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Protection of mice from fatal measles encephalitis by vaccination with vaccinia virus recombinants encoding either the hemagglutinin or the fusion protein

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ABSTRACT **Vaccinia virus recombinants encoding the hemagglutinin or fusion protein of measles virus have been constructed. Infection of cell cultures with the recombinants led to the synthesis of authentic measles proteins as judged by their electrophoretic mobility, recognition by antibodies, glycosylation, proteolytic cleavage, and presentation on the cell surface. Mice vaccinated with a single dose of the recombinant encoding the hemagglutinin protein developed antibodies capable of both inhibiting hemagglutination activity and neutralizing measles virus, whereas animals vaccinated with the recombinant encoding the fusion protein developed measles neutralizing antibodies. Mice vaccinated with either of the recombinants resisted a normally lethal intracerebral inoculation of a cell-associated measles virus subacute sclerosing panencephalitis strain.**

Measles virus is the causative agent of a highly infectious childhood disease commonly characterized by fever, upper respiratory tract symptoms, and an extensive rash, all of which disappear in conjunction with the development of the immune response. The high incidence of measles and the occurrence of major complications such as pneumonia, secondary infections, acute encephalitis, or, more rarely, subacute sclerosing panencephalitis (SSPE) have led to the widespread use of attenuated measles vaccines, which to a large extent have proved their efficacy. However, on the African continent and in other developing parts of the world, measles infections are often more severe and remain a major cause of mortality in children. The present-day measles vaccine lacks some of the features that enabled the success of the smallpox vaccine—namely, thermostability and convenience of production. We have therefore undertaken the construction of a recombinant smallpox vaccine that encodes measles virus surface proteins required for immunization. Recent cloning of the two measles virus envelope genes (1–4), the hemagglutinin (HA) gene and the fusion (F) gene, as well as the development of methods for insertion of foreign genes into the vaccinia virus (VV) genome (for review, see refs. 5 and 6) have set the background for this project. Both the HA gene and the F gene have been inserted separately into the VV genome. Expression of these measles virus proteins in VV recombinant infected cells as well as posttranslational processing and membrane transport have been demonstrated. The majority of mice vaccinated with a single dose of either of the recombinant viruses developed measles neutralizing antibodies, and mice inoculated with the VV recombinant expressing the HA gene developed anti-HA antibodies. Vaccinated mice also resisted a normally lethal intracerebral injection of a cell-associated measles SSPE strain.

MATERIALS AND METHODS

Cells and Viruses. VV Copenhagen strain and the temperature-sensitive mutant (N7) derived from it were used for construction of recombinants (7). VV stocks were made in chicken embryo fibroblasts. VV infections were also carried out in baby hamster kidney (BHK-21) cells or thymidine kinase-deficient mouse L cells (LTK^- cells), depending on the particular experiment. A mouse-adapted strain of a cell-associated measles SSPE virus was used for the challenge experiments (8). The measles virus Hallé strain produced in Vero cells was used in neutralization and hemagglutination inhibition tests.

Construction of VV Recombinants. Molecular cloning procedures were essentially as described by Maniatis *et al.* (9). Site-directed mutagenesis was conducted according to Zoller and Smith (10). VV recombinants were constructed following published techniques (11–13) with several modifications (14, 15). Briefly, cDNA coding for the measles HA or F gene from the Hallé strain was inserted downstream of the VV 7.5-kDa promoter previously inserted within the VV thymidine kinase gene (vector pTG186poly). VV thymidine kinase-deficient recombinants containing the foreign sequences were obtained by simultaneous infection of chicken embryo fibroblasts with the VV temperature-sensitive N7 mutant and cotransfection with the appropriate recombinant plasmid as well as VV wild-type DNA. An initial selection in chicken embryo fibroblasts at the nonpermissive temperature against the temperature-sensitive mutant was followed by selection for non-temperature-sensitive VV thymidine kinase-deficient recombinants on mouse LTK^- cells in the presence of 100 μg of 5-bromodeoxyuridine per ml. Recombinants were further subcloned before making viral stocks and purifying the viruses.

Immunoabsorption and Immunofluorescence of Measles Proteins. BHK-21 cells (10^6 cells in 35-mm Petri dishes) infected with VV recombinants or the wild-type virus at ≈0.1 plaque-forming unit (pfu) per cell for 15 hr were labeled for 4 hr with 50 μCi of [^{35}S]methionine (1000 Ci/mmol; 1 Ci = 37 GBq) per ml in a medium lacking unlabeled methionine. Cells were then scraped from the dish and centrifuged. Cell pellets were lysed in 400 μl of immunoabsorption buffer (16), disrupted by sonication, and centrifuged twice at 10,000 × *g* for 5 min, and the supernatants were immunoabsorbed to protein A-Sepharose using 5 μl of guinea pig anti-measles serum and 4 mg of protein A-Sepharose. The guinea pig serum and protein A-Sepharose had been previously incu-

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Abbreviations: HA, hemagglutinin; F, fusion; VV, vaccinia virus; SSPE, subacute sclerosing panencephalitis; pfu, plaque-forming units.

bated with extracts from wild-type VV-infected cells to saturate nonspecific binding sites. Material bound to the protein A-Sepharose immunoglobulin complex was washed in appropriate buffer (16) and then was removed by boiling in an ionic detergent. Immunoabsorbed proteins were analyzed by $NaDodSO_4$/PAGE followed by autoradiography. For immunofluorescence, cells on glass slides were infected with ≈0.1 pfu per cell for 15 hr, then fixed with acetone, rehydrated with phosphate-buffered saline (PBS; 135 mM NaCl/26 mM KCl/3 mM $CaCl_2$/5 mM $MgCl_2$/70 mM Na_2HPO_4/15 mM KH_2PO_4, pH 7.5), and incubated for 30 min at room temperature with a 1:200 dilution of the anti-HA monoclonal antibody CL55 (17) or the anti-F monoclonal antibody 19FF10 (a gift of E. Norrby, Karolinska Institüt, Stockholm). The slides were washed with PBS and incubated again for 30 min at room temperature with a 1:100 dilution of an anti-mouse antibody conjugated with fluorescein isothiocyanate. After extensive washing with PBS, the cells were observed with a Nikon fluorescent microscope. Under these conditions, no fluorescent staining was observed in uninfected cells or cells infected with wild-type VV.

Immunogenicity and Protection of Mice. BALB/c mice (4–5 weeks old) were scarified with 4×10^7 pfu of purified VV recombinants. Sera were assayed for hemagglutination inhibition (18) or neutralization (17). Mice were challenged by intracerebral injection of 50 μl of a 10% suspension of mouse brain previously inoculated with a cell-associated measles virus SSPE strain (8).

RESULTS

Construction of VV Recombinants. Full-length cDNAs encoding the HA protein or the F protein of the measles virus Hallé strain have been previously isolated, and their sequences have been determined (2, 3). As a first step prior to transferring these genes into the VV genome, they were inserted into the single-stranded phage vector M13 (Fig. 1). The HA cDNA carried on the vector designated M13TG2101 was modified by localized mutagenesis to contain a *Pst* I site 11 nucleotides preceding the translation initiation codon, thus creating the sequence **CTGCAGATCATCCACAATG**. Similarly, the 5′ portion of the F cDNA (vector designated M13TG2103-5′) was modified to contain an *Nsi* I site four

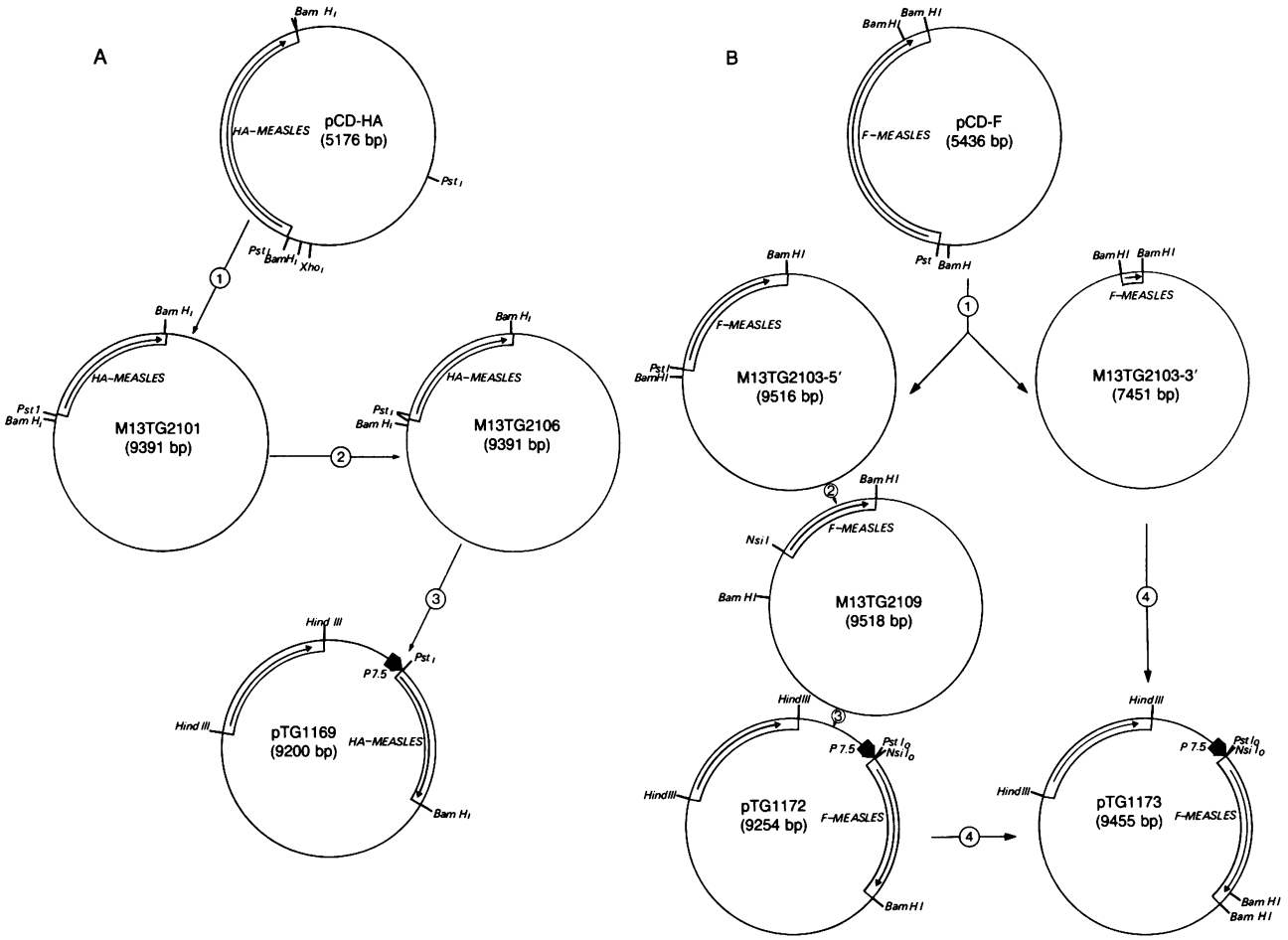

FIG. 1. Strategy for the construction of recombinant plasmids used in the transfer of the measles HA and F genes to the VV genome. (*A*) The cDNA encoding measles HA protein, flanked by *Bam*HI sites in plasmid pCD-HA, was excised with *Bam*HI and inserted into the *Bam*HI site of an M13 vector (step 1). A new *Pst* I site was introduced by site-directed mutagenesis 11 nucleotides upstream from the first codon of the measles HA gene (step 2). The entire measles HA coding sequence between the new *Pst* I site and the *Bam*HI site was inserted downstream of the 7.5-kDa promoter on the VV transplacement vector pTG186poly (step 3). (*B*) The cDNA encoding measles F protein contained in pCD-F was inserted in two separate pieces into an M13 vector using the *Bam*HI sites (step 1). The 5′ portion of the cDNA was then modified by site-directed mutagenesis to contain an *Nsi* I site as described in the text (step 2). The modified 5′ fragment was then excised with *Nsi* I and *Bam*HI and was inserted downstream of the 7.5-kDa promoter on the VV transplacement vector pTG186poly previously cut with *Pst* I and *Bam*HI (step 3). The 3′ fragment of the F gene was added to the pTG1172 plasmid using the *Bam*HI site available (step 4). In the three plasmids generated (pTG1169, pTG1172, and pTG1173), the 7.5-kDa promoter is represented by a thick arrow. Immediately upstream from the promoter is the left portion of the VV *Hin*dIII J fragment. Downstream of the F gene and the HA gene is the right portion of the VV *Hin*dIII J fragment. pTG 1H, flanked by *Hin*dIII sites, was the bacterial replicon used for propagation of the plasmids. bp, Base pair(s).

nucleotides upstream of the second of the two ATGs that could be used in translation initiation, creating the sequence **ATGCATATCATG**. The second ATG was chosen because the signal sequence generated was estimated to be of sufficient size as compared to signal sequences from other viral transmembrane proteins. The procedure followed was also designed to allow the removal of a long stretch of 580 noncoding nucleotides that might be responsible for the low level of synthesis of the F protein in measles-infected cells from the F mRNA. The modified HA and F genes were then inserted, by way of the newly created *Pst* I site or *Nsi* I site, immediately downstream of the VV 7.5-kDa promoter contained on the vaccinia transplacement vector pTG186poly. The recombinant plasmids pTG1169 and pTG1173, which contained the measles HA gene and F gene, respectively, flanked by VV sequences, were then used to insert the foreign sequences into the VV genome by *in vivo* homologous recombination resulting in the generation of VV thymidine kinase-deficient recombinants designated VVTGHAM2-2 and VVTGFM1173.

Synthesis of Measles Proteins in Cells Infected with the VV Recombinants. BHK-21 cells were infected with either of the VV recombinants for 15 hr; then newly synthesized proteins were labeled for 4 hr with [^{35}S]methionine. Infected-cell lysates were prepared, and proteins were immunoabsorbed to protein A-Sepharose beads using a guinea pig serum directed against measles virus antigens. PAGE of the immunoabsorbed proteins followed by autoradiography demonstrated that the VVTGHAM2-2 recombinant containing the HA gene induced the synthesis of proteins of approximately 80 and 75 kDa (Fig. 2). The largest protein comigrated with the fully glycosylated species of HA protein from measles virus (data not shown). The smaller polypeptide corresponds to incompletely glycosylated HA protein. When PAGE was carried out on samples devoid of mercaptoethanol, HA protein dimers migrating with the same electrophoretic mobility as authentic measles HA protein dimers could be observed (data not shown). Cells infected with the VVTGFM1173 recombinant encoding the F gene yielded three polypeptides that were immunoabsorbed with the anti-measles serum. The polypeptide of ≈60 kDa displays the electrophoretic mobility expected of the uncleaved translation product of the F gene, also referred to as F_0. The two smaller polypeptides (40 and 20 kDa) correspond to the natural products that appear in measles infection after cleavage of F_0. The heterogeneity in size of the F_2 polypeptide is also characteristic of natural F_2 and is best explained by variation in its carbohydrate content. To investigate whether the HA and F proteins induced by the VV recombinants appeared on the infected cell surface, LTK$^-$ cells were infected and were tagged 15 hr later with either HA-specific or F-specific mouse monoclonal antibodies followed by an anti-mouse fluorescent antibody. Characteristic membrane fluorescence was apparent on cells infected with either of the recombinants (Fig. 3). Moreover, cells infected with the HA-expressing recombinant absorbed African green monkey erythrocytes, which further demonstrates the external presentation of the HA protein. Thus the VV recombinants induced the synthesis of measles proteins of the correct size and were apparently both processed and transported to the cell surface just as authentic measles proteins.

Immunization of Mice with the VV Recombinants. Mice (4–5 weeks old) were scarified on the tail with 4×10^7 pfu of either of the VV recombinants. Three weeks later, blood samples were taken from the animals, and the neutralizing antibodies or anti-HA antibodies were assayed. The majority of the animals displayed a significant level of neutralizing antibodies when vaccinated with either of the recombinants (Table 1). In addition, most animals inoculated with the VVTGHAM2-2 recombinant developed anti-HA antibodies

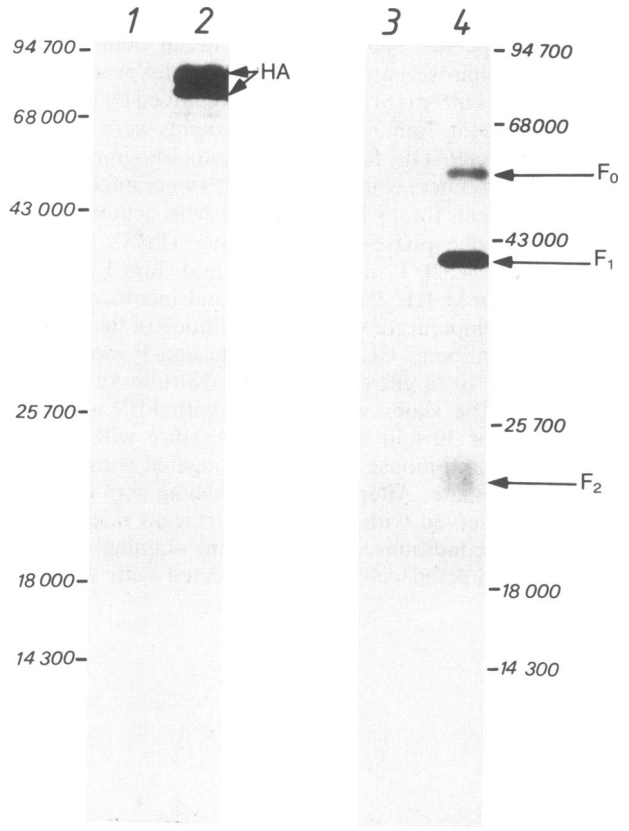

FIG. 2. Measles virus proteins synthesized in BHK-21 cells infected with VV recombinants. Cells infected for 15 hr with wild-type VV (lanes 1 and 3), VVTGHAM2-2 (lane 2), or VVTGFM1173 (lane 4) were labeled with [^{35}S]methionine for 4 hr. Cell lysates were then immunoabsorbed with antiserum directed against measles proteins in the presence of protein A-Sepharose. Immunoabsorbed proteins were analyzed by PAGE followed by autoradiography. The positions of molecular mass markers (in Da) used for calibration and the position of measles proteins are indicated.

(Table 1). Three weeks after vaccination, mice were challenged intracerebrally with a lethal dose of a cell-associated measles SSPE strain. Several days later, the unvaccinated animals or mice vaccinated with wild-type VV displayed characteristic symptoms (running in circles, weight loss, and ruffled coats). Deaths in control animals occurred from the 5th day after challenge up to the 12th day. Out of a total of 24 control animals, only 3 survived, whereas all animals previously vaccinated with either of the recombinants showed no signs of disease and survived until day 50 postinfection. Afterwards, three mice inoculated with VVTGHAM2-2 displayed encephalitis symptoms and succumbed. All others, however, remained healthy (Table 2).

DISCUSSION

This work has demonstrated expression of the two envelope antigens of measles virus in cells infected with recombinant VV containing the corresponding genes. Both HA and F proteins were not only processed but also transported to the cell surface as in a natural measles virus infection. Moreover, either of the VV recombinants induced the appearance of measles neutralizing antibodies in most of the vaccinated mice and protected all of them against fatal acute encephalitis. The few deaths that occurred among animals vaccinated with VVTGHAM2-2 >50 days after challenge may be explained by the nature of the mouse model employed in which the HA protein is expressed at low levels. Alterna-

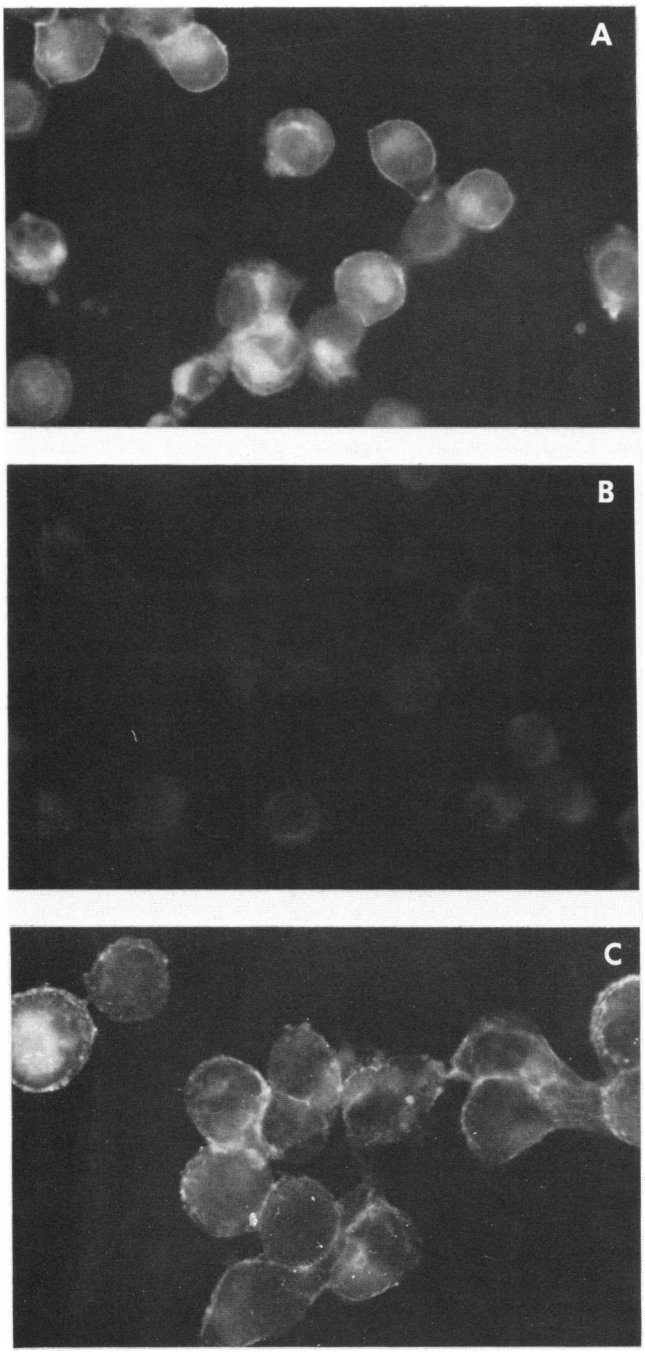

FIG. 3. Fluorescent antibody labeling of the measles HA or F proteins in LTK$^-$ cells infected with VV recombinants. (*A*) Cells were infected with VVTGHAM2-2 for 15 hr and incubated with monoclonal anti-HA antibody followed by fluorescein isothiocyanate anti-mouse antibody. (*B*) Cells were infected as in *A* and then incubated with monoclonal anti-F antibody followed by fluorescein isothiocyanate anti-mouse antibody (negative control). (*C*) Cells were infected with VVTGFM1173 for 15 hr and incubated with monoclonal anti-F antibody followed by fluorescein isothiocyanate anti-mouse antibody.

tively, immunity induced by the HA antigen may be intrinsically poorer than immunity induced by the F antigen. Retarded neurological disease despite the prior presence of antibodies against the HA protein has been previously reported (17, 19).

Recent work with two other members of the Paramyxovirus family has shown that each of the two envelope proteins from human respiratory syncytial virus (20–23) or from simian virus 5 (24) is able to induce neutralizing antibodies and prevent viral replication in the lungs of susceptible animals. In respiratory syncytial virus infection, the fusion protein was slightly more protective than the major glycoprotein (22), whereas in simian virus 5 infection, the contrary occurred (24). However, in these experimental systems, unvaccinated animals do not display symptoms and do not succumb to the challenge infection. In the measles model we studied, control mice exhibit symptoms characteristic of encephalitis and die from the viral infection. Protection by a single inoculation of the VV recombinants is consequently compelling evidence for the potency of the recombinant vaccines.

Table 1. Immune response in mice vaccinated with VV recombinants

VV recombinant	HI titer (*n*)	NT (*n*)
VVTGHAM2-2	<20 (1)	<40 (1)
	40 (4)	40 (1)
	80 (8)	80 (8)
	160 (9)	160 (3)
	320 (2)	320 (7)
		640 (1)
		1280 (2)
VVTGFM1173		20 (3)
		40 (5)
		80 (3)
		160 (10)
		320 (3)

Hemagglutination inhibition (HI) titers are expressed as the reciprocal of the highest serum dilution that completely prevented hemagglutination of African green monkey erythrocytes. Sera from uninfected animals or wild-type VV-infected controls gave titers of <20. Neutralization titers (NT) are expressed as the reciprocal of the highest serum dilution that completely neutralized 50 pfu of measles virus during a 1-hr incubation at 37°C. The titers from uninfected or wild-type VV-infected controls were <40. *n*, Number of mice.

Measles virus belongs to the *Morbillivirus* genus within the Paramyxoviridae family and therefore shares many properties with three other members of the genus: canine distemper virus (CDV), rinderpest (RPV), and peste des petits ruminants (PPRV). Cross-reactive F antigens between measles, CDV, and RPV have been well documented (25–29). Measles HA is also clearly cross-reactive with HA from RPV (26, 28), and some monoclonal antibodies directed against measles HA can neutralize, albeit poorly, CDV (17). Such homologies suggest that the VV recombinants expressing measles proteins might protect animals against other *Morbillivirus* infections.

Protection against a challenge measles infection could be made still more effective by inserting both the HA and F genes into the same VV recombinant. In this respect, it is

Table 2. Protection of mice from fatal measles encephalitis

Vaccine	Animals challenged, no.	Animals surviving, no.
	Experiment 1	
Wild-type VV	12	2
VVTGHAM2-2	19	19*
	Experiment 2	
None	9	1
Wild-type VV	3	0
VVTGFM1173	22	22

Three weeks after scarification with 4×10^7 pfu of either recombinant virus, mice were challenged intracerebrally with a lethal dose of a cell-associated measles SSPE strain. Animals that died within the first 3 days were not included in the results, as deaths could be attributed to damage caused by the inoculation route.
*After day 50, three animals in this group succumbed.

important to recall that the failure of killed measles virus to stably protect from measles has been attributed to an antigenically inactive F component in the vaccine (30). Thus, although anti-HA antibodies alone are protective in an animal model (17) and a purified *Morbillivirus* F protein is also protective (31), a more reliable genetically engineered vaccine should combine the two proteins.

The usefulness of the mouse encephalitis model in predicting the efficacy of a measles vaccine for humans is largely unknown. Other animal models that more closely reproduce the human disease will therefore be sought. If these prove successful, VV recombinants expressing measles antigens in association with antigens of other pathogens could contribute significantly to solving major health problems in developing countries. The ability to insert large amounts of DNA into the VV genome (32) and to express multiple foreign antigens (33) should lead to a serious consideration of the potential for vaccinating against a number of important diseases with a single dose of live recombinant VV.

Note Added in Proof. During continued observation of animals for up to 270 days, additional deaths occurred in the group vaccinated with VVTGHAM2-2 and challenged with measles virus, in contrast to the group vaccinated with VVTGFM1173, which remained fully protected as of 180 days after challenge.

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