Acyclovir Efficiently Inhibits Oropharyngeal Excretion of Epstein–Barr Virus in Patients with Acute Infectious Mononucleosis

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SUMMARY

Shedding of Epstein–Barr virus (EBV) into saliva was studied in 31 patients with verified acute infectious mononucleosis. The patients had been randomized for intravenous treatment with acyclovir (ACV) at 10 mg/kg body weight at 8 h intervals for 7 days, or placebo, in a double-blind trial. EBV in centrifuged throat washings was detected by transformation of umbilical cord lymphocytes and by immunofluorescence staining for EBV-associated nuclear antigen in fixed cell smears. Saliva samples were obtained before and during treatment, and after 4 weeks and 6 months, respectively. ACV effectively but transiently interrupted EBV production (P < 0.001), but virus shedding resumed at the initial level within 3 weeks of cessation of the treatment. Initially, 93.5% of the patients had detectable EBV in the saliva compared with 83% in the 4th week and 58% after 6 months.

At pharmacological doses acyclovir (ACV) is a non-toxic antiviral nucleoside analogue (Elion et al., 1977) which efficiently inhibits not only herpes simplex virus (Schaefler et al., 1978) but also varicella-zoster virus (Biron & Elion, 1980) and Epstein–Barr virus (EBV) (Colby et al., 1979; Lin et al., 1984) replication in vitro. Both human herpes simplex virus and varicella-zoster virus direct the initial phosphorylation of ACV by the virus-specific thymidine kinase, and after two additional phosphorylations mediated by the cell, the biologically active triphosphorylated form is produced (Elion et al., 1977). This form of ACV inhibits viral DNA polymerase competitively (Pagano & Datta, 1982), and also terminates the viral DNA chain because the drug is incorporated in the viral DNA molecule in place of deoxyguanosine monophosphate (Furman et al., 1979). Since an EBV-coded thymidine kinase has recently been identified (E. Littler, J. Zeuthen, A. McBride, E. Trost-Sorensen, K. L. Powell, J. E. Walsh-Arrand & J. Arrand, unpublished results), it is reasonable to assume that the inhibition of the lytic cycle and the 100-fold greater inhibition of the EBV DNA polymerase compared to host cell DNA-polymerase α (Datta et al., 1980) is due to a similar mechanism of action.

The only known source of biologically active EBV in infected human beings is the oropharynx. Evidence has been presented that the virus is produced in epithelial cells in salivary glands (Wolf et al., 1984), in the oropharyngeal surface epithelial layers (Sixbey et al., 1984) and in oral leukoplasias of immunosuppressed patients (Greenspan et al., 1985). In these studies the virus-carrying cells were virus-producing, showing both a high number of viral DNA copies and late antigens. EBV production can be demonstrated during the course of primary EBV infection, and also regularly in asymptomatic virus carriers (Chang & Golden, 1971; Gerber et al., 1972; Miller et al., 1973; Niederman et al., 1976), a state known to persist for life after primary EBV infection (Yao et al., 1985).

Thirty-three patients with acute infectious mononucleosis (IM) were included in a double-blind, placebo-controlled, clinical trial for ACV treatment (Andersson et al., 1986). The patients were aged between 15 and 25 years. Of the 33 patients, two were excluded due to false diagnosis. The entering criteria were clinical indications of IM with symptoms not exceeding 7 days, a
positive heterophile antibody test and/or absolute lymphocytosis with 5% atypical lymphocytes in peripheral blood, and later confirmation by EBV-specific serology. All of the patients had symptoms so severe that they required hospitalization. They were treated with acyclovir (10 mg/kg body weight) or placebo, as a 1 h intravenous infusion every 8 h for 7 days, after computerized randomization.

While in hospital the patients were checked daily according to separate case protocols for objective (by a physician) and subjective (by the patient himself) general health, and by the clinician for rash, sore throat, tonsillar enlargement, liver and spleen enlargement, temperature (rectally) and body weight. Laboratory tests were followed by tests of complete haematological status, including differential counts, determination of the proportion of atypical lymphocytes and liver enzymes. These tests were done on days 0, 3, 7 and 14 and then weekly until total recovery. Serum was collected for determination of Paul-Bunnell-Davidsohn and EBV antibody titres on days 0, 14, 90 and 180 after admission (Andersson et al., 1986).

Throat washings were obtained on entry and on days 3, 7, 28 and 180 after admission by letting the patients rinse their mouths with 10 ml RPMI 1640. Bacteria and debris were removed by centrifugation at 10000 g for 10 min. This resulted in less reduction of titre than filtering through 0.8 μm or 0.45 μm Millipore filters (Chang & Golden, 1971; Niederman et al., 1976), and no bacterial contamination was experienced during the testing. Before freezing, 10% foetal calf serum was added, and the samples were stored at −70 °C until tested.

The presence of virus in oropharyngeal washings was tested in two ways: by transformation of cord blood cells with 10-fold dilutions of the mouth wash (Table 1), and by screening for the induction of EBV-associated nuclear antigen (EBNA) in the same cord blood cells 7 days after infection with undiluted samples. In the transformation assay, the virus titre providing 50% transformation was determined from the virus dilution curves (TT50). Thus, a measurement of the amount of virus in each sample was obtained with both assays. The correlation between the TT50 and the percentage of EBNA-positive cells was high (Fig. 1, r = 0.67).

With the transformation assay, virus was detected in 87% (27/31) of the samples at admission (Table 2). The viral titres in the mouth washes varied over a 600-fold range between patients at
Table 1. *Titres of EBV transforming activity (TT_{50}) in mouth washes from IM patients at different times after admission*.

<table>
<thead>
<tr>
<th>Placebo-treated patients</th>
<th>ACV-treated patients</th>
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<tr>
<td></td>
<td>Days after admission</td>
</tr>
<tr>
<td></td>
<td>0</td>
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<td>Patient no.</td>
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<td>4</td>
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* The transformation assay was performed by adding thawed mouth wash preparations to cord blood cells isolated in Ficoll–Isopaque. The mouth washes were diluted 10^0, 10^-1 and 10^-2. For each dilution, 2 x 10^6 cells were exposed to 2 ml of the mouth wash samples for 1 h, washed, and distributed in 10 wells in a microplate (Falcon) with 2 x 10^5 cells/well in RPMI 1640 containing 10% foetal calf serum, supplemented with streptomycin (100 mg/ml) and penicillin (100 U/ml). The cells were fed and screened weekly for 4 to 6 weeks for transformation (Zerbini & Ernberg, 1983). Transformation was easily seen as an overgrowth of blasts. Uninfected cells and cells exposed to B95-8 virus (Miller & Lipman, 1973) diluted 10^-2, 10^-3 and 10^-4 (1 ml diluted virus/10^6 cells) were used as controls. The results were plotted in a log-log plot of titre versus percentage of wells showing transformation, and from these plots the dilution of virus causing 50% of the wells to contain transformants (TT_{50}) was calculated. The reciprocal titres are given in the Table.

† neg, Negative.
ND, Not done.

Table 2. *Proportion of oropharyngeal EBV shedders* in IM patients treated with acyclovir or placebo for 7 days.

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>0</th>
<th>4</th>
<th>7</th>
<th>28</th>
<th>180</th>
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<tbody>
<tr>
<td></td>
<td>Acyclovir</td>
<td>14/15</td>
<td>1/14†</td>
<td>1/15†</td>
<td>12/15</td>
<td>8/15</td>
</tr>
<tr>
<td></td>
<td>Placebo</td>
<td>15/16</td>
<td>14/16</td>
<td>16/16</td>
<td>13/15</td>
<td>10/16</td>
</tr>
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* The presence of EBV was determined by the transformation assay (see Table 1) and EBNA induction in cord blood cells. Staining for EBNA was performed by anti-complementary immunofluorescence according to Reedman & Klein (1973). Five x 10^5 cells were incubated with undiluted mouth wash or control B95-8 virus and harvested on day 7. All samples were stained with an EBNA-positive serum, and with an EBNA-negative serum as control. The preparations were examined for positive and negative cells at 650 x magnification in a Leitz fluorescence microscope equipped with Ploem-Opaque epi-illumination.

† Acyclovir is significantly more effective in reducing viral shedding than placebo (P < 0.001; Student’s t-test).

admission, as measured by the transformation assay. With the EBNA assay, virus was detected in 90% (28/31) of the donors at enrolment. If the two tests are considered together, virus was detected in 29/31 donors (93.5%). Only in one donor was no virus detected at any time point, although this patient clearly had acute IM, as judged by clinical and laboratory signs and EBV serology. The EBNA assay is more rapid (1 week) but less sensitive for quantification of virus, and would thus be less suitable for titration of the virus.

In the majority of the ACV-treated patients (93%; 14/15) no virus activity at all was detected
During the period of treatment (1 to 7 days), while 87.5 to 100% (14 or 16/16) in the placebo group had detectable virus titres in mouth wash samples during the same period.

During the period of treatment there was a significant reduction of virus titre (geometric mean titre; 40-fold; \( P < 0.001 \)), which returned to initial values after the treatment (Table 2, Fig. 2). The exact time at which virus production returned to the initial level was not determined, but in general the titres seen after cessation of the treatment were comparable to the levels of virus production at admission both for each patient individually, and as the geometric mean titre of the treated group. Thus, it seems that the drug prevented virus production/replication \textit{in vivo}, but did not eradicate the viral genetic information. It is likely, therefore, that the virus is present in cells in the oropharynx in a latent form, inaccessible to the effects of the drug. As soon as the drug is withdrawn these viral genomes are fully competent to resume virus production.

The efficient inhibition of EBV production \textit{in vivo} during treatment of patients with IM agrees well with the documented effect of the drug on EBV replication in virus-producing cells \textit{in vitro} (Pagano & Datta, 1982) and has also been suggested by an earlier preliminary study (Pagano et al., 1983). The effective dose for inhibiting 50\% (ED\(_{50}\)) of EBV DNA replication \textit{in vitro} has been determined to be 7 \(\mu\)M in Raji cells and 6 \(\mu\)M in P3HR-1 cells (Colby et al., 1982). In a later study, the ED\(_{50}\) was determined to be 0.3 \(\mu\)M after continuous exposure of P3HR-1 cells to ACV for 14 days (Lin et al., 1984). The higher estimate was based on a shorter culture period with ACV (7 days) which in fact may be more relevant as our patients were treated with ACV intravenously for 7 days. However, complete inhibition \textit{in vitro} was only obtained with 100 \(\mu\)M-acyclovir (Lin et al., 1984). The pharmacokinetics of ACV shows that after infusion of 10 mg/kg for 30 min there is a peak serum concentration of 60 to 80 \(\mu\)M which declines to 7 to 8 \(\mu\)M after 2-5 h (De Miranda & Blum, 1983). In our study, the plasma ACV concentrations assessed immediately after infusion on day 3 and day 7 showed mean peak levels of 78 \(\mu\)M (range 44 to 100) and 69 \(\mu\)M (range 39 to 120), respectively. Thus, the ACV concentration obtained in serum is of the same order as that which is inhibitory to EBV DNA replication \textit{in vitro}. The possible presence of ACV in our salivary samples is not considered to be a disturbing factor in our test system, since the drug does not affect the viral transformation at non-toxic doses, but only the productive virus cycle (I. Ernberg & S. Andersson, unpublished results; Pagano et al., 1983).
In contrast to the dramatic antiviral effect, the clinical response to the ACV treatment was less pronounced. There was no statistically significant effect for any single symptom, but trends towards regression of fever, weight loss, pharyngitis and tonsillar swelling were noticed in particular. However, if these data were combined with the patients' self-assessment of general health, a significant effect could be demonstrated in the ACV-treated patients compared to the placebo-treated group by the log rank test ($P \leq 0.01$; Andersson et al., 1986).

Interrupting the production of virus by ACV treatment may be a useful means of elucidating the pathogenic mechanism of IM, and in particular the interplay between the oropharyngeal EBV production, the circulating EBV-infected B lymphocytes and the immune regulatory and immunological mechanisms acting on these transformed cells. One obvious question which might be answered is whether the infected B lymphocyte pool is continuously supplied by newly infected cells from the oropharyngeal tissues.

Our study shows that ACV is effective in inhibiting EBV production, and thus eliminates the initial pathogen of IM. We think that this finding opens promising possibilities towards treatment of more extended or severe courses of IM, as is occasionally seen (Sullivan et al., 1984).

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REFERENCES
Short communication


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