Effects of Acyclovir on Herpes Simplex Virus Type 1 Infection in Mice Treated with 12-O-Tetradecanoylphorbol 13-acetate

By P.-A. LARSSON, 1* S. L. JOHANSSON, 3 J.-M. HIRSCH 2 AND A. VAHLNE 1

Departments of 1Clinical Virology, 2Oral Surgery, University of Göteborg, Guldheds gatan 10b, S-413 46 Göteborg, Sweden and 3Department of Pathology and Microbiology, University of Nebraska Medical Center and Eppley Institute for Research on Cancer and Allied Diseases, Omaha, Nebraska 68182, U.S.A.

(Accepted 6 March 1989)

SUMMARY

The purpose of this study was to determine whether infectious herpes simplex virus type 1 (HSV-1) has tumorigenic properties and, if so, whether inhibition of the cytolytic replicative cycle of the virus after infection enhances tumour development. Eighty mice were subjected to repeated inoculation of HSV-1 on their upper lips after scarification, and systemic administration of acyclovir (ACV). 12-O-tetradecanoylphorbol 13-acetate (TPA) was used as the tumour promoter. The tumour incidence was compared to control groups each of 40 mice that were either not treated with ACV, not treated with TPA, not infected with HSV or only scarified. In the virus-infected group treated with ACV and TPA, 25% of the animals developed tumours. In the HSV-infected group treated with TPA only, 25% of the animals also developed tumours. The uninfected animals which were not treated with TPA, not infected with HSV or only scarified. In the virus-infected group treated with ACV and TPA, 25% of the animals developed tumours. In the HSV-infected group treated with TPA only, 25% of the animals also developed tumours. The uninfected animals which were not treated with TPA developed tumours to a significantly lesser degree. In conclusion, the combined effects of HSV-1 and TPA, with or without ACV treatment, resulted in a significant increase in the number of tumours in comparison to the control groups.

INTRODUCTION

The role of herpes simplex virus (HSV) in human cancer has been the subject of great interest and controversy. It has clearly been established that inactivated HSV-1 is capable of transforming cells in vitro (Rapp, 1980) and circumstantial evidence supports the conclusion that HSV is associated with some human cancers (Shillitoe & Silverman, 1979).

Burns & Murray (1981) reported that inoculation of HSV-2 on abraded mouse lips followed by u.v. irradiation and exposure to 12-O-tetradecanoylphorbol 13-acetate (TPA) application resulted in the development of both squamous cell carcinomas and papillomas of the lip. They concluded that the inactivation of HSV by u.v. irradiation after infection allowed the virus to express its inherent oncogenic capacity when combined with the tumour promoter. Repeated exposure or persistent infection by HSV has previously been suggested as a possible mechanism for oncogenic transformation but reports confirming this are lacking. However, long term administration of snuff in combination with repeated HSV infections results in the development of squamous cell carcinomas both in the oral cavity of rats (Hirsch et al., 1984a) and in the cheek pouch of Syrian hamsters (Park et al., 1985). The carcinogenic effects of the combination of tobacco and HSV have been explained by the inhibitory capacity of tobacco extracts on cytolytic HSV infections (Hirsch et al., 1984b). The tumorigenic effect of the combination of HSV and tobacco is not localized at the site of application as tumours are also found at distant sites in rats exposed to snuff after HSV infection (P.-A. Larsson et al., unpublished results).

Acyclovir (ACV) is a specific antiviral drug that blocks viral replication by acting as a terminator of DNA elongation. Long term bioassay studies of ACV have not demonstrated any
carcinogenic properties in rodents, nor has it shown any transforming effects in eukaryotic cells (Tucker, 1982).

The present study was designed to determine whether exposure of mouse lips to infectious HSV-1, with or without the administration of a tumour promoter, would result in tumour development and whether inhibition of the cytolytic replicative cycle of the virus would then enhance tumour incidence. Systemic ACV treatment was used for inhibition of HSV-1 replication and local application of TPA was used for tumour promotion.

METHODS

Mice. Seven-week-old female and male mice (Swiss Albino, own breed) were kept in plastic cages, in 12 groups of 20. Males and females were kept separately. The animals were fed a standard pelleted diet (Astra Ewos) and tap water ad libitum. Temperatures in the animal quarters were kept at 22 ± 2 °C and the light/dark cycle was 12 h.

Chemicals. ACV was kindly supplied by Burroughs-Wellcome and TPA (1.425 mg/ml) was purchased from Sigma.

Virus. HSV-1 strain F (1.3 × 10⁷ p.f.u./ml) was kindly supplied by B. Roizman. The techniques for preparation of virus stock suspensions and for the plaque assay of virus in GMK cells have been described in detail elsewhere (Vahlne et al., 1981).

Experimental design. This is summarized in Table 1. The experiment started when the animals were 7 weeks old and their initial weight was, on average, 20 g. The 240 animals were divided into four groups (II to V) of 40, with 20 of each sex in each group, and one group (I) of 80 mice, 40 males and 40 females. All animals were scarified on both sides of the upper lip once a month. In groups I, II and III, virus suspension was thereafter applied to the scarified area of the lip; the animals in groups IV and V were mock-infected. The virus suspension was applied topically in a total volume of 25 µl at each application. Two of the virus-exposed groups (I and III) and one of the mock-infected groups (IV) received 0.5 mg ACV by intraperitoneal injection, at days 2, 4 and 6 after each inoculation. Two of the virus-infected groups (I and II) and one of the mock-infected groups (IV) were also exposed to TPA which was applied every 2nd, 4th and 6th day during weeks 2, 3 and 4 of each month. Ten µl of the drug was applied topically to the lip each time. TPA treatment was continued for 13 months in total. All mice were observed every other day until the experiment was terminated after 15 months when the mice were sacrificed by ether anaesthesia.

Morphological methods. The mice were killed at the termination of the study and underwent a complete post-mortem examination. Microscopic examination was performed on the lip, heart, lung, forestomach, liver, urinary bladder, kidneys, spleen and grossly abnormal organs or tissue. The specimens for this were fixed in 4% buffered formalin solution and embedded in paraffin. Sections (5 µm) were stained with haematoxylin and eosin according to the Weigert-van Gieson method.

Serological analyses. At the end of the experiment 1 ml of blood was drawn by cardiac aspiration from each animal for the determination of antibodies against HSV-1. The titres were established by an ELISA-based technique as described previously (Svennerholm et al., 1984). Titres less than 1/100 were regarded as negative.

Statistics. The tumour incidence was calculated in each experimental group and factor analysis was done for the studied chemical compounds, as well as the combinations of these. Statistical significance of the results was calculated with Fisher's exact test (two-tailed). P ≤ 0.05 was regarded as statistically significant.

RESULTS

Mice which had been inoculated with virus without subsequent ACV treatment developed typical vesicular–ulcerative lesions within 3 days. Seven to 10 days after inoculation the lesions were crusted and proceeded to the state of complete healing. The mice inoculated with virus

Table 1. Experimental design, showing the monthly treatment of animals during 13 months of the 15 month experimental period

<table>
<thead>
<tr>
<th>Week</th>
<th>Day</th>
<th>Group I (80)*</th>
<th>Group II (40)</th>
<th>Group III (40)</th>
<th>Group IV (40)</th>
<th>Group V (40)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>Scarified + HSV-1</td>
<td>Scarified + HSV-1</td>
<td>Scarified + HSV-1</td>
<td>Scarified</td>
<td>Scarified</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>ACV</td>
<td>TPA</td>
<td>ACV</td>
<td>ACV</td>
<td>ACV</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>ACV</td>
<td>TPA</td>
<td>ACV</td>
<td>ACV</td>
<td>ACV</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>ACV</td>
<td>TPA</td>
<td>ACV</td>
<td>ACV</td>
<td>TPA</td>
</tr>
<tr>
<td></td>
<td>(2,4,6)</td>
<td>TPA</td>
<td>TPA</td>
<td>TPA</td>
<td>TPA</td>
<td>TPA</td>
</tr>
</tbody>
</table>

* Number of mice in each group is shown in parentheses.
and treated with ACV developed simple superficial wounds on their lips, as did those which were only scarified. These wounds healed within 4 days.

Owing to extensive cannibalism we were able to perform autopsies on only 140 of the 240 mice, which is therefore the effective number of animals in the study.

The type and location of all tumours found are presented in Table 2. Tumours of skin, lips and breast were all obvious at gross inspection. All eight lip tumours in groups I (six) and II (two) were squamous cell papillomas characterized by epithelial proliferation with hyper- and parakeratosis (Fig. 1). The squamous epithelial cells varied from mature to moderately dysplastic (Fig. 2). Evidence of invasion was not present. The two skin tumours found in group I were both early squamous cell carcinomas (Fig. 3) with one of these localized on the cheek close to the lower lip. The skin tumour in group II was a tail squamous cell papilloma with slight dysplasia. The lung tumours found in groups II and V were both in the range of moderately to well differentiated papillary adenocarcinomas. The two kidney tumours in groups I and V were both renal cell tumours composed of clear and granular cells. The four breast tumours in group I were moderately differentiated invasive adenocarcinomas. In group II one breast tumour was an adenocarcinoma similar to those in group I and the other was a squamous cell carcinoma. The breast tumours in groups III and V were both relatively poorly differentiated adenocarcinomas.

Histological examination of the lip specimens also revealed that the lips of almost all the animals in groups I, II and IV had focal hyperplasia of squamous epithelium with pronounced hyperkeratosis (Fig. 4). All groups had a moderate inflammatory reaction and a pronounced fibrosis in the subepithelial layers of their lips without any obvious differences between the five groups.

Serological analysis of the animals for antibodies against HSV-1 revealed positive titres in all mice of groups I, II and III, whereas for all animals of groups IV and V analysis revealed negative titres. There was no significant difference in antibody titres between the animals of groups I, II and III.

**DISCUSSION**

The present study has shown that repeated HSV-1 infection in the lips of mice treated with ACV followed by TPA administration for up to 13 months results in squamous cell tumours in the treated area. However mice subjected to HSV-1 infection and TPA treatment only also developed squamous cell lip tumours. Animals which were not treated with the combination of HSV-1 and TPA developed no lip tumours. The difference in tumour incidence between groups I and II was not significant. However, if the results for the animals that received HSV inocula and TPA, with or without acyclovir (groups I and II), are added together, the differences in incidence of lip tumours as well as of all tumours are statistically significant ($P \leq 0.05$) when compared with animals not receiving this combined treatment (groups III, IV and V).

Burns & Murray (1981) used a classical model of initiator, cofactor and promoter to study the incidence of tumours in the treated area, i.e. the mouse lip. When HSV-2 infection was followed by u.v. irradiation for inactivation of virus infectivity, a significant number of lip cancers was found. The latent period for development of tumours after virus inoculation and u.v. irradiation

---

**Table 2. Total distribution of tumours found in the different groups**

<table>
<thead>
<tr>
<th>Organ</th>
<th>I  (n = 52)</th>
<th>II (n = 23)</th>
<th>III (n = 28)</th>
<th>IV (n = 13)</th>
<th>V  (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lip</td>
<td>6 (0)*</td>
<td>2 (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Skin</td>
<td>2 (2)</td>
<td>1 (0)</td>
<td>0</td>
<td>0</td>
<td>1 (0)</td>
</tr>
<tr>
<td>Lung</td>
<td>0</td>
<td>1 (1)</td>
<td>0</td>
<td>0</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Kidney</td>
<td>1 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Breast</td>
<td>4 (3)</td>
<td>2 (2)</td>
<td>1 (1)</td>
<td>0</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Total</td>
<td>13 (6)</td>
<td>6 (3)</td>
<td>1 (1)</td>
<td>0</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Animals with tumours (%)</td>
<td>25</td>
<td>25</td>
<td>4</td>
<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>

* Numbers of malignant tumours are shown within brackets.
Fig. 1. Squamous cell papilloma of the upper lip in a mouse treated with HSV-1 followed by ACV and TPA (group 1). Bar marker represents 0.5 mm.

Fig. 2. Higher magnification of Fig. 1. Note the mildly dysplastic epithelium with hyper- and parakeratosis. Bar marker represents 100 μm.

varied between 10.8 and 24 weeks. In the present study, lip, skin and breast tumours developed after 11 to 14 months. Consequently, one problem in the present study was a high rate of loss of mice due to decreasing general health in the ageing animals and cannibalism. However, we considered the long-term treatment to be important in studying the oncogenic properties of ACV-treated herpes infection in mice and therefore we accepted the high mortality among the animals. In the present study, TPA administration was discontinued after 13 months, 2 months
ACV and tumour promotion in HSV-1 infection

Fig. 3. Invasive squamous cell carcinoma of the skin in a group I mouse. Bar marker represents 100 μm.

Fig. 4. Section of the lip in a group II mouse without a tumour. Note the marked epithelial hyperplasia and hyperkeratosis. Bar marker represents 100 μm.

before the termination of the study, which may have resulted in the disappearance of reversible lesions induced by TPA. On the other hand, with increased time of latency, irreversible and malignant lesions would have had a better chance to develop. The difference in tumour incidence between this study and the one by Burns & Murray (1981) might indicate that HSV-2 has a more potent initiating capacity than HSV-1. However, the different methods of inactivation of HSV after infection might well have been an important factor.
The present study was extended to the tumour response in the whole body, in addition to the lip area. The animals in groups I and II developed more tumours in the lung, kidney and breast compared to the animals in groups III, IV and V which, although the numbers are low, might suggest that the tumour formation after treatment with HSV-1 and TPA may take place at sites other than the site of application. We have also found that in rats, intra-orally exposed to the combination of HSV-1 and tobacco, tumours distant to the oral cavity are induced (P.-A. Larsson et al., unpublished results). Most cells in mice are permissive to HSV infection, and in mice inoculated orally with HSV the virus will probably spread systemically. Also, locally applied chemicals are absorbed and generally spread within the body. Thus, both HSV and TPA, as well as systemically administered ACV, might exert their effects on different cells distal to the site of application. Another explanation could be that a generalized HSV infection might depress the immune response of the animals, thereby facilitating spontaneous tumour development.

In this study we were not able to show that blocking of the lytic replicative cycle of HSV-1 by ACV results in an increased tumour incidence, even when followed by tumour promoter treatment. In fact the difference in tumour incidence outside the oral cavity between the different groups of mice might indicate a possible anti-tumorigenic effect of ACV, although the number of these tumours was too small to allow statistical evaluation. However our data are the first to provide evidence that infectious HSV-1 has tumorigenic properties. This has not been shown before and will require further study.

This study was supported by the Swedish Tobacco Company, the Swedish Dental Association and the Swedish Cancer Society.

REFERENCES


(Received 28 October 1988)