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Effects of therapy with an immunomodulator (imiquimod, R-837) alone and with acyclovir on genital HSV-2 infection in guinea-pigs when begun after lesion development

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Summary

We evaluated the effects of the immunomodulator, imiquimod (R-837) applied topically, alone and in combination with i.p. acyclovir (ACV) on acute, recurrent and neural HSV-2 genital infection in guinea-pigs when 10 days of therapy was begun after HSV lesions developed. The combined therapy was most effective, significantly reducing the severity of the acute disease, as early as 2 days ($P < 0.05$), and vaginal viral shedding ($P < 0.05$) as early as 1 day after therapy was begun. The total lesion score for the acute disease was also significantly less in the group receiving imiquimod and ACV (5.4 ± 0.5) compared to controls (13.1 ± 1.2, $P < 0.001$) or imiquimod alone (9.8 ± 1.2, $P < 0.05$). Therapy, however, had no significant effect on the number of days with recurrent lesions. Imiquimod increased the lymphoproliferative response to HSV ($P < 0.01$), while combined therapy reduced HSV antibody titers ($P < 0.01$) on day 28 compared to placebo and also reduced the effect of imiquimod alone on the lymphoproliferative response. The combination of this effective immunomodulator, imiquimod, and acyclovir appears to provide effective therapy for acute genital HSV-2 infection even when begun after lesion development.

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Herpes simplex virus; Immunomodulator; Imiquimod; Genital HSV

Introduction

Herpes simplex virus type 2 (HSV-2) genital infections are the major cause of genital ulcer disease among patients seeking medical attention in the United States. Current estimates are that 14–19% of the U.S. population has been infected by HSV-2, almost all through sexual contact (Johnson et al., 1989). Treatment of acute genital HSV-2 infection is currently limited to acyclovir (ACV). When ACV is initiated early after infection, therapy significantly reduces several clinical and virologic aspects of the acute infection, including time to crusting and healing of lesions, disappearance of pain and cessation of viral shedding (Bryson et al., 1983; Mertz et al.,1984). Except for one report (Bryson et al., 1985), treatment of the acute disease, however, does not appear to decrease subsequent recurrent disease (Mertz et al., 1984; Corey et al., 1985; Mindel et al., 1986; Peacock et al., 1988). In experiments using the guinea-pig model of genital herpes, ACV therapy also did not reduce recurrences even when administered 12 h after HSV inoculation (Bernstein et al., 1986). ACV resistance is also becoming an increasingly common and serious problem (Erlich et al., 1989; Safrin et al., 1991; Gately et al., 1990).

Imiquimod, also known as R837, is an immune modulator with no direct in vitro antiviral activity (Harrison et al., 1989). Using the guinea-pig model of genital HSV, we have shown that early treatment markedly reduces the severity of the acute disease and decreases viral shedding in the vagina and viral titers in neural tissues (Harrison et al., 1989; Bernstein and Harrison, 1989). Further, unlike ACV treatment in this model (Bernstein et al., 1986), early treatment of the acute disease with imiquimod significantly reduced subsequent episodes of recurrent disease (Harrison et al., 1989; Bernstein and Harrison, 1989). The present evaluations were performed to determine whether imiquimod with or without ACV would effect the clinical and virological course of the acute disease and the recurrence pattern when begun after the development of lesions because patients with primary genital HSV infection do not present for treatment until after lesions appear. Because neural tissues are the site of HSV latency, the effects of therapy on neural infection in this model were also examined. The effects of imiquimod, at least in part, are related to the induction of endogenous α-interferon and the up-regulation of cell mediated, but not humoral immunity (Harrison et al., 1989; Bernstein and Harrison, 1989; unpublished data). Clinical trials with imiquimod are currently underway.

Materials and Methods

Drugs

Imiquimod was provided by 3M Pharmaceuticals (St. Paul, MN) as a 1% cream for topical intravaginal administration (Harrison et al., 1989; Bernstein and Harrison, 1989). The vehicle without active drug was administered intravaginally as placebo. Acyclovir (Burroughs Wellcome, Research Triangle, NC) was administered by intraperitoneal (i.p.) injection.

Virus

HSV-2 strain 333 was prepared as clarified supernatant from rabbit kidney cells 24 h following infection as previously described (Harrison et al., 1989).

Experimental design

Hartley female guinea-pigs weighing 250–300 g (Charles River Breeding Laboratories, Wilmington, MA) were inoculated intravaginally with 5×10^5 pfu of strain 333 HSV-2. Animals were then evaluated twice per day until the first lesion was observed. At the time a lesion was seen the animal was randomized to receive either topical intravaginal imiquimod alone at a dose of 5 mg/kg/day given qd, topical imiquimod as above plus ACV (combined group) at a total dose of 60 mg/kg/day administered i.p. twice daily, or topical placebo. No vaginal irritation was observed in treated animals. Lesions developed between day 2 and 5. Treatments were continued for 10 days.

The evaluation of acute and recurrent disease was performed on 44 animals that developed genital lesions. Of these, 15 received placebo, 14 imiquimod, and 15 combined therapy. The animals were scored on a scale of 0–4 for the acute disease (days 1–14) as previously described (Bernstein et al., 1986). The total acute lesion score is the sum of the daily scores from days 1–14 (Bernstein et al., 1986). Recurrent disease was evaluated daily from day 15, when the acute disease had resolved, until day 60. Recurrent lesion days are the total number of days animals exhibited a recurrent lesion (Bernstein et al., 1986). Samples of vaginal secretions were collected for days 1–10 and vaginal viral shedding quantified by plaque assay as previously described (Bernstein et al., 1986). Animals were bled by intracardiac puncture on days 14, 28, and 60 for HSV antibody and the peripheral blood mononuclear cells (PBMC) purified and used to measure lymphocyte proliferation to HSV antigen.

Another 36 animals (12 in each group) that developed genital lesions were randomized to the three groups as described above and used to determine the effects on viral titers in neural tissues during the acute disease. Six animals per group were killed 2 days after therapy was begun and the remaining six from each group 3 days after therapy was begun. After death, the lumbrosacral dorsal root ganglia and spinal cord were removed and homogenized on ice in 1.0 ml of Earle's minimal essential medium. After centrifugation to remove cell

debris, the supernatant was used to determine viral titers in duplicate (Bernstein and Harrison, 1989).

Enzyme-linked immunosorbent assay for HSV-2 antibodies

The ELISA assay was performed as previously described using lectin purified HSV-2 glycoproteins as the solid phase and peroxidase-conjugated rabbit anti-guinea-pig immunoglobulins (Accurate Chemical, Westbury, NY) for detection (Harrison et al., 1989; Bernstein and Harrison, 1989). The absorbance from serial dilutions of test sera were then compared to a standard curve, obtained from a high-titered HSV-2 serum arbitrarily assigned a value of 10 000 units.

Lymphocyte proliferation assay

This assay was performed as previously described (Bernstein et al., 1991). Briefly triplicate cultures of guinea-pig PBMC were incubated for 5 days with phytohemagglutinin (16 μg/ml), HSV-2 antigen [1:16 dilution] or control antigen [1:16 dilution]. Cultures were then pulsed with [H^3]TdR (1 μCi/well) for 18 h, and the contents of the wells were then collected onto glass-fiber filters. Incorporated radioactivity was expressed as cpm of antigen stimulated wells minus cpm from cells stimulated with control antigen.

Statistics

Comparison of means was performed by utilizing a two-tailed ANOVA evaluation with a Bonferroni correction for multiple groups. Data are presented as the mean $\pm$ standard error (S.E.).

Results

Acute disease

Of the 44 animals that developed lesions following HSV-2 inoculation, two developed lesions on day 2 (one placebo, one combined therapy), two on day 3 (one placebo, one combined therapy), 35 on day 4 (11 placebo, 14 imiquimod, 10 combined therapy) and five on day 5 (2 placebo, 3 combined therapy). Thus, there were no differences between groups.

As seen in Fig. 1. the severity of the genital disease that developed was similar for all the groups through day 4, but was significantly different on days 6–11, with the most significant difference on days 7–9 ($P < 0.0004$ by ANOVA). By day 6, two days after the majority of animals had begun therapy, the disease in the combined group was significantly less severe than the placebo group ($P < 0.05$). By day 7, this difference had increased ($P < 0.001$) and on this day the combined group also had less severe disease than the imiquimod alone group ($P < 0.05$). The imiquimod alone group developed less severe

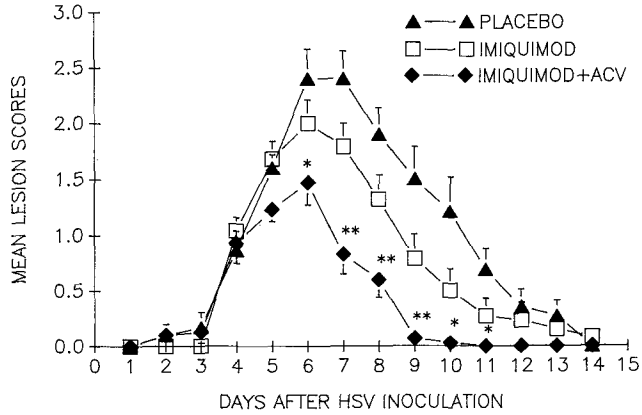

Fig. 1. Effects of imiquimod and ACV on the clinical severity of genital HSV-2 infection. Therapy with either placebo, topical imiquimod (5 mg/kg/day) or topical imiquimod plus ACV (60 mg/kg/day) was begun after the development of lesions and continued for 10 days. Differences between the combined and placebo group were significant at $P < 0.05$ (*) or $P < 0.001$ (**)

disease than placebo recipients on days 6–11; however, differences approached but did not reach significance, using the Bonferroni correction. When the total lesion score for the acute disease was compared, the combined group had significantly milder disease (5.4 ± 0.5) compared to the placebo group (13.1 ± 1.2, $P < 0.001$) and also compared to the imiquimod group (9.8 ± 1.2, $P < 0.05$).

Vaginal viral shedding was also reduced in the treated groups (Fig. 2). Even on day 5, one day after therapy was begun in the majority of animals, the combined group was shedding significantly less virus than the placebo group (P

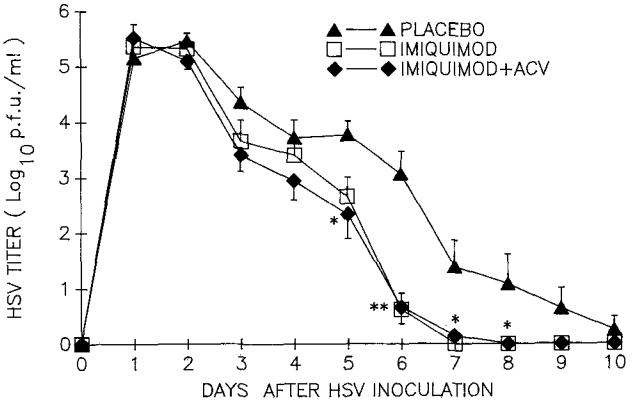

Fig. 2. Effects of imiquimod and ACV on vaginal HSV-2 shedding when therapy is begun as described in Fig. 1. The geometric mean log 10 titers ± SE were obtained by plaque titration of vaginal swab specimens collected daily. Differences between groups on that day were significantly different at $P < 0.05$ (*) or $P < 0.001$ (**). The day 5 differences are between the combined and placebo groups. The days 6–8 differences are between either treatment group and placebo.

<0.05). By day 6 and through day 8, both the treated groups were shedding significantly less virus than placebo recipients. No significant differences were detected comparing the two treatment groups.

Recurrent disease

The number of recurrent lesion days did not differ significantly among the groups ($P = 0.28$ by ANOVA). Recurrent lesion days per animal during the day 15 to day 60 observation period were highest in the combined group (2.8 ± 0.8) compared to placebo (1.6 ± 0.5, $P = 0.20$ by uncorrected Student's t-test) and imiquimod alone (1.8 ± 0.2).

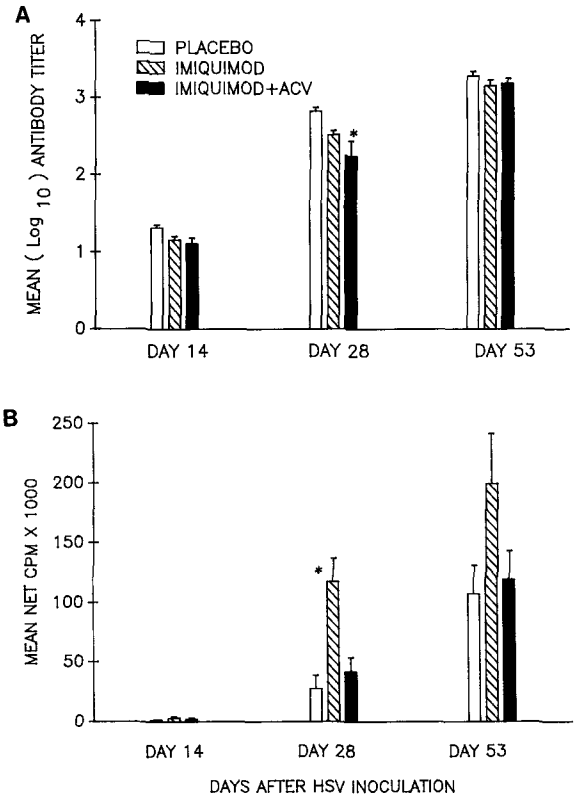

Fig. 3. Immunologic effects of imiquimod and ACV therapy. In (A), the geometric mean antibody titer $\pm$ SE determined by ELISA against HSV-2 glycoproteins is shown. Difference between the combined and placebo group was significant at $P < 0.01$ (*). In (B), the PBMC lymphoproliferation response to HSV-2 is shown. The results are expressed as the mean cpm + SE for HSV-2 antigen minus control antigen stimulated cells. Difference between the imiquimod alone group and either the placebo or combined group was significant at $P < 0.05$ (*).

Immune responses

The antibody response to HSV (Fig. 3A) was decreased in the combined group on day 28 compared to placebo ($P < 0.01$). By day 53, antibody titers were essentially equivalent between groups. Lymphoproliferative responses to HSV-2 (Fig. 3B) were increased significantly in the imiquimod group on day 28 compared to the placebo group ($P < 0.01$ and remained elevated through day 53 in this group (N.S.). The addition of ACV appeared to reduce the effect of imiquimod so that on day 28 the combined group was only slightly higher than the placebo group and significantly less than the imiquimod alone group ($P < 0.01$).

Viral titers in neural tissue

The three groups used in this experiment were identical to those previously discussed. All groups were equivalent in that all animals developed lesions between days 4 and 5 and vaginal viral shedding on day 2 ranged from 5.6 to 5.8 log 10 pfu/ml for each group (data not shown). After 2 days of therapy no significant differences were detected in the viral titers in either dorsal root ganglia or spinal cord (Fig. 4). After 3 days of therapy, however, the group receiving combined therapy had significantly lower viral titers in the dorsal root ganglia ($P < 0.05$) and the lowest titers in the spinal cord (N.S.). The combined therapy may have been more effective in the dorsal root ganglia compared to the spinal cord because of the higher concentration of ACV in the ganglia (Myerson and Hsiung, 1983).

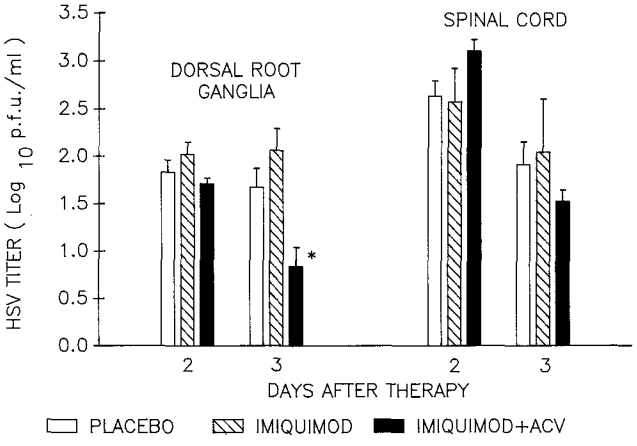

Fig. 4. Effects of imiquimod and acyclovir on acute neural HSV-2 infections when therapy is begun as described in Fig. 1. Animals were killed 2 or 3 days after therapy was begun and the titer of HSV-2 in lumbosacral dorsal root ganglia and spinal cord determined. Difference between combined and placebo group was significant at $P < 0.05$ (*).

Discussion

In this study, the combination of imiquimod and ACV effectively reduced clinical genital HSV-2 disease and vaginal viral shedding within 1–2 days of initiating therapy, even though therapy was begun after the development of lesions. At this time, the disease in the combined group was less severe than in the placebo group ($P < 0.05$) while viral shedding was decreased by over 99%. Overall, the total lesion scores were reduced by 59% in the combined group compared to placebo. Other studies have shown synergy in vivo between DHPG, another acyclic nucleoside, and α-interferon (Fraser-Smith et al., 1984) which is induced by imiquimod therapy (Harrison et al., 1989; Bernstein and Harrison, 1989) against a lethal HSV-2 infection in mice.

Although not included in this study, previous evaluations in guinea-pigs of ACV alone showed that oral ACV therapy begun at 24, 48, or 96 h after viral inoculation had no effect on vaginal viral shedding at any time and only decreased mean lesion scores when given at $\leqslant 48$ h (Kern, 1982). ACV therapy given i.m. reduced mean lesion scores by about 45% when given within 48 or 96 h after infection, but not vaginal viral titers. In other studies (Landry et al., 1982; Pronovost et al., 1982), ACV therapy begun at 3 days after HSV inoculation had no significant effect on vaginal viral shedding of either HSV-1 or HSV-2, but therapy did decrease the clinical disease in HSV-2, but not HSV-1-infected animals. No significant effects on viral titers in neural tissues obtained during the acute disease were detected in these studies. In our studies of ACV therapy in the guinea pig, even when ACV was administered at 12 h after HSV-2 inoculation, there was no effect on vaginal viral shedding, but a decrease in mean lesion scores was seen. Thus, it appears unlikely that the effects we noted in the combined group, especially against viral shedding, were due primarily to ACV.

We have previously reported that imiquimod given either 12 h or 36 h after HSV inoculation significantly reduced the recurrence rate (Harrison et al., 1989; Bernstein and Harrison, 1989). Initiating treatment before lesions also significantly limited the acute neural infection, and the development of latency (Harrison et al., 1989; Bernstein and Harrison, 1989). In the study reported here where treatment was begun after lesion development, neither imiquimod alone nor imiquimod in combination with ACV reduced subsequent recurrences. It appears that initiating therapy after herpetic lesions develop, and thus after virus had reached the dorsal root ganglia (Bernstein and Stanberry, 1986; Stanberry et al., 1982), the site of latency, eliminated the effects on subsequent recurrences. It should be noted, however, that for unknown reasons the recurrence rates in this study were lower than we have previously observed.

The host–virus interaction in the neural tissues early in infection, that leads to either a productive or a latent infection, is not understood. It appears that HSV can establish a latent infection shortly after reaching the ganglia (Steiner et al., 1990) even with viral mutants that are defective in viral replication

(Steiner et al., 1990; Leib et al., 1989; Katz et al., 1990) or under conditions (Speck and Simmons, 1991) that produce little or no acute ganglionic viral replication. The immune response may also play a role in the induction of latency. In mouse models, there appears to be a shift from a productive to a latent infection when HSV antibody or immune lymphocytes are administered soon after infection (Price and Schmitz, 1979; Schneweis et al., 1988). Therefore it is possible that, once virus reaches the ganglia, it can initiate a latent infection even if host immune responses are increased and concentrations of antivirals are sufficient to prevent active viral replication. Latent virus may reactivate later, producing recurrent disease. This may explain why treatment of the acute infection with imiquimod alone or combined with ACV, which so effectively reduced peripheral HSV replication and even HSV titers of replicating virus in the ganglia, did not reduce the subsequent recurrence rate.

In the experiment reported here, imiquimod increased cell mediated immunity but had little effect on antibody levels, consistent with our previous reports (Harrison et al., 1989; Bernstein and Harrison, 1989). The addition of ACV significantly decreased antibody titers, which is also consistent with reports in guinea-pigs (Bernstein et al., 1986) and humans (Bernstein et al., 1984; Ashley and Corey, 1984). Further, ACV inhibited the increased cell-mediated immune response induced by imiquimod. Previous reports in humans have also shown that ACV can inhibit the lymphoproliferative response to HSV (Lafferty et al., 1984). It is intriguing to speculate that the decreased immune responses produced by ACV could be related to the increased number of recurrences seen in the combined group.

The combination of the effective immune modulator, imiquimod, and ACV appears to provide effective therapy for acute genital HSV-2 infection. Although the therapy schedule we evaluated did not reduce recurrent lesion days, we have observed that prolonged imiquimod therapy (21 days) given to latently infected guinea-pigs reduces recurrences not only during the 3 weeks of therapy, but for several weeks afterwards (Harrison et al., 1991). It is, therefore, possible that prolonging the imiquimod portion of the combined therapy after ACV is discontinued will increase the immune response and thereby decrease recurrences. Further evaluation of imiquimod alone or combined with an antiviral for treatment of HSV and other viral infections appears warranted.

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