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Posttherapy Suppression of Genital Herpes Simplex Virus (HSV) Recurrences and Enhancement of HSV-Specific T-Cell Memory by Imiquimod in Guinea Pigs

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Imiquimod, an immunomodulator with no direct in vitro antiviral activity, has in vivo anti-herpesvirus activity by inducing interferon and enhancing other only partially defined immune responses. Imiquimod treatment of primary genital herpes simplex virus (HSV) infection in guinea pigs reduces the level of genital disease by 90%. We further investigated its utility as suppressive therapy of recurrent disease in animals that had recently recovered from primary genital HSV-2 disease. Imiquimod administered intravaginally once per day for 5 days reduced the number of recurrences only during treatment, while a 21-day regimen reduced the number of recurrences for 8 weeks. For the entire 10 weeks of observation, overall numbers of recurrences were reduced 67% by the 21-day imiquimod treatment ($P < 0.0001$). Latent HSV in ganglia was not affected by either regimen. Increased circulating alpha interferon activity was observed during therapy with both regimens. Interferon levels rapidly returned to baseline with cessation of treatment. Posttreatment, 5-day imiquimod treatment did not provide clinical benefit or enhancement of cell-mediated or cytokine responses. Twenty-one-day imiquimod treatment reduced both the number of clinical recurrences and levels of HSV antibody for 5 to 6 weeks posttreatment compared with the placebo. Additionally, 21-day imiquimod treatment enhanced HSV antigen-specific interleukin 2 production and proliferative responses by mononuclear cells ($P < 0.001$) for 4 weeks after treatment. Twenty-one-day imiquimod therapy suppressed recurrent HSV genital disease during and for weeks after therapy, enhanced memory-dependent cytokine and T-cell responses, and reduced the level of antibody responses.

Current therapy for herpes simplex virus (HSV)-induced genital disease is suboptimal. Successful management of frequently recurrent genital HSV-2 disease in humans requires daily doses of acyclovir (ACV) in suppressive regimens lasting for months to years (12). Although this approach is effective during the period of ACV administration, recurrences usually happen when treatment is discontinued (14). Furthermore, some data suggest that ACV therapy of primary infections may limit development of the full extent of immune responses to HSV (3, 6). Another problem with currently available therapy is the increasing frequency of ACV-resistant HSV strains usually associated with chronic or repeated courses of ACV (7, 11), particularly in immunocompromised patients. Thus, it appears that mutant selection is enhanced when the host immune system is less capable of HSV-specific responses. Therefore, new strategies that incorporate both antiviral effects and enhancement of HSV-specific immune responses would be attractive as alternative or additive therapy for HSV-induced disease, particularly when the clinical situation involves an increased risk of ACV-resistant isolates.

We have previously shown that imiquimod (formerly R-837) induces in vivo antiviral effects principally as an immunomodulator that induces alpha interferon (IFN-α) and upregulates selected cell-mediated immune (CMI) responses to HSV in the guinea pig model (1, 9). Short-term (5-day) imiquimod regimens initiated soon (<36 h) after vaginal HSV-2 inoculation reduced the level of acute primary genital HSV disease and, unlike ACV, appeared to produce a partial reduction in the number of genital HSV recurrences after discontinuation of treatment (1, 9) in the guinea pig model.

In other guinea pig studies, we noted that imiquimod produces an adjuvant effect with an HSV mixed-glycoprotein vaccine against primary HSV infection, apparently by enhancing antigen presentation and upregulating certain HSV-specific T-cell and cytokine responses (4). We had previously correlated enhancement of these responses to inhibition of recurrent HSV disease in guinea pigs (2, 13). The experiments reported here are the first to evaluate imiquimod as suppressive therapy for recurrent HSV genital disease.

MATERIALS AND METHODS

Drug. Imiquimod (1-[2-methylpropyl]-1*H*-imidazo-[4,5-*c*] quinolin-4 amine [formerly R-837]) in a 1% cream for intravaginal administration was given once per day at 50 μl/100 g of animal body weight beginning on days 14 to 15 after HSV-2 intravaginal inoculation (soon after recovery from primary genital disease). Vehicle without imiquimod was administered as a placebo in the same fashion to animals. In the first experiment, animals received 5 consecutive days of treatment, and in the second experiment animals received 21 consecutive days of treatment.

Virus and viral cultures. Strain 333 of HSV-2 was prepared as previously described (9) and maintained frozen at −70°C at a titer of 1.2×10^7 PFU/ml. Cultures of minced dorsal root

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ganglia were made to document latent infection by triplicate cocultivation and incubation with rabbit kidney cell monolayers (1, 9).

Experimental design. Hartley albino 350- to 450-g female guinea pigs (Charles Rivers Laboratories) were inoculated intravaginally with 5×10^5 PFU of HSV-2 in 0.1 ml after being preswabbed with a calcium alginate swab. Acute genital disease was scored daily from 0 to 4 for each animal (1, 9) for the first 14 days. Randomization to imiquimod or placebo was performed after recovery from primary genital disease on day 14. Short-course (5-day) animals were treated on days 15 to 19, and longer-course (21-day) animals were treated on days 15 to 35. All animals were scored daily for clinical recurrences until 10 to 12 weeks after viral inoculation.

Subsets (minimum, $n = 12$ per assay day per group) of animals from each treatment group were bled serially for plasma to be used in assays for HSV antibody and circulating IFN. Subsets of animals ($n = 6$) were also bled for peripheral blood mononuclear cells (PBMCs) to be used in assays for PBMC-mediated cytolysis of HSV-infected targets and for proliferative responses to HSV-2 antigen or mitogen. A group of five uninfected untreated animals served as negative controls for HSV-infected human foreskin fibroblast (HFF) cytolysis assays. At the end of each study, seven animals from each group were dissected to obtain dorsal root ganglia and spinal cord, which were minced and cocultivated on rabbit kidney monolayers to detect latent HSV.

Immune assays. Enzyme-linked immunosorbent assay (ELISA) antibody to HSV was assayed as previously described (5). Total IFN activity was assayed as previously described (9) by a standard vesicular stomatitis virus plaque reduction assay with a human IFN-α standard (Hoffmann-La Roche). In multiple assays, the protective end point was consistently produced by 0.3 to 1 U of the human IFN-α standard. Results are expressed in equivalence units of human IFN-α per milliliter of plasma.

Proliferation to whole HSV-2 antigen. HSV antigen was produced as previously described (1, 9) from HSV-2-inoculated rabbit kidney cells, and control antigen was produced in the same manner from uninfected cells. PBMC proliferation was assayed as previously described (1, 9). Briefly, the assay measured [^{3}H]thymidine incorporation after an 18-h pulse in triplicate wells of 5×10^5 PBMCs cultured for 5 days with HSV antigen (1:16) versus control antigen (1:16) or 16 μg of phytohemagglutinin versus media alone. The values for HSV-2 antigen are the mean counts per minute in HSV-2-stimulated wells minus the mean counts per minute in control antigen wells. For mitogens, the values are the mean counts per minute of mitogen-stimulated wells minus the mean counts per minute of wells with media and PBMCs alone.

In vitro IL-2 production assays. Supernatants were collected at 48 h of incubation of PBMCs with whole HSV antigen and control antigen for assays of interleukin 2 (IL-2) activity. The IL-2 bioassay involved measuring [^{3}H]thymidine incorporation into 4×10^3 cells of the IL-2-dependent murine cell line CTLL-2 after 20 h of culture and a 4-h thymidine pulse as previously described (1, 9). Relative IL-2 production was measured as the activity in HSV antigen-stimulated wells minus that in control antigen-stimulated wells compared with a known standard of 100 U of IL-2 (Cetus Corporation).

PBMC-mediated MHC-unrestricted cytolysis assays of HSV targets. Major histocompatibility complex (MHC) unrestricted cytolytic activity against HSV-infected HFF targets was assayed (in the 5-day experiment only) by standard ^{51}Cr release assays as previously described (9, 10). Briefly, uninfected and HSV-infected HFFs were used as targets for HSV-specific MHC-unrestricted cytolytic activity. Single-cell suspensions of ^{51}Cr-labeled HFFs, either uninfected or 2 h after infection with McIntyre HSV-1, were incubated with PBMCs (3.2×10^5) in triplicate for 12 h at an 80:1 effector/target ratio. The percentage of cytolysis was calculated with a standard formula (5). MHC-unrestricted HSV-specific cytolysis was defined as cytolysis of HSV-infected HFFs minus that of uninfected HFFs.

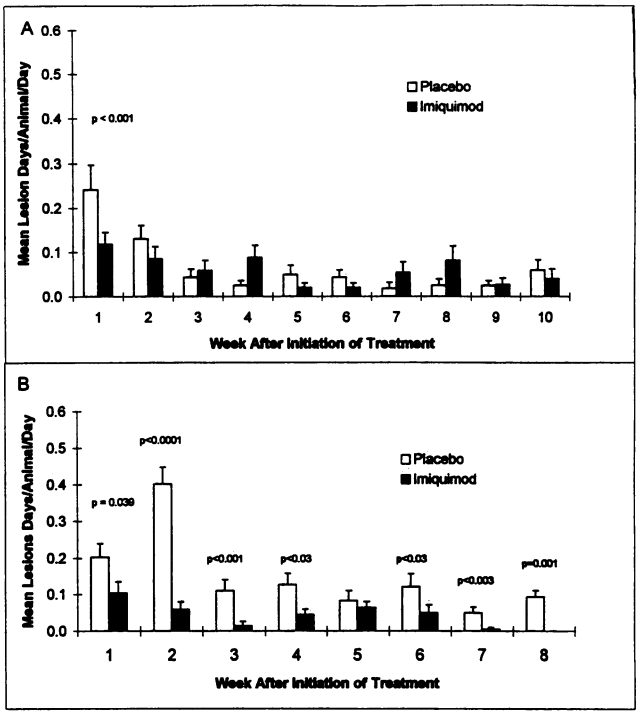

FIG. 1. Mean lesion days per animal per day, combined by week, for each experimental group. Animals in the 5-day treatment groups (A) received imiquimod or placebo intravaginally once per day on days 15 to 20 after HSV vaginal inoculation. Animals in the 21-day treatment groups (B) received imiquimod or placebo intravaginally once per day on days 15 to 35 after HSV vaginal inoculation. Statistical evaluation was by the two-tailed Student's t test.

Statistical analysis. Mean values in the text and the tables were compared by using Student's t test or analysis of variance, depending on the number of groups and days to be analyzed.

RESULTS

Primary HSV infection prior to treatment. In the study of short-course suppression (5-day therapy), randomization of 48 HSV-infected animals resulted in 24 placebo and 24 imiquimod recipients. In the study of longer-course suppression (21-day therapy), 29 animals received imiquimod and 26 received placebo. Randomization on day 14 after vaginal inoculation to placebo or imiquimod treatment resulted in similar mean areas under the curve for acute lesion scores for each randomized group (data not shown). Similar mean peak lesion scores for prerandomization primary disease (days 1 to 14 after inoculation) were also observed in the randomized placebo and imiquimod groups for both the 5- and 21-day treatment studies (data not shown).

Effect on recurrent genital disease. As shown in Fig. 1, the 5-day treatment groups exhibited a significant difference only during imiquimod treatment (days 15 to 20). In contrast, the 21-day treatment groups exhibited differences in recurrent

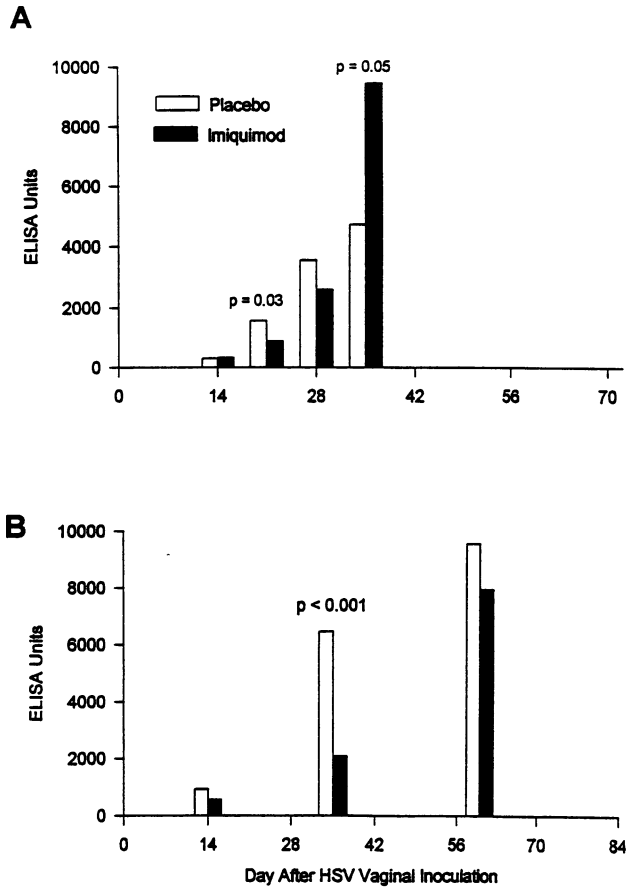

FIG. 2. ELISA antibody to HSV, by day, after viral vaginal inoculation. Blood specimens were obtained from the HSV-infected placebo recipients and the HSV-infected imiquimod recipients. (A) Data from the 5-day treatment experiment. (B) Data from the 21-day treatment experiment. Statistical evaluation was by the two-tailed Student's *t* test.

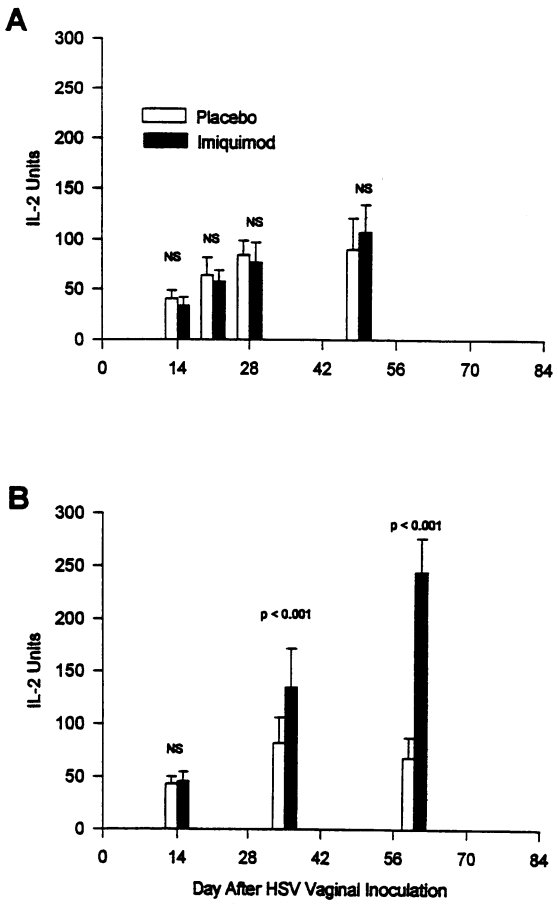

FIG. 3. IL-2 produced by in vitro HSV antigen-stimulated PBMCs obtained from HSV-infected animals. Blood specimens were obtained from the placebo recipients and imiquimod recipients. (A) Data from the 5-day treatment experiment. (B) Data from the 21-day treatment experiment. Statistical evaluation was by the two-tailed Student's *t* test. NS, not significant.

disease during treatment and in 4 of the subsequent 5 weeks of observation. Similarly, when recurrences were analyzed for the entire observation period, the overall reductions in mean recurrent disease were 16% with imiquimod for 5 days (0.29 ± 0.14 versus 0.35 ± 0.14 lesion days/week for imiquimod and placebo recipients, respectively [$P > 0.2$]) and 67% with imiquimod for 21 days (0.35 ± 0.17 versus 1.05 ± 0.19 lesion days/week for imiquimod and placebo recipients, respectively [$P < 0.0001$]).

HSV-specific immune responses. Both 5- and 21-day imiquimod recipients exhibited lower HSV-specific antibody levels in the period soon after cessation of suppressive therapy (Fig. 2). The 5-day imiquimod group exhibited a rebound in antibody response eventually surpassing that for the placebo group (Fig. 2A). In contrast the 21-day imiquimod group had persistently lower antibody titers at each assay point (Fig. 2B).

Assays for MHC-unrestricted cytolytic activity against HSV-infected targets were performed only in animals from the 5-day experiment. There were no differences in cytolytic activity between HSV-infected groups, whether they received imiquimod or not, but all HSV-infected animals (both placebo and imiquimod recipients) exhibited significantly higher levels of activity against HSV targets than uninfected control animals receiving placebo (data not shown).

In vitro IL-2 production by phytohemagglutinin-stimulated PBMCs did not differ significantly between any HSV-infected treatment groups at any time in the study (data not shown). HSV antigen-stimulated PBMCs from uninfected placebo controls produced <5 U of IL-2 on each assay day (data not shown). HSV antigen-stimulated PBMCs from HSV-infected 5-day imiquimod recipients produced IL-2 activity similar to that of HSV-infected 5-day placebo recipients on days 14, 21, 28, and 49 after infection (Fig. 3A); i.e., there was increasing in vitro IL-2 production with time after infection in both HSV-infected groups. This differed from the results in the 21-day therapy groups, where pretreatment (day-14) IL-2 activity in supernatants of HSV antigen-stimulated PBMCs did not differ, but IL-2 activity was significantly higher in 21-day imiquimod recipients (Fig. 3B) than in 21-day placebo recipients on days 35 and 60 ($P < 0.001$).

Despite randomization, HSV-stimulated proliferative response levels in the HSV-infected 5-day placebo recipients were higher than those in the HSV-infected imiquimod recipients prior to treatment ($P < 0.01$) (Table 1). Although none of the differences in HSV proliferative responses between 5-day treatment groups were significant, the 5-day imiquimod group exhibited lower levels of initial responses on days 21 to 28 and higher levels of late responses on day 49 compared with the 5-day placebo group (Table 1). In contrast, the 21-day placebo

TABLE 1. Specific proliferative responses to HSV antigen in animals receiving 5 or 21 days of imiquimod therapy or placebo beginning on day 15 after HSV vaginal inoculation

Treatment	Specific proliferative response (cpm [10^3] ± SE) in:					
	5-day treatment group				21-day treatment group[a]	
	Day 14	Day 21	Day 28	Day 49	Day 35	Day 60
Placebo	35.2 ± 4.9[b]	46.8 ± 11.1	124 ± 21	186 ± 31	46.0 ± 14.8	60.9 ± 16.7
Imiquimod	16.1 ± 3.9	33.2 ± 9.6	79 ± 20	276 ± 20	133.5 ± 11.8[c]	96.7 ± 18.4[c]

[a] Pretreatment (day-14) HSV proliferative responses to HSV antigen were not significantly different for the day-14 pretreatment assay.
[b] $P < 0.01$ compared with placebo recipients by two-tailed Student's t test.
[c] $P < 0.001$ compared with placebo recipients by two-tailed Student's t test.

and imiquimod recipients exhibited similar responses to HSV antigen only in the pretreatment assay. After treatment, the mean proliferative response level to HSV antigen was significantly higher in the imiquimod group than in the placebo group on day 35 ($P < 0.001$) and on day 60 ($P < 0.001$) (Table 1).

Circulating IFN. Pretreatment levels of circulating IFN activity were similar and averaged below 20 U/ml in all groups. Elevated circulating IFN activity levels were detected in both imiquimod groups only during treatment ($P < 0.001$) on each treatment day assayed, i.e., days 15 to 20 for the 5-day group and days 28 and 35 for the 21-day group, returning to baseline soon after cessation of therapy (Table 2).

Latent neural HSV in tissue obtained after treatment. Five-day recipients of imiquimod and placebo had similar rates of detectable latent HSV-2 by classic cocultivation of ganglia or spinal cord when sacrificed on day 90 after HSV inoculation: 75% (6 of 8) for drug recipients compared with 88% (7 of 8) for placebo recipients. This was also true for 21-day recipients: 78% (7 of 9) for imiquimod recipients and 89% (8 of 9) for placebo recipients.

DISCUSSION

One goal of suppressive therapy for frequently recurrent genital HSV is continued suppression after therapy is discontinued. Our initial investigations of imiquimod treatment of primary HSV-2 genital disease showed that, unlike ACV (5, 6), imiquimod not only reduced the level of acute HSV genital disease but also reduced the number of subsequent recurrences of the disease. While imiquimod-treated animals developed reduced HSV-2 antibody responses (1, 9), HSV-specific cell-mediated and cytokine (IL-1 and IL-2) responses were enhanced, despite reduced duration and quantity of HSV replication. We therefore evaluated imiquimod for treatment of recurrent HSV disease, postulating that the level of disease would be reduced by IFN induced during therapy and later by upregulated HSV-specific cell-mediated immune responses. In this study, we showed that 21 days of imiquimod therapy not only reduced the number of recurrences during therapy but also reduced the number of recurrences for 5 of the 6 weeks after therapy was stopped.

The reduced level of recurrent genital disease observed in the 21-day imiquimod recipients appeared associated with upregulation of selected cell-mediated and IL-2 responses to HSV, similar to that which we have reported with vaccine immunotherapy (2, 13). No overall disease reduction or posttreatment effect was noted with the shorter (5-day) imiquimod suppressive regimen. However, recurrence rates in the 5-day placebo control group were less than half of the normal historically observed rate in placebo-treated HSV-infected animals (1, 2, 5, 9, 13). On the basis of this and the significantly higher level of pretreatment HSV-specific proliferative T-cell responses, the animals randomized to the placebo group inadvertently constituted a group of relative hyperresponders to HSV compared with the imiquimod group, despite similar primary clinical disease profiles. This appeared to reduce the level of disease in the control population and may have reduced our power to detect a clinical posttreatment beneficial effect with the 5-day drug treatment. However, no immunologic enhancement was detected after short-course imiquimod therapy except for a late antibody enhancement which did not correlate with clinical benefit. Reasons for the unusually high percentage of hyperresponding naturally HSV-resistant animals in one study arm despite randomization are unclear. The number of such naturally HSV-resistant animals among our study groups is usually <10% of all animals from this supplier. Another potential explanation of the difference between background recurrences in the 5- versus 21-day experiments is that the 5-day study was also performed temporally before the 21-day study because of housing limitations. Thus, while all animals were from the same supplier, the 5- and 21-day groups were obtained at different times. It is possible that some posttreatment benefit might have been detected after 5 days of imiquimod therapy if the hyperresponsive animals had been evenly distributed to both the placebo group and the imiquimod group, but our current data do not support such a conclusion.

The mechanism for the posttreatment protective effect, observed after 21 days of imiquimod therapy, cannot be definitively understood from the selective survey of immune function provided by the two experiments reported here. However, it seems reasonable to attribute most if not all of the reduction in number of recurrences during therapy with either imiquimod regimen to the endogenous IFN induced by treatment. Another mechanism must be postulated for the benefit observed in the 21-day imiquimod recipients, because circulating IFN levels rapidly returned to baseline less than a week after drug administration was stopped.

The mechanism for this longer posttherapy suppression could have been drug-induced reduction in the level of latent HSV in ganglia, yielding fewer reactivation events. However, the posttreatment protection in the 21-day imiquimod recipients did not correlate with detectable differences in latent infection of ganglia or spinal cord by classic cocultivation methods. This suggests that immune modification played a role in the extended beneficial effect. We postulate that the posttreatment effect resulted from imiquimod's inducing an immune modulation weighted toward protective responses not ordinarily produced by natural infection. Possible avenues of such immune modulation include humoral, cytokine, and/or cell-mediated responses. From the current data and our previous studies (2, 13), we further postulate that enhanced cytokine and cell-mediated responses are most likely responsible for the extended benefits.

TABLE 2. Circulating IFN-α/β concentrations in sera of animals receiving placebo or imiquimod from days 14 to 20 or 15 to 35 after HSV-2 vaginal inoculation

Treatment	Circulating IFN-α/β concn (human IFN-α eq U) in:											
	5-day treatment group						21-day treatment group					
	Day 14	Day 15	Day 16	Day 17	Day 20	Day 28	Day 60	Day 14	Day 28	Day 35	Day 42	Day 60
Placebo	15.6 ± 3.7	8.9 ± 1.9	7.3 ± 2.0	8.7 ± 3.5	7.9 ± 2.4	9.1 ± 2.7	8.1 ± 3.2	14.9 ± 4.2	19.1 ± 4.7	11.0 ± 2.9	13.1 ± 3.4	11.6 ± 2.2
Imiquimod	9.8 ± 1.8	327.1 ± 51.6	483.8 ± 30.4	355.6 ± 54.8	405.8 ± 60.1	11.7 ± 3.3	7.7 ± 2.9	12.7 ± 3.5	163.4 ± 27.4	112.6 ± 35.8	12.0 ± 3.6	9.4 ± 3.6
P^a	>0.4	<0.0001	<0.0001	<0.0001	<0.0001	NS[b]	NS	>0.4	<0.001	<0.001	NS	NS

[a] Statistical analysis was performed by two-tailed Student's *t* test.
[b] NS, not significant.

We previously observed in HSV vaccine trials in this animal model (2, 13) that ELISA antibody to HSV-2 did not correlate with protection. The imiquimod group that had the least number of recurrences in the currently reported experiment (21-day recipients) produced less HSV antibody than the placebo recipients. The decreased humoral response probably resulted from there being less HSV antigen available from recurrent disease for processing and humoral immune boosting in the imiquimod recipients. Our previous experience with imiquimod treatment of primary genital HSV-2 disease also revealed less disease in the face of a reduced antibody response (1, 9). We postulate that, like ACV (5, 6), imiquimod quantitatively reduced HSV antigen levels in acute disease and in recurrent disease (this study), thus reducing the level of humoral response. The induction of IFN-α may also alter immunoglobulin production (8).

Reduced HSV genital disease levels have been associated with cytokine-enhanced cytolytic activity against HSV targets (15). Enhancement of this response might also have been expected in animals with high levels of circulating IFN. However, we failed to detect enhanced activity against HSV targets in the 5-day-treated group. This suggests that IFN-induced enhancement may be lost if IFN levels exceed what may be a threshold for stimulation of cell-mediated cytolysis. There are precedents for a limited dose range for efficacy with other proinflammatory cytokines on cytolytic CMI (15). Other cell-mediated or cytokine responses appeared more likely candidates for induction of the beneficial posttreatment clinical effect.

HSV-specific T-cell and cytokine responses were also assayed in these experiments. We detected enhanced HSV-specific cell-mediated and IL-2 responses well beyond the duration of treatment in the 21-day imiquimod group, which also exhibited the posttreatment beneficial clinical effect. These HSV-specific responses may provide or be surrogate markers for immune protection not ordinarily induced by unmodified HSV infection. The correlation of protection with enhanced IL-2 and proliferative responses to HSV suggests that memory-dependent Th1 subpopulations of $CD4^+$ cells are involved. Additional evidence for this is our preliminary observation of a transient increase in the number of circulating $CD4^+$ cells within 5 to 7 days of initiating imiquimod treatment (unpublished observations).

In the studies reported here, a posttreatment beneficial clinical effect was observed with 21-day but not 5-day suppressive imiquimod therapy. This posttreatment effect could not be explained simply by induced IFN and was most likely due to upregulation and enhancement of memory-dependent CMI responses to HSV. To our knowledge, this is the only drug treatment with continued benefits beyond the time of administration. Further trials of prolonged therapy with imiquimod coupled with other antiviral agents or as an adjuvant during immunotherapy of recurrent HSV genital disease also appear warranted.

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