mean LP (in weeks) for particular type of immunosuppression</th>
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<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>RE1</td>
<td>17</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>52</td>
<td>80–95</td>
<td>-</td>
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<tr>
<td>None</td>
<td>1</td>
<td>RE1</td>
<td>21</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>4</td>
<td>75–82</td>
<td>75·6</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>RE1</td>
<td>19</td>
<td>$1 \times 10^6$</td>
<td>i.p.</td>
<td>4</td>
<td>75</td>
<td>83·6</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>RE7</td>
<td>23</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>5</td>
<td>74–82</td>
<td>79·3</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>RE7</td>
<td>20</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>1</td>
<td>58</td>
<td>70–80</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>RE-HSV-2-333</td>
<td>17</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>5</td>
<td>82–99</td>
<td>83·6</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>RE-HSV-2-333</td>
<td>19</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>4</td>
<td>74–82</td>
<td>79·3</td>
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<tr>
<td>None</td>
<td>1</td>
<td>RE-HSV-2-333</td>
<td>23</td>
<td>$1 \times 10^6$</td>
<td>i.p.</td>
<td>3</td>
<td>63–80</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>Hood control</td>
<td>19</td>
<td>$1 \times 10^6$</td>
<td>s.c.</td>
<td>5</td>
<td>--</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

* In addition 10 rats with no immunosuppressive treatment were observed over 2 to 2 ½ years and no tumours were detected. Rats injected with all these cell types were killed at 30 weeks after injection and examined for tumours. Although no tumours were found their littermates developed tumours at later dates. Abbreviations as in footnote to Table 1.
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of the neck, as it was thus easier to state definitely that the tumour arose at the site of inoculation. With s.c. inoculation of cells it was also easier to define true tumour metastases.

Hood cell lines (derived independently by serially passaging different rat embryo cultures) were used as control cell lines in these experiments. These cells were passaged a comparable number of times to the transformed cells and were injected into animals which had received high, medium or no immunosuppression. Observation of these animals over a period of 2 years showed no tumours arising due to inoculation of control cells. Animals subjected to each of the different forms of immunosuppression were also maintained in the laboratory but these did not show any signs of spontaneous tumours nor did they appear to be any more susceptible to disease than non-immunosuppressed animals kept similarly.

An attempt to make exactly comparable control cell lines was made on several occasions by picking clones of rat embryo cells from control plates in transformation experiments and passaging these in tissue culture. However, for various reasons these control cell lines were almost always lost, most probably due to the length of time the control plates are kept in transformation assays. No established control clones survived for any length of time. At this stage it was decided to make control lines by passaging mass culture of rat embryo cells. Little difficulty was found in forming control Hood lines in this way. Five Hood control cell lines were made but only two were tested in rats for tumorigenicity.

Transplantibility of the tumour cells and tumour cell lines

Three different tumours (Table 4) were examined by injecting $1 \times 10^5$ cells, produced as a single cell suspension by trypsinization of a tumour mass. One hundred per cent (29/29) of newborn injected animals developed tumours within 3 weeks. Animals up to a week old also gave 100% tumours on transplantation. Three tumours which were passaged twice in cell culture in vitro were similarly examined, i.e. at passage 3 for tumour induction, and they produced tumours in 100% of the animals within 3 to 4 weeks. The immunosuppressive treatment used in the rat which produced the original primary tumour did not appear to affect the transplantability of the actual tumour cells in newborn rats, i.e. tumour cells from highly immunosuppressed animals transplanted as well as those from non-immunosuppressed animals at passages up to passage 2.

However, three different tumours, derived from inoculation of RE1 cells into non-immunosuppressed animals were passaged for 10 to 12 passages in the laboratory, frozen in glycerol for 6 months and subsequently thawed and passaged a further 10 to 12 times. These cells, when injected s.c. at $1 \times 10^6$ cells per newborn animal, did not produce tumours until 6 to 12 months after injection of the cells. These results, although on a very small sample would seem to indicate that HSV tumour cells from ts transformed lines become less oncogenic with increasing passage in vitro. This result contradicts the result found with the transformed cells themselves. The result might also suggest that there are two steps involved in tumour production, i.e. the virus HSV and also a possible rat virus.

However, in all cases the primary and transplanted tumours were progressive, highly invasive and many showed metastases. Tumour regression was never seen in primary or transplanted tumours.

Routine post mortem

All tumour-bearing animals were given a routine post mortem. All highly immunosuppressed animals (Table 1) had small lymph nodes but no detectable metastases (as determined by the naked eye at post mortem), although their tumours were large and invasive. Confirmation of successful thymectomy was seen at post mortem. All animals
Table 4. Transplantability of tumour cells into non-immunosuppressed rats either as a single suspension after trypsinization of the tumour or after passage three times in tissue culture after formation of a tumour cell line

<table>
<thead>
<tr>
<th>Immunosuppressive treatment of animal in which tumour originally arose</th>
<th>Age of rat on inoculation of cells (in days)</th>
<th>Tumour cell type injected</th>
<th>No. of cells injected</th>
<th>Passage no. of tumour cell line</th>
<th>Route of inoculation</th>
<th>No. of tumours observed</th>
<th>No. of rats inoculated</th>
<th>LP for tumour (in weeks)</th>
<th>Mean LP (in weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>REI tumour</td>
<td>$1 \times 10^5$</td>
<td>—</td>
<td>s.c.</td>
<td>$\frac{10}{10}$</td>
<td>2-3</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Thymectomy + 400 R* (with shoes) + ALS + ATS</td>
<td>1</td>
<td>REI tumour</td>
<td>$1 \times 10^4$</td>
<td>3</td>
<td>s.c.</td>
<td>$\frac{10}{10}$</td>
<td>2-3</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>REI tumour</td>
<td>$1 \times 10^5$</td>
<td>—</td>
<td>s.c.</td>
<td>$\frac{10}{10}$</td>
<td>2-3</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>1</td>
<td>REI tumour</td>
<td>$1 \times 10^5$</td>
<td>—</td>
<td>s.c.</td>
<td>$\frac{10}{10}$</td>
<td>3-4</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Thymectomy + 400 R* (with shoes) + ALS + ATS</td>
<td>1</td>
<td>REI tumour</td>
<td>$1 \times 10^5$</td>
<td>3</td>
<td>s.c.</td>
<td>$\frac{10}{10}$</td>
<td>3-4</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>ALS</td>
<td>7</td>
<td>REI tumour</td>
<td>$1 \times 10^5$</td>
<td>—</td>
<td>s.c.</td>
<td>$\frac{10}{10}$</td>
<td>3-4</td>
<td>3.4</td>
<td></td>
</tr>
</tbody>
</table>

* R = X irradiation in rad emitted from a Cs 137 source of standard dose rate 112 rads/min. Shoes = small lead strip attached to leg to prevent total irradiation and allow regeneration of bone marrow cells. Other abbreviations as in footnote to Table 1.
Oncogenicity of herpesvirus transformed lines

with a medium degree of immunosuppression (Table 2), i.e. ALS or ATS, also had large invasive tumours but, again, true metastases were not seen as observed on macroscopic examination by the naked eye.

In the non-immunosuppressed animals, about 50% showed metastases in the lungs on macroscopic examination, a finding confirmed by histological examination of a sample of lung tissue by Dr A. Cochran, the primary tumour being an s.c. one in the back of the neck. Tumours frequently invaded skin, muscle and spinal cord.

In three animals, a small discrete s.c. tumour was removed under anaesthesia. All tumour tissue was apparently removed; however, in all cases, the tumour regrew within 3 to 5 months at the same site, i.e. s.c. in the back of the neck. Regrowth was in all cases invasive and the lungs were extensively invaded by tumour metastases.

Examination for tumours or metastases in animals showing no tumours
6 months after injection of transformed cells

A post mortem examination was carried out on about 20 rats which were injected when newborn with \(1 \times 10^6\) transformed cells and which showed no palpable tumours after 6 months. Post mortem examination revealed macroscopically no tumours either at the site of inoculation or in the lungs, liver, spleen or kidneys. These animals were littermates of rats which developed tumours after a latent period of 57 to 102 weeks. No tumours were detected at this stage, i.e. at 6 months after injection of the transformed cells, even in the animals where 100% of the remaining littermates developed tumours within 57 to 102 weeks.

Some rats were lost in these experiments due to middle ear infection, a condition which tends to affect this colony of inbred Hooded Lister rats.

Tumour cell lines

Twenty-one cell lines were established from different tumours representative of the different types of transformed cells and also of the different types of immunosuppression and of no immunosuppression. Lines grown from the highly immunosuppressed animals had the most regular appearance in monolayer cultures and were very similar to the transformed cell line from which they were derived. The three cell lines cultured from regrowth of the original tumour (after excision) presented the most bizarre morphological appearance, with many giant, polyploid and syncytial cells present when first cultured. It was found, however, that cultures with many bizarre cells when first explanted lost most of these abnormal cells on formation of a readily transferable line and the cells which passaged to form tumour cell lines were predominantly of fibroblastic morphology, resembling the transformed cells from which they were derived (Macnab, 1976) but with a greater amount of giant cells. No crisis phase was seen in the tumour cell lines in culture comparable to that described for transformed cell lines (Macnab, 1974).

In conclusion it may be said that the HSV transformed rat embryo cells showed invasive and frequently metastatic tumours in the inbred rat host whose time of expression was directly related to the degree of immunosuppression of the host rat.

Expression of herpes simplex information in the rat tumour cell lines
and in the tumour-bearing animals

It has already been stated that HSV transformed rat embryo cell lines express herpes simplex antigens as detected by immunofluorescence (Macnab, 1974, 1975, 1976).

Results of the immunofluorescence studies here were based on examination of 10 to 20 fields of each slide. Each experiment was done on a minimum of three and a maximum of seven different occasions.
Fig. 2. (a) Perinuclear and cytoplasmic fluorescence detected in RT13 tumour cell line (tumour induced by rat embryo cells transformed by u.v.-inactivated HSV-2-333) by an antiserum prepared in rats against the early polypeptides of HSV-2 HG 52 infected rat embryo cells. (b) Perinuclear and cytoplasmic fluorescence in T1 RT20 tumour cell line (transplanted RT20 tumour derived from original inoculation of rat embryo cells transformed by HSV-2 HG 52 ts 1) detected by an antiserum prepared in rats against the early polypeptides of HSV-2 HG 52 infected rat embryo cells.
Fig. 3. Surface fluorescence as detected in rat embryo cells 4 to 5 h after infection with HSV-2 HG 52 by antisera from a tumour bearing rat. (Cells had a 2 min fixation in glutaraldehyde before staining. This procedure shows surface fluorescence without penetration of the cell.)

(1) Detection of HSV antigens in fixed transformed tumour cell lines by the rat antiserum directed against the early proteins of HSV-2 infection. Ten out of 21 possible primary tumour cell lines were examined and showed perinuclear and cytoplasmic fluorescence of the type seen in Fig. 2(a) which is of the RT13 tumour cell line at passage 10. This tumour arose 36 weeks after the s.c. injection of rat embryo cells transformed by u.v.-irradiated HSV-2-333. The rat had been immunosuppressed by ALS. Fig. 2(b) demonstrates fluorescence of T1 RT20, a tumour cell line at passage 3 which arose one year after injection of the RT20 tumour cell line at passage 20. The RT20 tumour arose from the s.c. injection of rat embryo cells transformed by HSV-2 ts 1 (RE1 line) after a latent period of 84 weeks. The fluorescence in T1 RT20 is dispersed more throughout the cytoplasm than with the RT13 tumour cell line. Rat embryo control cells did not fluoresce with this antiserum, neither did control rat antiserum fluoresce HSV-transformed or control cells. The rabbit antisera, 4740 and 4741, prepared from cells infected by HSV-2 HG 52 ts 1 gave a similar pattern of fluorescence. However, control rabbit sera showed some degree of non-specific fluorescence with transformed cells. Eight other tumour cell lines which were examined gave similar patterns of immunofluorescence.

Immunofluorescence tests on the surface of four unfixed tumour cell lines showed that about 60% of the cells gave surface fluorescence in tests using the 4740 and 4741 rabbit antiserum prepared against HSV-2 HG 52 ts 1 infected cells. Control rat embryo cells did not fluoresce with this antiserum. Neither did control rabbit antiserum fluoresce with the surface of the tumour cell lines.

(2) Detection of HSV antibody in sera from tumour-bearing rats using the surface of unfixed or glutaraldehyde fixed rat embryo and human embryo lung cells infected with 1 to 2 p.f.u./cell of HSV-2 HG 52 4 to 5 h after release from a cycloheximide block. The
four different sera from tumour bearing animals which were tested showed surface fluorescence (Fig. 3) with both infected rat embryo and human embryo lung cells. These sera showed no fluorescence with control human embryo lung or rat embryo cells. Neither did control rat serum fluoresce the surface of rat embryo or human embryo lung cells.

Polyoma antiserum (kindly supplied by Dr J. F. Williams) and used as a control showed no immunofluorescence when tested with the 10 HSV transformed tumour cell lines, but gave bright fluorescence when reacted with polyoma transformed cells (Steinhauer, 1976).

In conclusion the cultured tumour cells showed cytoplasmic and surface fluorescence with two different HSV antisera and the sera from tumour-bearing animals reacted with HSV infected rat embryo and human embryo lung cells showing surface fluorescence.

Histology

Tumours which arose in animals subjected to different types of immunosuppressive treatment were histologically examined by Dr Alistair Cochran, University of Glasgow. All tumours were classified as pleomorphic spindle cell sarcomas with an abundance of collagen and reticulin. The lung metastases were histologically indistinguishable from the original primary tumour cells.

Examination of sections of tumours and of metastatic cells in the electron microscope by Dr Ian More, University of Glasgow, detected no C-type particles nor did a study of electron microscope sections in this department. No C-type particles were detected previously in the transformed cells when examined by electron microscopy or by assay for reverse transcriptase (Macnab, 1974, 1976).

DISCUSSION

The results presented in this paper (Tables 1 to 3) show a correlation between the immunocompetence of the host rat and the time taken (latent period) for a tumour to be formed after injection of HSV transformed cells. A high degree of immunosuppression in addition increased the incidence of tumours. A most unexpected and interesting finding was that tumours appeared in rats after a latent period of 2 years. The majority of tumour studies were in rats injected s.c. into the back of the neck and it was at this site of inoculation that the tumours arose even after the long latent period of 2 years. The fact that they arose at the site of inoculation would argue against the possibility that the tumours arose spontaneously. Preliminary karyological analysis (Macnab, 1976) indicates that, despite the long latent period in the immunocompetent rat, the tumour cell has markers corresponding to those in the injected transformed cell. All the tumours seen in this study were invasive and in 50% of the non-immunosuppressed rats metastases were seen by the naked eye in the lungs. Since the metastases were seen in animals bearing s.c. tumours in the back of the neck they were true metastases and not tumour spread, which is sometimes seen when transformed cells are injected intraperitoneally. Regression of tumours was never seen. As stated in the Results section, a post mortem examination of 20 rats showed no signs of tumours 6 months after injection of $1 \times 10^6$ transformed cells (injected as newborn rats). Littermates of these animals inoculated at the same time (in some cases 100% of the remaining litter), developed tumours within 57 to 102 weeks of injection of $1 \times 10^6$ transformed cells (again injected as newborn rats). Animals injected with RE7 cells sometimes died without developing tumours. The reason for this has not been determined. Kucera et al. (1977) have shown increased oncogenicity of HSV transformed cells selected in agar. Early work in transformation (Macnab, 1976 and unpublished results) showed that both transformed and control Hood (Hooded Lister rat embryo) cell lines eventually, but not initially,
developed the capacity to form colonies in soft agar. It was thus felt that selection in soft agar was not specific for HSV transformation in the system in use in these experiments.

The most striking differences in this HSV rat transformation system compared with the hamster system (Duff & Rapp, 1971, 1973; Macnab, 1974; Kimura et al. 1975) is that HSV transformed rat cells at fairly low passage numbers have the capacity to remain dormant or latent in vivo for lengths of time of 2 years (about two-thirds of the life span of the average laboratory rat) but that the eventual outcome of injection of such a cell line is almost always the formation of a tumour. It is not clear where these transformed cells remain in the rat during this long latent period, but the eventual tumour does arise at the site of inoculation. Since these results are produced in a highly inbred population of rats which is not susceptible to spontaneous tumours it may be that the genetic characteristics of this inbred strain are quite different from other experimental populations. The question which remains is whether or not transformation with HSV causes alterations in rat embryo cells, either by integration or some other mechanism, which confers on a small population of these cells an eventual tumorigenic potential. More tests on the eventual potential tumorigenicity of the rat embryo cell itself will be required. The tumorigenicity of the HSV transformed cell is being investigated at present at high passages and also in the nude athymic mouse.

No tumours have been seen to date in rats injected with either control Hood cell lines or with rat embryo cells at the passage numbers tested.

Immunofluorescence studies have shown the continuing expression of HSV information in at least 10 tumour cell lines as detected by an antiserum prepared in rats against the early proteins of HSV-2 HG 52 infection. Some cells show antigens accumulated in the nucleus and others show antigens spread throughout nucleus and cytoplasm.

Fig. 4. Rat embryo cells infected with HSV-2 HG 52 2 to 3 h after release from a cycloheximide block stained with antiserum made against the early proteins of HSV-2 HG 52 infection. Some cells show antigens accumulated in the nucleus and others show antigens spread throughout nucleus and cytoplasm.
polypeptides of HSV-2 infection. Antisera directed against these early proteins detect cytoplasmic and nuclear fluorescence in infected cells (Fig. 4). Perinuclear and cytoplasmic fluorescence is seen in the tumour cell lines (Fig. 2a, b). Fluorescence was similar in both but more perinuclear fluorescence was seen in the tumour cell line derived from the u.v.-inactivated transformed cell line. Almost 100% of the cells fluoresced. (The u.v.-inactivated HSV-2-333 used to transform the cell line was the same virus as that used by Duff & Rapp, 1971 and was kindly supplied by Dr Rapp.)

Sections of transformed and tumour cells examined by electron microscopy showed marginated chromatin (Macnab, 1976) as has previously been reported in the hamster transformed and tumour cells (Glaser et al. 1972) and has been seen in lytic infection (Rixon, 1977).

This margination of chromatin could account for the concentration of antigen detected around the nucleus in the transformed and tumour cell lines if such antigen was representative of DNA binding proteins. The early antiserum detects immediate early proteins, all of which are DNA binding proteins (R. T. Hay and J. Hay, unpublished). It will also, of course, detect later proteins since there is no block to HSV DNA synthesis after release from cycloheximide. These later proteins may also be DNA binding proteins. However, the location of HSV-specific antigens in the tumour cell lines (Fig. 2a, b) does not resemble that seen in lytic infection (Fig. 4) nor does it resemble nuclear antigens as detected in the thymidine kinase transformation system (Kurchak et al. 1977). Rixon (1977) has detected margination of chromatin in lytically infected cells as early as 3 to 5 h after infection. In the lytic cells here, however, fluorescence is detected throughout the nucleus at 2 to 3 h after release from the cycloheximide block. A similar problem in location of fluorescence has been cited by Flannery et al. (1977) who specifically detected an early non-structural antigen (VP143) in HSV-transformed and tumour hamster cells.

The method of preparation of the rat cells for fluorescence, which does not aim to swell or spread the nucleus, may not be suitable for detecting the real distribution of antigen within the transformed system cell lines. Further studies on this problem are in progress.

The rabbit antisera 4740 and 474I prepared against a 24 h infection of RK cells by HSV-2 HG 52 ts I gave a similar pattern of fluorescence to the other antisera. In addition, these rabbit antisera detected cell surface fluorescence of 60% of the tumour cells. These sera, 4740 and 474I, contain antibodies to immediate early, early and late proteins accumulated over 24 h: it has been shown that infection with ts I at NPT leads to an accumulation of immediate early antigens which are not processed or degraded to the same extent as wild type infection. (J. C. M. Macnab, unpublished results of fluorescence studies). The sera of four tumour-bearing rats fluoresced the cell surface of cells 4 to 5 h after infection with HSV-2, HG 52 implying the presence of early polypeptides being expressed in the tumour cells and detecting antibody response in the tumour-bearing animals. Within the rat system, antisera did not present problems of non-specific fluorescence and the controls were all negative. The results at present indicate that in the proportion of HSV tumour cell lines tested specific HSV information is expressed.

The continuing expression of HSV information in the tumour cell lines, however, does not resolve to any extent the actual role of HSV in oncogenic transformation although the results of these studies suggest that cells transformed by HSV have an eventual, if latent, oncogenic potential in vivo and also that lines formed from such tumour cells continue to express HSV information in almost 100% of the cells.

No studies are at present available as to the state of the HSV genome in these rat-transformed and tumour cell lines.
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