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Topical treatment with BV-araU of immunosuppressed and immunocompetent shaved mice cutaneously infected with herpes simplex virus type 1

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Summary

Effect of topical treatments with BV-araU was tested in cutaneous infections of shaved Balb/c mice with herpes simplex virus type 1. Evolution of zosteriform skin lesions associated with infection with a low virulent KOS(S) strain was almost completely suppressed by treatments with 5% BV-araU cream given 4 times daily for 5 days starting 1 day after inoculation. This effect was equivalent to that of Zovirax Cream including 5% acyclovir. One percent BV-araU cream was also effective in inhibiting progression of symptoms, while 0.2% cream was not effective. Five percent BV-araU cream significantly suppressed progression of skin lesion even if initiation of treatment was delayed to 2 days after infection. However, the efficacy was diminished by further delay in starting treatment. The effect of BV-araU cream was also evident during infection of immunosuppressed mice. Virus titers in the skin tissue encompassing the inoculation site of mice decreased the day after the first treatment. In the lower flank site, virus replication was almost completely suppressed by the treatment beginning 1 day postinfection. Topical application of BV-araU may be useful therapy for HSV-1 infections in humans, including immunocompromised patients.

BV-araU; HSV-1; Mouse skin infection; Topical treatment

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Introduction

1-β-D-Arabinofuranosyl-*E*-5-(2-bromovinyl)uracil (BV-araU) exhibited potent antiviral activity against herpes simplex virus type 1 (HSV-1) and varicella-zoster virus (VZV) but was weakly active against herpes simplex type 2 in cell cultures (Machida, 1986; Machida and Sakata, 1984; Machida et al., 1981). In clinical trials, oral administration of BV-araU to immunocompetent and immunocompromised patients with herpes zoster was effective in reducing the time of vesicles, erythema and pain (Hiraoka et al., 1991; Niimura et al., 1990). BV-araU also showed potent effects against intraperitoneal (i.p.) and intracerebral infections with HSV-1 in mice (Ijichi et al., 1990; Machida et al., 1990; Machida and Takezawa, 1990). We have previously reported that oral treatment with BV-araU at a dose of 20 mg/kg twice daily was effective in suppressing cutaneous symptoms and reducing mortality of both immunosuppressed and immunocompetent shaved Balb/c mice cutaneously infected with HSV-1 (Machida et al., 1992). The only study to demonstrate the effect of topical BV-araU was reported by De Clercq (1984), who showed that 10% BV-araU dissolved in DMSO was moderately effective in inhibiting the onset of cutaneous symptoms caused by HSV-1 infection in hairless mice. However, no study showed in detail the effect of topical treatment with BV-araU against HSV-1 skin infections. Since shaved mice develop clear skin lesions by cutaneous infection with HSV-1, this infection can mimic dermal herpes infections in human (Machida et al., 1992; Simmons and Nash, 1984), and was suggested to be use for pre-clinical evaluation of new antiherpes drugs (Kristofferson et al., 1988). In the current study, we have prepared formulations containing various concentrations of BV-araU and show the efficacy of BV-araU cream in HSV-1 infected shaved mice, the influence of delayed therapy, and the inhibition of virus growth in dermal tissues. Herpes virus infections are often seen in immunocompromised patients and these infections tend to be life-threatening. Therefore, antiherpesviral drugs are required to have potency even for these patients for the beneficial clinical application. We have also included examination of effect of BV-araU cream in immunosuppressed mice, which can mimic such immunocompromised patients (Ijichi et al., 1990).

Materials and Methods

Animals

Male Balb/c mice were bred in our laboratory starting from a colony obtained from Clea Japan or purchased from Clea Japan. To induce immunosuppression, mice were treated i.p. with 200 mg of cyclophosphamide (Aldrich Chemical) per kg body weight one day before virus inoculation according to the method of Ikeda et al. (1988). Degree of induced immunosuppression was described by Ikeda et al. (1988).

Viruses

Two strains of HSV-1 were used. One of the sub-strains of KOS strain, namely KOS(S) strain, was kindly supplied by Dr. Y. Ozaki, Shiga Medical College. Lethal infection did not occur in immunologically normal mice when infected i.p. with this strain (Ijichi et al., 1990). By the cutaneous infection, the infected mice developed severe zosteriform symptoms, but the lesions spontaneously resolved 2 to 3 weeks after inoculation (Machida et al., 1992). HSV-1 F strain, a gift from Dr. Hayashi, Public Health Research Institute of Kobe City, Kobe, was lethal for mice by the cutaneous infection with most of the infected mice dying between 6–9 days postinfection (p.i.). Both strains were susceptible to BV-araU: ED_{50}s for KOS(S) and F strains in mouse 3T3 cells were 0.137 and 0.125 μg/ml, respectively (Machida et al., 1992).

Viral inoculation

The right dorsum of the mice (usually 7–8 weeks of age) was shaved with a razor 1 or 2 days before virus inoculation and abraded with a needle, about 5 $\times$ 5 mm^2 in area. 15 μl of a viral suspension containing HSV-1 KOS(S) strain (2.2×10^7 PFU) or F strain (1.0×10^7 PFU) was inoculated onto the abraded dorsal flank according to the method of Nagafuchi et al. (1979). The infected mice were housed individually with their neck protected by a necklace-ring (Natsume Seisakusho) to prevent licking drug-containing cream.

Drugs and treatments

Five percent BV-araU cream, based in 25% polyethylene glycol 900, NF and 35% propylene glycol, USP, and containing several additives such as thickeners and emollients, emulsifiers, antifoam, and antioxidant, was prepared at Bristol-Myers Squibb Pharmaceutical Institute. The 5% BV-araU cream was diluted with a cream containing the same components without drug, to obtain 1 and 0.2% BV-araU cream. Mice were topically treated with BV-araU cream using cotton-applicator every 4 h, 4 times daily from 8.00 to 20.00 h, for 5 days beginning one day p.i. For delayed therapy assay, initiation of the treatment was delayed 2–5 days p.i. Control mice received placebo cream, containing the same components without drug. Both BV-araU cream and placebo cream did not show any skin toxicity for the shaved mice.

Evaluation of efficacy

Mice were observed for skin lesions and death twice a day. The severity of skin lesions was scored from 0 (for no lesion) to 8 (for most severe zosteriform ulceration with systemic symptoms such as paralysis) according to the criteria previously described (Machida et al., 1992). Morbidity rates were determined by dividing the number of mice with significant symptoms (lesion score; 3 or greater) by the total mice number. The differences between placebo-treated control and drug-treated groups in the mean symptom score at each time-point and maximum lesion score were statistically evaluated by the Mann-Whitney U test. Differences in the morbidity rate and mortality rate between placebo-

treated control and drug-treated groups were evaluated by chi-square test with Yates' correction.

Determination of viral titers in dermal tissues

To determine the inhibition of HSV-1 replication in dermal tissues of the infected mice, three or four mice from 5% BV-araU cream-treated or placebo-treated group were killed at appropriate times after inoculation with KOS(S) strain. The virus inoculation site and the lower flank site were obtained separately. The lower flank site consisted of skin between the inoculation site and the anterior midline, which was excluded from inoculation site, but showed zosteriform lesions. The skin specimens were washed three times with cold sterile phosphate buffer saline (PBS), minced, and homogenized with a Potter–Elvehjem homogenizer to make a 5% suspension with sterile PBS. The homogenates were centrifuged at 1500 × *g* for 10 min at 4°C and the supernatant was stored at −80°C until use. Virus titration of each sample was determined by a standard plaque assay on immortalized human embryo cells, KMST-6 (Machida and Takezawa, 1990). In the assay, the infected cells were carefully washed with Hanks' balanced salt solution after 30 min of adsorption period. Our preliminary experiment indicated that influence of persisting BV-araU in the dermal tissues originated from cream on the assay was almost completely minimized by the procedures washing the skin specimens and infected cells, probably because BV-araU is needed to continuously persist for exhibition of anti-HSV-1 effect (Machida et al., unpublished).

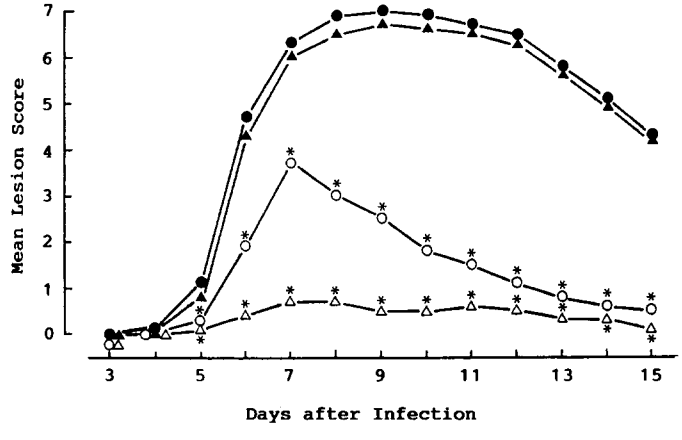

Fig. 1. Effect of BV-araU cream with various concentrations on cutaneous infection with HSV-1 KOS(S) strain. Mice were treated with each cream every 4 h, 4 times a day for 5 days starting 1 day p.i. (●) Placebo-treated; (▲) 0.2% BV-araU cream; (○) 1% BV-araU cream; (△) 5% BV-araU cream. *$P < 0.001$ by the Mann-Whitney U test.

Results

Antiviral effect of BV-araU cream

As shown in Fig. 1, treatments with 1 and 5% BV-araU cream displayed good efficacy in inhibiting the evolution of skin lesions associated with HSV-1 KOS(S) infection. None of the mice treated with 5% BV-araU cream showed any zosteriform symptoms. The morbidity rate decreased to 20% in mice treated with 5% BV-araU cream compared with 100% in placebo-treated group ($P < 0.001$ by χ^2-analysis with Yates' correction). Mean lesion score in mice treated with 1% BV-araU cream decreased after 7 days, while high mean scores were recorded from 6 to 15 days p.i. in placebo-treated group. The mean maximum lesion score was also lower in the groups treated with 1% and 5% BV-araU cream than that in placebo-treated group: the score in placebo-treated group was 7.0, and those in groups treated with 1% and 5% BV-araU cream were 3.9 and 1.3, respectively ($P < 0.001$ by the Mann-Whitney U test). In contrast, 0.2% BV-araU cream did not show any therapeutic effect. The effect of 5% BV-araU cream was comparable to that of 5% Zovirax Cream (Table 1). This was observed reproducibly. The data in Table 1 also confirmed that the treatment with 5% BV-araU cream was markedly effective in inhibiting the appearance of symptoms.

Effect of delayed therapy with 5% BV-araU cream

Fig. 2 shows the changes in mean lesion scores of cutaneous symptoms in KOS(S) strain-infected mice treated with 5% BV-araU cream beginning 1 to 5 days after infection. Progression of zosteriform symptoms was inhibited when treatments started as late as 2 days p.i. In the groups of mice that received 5% BV-araU cream beginning 3 or 5 days p.i., mean lesion scores were lower than those in placebo-treated group, but the scores increased again after cessation of

TABLE 1

Effect of BV-araU and Zovirax cream on the cutaneous infection with HSV-1 KOS(S) strain in mice

Treatment	Maximum lesion score (Mean ± SE)	Morbidity rate[a]
Exp. 1		
None (placebo)	5.60 ± 0.31	15/15
5.0% BV-araU	0.33 ± 0.19 ($P < 0.001$)[b]	0/15 ($P < 0.001$)[c]
5.0% Zovirax cream[d]	0.27 ± 0.21 ($P < 0.001$)	1/15 ($P < 0.001$)
Exp. 2		
None (placebo)	4.53 ± 1.41	14/15
5.0% BV-araU	1.57 ± 1.40 ($P < 0.001$)	4/14 ($P < 0.01$)
5.0% Zovirax cream	1.33 ± 1.35 ($P < 0.001$)	5/15 ($P < 0.01$)

7-week-old and 6-week-old Balb/c mice were used in Exp. 1 and Exp. 2, respectively.
[a]Number of mice with significant symptoms (lesion score; 3 or greater) per total.
[b]Significantly different from the placebo-treated control (the Mann-Whitney U test).
[c]Significantly different from the placebo-treated control (χ^2-analysis with Yates' correction).
[d]Containing 5% acyclovir.

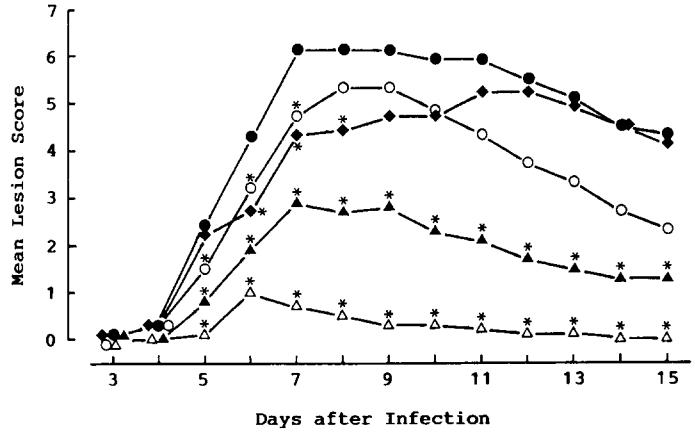

Fig. 2. Effect of BV-araU cream treatment on cutaneous infection with HSV-1 KOS(S) strain. Inoculated mice received placebo cream or 5% BV-araU cream 4 times a day for 5 days beginning various times after inoculation: placebo (control), day 1–5 (●); BV-araU cream, day 1–5 (△); BV-araU cream, day 2–6 (▲); BV-araU cream, day 3–7 (◆); and BV-araU cream, day 5–9 (○). $^{*}P < 0.01$ by the Mann-Whitney U test.

the treatment. Mean maximum lesion scores in groups treated from 1 and 2 days p.i. were 1.4 and 4.1, respectively, with significant reduction from placebo-treated group ($P < 0.001$ by the Mann-Whitney U test). Reduction in mean maximum lesion score was not found in group treated from 3 or 5 days p.i. However, the morbidity rate was significantly reduced only in the group treated from 1 day p.i. The morbidity rates in placebo-treated control group and groups treated from 1, 2, 3 and 5 days p.i. were 100%, 13% ($P < 0.001$ by χ^2-analysis with Yates' correction), 80%, 100% and 100%, respectively.

BV-araU cream (5%) was also effective in HSV-1 F strain infection (Table 2). Most of placebo-treated control mice died within 9 days p.i. (the mean

TABLE 2

Effect of delayed therapy with 5% BV-araU cream on the morbidity rate and mortality rate of HSV-1 F strain-infected mice

Duration of treatment with BV-araU	Maximum lesion score (Mean ± SE)	Morbidity rate[a]	Mortality rate
None (placebo)	6.00 ± 0.41	15/15	13/15
1–5 days p.i.	0.93 ± 0.39 ($P < 0.001$)[b]	4/15 ($P < 0.001$)[c]	1/15 ($P < 0.001$)[c]
2–6 days p.i.	4.13 ± 0.62 ($P < 0.05$)	10/15 ($P < 0.05$)	5/15 ($P < 0.01$)
3–7 days p.i.	4.93 ± 0.72	11/15	5/15 ($P < 0.01$)
4–8 days p.i.	5.00 ± 0.67	12/15	6/15 ($P < 0.05$)

[a]Number of mice with significant symptoms (lesion score; 3 or greater) per total.
[b]Significantly different from the placebo-treated control (the Mann-Whitney U test).
[c]Significantly different from the placebo-treated control (χ^2-analysis with Yates' correction).

survival time was 7.0 days). Treatments starting 1 day p.i. markedly suppressed the development of symptoms. Symptoms were also suppressed when treatments began 2 days p.i. The morbidity rates in groups treated from 1 and 2 days p.i. were significantly reduced. However, the effect was not significant when treatments began 3 and 4 days. The survival rate markedly increased in all treated groups irrespective of the day of initiation of the treatment.

Effect of 5% BV-araU cream on HSV-1 KOS(S) infection in immunosuppressed mice

Next, effect of 5% BV-araU cream in immunosuppressed mice infected with HSV-1 KOS(S) strain was tested. In immunosuppressed mice receiving placebo cream zosteriform erosion developed as did in immunocompetent mice, but this erosion hardly progressed to zosteriform ulceration. Ulceration was seen, if any, only in narrow area, and mice died with symptoms remaining at the state of zosteriform erosion (lesion score 4 to 5). Formation of the ulceration might be due to immune reaction by host defense mechanism, and low responsibility of the immune system in the immunosuppressed mice may participate hardly developing ulceration. Consequently, the severity of symptoms, scored according to previously determined criteria (Machida et al., 1992), in immunosuppressed placebo-treated control group were less than that in placebo-treated immunocompetent mice shown in Figs. 1 and 2. When the immunosuppressed mice received 5% BV-araU cream therapy starting 1 day p.i., a marked effect was observed, though it was slightly less effective than that observed in normal mice (data not shown). A significant reduction was also seen in the severity of symptoms of immunosuppressed mice throughout experiment period compared to the placebo-treated control group of mice even

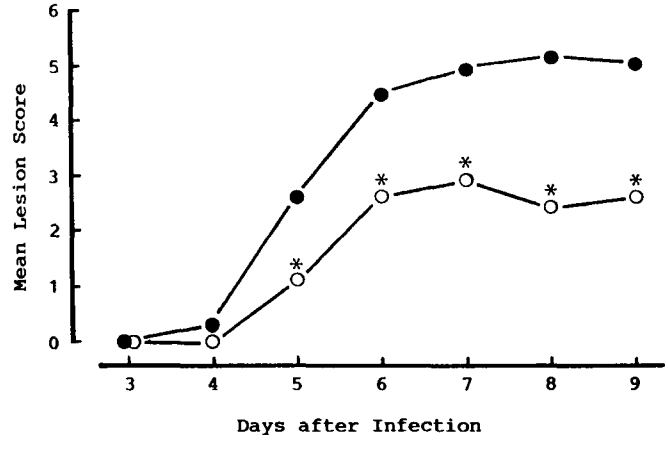

Fig. 3. Effect of BV-araU cream on cutaneous infection with HSV-1 KOS(S) strain in immunosuppressed mice. Mice were treated with 5% BV-araU cream every 4 h, 4 times a day for 7 days beginning 2 days p.i. (●) Placebo (control); (○) 5% BV-araU cream. $^{*}P < 0.001$ by the Mann-Whitney U test.

when initiation of the treatment with 5% BV-araU cream was delayed to 2 days p.i. (Fig. 3). BV-araU cream also reduced the maximum lesion score. The mean maximum lesion score in the placebo-treated group was 4.9 compared with 3.1 in the treated group ($P < 0.001$ by the Mann-Whitney U test). More than half of the placebo-treated control mice died within 10 days p.i. and the final mortality rate was 100% in this group. On the other hand, only 33% of the treated mice died ($P < 0.01$ by χ^2-analysis with Yates' correction), although all of the treated mice developed symptoms score of 3 or greater (morbidity rate was 100%).

Inhibitory effect of BV-araU cream on virus replication in dermal tissue

Effect of 5% BV-araU cream was also assessed by monitoring the change in virus titers isolated from dermal tissues. The fate of inoculated HSV-1 KOS(S) strain was first examined during the first 24 h following inoculation. The level of infectious virus recovered from the dermal tissue (inoculation site) was 1.3×10^3 PFU per tissue at zero time and gradually decreased and became below detectable level (10 PFU per tissue) 12 h after inoculation (Fig. 4A). Since a high titer of virus is observed at 24 h after inoculation, infectious virus detected at times over 24 h p.i. are progeny viruses. Changes in virus titers in dermal tissues were next determined during the course of therapy with 5% BV-araU cream. In the placebo-treated group, a high virus titer was found at the inoculation site for 4 days, before rapidly decreasing (Fig. 5B). On the other hand, a large amount of infectious virus was detected at the lower flank site of placebo-treated control mice 4 days i.p., showing a peak 5 days p.i. (Fig 5C). Infectious virus was no longer detectable in either the inoculation site or the lower flank site of any mouse 7 days p.i., although skin lesion gave a high score

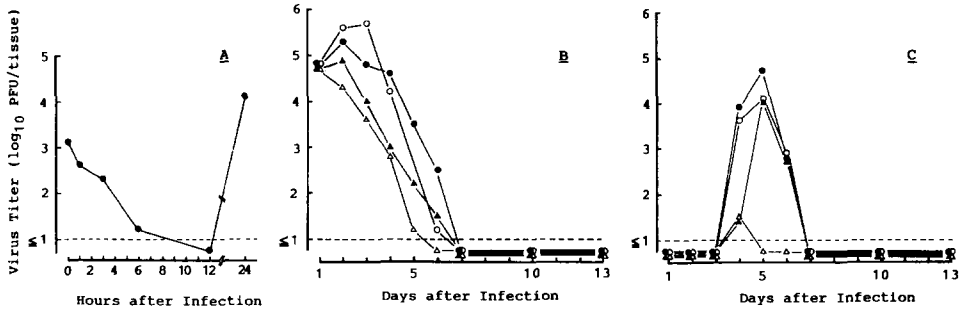

Fig. 4. Changes in virus titers found in dermal tissues of HSV-1 KOS(S) strain-infected mice. (A) Change in amount of infectious virus recovered from the inoculation site. Mice were cutaneously infected with HSV-1 and virus titers determined for the first 24 h post-inoculation as described in Materials and Methods. Each point represents geometric means obtained from four mice. (B) Changes in virus titers in the inoculation site of mice receiving 5% BV-araU cream therapy starting on different days after inoculation. Infected mice were treated with placebo for 5 days starting 1 day p.i. (●), or BV-araU cream 4 times a day for 5 days beginning 1 (△), 2 (▲), or 3 (○) days p.i. Virus titers are geometric means from a group of three or four mice. (C) Changes in virus titers In the lower flank site of mice receiving 5% BV-araU cream therapy starting on different days after inoculation. The same mice were used as in Fig. 4B.

on that day. In the treated groups which received therapy beginning 1 and 2 days p.i., the virus titer in the inoculation site decreased on the day following the first treatment. BV-araU treatments started 1 day p.i. almost completely suppressed virus replication in the lower flank site accompanying marked inhibition of progression of the skin lesions (results similar to that shown in Fig. 2). When treatments were started 2 and 3 days p.i., virus titers in the lower flank site were only 5 times lower (about 10^4 PFU per tissue) than that of placebo-treated mice. However, progression of the symptoms was suppressed in the treated groups compared to placebo-treated group (Fig. 2).

Discussion

We investigated the effects of topical treatments with BV-araU cream on HSV-1 cutaneous infections of mice using an animal model of HSV infections in human. Early treatments with 5% BV-araU cream showed excellent efficacy in blocking the progression of skin lesions caused by infection with HSV-1 KOS(S), and reducing the morbidity rate. Our study is the first finding on the effect of topical applications of BV-araU cream available for clinical use, although De Clercq (1984) showed a moderate effect of BV-araU dissolved in DMSO against the onset of symptoms in hairless mice. Topical treatments with cream-based BV-araU seems equivalently effective to those with 5% Zovirax, which is commercially available, and slightly more efficacious than oral administration in the cutaneous infections of mice (Machida et al., 1992). A moderate effect was seen when the treatment initiated at 2 days p.i. Five percent BV-araU cream was effective even when treatments were started as late as 3 or 5 days after infection with HSV-1 KOS(S). However, a significant reduction in the progression of symptoms was limited to the time during treatment. Thus, delayed therapy with BV-araU cream reduced its efficacy. A long-term treatment may be necessary for effective therapy with BV-araU cream in delayed therapy. Kristofferson et al. (1988) reported that topical treatment with foscarnet initiated 12 h, 24 h and 48 or 72 h p.i. were markedly effective, moderately effective, and inefficacious, respectively. Klein (1985) showed reduced effects of late therapy with other antiherpesvirus drugs as well.

We observed that the inoculum virus titer was reduced below detectable level following inoculation by 12 h, while Sydiskis and Schultz (1965) reported reduction of virus titer from inoculum levels within 6 h. Klein (1985) suggested that the inoculated virus immediately disseminates to a different site, probably sensory ganglia, and returns to the same site by 24 h p.i. The virus titer in the lower flank site reached maximum 5 days p.i. and then rapidly decreased. In contrast to our findings, Kristofferson et al. (1988) showed persisting of high titers of virus in both inoculation site and the lower flank from 4 to 7 days p.i. Nature of virus and the host immune system should affect the virus clearance in the murine infection model. In spite of the complete clearance of virus by 7 days p.i., severe ulceration continued from 6 to over 10 days p.i. in our

infection model. The cutaneous symptoms are not only caused by virus replication, but may also be related to non-specific cellular immune responses in the infected mice (Simmons and Nash, 1984).

The 5% BV-araU cream strongly inhibited virus replication in skin tissues. In particular, the virus titer in the lower flank site was nearly completely suppressed by treatments with BV-araU cream beginning 1 day p.i. The secondary virus dissemination would account for the zosteriform distribution of local skin lesions (Simmons and Nash, 1984; Stanberry et al., 1982). Complete suppression of the development of zosteriform symptoms by the BV-araU cream treatments should correlate with inhibition of the secondary virus dissemination. BV-araU cream was also effective in reduction of the symptoms at 7 and 8 days p.i. even when treatments began 5 days p.i., while infectious virus in placebo-treated group disappeared 7 days p.i. There is a possibility that some undetectable virus remained in the skin tissues, and caused the progression of symptoms during the late phase of infection. Delayed therapy might suppress these symptoms in later stage infection.

The effectiveness of BV-araU cream on cutaneous infection was also demonstrated in immunosuppressed mice, as observed when BV-araU was administered orally (Machida et al., 1992), although it was not as effective as seen previously in immunocompetent mice. Since BV-araU cream markedly suppressed progression of skin lesions and inhibited virus replication in skin tissues of mice cutaneously infected with HSV-1, it seems that BV-araU cream may have useful clinical effectiveness for human herpesvirus diseases, including those in immunocompromised patients such as AIDS.

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