Antiviral Research, 10 (1988) 209–224
Elsevier

AVR 00322

Modification of immunological responses and clinical disease during topical R-837 treatment of genital HSV-2 infection

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(Received 5 July 1988; accepted 15 September 1988)

Summary

R-837, a compound with no in vitro anti-HSV activity, was administered intravaginally to guinea pigs (5 mg/kg every 12 h) for five days beginning 12 h after genital HSV-2 inoculation. Drug treatment reduced vaginal viral replication ($P<0.0001$), completely protected against primary disease and reduced recurrent genital HSV disease ($P<0.0001$). Drug treatment also induced mild fever, weight loss, and decreased water intake. R-837 was a potent interferon inducer, which also induced variable enhancement of cell-mediated cytolytic activity against HSV-2 targets. Less than 36 h of vaginal HSV shedding was observed in animals with R-837 induced early enhancement of HSV-target cytolysis. Compared to placebo, R-837 decreased ELISA and ADCC antibody to HSV-2, but accelerated HSV-2 specific in vitro IL-2 production and peripheral blood mononuclear cell (PBMC) proliferation. R-837 exhibited potent anti-HSV activity in vivo apparently due to cytokine induction and enhancement of cell-mediated responses.

R-837; HSV-2 infection

Introduction

The only available treatment for primary and recurrent genital herpes is acyclovir (Bryson et al., 1985; Mertz et al., 1984), an inhibitor of viral replication which

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must be chronically ingested to affect recurrence patterns (Douglas et al., 1984; Straus et al., 1984). Alternative strategies for control of HSV infections include the administration of HSV glycoproteins as selective immune stimulators given either before (Stanberry et al., 1987) or after (Stanberry et al., 1988) infection. Attempts at modifying these increasingly common infections by use of immunomodulating agents such as inosine pranobex (Mindel et al., 1987) or interferon-alpha (Eron et al., 1986; Kuhls et al., 1986) have been disappointing.

R-837, a compound with no demonstrable in vitro activity against herpes simplex type-2 (HSV-2) is an immune response modifier that may be applied topically. It has previously been shown to inhibit primary in vivo genital herpes (Miller et al., 1985) in the guinea pig and to have modest in vitro and in vivo activity against guinea pig CMV probably due to interferon induction (Chen et al., 1988). Employing the guinea pig model of genital HSV-2 infection, which accurately predicted the human efficacy of antiviral compounds, including acyclovir, we investigated the effects of topical R-837 on initial and recurrent HSV-2 genital disease, HSV-2 vaginal shedding, and latent HSV-2 in neural tissues. Further, we describe the effects of R-837 on temporal cytokine, cell-mediated and antibody responses in relation to the severity of initial and recurrent genital HSV-2 disease.

Materials and Methods

Drug

R-837 (1-isobutyl-1H-imidazo [4,5-c] quinolin-4 amine) was provided by 3M Riker, 3M Health Care Group, St. Paul, MN, as a 1% suspension for intravaginal administration at a dose of 50 μl/100g of animal body weight (approximately 5 mg/kg/dose) every 12 h for five days beginning 12 h after initial sham or HSV-2 intravaginal inoculation. Drug was administered intravaginally by syringe and smearing the excess on genital skin. Vehicle without R-837 was administered as the placebo.

Tissue cultures

Primary fetal guinea pig cell (fgp) cultures were prepared as previously described and determined to be free of indigenous caviid viruses and mycoplasma (Myers et al., 1985; Harrison et al., 1988). For confirmation of viral isolates, first through third passage rabbit kidney cell monolayers (RK) were prepared as previously described (Bernstein et al., 1986). K562 cell (a gift from Waner, Oklahoma City) were maintained in RPMI with 10% FBS and gentamicin and were passed three days before use in cytotoxicity assays.

Virus strains and viral cultures

Pools of HSV-2 strains 333 (Stanberry et al., 1985) and MS strain (ATCC No VR-540) were prepared as clarified supernatant from rabbit kidney cells and maintained at −70°C with titers of 5.0×10^6 and 3.4×10^7 PFU/ml respectively. Cultures of vaginal swabs were titered by plaque assay on rabbit kidney cells in tri-

plicate (Stanberry et al., 1982; Stanberry et al., 1985). In vitro antiviral activity of R-837 was tested by plaque reduction on fgp and RK cells. Varying concentrations of R-837 between 0.02 and 20.0 μg/ml were incubated in triplicate with 50–60 PFU of Strain 333 for 30 min at room temperature before inoculating onto fgp of RK monolayers in 24-well plates (Corning, Corning N.Y.) Identical concentrations of R-837 in BME 2% were also inoculated onto RK and fgp monolayers for 14 h prior to inoculation with 50–60 PFU of Strain 333 HSV-2. At the end of the experiment, six to eight dorsal root ganglia and the lumbosacral spinal cord from six HSV-infected placebo recipients and eight HSV-infected R-837 recipients were minced, cocultivated as explants on rabbit kidney monolayers as previously described (Bernstein et al., 1986).

Indiana Strain VSV (ATCC VR-158) for use in interferon bioassays was prepared as clarified supernatant from Strain-2 fgp monolayers 72 h after infection and maintained frozen at −70°C with titers of 4.6×10^6 PFU/ml.

HSV-2 antigen

Antigen for use in proliferative assays and for skin testing was produced by inoculation of monolayers of rabbit skin cells with Strain 333 of HSV-2 at an MOI of 0.1. Twenty-four hours later when the monolayers exhibited 100% cytopathic effect, the cell layer was freeze-thawed three times and sonicated. The resulting viral/cell suspension was clarified by slow centrifugation at $400 \times g$ for 10 min. The supernatant was centrifuged at $100000 \times g$ to pellet virus which resulted in a titer of 5×10^7 PFU/ml. Virus was inactivated by U.V. light exposure for 15 min. Antigen was shown to be free of infectious virus by inoculation onto rabbit kidney monolayers. Control antigen was prepared identically except that uninoculated rabbit skin monolayers were used.

Animals

Fifty-six 400–500 g female Hartley guinea pigs (Charles Rivers Labs, Wilmington, Delaware) were randomly divided into four treatment groups: sham inoculated placebo recipients ($N=12$); sham inoculated R-836 recipients ($N=12$); HSV-2 inoculated placebo recipients ($N=12$); and HSV-2 inoculated R-837 recipients ($N=20$).

Animals were inoculated intravaginally with 5×10^5 PFU of Strain 333 HSV-2 intravaginally (0.1 ml) as previously described (Stanberry et al., 1982). Sham inoculated animals received the same volume of clarified supernatant from uninfected rabbit kidney cells. Daily weights, temperatures, and clinical genital scores (Stanberry et al., 1982; Bernstein et al., 1986) were determined for 28 days. Water intake was monitored daily by measuring water remaining in water bottles each morning during drug or placebo treatment. Recurrences were tabulated as the number of days with genital lesions on or after day 15 (lesion days) (Bernstein et al., 1986). Vaginal HSV replication was titered from daily vaginal swabs for the first 10 days. Subsets of animals from each treatment group were bled serially by intracardiac puncture for immunologic assays. On day three of treatment, total peripheral white blood cell counts were performed on citrated blood using a hemocytometer.

Six animals in each of the first three groups and four R-837 HSV-inoculated animals with less than 36 h vaginal HSV shedding and six with more than 36 h vaginal HSV shedding were rechallenged intravaginally with an LD_{50} of MS strain of HSV-2 (5×10^6 PFU) on day 60 after the initial Strain 333 HSV-2 or sham inoculation, and scored for mortality and genital lesions during the subsequent ten days.

HSV-seronegative control inbred Strain-2 animals (Children's Hospital Research Foundation, Cincinnati, Ohio) provided pooled PBMC used in antibody-dependent cell-mediated cytotoxicity (ADCC) assays.

Interferon (IFN) assay

IFN activity was assayed as antiviral activity induced by heat inactivated (56°C for 45 min) guinea pig plasma in primary cultures of fetal guinea pig monolayers in 96 well plates employing a standard VSV plaque reduction assay (Winship et al., 1984). Experimental plasma in two-fold dilutions was pre-incubated for 12 h on fetal guinea pig monolayers which were then challenged with 15–25 PFU of VSV, and the endpoint read as 50% reduction in CPE at 72 h. Standards used in this assay were human alpha interferon (Hoffman-LaRoche) and plasma (65 u/ml equivalence to human alpha interferon) obtained at 8 h after I.P. inoculation of Strain-2 guinea pigs with 1 mg of Poly-I Poly-C (Sigma, St. Louis). In multiple assays, a protective endpoint was consistently produced by 0.5–1 unit of the concurrently assayed human alpha interferon standard.

Interleukin-2(IL-2) assay

IL-2 was assayed as previously described (Jenski et al., 1987) by measuring ^{3}H-thymidine incorporation into 4×10^3 cells of the IL-2 dependent murine cell line, CTLL-2, during a 4 h thymidine pulse after 20 h of incubation with test supernatants. Relative IL-2 production was measured as the ratio of activity in supernatants of HSV-stimulated wells compared to that in wells with PBMC stimulated by control antigen.

Proliferation assay

Proliferation was assayed as the ^{3}H-thymidine incorporation after a 19 h pulse in triplicate wells of 5×10^5 PBMC cultured for five days with HSV-2 antigen (1:16) versus control antigen (1:16), or 16 μg PHA versus media alone in RPMI 1640 supplemented with 50 μg/ml gentamicin, 5×10^{-5} M 2-ME, 12.5 ml of 1M Hepes/500 ml RPMI, and 5% guinea pig serum. Response is presented as the mean counts per minutes (CPM) in HSV-2 stimulated wells divided by the mean CPM in control antigen wells. For PHA the response is represented as the mean CPM of PHA stimulated wells divided by that of wells with media and PBMC alone.

PBMC-mediated cytolysis assays

Cytolysis was assayed as ^{51}Cr release from viral infected and K562 erythroleukemia targets (Harrison et al., 1985). Primary Strain-2 fgp cultures were used as targets 14 h after infection with Strain 333 HSV-2 at an MOI of one in both PBMC-mediated cytolysis and ADCC assays. PBMC (3.2×10^5) were incubated for 12 h

with 4×10^3 ^{51}Cr-labeled targets (80:1 E:T ratio). Percent PBMC-mediated cytolysis was calculated as 100 times the ratio of CPM in experimental wells (effectors + targets) minus spontaneous release (SR) (targets + media), divided by total CPM (targets plus detergent) minus SR.

For ADCC assays, HSV-2 infected fgp targets were incubated for 30 min in the presence of heat inactivated plasma from experimental animals prior to addition of pooled PBMC from uninfected inbred Strain-2 animals. Percent ADCC was calculated as 100 times the ratio of the CPM in the presence of experimental plasma minus the CPM in the presence of HSV-negative guinea pig plasma, divided by total CPM minus CPM in the presence of HSV negative plasma.

ELISA HSV-2 antibody

The ELISA assay (Bernstein et al., 1987) employed clarified antigen prepared from Triton-X solubilized HSV-2 infected or uninfected control Hep-2 cells, peroxidase conjugated rabbit anti-guinea pig immunoglobulin (Accurate Chemical, Westbury, NY), and the substrate O-phenylenediamine. Positivity was defined as an absorbance at 490 nm more than three standard deviations greater than the mean of negative control serum against HSV-2 antigen and also more than twice the mean of the experimental sample against control Hep-2 cells.

Skin testing for reactivity to HSV-2 antigen. Thirty days after intravaginal HSV-2 or sham infection, 0.1 ml of control and HSV-2 antigen were administered intradermally on opposite sides of the dorsal paraspinous lumbar areas. Induration and erythema were measured with a micrometer at 24 and 48 h after intradermal administration of antigens. Greater than 0.5 mm induration was considered a positive response.

Results

In vitro antiviral effects of R-837

At concentrations ranging from 0.04 to 2.5 μg/ml, no decrease in plaque formation was observed, however, toxicity to both RK or fgp monolayers was noted at ≥2.5 μg/ml.

Effects on genital disease in HSV-inoculated animals

Compared to placebo treatment, R-837 completely protected all 20 drug-treated HSV-infected animals ($P<0.0001$) from primary genital disease during the first fourteen days after inoculation (Fig. 1, upper panel). Recurrent genital disease between days 14 and 28 was reduced by 93%, (mean lesion days/animal for placebo = 6.8 ± 1.0 and for R-837 = 0.4 ± 0.3, $P<0.0001$). Recurrent disease was observed in only two HSV-2 infected R-837 recipients. These two animals had previously exhibited no initial clinical disease but demonstrated 72 h of vaginal replication and each developed one recurrence with a concurrent positive vaginal culture in the third week after inoculation. However, HSV-2 was not recovered

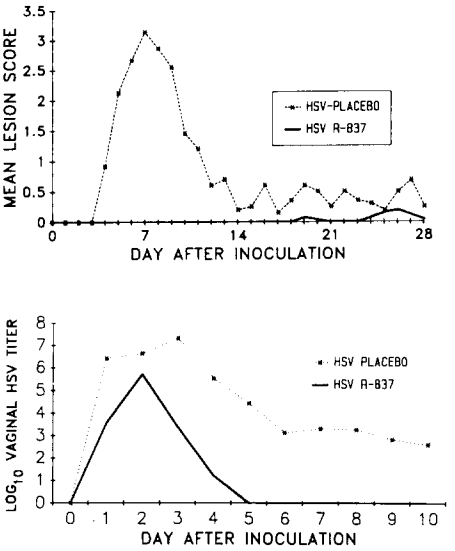

Fig. 1. Upper panel. Effects of topical R-837 on genital disease due to HSV-2. Treatment was initiated 12 h after inoculation and continued at 12 h intervals for five days. Lower panel. Duration of HSV shedding, peak virus titers, and area under the curve for titers were significantly ($P<0.0001$) reduced in R-837 treated animals compared to placebo recipients.

during 42 days of in vitro explant culture of the ganglia and spinal cords obtained from these two animals 60 days after inoculation.

Effects on vaginal virus shedding and latent HSV-2 in neural tissues

A lower mean peak vaginal titer (7.8×10^4 compared to 5.8×10^6 pfu, $P<0.0001$), a shorter duration of vaginal shedding (2.1 ± 0.9 compared to 10.0 ± 1.2 days, $P<0.0001$), and a smaller area under the vaginal virus titer/day curve (5.3 ± 3.3 compared to 35.5 ± 4.9, $P<0.0001$) were observed with R-837 compared to placebo treatment (Fig. 1, lower panel). Seven HSV-infected R-837 recipients had less than 36 hours of detectable HSV in vaginal secretions. Thirteen R-837 recipients and all placebo recipients had greater than 36 h of detectable HSV in vaginal secretions. HSV was not recovered during 42 days of cocultivation of ganglia and spinal cord tissues obtained on day 60 after vaginal inoculation from eight R-837 recipients compared to an 83.3% (5/6) recovery rate in placebo recipients, $P=0.015$, Fisher Exact Test.

Effects on immune responses to HSV

Topical R-837, was a potent inducer of interferon in both sham and HSV-2 inoculated animals (Table 1). Sham-inoculated R-837 recipients developed a peak geometric mean titer (GMT) of interferon of 1541.0 ± 9.9 on day two, considerably higher than we have observed with intraperitoneal injection of 5 mg of Poly-I Poly-C, 123.4 ± 11.2. Peak interferon titers in HSV-2 inoculated R-837 recipients

TABLE 1

Interferon titers in plasma[a] of placebo and R-837[b] recipients after HSV-2 or sham vaginal inoculation

Day	Placebo recipients		R-837 recipients	
	Sham	HSV-2	Sham	HSV-2
1	5.3 ± 1.4	24.2 ± 1.9	675.2 ± 4.9	274.2 ± 3.8
2	8.0 ± 1.6	15.9 ± 2.2	888.9 ± 4.5	550.0 ± 8.1
3	13.5 ± 1.7	84.4 ± 2.5	1541.0 ± 9.9	550.0 ± 3.8
4	27.9 ± 1.7	254.6 ± 7.9	387.6 ± 4.7	293.8 ± 10.4
6	32.1 ± 1.6	330.3 ± 10.4	192.5 ± 5.6	111.4 ± 3.4
7	32.1 ± 1.9	298.9 ± 9.1	168.8 ± 5.3	57.5 ± 2.2
8	10.0 ± 1.4	254.7 ± 16.1	63.9 ± 6.4	53.5 ± 4.5
10	9.1 ± 1.7	63.4 ± 1.7	42.5 ± 1.4	48.4 ± 5.4
20	8.3 ± 1.4	13.8 ± 1.7	20.0 ± 1.8	15.9 ± 2.0

[a] Plasma for interferon assays was obtained 12 h after previous dose.

[b] Intravaginal R-837 or placebo treatment was initiated 12 h after sham or HSV-2 inoculation and continued for five days at 12 h intervals.

occurred earlier and were higher than in HSV-2 inoculated placebo recipients (550.0 ± 8.1 on day two and 330.3 ± 10.4 on day six, respectively). The GMT of interferon activity in sham-inoculated placebo recipients was less than 20 except on days four and six (32.1 ± 1.6 and 32.1 ± 1.9 respectively). We have previously seen similar small rises in this activity in animals bled repetitively by intracardiac puncture (unpublished). Interferon levels in R-837 recipients decreased after day three in both treatment groups despite continued administration of R-837. Fever and weight loss occurred at times when plasma interferon titers were elevated in all groups. There was no significant difference in interferon titers when animals with less than 36 h HSV excretion were compared to those with greater than 36 h HSV excretion. R-837 induced interferon activity appeared to be alpha/beta and not gamma since activity persisted despite incubation of plasma at 37°C for 30 min and subsequent pH titration to a pH of 3.0 (data not shown).

Neither ELISA antibody nor ADCC antibody to HSV were detected in any sham-infected animals or in the seven HSV inoculated R-837 treated animals that had less than 36 h of vaginal viral shedding (Table 2). Antibody did develop in all other HSV-inoculated animals, but the magnitude of response measured by ELISA was lower in R-837 treated animals compared to recipients. On day 21, ADCC to HSV-2 targets peaked in placebo recipients but was lower in R-837 recipients. On day 28, there was no significant difference in ADCC between placebo recipients and R-837 recipients with more than 36 h vaginal HSV shedding (Table 3).

HSV-specific proliferative and IL-2 responses (Tables 3 and 4) were also not detected in any sham-inoculated animals, or in any HSV-inoculated R-837 recipients with less than 36 h vaginal viral shedding. However, proliferative responses to PHA were higher on each assay day for sham-infected R-837 recipients compared to placebo recipients. HSV-specific proliferation and in vitro IL-2 production were detected earlier (day 7 and 11 compared to day 21 and 25, respectively), but the magnitude of response was decreased in the HSV-infected R-837 recipients with

TABLE 2

ELISA antibody to HSV-2 and percent antibody dependent cell-mediated cytolysis (ADCC) of HSV-2 targets in HSV-2 inoculated placebo and R-837[a] recipients

Day	Placebo recipients		R-837 recipients			
			>36 h[b]		<36 h[b]	
	ADCC	/ ELISA	ADCC	/ ELISA	ADCC	/ ELISA
0	0.0	/ <10	0.0	/ <10	0.0	/ <10
5	9.5 ± 3.0	/ ND[c]	9.9 ± 7.2	/ ND	0.0[d]	/ ND
14	36.0 ± 6.4	/ 1995 ± 36	9.3 ± 5.1[d]	/ 79 ± 27[d]	0.0[d]	/ <10[e]
21	42.8 ± 8.1	/ ND	24.0 ± 4.5[d]	/ ND	0.8 ± 1.4[d]	/ ND
28	35.7 ± 7.6	/ 8318 ± 140	31.6 ± 6.8	/ 2291 ± 56[d]	1.1 ± 1.9[d]	/ <10[e]

[a] Treatment was initiated 12 h after sham or HSV-2 inoculation and continued for five days at 12 h intervals.
[b] Time period of vaginal HSV-2 shedding.
[c] Not done.
[d] $P<0.02$ compared to placebo recipients.
[e] $P<0.001$ compared to placebo recipients.

more than 36 h of vaginal HSV shedding compared to HSV-infected placebo recipients. In contrast, R-837 recipients with more than 36 h of viral replication had larger skin test responses to HSV-2 antigen than placebo recipients, 14.7 ± 0.7 mm compared to 8.8 ± 0.4 mm, $P<0.02$.

There were no differences in cytolytic activity against K562 targets among any of the groups (always less than 12%, data not shown). PBMC-mediated cytolysis of HSV-2 targets (Fig. 2) in sham-inoculated R-837 recipients was higher than that

TABLE 3

Proliferative responses[a] to HSV-2 antigen and PHA in placebo and R-837 recipients during the 28 days after HSV-2 or sham inoculation

Stimulus	Day	Sham Placebo	Sham R-837	HSV Placebo	HSV R-837 <36 h[b]	HSV R-837 >36 h[b]
HSV-2	7	0.8 ± 0.6	1.2 ± 0.2	1.5 ± 0.3	1.2 ± 0.2	3.3 ± 3.1
Antigen	14	1.3 ± 0.2	1.1 ± 0.4	1.9 ± 0.2	2.2 ± 0.3	6.9 ± 4.1
	21	0.9 ± 0.2	1.4 ± 0.2	8.6 ± 2.7	1.5 ± 0.4	7.2 ± 2.1
	28	1.2 ± 0.4	1.3 ± 0.3	23.4 ± 5.1	1.7 ± 0.3[c]	5.3 ± 1.3[d]
PHA	7	10.4 ± 3.1	21.1 ± 5.7[e]	17.0 ± 4.5	16.4 ± 4.9	13.9 ± 3.7
	14	12.1 ± 4.2	29.3 ± 6.3[e]	22.8 ± 3.4[e]	8.8 ± 3.4	15.3 ± 4.1
	21	10.1 ± 3.9	27.3 ± 6.1[e]	13.9 ± 3.7	11.0 ± 2.9	17.9 ± 4.8
	28	9.2 ± 2.7	33.7 ± 8.8[e]	11.0 ± 2.9	15.1 ± 4.1	13.2 ± 3.1

[a] Expressed as stimulation indices (S.I.± one S.D.). Mean CPM in control antigen and media wells was always less than 2000 CPM. A ratio of ⩾3 was considered positive.
[b] Duration of vaginal HSV-2 shedding.
[c] Compared to HSV-inoculated placebo recipients, $P<0.01$.
[d] Compared to HSV-inoculated placebo recipients, $P<0.05$.
[e] Compared to sham-inoculated placebo recipients, $P<0.01$.

TABLE 4

Interleukin-2 production in vitro from peripheral blood mononuclear cells of placebo or R-837[a] recipients during the 25 days after sham or HSV-2 inoculation

Day	Placebo recipients		R-837 recipients	
	Sham	HSV-2	Sham	HSV-2
0	1.4 ± 0.4	0.9 ± 0.3	0.8 ± 0.4	1.1 ± 0.1
4	1.1 ± 0.4	1.5 ± 0.4	1.7 ± 0.4	1.7 ± 0.7
11	1.3 ± 0.2	1.4 ± 0.6	1.0 ± 0.1	3.1 ± 1.4
18	0.9 ± 0.2	1.3 ± 0.1	0.6 ± 0.2	4.1 ± 0.6
25	0.9 ± 0.2	29.9 ± 11.4[b]	1.2 ± 0.1	9.6 ± 4.1[c]

[a] Treatment with R-837 or placebo was initiated 12 h after sham or HSV-2 inoculation and continued for five days at 12 h intervals. Interleukin production is expressed as the ratio of IL-2 activity in supernatant from HSV-2 stimulated wells compared to supernatant from control analyze wells. A ratio of ≥ 3 was considered positive.

[b] $P<0.01$ compared to Sham-placebo and Sham-R-837 recipients; $P<0.05$ compared to HSV-2 R-837 recipients.

[c] $P<0.05$ compared to Sham-placebo or Sham R-837 recipients.

of sham-inoculated placebo animals at the end of therapy, days 5 and 7. In HSV-inoculated placebo recipients, cytolysis of HSV-2 targets was enhanced compared to the control animals just prior to lesion eruption on day two, and also at the time of healing of genital lesions. In the HSV-infected R-837 group taken as a whole enhanced cytolysis of HSV targets was not observed until the end of therapy (Fig. 2, upper panel). However, the subset of R-837-treated animals with less than 36 hours of viral replication exhibited early enhanced cytolysis of HSV targets during R-837 treatment (Figure 2, lower panel).

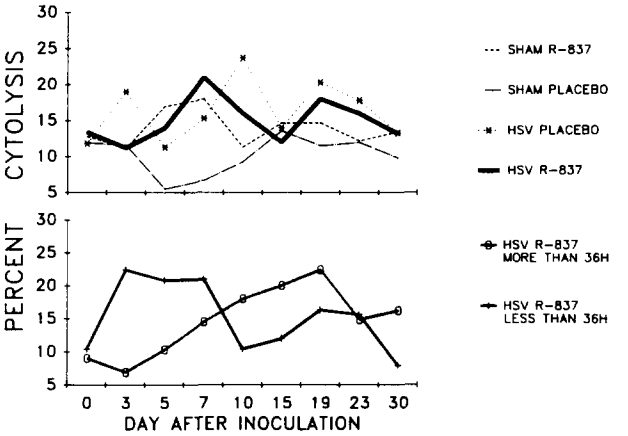

Fig. 2. Upper panel. Percent cytolysis of HSV-2 infected fetal guinea pig targets by peripheral blood mononuclear cells from the four experimental groups. Lower panel. Early enhancement of cytolysis of HSV targets during R-837 treatment was associated with early termination of vaginal shedding. The open circles represent R-837 recipients with more than 36 h vaginal HSV shedding and the cross-hatches represent those with less than 36 h shedding.

Toxic clinical effects of R-837 compared to unmodified HSV-2 infection

R-837 treated animals, whether sham-inoculated or HSV-inoculated, lost weight and had fever during the 5 days of treatment (Fig. 3). Sham-inoculated R-837, but not HSV-infected R-837 recipients had ruffled fur and were lethargic. Both sham-inoculated and HSV-inoculated R-837 groups decreased their water intake during treatment compared to sham-inoculated placebo animals (23.4 and 71.7 ml/animal/day compared to 128 ml/animal/day, respectively). Weight loss, decreased water intake (51.7 ml/animal/day) and fever observed in HSV-inoculated placebo recipients occurred on days five through 10 (when genital disease was most severe) compared to days one through five for R-837 recipients (during drug administration). On day three of R-837 treatment, total peripheral white blood cell counts were lower in R-837 recipients than in placebo recipients ($1.83 \pm 0.32 \times 10^3/mm^3$ compared to $4.51 \pm 0.86 \times 10^3/mm^3$ respectively, $P=0.001$).

Rechallenge with MS strain HSV-2

After HSV-2 rechallenge with an LD_{50} on day 60 after initial HSV-2 or sham inoculation, the previously sham-inoculated groups and the previously HSV-inoculated R-837 treated group with <36 h HSV shedding, exhibited typical genital disease, 100% incidence of hindlimb paralysis and/or urinary retention, and a 45% mortality rate. During the 28 days following rechallenge, the mean total lesion scores/animal was 20.2 for the originally sham-placebo group, 14.7 for the origi-

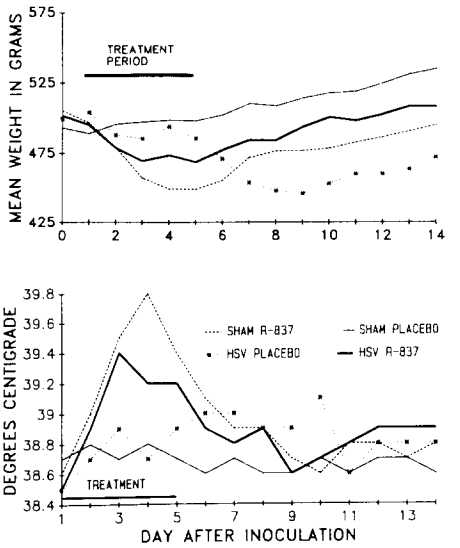

Fig. 3. Upper panel. Effect of HSV genital infection and/or R-837 topical treatment on body weight. R-837 induced weight loss during treatment and HSV infection caused weight loss during the period of genital disease. Lower panel. Changes in body temperature induced by genital HSV infection and/or R-837 topical treatment. R-837 induced fever during treatment and genital HSV infection induced fever during the time when lesions were present.

nally sham-inoculated R-837 group, and 21.4 for the originally HSV-inoculated R-837 recipients with less than 36 h HSV shedding. In contrast, minimal genital disease, no neurologic signs, and no mortality was seen in either the originally HSV-inoculated placebo group or the HSV-inoculated R-837 group with more than 36 h vaginal HSV-shedding. When compared with the sham-inoculated placebo group, both the HSV inoculated-placebo and the HSV-inoculated R-837 group with >36 h HSV shedding had lower mean total lesion scores/animal 2.1 ± 0.4 and 0.12 ± 0.07, $P<0.01$ and $P<0.0001$, respectively. The 2.1 mean total lesion score/animal rate is similar to a normal recurrence rate at this time after the initial inoculation in animals that have not been rechallenged.

Discussion

Attempts at providing effective therapy for initial and recurrent herpes have concentrated on the development of specific inhibitors of viral replication or augmentation of HSV-specific immune responses. Nonspecific immune modulators have generally been ineffective and in some cases have proven to be hazardous (reviewed in Corey et al., 1983), although IFN alpha administration early in primary infection has been shown to protect guinea pigs from initial but not recurrent genital disease (Kern, 1984). We were intrigued, therefore, that topical R-837, which lacks in vitro anti-HSV activity, provided complete protection from initial genital HSV-2 disease when treatment was initiated 12 h after HSV-2 inoculation. Drug administration also markedly reduced both the magnitude and duration of vaginal virus shedding. Thus, compared to our previous experience with oral acyclovir, also initiated 12 h after HSV-2 inoculation (Bernstein et al., 1987), topical application of R-837 was more effective against clinical disease, vaginal HSV-2 shedding and subsequent recurrent disease. Our results are also more dramatic than those reported during treatment of cutaneous footpad HSV-2 infection of guinea pigs with another biologic response modifier (Aurelian et al., 1987).

We have shown that topical R-837 induces higher interferon concentrations in plasma than intraperitoneal administration of Poly-I Poly-C, or intraperitoneal administration of R-837 at a dose of 3 mg/kg once a day (Chen et al., 1988). These differences may be due to differences in the bioassay used, a different route of administration or the higher daily dose in our experiments. The enhanced PHA responsiveness, earlier IL-2 and proliferative responses to HSV as well as enhanced HSV-2 delayed type hypersensitivity in R-837 treated HSV-infected animals differs from results reported in acyclovir treated humans, who exhibited both decreased and delayed proliferative responses to inactivated HSV antigen, presumably due to diminished production of HSV antigen in vivo (Lafferty et al., 1984). The decreased humoral responses to HSV appear similar to those reported in acyclovir treated guinea pigs (Bernstein et al., 1986, 1987) and humans (Ashely et al., 1984; Bernstein et al., 1984), and also are presumed to be due to truncated viral antigen production in vivo. Alternatively, decreased antibody production in R-837 recipients could be a direct effect of induced interferon on immunoglobulin synthesis by B-cells (Peters et al., 1986).

The enhanced cytolytic activity against HSV targets in animals with shortened vaginal HSV shedding is similar to that which we have previously observed in a small percentage of untreated HSV-inoculated animals that also subsequently developed no signs of clinical genital disease and truncated HSV-2 vaginal replication after vaginal HSV inoculation (7 of 84 animals, unpublished data). The effects on cytolysis of K562 targets were negligible, however, the activity was never very great. This suggests either that R-837 stimulates a different subset of NK cells than that recognizing K562 targets or that K562 cells are poor NK targets for guinea pigs despite previous reports (Altman et al., 1978; Debout et al., 1984).

The augmentation of in vitro cytolysis of HSV-2 targets by PBMC in R-837 recipients more than five days after R-837 treatment may be due to in vivo or in vitro cytokine induction of cytolytic cell populations. While cytokines might derive from monocytes and/or T-cells, both may be likely in view of the accelerated response of other cellular immune functions, for example, HSV-2 induced IL-2 production, proliferation, and the enhanced delayed type hypersensitivity. Increased or more efficient antigen presentation by monocyte/macrophages is suggested by the observed earlier in vitro HSV induced proliferative and IL-2 responses in drug recipients.

It appears that the toxicity associated with R-837 at the dose utilized, was due to interferon because the periods of fever and weight loss occurred at times when circulating interferon were above normal. R-837 induced higher titers of interferon sooner than unmodified genital HSV-2 infection, which also produced similar although later toxicity, i.e. fever and weight loss. Such toxicity has not been observed using a smaller dose once daily (Chen et al., 1988). Induction of IL-1 secretion by monocyte/macrophage subpopulations might also be involved in the febrile response. After day three of treatment, the decline in fever and interferon despite continued R-837 administration suggests tachyphylaxis, or exhaustion of an intermediary for in vivo secretion of interferon. Decreasing febrile responses have been seen in humans given interferon at frequent intervals (Breining et al., 1982).

Our data indicate that the development of immune-memory dependent functions (ELISA antibody, ADCC, IL-2 production, delayed type hypersensitivity reactions and proliferative responses to HSV-2) fail to develop without at least 36 h of vaginal HSV shedding. In previous studies, we have defined this as the approximate time needed for HSV to ascend the neural circuit to the ganglia (Bernstein et al., 1986). Therefore, it may be difficult to develop strategies in treating already infected hosts that would protect against the development of latency and still allow development of immune functions capable of protection from subsequent HSV challenge. This was underscored by the fact that HSV-inoculated R-837 recipients with less than 36 h vaginal shedding developed no immune-memory for HSV and remained as susceptible to genital rechallenge as previously uninoculated animals. In contrast, R-837 recipients with more than 36 h HSV shedding, suffered no initial genital disease, but developed immune memory for HSV-2 and were protected from genital disease during rechallenge, as were the infected placebo recipients which had undergone typical initial genital disease.

The mechanisms of protection against acute primary genital disease and of truncation of viral shedding remain undefined; however, both enhanced cell-mediated activity and cytokine induction may play a part. Enhancement of cytolytic activity and IL-2 induced immune responses have been implicated in protection against HSV-2 genital disease (Weinberg et al., 1986a,b, 1987). From our data it is not possible to conclude whether the decrease in recurrent genital disease was due to the up-regulation of HSV-specific cell-mediated immunity and production of cytokines which we detected after week two, or was due to decreased development of latent HSV-2 in the face of early nonspecific immunologic responses, or both. Regardless of the mechanisms, the absence of detectable latent HSV assayed by conventional explant cocultivation of ganglia and spinal cord from animals treated topically after HSV inoculation differs from that of acyclovir which had no effect on latency (Stanberry et al., 1982).

Investigation into the effects of even later initiation of treatment on the development of latency and resulting recurrent genital disease are underway. In view of the apparent differences in neural latency, it would also be interesting to assess movement of HSV-2 in neural tissues during acute infection (days 3–5) seeking quantifiable differences between placebo and R-837 treated animals after HSV-2 infection. Differences in the amount of the virus initially entering the neural tissues could explain the R-837 induced differences in both acute and recurrent clinical genital disease, as well as the decreased recovery of latent virus in ganglia.

In summary HSV-infected R-837 treated animals were completely protected from primary genital disease, and had reduced vaginal HSV-2 shedding, recurrent disease, and no detectable latent HSV-2 in neural tissues. Immunologically, there was immediate IFN production, variable enhancement of either early or late cytolysis of HSV-2 targets, and early induction of HSV-2 induced PBMC proliferation and IL-2 production. In contrast, ELISA and ADCC antibody were produced later and at lower levels. In some R-837 treated animals, HSV-2 replication was stopped so abruptly that no memory dependent immune functions resulted. Although R-837 has no in vitro anti-HSV activity, it was highly protective against HSV-2 disease in vivo apparently by virtue of its immune up-regulating capacity resulting in enhanced cytokine production and cell-mediated immune responses in guinea pigs. Studies are currently underway to determine less toxic and minimum protective dosage in guinea pigs which should allow trials of R-837 to begin in humans.

Acknowledgements

Animals used in this study were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Children's Hospital Research Foundation. We thank L.R. Stanberry for helpful discussions; M.G. Myers for critical review of the manuscript, Moira Haren and Gary Echler for technical assistance, and Charlotte Allen for manuscript preparation. This work was funded in part by grants HD-22214 and AI-23482 and a grant from 3M Riker, 3M Health Care Group.

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