Sensitivity of the Transforming and Replicative Functions of Epstein–Barr Virus to Inhibition by Phosphonoacetate

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SUMMARY

Disodium, phosphonoacetate (PA), at concentrations of 50 to 200 μg/ml, which still allowed continued growth of the EB virus-transformed B95-8 cell line on a routine culture regimen, was able to inhibit the production of virus capsid antigen and of virus particles by these cells down to very low but finite levels which persisted despite prolonged treatment. Further experiments measured the effects of these same drug concentrations on the EB virus-induced in vitro transformation of foetal cord blood lymphocytes and on the colony forming ability of already established EB virus-transformed foetal cell lines; in both types of culture, doses of PA up to and including 50 μg/ml did not affect cell growth within the 8-week observation period, whereas doses of 100 μg/ml and above were increasingly inhibitory. The cell lines established by EB virus-induced transformation in the continual presence of PA at 50 to 150 μg/ml contained multiple copies of the virus genome per cell just as did the corresponding cell lines established in control medium. The results argue against the existence of any PA-sensitive event unique to the EB virus-induced transformation process.

INTRODUCTION

Epstein–Barr (EB) virus has the capacity to infect human bone marrow-derived (B-) lymphocytes (Jondal & Klein, 1973; Pattengale et al. 1973; Greaves et al. 1975) and to induce their transformation in culture to permanent lymphoblastoid lines (Henle et al. 1967; Pope et al. 1968) in which essentially every cell carries multiple circular copies of the EB virus genome in some stable but non-integrated association with cellular DNA (zur Hausen et al. 1972; Lindahl et al. 1976), and expresses the virus-associated nuclear antigen (EBNA; Reedman & Klein, 1973). The precise sequence of events which must occur in an infected cell before the establishment of the transformed state is little understood, although it must clearly include the induction of EBNA and probably also the generation of multiple copies of the incoming virus genome by some process of amplification.

Although replication of the virus with the production of mature virus particles is certainly unconnected with the in vitro transformation process, in certain 'producer' lymphoblastoid cell lines at any one time a small fraction of cells ceases to proliferate and enters a cycle of virus production culminating in cell death and the release of infectious virus particles. In these producer lines, a proportion of the cells express early membrane antigen (EMA; Klein et al. 1966; Ernberg et al. 1974) and continue to proliferate until early antigen (EA; Henle et al. 1970) appears, at which stage host cell macromolecular syntheses are inhibited (Gergely et al. 1971) and cell death becomes inevitable. EA expression is followed
by the initiation of virus DNA synthesis and later by the appearance of virus capsid antigen (VCA; Henle & Henle 1966) and of late membrane antigen (LMA; Ernberg et al. 1974) as the cell begins to degenerate. However, movement into a productive cycle does not guarantee the complete synthesis of new virus particles as the infection can be aborted and the cells may die at any one of the intermediate stages.

The relationship between the virus-transformed state and the cycle of productive infection is delicately balanced and known to be subject to experimental manipulation (Gerber, 1972; Hampar et al. 1972). Recent work has shown that disodium phosphonoacetate (PA), which prevents herpes simplex virus replication in concentrations which are non-toxic for permissive monolayer cultures (Overby et al. 1974), inhibits EB virus replication (Summers & Klein, 1976; Nyormoi et al. 1976; Yajima et al. 1976); there are also suggestions that the EB virus-induced transformation process may include a step which is likewise unusually sensitive to the drug (Thorley-Lawson & Strominger, 1976). The present paper reports an investigation into the sensitivity of both the transforming and the replicative functions of EB virus to inhibition by PA and at the same time describes more exactly the effects of the drug on the growth rate of cells already established in culture following transformation by the virus.

METHODS

Stock cultures. Cultures of the B95-8 (Miller & Lipman, 1973), P3HR-1 (Hinuma et al. 1967), and Raji (Epstein et al. 1966) cell lines and of recently established lines of foetal cell origin were grown in RPMI 1640 medium with 2 mM-glutamine, 100 international units (i.u.)/ml penicillin, 100 µg/ml streptomycin and 10% foetal calf serum, in stoppered 50 ml flasks at 37 °C and passaged by weekly 1:1 sub-culture.

Phosphonoacetate. Disodium phosphonoacetate was a generous gift from Abbott Laboratories, Chicago, U.S.A. The powder was dissolved in culture medium at a concentration of 10 mg/ml and the solution stored in small volumes at 20 °C until required.

Immunofluorescence tests. Cells were stained for the EB virus-associated antigens VCA and EA by standard methods (respectively, Henle & Henle, 1966; Henle et al. 1970). The tests were carried out using a strong EA+/VCA+ serum (kindly supplied by Dr G de Thé, IARC, Lyon), two EA−/VCA+ sera and two EA−/VCA− control sera. Slides prepared from P3HR-1 cells (EA+/VCA+), bromodeoxyuridine-treated Raji cells (EA+/VCA−) and untreated Raji cells (EA−/VCA−) served as appropriate positive and negative controls. Counts to determine the incidence of VCA-positive and EA-positive cells in test preparations were made on at least 1000 cells per slide. The bromodeoxyuridine treatment of Raji cells was carried out by the method of Sugawara et al. (1972).

Preparation and titration of B95-8 culture supernatants. Supernatant fluid was carefully pipetted from B95-8 cultures, spun at 500 g for 10 min, and used either fresh or after storage in small volumes at −70 °C. The titre of EB virus in such preparations was determined by a foetal lymphocyte transformation assay carried out in microtest plate wells (Nunclon, A/S Nunc, Denmark) as described by Moss & Pope (1972).

Preparation of mononuclear cells. Twenty to 50 ml blood samples were taken into heparinized syringes (20 units heparin/ml) from the cord of full term deliveries and the mononuclear cells were separated as described by Böyum (1968). In some cases, thymus-dependent (T-) lymphocytes were selectively removed from foetal mononuclear cell suspensions by centrifugation after rosetting with sheep erythrocytes as described elsewhere (Rickinson et al. 1977a); the remaining T-cell-depleted population was then washed twice in culture medium and cell counts made using a Coulter counter (Model B).
Sensitivity of EB virus to phosphonoacetate

Infection with EB virus. Cells were resuspended at a concentration of 10^2/ml in a 1:10 dilution of supernatant fluid from 7-day-old cultures of the B95-8 cell line. After a 60 min incubation at 37 °C, during which the cell suspension was shaken at regular intervals, the cells were centrifuged at 150 g for 5 min, washed twice and resuspended in fresh culture medium at a known cell concentration.

Pokeweed mitogen (PWM). PWM (Cat. no 53G, Gibco Bio-cult Ltd, Paisley, Scotland) was reconstituted in the prescribed volume of sterile distilled water and the solution added to culture medium at a concentration of 5 μl/ml.

Radiolabelling. ³H-6-thymidine (sp. act. 5 Ci/mm; Radiochemical Centre, Amersham, England) was added to cultures at a concentration of 0.5 μCi/ml for the final 24 h of the culture period. The cells were collected on glass fibre filters and given two washes in 5% trichloroacetic acid, two washes in phosphate-buffered saline and two washes in methanol; all washing procedures were carried out at 4 °C. The filters were then placed in scintillation vials and exposed overnight to 0.4 ml Biosolv BBS-3 solubilizer (Beckman, Fullerton, California, U.S.A.) before the addition of 10 ml scintillation fluid and counting in an NE 8312 scintillation counter (Nuclear Enterprises, Edinburgh).

Hybridization. Hybridization procedures were kindly carried out by Dr M. Andersson-Anvret of the Department of Tumor Biology, Karolinska Institute, Stockholm. Preparations of DNA from the various cell samples and hybridization with pre-calibrated ³²P-labelled EB virus complementary RNA were carried out according to Lindahl et al. (1976). DNA preparations from the EB virus genome-negative Molt 4 cell line served as a negative control; artificial mixtures of Molt 4 DNA and purified EB virus DNA served as standards against which to assess the number of EB virus genome equivalents per test cell.

Experimental procedure

Effects of PA on B95-8 cell growth and EB virus production. B95-8 cells (10^5/ml) were cultured in medium containing PA at various concentrations up to 300 μg/ml and after 2, 4, 6 and 8 days of growth, cell counts were made on duplicate cultures from each drug dose. In addition, cells were cultured for 4 weeks as above in the presence of PA at various concentrations up to 200 μg/ml by harvesting the cells at weekly intervals and re-seeding half into fresh medium containing the appropriate drug concentration. The remaining cells from each harvest were immediately fixed on slides for EA and VCA testing, while the supernatant fluids were titrated for transforming EB virus.

Effects of PA on EB virus-induced and mitogen-induced cell proliferation. Cord blood mononuclear cells were either (a) depleted of T-lymphocytes, infected with EB virus, and seeded in vials (Sterilin, 5 cm by 1.5 cm diam.) at a density of 10^6 cells in 1 ml medium containing PA at various concentrations up to 300 μg/ml, or (b) cultured at a similar cell density in the presence of PWM in medium likewise containing various concentrations of PA. The cultures were labelled with ³H-thymidine either from day 3 to 4 or from day 6 to 7 and the degree of labelling measured by scintillation counting. Background labelling at each drug concentration was established from parallel cultures not exposed to EB virus or PWM.

Effect of PA on EB virus-induced transformation of foetal lymphocytes. Cord blood mononuclear cells were exposed to a range of tenfold dilutions of a standard EB virus preparation (see Rickinson et al. 1977a) and were then seeded into microtest plates at a density of 2.5 × 10^5 cells per well in 0.2 ml medium; uninfected control cells from the same foetal donor were set up in parallel. PA was added to the cultures at a range of concentrations up to 150 μg/ml either on the day of seeding or 6 days later. The plates were checked over 8 weeks for foci of transformed lymphoblastoid cells (Rickinson et al. 1974); confirmation
of transformation in selected cultures was demonstrated by growth after sub-culture. The efficiency of virus-induced transformation at each of the concentrations of PA was defined as the negative log$_{10}$ of that virus dilution which was sufficient to induce transformed foci in 50% of replicate cultures, calculated by the method of Reed & Muench (1938).

Effect of PA on colony-forming ability of EB virus-transformed foetal cell lines. The cell lines were obtained during the previous experiments by sub-culturing foci of transformed cells which arose in virus-infected PA-free cultures. Known numbers of these transformed cells (between $3 \times 10^3$ and 10) were seeded into microtest plate wells over a feeder layer of $10^5$ cord blood mononuclear cells. PA was added at a range of concentrations up to 150 μg/ml either on the day of seeding or 6 days later, and the cultures were observed over 8 weeks for the outgrowth of the transformed cells. The colony forming efficiency at each PA concentration was defined as 100 divided by that number of cells per well which was sufficient to produce successful outgrowth in 50% of cultures, calculated by the method of Reed & Muench (1938).

EB virus genome content of cell lines established in PA. The number of EB virus genome equivalents per cell was determined for lines established by virus-induced transformation of foetal cells in culture medium containing PA at concentrations up to 150 μg/ml. Groups of cell lines from each of two foetal donors were tested by nucleic acid hybridization.
**RESULTS**

**Effects of PA on B95-8 cell growth and EB virus production**

Growth curves of B95-8 cells cultured for 8 days in the presence of PA are shown in Fig. 1. A concentration of 100 µg/ml did not affect the increase in cell numbers, whereas higher doses were clearly inhibitory. Nevertheless it proved possible to maintain B95-8 cells on a weekly 1:1 sub-culture for many weeks in the presence of PA at concentrations up to 200 µg/ml and Fig. 2 shows the level of VCA-positive cells in these cultures. It can be seen that PA at 25 µg/ml had a moderate effect on VCA expression whereas drug doses of 50 to 200 µg/ml caused a much greater reduction which was apparent as early as 4 days from the beginning of treatment, but which never became complete. A few brightly fluorescent cells could be seen in cultures stained with EA−/VCA+ serum even after 28 days of high dose PA treatment, while no such cells were present in duplicate slides stained with EA−/VCA− control serum. Slides stained with EA+/VCA+ serum showed a 6 to 8% incidence of fluorescent cells irrespective of the strength or duration of drug treatment (data not shown).
The EB virus transforming titre of culture supernatants after 7 days treatment with PA at 50 to 200 μg/ml was reduced by 2 log units to about 1% control values (Fig. 3); it remained at this level with continued treatment at 50 μg/ml, but fell to 0.1% of control values after 28 days at 200 μg PA/ml (Fig. 3).

**Effects of PA on EB virus-induced and mitogen-induced cell proliferation**

EB virus-infected T-cell-depleted mononuclear cell cultures and PWM-stimulated total mononuclear cell cultures showed similar absolute levels of 3H-thymidine incorporation in the presence of PA, and in both cases this was much greater at 6 to 7 days than at 3 to 4 days (Fig. 4). EB virus-induced and PWM-induced cellular DNA synthesis thus appeared to be similar in their sensitivity to the drug and in both cases the counts fell significantly below control values once drug concentrations above 100 μg/ml were employed.

**Effect of PA on EB virus-induced transformation of foetal lymphocytes**

Table 1 gives detailed results from one experiment of the third set in which EB virus-infected foetal cells were grown in increasing concentrations of PA and observed to detect the incidence of transformation. It is evident that doses of 100 μg PA/ml or more impaired the emergence of transformed foci even where the drug was only added 6 days post-infection. These results were used to derive an efficiency of transformation for each type of culture and the values obtained in this and four other experiments, are presented in Fig. 5. Doses of PA up to 50 μg/ml did not affect the efficiency of transformation whilst doses of 100 μg/ml or more had an inhibitory effect; this was somewhat more pronounced where the drug was added at the initiation of culture rather than on day 6.
**Table 1.** Incidence of transformation in cultures of EB virus-infected foetal lymphocytes in the presence of PA either from the beginning (day 0) or from day 6 of culture

<table>
<thead>
<tr>
<th>Dilution of virus used in infection</th>
<th>Incidence of transformation in EB virus-infected cultures containing 10 μg PA/ml added on day 0</th>
<th>Incidence of transformation in EB virus-infected cultures containing 10 μg PA/ml added on day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0  25  50  75  100  150</td>
<td>0  25  50  75  100  150</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>6/6  6/6  6/6  6/6  6/6  3/6</td>
<td>6/6  6/6  6/6  6/6  6/6  6/6</td>
</tr>
<tr>
<td>10⁻²</td>
<td>6/6  6/6  6/6  6/6  6/6  4/6</td>
<td>6/6  6/6  6/6  6/6  6/6  6/6</td>
</tr>
<tr>
<td>10⁻³</td>
<td>6/6  6/6  5/6  3/6  6/6  0/6</td>
<td>6/6  6/6  6/6  6/6  6/6  6/6</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>2/6  3/6  3/3  2/6  0/6  0/6</td>
<td>1/6  1/6  1/6  0/6  0/6  0/6</td>
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<tr>
<td>10⁻⁵</td>
<td>0/6  0/6  0/6  0/6  0/6  0/6</td>
<td>0/6  0/6  0/6  0/6  0/6  0/6</td>
</tr>
</tbody>
</table>

**Fig. 5.** Efficiency of transformation (negative log₁₀ 50% transforming virus dilution) of freshly prepared foetal mononuclear cells infected with EB virus and then either (a) cultured in the presence of PA at various concentrations from the beginning of culture (day 0) or (b) cultured in normal medium, and then PA added on day 6 of culture.

**Effect of PA on colony forming ability of EB virus-transformed foetal cell lines**

The results from the fourth set of experiments are presented in a somewhat similar way in Table 2 and Fig. 6. Where progressively smaller numbers of cells from a recently established cell line were seeded in the presence of increasing PA concentrations, the incidence of successful colony formation by the transformed cells did not appear to be seriously affected until a dose of 100 μg PA/ml had been reached (Table 2); again the effect was more marked...
Table 2. Incidence of successful colony formation by EB virus-transformed cells cultured in the presence of PA either from the beginning (day 0) or from day 6 of culture

<table>
<thead>
<tr>
<th>Number of transformed cells seeded per culture*</th>
<th>Incidence of colony formation by EB virus-transformed cells in cultures containing</th>
<th>µg PA/ml added on day 0</th>
<th>µg PA/ml added on day 6</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>0 25 50 75 100 150</td>
<td>0 25 50 75 100 150</td>
</tr>
<tr>
<td>$3 \times 10^3$</td>
<td></td>
<td>6/6 6/6 6/6 6/6 6/6 6/6</td>
<td>6/6 6/6 6/6 6/6 6/6 6/6</td>
</tr>
<tr>
<td>$10^3$</td>
<td></td>
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<td>6/6 6/6 6/6 6/6 6/6 6/6</td>
</tr>
<tr>
<td>$3 \times 10^2$</td>
<td></td>
<td>6/6 6/6 6/6 6/6 6/6 6/6</td>
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<tr>
<td>$10^2$</td>
<td></td>
<td>5/6 5/6 5/6 5/6 5/6 5/6</td>
<td>5/6 5/6 5/6 5/6 5/6 5/6</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>1/6 1/6 1/6 1/6 1/6 1/6</td>
<td>1/6 1/6 1/6 1/6 1/6 1/6</td>
</tr>
</tbody>
</table>

* Known numbers of EB virus-transformed cells were co-cultivated in microtest plate wells with a feeder layer of $10^5$ freshly prepared foetal mononuclear cells.

Fig. 6. Efficiency of colony formation (100/number of cells required to produce colonies in 50 % of wells) of EB virus-transformed foetal cell lines when seeded at low density in microtest plate wells and then either (a) cultured in the presence of PA at various concentrations from the time of seeding (day 0) or (b) cultured in normal medium, and then PA added on day 6 after seeding.
Table 3. **Number of EB virus genome equivalents per cell in lines established by in vitro transformation of foetal cells in the presence of PA**

<table>
<thead>
<tr>
<th>Source of cell line</th>
<th>Concentration of PA (μg/ml)</th>
<th>Ct/min EB virus cRNA hybridized to cell DNA*</th>
<th>EB virus genome equivalents/cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor R + EBV</td>
<td>0</td>
<td>1490, 1727</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1905, 1620</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1978, 1865</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>2151</td>
<td>14</td>
</tr>
<tr>
<td>Donor P + EBV</td>
<td>0</td>
<td>3799, 3649</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1761, 2163</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>1720</td>
<td>11</td>
</tr>
</tbody>
</table>

* Ct/min after subtraction of a blank (counts retained by filter-containing Molt 4 DNA) from the standard mixtures of Molt 4 and EB virus DNA included in the assay; one EB virus genome equivalent per cell gives 156 ct/min.

in cultures given the drug from the beginning of the experiment. The efficiency of colony formation for each type of culture in this and three further experiments is shown in Fig. 6.

**EB virus genome content of cell lines established in PA**

The nucleic acid hybridization studies on foetal cells transformed *in vitro* by EB virus in the continual presence of PA at concentrations up to 150 μg/ml show that there was either no difference or only a slight reduction in the number of genome equivalents per cell as compared to the number in cells of the control cultures (Table 3).

**DISCUSSION**

Non-cytotoxic concentrations of phosphonoacetic acid (PAA), of the order of 100 μg/ml, appear to block the replication of several herpesviruses by inhibiting a virus-induced DNA polymerase activity (Mao *et al.* 1975; Huang *et al.* 1976; Leinbach *et al.* 1976). More recent studies have shown that EB virus replication is also sensitive to PAA at concentrations of 30 to 100 μg/ml, although the degree of inhibition and its dose dependence have not been accurately defined (Summers & Klein, 1976; Nyormoi *et al.* 1976; Yajima *et al.* 1976).

The results of the first set of experiments in the present investigation confirm that 50 to 200 μg/ml concentrations of PA (equivalent to 40 to 160 μg PAA/ml), which still allowed the continued growth of B95-8 cells (Fig. 1), did indeed drastically reduce the synthesis of both EB VCA and infectious virus particles (Fig. 2 and 3), but show for the first time that this inhibition of virus production is not complete. A very small number of unequivocally VCA-positive cells could always be found in cultures sampled during 4 weeks of continuous drug treatment (Fig. 2) and supernatant fluids from the cultures continued to show residual low levels of lymphocyte transforming ability (Fig. 3). Measurement of these low levels of virus replication can be confidently made using the lymphocyte transformation assay (Moss & Pope, 1972) which thus has a great advantage in these circumstances over the less sensitive ³H-thymidine uptake assay adopted by Nyormoi *et al.* (1976). The present demonstration of the incomplete nature of the PA inhibition raises the interesting possibility of selecting for PA-resistant mutants of EB virus by growing producer cells for long periods in medium containing the drug.

The ability of PA to inhibit EB virus replication at apparently non-cytotoxic concentrations suggested that this drug might be important for the experimental elucidation of certain aspects of EB virus-cell relationships (Rickinson *et al.* 1977b, c). For this, however, the
effects of the drug on cell growth needed to be carefully defined. The results from the second
set of experiments (Fig. 4) show that the inhibitory effects of PA on cellular DNA synthesis
induced by EB virus or PWM are essentially similar and appear at doses above 100 μg PA/ml,
whether assayed after 3 to 4 or 6 to 7 days of culture. In none of the four experiments in
this set was there any indication that virus-induced proliferation was especially sensitive
to inhibition by PA. This is in direct contrast to the findings of Thorley-Lawson & Stromin-
ger (1976) who reported that the EB virus-induced transformation process was much more
sensitive to inhibition by PAA added within 3 days of infection than was mitogen-induced
lymphocyte proliferation and suggested that some special PAA-sensitive transformation
event was occurring in the cells in the first 3 days after infection. This discrepancy in results
is difficult to explain on technical grounds, except that in the present experiments the response
to PWM was measured using unfractionated foetal mononuclear cells whilst Thorley-
Lawson & Strominger (1976) used pure peripheral B-cell populations from adult donors.
Further confirmation of the results reported here was obtained from the experiments in
which foetal lymphocytes from five separate cord blood samples were infected with EB
virus and then observed for transformation while growing for 8 weeks in the presence of
PA (Fig. 5). Thus PA doses up to and including 50 μg/ml clearly did not affect the efficiency
of transformation, whereas doses of 100 μg/ml and more had an increasingly inhibitory
effect. The data from an individual experiment of this type, shown in Table 1, demonstrate
that the establishment of transformed foci can indeed be prevented by the presence of PA
at 100 μg/ml, the very result obtained first by Thorley-Lawson & Strominger (1976) and
more recently by Lemon et al. (1978), but show that this effect is only clear-cut at particular
virus dilutions. Moreover, the present results fail to reveal any dramatic difference between
cultures receiving the drug immediately after infection and those to which the drug was
added 6 days later thus arguing against the existence of some PA-sensitive event crucial to
EB virus-induced lymphocyte transformation occurring within a few days of infection
(Thorley-Lawson & Strominger, 1976).

When, in the next set of experiments, the effect of PA on the growth of established EB
virus-transformed cells was measured using a sensitive 8-week colony forming assay, the
results (Table 2 and Fig. 6) were, in all important respects, similar to those obtained from the
transformation assay; in particular, cell growth is not significantly affected by PA doses up
to 50 μg/ml but is increasingly inhibited at doses of 100 μg/ml and above. Here again,
delaying the addition of PA until day 6 of culture made no dramatic difference to the subse-
quent yield of colonies, although it did allow slightly better outgrowth in cultures maintained
in the higher drug concentrations.

The close similarity in results from the transformation assay with EB virus-infected foetal
lymphocytes (Fig. 5) and from the colony forming assay with EB virus-transformed EBNA-
positive lymphoid cell lines recently established from the same foetal donors (Fig. 6) strongly
suggests that the inhibitory effect of PA on virus-induced transformation is not achieved by
blocking some special event in the transformation process, but merely reflects a capacity of
the drug to impair the outgrowth of virus-transformed EBNA-positive cells into recogn-
zible foci. This view is consistent with the observation that when PA inhibits virus-induced
transformation, it does so by interfering with some event other than the induction of
EBNA in the infected cells (Thorley-Lawson & Strominger, 1976). Clearly the ability of the
drug to impair the growth of virus-transformed cells is only revealed fully by colony-forming
assays of the type reported here and not by the short-term growth assays adopted in earlier
work (Nyormoi et al. 1976; Thorley-Lawson & Strominger, 1976; Lemon et al. 1977;
Fig. 1 and 4 of the present paper).
Sensitivity of EB virus to phosphonoacetate

It must be stressed that, just as EB virus-transformed cells from established lines will continue to grow and give colonies in PA at 100 to 150 μg/ml if seeded initially in sufficient numbers (Table 2), so too freshly prepared mononuclear cells infected with EB virus and cultured at these same drug doses, will give rise to transformed foci and yield a cell line (Table 1) providing the initial virus dose is sufficiently high (i.e. multiplicities of 0.01 to 0.1 transforming units per cell).

When the cell lines established by EB virus-induced transformation in the presence of PA at 50 to 150 μg/ml were investigated by nucleic acid hybridization, they were found to be carrying multiple copies of the virus genome per cell just as were the corresponding lines established in control medium (Table 3). This makes it clear that concentrations of PA which are sufficient to inhibit EB virus replication almost completely do not affect those events whereby the incoming virus genome is amplified during virus-induced transformation. If PA does indeed block EB virus replication by inhibiting the virus-induced DNA polymerase activity recently recognized in productivity infected cells (Miller et al. 1977), then this enzyme seems unlikely to be involved in the amplification event referred to above. The timing of this amplification of the virus genome and its relationship to the overall transformation process remain fascinating but unsolved problems.

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