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Therapeutic Periocular Vaccination with a Subunit Vaccine Induces Higher Levels of Herpes Simplex Virus-Specific Tear Secretory Immunoglobulin A Than Systemic Vaccination and Provides Protection against Recurrent Spontaneous Ocular Shedding of Virus in Latently Infected Rabbits

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Rabbits latently infected with herpes simplex virus type 1 (HSV-1) were vaccinated either periocularly or systemically with a subunit vaccine (gB2 + gD2) plus adjuvant or adjuvant alone. Tear films were collected daily to measure recurrent infectious HSV-1 shedding. After systemic vaccination, the latently infected rabbits were not protected against recurrent ocular viral shedding (HSV-1-positive tear film cultures/total cultures) compared with either the systemic or periocular adjuvant controls (systemic vaccination = 49 of 972, 5.0%; systemic control = 46 of 972, 4.7%; periocular control = 43 of 930, 4.6%; $P > 0.8$). In contrast, latently infected rabbits vaccinated periocularly with the same vaccine had significantly reduced recurrent shedding (20 of 1026, 2.0%) compared with controls ($P < 0.001$) or systemic vaccination ($P = 0.0002$). Thus, recurrent HSV-1 shedding was significantly reduced by therapeutic local periocular subunit vaccination but not by therapeutic systemic subunit vaccination. Neutralizing antibody titers in the serum of systemically and ocularly vaccinated rabbits was similar. In contrast, HSV-specific tear secretory immunoglobulin A was significantly higher in the ocularly vaccinated group ($P < 0.01$). These results strongly suggest that in the rabbit, and presumably in humans, the local ocular (mucosal) immune response is much more important than the systemic immune response for therapeutic protection against recurrent ocular HSV-1 Thus development of a therapeutic vaccine against recurrent ocular HSV-1 should be directed at enhancing the local ocular (mucosal) immune response.

Key Words: corneal disease; HSV-1; latency; recurrent disease; vaccine.

INTRODUCTION

Seventy to 90% of adults in the United States harbor latent herpes simplex virus (HSV) infections. In addition to producing genital and oral infections, HSV can infect the eye. Approximately 90% of ocular HSV in humans is due to HSV type 1 (HSV-1). After initial ocular infection, HSV-1 establishes life-long latency in neurons of the trigeminal ganglia. Spontaneous reactivations, in which infectious virus returns to the eye and can be detected in tear films, occurs sporadically throughout life, with or without recurrent eye disease (herpetic keratitis). Once HSV has recurred in the eye, corneal disease, stromal scarring, and blindness can follow as the result of an incompletely defined immune response to the virus (Newell *et al.,* 1989; Rouse *et al.,* 1988). In the United States, recurrent ocular HSV-1 infection is the leading cause of corneal blindness due to an infectious agent (Nesburn, 1974, 1983; Smith *et al.,* 1980).

HSV-1 is usually acquired as a childhood infection. Thus widespread prevention of primary ocular herpes by prophylactic vaccination (i.e., a vaccine given to naive individuals to prevent primary infection) would require universal vaccination of infants and children and would not help those with existing latent infection. In contrast, a therapeutic vaccine (i.e., a vaccine given to latently infected individuals to decrease or prevent recurrent infections) could be targeted at individuals who have experienced primary ocular HSV-1 infection or who have a history of HSV-1 ocular recurrences. In addition, elimination of recurrent ocular HSV-1 by eliminating primary ocular HSV-1 via prophylactic vaccination would require many decades because of the large pool of latently infected individuals.

The development of therapeutic vaccines against viruses that normally produce multiple recurrent infections is complicated. With these viruses, the immune response to primary and recurrent infections is not sufficient to eliminate subsequent recurrent infection. Thus a vaccine that simply mimics the natural immune response is unlikely to provide significant protection against recurrent infection. A successful therapeutic HSV-1 vaccine may therefore have to elicit an immune response that is either much stronger than or different from that after natural infection.

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0042-6822/98 $25.00

TABLE 1

Vaccines

Vaccine[a]	Route	Inocula	No. of rabbits
Ocular gB2 + gD2 (periocular vaccine)	Subconjunctival[b]	7.5 μg of gB2,[d] 7.5 μg of gD2,[d] MF59 + MTP-PE	19
Systemic gB2 + gD2 (systemic vaccine)	Intramuscular[c]	25 μg gB2,[e] 25 μg gD2,[e] MF59 + MTP-PE	18
Ocular control (periocular mock vaccine)	Subconjunctival[b]	PBS, MF59 + MTP-PE	17–18[f]
Systemic control (systemic mock vaccine)	Intramuscular[c]	PBS, MF59 + MTP-PE	18

[a] All vaccines were administered three times at 3-week intervals. The first vaccination was given 3 weeks after initial ocular infection, at which time all rabbits harbored bilateral latent infections of the trigeminal ganglia.

[b] 0.1 ml per eye.

[c] 0.1 ml per rabbit.

[d] Per eye, 15 μg of each protein per rabbit.

[e] Per rabbit.

[f] Study began with 18 rabbits. One rabbit died after tear collection on day 5.

Vaccination before infection (i.e., prophylactic vaccination) can protect mice and rabbits against primary HSV-1-induced corneal disease (Foster *et al.,* 1988; Ghiasi *et al.,* 1995, 1996; Heiligenhaus *et al.,* 1994, 1995; Inoue *et al.,* 1990; Irie *et al.,* 1993; Keadle *et al.,* 1997; Morrison and Knipe, 1996; Nesburn *et al.,* 1990). In mice, prophylactic vaccination can decrease the rate of induced *in vivo* reactivation (Keadle *et al.,* 1997). Prophylactic vaccination of mice and rabbits can also decrease the rate of induced *in vitro* reactivation (Ghiasi *et al.,* 1994, 1995, 1996; Nesburn *et al.,* 1990). The decreased reactivation rates were almost certainly a result of the prophylactic vaccines providing some protection against primary ocular challenge, thereby resulting in a decrease in the amount of latency that was established (Ghiasi *et al.,* 1994, 1995, 1996; Nesburn *et al.,* 1990). To obtain a therapeutic vaccine effect, the vaccination must be given to already infected individuals, not to naive individuals.

Using a rabbit model, we previously demonstrated a vaccine and adjuvant combination that produces a therapeutic reduction of spontaneous recurrent ocular HSV-1 shedding (Nesburn *et al.,* 1994). Latently infected rabbits were vaccinated via a local periocular route using highly purified gD2 + gB2 as antigen and MF59 (microfluidized formulation #59 emulsion)–MTP-PE (*N*-acetyl-muramyl-l-alanyl-d-isoglutaminyl-l-alanine-2–1,2-dipalmitoyl-*sn*-glycero-3-(hydroxyphosoryloxy)ethylamide) as adjuvant. This therapeutic vaccination resulted in a 2- to 3-fold decrease in spontaneously reactivated HSV-1 in tear films. We also recently showed that it is even easier to protect against recurrent herpetic corneal disease than against recurrent virus shedding (Nesburn *et al.,* 1998a). Vaccine efficacy lasted longer against herpetic keratitis than against recurrent spontaneous shedding. In addition, homotypic vaccination was much more efficacious than heterotypic vaccination against recurrent HSV-1 shedding, whereas heterotypic and homotypic vaccinations were equally efficacious against recurrent herpetic keratitis. Because the ability of a therapeutic vaccine to reduce recurrent shedding appears to be a more stringent measure of vaccine efficacy than the ability to decrease corneal disease and because measuring recurrent shedding is much more objective than measuring corneal disease, in this report we used decreased recurrent viral shedding as the end point for therapeutic vaccine efficacy against ocular herpes recurrence.

We performed a number of studies in which rabbits latently infected with HSV-1 were vaccinated with a variety of vaccine preparations to look for therapeutic vaccine efficacy against recurrent ocular shedding, recurrent corneal disease, or both. In some studies, candidate therapeutic vaccines were delivered systemically (intramuscularly or subcutaneously). In other studies, candidate therapeutic vaccines were delivered periocularly (subconjunctival injection or topical application to the cornea). Some, but not all, of the vaccines delivered periocularly were efficacious against spontaneous shedding (Nesburn *et al.,* 1994) and recurrent corneal disease (Nesburn *et al.,* 1998a). In contrast, none of the vaccines delivered systemically provided significant reductions in recurrent shedding or disease (unpublished results). Although none of the systemic vaccination studies were done with vaccines that provided protection when delivered periocularly, these findings nevertheless led to the hypothesis that periocular therapeutic vaccine delivery may be more efficacious against recurrent HSV-1 than systemic delivery. Testing this theory is the focus of this report.

We report here that in cohorts of rabbits latently infected with HSV-1 and vaccinated either periocularly or systemically with the same vaccine preparation, the periocular vaccine had a vaccine efficacy during the 27-day

monitoring period of 57% (as measured by decreased spontaneous ocular shedding of HSV-1), whereas the systemic vaccine showed no vaccine efficacy.

RESULTS

Ocular vaccination of rabbits with preexisting HSV-1 latent infection

To establish cohorts of rabbits with HSV-1 bilateral latent infections of the trigeminal ganglia, rabbits were infected in both eyes with HSV-1 strain McKrae as described under Materials and Methods. Twenty-eight days p.i., the surviving latently infected rabbits were divided into four groups. As described under Materials and Methods and Table 1, the rabbits in the periocular vaccine group were vaccinated subconjunctivally with gB2 + gD2/MF59–MTP-PE, whereas the rabbits in the periocular mock vaccine group were vaccinated with the adjuvant MF59–MTP-PE alone. The systemic vaccine and the systemic mock vaccine groups were vaccinated intramuscularly with the same vaccines. Three vaccinations were given at 3-week intervals.

Recurrent viral shedding in tears during the initial 27-day monitoring period

Beginning 3 weeks after the final vaccination, tear films were collected daily from all eyes and individually plated on indicator cells to assay for the presence of reactivated (recurrent) virus. The cumulative number of positive cultures during the 27 days of collection is shown in Fig. 1. The data were standardized to represent cumulative positive cultures per eye. The periocular mock-vaccinated control group, the systemic mock-vaccinated control group, and the systemic vaccine group each had an average of 1.26–1.36 positive cultures per eye for the observation period. In contrast, the periocular gB2 + gD2/MF59–MTP-PE-vaccinated rabbits had, on average, only 0.53-positive cultures per eye.

A statistical analysis of positive versus total tear film cultures is shown in Table 2. During the 27-day monitoring period, only 2.0% of the tear films from rabbits vaccinated periocularly with gB2 + gD2 plus adjuvant were positive for virus compared with 4.6% of the tear films from rabbits mock-vaccinated periocularly. This difference was significant (Table 2, column 3; $P = 0.0008$, χ^2). In contrast, 5.0% of the tear films from rabbits vaccinated systemically with the same gB2 + gD2 plus adjuvant vaccine contained recurrent virus, which was similar to the 4.7% of positive tear films in the systemic mock-vaccinated group ($P = 0.83$). Thus compared with its control, periocular vaccination provided protection against recurrent ocular shedding, whereas compared with its control, systemic vaccination did not. A direct comparison of periocular gB2 + gD2 plus adjuvant vaccination with systemic gB2 + gD2 plus adjuvant vaccination indicated that the periocular vaccination was much more efficacious at reducing spontaneous recurrent shedding ($P = 0.0002$). The vaccine efficacy of the periocular gB2 + gD2 plus adjuvant vaccine calculated as described under Materials and Methods was 57% compared with no vaccine efficacy for the same vaccine delivered systemically (Table 2, column 5).

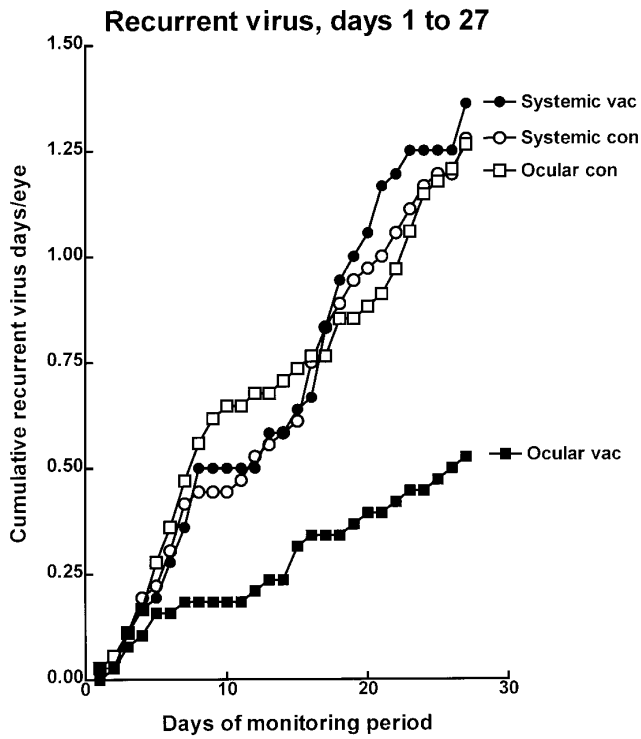

FIG. 1 Effect of therapeutic local vaccination versus therapeutic systemic vaccination on HSV-1 spontaneous ocular shedding over a 27-day monitoring period. Rabbits were ocularly infected with HSV-1 McKrae as described under Materials and Methods. Once latency had been established, groups of rabbits were vaccinated either periocularly (subconjunctivally) or systemically (intramuscularly on the back) with gB2 + gD2/MF59–MTP-PE or mock vaccinated with the adjuvant alone (control) as described under Materials and Methods. Identical vaccinations were given 21 and 42 days later for a total of three vaccinations. Beginning 21 days after the final vaccination, tear films were collected daily from all eyes as described under Materials and Methods. The cumulative number of HSV-1-positive cultures for each group, divided by the number of eyes in that group, are shown. Ocular vac indicates periocular subconjunctival vaccination; systemic vac, systemic intramuscular vaccination; ocular con, control periocular subconjunctival vaccination; systemic con, control systemic intramuscular vaccination.

The above analyses are commonly used for the evaluation of these types of experiments. However, these approaches do not take into account the number of eyes in each group and therefore do not distinguish among (1) large numbers of eyes observed over a short period of time, (2) moderate numbers of eyes over a moderate time, or (3) small numbers of eyes over a long period of time. Therefore, we also analyzed the data by another approach. For each eye in each group, the fraction of virus-positive days was determined (total virus-positive

TABLE 2

Recurrent Ocular HSV-1 Shedding Days 1–27

	Positive/total eye cultures[a]	*P* (less stringent)[b]	*P* (more stringent)[c]	Vaccine efficacy[d]
Ocular gB + gD	20/1026 (2.0%)			57%
Systemic gB + gD	49/972 (5.0%)			0%
Ocular adj	43/930 (4.6%)			
Systemic adj	46/972 (4.7%)			
Ocular gB + gD vs ocular control		0.0008	0.06	
Systemic gB + gD vs systemic control		0.83	0.31	
Ocular gB + gD vs systemic gB + gD		0.0002	0.004	
Ocular gB + gD vs systemic control			0.14	
Systemic gB + gD vs ocular control			0.48	

[a] Tears were collected daily from all eyes for 27 days 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] Chi-squared test (two-sided) based on total number of positive and negative cultures in each group.

[c] The fraction of time each eye was positive for recurrent HSV-1 (number of positive tear film cultures/total number of cultures) was determined, producing a fraction for each eye. The sets of fractions were compared using the Mann-Whitney rank sum test (two-sided).

[d] Vaccine efficacy was determined as described under Materials and Methods.

tear film cultures/total tear film cultures for each individual eye). The resulting fractions (one for each eye) then were subjected to statistical analysis (Mann–Whitney rank sum tests). Because each eye is now represented by the fraction of virus-positive days, this analysis takes into account the number of eyes in each group. By this more stringent analysis, periocular vaccination with gB2 + gD2 was also more efficacious in decreasing recurrent shedding than systemic vaccination with the same material (Table 2, column 3; $P = 0.004$). The decrease in recurrent shedding for the group vaccinated periocularly with gB2 + gD2 approached significance compared with the mock-vaccinated periocular control using a two-sided analysis ($P = 0.06$; Table 2; column 4) and reached significance using a one-sided analysis ($P = 0.03$, not shown). Finally, systemic vaccination did not decrease recurrent shedding compared with its control ($P = 0.31$). Thus during the 27-day monitoring period, periocular vaccination with gB2 + gD2 was efficacious compared with its control and compared with systemic vaccination with the same vaccine, whereas systemic vaccination was not efficacious compared with its control.

Recurrent viral shedding in tears during an extended 47-day monitoring period

To examine longer-term therapeutic protection against recurrent ocular shedding, the monitoring period for the periocular gB2 + gD2 vaccine group and its periocular mock vaccine control was extended to a total of 47 days (Fig. 2). During this longer period, the periocular gB2 + gD2 vaccine group had an average of 13 positive tear

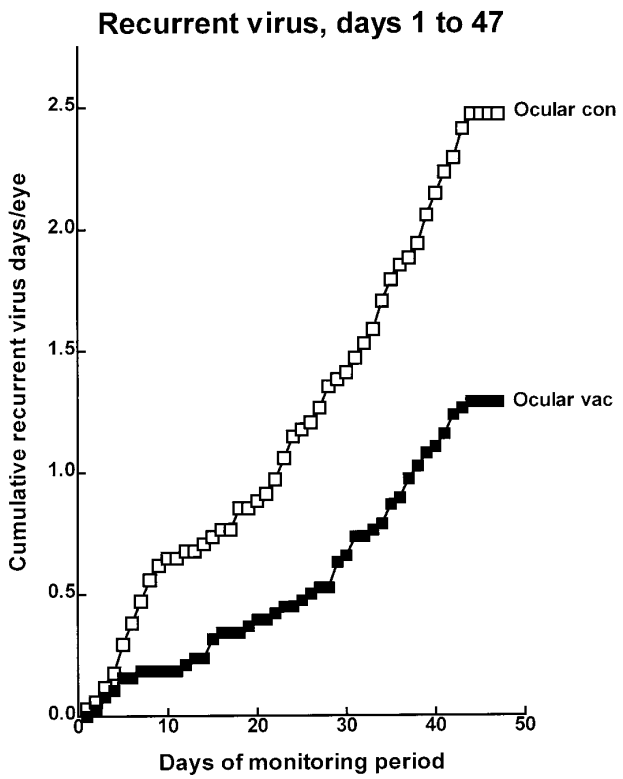

FIG. 2. Effect of vaccination on recurrent HSV-1 shedding over an extended 47-day monitoring period. Tears were collected daily from the ocular vaccinated and control groups for an additional 20 days and assayed, and the results are plotted as described in the legend to Fig. 1

TABLE 3

Recurrent Ocular HSV-1 Shedding Days 1–47

	Positive/total eye cultures[a]	*P* (less stringent)	*P* (more stringent)	Vaccine efficacy
Ocular gB + gD	49/1786 (2.7%)	0.0002	0.02	48%
Ocular control	84/1610 (5.2%)			

[a] Tears were collected daily from all eyes for 47 days and cultured on indicator cells for the presence of reactivated HSV-1. Analyses are as described for Table 1.

film cultures per eye compared with an average in the periocular mock vaccine control group of almost twice this number or ~2.5-positive cultures per eye. Furthermore, 5.2% of the tear films contained spontaneously reactivated virus in the control group compared with only 2.7% in the gB2 + gD2 vaccine group (Table 3). This difference was significant even by the more stringent statistical analysis (Table 3; column 4). The vaccine efficacy over the entire 47-day monitoring period was 48% (Table 3). This was slightly lower than the 57% vaccine efficacy during the first 27 days, suggesting that there was a small decrease in therapeutic vaccine efficacy between days 28 and 47 of the monitoring period corresponding to days 49 to 68 after the final vaccination. This was confirmed by analysis of just the last 20 days of the 47-day extended monitoring period (days 28–47) rather than the entire 47-day period. The reduction in the percent of tear films that contained spontaneously reactivated virus in the gB2 + gD2 periocular vaccine group (3.8%) compared with the mock vaccine group (6.0%) approached but did not reach significance, even using the less stringent analysis ($P = 0.06$, not shown).

Vaccination-induced neutralization titers

Sera were obtained from five rabbits in each vaccination group 3 weeks after the initial infection (just before the first vaccination) or 3 weeks after the final vaccination. Anti-HSV-1 serum neutralization titers were determined on individual sera as described under Materials and Methods. Before vaccination, four of five sera in each group had neutralization titers of <50 (the base level of the assay) (Table 4; p.i.–prevaccination). After the final vaccination, the systemic gB2 + gD2 vaccine group had a mean neutralization titer of 291 ± 52 and the periocular gB2 + gD2 vaccine group had a mean titer of 243 ± 28 (Table 4; postvaccination). These postvaccination titers were significantly higher than the prevaccination titers ($P < 0.008$, Mann–Whitney test). Because in previous studies we showed that mock vaccination with the same adjuvant used here does not alter serum neutralization titers (Nesburn *et al.,* 1994, 1998a), these results indicated that the rabbits developed an immune response after both systemic and periocular vaccinations. There was no significant difference between the

TABLE 4

Effect of Vaccination on Serum HSV-1 Neutralizing Antibody Titers

	Neutralizing antibody titers[a]			
	Ocular vaccination		Systemic vaccination	
	Postinfection (prevaccination)	Postvaccination	Postinfection (prevaccination)	Postvaccination
	<50	222	<50	400
	<50	163	<50	250
	<50	300	<50	375
	<50	314	<50	111
	64	217	80	320
Mean ± SEM[b]	53 ± 3	243 ± 28	56 ± 6	291 ± 52
P, postinfection vs postvaccination[b]		0.008		0.008
P, ocular vs systemic postvaccination[c]				0.31

[a] Sera were collected 3 weeks p.i. just before the first vaccination (postinfection-prevaccination) or 3 weeks after the final vaccination (postvaccination), corresponding to 12 weeks p.i. Serum neutralization titers were determined on individual sera from five randomly selected rabbits/group p.i. and five randomly selected rabbits/group postvaccination as described under Materials and Methods.

[b] For statistical analysis, 50 was substituted for <50, Mann-Whitney test.

[c] Mann-Whitney test.

postvaccination HSV-1 neutralization titers of the systemic and periocular vaccination groups ($P = 0.31$). Thus the higher vaccine efficacy of the periocular vaccination was not due to induction of a higher serum neutralization titer.

Vaccination induced anti-HSV-1 tear secretory immunoglobulin A

Tears were collected 3 weeks after the final therapeutic vaccination. Anti-HSV-1 secretory immunoglobulin A (sIgA) in individual tear samples was evaluated as described under Materials and Methods using purified gD2 as the capture antigen (Fig. 3A). The average anti-gD tear sIgA titer was significantly greater in the ocular vaccine group than in the systemic vaccine group ($P < 0.01$). Both the ocular and systemic vaccine groups had higher gD-specific tear sIgA than the mock vaccine groups ($P < 0.01$). Similar results were obtained using gB2 as the capture antigen (Fig. 3B), but the difference between the ocular and systemic vaccines did not reach statistical significance ($P = 0.14$, Student's *t* test). Taken together, these results indicate a correlation between therapeutic subunit vaccine efficacy against HSV-1 ocular recurrence and induction of anti-HSV tear sIgA.

DISCUSSION

We previously reported that subunit therapeutic local periocular vaccination of rabbits harboring bilateral latent infections in both trigeminal ganglia was able to reduce recurrent HSV-1 ocular shedding (Nesburn *et al.*, 1994, 1998a). However, the requirement for periocular delivery compared with systemic delivery to achieve this therapeutic efficacy had not been determined. Prophylactic vaccine efficacy against primary ocular challenge with HSV-1 has been obtained in the rabbit by systemic vaccination (a prophylactic vaccine in contrast to the therapeutic vaccine used in this study) (Nesburn *et al.*, 1990). Recently, however, we demonstrated that periocular vaccination provided more efficacious protection against primary ocular HSV-1 challenge in the rabbit than does systemic vaccination (Nesburn *et al.*, 1998b). Based on this finding, it appeared likely that periocular vaccination also would be more efficacious for therapeutic protection. However, because the immune responses involved in prophylactic protection may differ from those involved in therapeutic protection, this assumption required experimental confirmation as reported here. The adjuvant MTP-PE, used in the above studies and in the studies reported here, was selected because at the time these studies started, it appeared that MTP-PE was likely to be approved for human use. Unfortunately, unacceptable local side effects occurred with MTP-PE in human studies, and it is no longer considered to be a candidate for human use.

In the studies reported here, therapeutic local periocular vaccination with gB2 + gD2/MF59–MTP-PE resulted in a vaccine efficacy against spontaneous recurrent ocular virus shedding of 57% during a 27-day monitoring period. In contrast, systemic vaccination with the same vaccine resulted in no measurable vaccine efficacy. The protection afforded by periocular delivery is even more impressive because to strengthen the likelihood that any increased protection observed with ocular vaccination would be meaningful, the vaccinations were biased toward systemic vaccinations. Thus a lower dose of gB2 +

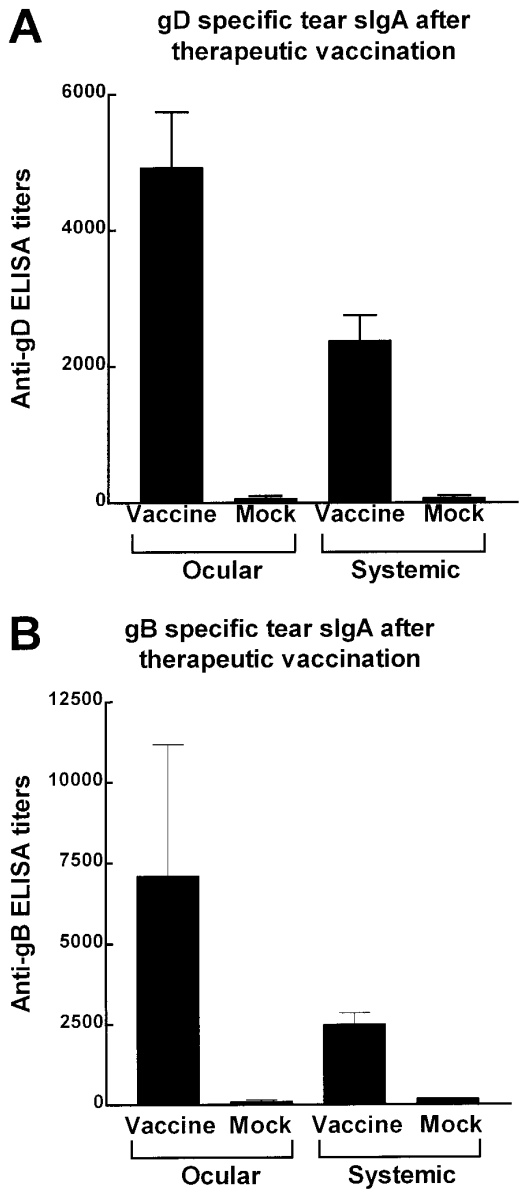

FIG. 3. Effect of therapeutic vaccination on HSV-specific tear sIgA. Tears were collected 3 weeks after the final vaccination and were individually assayed for HSV-specific sIgA by ELISA using gD2 (A) or gB2 (B) as the capture antigen as described under Materials and Methods. (A) Ocular vaccine: average of sIgA titers from 12 eyes; systemic vaccine: 12 eyes; ocular mock: 4 eyes; systemic mock: 2 eyes. (B) Ocular vaccine: 6 eyes; systemic vaccine: 11 eyes; ocular mock: 2 eyes; systemic mock: 1 eye.

gD2 was used periocularly (7.5 μg/eye of each glycoprotein versus 25 μg of each glycoprotein given systemically). Because subconjunctival (periocular) vaccination should induce much stronger mucosal immune responses at the eye than systemic vaccination, one explanation for the effectiveness of therapeutic periocular vaccination compared with systemic therapeutic vaccination is that induction of mucosal immune responses was required for therapeutic vaccine efficacy against ocular HSV-1 recurrences in this system.

Interestingly, although greater vaccine efficacy against recurrent shedding was achieved via the ocular route than by the systemic route, the average serum neutralization titers induced by the two different vaccine routes were similar. Thus therapeutic vaccine efficacy did not correlate with the induction of increased serum neutralizing antibody by the efficacious vaccine. In this study, using a subunit vaccine, the ability of vaccination to induce serum neutralization titers therefore was not predictive of vaccine efficacy against recurrent ocular shedding of spontaneously reactivated virus. In contrast to the serum neutralizing antibody titers, therapeutic protection against recurrent HSV-1 ocular shedding correlated with HSV-specific tear sIgA when groups were examined as a whole. However, there did not appear to be a strong correlation between sIgA and protection for specific eyes or rabbits (analysis not shown). Nevertheless, these results support the notion that for therapeutic efficacy against recurrent HSV-1 in the eye, local mucosal immunity is more important than systemic humoral immunity.

Mucosal immunity (or secretory immunity) is the first line of defense for protecting mucosal surfaces, including the eye, against microbial invasion. The major protective action of mucosal immunity is mediated by sIgA (Sullivan, 1994), which is the main immunoglobulin in tears. sIgA can inhibit the attachment and internalization of viruses (Malaty *et al.,* 1988) and therefore is likely to be important in protection against ocular HSV-1 infection. The lacrimal gland is the main source of tears and tear sIgA. The conjunctiva contains T, B, and plasma cells expressing IgA and may also be involved in ocular mucosal immunity (Sullivan, 1994).

Mucosal immunity is distinct from systemic immunity. Vaccination at mucosal sites often induces systemic, as well as mucosal, immune responses. In contrast, systemic vaccination rarely induces significant mucosal immunity (Sullivan, 1994). Thus for a vaccine to induce significant mucosal immune responses, it usually has to be delivered mucosally. In human studies of a prophylactic HSV vaccine, systemic vaccination with gB2 + gD2-induced high serum neutralization titers in HSV-seropositive persons (6- to 13-fold higher than natural infection after vaccination of latently infected individuals) (Straus *et al.,* 1993, 1994), yet it produced no significant vaccine efficacy against primary or recurrent genital herpes (R. L. Burke, personal communication). More recently, a similar vaccine also induced high serum neutralization titers without decreasing the rate of genital herpes recurrences during a 1-year period (Straus *et al.,* 1997). These studies may suggest the importance of local/mucosal immunity rather than systemic immunity in protecting against recurrent herpes simplex virus.

The results seen here in the rabbit ocular model of HSV-1 spontaneous reactivation differ from that recently reported in the mouse ocular model of UV-induced reactivation (Walker *et al.,* 1998). We showed here that in rabbits, therapeutic ocular vaccination, but not therapeutic systemic vaccination, significantly reduced the amount of spontaneous recurrent shedding, strongly suggesting that vaccine efficacy against recurrent ocular shedding required induction of local/mucosal immunity. In contrast, in mice, therapeutic intraperitoneal vaccination was able to decrease induced recurrent ocular shedding. These difference could be due to the use of a subunit vaccine in the rabbit compared with an attenuated live virus vaccine in the mouse or the use of spontaneous reactivation in the rabbit compared with induced reactivation in the mouse. More likely, however, these results suggest either less ocular mucosal surface immune privilege in the mouse or that in the mouse therapeutic ocular protection can be achieved by immune responses that do not protect rabbit eyes. In addition, in mice, therapeutic ocular vaccine efficacy correlated well with induction of high serum neutralizing antibody titers (Walker *et al.,* 1998). In contrast, in the current study, serum neutralizing antibody titers did not correlate with therapeutic ocular vaccine efficacy in the rabbit. In humans, serum neutralizing antibody titers also do not correlate with protection against recurrent ocular HSV-1 because individuals with continuing recurrent disease can develop extremely high serum neutralizing antibody titers while remaining susceptible to continuing recurrences.

The ability of the mouse and the inability of the rabbit to be protected against recurrent ocular HSV-1 by systemic immunity have also been seen for vaccine protection against primary ocular infection. In the mouse, systemic immunization (Ghiasi *et al.,* 1992, 1995) and even intraperitoneal injection of neutralizing antibody (Walker *et al.,* 1998) can protect against ocular HSV-1 challenge. In contrast, we recently showed that in rabbits, periocular vaccination with either subunit or live virus vaccines, was much more efficacious than systemic vaccination in protecting against ocular HSV-1 challenge (Nesburn *et al.,* 1998b).

We theorize that the ability of serum antibody to protect the mouse, but not the rabbit or human eye, may be due to eye size. In rabbits and humans, capillaries are only seen in the outer 1 mm of the cornea, effectively isolating the central cornea from circulating immune factors. In the mouse, capillaries are also confined to the outer 1 mm of the cornea. However, because of the small

size of the mouse cornea compared with rabbits and humans, it is likely that circulating immune factors can rapidly diffuse from these peripheral capillaries into the central cornea, thus allowing serum antibody to protect the mouse cornea. The ability of nonmucosal immunity (i.e., serum antibody) to protect the mouse eye may complicate deciphering the role of mucosal immunity in protection against recurrent ocular HSV-1, thereby resulting in an underestimation of the importance of vaccine-induced local/mucosal immunity in humans. Thus although rabbit immunology still lags behind that of the mouse, the rabbit is an important model for studying vaccine efficacy against ocular HSV-1 as it may apply to humans.

Whether therapeutic efficacy in the rabbit ocular model can also be achieved with a live virus vaccine and, if so, whether as seen here for a subunit vaccine, an ocular vaccine route would be more efficacious than a systemic vaccine route remains to be determined. Regardless, the results reported here suggest that therapeutic vaccine efficacy in the rabbit model of ocular HSV-1 spontaneous reactivation was due to mucosal rather than systemic immunity because (1) therapeutic vaccine efficacy was achieved with periocular (subconjunctival) vaccination, but not by systemic vaccination, even with a larger dose of the same vaccine; and (2) therapeutic efficacy correlated with induction of HSV-specific tear sIgA but not with induction of serum HSV-1 neutralizing antibody titers. Studies are planned to determine whether other mucosal routes of vaccination, such as intranasal or enteric, may prove equally efficacious as ocular vaccination and to determine whether other local ocular mucosal immune responses in addition to sIgA correlate with therapeutic vaccine efficacy.

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 2×10^8 pfu/ml.

Rabbits

New Zealand White male rabbits (Irish Farms) were 8–10 weeks old at the start of the procedure. Rabbits were treated in accordance with Association for Research in Vision and Ophthalmology (ARVO), American Association for Laboratory Animal Care (AALAC), and National Institutes of Health (NIH) 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) in a total volume of 50 μl into the conjunctival cul-de-sac, closing the eye, and rubbing the lid gently against the eye. As we have previously described, by day 21 p.i., all trigeminal ganglia in the surviving rabbits harbor a latent HSV-1 infection, resulting in a high group rate of spontaneous reactivation (~5–10% of tear film cultures are positive for HSV-1 on any given day) (Nesburn *et al.,* 1994; Perng *et al.,* 1994, 1996a, 1996b). Acute ocular infection was confirmed by HSV-1-positive tear film cultures collected on days 3–4 p.i. To control for a possible bias introduced by the severity of the initial infection, the latently infected rabbits were divided into four similar groups based on the severity of corneal disease observed during the acute phase of HSV-1 infection. Thus each group contained rabbits with a similar spectrum of acute eye disease severity. The resulting groups then were randomly assigned to receive one of the four vaccine treatments: (1) periocular gB2 + gD2 plus adjuvant, (2) systemic gB2 + gD2 plus adjuvant, (3) periocular adjuvant alone, or (4) systemic adjuvant alone.

Glycoproteins for vaccination

HSV-2 glycoproteins D (gD2) and B (gB2) were prepared by expression of the carboxyl-terminal truncated gene in Chinese hamster ovary cells followed by purification to near-homogeneity using a series of traditional chromatographic steps as previously described and as previously used by Chiron Corporation in clinical trials measuring protection from HSV-2 infection (Langenberg *et al.,* 1995). gD2 is a carboxyl-terminal truncation composed of amino acids 1–294 with three additional non-gD2 amino acids, Leu-Thr-Asn, at the carboxyl-terminus. The entire extracellular domain composes amino acids 1–313. gB2 is a transmembrane deletion composed of amino acids 1–702 (the entire extracellular domain), fused at the carboxyl-terminus to amino acids 777–882 (the entire cytoplasmic domain). Both HSV-2 genes were obtained from strain 333.

Adjuvant and vaccine preparation

The adjuvant MF59 with MTP-PE was prepared as follows. MF59 contained 5% (v/v) squalene (E. Merck, Germany) a natural metabolizable oil, and 0.5% (v/v) each of the surfactants Tween 80 (polyoxyethylene sorbitan mono-oleate) and Span 85 (sorbitan trioleate) (ICI America, DE). The mixture was emulsified by mixing at high pressure (~10,000 lb/in^2) using a Microfluidics emulsifier (Model 110Y, Newton, MA). The resulting emulsion was sterile filtered and stored at 4°C. MTP-PE obtained as a dry powder from Ciba-Geigy (Basle, Switzerland) was dissolved in the aqueous phase of the emulsion mixture before homogenization. This adjuvant was previously used in human clinical trials against genital herpes (Lan-

genberg *et al.,* 1995). The vaccine, containing MF59 with 50 μg of MTP-PE per dose (MF59–MTP-PE), was prepared just before immunization by mixing 1 volume of a mix of gB2 and gD2 in 2$\times$ PBS with 1 volume of emulsion.

Systemic vaccination

All rabbits received three inoculations at 3-week intervals. Each inoculation with gB2 + gD2/MF59–MTP-PE was delivered by a single intramuscular injection on one side of the lower back. Each dose contained 25 μg of each glycoprotein (gB2, gD2) in a total volume of 0.1 ml. Control, mock-vaccinated rabbits were inoculated identically and on the same schedule. The control vaccine consisted of MF59–MTP-PE plus PBS and was prepared as described above except that the 2$\times$ PBS contained no glycoproteins. The control vaccine contained the adjuvant alone without glycoproteins.

Periocular vaccination

Before inoculation, the rabbits were anesthetized with an intramuscular injection of ketamine/xylazine, and 1–2 drops of a 1% solution of proparacaine was applied topically, as eye drops, for local anesthesia. The vaccine was delivered by subconjunctival inoculation in the upper cul-de-sac using a 30-gauge needle and a disposable insulin syringe. Each eye received three inoculations of gB2 + gD2/MF59–MTP-PE at 3-week intervals. Each vaccine dose contained 7.5 μg of each glycoprotein in a final volume of 0.1 ml. Vaccination resulted in $\sim$25% of the eyes showing mild-to-moderate conjunctival and eye lid inflammation for up to 7 days.

Tissue culture assay for HSV-1 ocular shedding

As previously described (Nesburn *et al.,* 1994), beginning 3 weeks after the final vaccination, tear film specimens were collected daily from each eye using a nylon-tipped swab. The swab was placed in 0.5 ml of tissue culture medium containing antibiotics and antifungal agents, and the inoculated medium was used to infect rabbit skin (RS) cell monolayers. Cell monolayers were observed in a masked fashion by phase contrast microscopy for HSV-1 cytopathic effects. Positive cultures were blind passaged onto fresh RS cells for confirmation.

HSV-1 serum neutralization assay

Neutralizing antibody titers were done by 50% plaque reduction assays on CV-1 cells as described previously (Nesburn *et al.,* 1990).

ELISA assays for tear sIgA

Tears were collected with a microcapillary pipet and ELISAs were performed as previously reported (Mertz *et al.,* 1990) using either gD2 or gB2 as the capture antigen, goat anti-rabbit sIgA (Cappel, West Chester, PA) as the primary antibody, and anti-goat IgG alkaline phosphatase as the secondary antibody (Cappel).

Percent vaccine efficacy

Vaccine efficacy was calculated as 1 minus (the fraction of virus-positive tear films in the vaccine group, divided by the fraction of virus-positive tear films in the control group). This number then was multiplied by 100% to obtain the percent vaccine efficacy.

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