Vaccine Therapy for Ocular Herpes Simplex Virus (HSV) Infection: Periocular Vaccination Reduces Spontaneous Ocular HSV Type 1 Shedding in Latently Infected Rabbits

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Periocular vaccination of rabbits with preexisting herpes simplex virus type 1 (HSV-1) latent infection with recombinant HSV-2 glycoproteins B and D (gB2 and gD2) plus adjuvant significantly reduced ocular viral shedding. Rabbits were infected in both eyes with HSV-1 strain McKrae. Following HSV-1 infection and the establishment of latency (28 days postinfection), rabbits were given a periocular subconjunctival vaccination three times at 3-week intervals. Beginning 3 weeks after the final vaccination, tear films were collected daily and cultured to detect the presence of HSV-1 and determine the spontaneous HSV-1 ocular shedding rates. Periocular vaccination increased the mean HSV-1 serum neutralizing antibody titer to fivefold above that seen in mock-vaccinated latently infected rabbits. gB enzyme-linked immunosorbent assay (ELISA) antibody titers were increased approximately 8-fold, and gD ELISA antibody titers were increased 60-fold. These increases were all statistically significant ($P < 0.0001$). In two independent experiments, vaccination reduced the spontaneous shedding rate by approximately 2.5-fold ($P < 0.0004$). In addition, the percentage of eyes that never shed virus during the 6 week postvaccination test period increased threefold (20% in controls versus 60% in vaccinated animals; $P < 0.007$). These results show that spontaneous ocular shedding of HSV-1 in latently infected rabbits can be significantly reduced by local periocular vaccination. This is the first report in any animal model of a successful therapeutic vaccine against recurrent HSV-1 ocular shedding. These results support the concept that development of a therapeutic vaccine for ocular HSV-1 recurrence in humans is possible.

Herpes simplex virus (HSV) infections are extremely common in humans and can occur at diverse sites with a wide range of symptoms from inapparent to life-threatening encephalitis (3, 4). These infections are caused by two closely related viruses, HSV-1, often acquired in early childhood as an oral infection, and HSV-2, most often acquired as a genital infection after the onset of sexual activity. Following the initial infection, HSV replicates in the skin or mucosal epithelium, infects sensory nerves, and is transported to the involved sensory ganglia. Here HSV establishes a life-long latent infection that is punctuated by periodic reactivations.

HSV can infect the eye and produce recurrent ocular infections in a manner similar to that of oral and genital HSV infections. Following ocular infection, HSV-1 latency is established in neurons of the trigeminal ganglion. Reactivated HSV-1 can travel back to the eye via axonal transport and be detected by culturing tear films (virus shedding). Reinfection of the eye can produce corneal disease and stromal scarring as the result of an incompletely defined immunological response to the virus (23, 26). The scarring that occurs as a result of HSV ocular recurrence is a major cause of corneal blindness (15, 31). In developed nations, HSV is the most frequent serious viral eye infection and is the most common cause of infectious blindness (15). In the United States, almost 500,000 people per year suffer ocular HSV episodes requiring doctor

visits and medication (15). Over 1,000 corneal transplants per year are done in the United States as a direct result of HSV scarring (15, 31). More than 90% of ocular HSV infections are due to HSV-1.

Despite the seriousness of recurrent ocular herpes, the only approved treatments are for acute primary and acute recurrent infection; these treatments consist of the topical antiviral agents idoxuridine and trifluorothymidine. No drug is as yet approved for prevention of ocular recurrences. Given the success of acyclovir suppression therapy for recurrent genital HSV, practitioners have used such therapy for patients with recurrent ocular herpes. A multicenter, placebo-controlled trial of oral acyclovir for prevention of ocular recurrence is under way to determine if such therapy is effective (4a).

A more cost-effective alternative to antiviral drugs, the development of vaccines for HSV, has focused entirely on the problem of genital HSV-2. Since HSV-1 is usually acquired as a childhood infection, widespread prevention of ocular herpes by immunization would require universal prophylactic vaccination of infants.

Recently, there has been renewed interest in the concept of therapeutic vaccines for persistent infections, especially for intervention for human immunodeficiency virus infection (1, 24, 27, 29). An immunotherapeutic vaccine to reduce or prevent ocular recurrences would be of great value since most adults already harbor latent HSV-1 infection. However, there is a valid concern that vaccination of an individual with recurrent ocular herpes and subsequent boosting of the anti-HSV immune response could exacerbate the undefined immune response that produces the corneal scarring associated with ocular HSV recurrences.

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Therapeutic vaccination for oral or genital herpes has been tested previously with crude vaccines, resulting in claims of success in open trials. However, these claims were not substantiated in masked, placebo-controlled trials (reviewed in references 2 and 13). More recently, well-defined herpesvirus glycoprotein subunit vaccines have been developed by using recombinant DNA technology. These vaccines are very immunogenic in animals and afford protective immunity when used prophylactically in mouse and guinea pig models of herpes disease (5–8, 28, 32, 35). More importantly, in a guinea pig model of recurrent genital herpes, when subunit glycoprotein vaccines were used to immunize animals that had previously been infected with HSV and were experiencing spontaneous recurrent disease, both the frequency and the severity of recurrent disease were reduced (33). The magnitude of the reduction varied from 40 to 80% for effective vaccine formulations, depending primarily on the adjuvant included (9, 10, 34). Not all formulations were effective.

More recently, in a preliminary human clinical trial in HSV-seropositive individuals, a glycoprotein D2 (gD2) subunit vaccine elicited increases in gD2 antibody titers of 13-fold above that generated by natural infection and increases in neutralizing antibody titer of 6-fold (36). Cellular immune responses to gD2, measured by lymphoproliferation, were also increased above those present at study entry (38). This formulation has now been tested as an immunotherapeutic vaccine in a masked, placebo-controlled trial in humans with 4 to 14 genital herpes recurrences per year (12, 35a). For the 1-year period of observation, there was a modest (up to 36%) but statistically significant reduction in recurrent disease, as assessed by several different endpoints.

The immunotherapeutic effect demonstrated for genital herpes in humans and in the guinea pig model, combined with the efficacy of a prophylactic subunit vaccine against primary ocular infection in the rabbit (11), suggested that a successful therapeutic effect might be achieved for ocular herpes. We report here the use of a gB2-plus-gD2 (gB2+gD2) subunit vaccine to vaccinate latently infected rabbits against HSV-1 ocular recurrences. Since local immunity is likely to be especially important for mucosal surfaces and particularly for the eye, we vaccinated the rabbit eyes by injecting the vaccine periocularly, directly beneath the conjunctiva, rather than systemically. Our choice of periocular rather than systemic vaccination was also based on our preliminary studies indicating that local ocular vaccination appeared to protect rabbit eyes against primary HSV-1 challenge more effectively than did systemic vaccination.

Following vaccination of latently infected rabbits with the gB2+gD2 subunit vaccine, neutralizing antibody titers were increased 5-fold and gB2 and gD2 enzyme-linked immunosorbent assay (ELISA) antibody titers were increased approximately 8- and 60-fold, respectively. In two separate experiments, we observed no exacerbation of disease in vaccinated compared with control animals. Instead, spontaneous ocular HSV-1 shedding was reduced by two- to threefold compared with mock-vaccinated rabbits. This protection was highly significant.

MATERIALS AND METHODS

Virus. The HSV-1 strain McKrae was used for all experiments. The virus was triple plaque purified and passaged twice in CV-1 cells to produce a virus stock with a titer of 1.2×10^8 PFU/ml. In rabbits, the McKrae strain of HSV-1 causes severe corneal infection and has a high spontaneous reactivation rate (see below).

Rabbits. New Zealand White female rabbits (Irish Farms) were used for all experiments. Rabbits were 8 to 10 weeks old at the start of the procedure. Rabbits were treated in accordance with Association for Research in Vision and Ophthalmology, American Association for Laboratory Animal Care, and National Institutes of Health guidelines.

Rabbit model of ocular HSV-1 infection, latency, and spontaneous reactivation. To establish a cohort of rabbits with spontaneous recurrent ocular HSV-1 infections, naive rabbits were bilaterally infected without scarification or anesthesia by placing 2×10^5 PFU (HSV-1 strain McKrae) into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye for 30 s (25). Although this dose of virus results in the death of approximately 30 to 50% of the rabbits, this is the lowest dose at which all eyes develop a primary infection that mimics HSV keratitis in humans (16–19, 30). More importantly, all trigeminal ganglia in the surviving rabbits harbor a latent HSV infection resulting in a high group rate of spontaneous reactivation. On any given day during the first 4 months of latency, approximately 5 to 10% of eyes have virus-positive tear film cultures (see below for results for mock-vaccinated rabbits). Acute ocular infection was confirmed by HSV-1-positive tear film cultures collected on days 3 to 4 postinfection. At 28 days postinfection, all surviving rabbits are assumed to harbor a bilateral HSV-1 latent infection of both trigeminal ganglia.

Vaccine. The vaccine contained recombinantly expressed derivatives of HSV-2 gD and gB (gD2 and gB2). The glycoproteins were prepared by expression of carboxyl-terminal truncated derivative genes in Chinese hamster ovary cells, using methods similar to those described for the HSV-1 homologs (28, 37). gB2 lacks only the last 5 residues of the extracellular domain, while gD2 lacks the last 11 residues. Since both derivative proteins lack the transmembrane domains, they are constitutively secreted into the medium. The proteins were purified to near homogeneity from conditioned medium by column chromatography. The adjuvant, microfluidized formulation 59 (MF59) with *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-*sn*-glycero-3-(hydroxyphosphoryloxy)] ethylamide (MTP-PE), was prepared as follows. MF59 emulsion contained 5% (vol/vol) squalene (E. Merck, Darmstadt, Germany), a natural metabolizable oil, and 0.5% (vol/vol) each of the surfactants Tween 80 (polyoxyethylene sorbitan monooleate; ICI America, Wilmington, Del.) and Span 85 (sorbitan trioleate; ICI America). The mixture was emulsified by mixing at high pressure (approximately 10,000 lb/in²), using a Microfluidics emulsifier (model 110Y; Microfluidics, Newton, Mass.). The resulting emulsion was sterile filtered and stored at 4°C. MTP-PE, obtained as a dry powder from Ciba-Geigy (Basel, Switzerland), was dissolved in the aqueous phase of the emulsion mixture prior to homogenization. The vaccine, containing MF59 with 50 µg of MTP-PE per dose (MF59-MTP-PE), was prepared just prior to immunization by mixing 1 volume of gB2+gD2 in 2× phosphate-buffered saline (PBS) with 1 volume of emulsion. The final volume per dose was 0.1 ml. Each dose contained 7.5 µg each of gB2 and gD2. Control vaccinations consisted of either 0.1 ml of PBS (experiment A, mock control) or 0.1 ml of MF59-MTP-PE without gB2 or gD2 (1 volume of MF59-MTP-PE mixed with 1 volume of 2× PBS) (experiment B, adjuvant control).

Vaccinations. Prior to inoculation, topical proparacaine was given for local anesthesia. The vaccine was delivered by subconjunctival inoculation in the lower cul-de-sac with a 30-gauge needle and a disposable insulin syringe. The subconjunctival route allows the use of adjuvant and ensures that the

vaccine is delivered and retained at the local site. Subconjunctival injection is routinely used in clinical ophthalmology and would be an acceptable route of vaccination for individuals with severe recurrent ocular HSV. All eyes received three single dose inoculations on days 28, 49, and 70 postinfection, corresponding to three vaccinations at 3-week intervals. Control vaccinations were given on the same schedule. Vaccination resulted in approximately 25% of the eyes showing temporary, mild to moderate conjunctival inflammation.

Ocular shedding. Beginning 3 weeks after the final vaccination (91 days postinfection), tear film specimens were collected daily from each eye as previously described (20), using a nylon-tipped swab (catalog A5005-1, lot 4427; Baxter). The swab was then placed in 0.5 ml of tissue culture medium and squeezed, and the inoculated medium was used to infect primary rabbit kidney cell monolayers. These cell monolayers were observed in a masked fashion by phase light microscopy for up to 30 days for HSV-1 cytopathic effects. All positive monolayers were blind passaged onto fresh cells to confirm the presence of virus. Because of the large number of tear film samples handled daily (52 in experiment A and 68 in experiment B), it was not feasible to assay samples for potential reductions in virus titer following vaccination. Therefore, our observations were limited to the presence or absence of reactivated virus.

Determination of titers. Serum neutralizing antibody titers were done by 90% plaque reduction assays as previously described (14). Serum ELISA titers were determined as described previously (14), using either expressed and purified gB2 or gD2 as the capture antigen.

Other analyses. Primary eye disease was determined on a scale of 0 to 4 (0 = no disease, 4 = involvement of the entire eye) by examining the rabbit eyes on days 1, 3, 5, 7, 10, and 14 postinfection for herpes simplex dendritic and geographic ulcers and for iritis by slit lamp biomicroscopy using 1% methylene blue (21, 22).

Corneal scarring was determined by slit lamp biomicroscopy three times per week (21, 22).

Statistical analyses were performed by using Instat, a personal computer software program. Analyses included the Student *t* test, Mann-Whitney rank sum test, chi-square test,

Fisher exact test, and linear regression. Results were considered statistically significant when the *P* value was <0.05.

RESULTS

Ocular vaccination of rabbits with preexisting HSV-1 latent infection. Two independent but similar therapeutic vaccine experiments, with similar results, are reported here. Experiment B (including an adjuvant control) was performed after analysis of the results of experiment A (which used a PBS control) indicated a therapeutic effect. More rabbits were used in experiment B (17 vaccinated rabbits and 17 control rabbits) than in experiment A (13 vaccinated rabbits and 10 control rabbits).

Experiments A and B used the same vaccine and the same vaccination regimen. As described in Materials and Methods, in both experiments rabbits were infected in both eyes with HSV-1 strain McKrae in order to establish cohorts with HSV-1 latent bilateral infections of the trigeminal ganglia. In experiment A, the latently infected rabbits were randomly divided into a control group (10 rabbits) and a vaccine group (13 rabbits). To control for possible bias introduced by severity of initial infection, in experiment B the latently infected rabbits were divided into control (17 rabbits) and vaccine (17 rabbits) groups based on the severity of eye disease observed during the acute phase of HSV-1 infection. Thus, in experiment B, the control and vaccine groups were known to contain rabbits with similar spectra of acute eye disease severity.

All vaccine group eyes were inoculated subconjunctivally with HSV-2 gB2 plus HSV-2 gD2 in the adjuvant MF59-MTP-PE (see Materials and Methods). In experiment A, control eyes were mock vaccinated with an equal volume of PBS. In experiment B, control eyes were mock vaccinated with an equal volume of MF59-MTP-PE adjuvant alone. Three vaccinations were given at 3-week intervals. Beginning 3 weeks after the third vaccination, tear films were collected daily from all eyes and individually plated on indicator cells to assay for the presence of reactivated (recurrent) virus.

TABLE 1. Spontaneous shedding in vaccinated latently infected rabbits

Expt	No. positive/ total ^a (%)	Fraction of positive cultures/eye ^b (no. of eyes)	Fraction of positive cultures/rabbit ^c (no. of rabbits)	No. of eyes that never shed/total (%) ^d	No. of rabbits that never shed/total (%) ^e	No. of recurrent episodes ^f
A						
Vaccine	30/1,146 (2.6)	0.027 (26)	0.027 (13)	17/26 (65)	7/13 (54)	18
Control (saline)	49/850 (5.8)	0.066 (20)	0.066 (10)	4/20 (20)	1/10 (10)	25
<i>P</i> , vaccine vs control	0.0006 ^g	0.006 ^h	0.008 ^h	0.003 ⁱ	0.04 ^j	0.02 ^h
B						
Vaccine	34/1,340 (2.5)	0.028 (34)	0.026 (17)	19/34 (56)	12/17 (71)	26
Control (MF59-MTP-PE)	101/1,461 (6.9)	0.074 (34)	0.077 (17)	7/34 (21)	0/17 (0)	53
<i>P</i> , vaccine vs control	<0.0001 ^g	0.003 ^h	0.001 ^h	0.006 ⁱ	0.04 ⁱ	0.007 ^h

^a Tears were collected daily from all eyes for 43 days (experiment A) or 40 days (experiment B) and cultured on indicator cells for the presence of reactivated HSV-1. The number of HSV-1 positive cultures/total number of cultures is shown for each group of rabbits.

^b Average for all eyes of the total HSV-1-positive cultures for eye *x*/total number of cultures for eye *x*.

^c Average for all rabbits of the total positive cultures for rabbit *x*/total cultures for rabbit *x*.

^d Number of eyes that never produced an HSV-1-positive tear film culture/total number of eyes.

^e The number of rabbits that never produced an HSV-1-positive tear film culture/total number of rabbits.

^f Total number of reactivations in each group, assuming that consecutive days of HSV-1-positive tear film cultures from a single eye are the result of a single reactivation event.

^g Chi square, two sided.

^h Mann-Whitney rank sum test, two sided.

ⁱ Fisher exact test, two sided.

^j Fisher exact test, one sided.

FIG. 1. Cumulative days of recurrent ocular virus shedding following vaccination. Rabbits bilaterally latently infected with HSV-1 were vaccinated subconjunctivally three times at 3-week intervals starting 28 days postinfection as described in Materials and Methods. The vaccine consisted of 7.5 μ g each of expressed and purified gB2 and gD2 in the adjuvant MF59-MTP-PE. In panel A, the control vaccine was PBS; in panel B, the control vaccine was the adjuvant alone. In panel A, there were 13 rabbits in the vaccinated group and 10 rabbits in the control group; in panel B, there were 17 rabbits in each group. Tear films were collected daily for 43 (A) or 40 (B) days beginning 3 weeks after the final vaccination. The tear films were plated on indicator cells and observed for up to 30 days for the presence of reactivated virus as described in Materials and Methods. The results are plotted as the cumulative number of virus-positive cultures divided by the total number of cultures divided by the number of eyes. This gives the cumulative number of the average positive tear film cultures per eye on each day. At the end of each experiment, the vaccinated eyes averaged approximately one positive culture each. The control eyes averaged approximately three positive cultures each. The total numbers of cultures and positive cultures are shown in Table 1.

Positive versus total tear film cultures. The cumulative numbers of positive cultures during 43 days (experiment A) or 40 days (experiment B) are shown in Fig. 1. Because of the different numbers of rabbits (and eyes) in the two experiments and between the control and vaccine groups in experiment A, the data were standardized to represent cumulative positive cultures per eye. In both experiments, the control groups had approximately three positive cultures per eye for the observation period. In contrast, the vaccinated rabbits had, on average, only about one positive culture per eye.

A statistical analysis of positive versus negative tear film cultures is shown in Table 1. In experiment A, 5.8% (49 of 853) of the control tear films contained recurrent virus. In contrast, only 2.6% (30 of 1146; 2.2-fold fewer) of the tear films from vaccinated eyes were positive for virus ($P = 0.0006$, chi-square analysis). Similar results were obtained in experiment B. Virus was detected in 2.5% (34 of 1340) of the vaccine tear film cultures, approximately 2.8-fold less than in the control cultures (101 of 1461; 6.9%) ($P < 0.0001$).

Additional analyses. The two analyses presented above are commonly used for the evaluation of these types of experiments. However, these approaches do not take into account the number of rabbits (or eyes). They therefore do not distinguish among (i) large numbers of eyes cultured over a short period of time; (ii) moderate numbers of eyes cultured over a moderate time; or (iii) small numbers of eyes cultured

over a long period of time. Therefore, we also analyzed the data by other approaches.

For each eye in each group, the fraction of positive cultures was determined (total positive cultures/total cultures for each eye) (Table 1). The resulting fractions (one for each eye) were then subjected to statistical analysis (Mann-Whitney nonparametric test). Because each eye is represented by the fraction of positive cultures, this analysis takes into account the number of eyes in each group. The difference between the control and vaccine groups in each experiment remained statistically significant (experiment A, $P = 0.006$; experiment B, $P = 0.003$).

Because of the possibility of a small correlation or relationship between the two eyes in a given rabbit, we also compared the fraction of positive cultures per rabbit rather than per eye (Table 1). Despite the potential loss of statistical power due to 50% fewer datum points, there was still a statistically significant difference between the vaccinated and control groups in both experiments (A, $P = 0.008$; B, $P = 0.001$). This result confirmed that the statistical significance of the reduced rate of spontaneous ocular shedding in the vaccinated latently infected rabbits was not an artifact of assuming that each eye of a single rabbit is an independent entity.

The numbers of virus-positive tear film cultures for each eye (Fig. 2A and B) and each rabbit (Fig. 2C and D) were determined. Each square in Fig. 2 represents an eye or a

FIG. 2. Virus-positive cultures from individual eyes or individual animals following vaccination of latently infected rabbits. The virus-positive tear film cultures (representing reactivated virus from the latently infected and vaccinated rabbits in Fig. 1) are shown graphically. Each square represents a single eye (A and B) or a single rabbit (C and D). The vertical location of each square indicates the total number of recurrent virus-positive cultures for that eye or rabbit. The mean of each group is indicated by an arrow. In all four panels, the vaccine groups showed significant protection against recurrent virus, as indicated by the *P* values (boxed) derived from Mann-Whitney rank sum tests. (A) Individual eyes from experiment A; (B) individual eyes from experiment B; (C) individual rabbits from experiment A; (D) individual rabbits from experiment B. Solid squares, vaccinated; open squares, mock vaccinated. MF59-50 is MF59 with 50 µg of MTP-PE.

rabbit, with the vertical location of each square indicating the number of positive cultures. The differences between the means of the vaccinated and mock-vaccinated eyes and rabbits were statistically significant in both experiments ($P < 0.01$, Mann-Whitney). Thus, the vaccine reduced the mean number of positive cultures regardless of whether the analysis was on a per-eye or per-rabbit basis.

Another important measure of the success of a vaccine is the fraction of subjects that remain asymptomatic, in this analysis equivalent to the fraction of eyes that never shed virus (Table 1). In experiment A, 65% of the vaccinated eyes never shed virus, compared with only 20% of the control eyes ($P = 0.003$, Fisher exact test). Similar results were seen in experiment B. In the vaccine group, 56% of the eyes never shed virus, compared with 21% of the control eyes. Again, more vaccinated eyes remained free of detectable virus ($P = 0.006$, Fisher exact test). The fraction of rabbits that never shed virus was also significantly enhanced by vaccination (Table 1). In experiment A, 1

of 10 control rabbits compared with 7 of 13 vaccinated rabbits were protected from detectable shedding in both eyes ($P = 0.038$). In experiment B, none of 17 control rabbits compared with 5 of 17 vaccinated rabbits never shed detectable virus ($P = 0.038$). Thus, in both experiments, vaccination increased the number of eyes and rabbits that were protected against virus shedding.

A final analysis was done to compare the number of recurrent episodes, i.e., the number of episodes in which reactivated virus was detected in the tears, with consecutive days of positive cultures being treated as a single event. Thus, an eye that sheds virus for a single day and an eye that sheds virus for 5 consecutive days would both constitute one episode of reactivation or recurrence (Table 1). In experiment A, the vaccine group had 18 recurrent episodes, compared with 25 in the control group ($P = 0.02$, Mann-Whitney rank sum test). In experiment B, there were 26 events in the vaccine group and 53 in the control group ($P = 0.007$). Thus, the vaccine reduced not

only the total number of days of shedding (see above) but also the number of recurrent episodes.

Neutralization and ELISA titers. In experiment A, serum HSV-1 neutralization titers were compared between the vaccinated and control groups. Three weeks after the final vaccination, serum was obtained from each rabbit, and plaque reduction (90%) neutralization titers were determined for each serum sample (Fig. 3A). The latently infected vaccinated rabbits had a geometric mean serum neutralization titer that was five times higher than that of the latently infected mock vaccinated rabbits (140.1 versus 27.9). This difference was significantly different ($P < 0.0001$, Student t test), indicating that vaccination of HSV-1 latently infected rabbits with the gB2+gD2+MF59-MTP-PE subunit vaccine was capable of producing a large increase in the HSV-1 neutralization titer.

In experiment B, ELISA titers were measured individually

against gB2 and gD2. Again, serum was obtained from each rabbit 3 weeks after the final vaccination. Each serum was analyzed for ELISA titer, using either gB2 or gD2 as the capture antigen. The vaccinated rabbits had a gB2 ELISA titer approximately eight times higher than that of the mock-vaccinated latently infected rabbits ($P < 0.0001$) (Fig. 3B). The increase in gD2 ELISA titer (60-fold) was even greater than that for gB2 (Fig. 3C) and was also statistically significant ($P < 0.0001$). As with the increase in neutralization titers, the ELISA titer increases indicated that the anti-HSV immune response in latently infected rabbits could be significantly enhanced by vaccination with the gB2+gD2+MF59-MTP-PE subunit vaccine.

Although vaccination of latently infected rabbits increased both neutralization titers and ELISA titers, it did not appear that these immune responses correlated with the vaccine's

FIG. 3. Increased neutralization and ELISA titers following vaccination of latently infected rabbits. The latently infected rabbits were bled 3 weeks after the final vaccination. (A) Individual neutralization titers from experiment A, as determined by a 90% plaque reduction assay; (B) individual gB2 ELISA titers from experiment B; (C) gD2 ELISA titers from experiment B. The *P* values comparing the vaccinated and mock-vaccinated titers were determined by the Student *t* test.

ophthalmoscopy revealed no changes in the vitreous humor, retina, or choroid. However, in experiment A, 5 of 26 (19%), and in experiment B, 7 of 34 (21%) of the eyes in the vaccine (plus adjuvant) group showed moderate temporary localized conjunctival inflammation. In contrast, only 2 of 34 eyes (6%) in the control (adjuvant only) group (experiment B) and none of the eyes in the saline control group (experiment A) showed moderate disease. In both experiments, statistical analysis comparing the amount of local reaction to adjuvant plus glycoproteins, versus adjuvant alone, approached significance ($P = 0.07$, $P = 0.06$), suggesting that the HSV glycoproteins rather than the adjuvant alone were responsible for the inflammation following vaccination.

Long-term observation revealed no statistically significant difference in corneal scarring in the vaccinated compared with the mock-vaccinated rabbits (experiment A, $P = 1.0$, Fisher exact test; experiment B, $P = 0.48$, chi-square test).

DISCUSSION

In these experiments, expressed and purified HSV-2 glycoproteins gD and gB were used as a vaccine against HSV-1. The HSV-2 glycoproteins were used because they had already been shown to be partially therapeutic against HSV-2 recurrences in nonocular systems. The HSV-2 and HSV-1 gD and gB glycoproteins are approximately 85% homologous and have a high degree of cross-reactivity. We felt that the advantages of using a vaccine preparation that had already proven successful (albeit in different systems) outweighed the potential disadvantages of using HSV-2 rather than HSV-1 glycoproteins as a therapeutic vaccine against HSV-1. Future studies are planned

therapeutic effect. Within each group, we were unable to detect significant correlation between neutralization titers and the number of positive tear film cultures (by linear regression; $r < 0.55$, $P > 0.05$). Likewise, within each group, we were unable to detect a correlation between ELISA titers and the number of positive tear film cultures ($r < 0.41$, $P > 0.05$).

Vaccine safety. There were no corneal changes associated with the periocular inoculations. In addition, slit lamp examination revealed no abnormalities in the lens, and indirect

to determine the therapeutic efficacy of HSV-1 glycoproteins against ocular reactivation.

We found that subconjunctival vaccination of latently infected rabbits with the gB2+gD2+MTP-PE subunit vaccine significantly reduced HSV-1 ocular shedding. Two separate experiments were done with similar results. The data were analyzed for (i) total virus shedding; (ii) the fraction of positive cultures per eye; (iii) the fraction of positive cultures per rabbit; (iv) the number of positive cultures per eye and per rabbit; (v) the number (fraction) of eyes that never shed; (vi) the number of rabbits that never shed from either eye; and (vii) the number of recurrent events (assuming that consecutive days of shedding represented a single event). In all cases, the vaccinated groups showed statistically significant protection. Thus, the vaccine was therapeutically effective regardless of the parameters analyzed and regardless of whether the analyses were on a per-eye or per-rabbit basis. This is the first report in any system of an effective therapeutic vaccine against ocular HSV-1 recurrences. In fact, this is the first report in any animal of an effective therapeutic vaccine against any ocular disease.

Both neutralization titers and gB2 and gD2 ELISA titers were significantly increased (5- to 60-fold) by vaccination. However, when we analyzed neutralization or ELISA titers versus reduced spontaneous reactivation in individual rabbits within each group, there was no correlation. Thus, even though as a group the vaccinated rabbits had significantly increased neutralization and ELISA titers, on an individual rabbit basis, these elevated titers did not appear to be related to reduced ocular shedding. It is therefore likely that this vaccination regimen protected latently infected rabbits against ocular recurrence via an immune response other than neutralizing antibody (or other systemic antibody responses). This is in agreement with preliminary results from human vaccine studies in which boosted serum neutralization or ELISA titers did not correlate with protection against recurrent HSV-2 genital infections (35a) and with our previous study in the guinea pig (2a).

In experiment A, the latently infected rabbits were randomly assigned to the vaccine and control groups. Although we have not previously noticed any correlation between severity of acute eye disease and spontaneous reactivation, in experiment B, the assignments were made prospectively on the basis of the severity of eye disease during the acute infection. This produced a vaccine group and a control group that were known to contain rabbits with similar levels of primary ocular disease (and therefore presumably infection). This type of assignment was done to try to minimize potential random differences between the two groups. After obtaining all of the experimental data, we looked for potential correlation between the severity of acute eye disease and the rate of spontaneous ocular shedding. No correlation was seen. This corroborates our previous unpublished observations with HSV-1 strain McKrae in rabbits, namely, that at a single infectious dose, the observed severity of acute eye disease does not appear to be predictive of subsequent reactivation rates.

As reported above, some of the rabbits developed moderate local inflammation reactions following periocular vaccination. Such a vaccine reaction might not be acceptable for prophylactic vaccine in unaffected humans. However, for those with a history of severe ocular HSV-1 recurrences (the population at which a therapeutic ocular vaccine would be aimed), the type of vaccine reaction described here may be acceptable. MTP-PE, which was expected to be a safe adjuvant for use in humans at the time these experiments were begun, is now not considered suitable for use in an HSV vaccine for humans because of side effects noted in clinical trials. Experiments are in progress

to identify an effective and better-tolerated adjuvant, since our ultimate goal is to develop an effective vaccine without significant side effects.

The results reported here are also important from a safety standpoint. HSV-1-induced corneal scarring is thought to be the result of an immune response to the virus. For this reason, corneal scarring is more prevalent during ocular recurrences when the immune response has already been primed (and boosted) than during primary ocular infection. Thus, it is possible that a vaccine directed at genital HSV-2 and given periodically could enhance the harmful ocular immune response to recurrences of HSV-1. We therefore monitored corneal scarring during the time shedding was studied. No statistically significant differences were seen. Because of (i) the apparent safety and efficacy of this therapeutic vaccine in the rabbit, (ii) the similarity between ocular HSV in rabbits and humans, and (iii) the current success in HSV-2 genital vaccine studies in animals and humans, we are encouraged that a successful human ocular therapeutic vaccine may be possible.

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