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Influence of Mucosal and Parenteral Immunization with a Replication-Defective Mutant of HSV-2 on Immune Responses and Protection from Genital Challenge

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Herpes simplex virus (HSV) most frequently initiates infection at a mucosal surface; thus mucosal immune responses are likely to be important in defense against HSV infection. We have examined the effects of eliciting mucosal as well as systemic immune responses on protection against genital challenge infection with virulent HSV-2 in mice immunized with a replication-defective mutant of HSV-2. In addition, we have examined the types of immune responses elicited by immunization by the different routes under conditions known to provide protection. We observed that immunizations at parenteral and distal mucosal sites generate immune responses that have an additive effect in protection against challenge infection with virulent HSV-2. Immunization at either of these sites alone prevented paralysis and death after challenge virus infection and reduced replication of the challenge virus in the genital mucosa, although subcutaneous immunization was more effective in reducing virus replication. Simultaneous immunization at the two sites led to the greatest reduction in mucosal replication of challenge virus. The type of response generated was also affected by the route of immunization. Subcutaneous immunization results in a strong systemic immune response that is somewhat biased toward a Th1 T cell response, while intranasal immunization induces mucosal as well as systemic immunity, as evidenced by HSV-specific IgA in vaginal secretions, and a stronger bias toward a Th1 response. These results suggest that mucosal immunization may complement protective immunity against HSV-2 genital infection generated by parenteral immunization with replication-defective mutant virus. © 1998 Academic Press

INTRODUCTION

Many human pathogens enter and replicate at a mucosal surface before causing systemic infection. For pathogens that have the capacity to persist in an infected individual, it is particularly important to curtail infection at the mucosal surface before persistent infection of systemic sites of latency or chronic infection is initiated. HSV infection of the oral or genital mucosa generates a mucosal immune response with IgA and IgG present in genital secretions (Ashley *et al.,* 1994a,b). This primary infection via the mucosa renders individuals at least partially resistant to infection by a second, heterologous strain (Mertz *et al.,* 1992). These observations suggest that mucosal immune responses may be an important component of host defense against HSV, and their induction in addition to systemic immune responses may increase the efficacy of prophylactic vaccination.

Systemic immunization typically does not elicit mucosal immune responses (McDermott *et al.,* 1990), although mucosal immunization with HSV can elicit modest systemic immunity (Irie *et al.,* 1992; McDermott *et al.,* 1990; Milligan and Bernstein, 1995a). Induction of mucosal immune responses by immunization of the genital tract with subsequent protection from genital challenge generally has proven effective only with replicating virus (Milligan and Bernstein, 1995a; McDermott *et al.,* 1984); intravaginal (i.vag.) immunization with HSV glycoprotein D (gD) in adjuvant is inefficient (Drew *et al.,* 1992). These observations suggest that the genital mucosa, which lacks organized lymphoid accumulations (Parr *et al.,* 1994), may be a poor immune induction site. The genital tract can also be populated with memory cells by infection or immunization of distant mucosae (McDermott and Bienenstock, 1979; Haneberg *et al.,* 1994) and distal immunization can elicit immune responses in the genital tract of greater magnitude than i.vag. immunization (Haneberg *et al.,* 1994). Individuals with HSV-1 infection of the oral mucosa have HSV-specific IgA present in genital secretions (Ashley *et al.,* 1994a) and mount anamnestic responses to subsequent genital HSV infection (Ashley *et al.,* 1994a). In a mouse model, intranasal (i.n.) immunization with a replication-competent adenovirus vector expressing HSV gB has been shown to protect against genital challenge with HSV (Gallichan and Rosenthal, 1995).

HSV mutant strains defective for essential viral functions constitute a vaccine approach that stimulates broad spectrum immune responses to numerous HSV gene products without the risks associated with attenuated virus vaccines. These mutant viruses are live but

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genetically compromised for some step of infectious progeny virus production. Mutants containing a deletion in the ICP8 gene essential for viral DNA replication are truly defective for spread in a vaccinated host in that they can neither replicate their DNA nor produce progeny virions (Gao and Knipe, 1989). We have previously shown that parenteral immunization of guinea pigs with a replication-defective mutant of HSV-2, 5BlacZ, decreases severity of genital lesions, replication of HSV-2 challenge virus in the genital mucosa, and the incidence of recurrent disease (Da Costa *et al.,* 1997). The observation that a replication-defective HSV-1 mutant stimulates immune responses of nearly the same magnitude and duration as those stimulated by immunization with the wild-type parental strain (Morrison and Knipe, 1996) suggested that these viruses would elicit immune responses if inoculated via the mucosa as well. Support for this conjecture has come from a second type of HSV mutant defective for an essential viral function, the single cycle gH^- mutant (Farrell *et al.,* 1994). This mutant virus undergoes a round of replication in the host, but the virions produced are defective for entry into new cells. Intraepithelial immunization with an HSV-1 gH^- mutant can reduce primary lesion scores and incidence of recurrent infection in a guinea pig model of genital infection with HSV-2 (McLean *et al.,* 1994). In addition, i.n. immunization with this gH^- HSV-1 mutant has been shown to protect guinea pigs against HSV-2 genital challenge more effectively than the i.vag. route (McLean *et al.,* 1996). However, because gH^- mutants undergo a round of replication and likely dissemination in the host, the capacity to immunize a mucosal surface with replication-defective mutants was by no means certain.

We sought to determine whether immunization with a replication-defective mutant of HSV-2 is efficacious in protecting mice from genital challenge infection with HSV-2 and whether immunization of a distal mucosal surface with this mutant would elicit a mucosal immune response in the genital tract. We hypothesized that, under conditions of protective immunity generated by immunization with replication-defective HSV-2, the existence of mucosal as well as systemic immune responses would augment protection of the genital mucosa. We therefore chose the intranasal route for distal mucosal immunization and compared primary replication of challenge virus and protection against disease under conditions of mucosal, systemic, or combined mucosal and systemic immune responses. Second, we determined the type of helper T cell response associated with mucosal and systemic routes of immunization. Third, we examined the importance of live virus to effective mucosal immunization under circumstances where *de novo* synthesis of viral proteins in the mucosa and simple exposure of the mucosa to viral proteins can be compared. Answers to these questions will help to define the parameters by which effective immunization against HSV infection may be achieved.

RESULTS

We chose a mouse model of genital infection with HSV-2 (Bourne *et al.,* 1996; Parr *et al.,* 1994) because the inbred mouse model provides a large number of immunological reagents for analysis of protective immune mechanisms generated by mucosal and parenteral immunization with replication-defective mutants of HSV-2. Our mutant virus, 5BlacZ, has been engineered from the 186 strain of HSV-2 (Da Costa *et al.,* 1997); thus, as a heterologous challenge virus we used the virulent HSV-2 strain G (Larica *et al.,* 1968), a limited passage clinical isolate. I.vag. inoculation of 10^5–10^6 PFU of G virus by this protocol resulted in inflammation in the genital area and genital lesions by 5 days postinfection of naive mice. Infectious virus was shed from the genital tract for at least 7 days and ascending paralysis followed, leading to death of the animals between 8 and 15 days postinfection. Dose studies demonstrated that the LD_{50} of HSV-2 G inoculated i.vag. into BALB/c mice was 10^4 PFU (data not shown). This mouse model system was used to evaluate the capacity of the 5BlacZ HSV-2 replication-defective mutant to induce protective immunity and the effect of mucosal immunization on the resulting immune response and subsequent protection against genital infection.

Protection from genital infection by mucosal immunization with replication-defective virus

To determine the ability of immunization by distal mucosal or parenteral routes to protect against genital challenge, we immunized mice twice at 1-month intervals with 5BlacZ or control lysate of uninfected cells by i.n. or subcutaneous (sc) routes or by simultaneous i.n. and sc administration of virus such that the total dose of virus was the same in all cases. One month later, all groups were challenged i.vag. with HSV-2 strain G. Primary replication of challenge virus in the genital mucosa was reduced in mice immunized by any route with the replication-defective virus compared with mice inoculated with control cell lysate (Fig. 1). Mice immunized sc with the replication-defective virus had significantly lower titers of virus shed from the vaginal mucosa beginning 1 day after challenge and had nearly resolved the infection by day 3 (Fig. 1B). Mice immunized i.n. did not show a significant decrease in titer compared to control mice until more than 3 days after challenge. However, they, too, had resolved mucosal infection by 5 days after challenge, when the titer of virus in control mice was still $>10^3$ PFU/sample (Fig. 1A). The combination of i.n. and sc administration of virus resulted in the most dramatic reductions of primary replication in the genital tract, with a peak titer on day 1 postchallenge of only 10 PFU/sample (Fig. 1C).

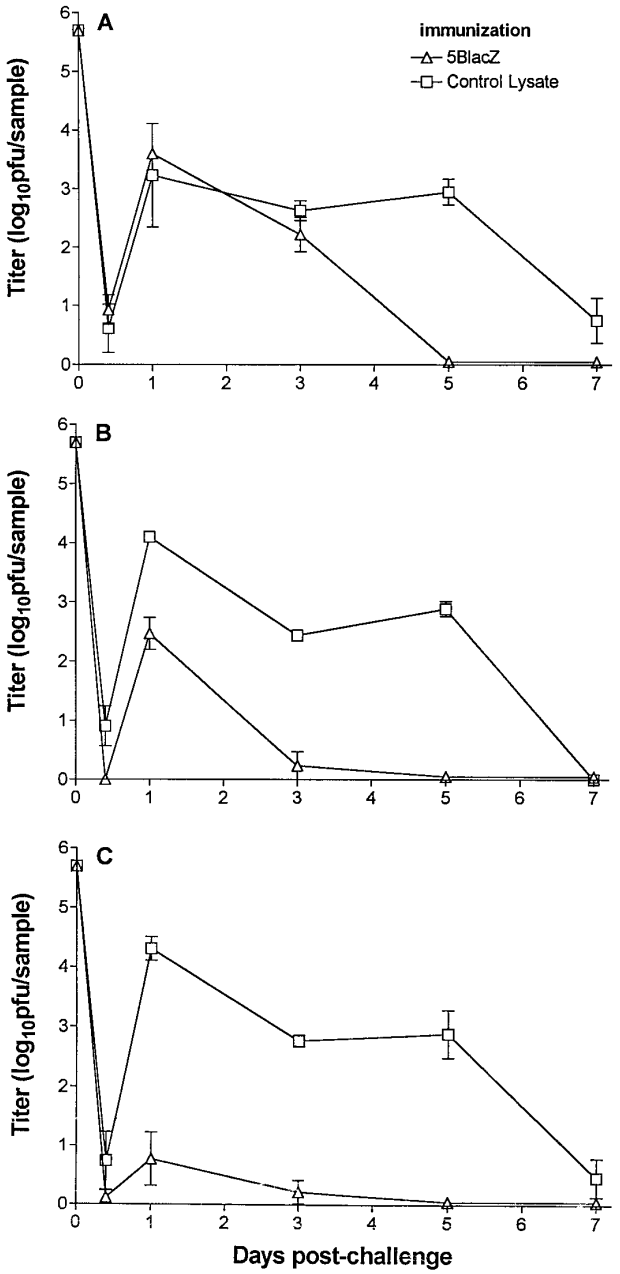

FIG. 1. Titers of HSV-2 challenge virus shed from the vaginal mucosa of mice immunized by different routes. Groups of five mice were immunized twice at 1-month intervals with mutant virus or control cell lysate by i.n. (A), sc (B), or simultaneous i.n. and sc routes (C) and then challenged i.vag. Vaginal swabs were collected at the indicated times postchallenge and titered by standard plaque assay. Values represent the mean ± SEM for each group. Data are from one representative experiment of four.

Mice immunized sc or i.n./sc with 5BlacZ were completely protected from development of local genital lesions and exhibited no signs of systemic disease, even though the dose of challenge virus represented approximately 50 LD_{50}. Mice immunized i.n. with 5BlacZ were protected from development of genital disease, showing only mild and transient signs of genital inflammation and

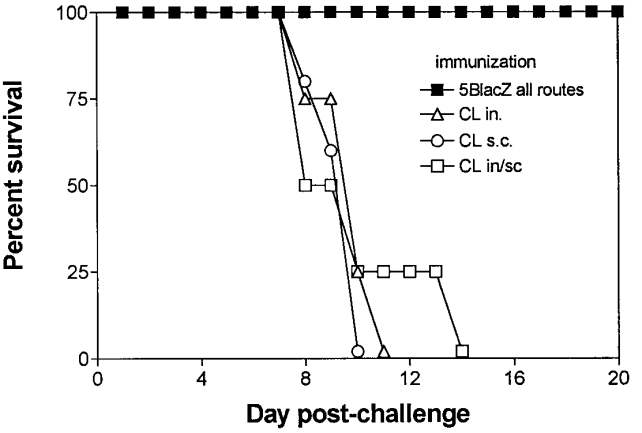

FIG. 2. Immunization with replication-defective virus promotes survival of mice after lethal i.vag. challenge with HSV-2. Mice were immunized twice with 5BlacZ or control lysate by the indicated route and monitored over time for survival after i.vag. challenge with approximately 50 LD_{50} HSV-2 G. Survival in groups of mice immunized with 5BlacZ was 100% regardless of the immunization route. Data are from the same experiment described in Fig. 1 (1 of 4 experiments).

ruffled fur at days 5–7 postchallenge (Fig. 2). In contrast, 100% of mice in all control groups developed severe genital lesions and hind limb paralysis (Fig. 2) and eventually succumbed to the challenge infection (Fig. 3). Interestingly, mice immunized i.n. with 5BlacZ and then infected i.vag. developed zosteriform lesions beginning approximately 8 days after challenge, suggesting that the immune response generated in i.n.-immunized mice is not sufficient to protect them from infection of peripheral sensory neurons innervating the genital tract.

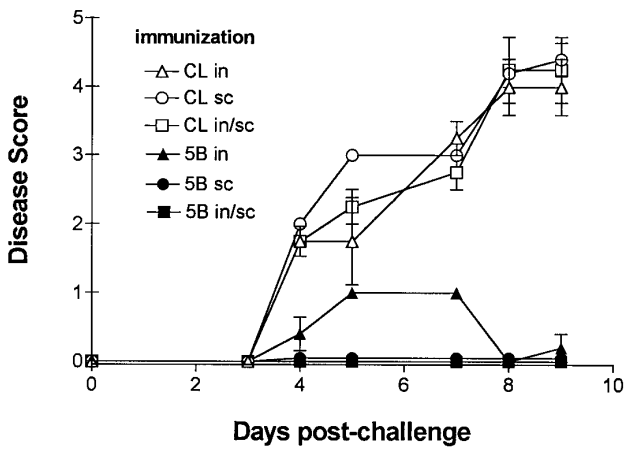

FIG. 3. Incidence of genital and neurologic disease in mice immunized with 5BlacZ by different routes. Groups of five mice were immunized twice at 1-month intervals with mutant virus (5B) or control cell lysate (CL) by the indicated routes, challenged 1 month after the second immunization, and observed daily thereafter for signs of clinical disease. Scores were given to individual mice based on the 5-point system described under Materials and Methods. Data points, representing mean score ± SEM for each group, are from the same experiment described in Fig. 1 (1 of 4 experiments).

TABLE 1

HSV-Specific Serum and Secretory Antibodies in Immunized Mice

Experiment No.	Immunization group	Serum neutralizing activity ($\log_2$)[a]	Serum ELISA IgG titer (ng/ml)	Vaginal wash ELISA titer (ng/ml)	
				IgG	IgA
1[b]	Control sc	<4	n.d.[c]		
	5BlacZ i.n.	<4	5 ± 2		
	5BlacZ sc	9.8	980 ± 300		
	5BlacZ in/sc	9.8	530 ± 160		
2[d]	Control i.n.		<0.02	<0.02	<0.3
	5BlacZ i.n.		11 ± 4	0.02 ± 0.02	6.2 ± 1.5
	5BlacZ sc		1790 ± 380	0.35 ± 0.15	<0.3

[a] Determined by CPE inhibition assay.
[b] Serum for Experiments 1 and 2 collected 16 and 20 days after second immunization, respectively.
[c] Not determined.
[d] Data are representative of three experiments.

Antibody responses in mice after mucosal or systemic immunization

We determined the serum neutralizing antibody titers and HSV-specific IgG ELISA titers in stored sera from the same group of mice challenged above. Mice immunized sc or i.n./sc had equivalently robust neutralizing antibody responses 16 days after the second immunization (Table 1, Experiment 1). Serum antibody neutralization titers in mice immunized i.n. with 5BlacZ were below the level of detection, suggesting that the protective effect of i.n. immunization was independent of serum neutralizing antibody. Furthermore, simultaneous i.n. administration of immunizing virus did not significantly boost systemic antibody titers elicited by sc antigen administration. HSV-specific IgG was detectable by ELISA in serum of i.n.-immunized mice, but was still very low compared with mice immunized sc or i.n./sc (Table 1, Experiment 1). In a separate experiment, HSV-specific antibodies in the vaginal secretions of mice immunized i.n. or sc were evaluated as a measure of the mucosal response to immunization. Vaginal wash samples were collected from mice 21 days after the second immunization with 5BlacZ and tested by quantitative ELISA for the presence of HSV-specific IgA and IgG (Table 1, Experiment 2). While i.n. immunization with 5BlacZ again elicited only low levels of HSV-specific serum IgG, virus-specific IgA was found in the vaginal wash samples (and in fecal samples in equivalent amounts; data not shown). This contrasts with the antibody response to sc immunization with 5BlacZ, which consisted of high titers of IgG in the serum and very low levels of IgG and no detectable IgA in vaginal wash samples.

Serum antibody responses following sc immunization with the HSV-1 ICP8$^-$ mutant, *d*301, show a relatively high ratio of IgG2a to IgG1 characteristic of a Th1 type of response to either HSV or HSV-encoded β-galactosidase (β-gal) (Brubaker *et al.,* 1996). We asked whether immunization with replication-defective virus via the nasal mucosa would also elicit a Th1-like response or would resemble the Th2 type of response that prevails after mucosal immunization with protein antigens. Surprisingly, mice that had been immunized i.n./sc had a higher serum IgG2a to IgG1 ratio than mice immunized sc with 5BlacZ (Table 2, Experiment 1), indicating an augmentation of the systemic Th1 response with simultaneous mucosal administration of mutant HSV. Although the total IgG response in the serum of mice immunized i.n. was much lower, the IgG2a to IgG1 ratio was skewed still further in the direction of a Th1 response. The relationship between the HSV-specific IgG2a to IgG1 ratios was maintained in a second experiment comparing mice immunized with 5BlacZ by sc or i.n. routes (Table 2, Experiment 2). The antibody response to virus-encoded β-gal in these same sets of sera also was examined to permit comparison with sera from mice immunized sc with β-gal in alum as a Th2 control. An exaggerated Th1 type response to virus-encoded β-gal again was observed in i.n.-immunized mice (Table 2, bottom). Taken together, these observations using IgG isotype ratios as a surrogate marker for Th responses indicate that mucosal immunization with replication-defective virus exaggerates the disposition toward a Th1 response seen after parenteral immunization and suggests that immunization with live but replication-defective virus stimulates immune responses through active infection rather than mere exposure to viral protein antigens.

Requirement of live virus for induction of the mucosal antibody response

We assessed whether active infection with *de novo* expression of viral proteins in infected cells indeed plays an important role in the stimulation of immune responses by mucosal immunization with replication-defective virus. Use of replication-defective virus for mucosal immunization likely limits exposure more precisely to mucosal

TABLE 2

IgG Isotype Ratio in Sera of Mucosally Immunized Mice

Experiment No.	Immunization group	Geometric mean titer ($\log_{10}$ ng/ml)[a]		
		IgG_{2a}	IgG_1	IgG_{2a}/IgG_1
HSV-specific antibodies				
1	5BlacZ sc	4.36 ± 1.07	5.99 ± 0.34	0.02
	5BlacZ in/sc	3.85 ± 0.15	4.67 ± 0.10	0.15
	5BlacZ i.n.	2.17 ± 0.24	3.35 ± 0.09	0.07 (3.5x)[b]
2[c]	5BlacZ sc	1.51 ± 0.11	2.93 ± 0.05	0.04
	5BlacZ i.n.	2.87 ± 0.09	3.07 ± 0.13	0.62 (15.5x)
	Control i.n.	u.d.[d]	u.d.	
β-Gal-specific antibodies				
1	5BlacZ sc	5.56 ± 0.21	6.53 ± 0.20	0.11
	5BlacZ in/sc	5.13 ± 0.12	5.82 ± 0.20	0.20
	5BlacZ i.n.	3.40 ± 0.64	3.93 ± 0.45	0.30 (2.7x)
2	5BlacZ sc	4.44 ± 0.38	5.04 ± 0.13	0.25
	5BlacZ i.n.	3.42 ± 0.24	2.72 ± 0.71	5.04 (20.2x)
	Control i.n.	u.d.	u.d.	
	β-Gal/alum sc	5.72 ± 0.22	7.19 ± 0.21	0.04

[a] Sera were collected 21 days after the second immunization and analyzed by ELISA.
[b] Fold increase in IgG_{2a}/IgG_1 ratios in i.n.-immunized mice compared with sc.
[c] Data are representative of four experiments.
[d] Undetectable.

tissues than would be possible with replication-impaired virus (because no progeny virus is produced), and use of partially purified virus stocks eliminates the immunizing potential of infected cell proteins. Mice were immunized twice i.n. with partially purified live or UV-inactivated 5BlacZ, a partially purified tk^- mutant of HSV-2 (positive control), or an equivalent preparation from uninfected cells (negative control), and the quantity of IgG in serum and IgA in vaginal washes was determined by ELISA. Immunization with live 5BlacZ generated IgA in vaginal washes and a low titer of IgG in serum (Fig. 4). Titers of both IgG and IgA were lower than that seen with the tk^-, replication-competent control virus but strongly contrasted with mice immunized with UV-5BlacZ, whose titers all were below the level of detection. These results suggest that the capacity to infect cells and synthesize viral proteins is an integral component of stimulation of mucosal as well as systemic immunity using replication-defective virus.

DISCUSSION

In this work we have shown that immunization of mice with a replication-defective HSV-2 mutant virus independently at parenteral or mucosal sites generates complementary immune responses whose protective effects are apparently additive when elicited together. Subcutaneous immunization led to a strong systemic immune response that is somewhat biased toward a Th1 T cell response, while intranasal immunization induced mucosal immunity, as evidenced by IgA in vaginal secretions, and a stronger bias toward a systemic Th1 response. Immunization at either of these sites alone could prevent paralysis and death after challenge virus infection and reduce genital replication of challenge virus, although subcutaneous immunization was more effective in reducing virus replication. Simultaneous immunization at the two sites led to the greatest reduction in challenge virus infection, suggesting that the types of immune responses induced by the two routes of immunization are additive in terms of protecting the genital mucosa. Finally, our data indicate that viral gene expression in mucosal cells infected by live, replication-defective virus is essential to the immune inductive capacity of the mutant virus.

Determination of the types of vaccine-induced immune responses that protect against virus infection is important to the design and implementation of any effective vaccine strategy. Immune responses generated by parenteral immunization with replication-impaired viruses have previously been shown to protect against systemic HSV-1 infection (Farrell *et al.,* 1994; Nguyen *et al.,* 1992) and corneal challenge (Morrison and Knipe, 1996, 1994) in mice and to reduce replication and disease associated with HSV-2 infection of the genital tract in guinea pigs (McLean *et al.,* 1994; Da Costa *et al.,* 1997). Immune responses generated by mucosal immunization of guinea pigs with a single cycle gH^- mutant reduces lesion incidence and severity but does not significantly alter primary replication of challenge virus in the mucosa (McLean *et al.,* 1996; Boursnell *et al.,* 1997). I.vag. and

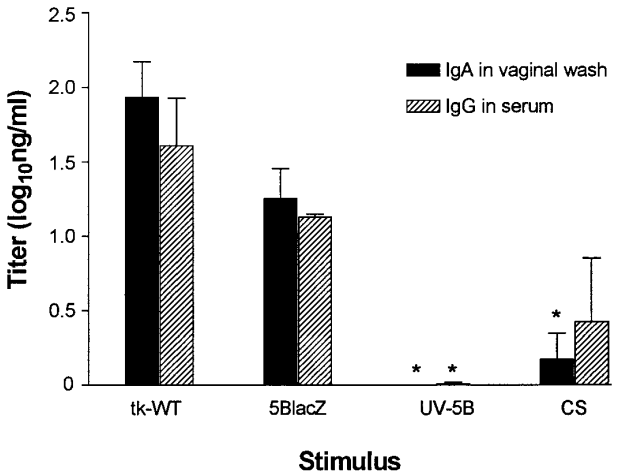

FIG. 4. HSV-specific antibody titers in serum and vaginal washes of mice immunized with different forms of virus. Groups of three to four mice were immunized twice with supernatant preparations of live or UV-inactivated 5BlacZ virus, its attenuated (tk^-) parental strain 186tkΔKpn, or control supernatant and then were challenged i.vag. with HSV-2 G. Virus-specific serum IgG and vaginal wash IgA titers were determined by ELISA of samples collected 21 days after the second immunization and 7 days after challenge, respectively. Data represent geometric mean titers ± SEM of a single experiment. Asterisks indicate titers significantly different from those of the live 5BlacZ-immunized group. One of 4 samples in the CS group was anomalously high; 3 of 4 were below the level of detection.

peroral routes of immunization are inferior to i.n. for both gH^- single cycle (McLean *et al.,* 1996) and $ICP8^-$ replication-defective (L. Morrison, unpublished observation) mutants. Comparison of i.vag. and sc routes of immunization with HSV-1 or HSV-2 single cycle mutants revealed that the parenteral route provides more significant protection of the mucosa (Boursnell *et al.,* 1997; McLean *et al.,* 1994). Our study is the first to assess the immune protection afforded by simultaneous mucosal (i.n.) and parenteral (sc) immunization. We found that mucosal immunization is not interchangeable with parenteral immunization. Sc immunization clearly gave superior protection in that mice showed no adverse clinical signs after challenge and had lower titers of challenge virus in the genital tract compared to mice immunized i.n. alone. Even a single sc immunization with 5BlacZ lowers primary titers and protects against disease (Da Costa *et al.,* in preparation). Nevertheless, it should be emphasized that i.n. immunization was able to significantly affect primary replication and development of disease even at the high challenge dose (50 LD_{50}) of HSV-2 strain G used in these experiments. Intranasal immunization contributed a mucosal immune response that parenteral immunization did not induce, as evidenced by sIgA in the genital tract. Notably, the systemic antibody response was lower in mice immunized i.n./sc than in those immunized sc, yet i.n./sc immunization afforded equivalent protection from disease and greater reduction of challenge virus replication in the genital mucosa. Our data suggest that i.n. immunization could be used to enhance the protective immunity generated by sc immunization. Whether simultaneous administration by i.n. and sc routes is optimal for induction of systemic and mucosal immune responses is a matter for further exploration.

All routes of immunization reduced primary replication to some extent in the genital mucosa, suggesting that preexisting immune effectors may act within the mucosa itself. The rapidity with which replication-defective virus-immune mice reduced challenge virus replication supports this contention. The immune components stimulated by replication-defective virus that provide protection of the genital tract at each stage of infection remain to be determined, but these issues can be readily addressed in the mouse model of genital HSV infection. The precise contribution of sIgA to protection may be difficult to assess, however, because of known compensatory mechanisms in mice genetically deficient in IgA production.

Antibody responses to the replication-defective mutants were monitored as an indicator of immune stimulation and type of Th response. They may correlate with level of protection but are not necessarily responsible for protection. $A_b^{0/0}$ (MHC class II^-) mice have been used to demonstrate the dependence of glycoprotein vaccine-induced protection on immune responses restricted by MHC class II (Ghiasi *et al.,* 1997). Our work identifying immune subsets elicited by replication-defective virus that are protective against HSV-1 infection of the cornea indicate an important contribution of T cells and in particular $CD4^+$ cells (Morrison and Knipe, 1997). Moreover, the data of Milligan and Bernstein (1995b) argue that memory $CD4^+$ T cells of the Th1 type are likely the important defenders of the genital tract against HSV infection, an interpretation that fits with our observation of a Th1-like antibody response. One role of $CD4^+$ Th1 cells is provision of help for IgG2a production, but the observation that IFNγ produced by $CD4^+$ T cells is required for rapid virus clearance from the genital tract (Milligan and Bernstein, 1997) indicates another role for the Th1 cytokine that could explain in large part the importance of Th1 responses to protection.

Although the titer of IgG in the serum of mice immunized by the i.n. route was low, the high ratio of IgG2a: IgG1 clearly indicated induction of a Th1-like systemic antibody response when virus is administered mucosally. Indeed, to our knowledge, this is the first indication that mucosal immunization can bias the systemic immune response even further toward a Th1 response than what is seen with parenteral immunization of virus. A Th1-like response induced via the mucosa contrasts with the prevailing view of Th2-mediated help for antibody production, at least in the gastrointestinal mucosa (Okahashi *et al.,* 1996; Fujihashi *et al.,* 1993). Our data are consistent, however, with observations on mucosal (lung) immunization or infection with influenza (Tomoda

et al., 1995) or infection of the gastrointestinal mucosa with recombinant salmonella (VanCott *et al.,* 1996). One interpretation of these findings is that a fundamental difference exists between mucosal immune stimulation by protein antigen and whole, infectious organisms that may actively stimulate cytokines and/or chemokines that lead to a Th1 type of systemic response. Alternatively, types of antigen presenting cells predominating at different mucosal surfaces may influence the type of T cell help induced at each and consequently the type of systemic response detected. If i.n. immunization with replication-defective HSV-2 also exaggerates the Th1-like nature of responses in the genital mucosa, this could be a mechanism whereby i.n./sc immunization resulted in the greatest reduction of challenge virus replication in the genital mucosa, exclusive of a contribution by sIgA.

The serum antibody response to β-gal also clearly indicated a ratio of IgG2a:IgG1 consistent with a Th1-like response, in contrast to the Th2-like response elicited by sc immunization with β-gal in alum. A Th1-like response to β-gal had been seen previously upon parenteral immunization with replication-defective virus (Brubaker *et al.,* 1996), and we have now shown this to be true after i.n. immunization as well. These results suggest that proteins expressed in the context of HSV infection will elicit immunity dominated by cell-mediated responses even though they would by themselves elicit a more humoral Th2 response, whether the response initiates systemically or at a mucosal surface. Thus, replication-defective mutants of HSV may be useful as a vector for stimulation of immune responses to foreign proteins whenever cell-mediated responses are desirable.

Our results with i.n. immunization are similar to those with single cycle gH^- virus (McLean *et al.,* 1996) in that a stronger mucosal IgA response was observed after immunization with live replication-defective virus than with UV-inactivated, replication-defective virus. This observation suggests that live virus is important to the inductive capacity of replication-impaired mutants. However, our results differ in that we observed (1) a very low systemic IgG response with live virus immunization, (2) little IgG transudate in vaginal wash samples, and (3) no systemic or mucosal antibody response after immunization with UV-inactivated 5BlacZ. gH^- virus may stimulate a greater systemic response after mucosal immunization due to dissemination into the systemic circulation of noninfectious progeny virions from the basolateral surfaces of infected mucosal cells. The distribution of 5BlacZ, on the other hand, may remain much more localized to the mucosa since no progeny virus is produced. Efficient stimulation of systemic immunity may, in turn, be necessary to generate enough IgG to detect as transudate. Regarding the third difference, i.n. immunization with inactivated gH^- virus may have elicited a weak mucosal response because of the much larger quantity of infected cell proteins in the whole cell lysate that was used or because of incomplete virus inactivation. Against this backdrop, lack of response to i.n. immunization with partially purified, UV-inactivated 5BlacZ clearly demonstrated that active infection and not mere antigen uptake is required to promote immune responses to replication-defective virus introduced via the mucosa. Whether use of live virus promotes immune responses through endogenous presentation of newly synthesized viral proteins in infected cells or simply by increasing the amount of total viral protein is not known.

The importance of mucosal responses to protection against genital HSV infection remains to be established. However, any mechanism that would enhance the numbers of HSV-specific memory cells in genital lymph nodes and tissues could be expected to enhance the host's preparedness to meet a genital challenge with HSV-2 and curtail infection before virus can enter the nervous system. Gallichan has demonstrated virus-specific ASC in the tract after i.n. immunization with an adenovirus vector expressing HSV gB (Gallichan and Rosenthal, 1996), and McDermott has demonstrated the protective capacity of HSV-immune T cells derived from genital but not distal lymph nodes to protect against genital challenge (McDermott *et al.,* 1989). A combination of parenteral and mucosal immunization with replication-defective virus may optimize induction of memory cells that home to the genital tract to provide maximum defense against challenge. Elucidation of mechanisms whereby mucosal immune responses are induced may be best answered through the use of the truly replication-defective mutants where immune induction is more likely to be confined to mucosal tissues and regional lymph nodes with little systemic response. Thus, replication-defective virus may prove useful as a tool for investigating immune responses to HSV at the mucosa and their importance as a defense mechanism, as well as potentially constituting a safe and effective vaccine strategy.

MATERIALS AND METHODS

Cells and viruses

5BlacZ, a replication-defective mutant of HSV-2, strain $186syn^+$, is defective in the U_L29 gene encoding ICP8 due to insertion of the lacZ gene (Da Costa *et al.,* 1997). A replication-competent, thymidine kinase-negative (tk^-) mutant of strain $186syn^+$, 186ΔKpn, has also been constructed (Taylor, Jones, and Knipe, unpublished results). Stocks of tk^- virus were prepared by growth in Vero cells, and 5BlacZ by growth in S-2 (Gao and Knipe, 1989) or V8-27 cells [stably transfected with the U_L29 and U_L54 genes; (Da Costa *et al.,* 1997)] in Medium 199 supplemented to contain 1% FCS by standard methods (Morrison and Knipe, 1996). HSV-2 strain G is a limited passage clinical isolate (Larica *et al.,* 1968). Supernatant virus stocks were prepared from infected cell cultures that were frozen and thawed once. Cells were removed by

low-speed centrifugation, and then virions in the supernatant were pelleted by centrifugation at 25,000*g* for 1 h at 4°C. Virus pellets were resuspended in a small volume of Medium 199 supplemented with 1% normal mouse serum, sonicated, and stored at −80°C. Control supernatant was prepared from frozen and thawed, uninfected cell cultures. A portion of the supernatant virus stock of 5BlacZ was inactivated by exposure to UV light as previously described (Morrison and Knipe, 1994), resulting in >6 $\log_{10}$ reduction in titer.

Mice and inoculations

Female BALB/c mice, purchased from Taconic Laboratories and the National Cancer Institute, were housed and cared for in accordance with PHS (Anonymous, 1985) and institutional guidelines and used at 7 weeks of age. For i.n. immunization, mice were anesthetized with nembutal (0.07 mg/g body wt) and 1×10^5–1×10^6 PFU in a 2.5-μl volume per nostril of virus or control preparation was applied with a pipette tip. For sc immunization, unanesthetized mice were injected in the hind flank with 2×10^6 virus or control preparation in 20-μl volumes using a 1-ml syringe fitted with a 26-gauge needle. For simultaneous i.n./sc immunization, 1×10^6 PFU was applied to one nostril and 1×10^6 PFU was injected in the flank. Immunizations were repeated after 4 weeks, and mice were challenged 4 weeks after the second immunization. Seven days and 1 day prior to challenge, mice were injected sc in the neck ruff with depoprovera (3 mg per mouse in a 100-μl volume). For i.vag. challenge, mice were anesthetized, preswabbed with a type 1 calcium alginate swab (Spectrum), and inoculated with 5×10^5 PFU of HSV-2 strain G (approximately 50 LD_{50}) in 5-μl volumes using a pipette tip.

Assay of acute infection

Ten hours and 1–5 days postchallenge, vaginal mucosae of mice were swabbed with two successive swabs. The swabs were placed together into vials containing 1 ml assay medium (PBS plus glucose and 1% FCS) and stored at −80°C. Viral titers were determined by standard plaque assay (Morrison and Knipe, 1996).

Clinical observations

Mice were observed daily for signs of genital lesions and systemic illness. The severity of disease was scored as follows: 0, no sign; 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, purulent genital lesions; 4, hind-limb paralysis; 5, death.

Serum collection and vaginal washes

Blood was collected from tail veins 3 weeks after primary immunization and 2–3 weeks after secondary immunization. Serum was prepared as previously described (Morrison and Knipe, 1994). Vaginal washes were performed at the time of serum collection by thrice titurating 25 μl PBS in the vaginal vault using a pipette tip. Washes were pooled, bestatin and 4-(2-aminoethyl)-benzenesulfonyl fluoride (Sigma) were added to a final concentration of 0.2 mM and 3.25 μM, respectively, and samples were frozen at −20°C.

Antibody neutralization assay and quantitative ELISA

Virus neutralization assays were carried out as previously described, except that HSV-2 G was used as the virus strain to be neutralized. A quantitative ELISA was developed by a modification of the technique of Zollinger and Boslego (Zollinger and Boslego, 1981). In brief, quantitation standards were established by titration of a positive control serum (pooled from mice immunized with HSV-2) on Immunlon 2 plates (Dynatech) coated with HSV-2 G lysate (Advanced Biotechnologies) in parallel with purified IgG and IgA (Sigma) or IgG_1 and IgG_{2a} (gift of P. Yaciuk) titrated on Maxisorp plates (Nunc) coated with goat anti-Kappa light chain (Caltag). OD values of positive control serum dilutions in the range of 1 to 2 units were used to determine antibody concentration by comparison to the purified antibody concentration curves. A dilution series of this positive control serum was then run in each assay to generate a standard curve for comparison of serum or vaginal wash samples. Goat anti-mouse IgG and IgA conjugated to biotin (Caltag) were used as secondary antibodies for assays of IgG and IgA, followed by addition of streptavidin–HRP (Sigma). HRP-conjugated goat anti-mouse IgG_1 and IgG_{2a} (Southern Biotechnologies) were used in isotype-specific assays. All assays were developed by addition of OPD (Sigma) for 30 min and then read at OD_{490}. Disparity between total and subtype-specific serum IgG titers (Tables 1 and 2) is attributable to use of sera from different experiments and to different sources of standards. Total antibody concentrations were determined by capture ELISA using anti-Kappa-coated plates.

Statistical analyses

Significance of the difference in vaginal wash titers was determined by comparison of groups on each day using the Student's *t* test, with no correction for multiple comparisons. Antibody titers were also compared using the Student's *t* test, with no correction for multiple comparisons.

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