Inhibition of Herpes Simplex Virus Replication by Cobra α-Neurotoxoid

By J. E. YOURIST,* H. G. HAINES AND K. D. MILLER

Departments of Microbiology, Medicine and Pathology, University of Miami School of Medicine, Miami, Florida 33101, U.S.A.

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SUMMARY

A peptide, prepared by gentle oxidative detoxification of a methionine-free cobra α-neurotoxin, was evaluated for antiviral activities in vivo and in vitro. When added to BHK cells before infection the peptide reduced herpes simplex virus type 1 (HSV-1)-induced TCD₅₀ in a dose- and time-dependent manner. The antiviral effect was also expressed on initiation of cell treatment 1 h after infection if the peptide was left in contact with cells. Single, intracerebral doses of the toxoid in suckling mice significantly increased 50% survival times, and reduced virus replication in the infected brain tissues. Cutaneous lesions and resultant scar formation induced by HSV-1 in hairless mice were also significantly reduced by subcutaneous peptide injections.

INTRODUCTION

Elapidae venoms, particularly their principal neurotoxins, have been reported to interfere with viral infections in vivo and in vitro. An early study by Sanders et al. (1958) demonstrated that detoxified cobra venom reduced the mortality of primates infected with poliovirus. Recently, the purified, major α-neurotoxin of the Thailand cobra (Naja naja siamensis), after oxidative detoxification, has been shown to have antiviral activity in vitro against an RNA virus, Semliki Forest virus (SFV) (Miller et al., 1977). A structurally related toxin from the Formosan krait (Bungarus multicinctus) was reported to block infectivity of rabies virus in cultured chick myotubes (Lentz et al., 1982). Otherwise, little is known of the antiviral effect of snake venom proteins.

The major α-neurotoxins of cobra and bungarus venoms have post-synaptic ‘curare-like’ actions (Tu, 1973). Those toxins are short chain peptides containing four or five disulphide bonds that maintain molecular conformation. When the bonds are oxidized or reduced and alkylated, toxicity is lost (Tu, 1973).

In preliminary studies we found that the oxidized cobra neurotoxin inhibited the replication of herpes simplex virus type 1 (HSV-1) both in vivo and in vitro (Yourist et al., 1979). HSV-1 was investigated because of its clinical significance and its pathogenesis which involves neurotropic mechanisms. The present work was undertaken to characterize further the antiviral activity of the oxidatively detoxified cobra α-neurotoxin against HSV-1.

METHODS

Neurotoxoid. N. siamensis venom was obtained from Biologicals Unlimited (Baltimore, Md., U.S.A.). The major α-neurotoxin of this venom was isolated by a slight modification (Miller et al., 1977) of the chromatographic method of Chatman & DiMari (1974). The purity of each preparation was established by polyacrylamide gel electrophoresis at both high and low pH (Gabriel, 1972; Bonner et al., 1968), and in the presence of SDS (Weber & Osborn, 1969). The toxin was assayed spectrophotometrically as A₂₈₀ [1 mg/ml = 1-06; Karlsson et al. (1971)]. The LD₅₀ values were determined in mice weighing 20 to 25 g.

Oxidative detoxification of the purified toxin was carried out and monitored as described by Miller et al. (1977). Excess reactants and reaction products were separated from the oxidized peptide on polyacrylamide P-2 gel columns equilibrated and eluted with 15 mm phosphate buffer pH 6-8, containing 0-12 m NaCl. Preparations were filter-sterilized before use. The peptide concentrations were measured spectrophotometrically as A₂₈₀ (1 mg/ml = 0-48) and by ninhydrin analysis (Moore & Stein, 1954).

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Cell and virus preparations. Baby hamster kidney cells (BHK-21) and rabbit kidney cells (RK) were obtained, respectively, from the American Type Culture Collection (Rockville, Md., U.S.A.) and Flow Laboratories. Cells were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% foetal calf serum, 0.75 mg/ml NaHCO₃, 29.5 mg/ml tryptose phosphate broth, 3.5 mg/ml glucose, 0.3 mg/ml glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and 20 units/ml mycostatin. BHK-21 cells, in MEM containing 10% foetal calf serum and 10% dimethyl sulphoxide, were stored at −70 °C. RK cells were maintained and utilized through no more than ten passages.

Stock preparations of HSV-1 (strain Dorr) were propagated on both BHK and RK cells by inoculation at a multiplicity of infection (m.o.i.) of 0.1. Monolayers showing 90% cytopathic effect (c.p.e.) were suspended in MEM, freeze–thawed three times, and centrifuged at 225 g. The supernatants were sonicated for 45 s and stored at −70 °C until titrated by plaque assays and employed in experiments.

Antiviral effects of neurotoxoid in vitro. TCD₅₀ assays were performed in Linbro 96-well tissue culture plates containing confluent monolayers of BHK cells treated with 0-15 ml of MEM–toxoid mixtures.

Aliquots (0-05 ml) of HSV-1 were employed for infection of treated cell cultures. Control uninfected cell preparations were treated with 10-fold dilutions of the peptide in MEM. Experimental and control preparations, in triplicate, were incubated at 37 °C for 72 h. The cultures were then scored, and the TCD₅₀ values calculated by the method of Reed & Muench (1938).

Antiviral effects of neurotoxoid in vivo. Groups of twelve 3-day-old suckling mice were treated intracerebrally (i.c.) with 0-05 ml aliquots of neurotoxoid (210 μg). Twenty-four h later the mice were challenged with 0-05 ml aliquots of HSV-1. Deaths were recorded daily. None of over 1000 animals given the two injections died within the subsequent 24 h period. The 50% survival times of the treated animals at each virus challenge dose were determined. Overall survivals were also noted at termination of the experiment (14 days). Significance of the peptide effects was determined by Student’s t-test. Additional i.c. peptide treatments 24 h after virus challenge were attempted, but those third injections into the infected brains produced traumatic deaths within 24 h.

Brains of the treated and untreated suckling mice were removed and frozen. For titrations of infectious HSV-1, brains from three animals were separately minced and homogenized (Potter–Elvejem; 25 strokes) in 2 vol. MEM. Each suspension was freeze–thawed three times, then centrifuged at 225 g. The respective supernatants were sonicated for 45 s and either stored at −70 °C or evaluated immediately by plaque assay.

Plaque assays were performed separately for individual animals on 10 × 35 mm plates containing confluent monolayers of RK cells infected with aliquots (0.1 ml) of serial 10-fold dilutions of each HSV-1 sample, all in duplicate. Adsorption times were 90 min. All infected monolayers were covered with 2 ml 5% methylcellulose in MEM and incubated for 72 h at 37 °C in 5% CO₂. Subsequently, the methylcellulose was removed, and each monolayer was washed once in phosphate-buffered saline (PBS). The cells were then stained with 0-2% crystal violet (in 95% methanol and 1% ammonium oxalate) for 60 s, rinsed with tap water, dried, and the plaques counted.

The effect of the peptide on HSV-1-induced skin lesions in hairless mice was also examined. Each mouse was scarified on the right flank, and the area was then infected by spreading a droplet of HSV-1 containing approximately 10³ p.f.u. Treated animals received 400 μg toxoid subcutaneously every 48 h for 12 days, commencing 24 h after infection. Control animals received similar treatments of sterile, buffered saline. The severity of cutaneous lesions that developed in the treated and untreated animals was rated daily. The scoring system, based on the development and regression of the cutaneous lesions, was that of Klein et al. (1974). An additional, objective criterion was the incidence of scar formation.

RESULTS

Neurotoxoid effects in vitro

BHK-21 cells treated with increasing concentrations of toxoid prior to virus challenge, with removal of residual peptide before infection, were protected from HSV-1 in a dose-related manner (Fig. 1). Initiation of treatment 1 h after infection with HSV-1 also produced this dose-dependent protection when the peptide remained in contact with the infected cells. However, there was diminished protection when the same concentrations of neurotoxoid were removed after 1 h of cell treatment and prior to virus inoculation (Fig. 1). Under the latter conditions peptide concentrations above 100 μg/ml did not fully inhibit viral replication. This indicates that the inhibitory action of the peptide is time-dependent.

In other experiments, constant concentrations of peptide (34-5 μg/ml) were added to cells at various intervals before and after infection. Antiviral effects were noted in those cultures treated for 1 h prior to infection, but only when the peptide was left in place. Cell treatments initiated 1 h after infection also reduced the TCD₅₀ when the toxoid remained in the cultures (Table 1).
Inhibition of HSV replication

Fig. 1. Effect of oxidized α-neurotoxin on HSV-1 replication in BHK-21 cells. The cells were treated with a range of concentrations of α-neurotoxoid (50 to 350 μg/ml) at 24 h prior to HSV-1 infection (Δ), 1 h prior to HSV-1 infection (O), or 1 h after HSV-1 infection (●). Prior to HSV-1 infection the cells were washed with PBS. The cells were inoculated with various doses (10^{-2} to 10^{-7} p.f.u.) of HSV-1. After 90 min the cells were washed with PBS and 0.1 ml of MEM containing 3% foetal calf serum was added. After 72 h of incubation at 37°C, c.p.e. was graded. Titrations were done in triplicate.

Fig. 2. Effect of a single intracerebral dose of oxidized α-neurotoxin (210 μg) on HSV-1 titres in individual brains of suckling mice challenged i.c. with 3.8 × 10^3 LD_{50} (●) compared to infected mice treated with buffered saline (○).

Table 1. Relationships between cell-neurotoxoid exposure times and HSV-1 replication in BHK-21 cells

<table>
<thead>
<tr>
<th>Treatment initiated*</th>
<th>Cells washed†</th>
<th>log_{10} TCD_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>-</td>
<td>6.50</td>
</tr>
<tr>
<td>1 h before infection</td>
<td>-</td>
<td>4.00</td>
</tr>
<tr>
<td>1 h before infection</td>
<td>+</td>
<td>4.25</td>
</tr>
<tr>
<td>24 h before infection</td>
<td>+</td>
<td>5.50</td>
</tr>
<tr>
<td>48 h before infection</td>
<td>+</td>
<td>6.25</td>
</tr>
<tr>
<td>72 h before infection</td>
<td>+</td>
<td>6.50</td>
</tr>
</tbody>
</table>

* 34.5 μg/ml neurotoxoid.
† Cells washed with PBS prior to infection.
Table 2. Effects of neurotoxoid on survival of suckling mice challenged with different doses of HSV-1

<table>
<thead>
<tr>
<th>Virus challenge (LD$_{50}$)</th>
<th>50% survival (days)</th>
<th>Survival (%) on day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Treated</td>
</tr>
<tr>
<td>$3.8 \times 10^4$</td>
<td>3-0</td>
<td>4-4</td>
</tr>
<tr>
<td>$3.8 \times 10^3$</td>
<td>4-0</td>
<td>5-4</td>
</tr>
<tr>
<td>$3.8 \times 10^2$</td>
<td>4-8</td>
<td>6-5</td>
</tr>
<tr>
<td>$3.8 \times 10^1$</td>
<td>5-0</td>
<td>14-0</td>
</tr>
<tr>
<td>$3.8$</td>
<td>6-4</td>
<td>No deaths</td>
</tr>
</tbody>
</table>

Table 3. Effects of different doses of neurotoxoid on survival of suckling mice challenged with HSV-1 ($6.0 \times 10^3$ LD$_{50}$)

<table>
<thead>
<tr>
<th>Toxoid (µg)</th>
<th>50% survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2-8</td>
</tr>
<tr>
<td>3-3</td>
<td>2-5</td>
</tr>
<tr>
<td>6-6</td>
<td>3-8</td>
</tr>
<tr>
<td>13-2</td>
<td>4-2</td>
</tr>
<tr>
<td>26-4</td>
<td>5-0</td>
</tr>
<tr>
<td>52-8</td>
<td>4-8</td>
</tr>
<tr>
<td>105-6</td>
<td>4-2</td>
</tr>
<tr>
<td>52-8*</td>
<td>4-5</td>
</tr>
</tbody>
</table>

* Heat-treated at 100 °C.

Optimum reduction in TCD$_{50}$ was achieved when cells were pretreated with peptide for 24 h, then washed with PBS before HSV-1 challenge. This antiviral effect was progressively diminished in monolayers treated with the same concentration of peptide for 48 and 72 h prior to infection.

The difference between reduction of TCD$_{50}$ achieved with 50 µg/ml peptide (Fig. 1) and that achieved with 34.5 µg/ml under similar conditions (Table 1) probably reflects the steep concentration dependence of the antiviral activity in that peptide range (Fig. 1).

During these investigations, small amounts of active toxin (<1 mouse LD$_{50}$) were purposely added to the cell culture control systems to determine effects of trace amounts of active toxin on viral inhibition by the toxoid. In every case the cultured cells were killed.

Effect of toxoid on i.c. herpes infections

Experiments with groups of 25 to 30 suckling mice indicated that single i.c. neurotoxoid pre-treatments (210 µg) reduced mortality following i.c. challenge 24 h later with $4.0 \times 10^4$ LD$_{50}$ of HSV-1 ($P < 0.05$). That peptide dose was non-toxic to a control group of uninfected animals. Neither intraperitoneal nor subcutaneous peptide administrations increased survival.

For quantitative evaluation of the therapeutic effect of the neurotoxoid, constant i.c. doses (210 µg) were administered to groups of 8 to 12 suckling mice 24 h prior to challenge of each group with a different HSV-1 concentration. Fifty percent survival times and the percentage of survivors in each group are recorded in Table 2. Fifty percent of the treated animals survived challenge with 38 LD$_{50}$ of virus, while all control animals died within 5 days of challenge, a finding significant at $P < 0.001$. At an HSV-1 challenge of 3-8 LD$_{50}$, all treated animals survived with no survivors in the control group.

This therapeutic response was dose-dependent as determined by changes in 50% survival times of groups of animals given different amounts of the peptide 24 h before challenge with $6 \times 10^3$ LD$_{50}$ of HSV-1 ($P < 0.05$). At the optimal dosage of 26-4 µg, 50% survival times were extended by 2-2 days following the large virus challenge employed.

Twelve mice were then treated with a large dose of toxoid (210 µg) mixed with a trace (<1 LD$_{50}$) of active toxin, followed by i.c. challenge with HSV-1. All of these animals died within 24 h, probably due to combined toxin and virus effects.
Inhibition of HSV-1 replication

Inhibition of HSV-1 replication in brain tissue

Suppression of HSV-1 replication in individual brains of the treated suckling mice was confirmed by plaque assays. Brains of suckling mice pretreated with single doses of toxoid (210 μg; i.c.) 24 h prior to the substantial challenge with $3.8 \times 10^3$ LD$_{50}$ of HSV-1 contained significantly lower HSV-1 titres on the second, fourth, and sixth days than did the untreated controls (Fig. 2).

Treatment of herpetic skin lesions

Cutaneous HSV-1 infections of hairless mice provided a model system which permitted sequential schedules with the peptide, a process necessary to maintain optimal levels of the toxoid. Subcutaneous neurotoxoid administrations every alternate day significantly ameliorated the acute cutaneous herpesvirus infections induced in the mice. Treated groups demonstrated maximum mean lesion scores slightly greater than 1·0, while the mean for the placebo group approximated 3·0 ($P < 0·001$; Fig. 3). Only two of 35 treated animals developed scars, compared to 18 out of 20 in the control group.

DISCUSSION

These studies concern effects of cobra α-neurotoxoid on the replication of HSV-1 in cultured cells and on the mean survival times of suckling mice infected intracerebrally. A model for cutaneous lesions in hairless mice was also utilized. A distinct antiviral effect was demonstrated in each of the three systems.
In BHK-21 cells, the antiviral effect was both time- and dose-dependent. Initiation of cell treatment after infection with HSV-1, leaving the peptide in contact with the infected cells, produced nearly the same antiviral effect as pretreatment of the cells 24 h prior to infection. Removal of the peptide by washing after 1 h of cell treatment prior to infection diminished the antiviral effect. The ability of the peptide to reduce viral replication when applied to the cells after infection suggests an inhibitory effect other than direct inactivation of viral adsorption or penetration. Treatment of the cells with single peptide doses 48 and 72 h before infection produced significantly and progressively less viral inhibition than did treatments 24 h prior to infection. This suggests catabolism of the peptide or some other intracellular turnover mechanism.

The studies in vivo indicated that single i.c. doses of neurotoxoid significantly prolonged survival times of suckling mice challenged with different LD₅₀ of HSV-1. This effect was dose-dependent and was unaffected by heat treatment of the peptide. Plaque assays of brain homogenates prepared from the individual infected mice pretreated with single doses of peptide confirmed the inhibition of virus replication in vivo. While the rate of virus replication in the brain tissues was reduced, not blocked completely, the plaque reduction of almost 2 log₅₀ was significant because of the large challenge doses of virus administered to the mice (3.8 × 10⁵ LD₅₀). Maintenance of high peptide levels in the suckling mouse brains was not possible because additional toxoid injections into the infected brains produced traumatic deaths. Intraperitoneal and subcutaneous treatments had no influence on survival of the mice with i.c. HSV-1 infections, perhaps due to turnover or to inaccessibility of the material to the brain from those routes of administration.

The hairless mice provided a model system for sequential peptide treatments to maintain high levels of the drug. In those mice neurotoxoid treatments significantly reduced mean scores of lesion severity due to cutaneous HSV-1 infections. The incidence of scar formation was also reduced, a potential objective criterion for evaluation of the therapeutic effect in vivo.

The primary, secondary and tertiary chemical structures of the α-neurotoxin employed for these studies are known (Tu, 1973; Walkinshaw et al., 1980), and oxidation of that toxin yields a non-toxic antiviral peptide against SFV (Miller et al., 1977). This information should be of value in designing studies to determine the exact mechanism of the antiviral effect against HSV-1. The absence of methionine in the N. siamensis toxin employed for these studies (Karlsson et al., 1971; Tu, 1973), and the mild pH maintained during oxidative detoxification (Miller et al., 1977), suggest that the primary structure of the peptide is relatively unaltered. Since the reduced, carboxymethylated toxin and the heat-stable, oxidized peptide also exhibited antiviral activity (unpublished results) the combined phenomena strongly suggest that the antiviral potential of the peptide is dictated by its primary structure. The anti-parallel β-pleated sheet structure, identified by Walkinshaw et al. (1980) as including the highly conserved region of the same N. siamensis neurotoxin employed for the present studies, may give stability to the toxoid after cleavage of the disulphide bonds. Also, the present studies demonstrate that sublethal doses of the toxin inhibit rather than enhance the antiviral effect in vitro and in vivo. This indicates that the toxoid rather than the toxin is the antiviral form of the peptide. Therefore, on the subject of structure-function relationships, a major question concerns how a peptide, on loss of secondary structure with resultant conformational changes, can assume an antiviral function.

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Inhibition of HSV replication


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