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The Antiviral Effectiveness of Butylated Hydroxytoluene on Herpes Cutaneous Infections in Hairless Mice (41425)

ALEC D. KEITH, DORIS ARRUDA, WALLACE SNIPES,[1] AND PHILLIP FROST*

*Biophysics Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802, and *Dermatology Department, Mount Sinai Hospital, Miami, Florida 33140*

Abstract. Hairless mice, cutaneously infected with herpes simplex virus type 1 (HSV-1), were treated topically with butylated hydroxytoluene (BHT). The effectiveness of BHT in shortening the duration of infections was assayed under three conditions. In the first experiments, mice undergoing primary infections with no prior immunity to HSV-1 were utilized. These animals tended to develop deep lesions that were not typical of recurrent HSV-1 infections in humans. A second set of experiments utilized mice that had recovered from a primary infection and that were immunosuppressed by γ irradiation. Immunosuppression was essential for the full development of lesions upon reinfection. The lesions in these animals remained more localized with less tendency to spread into deep tissues. A third set of experiments utilized animals that were subcutaneously inoculated with human serum γ-globulin 24 hr prior to infection. Lesions on these animals also remained localized and did not penetrate into deep tissues. Under all three conditions, BHT was found to be effective in reducing the clearance time of HSV-1 cutaneous lesions when applied topically to the infected area.

The agents reported to be clinically effective against HSV, or which have sufficient animal investigations to establish their clinical potential, usually interfere with viral nucleic acid metabolism in some way. The nucleoside analogs iododeoxyuridine (1), adenine arabinoside (2), and cytosine arabinoside (3) block the synthesis of viral (and to some extent cellular) DNA. Phosphonoacetic acid inhibits the HSV-specified DNA polymerase (4). Other compounds have also been advanced and may prove useful (5). Several intercalating dyes have been used in conjunction with light as photodynamic agents designed to function against HSV infections.

An alternative approach to the control of HSV infections is being explored in our laboratory. We have chosen to concentrate on molecules which have a high affinity for biological membranes and which perturb membrane structure. The rationale is based on the expectation that certain viral membrane functions may be more sensitive to perturbers than are cellular membrane processes. The subject of the present investigation, butylated hydroxytoluene (BHT), is a hydrophobic molecule that satisfies these criteria. BHT, which is extensively used as an antioxidant in foods, has been found to be noncarcinogenic (6), nonteratogenic (7), and nonmutagenic (8) in laboratory animals. The extensive literature on BHT has generally established its nontoxic properties (9). Our previous investigations (10–12) have shown that BHT is a potent inactivator of HSV and other lipid-containing viruses *in vitro*. The present paper deals with the effectiveness of BHT against type 1 HSV infections in hairless mice.

In an attempt to produce in hairless mice HSV-1 lesions that are similar to those of recurrent HSV infections in humans, we developed a procedure based on partial passive immunization with human serum γ-globulin. This approach was utilized in some of the experiments with BHT reported here and may provide a very useful model system for evaluating antiviral agents.

Materials and Methods. *Laboratory*

[1] To whom reprint requests should be addressed.

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mice. Hairless mice from Jackson Laboratories (Bar Harbor, Maine), 10–24 weeks old, were used in all experiments reported here. The mice were maintained in separate cages to prevent spread of infections between or among mice. The mice were kept in quarters with constant temperature (22°), uniform ventilation, and a 12-hr light/dark period, and were maintained on a diet of rat chow. No oral administration of BHT was carried out in these experiments.

HSV infections. Hairless mice were infected by making two parallel scratches on the right midthoracic area with a 27-gauge hypodermic needle. The scratches were approximately 1 cm long and of a depth such that, upon stretching the skin slightly, reflected light could be seen coming from tissue fluid collected in the scratch sites. HSV-1, strain KOS, at a titer of 7×10^7 PFU/ml was directly applied with a cotton swab and massaged in for about 10 sec.

By the second to third day after infection two parallel rows of blisters could be seen. These early lesions either developed progressively, resulting in crusted lesions on the fifth to seventh day, or regressed, leaving a reddened zone for 1–2 days. Lesions of this latter type usually cleared between 4 and 8 days, but occasionally reappeared as more extensive lesions in underlying tissue.

Scoring lesions. Beginning on Day 3 postinfection, mice were scored daily for the presence and severity of lesions. The presence of blisters, redness along the infection site, and crusted areas over the infection site were scored as positive, and the severity of these lesions was designated +, ++, or +++. Lesions that appeared early were scored as clear (0) when blisters and redness disappeared. Crusted lesions that developed later were scored as clear when the crust was lost and the skin color returned to normal.

BHT treatment. BHT was dissolved in a purified mineral oil (Drakeol, Penreco Div., Pennzoil Corp., Butler, Pa.). Initially, a preparation of 5% BHT was applied using three drops from a glass applicator directly to the infected site. This application corresponds to about 40 mg of preparation or 2 mg BHT for the 5% solution. An improved procedure involved the use of 15% BHT in mineral oil applied with a cotton-tipped applicator. This reduced the volume of preparation to about one-fifth that used in the drop method, restricted the preparation more closely to the infected site, and eliminated the toxic effects occasionally observed by the drop method.

γ irradiation. Mice were irradiated for 4 min in a ^{60}Co γ source at a dose rate of 156 rad/min. This dose corresponds to about the LD_{25}. Deaths began to occur about 12–14 days after exposure.

Human serum γ-globulin injections. In order to provide initial partial immunity to HSV-1, mice were subcutaneously injected with filter-sterilized human serum γ-globulin (Sigma Chemical Co., St. Louis, Mo.) dissolved in isotonic saline solution at a concentration of 1 mg/ml. After injection the skin area was massaged. This concentration was arrived at by titrating the injected dose to the level where lesions were visible but localized. Controls were injected with saline.

Results. *Toxic effects of BHT*. When either uninfected or infected mice were treated three times daily at the same site for several days by the drop procedure described under Materials and Methods, toxic effects were sometimes observed. These appeared initially as a skin irritation developing into erythema and eventually some sloughing of the skin. These effects were readily distinguishable from the early blisters and late crusted lesions in infected mice, but were not very dissimilar to the red zones that appear from Days 4 to 7 in mice undergoing primary infections without prior immunization. The toxic effects of BHT were not apparent when the more localized application of a smaller volume was applied with a cotton-tipped applicator.

Primary infections. Initial experiments established the effectiveness of BHT against primary HSV-1 infections in mice that had no prior immunity to the virus. Forty-two mice were infected and divided randomly into three groups of 14 mice each. One group was maintained as untreated controls, a second group was treated with mineral oil only, and a third group was

treated with mineral oil containing 5% BHT. Two treatments per day were given for 3 days starting 24 hr after infection. Applications were made by the drop procedure, described under Materials and Methods. In these initial experiments, the scorer was aware of which animals belonged to control, mineral oil, and mineral oil plus BHT groups.

Table I shows data for these experiments. The last column gives the percentage of animals scored positive for Days 3 through 10 postinfection. Statistical treatment of these data by the χ^2 analysis shows that the BHT-treated group had significantly fewer lesions than the control group at $P = 0.05$ or better on Days 8–10. On Day 7, the BHT-treated group had fewer lesions at $P = 0.10$. The group treated with mineral oil only was not significantly different from untreated controls in these experiments. In later experiments (see below) when more expertise was achieved in making small reproducible scratches, statistically significant reductions in the number of animals with lesions were seen at earlier times.

Time of treatment. Additional experiments were carried out to determine the optimal time to initiate BHT treatment. These experiments indicated that BHT treatment in the early phases of infection was more effective than initiating the treatment after the lesions developed. Seven groups of mice were infected; one group was maintained as an untreated control, and treatments were initiated on Days 1–6 for the remaining groups. In each case, treatments were twice daily for 3 days. Table II shows pooled data for the groups whose treatment began on Days 1–3

TABLE I. TREATMENT OF PRIMARY HSV-1-INDUCED LESIONS IN HAIRLESS MICE WITH MINERAL OIL AND MINERAL OIL CONTAINING 5% BHT

Treatment	Day after infection	Number with lesions[a] of severity				Percentage with lesions
		0	+	++	+++	
None	3		14			100
	4		6	7	1	100
	5		4	5	5	100
	6		6	4	4	100
	7	1	7	3	3	93
	8	1	6	4	3	93
	9	2	8	3	1	86
	10	2	7	4	1	86
Mineral oil only	3		14			100
	4		8	4	2	100
	5		6	5	3	100
	6	1	5	4	4	93
	7	2	6	4	2	86
	8	2	6	4	2	86
	9	2	6	3	3	86
	10	3	6	2	3	79
Mineral oil plus 5% BHT	3	2	12			86
	4	2	9	3		86
	5	1	7	4	2	93
	6	2	8	2	2	86
	7	4	6	2	2	71*
	8	7	2	3	2	50**
	9	8	3	2	1	43**
	10	8	3	2	1	43**

[a] Out of 14 animals used in each group.
* χ^2 significant at 10% level.
** χ^2 significant at 5% level.

TABLE II. EVALUATION OF TIME OF INITIATING TREATMENT ON THE EFFECTIVENESS OF 5% BHT PREPARATION ON PRIMARY HSV-1 LESIONS IN HAIRLESS MICE

Treatment	Day after infection	Number with lesions[a] of severity				Percentage with lesions
		0	+	++	+++	
None	3		12			100
	4		12			100
	5		8	3	1	100
	6	2	6	2	2	83
	7	3	4	3	2	75
	8	3	5	2	2	75
	9	3	6	2	1	75
	10	4	7	1		67
5% BHT (early[b])	3	2	10			83
	4	1	10	1		92
	5	3	9			75
	6	7	4	1		42*
	7	6	3	3		50*
	8	7	4	1		42*
	9	7	4	1		42*
	10	7	4	1		42*
5% BHT (late[c])	3		12			100
	4		11	1		100
	5		9	1	2	100
	6	2	7	2	1	83
	7	2	5	4	1	83
	8	4	5	2	1	67
	9	5	6	1		58**
	10	5	6	1		58**

[a] Out of 12 animals used in each group.
[b] Four animals' treatment was initiated on Day 1, four on Day 2, four on Day 3; data pooled.
[c] Four animals' treatment was initiated on Day 4, four on Day 5, four on Day 6; data pooled.
* χ^2 significant at 5% level or better.
** χ^2 significant at 10% level or better.

(early), and for the groups whose treatment began on Days 4–6 (late). The degree of statistical significance in the control *vs* treated groups for the percentage scored positive is indicated in Table II. The results show that BHT treatment was most effective when initiated early, under the conditions of these experiments. When treatment was initiated at later times (Days 4–6), the effectiveness of BHT in reducing the number of lesions was only seen near the end of the experiment when some of the untreated animals were beginning to clear. In all subsequent work treatments were started on Days 1–3 after infection.

Secondary infections in healed, γ-irradiated mice. These experiments were carried out in an attempt to produce HSV-1 lesions that remained more localized to the site of infection and more similar in appearance to herpes labialis in humans. Mice once infected with HSV-1 and since cleared were reinfected in the same manner except on the left side. Figure 1 shows that these mice did become infected but that the lesions were only transient. Cleared mice reinfected 18 days after a primary infection (closed circles) cleared more rapidly than did mice reinfected 70 days after a primary infection (open circles). It was possible, however, to establish infections in which lesions were present for more than 10 days by administering an immunosuppressive dose of radiation just prior to reinfection (triangles). The lesions obtained by this procedure were well contained to the site of reinfection and did not spread to deep tissues.

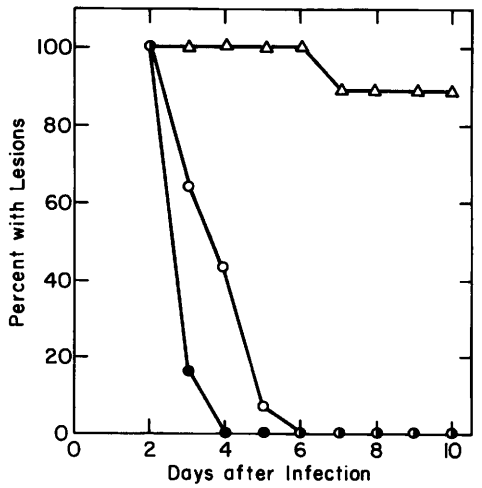

FIG. 1. Transient nature of the lesions in hairless mice that are reinfected with HSV-1 after recovery from an earlier primary HSV-1 infection. Nineteen animals were reinfected 18 days after the initial infection (closed circles). Twenty-eight animals were reinfected 70 days after the initial infection (open circles). Eight mice reinfected 40 days after the initial infection were exposed to γ rays prior to reinfection (triangles). Data are expressed as percentage with lesions vs days after the reinfection was administered.

Table III shows data from mice γ irradiated and reinfected 40 days after a primary infection. The animals were treated with 5% BHT in mineral oil by the drop procedure. Treatments were initiated on Days 2 and 3 for equal numbers of mice, and continued for 3 days in each case. It is apparent from Table III that BHT-treated mice were cleared of lesions much earlier than the untreated controls, and that the severity of the lesions in treated animals was less than that for the untreated controls prior to clearing. The level of statistical significance for the percent scored positive is indicated.

Human γ-globulin-treated mice. A more convenient procedure for obtaining localized cutaneous HSV lesions was developed with human γ-globulin injections. The optimum procedure to allow reproducible infection of mice near the 100% level, and at the same time result in localized lesions, was first established. This procedure consisted of injecting 50 μl of human serum γ-globulin (1 mg/ml) subcutaneously, on the same side to be infected, 24 hr prior to infection. The lesions remained more localized and of less severity in these passively immunized animals, without the tendency to form deep, ulcerated lesions.

Using this procedure for obtaining localized cutaneous HSV lesions, we carried out an experiment to evaluate the effectiveness of BHT in treating HSV infections. A total of 40 mice were injected with 50 μl each of human serum γ-globulin (1 mg/ml) and infected 24 hr later with HSV. Of these, 20 mice were treated with 15% BHT in mineral oil applied with a cotton-tipped applicator and 20 control mice were treated with mineral oil only. The treatments were initiated 24 hr after infection and continued twice daily for 5 days. The scorer had no knowledge of which mice were treated with BHT and which were controls. Table IV shows the results of these experiments. For Days 5–10, the BHT-treated animals had fewer lesions than the controls treated with mineral oil only, significant at the 1% level or better. The collective data of Tables I through IV establish that BHT, applied topically, is effective in shortening the duration of cutaneous HSV-1 lesions in hairless mice.

Discussion. Hairless mice are susceptible to cutaneous infections by HSV after skin injury. These animals were first used in this way by Lieberman *et al.* (13) to test the effectiveness of antiherpetic drugs. Guinea pigs (14) and rabbits (15) have also been used as experimental animals to test the effects of potential antiviral compounds on HSV cutaneous infections. Mice have been reported to develop deep, spreading lesions unlike the recurrent HSV infections in humans (13). We tried to overcome the appearance of deep lesions in several ways. First, by carefully controlling the size of the skin injury the lesions could initially be kept small. Some of these still underwent spreading and undoubtedly penetrated deeper than is characteristic of recurrent HSV in humans. Second, we used γ irradiation to partially suppress the immune system of mice that had recovered from a primary HSV infection. This approach appears to be promising as these secondary lesions of mice were more contained than the corre-

TABLE III. TREATMENT OF HEALED, γ-IRRADIATED, REINFECTED HAIRLESS MICE WITH 5% BHT PREPARATION

Treatment	Day after reinfection	Number with lesions[a] of severity				Percentage with lesions
		0	+	++	+++	
None	3		8			100
	4		4	3	1	100
	5		4	1	3	100
	6		5	1	2	100
	7	1	4	1	2	88
	8	1	3	3	1	88
	9	1	4	3		88
	10	1	5	2		88
5% BHT	3		8			100
	4		7	1		100
	5	5	3			38
	6	6	2			25*
	7	3	5			63
	8	6	2			25*
	9	8				0*
	10	8				0*

[a] Out of eight animals in each group.
* χ^2 significant at 1% level or better.

sponding primary lesions. Third, we inoculated mice with human serum γ-globulin 24 hr before infection to help prevent the infections from spreading and penetrating into deep tissues. This approach is particularly promising and may be an improved model system for recurrent HSV infections in humans.

We reasoned that mice undergoing primary infections initially have no immunity

TABLE IV. TREATMENT OF HUMAN γ-GLOBULIN-INOCULATED HAIRLESS MICE WITH 15% BHT PREPARATION

Treatment	Day after infection	Number with lesions[a] of severity				Percentage with lesions
		0	+	++	+++	
Mineral oil only	3	11	9			45
	4		19	1		100
	5		19	1		100
	6	5	13	2		75
	7	4	16			80
	8	4	16			80
	9	4	16			80
	10	5	15			75
Mineral oil plus 15% BHT	3	2	18			90
	4	4	16			80
	5	10	10			50
	6	15	5			25*
	7	15	5			25*
	8	15	5			25*
	9	15	5			25*
	10	15	5			25*

[a] Out of 20 animals used in each group.
* χ^2 significant at 1% level or better.

to prevent spread of the virus. BHT is used as a topical agent and as a result will be present in lower concentrations in deeper tissues. Therefore, BHT probably has its greatest effectiveness on superficial skin areas. We further reasoned that humans undergoing recurrent HSV infections usually have a competent immune system which may prevent the HSV infection from spreading. In order to make the hairless mouse model system more relevant to human infections, we treated hairless mice with human serum γ-globulin.

Having established an infection procedure, we tested the effectiveness of BHT as a topical agent for cutaneous HSV-1 infections. The data show that, when treatment is initiated early, BHT is effective in reducing the time required for recovery. While the mechanisms whereby BHT exhibits its effects in this system are as yet unknown, there is good reason to believe that it acts at the level of membrane-associated processes. BHT is known to modify the physical properties of model membranes and the membranes of yeast (16) and sperm (17) cells. *In vitro,* BHT has virucidal activity against lipid-containing viruses but is inactive against lipid-free viruses under comparable conditions (10). It is particularly effective against HSV, both type 1 (10) and type 2 (18), and against the enveloped bacteriophage φ6 (12). Other investigators have also reported that BHT is effective *in vitro* against Newcastle disease virus (19), cytomegalovirus (20), and Semliki Forest virus (20). Furthermore, Brugh found that BHT has activity *in vivo* against Newcastle disease virus infections in chickens (19).

In studies of the effects of BHT and similar molecules on φ6, we have found that inactivation is due to the removal of a specific envelope protein that is essential for adsorption of the virus to the host cell surface (12, 21). Studies to elucidate the mechanism of action of BHT against HSV and to further establish its effectiveness as a topical agent are currently underway in our laboratory.

Although the idea that BHT may have antiviral activity due to its ability to localize in and perturb the hydrocarbon zones of membranes may be true, we realize that other mechanisms are possible. BHT may well bind to hydrophobic sites on proteins and could modify the activity or binding properties of such proteins. BHT may modify fundamental lipid–protein interactions and thereby have its mechanism of action in an indirect manner. Combinations of all three of these mechanisms may contribute to its antiviral properties. Recently, we have found that HSV-1 virions inactivated by BHT are incapable of recombining with HSV-1 mutants during coinfections of human embryonic lung cells *in vitro* (data to be published).

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