Calcium Phosphate Nanoparticles Induce Mucosal Immunity and Protection against Herpes Simplex Virus Type 2

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Previously we reported that calcium phosphate nanoparticles (CAP) represented a superior alternative to alum adjuvants in mice immunized with viral protein. Additionally, we showed that CAP was safe and elicited no detectable immunoglobulin E (IgE) response. In this study, we demonstrated that following mucosal delivery of herpes simplex virus type 2 (HSV-2) antigen with CAP, CAP adjuvant enhanced protective systemic and mucosal immunity versus live virus. Mice were immunized intravaginally and intranasally with HSV-2 protein plus CAP adjuvant (HSV-2+CAP), CAP alone, phosphate-buffered saline, or HSV-2 alone. HSV-2+CAP induced HSV-specific mucosal IgA and IgG and concurrently enhanced systemic IgG responses. Our results demonstrate the potency of CAP as a mucosal adjuvant. Furthermore, we show that systemic immunity could be induced via the mucosal route following inoculation with CAP-based vaccine. Moreover, neutralizing antibodies were found in the sera of mice immunized intranasally or intravaginally with HSV-2+CAP. Also, the results of our in vivo experiments indicated that mice vaccinated with HSV-2+CAP were protected against live HSV-2 infection. In conclusion, these preclinical data support the hypothesis that CAP may be an effective mucosal adjuvant that protects against viral infection.

Since mucosal surfaces act as the primary point of entry for most pathogens and the first line of defense against them, vaccines inducing effective mucosal immunity may reduce rates of infection and decrease the morbidity and mortality of infectious diseases. Currently, no safe and effective mucosal vaccine adjuvants are approved for human use.

Mucosal vaccine delivery is a promising strategy. Mucosal vaccines administered in one part of the body can elicit an antibody response in mucosal tissues remote from the site of initial antigen exposure. This effect occurs because of the common mucosal immune system (13). A major obstacle to developing a mucosal vaccine in humans is finding a safe and effective adjuvant. Experimental mucosal adjuvants include cholera toxin, heat-labile enterotoxin, mutant toxins (LT, K63 and LTR72), CpG oligodeoxynucleotide, polymerized liposomes, microparticles, and interleukins or immune modulators. None of these adjuvants is approved for use in humans (3, 12, 18).

Biodegradable calcium phosphate particles have been investigated as an alternative to aluminum adjuvants for parenteral vaccines. Clinical studies conducted in France described the use of a calcium phosphate adjuvant for secondary or booster immunizations against diptheria and tetanus (8). Calcium phosphate has also been used for allergen desensitization (6, 16). Early studies indicated that calcium phosphate particles produce strong adjuvant effects, induced less immunoglobulin E (IgE) than aluminum adjuvants, and elicited only minimal local irritation in animal experiments and human clinical trials (6, 8, 11, 17).

Here, we describe a unique formulation of calcium phosphate nanoparticles (CAP) which is distinct from the formulations of calcium phosphate described by European scientists (16) and demonstrate its use as an effective mucosal adjuvant. Our results indicate that following viral challenge, mice immunized with CAP-based formulations of herpes simplex virus type 2 (HSV-2) glycoprotein exhibited significantly increased survival rates and less severe clinical infection than controls. These findings demonstrate that CAP delivered as a mucosal adjuvant confers protective antiviral immunity.

MATERIALS AND METHODS

Formulation of subunit vaccine. The preparation of partially purified HSV-2 glycoproteins has been described previously (7, 19). Briefly, infected cells were collected and sonicated. The viral suspension was centrifuged at 5,500 × g for 15 min. Supernatant was collected and stored with 1% IGEPAL (Sigma Chemical Co., St. Louis, Mo.) lysis buffer for 30 min on ice. The solution was centrifuged at 18,500 × g for 2 h. The supernatant was dialyzed against phosphate-buffered saline (PBS) at 4°C and stored at −80°C. Then 1 mg of HSV-2 protein was added to 7.5 ml of 12.5 mM calcium chloride, followed by the addition of 7.5 ml of 12.5 mM dibasic sodium phosphate and 1.5 ml of 15.6 mM sodium citrate. The solution was stirred until the final average particle size was less than 1.2 μm, as determined with a Coulter N4Plus Submicron particle sizer, and was treated with 129 mM cellulose overnight. The total protein inside CAP was 123 μg. The particle containing HSV-2 protein was coated again with 3.877 mg of HSV-2 proteins by coinubcation for 1 h at 4°C. The final concentration of CAP plus HSV solution was 2 mg of HSV/ml and 10 mg of CAP/ml. The control vaccines were PBS, CAP alone, and HSV-2 protein alone.

Animals. Female BALB/c mice, 6 to 8 weeks old and weighing 25 g, were obtained from Charles River Laboratories. The mice were maintained in standard housing with a normal diet of Purina rodent chow 5001.

Immunization and sample collection. Eight groups of five female BALB/c mice were inoculated intravaginally or intranasally with HSV-2+CAP (20 μg of viral protein plus 100 μg of CAP per dose per mouse), HSV-2 alone (20 μg per dose per mouse), or CAP alone (100 μg per dose per mouse) in a total volume of 50 μl (intravaginally) or 10 μl (intranasally).

The mice received two inoculations, on days 0 and 7. Samples were collected 7, 14, and 38 days after the last immunization. Blood was obtained from the orbital sinus, and the serum samples were stored at −20°C. Mucosal samples were collected 14 days after the last immunization by vaginal lavage with 100 μl of PBS. The sediments were removed by centrifugation, and samples were pooled and stored at −20°C.

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ELISA. HSV-specific antibodies were detected by an end-point dilution enzyme-linked immunosorbent assay (ELISA) as described previously (3). Titers for IgG in plasma samples were expressed as group mean ± standard error of the mean of values for individual animals. Titers for IgA and IgG in mucosal samples were expressed as the mean of triplicate assays from pooled mucosal samples.

**HSV-2 challenge experiment.** Using methods reported previously (7), mice were injected subcutaneously with DepoProvera (Upjohn, Kalamazoo, Mich.) at a concentration of 2 mg/mouse in 50 μl of distilled water on the 45th day following primary immunization. Five days later, the mice were challenged intravaginally with 10^6 PFU of HSV-2. Mice were examined daily for genital pathology, and the clinical scoring was performed by an investigator blinded to the animal's immunization status. Clinical pathology was scored on a 5-point scale: 0, no apparent infection; 1, slight redness of external vagina; 2, severe redness and swelling of external vagina; 3, genital ulceration with severe redness, swelling, and hair loss of genital and surrounding tissue; 4, severe ulceration of genital and surrounding tissue and paralysis; and 5, death.

**Neutralization assay.** As reported previously (7), Vero cells were propagated in culture plates. Pooled mouse serum samples from day 38 after the last immunization were incubated with HSV-2 and assessed for the presence of HSV-2-specific neutralizing antibodies by plaque assay. The titer is the reciprocal of the serum dilution required to inhibit the cytolysis of a confluent monolayer of Vero cells by 50%.

**Statistical analysis.** Pathological data were analyzed by analysis of variance to determine the difference between groups.

**RESULTS**

As indicated in Fig. 1, both the intranasal and intravaginal HSV-2+CAP-vaccinated mice showed a high titer of HSV-specific mucosal IgA and IgG in vaginal lavage fluid at 14 days after the last immunization.

Serological IgG and IgG2a titers determined on day 38 after the last immunization showed a systemic response in the mice after intranasal or intravaginal immunization with HSV-2+CAP compared to PBS, CAP alone, or HSV-2 alone (Fig. 2).

The neutralization assay was performed at day 38 following secondary immunization. Neutralizing antibodies were found in both the intranasally and intravaginally HSV-2+CAP-immunized mice at titers of 1:40 and 1:80, respectively. Notably, neutralizing antibodies were absent in the mice inoculated with PBS alone, CAP alone, or HSV-2 alone.

Resistance to HSV-2 infection was evaluated by monitoring clinical pathology. On days 6, 8, and 10, the reduced clinical severity in mice intravaginally immunized with HSV-2+CAP
achieved statistical significance \( P < 0.05 \) compared to mice immunized with PBS, CAP alone, or HSV-2 alone (Fig. 3, right panel). One of five mice intravaginally inoculated with HSV-2/CAP died from HSV-2 infection, whereas all of the mice intravaginally vaccinated with PBS, HSV-2 alone, and CAP alone developed severe disease and died by day 8 or 10. Similarly, the mice vaccinated intranasally with HSV-2/CAP showed reduced clinical severity compared with mice immunized with PBS, CAP alone, or HSV-2 alone at days 8 and 10 (Fig. 3, left panel). Two of five mice intranasally vaccinated with CAP/HSV-2 died, compared with the controls (i.e., recipients of PBS, CAP alone, and HSV-2 only), all of which died eventually. All surviving mice were kept for 2 more weeks and recovered gradually.

**DISCUSSION**

The mucosal tissues are the primary routes of entry into the body for microbial pathogens. Vaccines inducing mucosal immunity prevent the transmission of infection via mucosal surfaces. However, no mucosal vaccine adjuvant is currently approved for human use. Because of the weak inherent immunogenicity of some antigens targeted for vaccine development, such as epitope subunits and recombinant peptides, there is a great need for safe and efficient mucosal adjuvants. The only adjuvants used in licensed vaccines in the United States are aluminum compounds, which effectively enhance immune responses (2, 5, 14). However, human studies have shown them to be weak adjuvants for inducing cell and humoral immunity to some virus protein subunits (S. J. D. Bell, personal observation). Additionally, alum can elicit an IgE antibody response that increases the risk of allergic reactions.

We have reported previously that CAP delivered intraperitoneally with HSV-2 and Epstein-Barr virus proteins induced high titers of IgG2a antibody and neutralizing antibody and facilitated a high degree of protection against viral infection in a murine model (7). In this study, using HSV-2 protein as a model antigen, we evaluated the immunity and efficacy of an HSV-2/CAP experimental vaccine. Our results indicated that mice vaccinated either intravaginally or intranasally with HSV-2/CAP had high antibody levels at mucosal surfaces and effective neutralizing antibody titers and were protected against virus infection. We assumed that the neutralizing antibody prevented the attachment of pathogens to the epithelial surfaces and conferred protection against subsequent viral infection. Our findings also confirmed the previous studies (4, 15) showing that antibodies can efficiently neutralize virus in mucosal areas.

The immune system within the female lower genital tract is the initial defense against sexually transmitted diseases. Our study suggested that intravaginal immunization induced relatively higher levels of mucosal IgG and IgA than intranasal immunization, providing optimal protection against HSV-2 infection. This observation supports the findings of others (1, 9, 10) and suggests that genital local immunity and Th1 response in association with other protecting factors, such as local production of antibodies and viral clearance from the vaginal mucosa, play a major role in HSV-2 infection in mice. Our next step is to prove that CD4\(^+\) T cells secreting gamma interferon and B cells or natural antibodies are critical for immune protection against lethal genital HSV-2 reinfection.

The exact mechanism of the adjuvant action of CAP is not fully understood. M cells in the mucosal tissues are known to reside exclusively in the epithelium and deliver foreign material by transepithelial transport from the lumen to the underlying mucosa-associated lymphoid tissue. Particulate antigens are desirable because they permit M cells to translocate across the tight epithelial barrier to mucosal dendritic cells. Therefore, the particulate mucosal vaccine created from the combination of soluble antigens formulated within CAP provides the
desirable size and functional attributes to induce effective mucosal immunity.

Recent comparative studies have indicated that microparticles are potent adjuvants for mucosal delivery (18). However, microparticles are not an ideal size for inducing cellular immunity because they tend to be too large, and it is believed that M cells, dendritic cells, macrophages, and local lymph nodes are more effective at taking up smaller particles. Advantageously, CAP are generally in the preferred size range (i.e., less than 1.2 μm, versus 1 μm-sized polymers) and also stimulate cellular immunity and cytokotic T lymphocyte responses (unpublished data). Based on these results, we conclude that (i) the CAP-based HSV-2 subunit vaccine appears to concurrently induce both systemic and mucosal immunity and (ii) CAP shows great potential as a safe and effective mucosal vaccine adjuvant for humans, given its relative absence of side effects and lack of IgE antibody induction.

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REFERENCES
