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Protection from Oral Herpes Simplex Virus Infection by a Nucleic Acid-Free Virus Vaccine

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The effect of immunization with inactivated herpes virus vaccines, including a vaccine free of all nucleic acid, was investigated in a mouse model system. Protection against oral lesions induced by herpes simplex virus type 1 was demonstrated by several criteria: (i) reduction in the incidence and severity of primary oral lesions; (ii) decrease in acute and latent infection of the regional sensory ganglia; and (iii) protection from viral encephalitis and death. The immune response of mice to the vaccine and to subsequent virus challenge was measured by following serum-neutralizing antibody titers.

The ubiquitous herpes simplex virus type 1 (HSV-1) is a common cause of primary and recurrent oral infections. During primary infection, the neurotropism and axonal spread of the virus results in latent infection of the regional sensory ganglia (2, 6, 11, 19). The latent virus can then be activated and cause periodic episodes of recurrent lesions, which may be associated with the development of neoplastic disease (1, 17, 18, 22).

A vaccine that offers safe and effective protection from primary or recurrent herpetic infections would constitute a major contribution toward control of human herpetic diseases. Conceptually, such a vaccine could prevent infection of the regional sensory ganglia and thus prevent latency and the potential neurological or neoplastic sequelae. Prior immunization of mice with live HSV-1 has been demonstrated to decrease the mortality as well as the incidence of latent ganglionic infection that followed epithelial infection with HSV-1 (20). In addition, rabbits have been protected from lethal infection with HSV-1 by prior immunization with an envelope-associated viral antigen preparation (26). A number of formalin-inactivated HSV vaccines have been used clinically in patients with latent HSV infection in attempts to prolong disease-free intervals between recurrent episodes (10, 12). However, because of the oncogenic potential and the propensity of herpesviruses to establish latency (2, 5-8, 19, 21, 24), there is an inherent risk with the use of a nucleic acid-containing vaccine from either an attenuated or inactivated virus. Therefore, the most appropriate HSV vaccine for human use would be free of viral nucleic acid yet retain the antigenic properties necessary to evoke a protective immunological response. In this report we present data to demonstrate that a nucleic acid-free virus vaccine can protect mice from primary lip lesions and the neurological sequelae that follow oral herpetic infection.

MATERIALS AND METHODS

Cells. Human epidermoid carcinoma (HEp-2) and green monkey kidney (Vero) cells were grown in Eagle minimal essential medium with Earle balanced salt solution supplemented with 10% fetal bovine serum, 50 μg of gentamicin per ml, and 0.15% $NaHCO_3$ (for cells in stoppered vessels) or 0.225% $NaHCO_3$ (for cells in 5% CO_2 atmosphere). Cultures were maintained in Eagle medium supplemented with 2% serum. Cultures were found to be free of mycoplasma on routine screening.

Virus production and assay. An oral isolate of HSV-1 (Patton strain) was obtained from William Rawls at Baylor College of Medicine, Houston, Tex. The virus was passaged three times in HEp-2 cells, after which it was plaque-purified three times. Stocks were then prepared by infecting cell monolayers at a low multiplicity of infection (0.1). The infected cells were incubated at 34°C and harvested when 80% of the cells demonstrated typical HSV-induced cytopathic effects. Virus infectivity was assayed for plaque-forming units (PFU) on Vero cells in 16-mm tissue culture plates (Linbro, New Haven, Conn.) using a 2% methylcellulose overlay (16).

Solutions and chemicals. Cesium chloride was obtained from Apache Chemicals (Seward, Ill.). Electrophoretically purified deoxyribonuclease I (18,300 U/mg) was obtained from Worthington Biochemicals Corp. (Freehold, N.J.). Stock solutions of sodium dodecyl sulfate and Sarkosyl (NL 97; Geigy) were made to 10% in distilled water. Tris(hydroxymethyl)aminomethane-NaCl buffer consisted of 0.1 M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, and 0.001 M $MgCl_2$.

Animals. Four-week-old male BALB/c mice were

obtained from Laboratory Supply Co. (Indianapolis, Ind.) and acclimated for 1 week before use in experiments. Infected and noninfected mice were kept 10 per cage in separate rooms.

Oral inoculation of BALB/c mice. Oral infection of anesthetized (methoxyfluorane inhalation) mice was accomplished by lightly abrading the oral mucosa on one side of the mouth with an emery board. The abrading process did not cause bleeding. The abraded area was then swabbed with a cotton swab saturated (approximately 0.1 ml) with virus solution containing 10^6 to 10^7 PFU/ml. Both control and immunized animals were anesthetized between days 3 and 7 after infection, and the lesions were examined with the aid of 2× magnification. In controlled experiments, uninfected mice housed in the same cage with infected mice did not acquire oral lesions through horizontal transmission. The inoculation site was categorized as exhibiting either large (>2 mm) or small (<2 mm) ulcerative or vesicular lesion, erythema only, or no visible lesion.

Preparation of HSV-1 vaccine. HEp-2 cells were infected with HSV-1 (multiplicity of infection = 1) and incubated at 34°C until 80% of the monolayer demonstrated viral cytopathic effects. The medium was decanted, and the cells were collected in sterile distilled water (1 ml/75-cm^2 flask) by scraping with a rubber policeman. The cells were disrupted with 50 strokes in a Dounce homogenizer and centrifuged at 1,000 × g, and the clarified supernatant portion was inactivated with formalin (final concentration, 1:400). The supernatant fluid, which was demonstrated to be free of infectious virus, was treated with 1% sodium dodecyl sulfate and 1% Sarkosyl to lyse the virions. It was then concentrated fourfold in a Millipore ultrafiltration apparatus. The retentate was dialyzed against physiological saline. To prepare the nucleic acid-free vaccine, the concentrated lysate was centrifuged to equilibrium in CsCl (median buoyant density = 1.700 g/cm^3) in an SW50.1 rotor at 40,000 rpm for 48 h (15). The top of the gradient, containing the nucleic acid-free protein preparation, was collected and dialyzed exhaustively against Tris-NaCl buffer. The dialysate was then hydrolyzed with 50 μg of deoxyribonuclease I per ml for 30 min at 37°C. This procedure completely removed exogenously added ^{3}H-labeled HSV-1 deoxyribonucleic acid from sodium dodecyl sulfate- and Sarkosyl-lysed HSV-1 virions (15).

Immunization procedure. Five-week-old mice were immunized at four sites (rear footpads and subcutaneously in both hind legs) twice at 14-day intervals. A mixture of HSV antigen preparation and complete Freund adjuvant was used for the first immunization; the second immunization was with antigen alone. The immunization dose contained 10^9 PFU of HSV-1 before inactivation and approximately 1.5 mg of protein. Ten days after the second immunization, mice were challenged orally with HSV-1 as described above.

Assays for acute and latent ganglionic infection. Regional sensory ganglia (trigeminal) were removed and assayed for infectious HSV-1 during both acute and latent phases of infection. During acute infection (4 to 6 days postinoculation), ganglia were removed and individually homogenized in 1 ml of maintenance medium with a mortar and pestle, and the cell-free homogenates were assayed for infectious virus (25). During the latent period of infection (30+ days after oral challenge), cell-free ganglionic homogenates and ganglionic explants were assayed for infectious virus (23). The explanted ganglia were co-cultivated on Vero cell monolayers using previously described methods (25). The presence of HSV-1 in the culture media was confirmed by viral neutralization tests.

Serum-neutralizing antibody assay. Serial twofold dilutions of heat-inactivated (56°C/30 min) sera (0.1 ml) were incubated with an equal volume of medium containing 40 PFU of HSV-1 and 20 hemolytic units of guinea pig complement (Colorado Serum Co., Denver, Colo.) in a 36°C water bath for 30 min. Residual virus was assayed for PFU on Vero cells as described above. Antibody titers, expressed in units, were interpolated graphically as the reciprocal of the dilution causing 50% plaque reduction. A standard rabbit anti-HSV-1 serum was titered simultaneously in each assay, with titers ranging from 400 to 600 U/0.1 ml.

Statistical procedures. Serum neutralization antibody titers of control and immunized mice were analyzed by Student's t test. The incidences of infection, development of encephalitis, or latent ganglionic infection were analyzed by the chi-square test with the Yates correction factor.

RESULTS

Oral infection. Oral herpetic lesions may be induced in young BALB/c mice (20). In our laboratory, between 95 and 100% of animals infected by swabbing an abraded oral mucosa developed local lesions at the inoculation site within 5 days. The severity of the oral lesions was maximal 5 to 6 days after infection, and deaths occurred between days 7 and 14. Deaths were documented to be due to herpetic encephalitis by clinical signs and by virus isolation from brain tissue. In surviving animals, the oral lesions resolved within 14 days, after which time HSV-1 could not be recovered from homogenates of oral tissues or trigeminal ganglia.

Effect of inactivated, HSV-1 whole-virus vaccine on oral herpetic lesions and their neurological sequelae. Immunization of BALB/c mice with the formalin-inactivated, detergent-lysed whole-virus vaccine markedly reduced the incidence and severity of oral lesions (Table 1). Almost all (96%) infected, nonimmunized mice developed vesicular or ulcerative lesions at the inoculation site, whereas half of the immunized mice remained lesion-free or developed only erythema. Virus was consistently isolated from vesicular or ulcerative lesions on day 5 after infection, but could not be recovered from swabs of inoculation sites that appeared

normal or exhibited only erythema. Immunization with the detergent-lysed virus vaccine also significantly reduced neurological sequelae after infection (Table 2). At the higher virus inoculum, 90% of the nonimmunized mice developed acute trigeminal ganglionic infection, and 66% died of encephalitis. In contrast, immunized mice developed neither acute ganglionic infection nor encephalitis. Immunized and nonimmunized mice that survived the HSV-1 infection were assayed 3 months later for active or latent virus infection. As indicated earlier, no evidence of active infection was ever obtained from homogenized ganglia more than 14 days after infection. The explanted trigeminal ganglia from both immunized and nonimmunized mice began to release virus between days 7 and 28 after explantation. In most instances, only the ipsilateral ganglion was latently infected, although occasionally the contralateral ganglion was also positive. Whereas 69% of nonimmunized mice showed latent ganglionic infection, only 10% of the immunized mice developed latent viral infection.

TABLE 1. *Effects of immunization with formalin-inactivated, detergent-lysed herpes simplex whole-virus vaccine on oral hepatic lesions*[a]

Gross description of inoculation site	Mice with described lesion/ total no. of mice examined[b]	
	Nonimmunized	Immunized
Ulcerative or vesicular herpetic lesion present	52/54 (96)[c]	26/53 (49)
Large (>2 mm)	25/54	11/53
Small (<2 mm)	27/54	15/53
No herpetic lesions present	2/54 (4)	27/53 (51)
Erythema only	2/54	14/53
No visible change	0/54	13/54

[a] Mice were infected with a solution of virus containing 10^7 PFU/ml as described in Materials and Methods.

[b] The mice were examined daily for oral lesions. The results ($P < 0.0005$) were obtained at the peak of the response on day 5.

[c] The numbers in parentheses represent the percentage of mice with the described lesion per total number of mice examined.

Effect of nucleic acid-free HSV-1 vaccine on oral herpetic lesions and their neurological sequelae. The previous data demonstrated vaccine-mediated protection using a detergent-lysed, whole-virus preparation. Experiments were undertaken to test protection provided by the same vaccine after removal of the viral nucleic acid. Immunization of mice with a nucleic acid-free preparation of viral antigens also reduced the incidence of oral lesions after virus infection (95% in nonimmunized mice to 67% in immunized mice, $P < 0.02$; Table 3). The oral lesions that appeared in immunized mice were less severe than in controls. Incidence and severity of oral lesions were assessed in a manner analogous to that used for Table 1. The number of mice developing either acute ganglionic infection or dying of encephalitis was also reduced in immunized mice. Furthermore, when latent infection with HSV-1 was monitored in survivors, the incidence of latent infection was significantly reduced ($P < 0.05$) by immunization.

Neutralizing antibody levels after immunization or oral infection. Individual immune mouse sera obtained after the first immunization, after the second immunization, at the

TABLE 2. *Effect of immunization with formalin-inactivated, detergent-lysed herpes simplex whole-virus vaccine on the neurological sequelae of oral herpetic infection*[a]

Treatment	Virus challenge (PFU/ml)[b]	Incidence of encephalitis (no. infected/no. examined)	Acute ganglionic infection (no. infected/ no. examined)[c]	Latent ganglionic infection (no. infected/ no. examined)[d]
Nonimmunized	4.2×10^6	13/76 (17)[e]	ND[f]	7/9 (78)
	1.0×10^7	31/47 (66)	9/10 (90)	9/13 (69)
Immunized	4.2×10^6	0/8 (0)	ND	0/8 (0)
	1.0×10^7	0/43 (0)	0/12 (0)	3/31 (10)

[a] Immunized mice were significantly protected from encephalitis ($P < 0.0005$), acute ganglionic infection ($P < 0.0005$), and latent ganglionic infection ($P < 0.0005$).

[b] Approximately 0.1 ml of virus challenge solution containing the indicated PFU/ml was applied to abraded oral surfaces (see Materials and Methods).

[c] Demonstrated by presence of infectious virus in trigeminal ganglia homogenates prepared 5 days after oral infection.

[d] Demonstrated by trigeminal ganglia explanation 3 months after oral infection.

[e] The numbers in parentheses represent the percentage of animals infected per number of animals examined.

[f] ND, Not determined.

TABLE 3. *Effect of prior immunization with a nucleic acid-free HSV vaccine on oral herpetic lesions and their sequelae*[a]

Description of disease state	Animals with disease state/total no. of animals	
	Nonimmunized	Immunized
Primary oral infection	38/40 (95)[b]	12/18 (67)
Acute ganglionic infection[c]	6/6 (100)	3/6 (50)
Encephalitis	15/34 (44)	3/12 (25)
Latent ganglionic infection[d]	13/14 (93)	4/9 (44)

[a] Mice were infected with a solution of virus containing 10^7 PFU/ml as described in Materials and Methods.

[b] The numbers in parentheses represent the percentage of animals with a disease state per total number of animals.

[c] Demonstrated by presence of infectious virus in trigeminal ganglia homogenates prepared 5 days after oral infection.

[d] Demonstrated by trigeminal ganglia explantation 1 month after oral infection.

time of oral challenge, on day 5 during acute infection, and at the time of ganglionic explantation were titered for HSV-1 neutralizing antibody. With either the inactivated whole virus or nucleic acid-free virus vaccine, no detectable neutralizing antibody (<10 U) was demonstrable by 14 days after the first immunization. Low levels of antibody (15 to 26 U) were occasionally detected at the time of oral challenge (10 days after the second immunization). Antibody levels remained low during acute infection (5 days after oral challenge). Antibody titers continued at a low level at the time of ganglionic explantation (21.1 ± 3.6 U for the detergent-lysed, whole-virus vaccine and 12.3 ± 1.0 U for the nucleic acid-free virus vaccine; Fig. 1). Antibody levels did not correlate with the presence or absence of latent ganglionic infection. In contrast to the results of mice immunized with either type of vaccine, neutralizing antibody titers were much higher (69.0 ± 9.1 and 55.1 ± 8.0 U) in surviving nonimmunized mice. Again there was no correlation of the neutralizing antibody titer with latent virus infection.

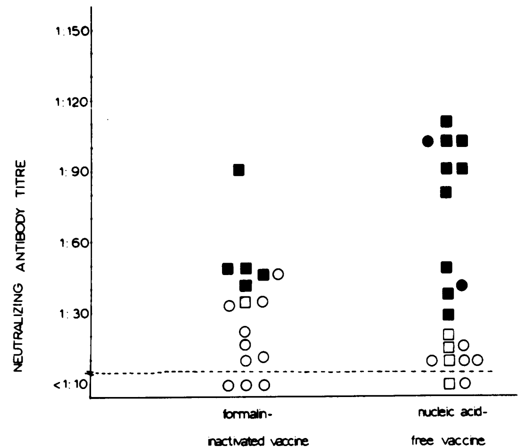

FIG. 1. *Serum-neutralizing antibody titers in immunized and nonimmunized control mice at the time mice were sacrificed to determine incidence of latent infection. Sera were obtained 3 months after infection in the formalin-inactivated vaccine group and 1 month after infection in the nucleic acid-free vaccine group. At this time, animals were sacrificed and explanted ganglia assayed for latent HSV-1 infection. Neutralizing antibody titers were determined as described in Materials and Methods. Positive = latent trigeminal ganglionic infection. Symbols: □, immunized/positive; ■, control/positive; ○, immunized/negative; ●, control/negative.*

DISCUSSION

Price et al. (20) have demonstrated that immunization of mice by prior intraperitoneal infection with viable HSV-1 could protect against encephalitis and latent infection that followed subsequent oral challenge with HSV-1. These observations were promising in that they indicated that persistent infection with herpesviruses could be prevented by immunization. However, because of the oncogenic potential of HSV (7, 8), it would be inappropriate to immunize humans with an attenuated virus. Hilleman has suggested that even the presence of potentially oncogenic viral nucleic acid would preclude vaccination (9).

The present results indicated that immunization of mice with inactivated herpes simplex whole-virus vaccine and, most importantly, with a vaccine free of nucleic acid protected mice from subsequent oral infection with HSV-1. The protective effect was demonstrated by several criteria. First, the incidence and severity of primary oral lesions was reduced. Second, fewer mice exhibited acute ganglionic infection or died of encephalitis. Finally, the incidence of latent trigeminal ganglionic infection was reduced. The nucleic acid-free vaccine did not appear to be as potent as the unfractionated, detergent-lysed virus vaccine. However, the efficacy of this vaccine may be improved by such procedures as protein aggregation or polymerization or combination with immunomodulators, methods that have been successful with experimental virus vaccines (4, 13). In addition, a more specific vaccine might be prepared by

the use of viral glycoprotein subunits (14). The use of these purified subunits should eliminate problems with cross-reactivity and possible autosensitization phenomena.

The mechanisms involved in the protective effect of the vaccine have not yet been defined. Immunization with the detergent-lysed, nucleic acid-free vaccine induced undetectable or very low levels of neutralizing antibody. Even after oral infection, the levels of neutralizing antibody remained relatively low in immunized mice. Furthermore, the levels of antibody were low in immunized mice whether they were latently infected or free of persistent virus infection. In contrast, oral infection produced significant levels of antibody in surviving nonimmunized control animals. These results suggested that the immunization procedure inhibited virus growth early in infection, so that insufficient virus antigen was produced for stimulation of lymphoreticular tissue. The data suggest that humoral immune responses, at least as measured by serum-neutralizing antibody, play a minor role in the protection afforded by immunization. This is consistent with previous observations that resistance to HSV appears to be related more closely to specific cell-mediated immune responses and to nonspecific resistance factors than to humoral immunity (3, 17).

It appears that once HSV-1 has established a latent infection, immunization may not prevent recurrence of lesions (10, 12). This may be attributed to the ability of HSV to exist in a latent state in ganglia and spread via the neuronal axons, protected from immunological effector systems. The protection afforded to mice by the nucleic acid-free HSV vaccine, however, suggests that a similar vaccine may be useful in protecting humans from primary infections and their neurological sequelae. Children or seronegative adults could be immunized with such a vaccine prior to infection with the pathogen. This approach would provide needed protection without the risk of infection with a potentially oncogenic virus.

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