Potent Adjuvant Activity of Cationic Liposome-DNA Complexes for Genital Herpes Vaccines

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Development of a herpes simplex virus (HSV) vaccine is a priority because these infections are common. It appears that potent adjuvants will be required to augment the immune response to subunit HSV vaccines. Therefore, we evaluated cationic liposome-DNA complexes (CLDC) as an adjuvant in a mouse model of genital herpes. Using a whole-virus vaccine (HVAC), we showed that the addition of CLDC improved antibody responses compared to vaccine alone. Most important, CLDC increased survival, reduced symptoms, and decreased vaginal virus replication compared to vaccine alone or vaccine administered with monophosphoryl lipid A (MPL) plus trehalose dicorynomycolate (TDM) following intravaginal challenge of mice. When CLDC was added to an HSV gD2 vaccine, it increased the amount of gamma interferon that was produced from splenocytes stimulated with gD2 compared to the amount produced with gD2 alone or with MPL-alum. The addition of CLDC to the gD2 vaccine also improved the outcome following vaginal HSV type 2 challenge compared to vaccine alone and was equivalent to vaccination with an MPL-alum adjuvant. CLDC appears to be a potent adjuvant for HSV vaccines and should be evaluated further.

Herpes simplex virus type 1 (HSV-1) and HSV-2 are two members of the HSV family of alphaherpesviruses, which establish lifelong latent infection in sensory neurons and lead to chronic herpes disease. HSV-1 infection causes facial/ocular disease, while HSV-2 is the leading cause of genital herpes, although both viruses can be found at oral and genital sites. Indeed, the incidence of HSV-1 genital disease is increasing and approximates that of HSV-2 in certain countries (17). Approximately 45 million people in the United States (20 to 30%) have genital herpes infections, and new infections occur at a rate of 1 million per year (17, 29). One of the most serious complications of genital herpes occurs when the virus is transmitted from mother to neonate. Infection of the neonate causes significant morbidity and mortality, even with proper antiviral therapy (25). Genital herpes infection also increases the risk of acquiring human immunodeficiency virus (HIV) infection and increases shedding of HIV in genital lesions (5, 40).

HSV-2 infection induces both humoral and T-cell-mediated immunity; however, the mechanisms that contribute to long-term control of genital herpes are not understood and could be different from those that will protect against primary infection or disease. Studies from animal models of HSV infection and human studies indicate that high levels of neutralizing antibodies, innate immunity natural killer (NK) cells, interferon (IFN), and macrophages contribute to protection from HSV infection, but the major determinants of HSV protection are both CD8+ and especially CD4+ T cells (7, 9, 23, 27, 30, 31). Clearance of virus from recurrent lesions is also more closely correlated to T-cell immunity. Thus, when a recurrent lesion occurs, mononuclear cells, primarily CD4+ T cells, infiltrate the lesion as early as 2 days after formation and are followed by an influx of CD8+ T cells at later times (10). Although both HSV-specific CD4+ and CD8+ T-cell responses are detected, clearance of HSV-2 from lesions correlates with a CD8+ cytotoxic T lymphocyte response (27, 45).

Vaccines for genital herpes have a long history, beginning in the 1940s, but only recently have some HSV-2 subunit vaccines shown partial efficacy in human trials (reviewed in reference 35). A gD2 vaccine (GlaxoSmithKline) formulated with a mixture of alum and 3-deacylated monophosphoryl lipid A (MPL) prevented clinical symptoms of primary HSV-2 infection (approximately 70% efficacy), but only in women who were HSV-1 and HSV-2 seronegative before vaccination (38). Protection against HSV-2 infection was approximately 40% in the HSV-seronegative women. The vaccine did not provide protection in men or in women who were previously infected with HSV-1. Another vaccine consisting of gD2 and gB2 formulated with the adjuvant MF-59 (Chiron) induced a strong neutralizing antibody and CD4+ T-cell proliferative responses but did not decrease primary genital infections or the frequency of subsequent recurrences (8). However, it should be pointed out that the results for this vaccine were not analyzed for protection only in HSV-1-seronegative women, thus making comparisons with the gD2 vaccine discussed above difficult. Some have attributed differences in the Chiron and GSK vaccines to the adjuvant used in the vaccines, thus highlighting the importance of adjuvants for HSV vaccines (9, 26).

Cationic liposome-DNA complexes (CLDC) were originally developed as a system of gene delivery of bacterial plasmid DNA for potential gene therapy (46). However, as part of preclinical and phase I studies of this technology, it became...
apparent that intravenous administration of CLDC profoundly activated innate immunity and inhibited gene expression. CLDC administration resulted in the release of particularly high circulating levels of alpha IFN (IFN-α), suggesting potent activation of plasmacytoid dendritic cells (DC), and interleukin-12, suggestive of conventional DC activation (11, 12, 15). This activation was independent of whether the plasmid contained any cDNA coding region (the "empty-vector" effect) and has subsequently been shown to occur with Toll-like receptor 3 (TLR3) agonists as well when the same mixture of cationic and neutral lipids is used (44). The empty-vector DNA used for CLDC gene therapy contains multiple unmethylated CpG motifs, and part of the robust induction of innate immunity is likely to reflect the internalization of the DNA into the endosomes of plasmacytoid DC, where these CpG motifs can engage TLR9. Further, addition of peptide or protein antigens to CLDC created a very potent adjuvant effect, with elicitation of strong T-cell and antibody responses (44). We therefore evaluated the use of CLDC as an adjuvant for a genital herpes vaccine using the well-established mouse model.

MATERIALS AND METHODS

Adjuvants. MPL (0.5 mg) plus trehalose dicycromoniumycytolate (TDM) (0.5 mg) in 2% oil (squalene)-Tween 80-water was purchased from Sigma-Aldrich Corp., St. Louis, MO, and administered as 50 μg/dose. The MPL-alum combination contained 50 μg of MPL (Sigma-Aldrich Corp., St. Louis, MO) and 200 μg of aluminum potassium sulfate (Sigma-Aldrich Corp., St. Louis, MO) per dose of vaccine.

CLDC. The plasmid (pVB7-100) (Juvaris BioTherapeutics, Inc., Burlingame, CA) was provided as a white, lyophilized powder manufactured from plasmid DNA complexed with liposomes. The plasmid (pMB75.6) was 4,242 base pairs in length and was in a Tris-HCl buffer. Liposomes were prepared from the cationic lipid DOTIM (1-[2-(oleoyloxy)ethyl]-2-oleyl-3-(2-hydroxyethyl)imidazolinium chloride) and the neutral lipid cholesterol. The plasmid DNA and liposome intermediates were each diluted with lactose in 1.4 mM Tris-HCl and then complexed under aseptic conditions to form the formulated drug substance. After reconstitution, the final drug product was a colloidal dispersion of 0.3 mg/ml DNA, 1.88 mg/ml MPL, and 1.05 mg/ml cholesterol at pH 7.0 containing 1.4 mM Tris-HCl and 10% (wt/vol) lactose (14). The formulated drug substance was placed into vials and lyophilized to produce the drug product. The lyophilized CLDC drug product was reconstituted in sterile water for injection.

Vaccines. (i) Whole Virus. The HSV vaccine (HVAC) was prepared by infection of Vero cells with HSV-2 strain 186. Infected cells were then washed and solubilized with 0.2% Triton X-100 detergent and clarified by centrifugation (5,000 × g). The HSV vaccine was tested before use in the vaccine studies for the presence of replicating virus, as determined by plaque assay, and was found to be negative for infectious virus. Animals received 100 μg of the solubilized protein in 200 μl by subcutaneous (SC) inoculation.

(ii) gD2. The gD2 vaccine was prepared by R. Eisenberg and G. Cohen (University of Pennsylvania) from SF9 (Spodoptera frugiperda) cells (Gibco BRL) infected with a recombinant baculovirus expressing gD2, as previously described (41). Five micrograms of gD2 was absorbed onto the alum and then combined with MPL. Animals received 5 μg of gD2 in 200 μl by SC inoculation.

Animals. Female Swiss Webster mice (18 to 21 g) were obtained from Harlan (Indianapolis, IN) and housed under AALAC-approved conditions.

Virus. HSV-2 strain 186 was prepared as previously described (4).

Experimental design. Mice were vaccinated SC twice 42 and 21 days prior to intravaginal challenge with a lethal dose of HSV-2 strain 186 (1 × 106 PFU). Prior to challenge, mice were pretreated with progesterone, as previously described (39). To determine the levels of replicating virus, vaginal swab specimens were collected on days 1 to 4 postchallenge, and the virus titer was subsequently quantified by plaque assay. Animals from which no virus was isolated were assigned a value of 0.6, the limit of detection for the assay. Animals were evaluated for the symptoms of herpesvirus infection (erythema, hair loss, and mortality) for 21 or 30 days after inoculation. Those animals alive after follow-up were deemed to have survived the herpesvirus infection.

Antibody. Antibody was measured by enzyme-linked immunosorbent assay (ELISA) using an HSV-2 glycoprotein-enriched lysate as the solid phase (3) and biotinylated anti-mouse immunoglobulin G (IgG), IgG2a, or IgG1 (Southern Biotechnology, Birmingham, AL) for detection. The plates were then developed by addition of horseradish peroxidase-conjugated goat anti-biotin antibody (Vector Laboratories, Burlingame, CA) followed by o-phenylenediamine dihydrochloride plus hydrogen peroxide (Sigma-Aldrich Corp., St. Louis, MO), as previously described (13). The absorbance at several dilutions was then compared to a standard curve, and the quantity of antibody was expressed as ng/ml. Seven to 12 animals were individually evaluated at each time point.

Splenocyte restimulation assays. Splenocytes (4 × 106 cells/well) isolated from vaccinated and control mice 14 days after the second vaccination with gD2 were cultured in fetal bovine serum-enriched RPMI media. CD4- and CD8-depleted cell fractions were obtained by magnetic bead labeling and column separation, as directed by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were restimulated with 2.5 μg HSV gD2 antigen. Supernatants were collected after 96 h of stimulation and assayed by ELISA for mouse IFN-γ (eBiosystems, San Diego, CA). Controls included spleen cells and CD4- or CD8-depleted fractions cultured 96 h without the addition of specific antigen.

Statistics. Mean values were compared by analysis of variance and then by the Bonferroni correction for multiple groups, including the vaccine alone and the vaccine with each adjuvant. Discrete variables were analyzed using Fisher's exact test. All comparisons were two-tailed.

RESULTS

In the initial efficacy experiment, 60 female outbred Swiss Webster mice (12 mice/group) were divided as follows: group 1, no HVAC/no adjuvant; group 2, HVAC alone; group 3, HVAC with CLDC; group 4, HVAC with MPL-TDM in 2% squalene; and group 5, CLDC alone.

HSV-specific antibodies were detectable after one immunization of HSV vaccine, HVAC with CLDC, or HVAC with MPL-TDM. Administration of CLDC enhanced the levels of detectable HSV-specific antibodies in the serum compared to immunization with vaccine plus MPL-TDM (1.8-fold-higher levels) or the HVAC alone (3.1-fold-higher levels) after the first dose, suggesting that CLDC enhances antigen-specific antibody production (Fig. 1A). Following the second immunization, the levels of HSV-specific antibodies remained highest in the CLDC group (2,195 ng/ml) compared to levels in the group receiving HVAC alone (1,503 ng/ml, P < 0.01) or with MPL-TDM (1,758 ng/ml, P < 0.05).

Animals were also assessed daily to monitor the development of symptomatic HSV infection. As shown in Fig. 1B, all of the mice that did not receive vaccine, 83% of the mice that received CLDC alone, and 80% of the mice that received HVAC alone developed local disease. In contrast, only 40% of the mice that received HVAC with MPL-TDM adjuvant (P = 0.170 versus mice that received vaccine alone) and 18% of the mice receiving HVAC with CLDC (P = 0.009 versus mice that received vaccine alone) developed disease. Similarly, as shown in Fig. 1C, mice that received no vaccine, CLDC alone, or vaccine alone were not protected from death (8 to 50% survival by 21 days). The HVAC with MPL-TDM protected 60% of the mice (P = 0.170 versus mice that received vaccine alone), while 100% of the mice that were vaccinated with HVAC with CLDC were protected from death (P < 0.001 versus mice that received vaccine alone and P = 0.035 versus mice that received HVAC–MPL-TDM).

To further document the antiviral effects of vaccination, effects on vaginal viral replication were examined. Immunization with HVAC with CLDC significantly reduced the level of infectious virus in the vagina on all 4 days and eliminated viral shedding by day 4 in 10 of 11 animals (Fig. 1D). These reductions in viral shedding compared to those with vaccine alone.
were significant on days 1 to 3 ($P < 0.01$) and compared to those with HVAC with MPL-TDM titers were significantly less on days 2 and 3 ($P < 0.01$).

A second study using 48 female outbred Swiss Webster mice ($n = 12$) was then performed to validate the first results and extend the observation to 30 days, as it appeared that animals in the MPL-TDM vaccine group continued to die after day 21. In agreement with the results of the first study, mice vaccinated with the HVAC alone were more likely to develop local disease (75%) than animals vaccinated in combination with CLDC (25%, $P = 0.039$ versus mice that received HVAC alone) (Fig. 2A). However, MPL-TDM was not effective in this study; 83% of MPL-TDM-vaccinated mice developed disease. Thus, in this study HVAC with CLDC provided significantly better protection than HVAC with MPL-TDM ($P = 0.012$). The difference in mortality rates between the HVAC with CLDC (25%) and HVAC with MPL-TDM (75%) groups was also significant ($P = 0.039$) (Fig. 2B).

In order to evaluate CLDC with a vaccine that more closely resembles the vaccine that previously showed some protection in clinical trials (38) and is being further evaluated in a large clinical trial, we evaluated the antibody and T-cell responses in mice immunized with gD2 with and without CLDC. Higher levels of total anti-gD2 IgG antibody were detected in the group immunized with CLDC plus gD2 than in the group immunized with unadjuvanted gD2 vaccine ($P < 0.01$) (data not shown). In addition, the ratio of IgG2a to IgG1 was increased in the CLDC group compared to that in the unadjuvanted group (data not shown). Further, splenocytes from animals immunized with gD2 and CLDC produced a significantly higher level of IFN-γ than splenocytes from animals immunized with gD2 alone ($P < 0.05$) (data not shown). When these splenocytes were depleted of CD4+ cells, they were no longer responsive, while in contrast, CD8+-depleted splenocytes remained IFN-γ reactive (data not shown).

We then compared the gD2 vaccine with CLDC to gD2 with MPL-alum to further match the vaccine and adjuvant to the vaccine proceeding in clinical trials. Splenocytes from animals immunized with gD2 and CLDC produced significantly more IFN-γ than animals immunized with gD2 alone ($P < 0.01$) (Fig. 3). To evaluate efficacy, 50 mice were divided equally to receive no vaccine or adjuvant;
gD2 alone, with CLDC, or with MPL-alum; or HVAC with CLDC. Mice receiving CLDC-gD2 or MPL-alum–gD2 produced considerably higher levels of anti-HSV antibody than those receiving gD2 alone (P < 0.01), although antibody levels induced by MPL-alum were higher than those induced by CLDC (P < 0.05) (Fig. 4). However, vaccination with CLDC-gD2 resulted in a higher ratio of IgG2a to IgG1 (0.86) compared to the MPL-alum–gD2 group (0.39), indicating that the CLDC adjuvant may be more effective than MPL-alum at promoting TH1 immunity (Fig. 4).

Vaccination with gD2 alone did not protect against disease or death, although death was delayed in mice receiving gD2 alone (mean day of death = 16.0) compared to the untreated group (mean day of death = 10.6, P = 0.015). As shown in Fig. 5A, mice receiving CLDC-gD2 or MPL-alum–gD2 were significantly protected from disease (40% in each group) compared to mice receiving the gD2 antigen alone (100% with disease, P = 0.011). Vaccination also decreased death in the adjuvant groups from 90% in the gD2-alone group to 20% and 10% for CLDC (P = 0.006) and MPL-alum (P = 0.001), respectively (Fig. 5B). Further, vaccination with CLDC-gD2 reduced vaginal viral titers on all days postchallenge compared to titers in the gD2-alone group (not significant), but titers were similar to those of the gD2-plus-MPL-alum group (Fig. 5C). Of interest, the virus titers of the HVAC-plus-CLDC group were less than titers of the gD2-plus-CLDC group on day 1, suggesting that additional HSV antigens may contribute to protection.

**DISCUSSION**

There is a clear need for a vaccine that would protect against genital herpes infection and disease. The most recent results of trials using subunit HSV-2 glycoprotein vaccines indicate that the vaccines were ineffective or effective with only a subset of
the population, HSV-seronegative women (8, 38). The effectiveness of vaccines can be improved with potent adjuvants, and there is a recent boom in the interest in adjuvants (19, 28, 44). The ability to induce a T-cell response may be especially important for a herpesvirus vaccine (7, 9, 23, 27, 30, 31).

The mechanism of action of adjuvants is complex and incompletely understood. However, the dominate mechanisms of some adjuvants are known. Aluminum hydroxide and MF59 are the only vaccine adjuvants currently widely licensed for use with humans (note that MF59 is not licensed in the United States). Both augment adaptive immune responses largely through enhancing antigen delivery, although induction of a local, proinflammatory environment is also contributory (32, 34). Two other promising adjuvants, MPL and CpG oligonucleotides, function primarily through activation of innate immunity, which rapidly evolves into an adaptive response (36). Specifically, MPL induces innate immunity via activation of TLR4, whereas the adjuvant effects of CpG oligonucleotides are mediated through interaction with TLR9 (32, 36, 37). CLDC operate via a more diverse mechanism. These complexes combine activation of innate and ultimately adaptive immunity via TLR9 and non-TLR9 pathways coupled with antigen delivery (11, 20, 42, 43). The use of cationic liposomes is thought to direct antigens to DC; enable entry into cells, specifically the endosome compartment; enhance entry into major histocompatibility complex class I pathways; and potentiate activation of innate immunity by TLR9 agonists, like plasmid DNA (11, 18, 22, 44). Thus, the combination of cationic liposomes and plasmid DNA should improve both innate and adaptive immune responses. Recently, CLDC was shown to effectively induce CD4+ and CD8+ T-cell responses against peptide and protein antigens in mice (44). Further, a CLDC-adjuvanted simian immunodeficiency virus (SIV) vaccine induced stronger SIV-specific T- and B-cell responses than an SIV vaccine without adjuvant in rhesus macaques (14).

In the present studies, we have shown that CLDC enhanced antibody responses to both HSV whole-virus vaccine and an HSV gD2 vaccine. Further, vaccination with CLDC resulted in a higher ratio of IgG2A to IgG1, indicating that CLDC may be a more effective inducer of TH1 immunity than MPL-alum, the adjuvant being used in a large phase III investigation of a genital herpes vaccine in young women. When IFN-γ production from splenocytes stimulated with gD2 was evaluated, the group immunized with gD2 and CLDC produced significantly

![Graph A](image1)

**FIG. 5.** Evaluation of vaccination with gD2 vaccine and CLDC clinical outcome through day 14 in mice (n = 10/group) challenged with HSV-2. Mice were followed daily for 30 days for signs and symptoms of genital disease (A) and for mortality (B). (C) Vaginal virus shedding was measured on days 1 to 4. Adj., adjuvant. Error bars are standard deviations.
higher levels of IFN-γ than the group immunized with gD2 alone or with MPL-alum.

The addition of CLDC also significantly decreased vaginal HSV replication compared to vaccine alone. Importantly, immunization with CLDC improved the outcome of vaccination with HVAC compared to vaccination with MPL-TDM and was equivalent to MPL-alum when used with a gD2 vaccine. In two experiments, survival of animals receiving two doses of HVAC with CLDC was significantly ($P < 0.05$) greater than survival in groups receiving HVAC and MPL-TDM.

The mouse model of genital HSV-2 infection has been used to evaluate therapies since 1977 (1, 24). In this model, virus first replicates in the vaginal epithelium and then ascends to the dorsal root ganglia, spinal cord, and brain (33). Death is due to encephalitis or, more rarely, to disseminated infection. Thus, unlike with disease in humans, this is not a self-limited infection. Nevertheless, vaccines can limit local replication in the vagina, suppress symptoms, and prevent death, making it a useful model for screening HSV vaccines and adjuvants.

Overall, as discussed above, significant protection was seen with the gD2 vaccine when administered with MPL and especially CLDC, but complete protection from symptoms or death was not seen, especially when evaluations were continued for 30 days. There is considerable debate about which antigens should go into a subunit HSV vaccine. The use of gD2 and gD2gB2 has provided some protection (8, 38), but this is not considered to be optimal. There is evidence to suggest that the addition of other glycoproteins could improve vaccine efficacy (16), while others would suggest that identifying and including the most potent inducers of T-cell immunity is the best approach (21).

In this regard, it is interesting to note that several CD4 and CD8 T-cell epitopes have been identified in gD2 (2, 6).

The CLDC adjuvant evaluated with HSV vaccines in the mouse genital herpes model demonstrated increased protection with two HSV vaccines and should be evaluated further. Future studies of CLDC will evaluate the adjuvant with candidate vaccine antigens in the guinea pig model of genital herpes prior to initiation of clinical trials for prophylactic and/or therapeutic HSV-2 vaccines.

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