Adjuvant Effects of Imiquimod on a Herpes Simplex Virus Type 2 Glycoprotein Vaccine in Guinea Pigs

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The adjuvant effects of imiquimod for a herpes simplex virus (HSV) vaccine were evaluated. Guinea pigs were immunized with 35 μg of lectin-purified HSV-2 glycoproteins 14 and 35 days before intravaginal HSV-2 inoculation. Immunizations were given either alone, with complete Freund's adjuvant, or with three different 5-day regimens of imiquimod. Although immunization alone decreased the severity of the acute disease and viral replication, in two separate experiments, the addition of imiquimod produced further decreases. The addition of subcutaneous imiquimod further decreased viral shedding by >3 logs on day 1 after virus inoculation ($P < .001$). All groups that received immunization and imiquimod also developed significantly fewer HSV recurrent lesion days ($P < .05$–$.001$) compared to immunization alone. No recurrent lesions were detected in the group that received immunization and subcutaneous imiquimod. Imiquimod enhanced the effectiveness of HSV-2 immunization, especially in reducing recurrent HSV disease, and should be evaluated further as an adjuvant.

The development of a herpes simplex virus (HSV) vaccine remains a high priority. Effective vaccines should prevent clinical disease and prevent or reduce the establishment of latent HSV infections and recurrent disease. The surface glycoproteins of HSV are considered good candidates for a subunit vaccine [1–5], although we [1] and others [2, 3, 6] have demonstrated the importance of adjuvants for the development of protective immune responses.

Adjuvants are immunostimulatory substances that potentiate the immune response induced by vaccines or other antigens beyond the level of the antigen alone. Aluminum salts (alum), the only adjuvant currently approved for human use in the United States, have certain disadvantages, perhaps most importantly the lack of effective induction of cell-mediated immunity [7]. Further, use of alum as an adjuvant for potential HSV vaccines has not provided as good protection as have other adjuvants [2, 3, 6]. Complete Freund's adjuvant (CFA) has provided good protection in these experiments, but it cannot be used because of its severe side effects.

Imiquimod, also known as R837 [8, 9] and S26308 [10], is an immunomodulator with no direct in vitro anti-herpes virus activity [8]. Imiquimod induces high levels of interferon-α in guinea pigs [8, 9] and humans [11] as well as β2-microglobulin and neopterin [11]. In our evaluations of imiquimod for treatment of genital HSV, we noted that guinea pigs receiving imiquimod shed significantly less virus than did controls, yet immune responses, especially cell-mediated responses, were as high or higher than in controls [8, 9]. Thus, it appeared that imiquimod could act as an adjuvant by inducing interferon or interleukin (IL)-2 production [8, 9], which could potentiate the effectiveness of an HSV vaccine [12].

To evaluate the potential of imiquimod to enhance the immunogenicity of an HSV-2 glycoprotein subunit preparation, we compared the protective efficacy to the glycoprotein preparation alone or combined with CFA. Because we were unsure when the induction of cytokines by imiquimod would most effectively boost immune responses, we evaluated both simultaneous and delayed administration of imiquimod. Because guinea pigs experience both acute and recurrent disease after HSV infection, the effects of immunization on both could be evaluated in this model.

Materials and Methods

HSV-2 glycoprotein preparation. The HSV-2 glycoprotein preparation was prepared using methods similar to those previously reported [1]. Briefly, HSV-2 strain MS–infected Vero cells were solubilized and the glycoproteins purified by lentil-lectin Sepharose chromatography. The glycoprotein preparation was diluted to contain 350 μg of total protein/mL.

Imiquimod. Imiquimod (1-[2-methylpropyl]-1*H*-imidazo-[4,5-c]quinolin-4-amine) 1% cream formulation (3M Pharmaceuticals) was administered intravaginally at 5 mg/kg/day [8–10]. The hydrochloride salt was administered subcutaneously in the upper back at 3 mg/kg/day.

Administration of adjuvants and glycoproteins. Hartley fe-

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male guinea pigs (Charles River Breeding Laboratory, Wilmington, MA), weighing 200–300 g, were immunized with 35 μg of HSV-2 glycoproteins in the hind footpad once 35 days before and again 14 days before vaginal inoculation with HSV-2. The CFA group was immunized with the glycoproteins mixed 1:1 with CFA. The groups given glycoprotein alone or imiquimod received the glycoprotein mixed 1:1 with saline. The imiquimod intravaginal groups also received imiquimod once daily for 5 days after each immunization beginning either simultaneously with glycoprotein administration (imiquimod[S] group) or 48 h after administration (imiquimod [D] group). Subcutaneous imiquimod was administered for 5 days beginning simultaneously with glycoprotein administration (imiquimod [Sub Q] group).

HSV-2 challenge and evaluation. Animals were inoculated intravaginally with $10^{5.7}$ pfu of either the 333 (first experiment) or MS strain (ATCC VR-540; second experiment) of HSV-2 14 days after the second immunization. HSV titers were determined from vaginal secretions as previously described [1]. During the acute infection (days 1–14), genital skin disease was quantitated on a scale of 0–4 [1]. Total acute lesion scores are the sum of these scores for days 1–14. The initial, but not the second, experiment was done in a blinded fashion. After recovery from the acute infection, animals were examined daily for recurrent herpetic disease from days 15 to 60. Sera were collected from immunized animals just before intravaginal inoculation, 14 days later, and again at either 44 or 60 days after inoculation.

ELISA for HSV-2 antibodies. HSV-2 antibodies were quantified by an ELISA as previously described [8]. Briefly, lectin-purified HSV-2 glycoproteins were used as the solid phase, and peroxidase-conjugated rabbit anti–guinea pig immunoglobulins (Accurate Chemical, Westbury, NY) were used for detection of guinea pig antibody. The absorbances were then compared to a control serum arbitrarily assigned a value of 10,000 units/mL.

Statistics. Comparison of lesion scores for acute disease, viral shedding, and recurrent lesion days were done by two-tailed analysis of variance with the Bonferroni correction to adjust for multiple groups. Data are expressed as mean ± SE. Discrete variables were analyzed using a two-tailed Fisher's exact test.

Results

To determine if imiquimod would increase the effectiveness of an HSV-2 glycoprotein vaccine, we performed two experiments. In the first, 55 guinea pigs were randomized into five equal groups: (1) unimmunized control, (2) glycoprotein alone, (3) glycoprotein + imiquimod(D), (4) glycoprotein + imiquimod(S), and (5) glycoprotein + CFA. Encouraged by the initial results, we repeated the original experiment to verify the results using the same five groups. Further, we added two groups to examine the effects of imiquimod when it is given systematically as glycoprotein + imiquimod(Sub Q) and to control for the effects of imiquimod alone (saline + imiquimod[S]). A total of 82 animals was used for this evaluation. Because we were especially interested in the effects on recurrent disease, we used a pool of HSV-2 MS strain that had previously produced milder acute disease but more frequent recurrences. Because the results of the experiments were similar, graphic data will be presented only for the second, more complete experiment.

Acute disease. In the initial experiment, immunization with the HSV-2 glycoproteins alone significantly reduced the total acute lesion score (days 1–14) per animal from 19.1 ± 3.2 in the control group to 4.0 ± 1.0 ($P < .001$). Because of the mild disease in the glycoprotein-alone group, no further significant reduction could be demonstrated for the adjuvant groups, although the total lesion score was less for each of the groups that received adjuvant (2.8 ± 0.7, 2.2 ± 0.6, 1.2 ± 0.6 for imiquimod[D], imiquimod[S], and CFA, respectively).

In the second experiment, the only groups to develop lesions acutely were the groups not immunized with glycoproteins (unimmunized, 11/11; imiquimod alone, 9/9) and the group that received glycoproteins without adjuvant (6/11) (figure 1A). Again, because of the significant effect of immunization with glycoprotein alone, only small additional effects of the adjuvant could be demonstrated on the severity of the acute disease (differences in total lesion score: $P < .05$ for each compared to glycoprotein alone). Imiquimod alone did not affect the severity of the acute disease when used as described in Materials and Methods.

Vaginal viral shedding. Immunization alone also reduced vaginal viral shedding. In the initial experiment, immunization with glycoproteins alone decreased viral shedding by >1 log on day 1 compared to controls (not significant). Groups that also received imiquimod, however, shed ~2 logs less virus on day 1 than did the unimmunized control ($P < .05$). The group receiving CFA had the lowest vaginal virus titers, which were ~2 logs less than the imiquimod groups on day 1 ($P < .05$).

In the second experiment, the addition of imiquimod again further decreased viral shedding compared to the glycoprotein-alone group (figure 1B). Viral shedding was decreased 1 log in the imiquimod(D) group (not significant), by another log in the imiquimod(S) group ($P < .05$), and by yet another log in the imiquimod(Sub Q) group ($P < .001$) on day 1. Thus, there was >99.9% reduction in the imiquimod(Sub Q) group compared to the glycoprotein alone group and a >99.99% reduction compared to the unimmunized control group. No virus was detected in animals that had received glycoprotein with CFA. Imiquimod alone had no significant effect on vaginal viral shedding.

Recurrent disease. Although immunization with the glycoproteins alone significantly reduced the acute disease and modified vaginal viral shedding in the first experiment, the recurrence pattern in this group was similar to unimmunized controls (4.3 ± 0.9 vs. 4.9 ± 0.9 recurrent lesion days, from days 15 to 60, respectively; table 1). The addition of imiquimod, however, significantly reduced recurrent lesion days to 0.8 ± 0.3 and 0.1 ± 0.1, respectively, for the delayed and simultaneous groups ($P < .001$ for each compared to glyco-

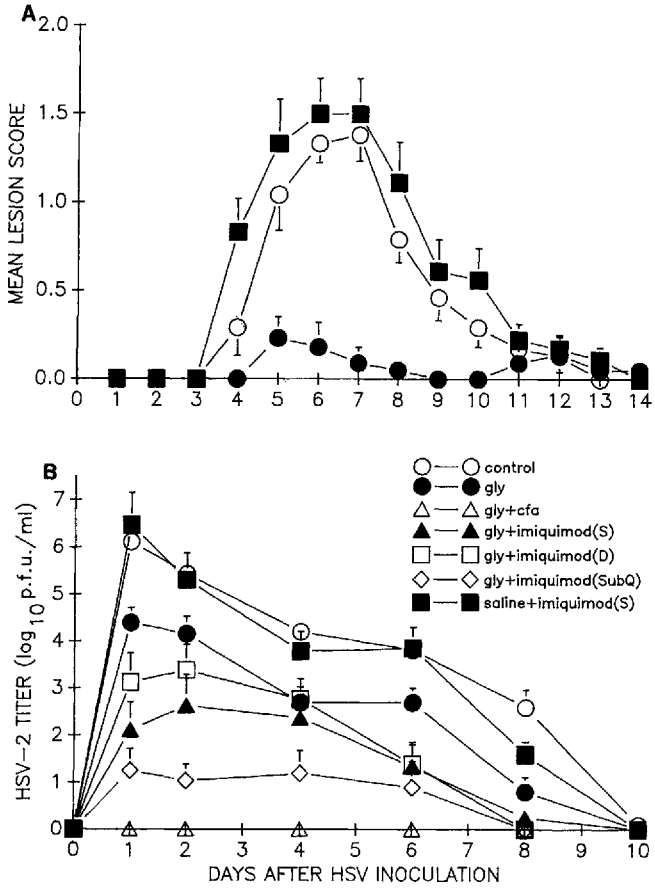

Figure 1. Clinical course of genital skin disease (**A**) and vaginal virus shedding (**B**) in female guinea pigs inoculated with HSV-2. Animals were immunized with HSV-2 glycoproteins (gly) alone or with complete Freund's adjuvant (cfa) in hind footpads 35 and again 14 days before HSV-2 inoculation. Groups that received immunization and imiquimod were treated with a 5-day course of imiquimod beginning either simultaneously with immunization or after a delay of 48 h. Control animals were not immunized. Genital skin disease developed only in 3 groups shown in **A**.

protein alone). Only 1 of 10 animals in the imiquimod(S) group developed a recurrence compared with 8 of 9 recipients of glycoprotein alone ($P < .002$).

In the second experiment, immunization with the glycoproteins alone significantly reduced recurrent lesion days compared to unimmunized controls ($P < .01$) but not the number of animals with recurrences (table 1). Compared to the group receiving glycoprotein alone, however, the addition of imiquimod further significantly reduced recurrent lesion days in each group. None of the animals in the group that received glycoprotein + imiquimod(Sub Q) developed recurrences. The group that received imiquimod alone developed significantly fewer recurrences than did the control group ($P < .01$).

Antibody response. Antibody titers were only marginally increased by the addition of imiquimod. Compared to the glycoprotein-alone group, the only imiquimod group that had significantly higher antibody levels was the imiquimod(S) group in the initial experiment (log geometric mean titer 4.4 ± 0.04 vs. 4.7 ± 0.04, $P < .05$). In contrast, CFA increased antibody titers by >1 log ($P < .001$) in both experiments. Of interest, animals that received imiquimod alone before challenge developed higher titers initially (day 14) than did the control group ($P < .05$). This may indicate that the imiquimod-alone group did develop a greater immune response after challenge, which could account for the decreased recurrence rate observed in this group.

Discussion

Effective HSV subunit vaccines may require the inclusion of potent adjuvants. Initial results of human trials have been disappointing, perhaps because a relatively weak adjuvant, alum, was used for vaccination [13]. An effective adjuvant for an HSV vaccine should augment the ability of the vaccine to decrease viral replication at the mucosal site, prevent clinical disease, and prevent or significantly decrease the number of recurrences that develop after infection. In the experiments reported here, imiquimod fulfilled all of these requirements.

The most effective imiquimod regimen evaluated, subcutaneous administration for five doses beginning at the time of immunization, decreased vaginal virus titers by >3 logs on day 1 compared to animals that received the glycoprotein without adjuvant and by >4 logs compared to unimmunized controls. None of the animals developed acute or recurrent

Table 1. Effect of adjuvant on recurrent genital HSV-2 disease in HSV-2 glycoprotein (Gly)–immunized guinea pigs.

Experiment, group	Animals with recurrent lesions	No. days with herpetic lesions*
One		
Unimmunized	6/7	4.9 ± 0.9
Gly	8/9	4.3 ± 0.9
Gly + imiquimod(D)	4/10	0.8 ± 0.3†
Gly + imiquimod(S)	1/10‡	0.1 ± 0.1†
Gly + CFA	3/10§	0.4 ± 0.2†
Two		
Unimmunized	10/10	5.7 ± 0.8
Gly	9/11	2.5 ± 0.9‖
Gly + imiquimod(D)	4/10	0.4 ± 0.2§
Gly + imiquimod(S)	3/10§	0.3 ± 0.1§
Gly + imiquimod(Sub Q)	0/11†	0‡
Gly + CFA	0/9†	0‡
Saline + imiquimod(S)	8/9	1.8 ± 0.5‖

NOTE. Imiquimod(D), -(S), and -(Sub Q), respectively, are 48-h delayed, simultaneous, and simultaneous subcutaneous administration; CFA, complete Freund's adjuvant.

* Mean ± SE/animal of days with recurrent herpetic lesions.

†, ‡, § $P < .001$, $P < .01$, $P < .05$, respectively, compared to Gly alone.

‖ $P < .01$ compared to unimmunized group.

disease in this group, and thus the results were comparable to using CFA as an adjuvant. Topical (intravaginal administration) of imiquimod was also effective as an adjuvant when given with glycoprotein immunization, reducing both vaginal viral shedding and the acute disease. Further, recurrent disease was decreased significantly compared to the animals that received glycoprotein alone. No significant differences were detected between simultaneous and delayed administration; however, the animals that received simultaneous administration of topical imiquimod had lower virus titers and fewer recurrent lesion days in both experiments. Topical administration as an adjuvant would have an advantage over systemic routes, especially if more than one dose is necessary for maximizing the adjuvant effect. An active oral formulation for imiquimod is also available and is being evaluated in early human trials [11].

Results of this experiment were similar to others that used the guinea pig model of genital HSV-2 to evaluate HSV vaccines [1–6]. We have previously shown that HSV-2 glycoprotein + CFA completely protected against acute disease and reduced recurrent disease by ~85% [1]. Sanchez-Pescador et al. [3] reported a decrease of 92% and Berman et al. [2] found complete protection from acute disease in animals immunized with HSV glycoprotein and CFA. Other adjuvants, such as MTP [3, 6] and IL-2 [12], have provided similar protection when combined with a subunit HSV vaccine. Thus, it appears that imiquimod enhances the effectiveness of HSV subunit vaccines as well as other adjuvants that have been evaluated.

The mechanism by which imiquimod increased the protective effects of this immunization is not clear. Previous studies using imiquimod to treat HSV infection suggest that topical imiquimod is absorbed and increases systemic levels of interferon [8, 9] and perhaps other cytokines as does systemic and oral administration of imiquimod [11] (unpublished data). Animals treated with imiquimod also developed increased lymphoproliferative but not humoral responses to HSV, presumably related to the induction of specific cytokines. In the experiments reported here, imiquimod also did not have a pronounced effect on antibody levels. It would appear most likely, therefore, that combining imiquimod with immunization increases protective cell-mediated immune responses. It is possible that imiquimod increased the levels of protective $CD4^+$ or $CD8^+$ immune responses or down-regulated suppressor responses through the induction of interferon or other cytokines.

The protective immune responses could reduce recurrences either by decreasing the number of neurons that become latently infected after challenge or through a continued up-regulation of immune functions responsible for limiting recurrent disease. Quantitative analysis of latent viral DNA should allow investigation of this question.

Because the adjuvant effects of imiquimod most significantly altered recurrent disease, future studies will evaluate its potential to act as an adjuvant when administered to animals with an established latent infection. We have previously shown that immunization with HSV glycoprotein of guinea pigs with an established latent infection can reduce subsequent recurrences and that this effect is dependent on the adjuvant used [14]. Up-regulation of selected cell-mediated immune function appears to be related to the decrease in recurrences [15], adding to the interest of using imiquimod as an adjuvant in this approach.

In these experiments imiquimod acted as an adjuvant by potentiating the effectiveness of a subunit HSV-2 vaccine. Further evaluations of the immune responses that are up-regulated by imiquimod to provide this protection are necessary and should provide insight into the immune mechanisms that protect from acute and recurrent HSV infections.

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Human Herpesvirus-6 (HHV-6) Infection in Allogeneic Bone Marrow Transplant Recipients: Evidence of a Marrow-Suppressive Role for HHV-6 In Vivo

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Sixteen adults were studied for the first 100 days after allogeneic bone marrow transplant to assess the pathogenic role of human herpesvirus-6 (HHV-6) infection in patients with unexplained febrile illnesses. HHV-6 was directly isolated from the blood of 6 patients. Analysis of the clinical courses of these 16 patients revealed otherwise unexplained posttransplant marrow suppression in 5 patients. Idiopathic marrow suppression occurred more frequently in patients with concurrent HHV-6 viremia (4/6) than in those from whom HHV-6 was not isolated from peripheral blood (1/10, $P < .05$). An etiologic role for the virus was also supported by isolation of HHV-6 from the bone marrow of all 4 patients at the time of marrow suppression and by in vitro colony-forming unit (cfu) assays that demonstrated that HHV-6 could inhibit cfu–granulocyte-macrophage and burst-forming unit–erythroid growth from human bone marrow. By restriction enzyme mapping, all clinical isolates were type B, suggesting that bone marrow transplant recipients may be preferentially infected with and reactivate this HHV-6 subtype. This study implicates HHV-6 as a novel cause of bone marrow suppression in marrow transplant recipients.

Human herpesvirus-6 (HHV-6), a recently discovered member of the herpesvirus family, was initially isolated from the peripheral blood of patients with lymphoproliferative disorders [1]. Despite bearing limited genomic sequence homology to cytomegalovirus (CMV), HHV-6 was found to be distinct from all members of the herpesvirus family by its ultrastructural characteristics, antigenic properties, and in vitro cell tropism [1, 2]. While the pathogenesis of HHV-6 has yet to be fully delineated, accumulating evidence suggests that this virus can cause clinical illness in humans. Early studies revealed HHV-6 to be the etiologic agent of exanthem subitum [3], a self-limited childhood illness characterized by fever and rash. Whether HHV-6 can infect and cause clinically significant disease in bone marrow transplant (BMT) recipients, however, is largely unknown. Recently, the virus was isolated early in the posttransplant period from the blood of pediatric BMT recipients, some of whom had skin rashes [4]. We have also shown that HHV-6 appears to play an etiologic role in selected cases of interstitial pneumonitis after BMT [5]. In this study, we evaluated a cohort of 16 adult BMT patients for the clinical manifestations associated with HHV-6 infection.

Materials and Methods

Patient population. Sixteen patients undergoing allogeneic BMT for leukemia or lymphoproliferative disorders were studied for the first 100 days after transplantation. Pretransplant conditioning was with a previously described regimen of high-dose cytosine arabinoside, cyclophosphamide, methylprednisolone, and fractionated total body irradiation (14 Gy) [6]. Graft-versus-host disease prophylaxis consisted of T cell depletion with an anti-CD3 monoclonal antibody, $T_{10}B_9$, and complement in conjunction with posttransplant cyclosporine administration [6]. All patients received prophylactic acyclovir (5 mg/kg

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