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Vaccination with Recombinant Vaccinia Viruses Expressing ICP27 Induces Protective Immunity against Herpes Simplex Virus through $CD4^+$ $Th1^+$ T Cells

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This study was designed to evaluate the efficacy and mechanisms of protection mediated by recombinant vaccinia viruses encoding immediate-early (IE) proteins of herpes simplex virus type 2 (HSV-2). Three mouse strains were immunized against the IE proteins ICP27, ICP0, and ICP4, and mice were challenged intracutaneously in the zosteriform model with HSV-2 strain MS. Protection was observed only following immunization with the ICP27 construct and then only in the BALB/c mouse strain. Protection in BALB/c mice was ablated by $CD4^+$ T-cell suppression but remained intact in animals depleted of $CD8^+$ T cells. Moreover, protection could be afforded to SCID nude recipients with $CD4^+$ but not $CD8^+$ T cells from ICP27-immunized mice. Only BALB/c mice developed a delayed-type hypersensitivity reaction to HSV-2, and in vitro measurements of humoral and cell-mediated immunity revealed response patterns to ICP27 and HSV that differed between protected BALB/c and unprotected mouse strains. Accordingly, BALB/c responses showed antigen-induced cytokine profiles dominated by type 1 cytokines, whereas C57BL/6 and C3H/HeN mice generated cytokine responses mainly of the type 2 variety. Our results may indicate that protection against zosterification is mainly mediated by $CD4^+$ T cells that express a type 1 cytokine profile and that protective vaccines against HSV which effectively induce such T-cell responses should be chosen.

Herpes simplex virus (HSV) infection is a common cause of human disease, and as yet we lack suitable vaccines for its control. Results of many studies with humans and animals suggest that aspects of T-cell immunity are crucial for clearing virus from infected tissues (16). Consequently, any vaccine must effectively stimulate T-cell immunity of the appropriate type. Evaluating any anti-HSV vaccine for inducing optimal immunity in humans is either extremely difficult or impossible, and so an animal model system must be used. Unfortunately, no animal model of HSV infection perfectly mimics the pathogenesis of human disease, especially with respect to spontaneous recrudescent disease associated with reactivation of virus from latency. Recrudescent lesions result from virus replication at peripheral sites following the dissemination of virus to such sites from nerve cell bodies via anteriograde spread along sensory nerves (17). An experimental system which perhaps best mimics this later scenario is the zosteriform spread model of Simmons and Nash (17). In this model, virus infection is initiated in the flank and virus spreads via retrograde axonal transport to the spinal ganglia. After replication at this site, virus moves via anteriograde axonal spread to the innervated dermatome, where lesions develop. The zosteriform model is useful for measuring immunity to cutaneous disease as well as spread within the nervous system (17). Previous observations have indicated that immunity to cutaneous lesions may be mediated mainly by $CD4^+$ T cells and that immunity within the nervous system may be mediated by $CD8^+$ T cells (18).

The HSV virion encodes more than 70 proteins, and choosing the most logical candidate for vaccines is problematic. We have chosen to investigate the value of immediate-early (IE) proteins in part on the basis of the logic that effective immunity to such proteins may curtail replication before a replication cycle is completed and the infected cells are destroyed (3). Moreover, IE proteins are recognized by human HSV-specific cytotoxic T lymphocytes induced by in vitro stimulation of peripheral blood mononuclear cells with HSV type 2 (HSV-2)-infected cells (11). To this end, we have expressed three IE proteins of HSV-2 in recombinant vaccinia viruses and, using the zosteriform model, have assessed their protective effects to virus challenge in three strains of mice. We observed protection only with one recombinant vaccinia virus construct (expressing ICP27) and then in only one of the three mouse strains investigated (BALB/c). In such mice, protection was shown to be a property of $CD4^+$ T cells and was considered to represent the activity of $CD4^+$ T cells of the type 1 cytokine profile.

MATERIALS AND METHODS

Mice. Female BALB/c (*H-2*^{d}), C57BL/6 (C57; *H-2*^{b}), and C3H/HeN (C3H; *H-2*^{k}) mice were obtained from Harlan Sprague-Dawley, Indianapolis, Ind. CB17 SCID mice were purchased from Taconic, Germantown, N.Y., and BALB/c *nu/nu* mice were purchased from Life Sciences, Inc., St. Petersburg, Fla. During the experimental procedures, the investigators adhered to the guidelines proposed by the Committee on the Care of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. The research facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

Viruses. Recombinant vaccinia viruses expressing IE proteins of HSV-2, designated vv ICP27, vv ICP0, and vv ICP4, and vtk$^-$ (control vaccinia virus) were grown in CV-1 cells (ATCC CCL81); HSV-2 (MS) was grown in Vero cells (ATCC CCL70).

Construction of plasmids and recombinant vaccinia viruses. The ICP27 vaccinia virus transfer plasmid was obtained by coligation of a synthetic oligonucleotide (encoding the first 19 amino acids of the ICP27 gene up to the natural *Xho*I site) and a 1,670-bp *Xho*I-*Mlu*I fragment isolated from plasmid f (obtained from D. J. McGeoch, Medical Research Council, Glasgow, United Kingdom) in a

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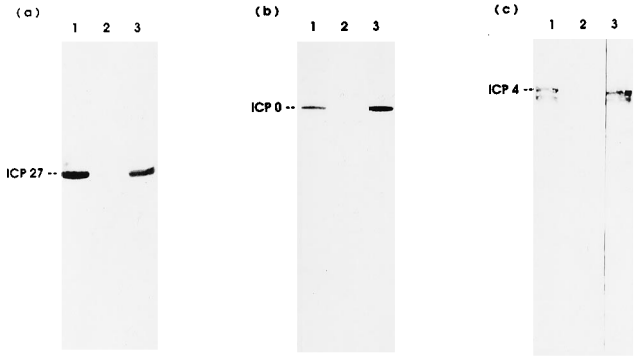

FIG. 1. Expression of ICP27, ICP4, and ICP0 by recombinant vaccinia viruses. Cell extracts were prepared and subjected to SDS-PAGE as described in Materials and Methods. The proteins were transferred to nitrocellulose and probed with antibodies specific for HSV-2 ICP27 (a), ICP0 (b), and ICP4 (c). (a) Lane 1, extract from BHK-21 cells infected with vv ICP27; lane 2, extract from BHK-21 cells infected with control vaccinia virus (vtk$^-$); lane 3, extract from BHK-21 cells infected with HSV-2. (b) Lane 1, extract from BHK-21 cells infected with vv ICP0; lane 2, extract from BHK-21 cells infected with control vaccinia virus (vtk$^-$); lane 3, extract from BHK-21 cells infected with HSV-2. (c) Lane 1, extract from BHK-21 cells infected with vv ICP4; lane 2, extract from BHK-21 cells infected with control vaccinia virus (vtk$^-$); lane 3, extract from BHK-21 cells infected with HSV-2.

pSC11 derivative plasmid. The recombinant ICP27 vaccinia virus vector was used to transfect wild-type vaccinia virus (strain WR)-infected CV-1 cells.

The ICP0 gene (HSV-2 strain HG52) was reconstituted without the two introns from two plasmids, Bam p and Bam g (provided by D. J. McGeoch). Plasmid Bam p carries the *Bam*HI P fragment of the HSV-2 genome cloned in pAT153. It contains the 3′ part of ICP0 gene. Plasmid Bam g corresponds to the *Bam*HI G fragment of the HSV-2 genome cloned in pAT153. It carries both the 5′ part of the ICP0 gene and the 3′-terminal sequence of the ICP4 gene. The 5′ part of the gene was reconstituted as a *Hin*dIII-*Bam*HI (327-bp) fragment which was generated by reverse transcriptase-mediated PCR using poly(A)$^+$ RNA from HSV-2 (HG52)-infected BHK-21 cells treated with phosphonoformate and harvested at 16 h postinfection. The whole cDNA of ICP0, without introns, was reconstituted by inserting the *Hin*dIII-*Bam*HI 327-bp PCR fragment in pRIT13564 digested with *Hin*dIII-*Bam*HI to create pRIT14071. A 2,515-bp-long *Bgl*II-*Asp*718 fragment containing the whole cDNA of ICP0 was inserted into the vaccinia virus transfer vector pUBL5212 (pSC11 derivative) cut by *Bgl*II-*Asp*718, resulting in pRIT14072. This plasmid was used to transfect wild-type vaccinia virus (strain WR)-infected CV-1 cells.

The complete sequence of ICP4 was constructed from two plasmids, pBB17 and Bam g (provided by B. C. Barnett and D. J. McGeoch, Medical Research Council). pBB17 is a pUC19-derived plasmid with the *Kpn*Ia-*Hin*dIII K fragment of the HSV-2 genome and therefore carried a large part of the 5′ sequence of the ICP4 gene. The cDNA coding for the 5′ part of ICP4 was isolated as a 3,493-bp-long *Bgl*II-*Asp*718 fragment from pRIT13643 (unpublished results) and ligated together with a 1,125-bp *Asp*718-*Sph*I fragment (3′ end of the ICP4 gene) derived from pRIT14069. This cloning eliminated the *a* sequence present 3′ downstream ICP4 coding sequence. These two ligated fragments were inserted into the *Bgl*II-*Sph*I-digested vaccinia vector, pULB5212 (pSC11 derivative). The recombinant ICP4 vaccinia virus vector pRIT14070 was used to transfect wild-type vaccinia virus (strain WR)-infected CV-1 cells.

Recombinant plaques were screened by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) coloration and subsequently tested for protein expression by Western blot (immunoblot) analysis as described below. After plaque purification twice, recombinant vaccinia virus stocks were made on BHK-21 cells infected at a multiplicity of infection (MOI) of 0.02. It should be noted that the titer of the recombinant ICP4 vaccinia virus stock was approximately 10-fold lower than the titer usually used for other recombinant vaccinia viruses.

SDS-PAGE and Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting were performed on cell extracts derived from BHK-21 cells infected for 24 h at an MOI of 10 with either vv ICP27, vv ICP4, vv ICP0, or vtk$^-$. A lysate of BHK-21 cells infected with HSV-2 (MS) (MOI of 10, 24-h infection) in the presence of phosphonoacetic acid (200 μg/ml, final concentration) was used as a positive control. Cell extracts were electrophoresed on SDS-polyacrylamide gels (7.5% for vv ICP4 and 10% for vv ICP27 and vv ICP0). The proteins were transferred to nitrocellulose and probed with an antibody specific for ICP27, ICP4, or ICP0 (supplied by H. Marsden, MRC) (Fig. 1). Presence of the expected ICP27 protein was detected in both the HSV-2- and vv ICP27-infected cells extracts (Fig. 1a, lanes 3 and 1, respectively) but not in the vtk$^-$-infected cell extract (lane 2). Similarly, recombinant ICP0 protein, with an electrophoretic mobility similar to that ICP0 synthesized in HSV-2-infected cells, was detected in vv ICP0- but not vtk$^-$-infected cell extract (Fig. 1b). Two bands of ICP4 were observed in an extract from cells infected with HSV-2 (Fig. 1c, lane 3). Similar immunoreactive bands were detected in the vv ICP4-infected cell lysate (lane 1) but not in the vtk$^-$-infected cell extract (lane 2).

Immunization of mice. Each mouse was immunized on days 0 and 7 with 10^7 50% tissue culture infective doses ($TCID_{50}$) of vaccinia virus strains in a 50-μl volume in the hind footpads. Immunization with UV-inactivated HSV-2 (MS) served as the positive control. For UV inactivation, 10^7 $TCID_{50}$ of HSV-2 (MS) was exposed to a germicidal UV lamp (G15T8; 15 W; Sylvania Electric Products, Danvers, Mass.) for 3 min at a distance of 3 cm. This procedure resulted in a reduction of 5 log units in virus titer. For some experiments, 10^7 $TCID_{50}$ of live HSV-1 (KOS) was used for immunization. The parent vaccinia virus (vtk$^-$) was used as the negative control.

Virus challenge. Prior to challenge, usually on day 14, the left flank area was depilated by a combination of hair clipping (Oster Animal Hair Clipper, Milwaukee, Wis.) and use of the chemical Nair (Carter-Wallace, Inc., New York, N.Y.). The animals were anesthetized with Metofane (methoxyflurane; Pitman-Moore, Inc., Mundelein, Ill.). Following depilation, a total of 20 scarifications were made in an approximately 4-mm^2 area. To such scarifications, 10 μl of virus was added. For the challenge, 5.2×10^3 $TCID_{50}$ (10 50% infective doses [ID_{50}]) and 2.6×10^5 $TCID_{50}$ (500 ID_{50}) were used as low and high doses, respectively. Mice were coded and observed for lesion development, encephalitis, and mortality. Animals that did not develop lesions until day 14 were considered protected. A few mice developed lesions that cleared before day 14, and the mice remained healthy afterwards; these were also considered protected mice. The mice that developed lesions and encephalitis and were subsequently moribund were designated the unprotected group. Severity of the lesions was scored as follows: 1+, vesicle formation; 2+, local erosion and ulceration of the local lesion; 3+, mild to moderate ulceration; 4+, severe ulceration, hind limb paralysis, and encephalitis.

MAbs. For T-cell depletion, anti-CD4 (G.K. 1.5 ascites fluid; ATCC TIB 207), anti-CD8 (2.43 ascites fluid; ATCC TIB 210), and anti-HLA-DR5 (HB151 ascites-negative control; ATCC HB151) monoclonal antibodies (MAbs) were used. Pharmingen (San Diego, Calif.) reagents, namely, rat anti-mouse CD4-fluorescein isothiocyanate (catalog no. 01064D), rat anti-mouse CD8-phycoerythrin (catalog no. 01045B), rat immunoglobulin G2a-k (IgG2a-k)-fluorescein isothiocyanate (isotype control; catalog no. 11024C), and rat IgG2a-k-phycoerythrin (isotype control; catalog no. 11025A) were used for the flow cytometry analysis.

In vivo T-cell depletion. Mice were vaccinated on days 0 and 7 and challenged on day 21. Depleting and control MAbs (0.5 mg per mouse) were administered intravenously on days 14, 16, 20, 22, 28, and 35. Two mice from each MAb-treated group were sacrificed on days 29 and 35, and the efficiency of depletion was assessed by two-color flow cytometry (Consort-30 FACScan Flow cytometer; Becton Dickinson, Mountain View, Calif.).

Adoptive transfer experiments. Naive BALB/c mice were immunized twice with either vv ICP27 or vtk$^-$ as described above. Fourteen days after the first immunization, spleens were collected and a single-cell suspension was prepared. Splenocytes were fractionated into either CD4$^+$ or CD8$^+$ T-cell subsets, using CD4- or CD8-enriching columns obtained from R & D Systems, Minneapolis, Minn. (catalog no. MCD4C-1000 for CD4 and catalog no. MCD8C-1000 for CD8). The procedure described by R & D Systems was adhered to exactly to obtain CD4$^+$ or CD8$^+$ T-cell subsets. Unfractionated and enriched T-cell subsets (10^7 cells in 100 μl of phosphate-buffered saline [PBS; pH 7.2]) were injected intravenously slowly into CB17 SCID or BALB/c nude mice. At 24 h after adoptive transfer, recipients were infected on the flank with HSV-2 (MS) as described above. Samples of the fractionated cell populations were checked for purity by fluorescence-activated cell sorting (FACS).

Antibody assays. On day 14 after the first immunization, sera were collected and checked for vaccinia virus-specific (IgG1 and IgG2a) and ICP27-specific (IgG) antibodies. Antibody produced against vaccinia virus was assayed by enzyme-linked immunosorbent assay (ELISA) as described by Nguyen et al. (15). Briefly, sucrose gradient-purified vtk$^-$ was heat inactivated (56°C for 30 min) and sonicated for 2 to 3 min, and an optimal quantity was used to coat ELISA plates. Serum dilutions from test and naive mice were added, after which washed plates were reacted with specific antibodies to the IgG1 and IgG2a isotypes (Pharmingen). The quantity of antibody of each isotype bound was computed from a standard curve run simultaneously, measuring the quantity of purified isotype bound to plates coated with polyspecific anti-IgG.

ICP27-specific antibodies were determined by enhanced chemiluminescence (ECL)-Western blot assay as described by Dalessio and Ashley (6). Briefly, baculovirus-infected cells expressing ICP27 were lysed with a mechanical glass homogenizer in 0.1× PBS at 4°C and spun for 45 min at 50,000 rpm. The supernatant was collected, and the protein concentration was measured by spectrophotometry. Each gel was loaded with 1,320 μg of this protein. Separated proteins were transferred to polyvinylidene difluoride (Millipore, Bedford, Mass.) paper; the whole paper was blocked with PBS containing 0.05% Tween 20 and 5% nonfat milk and cut into several strips. Each strip was allowed to react for 2 h at room temperature with various concentrations of mouse sera diluted in blocking buffer (PBS [pH 7.2] containing 5% nonfat milk). Then strips were washed four times with PBS-Tween 20 and allowed to react with goat antimouse IgG (catalog no. 1030-05; 1:5,000 dilution; Southern Biotechnology Associates,

Inc., Birmingham, Ala.). The strips were washed four times, ECL-Western blotting detection system reagents (Amersham, Arlington Heights, Ill.) were added, and the strips were incubated for 1 min. The strips were covered with a plastic sheet and exposed to X-ray film (Hyperfilm-ECL; Amersham) for 30 s. Film was developed with a Kodak X-Omat processor, and a band at 63 kDa (2) was detected (with the help of a prestained SDS-PAGE standard low-range molecular weight marker; catalog no. 161 0305; Bio-Rad, Hercules, Calif.).

Delayed-type hypersensitivity (DTH). Test antigens were injected in 20-μl volumes in the ear pinna, and ear thickness was measured with a screw gauge meter (Oditest, H. C. Kroeplin GHBH, Germany) 24, 48, and 72 h later as described in detail elsewhere (10). Test antigens included UV-inactivated HSV-2 (MS) partially purified by sucrose density gradient and with an infectivity titer of 10^5 $TCID_{50}$ prior to UV inactivation. UV-inactivated vv ICP27 (10^5 $TCID_{50}$) or Vero cell culture extract was also used. Mice received either HSV or vaccinia virus in one ear and tissue culture extract in the other. Results were expressed as the mean increase in ear thickness (five mice per group) that occurred before and after antigen injection. The values obtained were analyzed for statistical significance by Student's *t* test, using the Starview 512 (Macintosh) statistics program.

Cytokine profile of vv ICP27-vaccinated mice. One milliliter of spleen cells (10^6 cells per ml) from the immunized or control mice was suspended in RPMI 1640–10% PBS and stimulated with UV-inactivated HSV-1 (KOS) at an MOI of 1.5. The cells were incubated at 37°C in 12-well culture plates, and 72 h later, the cells and supernatant were collected. The supernatant was aliquoted and stored at −20°C for cytokine analysis (sample I). The pellet was resuspended in 1 ml of RPMI 1640–10% PBS, restimulated with HSV-1 (KOS) as described above, and incubated at 37°C. After 72 h, the cells were collected and centrifuged and the supernatant was collected and preserved as described above (sample II). Controls included unstimulated cells and concanavalin A-stimulated or UV-inactivated vv ICP27-stimulated culture supernatants. Samples I and II were screened for gamma interferon (IFN-γ) (by ELISA, using an ELISA kit from Gibco BRL, Gaithersburg, Md.), interleukin-4 (IL-4) (by CT4.S bioassay), and IL-2 (by CTLL-2 cell bioassay). For IFN-γ detection, 100-μl aliquots of serially diluted samples were added to antimouse IFN-γ-coated plates. RPMI 1640–10% PBS and recombinant mouse IFN-γ (standard, provided along with the kit) were included as negative and positive controls, respectively. The plates were covered, incubated for 1 h at 37°C, and then washed twice in wash buffer. One hundred microliters of detector complex (anti-mouse biotinylated IFN-γ plus streptavidin conjugated with peroxidase) was added, and the mixture was incubated for 1 h at 37°C. After three washings, 100 μl of *p*-nitrophenylphosphate substrate (provided with the kit) was added, and the mixture was incubated for 30 min at 37°C. The plates were read at 405 nm with an ELISA plate reader (ELISA AUTO reader, model EL 310; Bio-Tech Instruments, Inc., Burlington, Vt.). The standard curve was obtained by plotting the mean absorbance for each standard on the vertical (*y*) axis versus the corresponding IFN-γ concentration on the horizontal (*x*) axis. The concentration of IFN-γ in the sample was determined from the mean absorbance and the corresponding concentration.

IL-2 bioassay. CTLL-2 cells (generously provided by Cynthia Watson and William Paul, National Institutes of Health, Bethesda, Md.) were grown on modified RPMI 1640 (CTLL medium) containing 10% fetal bovine serum, 10% L-glutamine (100 mM), 500 μl of 2-mercaptoethanol, 10% penicillin (100 IU/ml), streptomycin (100 μg/ml), and 10% sodium pyruvate (0.1 M). Samples were diluted serially on 96-well round-bottom microtiter plates (Falcon no. 3077; Becton Dickinson, Paramus, N.J.). Specificity of the proliferation was determined by adding 10 μg of rat anti-mouse IL-2 (Pharmingen catalog no. 18161D) or IL-4 (Pharmingen catalog no. 18031D), or both, per ml. CTLL cells were washed four times in CTLL medium to remove any residual IL-2 and adjusted to a concentration of 10^5 cells per ml. Fifty microliters of this cell suspension was added to all wells, and the wells were incubated for 48 h at 37°C; 50 μl of [^{3}H]thymidine (catalog no. 2404105; ICN Radiochemicals, Irvine, Calif.) containing 1 μCi was added to all wells, and the wells were incubated for 18 h. The cells were harvested with a cell harvester (Inotech, Lansing, Mich.) and dried, and radioactivity (counts per minute) was measured with the Trace-96 program in a β counter (Inotech). Recombinant murine IL-2 (catalog no. CY-24-025; Biosource International, Camarillo, Calif.) was used as the standard, and RPMI 1640–10% PBS was used as a negative control. The concentrations of IL-2 in the samples were determined from the standard curve derived from recombinant murine IL-2. For IL-4 detection in culture supernatants, a similar CT4.S cell bioassay was used.

RESULTS

Protection against zosterification with recombinant vaccinia virus expressing HSV IE proteins. Three recombinant vaccinia viruses expressing IE proteins of HSV-2 were used to immunize three strains of mice, after which animals were challenged in the flank with HSV-2 (MS) and the percentage of animals developing zoster skin lesions was recorded. vv ICP27 was highly effective at protecting BALB/c mice but not the two other strains of mice tested (C57 and C3H) (Table 1). Moreover, neither vv ICP0 nor vv ICP4 protected any of the three mouse strains. The level of protection mediated by vv ICP27 in BALB/c mice was considered substantial, since almost all animals were protected against challenge with 500 ID_{50} of challenge virus.

TABLE 1. Protection against zosteriform lesions by recombinant vaccinia viruses in different mice strains[a]

Immunogen	% of protected mice on day 14 postchallenge			
	BALB/c		C3H, 10 ID_{50}	C57, 10 ID_{50}
	500 ID_{50}[b]	10 ID_{50}		
vv ICP27	90	100	0	0
vv ICP0	0	0	0	0
vv ICP4	0	0	0	0
HSV-2 (UV inactivated)	100	100	100	100
vtk$^-$	0	0	0	0

[a] Group of 10 mice were vaccinated on days 0 and 7, challenged on day 14 with HSV-2 (MS), and coded. Severity of the lesions was graded as described in Materials and Methods.

[b] Virus dose used for challenge.

To analyze the possible mechanism(s) of protection involved in the immunity mediated by vv ICP27, BALB/c mice were immunized with recombinant virus and then the function of the two major T-cell subtypes was suppressed by repeated administration of a subset-specific MAb. The antibody suppression regimen used was shown to be effective by FACS analysis measuring the fraction of splenocytes that expressed $CD4^+$ and $CD8^+$ T cells in sample animals after treatment (Table 2). Our results clearly indicate that the immunity induced by vv ICP27 involved $CD4^+$ T cells (Table 3). Accordingly, the immunity was completely abrogated in animals in which $CD4^+$ T cells were suppressed, but $CD8^+$-suppressed animals remained solidly immune.

Further evidence that the immunity induced by vv ICP27 involved the function of $CD4^+$ rather than $CD8^+$ T cells was obtained by immunizing BALB/c mice with vv ICP27 and then transferring either immune $CD4^+$- or $CD8^+$-enriched T-cell populations to SCID or nude mice and challenging recipient animals with HSV-2. The results shown in Table 4 are for an experiment performed with SCID mouse recipients. A similar pattern emerged from a repeat experiment with SCID mice as well as with nude mice recipients. It is clearly evident that protection was observed only in animals that received immune splenocytes and that upon fractionation into $CD4^+$ and $CD8^+$ T cells, protection occurred only in recipients of $CD4^+$ T cells.

Immune responses induced by vv ICP27. Our data indicate that solid protection against HSV-2 challenge is induced by

TABLE 2. Efficiency of T-cell depletion[a]

Mouse group	% of $CD4^+$ cells	% of $CD8^+$ cells
Nondepleted	30.9	11.8
$CD4^+$ depleted	0.6	26.6
$CD8^+$ depleted	34.0	2.1
HLA-DR5 treated	26.8	14.1

[a] Efficiency of depletion was determined by two-color flow cytometry analysis of splenocytes from vv ICP27-immunized sample mice on day 29 after the first immunization. Each mouse received 0.5 mg of MAb intravenously on days 14, 16, 20, 22, 28, and 35 after the first vaccination. In a second experiment, we achieved 98.2 and 94.8% depletion of $CD4^+$ and $CD8^+$ T cells, respectively, after MAb treatment. In this experiment again, all anti-$CD4^+$ MAb-treated mice developed lesions and the anti-$CD8^+$ MAb-treated mice remained solidly immune.

TABLE 3. Effect of T-cell subset depletion in vv ICP27-immunized BALB/c mice: abrogation of vv ICP27-induced protection after anti-CD4 MAb treatment[a]

Days post-challenge	% Developing lesions				
	Group A, anti-CD4	Group B, anti-CD8	Group C, anti-HLA-DR5	Group D, no antibody treatment	Group E, vtk$^-$ immune
3	0	0	0	0	0
5	60 (2.0)	20 (1.0)	0	0	20 (1.0)
9	100 (3.6)	0	0	0	40 (3.0)
11	100 (4.0)	0	0	0	100 (3.8)

[a] Groups A to D were immunized with vv ICP27 on days 0 and 7, and group E was immunized with vtk$^-$. Each mouse was injected with 0.5 mg of MAb on days 14, 16, 20, 22, 28, and 35 and challenged with 10 ID_{50} (5.2×10^3 $TCID_{50}$) on day 21. The values show percentages of mice that developed lesions in groups of five mice. One of the mice that received the anti-CD8 MAb developed a mild lesion on day 5 which eventually healed. Values in parentheses indicate the mean severity of the lesions, scored as described in Materials and Methods.

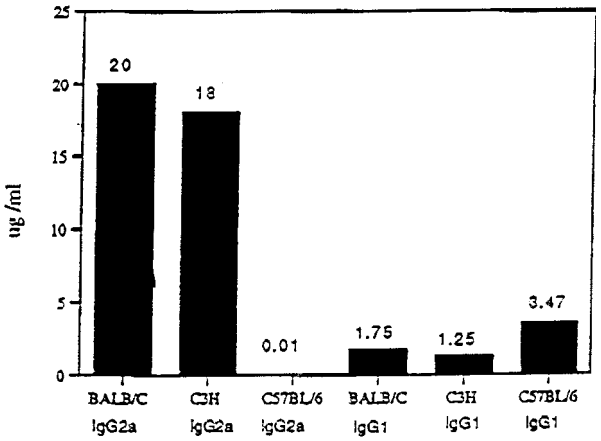

FIG. 2. Vaccinia virus-specific antibody response after vaccination with vv ICP27. Mice were immunized on days 0 and 7, and serum was collected, pooled (on day 14), and checked for vaccinia virus-specific antibodies by ELISA.

immunization with ICP27 but that such protection occurs only in BALB/c mice. Explanations for the failure to protect C57 or C3H mice against HSV-2 challenge could include an absence of an immune response to ICP27 in the two unprotected strains or perhaps immune responses which failed to induce adequately components which mediate protection. All three mouse strains immunized with vv ICP27 were tested for antibody production against both vaccinia virus and ICP27. The pattern of response against vaccinia virus was the same in both BALB/c and C3H (unprotected) mice. Both strains developed both IgG1- and IgG2a-specific antibodies, with the latter isotype present at a higher concentration than IgG1 (Fig. 2). Interestingly, C57 mice responded with a different isotype profile, with IgG1 antibodies predominating. With respect to anti-ICP27 IgG antibodies, both the protected BALB/c and non-protected C3H strains responded positively, as judged by a qualitative ECL-Western blotting analysis (Fig. 3). Attempts to measure IgG1 and IgG2a antibodies to ICP27 by ELISA were confounded by low titers and unreproducable results.

As documented previously, protection against zosteriform lesions was seemingly attributable to CD4$^+$ T-cell function (14). In consequence, various aspects of CD4$^+$ T-cell function were measured in the three mouse strains following immunization with vv ICP27. Our results clearly show differences in the nature of the CD4$^+$ T-cell response between the protected and unprotected strains. Mice of all three strains were tested for DTH responses to both HSV and vaccinia virus. Such responses against HSV were shown by others to be a property of CD4$^+$ T cells (14). As shown in Table 5, the patterns of DTH response to HSV differed between the protected BALB/c and unprotected mouse strains: whereas all three strains responded positively and approximately equally to vaccinia virus, responses to the HSV antigen occurred only in BALB/c mice.

TABLE 4. Prevention of zosteriform lesions in SCID mice by adoptive cell transfer[a]

Days post-challenge	% of animals that developed lesions					
	CD4$^+$	CD8$^+$	CD4$^+$ and CD8$^+$	vv ICP27-primed splenocytes	UV-inactivated HSV-2-primed splenocytes	vtk$^-$-primed splenocytes
3	0	0	0	0	0	0
6	0	100	0	0	0	100
9	0	100	0	0	0	100
14	0		0	0	0	100

[a] A total of 10^7 cells of either unfractionated T cells or T-cell subset collected from the spleen 14 days postimmunization were transferred into groups of four mice; 24 h later, animals were coded, challenged with 10 ID_{50} of HSV-2, and then observed for the development of zosterification. Mice given no cell transfer or transfer of splenocytes from nonimmune mice were not protected. Clinical severity of the lesions was scored as described in Materials and Methods.

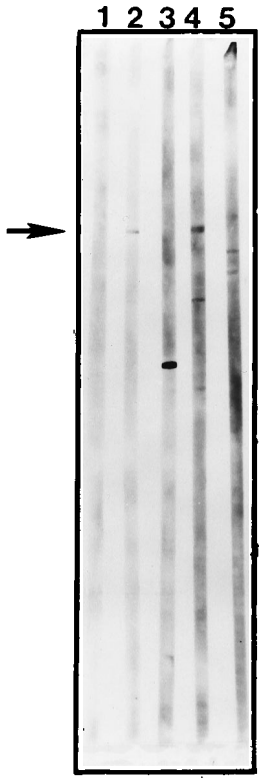

FIG. 3. IgG antibody response against ICP27 detected by ECL-Western blotting analysis. Mice were immunized and bled as described in Materials and Methods. The sera were diluted 1:500 before used for the assay. Lane 1, preimmune BALB/c serum; lane 2, serum from BALB/c mice vaccinated with vv ICP27; lane 3, BALB/c vtk$^-$-immune serum; lane 4, C3H vv ICP27-immune serum; lane 5, C3H vtk$^-$-immune serum. The ICP27 band (63 kDa) was detected with the help of a molecular weight marker. Mouse serum reacting to the ICP27 protein is indicated by an arrow.

TABLE 5. Development of DTH in different mouse strains vaccinated with vv ICP27[a]

Immunogen	Mean ± SD increase in ear thickness over the prechallenge thickness after 48 h (mm^2)					
	BALB/c		C3H		C57	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
vv ICP27	7.4 ± 1.0*	11.0 ± 1.4	2.6 ± 0.6**	16.4 ± 1.5	2.2 ± 1.3***	19.0 ± 3
vtk^-	1.6 ± 0.6	8.2 ± 1.1	1.2 ± 0.5	18.6 ± 1.7	1.2 ± 0.5	18.6 ± 2.1
HSV-1 (KOS)	13 ± 2	ND	13.6 ± 3.1	ND	14.8 ± 2.1	ND

[a] Each group of five mice was immunized with one of the immunogens on days 0 and 7. As a positive control, 10^7 $TCID_{50}$ of HSV-1 (KOS) was injected similarly. For the DTH assay, each mouse was injected with 10^5 $TCID_{50}$ of UV-inactivated HSV-2 in the right ear pinna and Vero cell extract in the left (group 1) or with 10^5 $TCID_{50}$ of UV-inactivated vv ICP27 in the right ear pinna and CV-1 cell extract in the left (group 2). Ear thickness in mice given cell extracts did not exceed 2.8 U. ND, not done. * versus **, $P = 0.0001$; * versus ***, $P = 0.0001$; ** versus ***, $P = 0.55$ (Student's t test).

Finally, the patterns of cytokine responsiveness in the mouse strains were measured in splenocytes stimulated in vitro with HSV as well as with control stimulants. The results shown in Table 6 again indicate differences in the patterns of responsiveness to HSV between ICP27-immune BALB/c mice and immune C57 and C3H mice: whereas immune BALB/c mice responded to HSV with a cytokine pattern dominated by type 1 cytokines (IFN-γ and IL-2 [13]), such responses in C57 and C3H mice were significantly less. However, in such mice, the IL-4 response was far greater than that seen in BALB/c mice. Whereas the three ICP27-immunized mouse strains showed apparent differences in cytokine stimulation with HSV, the responses of all three strains to vaccinia virus antigen were entirely of the type 1 cytokine profile. Moreover, mice immunized with HSV-1 generated a splenic T-cell response which had a type 1 cytokine profile.

DISCUSSION

We have shown that immunization of BALB/c mice with a recombinant vaccinia virus expressing the IE gene ICP27 of HSV-2 protects mice against zosteriform lesions and death caused by challenge with HSV-2. Although protection was evident in BALB/c mice, neither C57 nor C3H mice were protected following immunization with vv ICP27. Furthermore, none of the three strains immunized with recombinant vaccinia viruses expressing two other IE proteins, ICP0 and ICP4, were protected against zosteriform lesions. Two other IE regulatory proteins, ICP4 and ICP47 of HSV-1, were studied for the ability to confer protection against HSV-1 challenge in mice. Neither induced protection (1, 12).

One explanation for the failure to observe protection after immunization with a recombinant vector is that the encoded protein is not expressed in vivo. This explanation was shown not to be valid for ICP4 and ICP47, both of which induced some protein-specific immune responses (1, 12). In the present report, we failed to document whether the ICP0 and ICP4 proteins were expressed and recognized by the immune system in vivo, but at least in vitro, both vectors expressed proteins and mice immunized with them developed anti-vaccinia virus responses (data not shown). Moreover, failure of expression was clearly not the case with the vv ICP27 construct, since in BALB/c mice, this protein was a highly effective inducer of protective immunity. Why the same construct failed to induce protection in the two other mouse strains remains to be resolved. Possible explanations include the lack of recognition of ICP27 by the unprotected mice, an unlikely event given the large size of the ICP27 protein, or the generation of a response which failed to include components of immunity responsible for mediating protection in the model system used for analysis. Our results support the latter notion. Thus, the challenge system that we chose for measuring protection was the mouse zosteriform spread model, which is based on a cutaneous lesion that results from virus spreading to the site by way of the nervous system. The model is considered valuable since it

TABLE 6. Cytokine production by splenocytes from immune mice stimulated in vitro with antigen[a]

Immunogen	In vitro restimulation	Concn (pg/ml)								
		BALB/c			C3H			C57		
		IFN-γ	IL-2	IL-4	IFN-γ	IL-2	IL-4	IFN-γ	IL-2	IL-4
vv ICP27	HSV	2,450	1,800	15	350	100	130	100	100	280
	vtk^-	3,700	4,400	<10	5,700	4,200	<10	6,600	3,350	<10
	ConA	3,600	4,500	300	7,200	4,500	280	6,600	3,400	300
vtk^-	HSV	<100	<100	<10	<100	<100	25	<100	<100	<10
	vtk^-	4,000	4,250	10	7,200	3,300	<10	8,200	2,200	<10
	ConA	4,000	4,450	280	5,700	3,400	320	8,250	3,300	320
HSV-1 (KOS)	HSV	2,800	3,300	<10	3,400	3,200	<10	3,900	3,500	<10
	vtk^-	<100	<100	<10	<100	<100	<10	<100	<100	<10
	ConA	3,400	4,400	300	3,900	3,450	300	4,000	4,350	260

[a] Mice immunized on days 0 and 7 were killed on day 14. Splenocytes were in vitro restimulated with HSV-1 (KOS) (UV inactivated) or vtk^- (UV inactivated) at an MOI of 1.5 or with concanavalin A (ConA; 5 μg/10^6 cells per ml) and incubated for 72 h at 37°C in a 12-well plate. The cells were scraped and centrifuged, and the supernatants (sample I) were aliquoted and stored at −20°C. The pellet was restimulated with the antigens mentioned above and incubated for another 72 h. The supernatants (sample II) were collected and stored. Data were obtained from sample II except that for concanavalin A-treated cells, 72-h data are presented (since the cells died after 72 h of initial restimulation). IFN-γ was detected by ELISA; IL-2 and IL-4 by were detected bioassay. HSV-1 (KOS) (10^7 $TCID_{50}$) was used as the immunogen to serve as a positive control.

mimics what may occur during HSV recrudescence in humans (17). Moreover, in previous studies, protection and clearance of virus from the cutaneous site were shown to be mainly a property of $CD4^+$ T cells (14, 18). Our results indicate that viral clearance may represent predominantly the effects of type 1 $CD4^+$ T cells. In the present report, we provide further evidence that protection against zosterification involves principally $CD4^+$ T cells. Accordingly, BALB/c mice immunized with ICP27 lost immunity when $CD4^+$ T cells were removed by antibody suppression. This effect did not occur if $CD8^+$ T cells were suppressed. More convincing evidence that $CD4^+$ T cells subserved a protection role came from the results of experiments involving adoptive transfer of immune T-cell populations to immunocompromised mice. In such experiments, adoptive transfer of $CD4^+$ T cells, but not $CD8^+$ T cells, provided protection against zosterification.

Regarding the function of $CD4^+$ T cells responsible for cutaneous immunity, our results support the notion that cells with a type 1 cytokine profile are principally involved and, as a corollary, that C3H and C57 mice were not protected because they failed to generate adequate type 1 cytokine-producing T-cell responses; the data showed that only vv ICP27-immunized BALB/c mice developed a DTH response to HSV antigen. In most systems, including HSV, DTH responses are usually considered a measure of Th1 cells (4). In the HSV system, DTH responses are inhibited by anti-type 1 cytokine treatment (8), and in preliminary experiments using reverse transcriptase-mediated PCR, we have found predominantly type 1 cytokine mRNA in cells isolated from DTH lesions (9).

Further evidence that protection afforded by vv ICP27 in BALB/c mice correlated with the induction of cells with a type 1 cytokine profile came from measurements of splenic T-cell cytokine responses to HSV. It was clearly evident from such studies that immunized BALB/c mice responded with predominantly a type 1 profile, whereas the two unprotected strains examined generated responses that showed mainly a Th2 pattern. It will be of particular interest to understand mechanistically why both C3H and C57 mice responded to vv ICP27 with a type 2 cytokine response, since both strains responded to infection with vaccinia virus itself as well as to infection with HSV-1 with a type 1 cytokine response.

It has become apparent from many recent observations that the pattern of IgG isotype response to antigens reflects the major type of cytokine-producing type of CD4 cells induced (7, 15). For example, a predominance of type 1 cytokines, particularly IFN-γ, is associated with antibody responses in which IgG2a predominates over IgG1. This is a common pattern for most antibody responses to live virus infection, including HSV-1 infection in BALB/c mice. We have also noted that both BALB/c and C3H mice respond to both HSV-2 (data not shown) and vaccinia virus with mainly an IgG2a antibody response. However, C57 mice responded to both viruses with mainly an IgG1 response. The reason for such differences, which also have been noted by others (5), is not known. Attempts to establish if BALB/c and the unprotected C3H and C57 strains generated different isotype profiles to ICP27 following immunization were confounded by weak responses measured by ELISA. However, as measured by a sensitive ECL-Western blotting approach, both BALB/c and C3H mice produced IgG antibody, indicating once again that the reason for the failure to induce protection in C3H mice was not a failure to recognize the ICP27 protein.

In conclusion, our results demonstrate that a vaccinia virus construct expressing ICP27 of HSV-2 induces protection against virus challenge, although this occurred only in one of three mouse strains investigated. The protection was seen to be mediated by $CD4^+$ T cells which expressed mainly a type 1 cytokine profile. The reason why ICP27, when expressed by a recombinant vaccinia virus, induced a predominant Th1 response only in the BALB/c mice is under investigation. We are attempting to manipulate immunization conditions to determine if C3H mice can be induced to generate Th1 CD4 T-cell responses to ICP27 and if such mice are protected from challenge.

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