Expression of the F and HN Glycoproteins of Human Parainfluenza Virus Type 3 by Recombinant Vaccinia Viruses: Contributions of the Individual Proteins to Host Immunity

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cDNA clones containing the complete coding sequences for the human parainfluenza virus type 3 (PIV3) fusion (F) and hemagglutinin-neuraminidase (HN) glycoprotein genes were inserted into the thymidine kinase gene of vaccinia virus (WR strain) under the control of the P7.5 early-late vaccinia virus promoter. The recombinant vaccinia viruses, designated vaccinia-F and vaccinia-HN, expressed glycoproteins in cell culture that appeared to be authentic with respect to glycosylation, disulfide linkage, electrophoretic mobility, cell surface expression, and, in the case of the HN protein, biological activity. Cotton rats inoculated intradermally with vaccinia-HN developed serum neutralizing antibody titers equal to that induced by respiratory tract infection with PIV3, whereas animals receiving vaccinia-F had threefold lower neutralizing antibody titers. A single immunization with either recombinant vaccinia virus induced nearly complete resistance in the lower respiratory tract of these animals. With regard to protection in the upper respiratory tract, animals immunized with vaccinia-HN or vaccinia-F exhibited reductions in PIV3 replication of >3,000-fold and 6-fold, respectively. This large difference (>500-fold) in reduction of PIV3 replication in the upper respiratory tract was in contrast to the relatively modest difference (3-fold) in serum neutralizing antibody titers induced by vaccinia-HN versus vaccinia-F. This dissociation between the level of neutralizing antibodies and protection suggested that immunity to PIV3 is complex, and that immune mechanisms other than serum neutralizing antibodies make important contributions to resistance to infection. Overall, under these experimental conditions, vaccinia-HN induced a substantially more protective immune response than did vaccinia-F.

Human parainfluenza virus type 3 (PIV3), a member of the family *Paramyxoviridae*, is second only to respiratory syncytial virus (RSV) as a primary cause of severe lower respiratory tract disease in children under 1 year of age (4). Older children and adults are frequently reinfected, resulting in less severe upper respiratory tract (URT) illness. The virus occurs worldwide, and virtually all children are infected by age 4 (4).

Attempts to develop an effective PIV3 vaccine have been largely unsuccessful. Field evaluations of killed PIV3 vaccines demonstrated an increase in virus-specific serum antibodies in vaccinees without significant protection against disease (11, 28, 44). The primary immunogens of PIV3 are its two surface glycoproteins. The F protein, (*M_r* 60,000 to 63,000), mediates viral penetration, hemolysis, and syncytium formation, which is a characteristic cytopathic effect and may be important in cell-cell spread (5, 13, 21, 33, 43, 46). The F protein is synthesized as an inactive precursor *F*₀, which is activated by proteolytic cleavage into two disulfide-linked subunits, *F*₁ and *F*₂ (12, 25, 36). The second surface glycoprotein, HN (*M_r* 69,000 to 72,000), is responsible for viral attachment and exhibits both hemagglutination and neuraminidase activities (13, 33, 35, 43, 46). Ray et al. have shown that subcutaneous inoculation of hamsters with solubilized envelope glycoproteins can protect their lower respi-

ratory tract against challenge with PIV3 (32). However, immunization with the individual glycoproteins has not been described, and the relative contributions of the individual glycoproteins in eliciting a protective immune response was unknown.

The development of techniques for producing live vaccinia virus (VV) recombinants expressing foreign genes has provided a novel method for studying immune responses to individual proteins (19). Several viral surface antigens, including those of the pneumovirus RSV, have been successfully expressed by VV recombinants and shown to elicit protective neutralizing antibodies in experimental animals (9, 27). Recently, the nucleotide sequences of the PIV3 F and HN mRNAs were determined from virion RNA and cDNA clones (8, 41). We have constructed cDNAs which contain the complete coding regions of the F and HN protein genes under the control of the early-late P7.5 VV promoter, and we have inserted each of these genes into the thymidine kinase (TK) gene of the WR strain of VV. The resulting recombinants, designated vaccinia-F and vaccinia-HN, were then used to inoculate cotton rats, an experimental animal permissive for PIV3 infection. In this paper, we describe (i) the expression of the F and HN glycoproteins by VV recombinants in cell culture, (ii) the development of serum neutralizing antibodies to both F and HN glycoproteins by cotton rats inoculated with vaccinia-F or vaccinia-HN, (iii) the induction by either VV recombinant of resistance to viral replication in the lower respiratory tract (LRT) of cotton rats

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challenged with PIV3, and (iv) a 500-fold greater restriction of PIV3 replication in the URT of animals previously immunized with vaccinia-HN compared with those immunized with vaccinia-F, despite the development of only a 3-fold greater neutralizing antibody titer by the vaccinia-HN group.

MATERIALS AND METHODS

Viruses and cells. Purified stocks of VV (WR strain) and recombinant VV were prepared from infected CV-1 monolayers as described previously (9). PIV3 strain 47885 was plaque purified three times and propagated in HEp-2 cells. Assays for biological activity of expressed proteins were performed in HEp-2 cells. Recombinant VV vSC-14 (3) contains the hepatitis B virus surface antigen gene under the control of the P7.5 promoter and was used as a negative control.

Construction of VV recombinants. Two previously described overlapping F cDNA clones were cut at a unique *Bgl*I site and ligated to form a complete cDNA of the F mRNA (41). To remove noncoding sequences and homopolymer tails, F and HN cDNAs were inserted into the *Pst*I sites of pTZ18R (Pharmacia Fine Chemicals, Piscataway, N.J.) and the replicative form of M13 mp18, respectively. A single-stranded template for mutagenesis was prepared according to the supplier's protocols for pTZ18R and conventional methods for M13. Oligonucleotide-directed mutagenesis (49) was utilized to introduce *Bam*HI sites within 3 to 6 nucleotides of the translational initiation and termination sites. The oligonucleotides (which were antisense) were as follows, identified according to gene and gene end, with the *Bam*HI sites and termination or initiation codons in boldface type: F-3', 5'TAGAGTCGACCTGCAGGATCCCTGTCTTTGTTTGAAT; F-5', 5'TATTGAGGTTGGCATGGATCCATGCAAGCTTGGCACT; HN-3', 5'GTGCCAAGCTTGCATGGATCCTTATGATTAAGTGCAGCTTGG; HN-5', 5'CCAGTATTCATCTCGGATCCCCGGGTACCGAGCTCG.

After the mutagenesis, the sequences of the HN and F cDNAs were confirmed in their entirety by dideoxynucleotide sequencing with synthetic oligonucleotides as primers (48).

Construction of the VV recombinants. The resected F and HN cDNAs were isolated by digestion with *Bam*HI, made blunt ended with T4 DNA polymerase, and inserted into the *Sma*I site of the VV plasmid coexpression vector pSC11 (3). Features of this construct include the following: (i) the foreign cDNA is under the control of the early-late VV promoter P7.5; (ii) the vector contains the *Escherichia coli* β -galactosidase gene under the control of the late VV promoter P11; and (iii) both chimeric genes are flanked by VV TK gene sequences. Upon transfection of VV-infected cells, the TK gene sequences direct homologous recombination into the TK locus of the VV genome, yielding live recombinant VV that are identified colorimetrically by the presence of β -galactosidase activity. Methods for obtaining such recombinants have been described in detail (3, 9). The mRNAs expressed from the chimeric 7.5K-F and 7.5K-HN genes would each contain a 5' noncoding sequence of 52 nucleotides consisting of 44 nucleotides representing the start of the VV 7.5K mRNA, 5 nucleotides representing the filled-in *Bam*HI site, and the original 3 PIV3-specific nucleotides preceding the PIV3 translational start codon (3, 9, 38). In comparison, F and HN mRNAs expressed by PIV3 contain 5' noncoding regions of 193 and 73 nucleotides, respectively (8, 40, 41).

Radiolabeling and immunoprecipitation of PIV3-infected cell lysates. CV-1 cells were infected with VV recombinants (30 PFU per cell) for 2 h and then incubated for 4 to 6 h with 20 μ Ci of [35 S]methionine per ml or 16 h with 50 μ Ci of [3 H]glucosamine per ml. Similar conditions were used to label cells infected with PIV3 (5 PFU per cell), except that the labeling period for [35 S]methionine was 24 to 28 h postinfection. Infected cell lysates were prepared as described previously (26), and immunoprecipitations were carried out with anti-PIV3 hyperimmune cotton rat sera and protein A-Sepharose (Sigma Chemical Co., St. Louis, Mo.). Samples were boiled in electrophoresis sample buffer with or without 2-mercaptoethanol and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18).

Indirect immunofluorescence. CV-1 cells were grown on glass cover slips and infected with 1 PFU of PIV3 or a VV recombinant per cell. Cells were incubated for 24 h and fixed with 3% (vol/vol) Formalin in phosphate-buffered saline for cell surface immunofluorescence. Fixed cells were reacted sequentially with anti-PIV3 hyperimmune cotton rat serum, rabbit anti-cotton rat serum, and goat anti-rabbit immunoglobulin G fluorescein-conjugated antibody (Cooper Biomedical, Inc., West Chester, Pa.) (27).

Immunological response to immunization and protective efficacy. Young adult cotton rats (*Sigmodon fulviventer*) were immunized by intradermal (i.d.) inoculation with one or both VV recombinants ($10^{8.0}$ PFU each) or by intranasal (i.n.) inoculation with PIV3 ($10^{6.0}$ PFU); 28 days after immunization, animals were bled and then challenged with PIV3 i.n. ($10^{5.0}$ PFU). Animals were sacrificed 4 days after challenge, at the peak of PIV3 replication in the respiratory tract (unpublished observation). PIV3 specific antibody responses were measured by an enzyme-linked immunosorbent-assay (ELISA) with purified PIV3 as the antigen (31). Hemagglutination inhibition assays were performed as described previously (6). PIV3 serum neutralizing antibody titers were determined by a complement-enhanced 60% plaque reduction neutralization assay. PIV3 titers in the nasal turbinates and lungs were determined by 50% tissue culture-infective dose (TCID₅₀) assays. Each assay was done in MK-2 cells and included two wells per dilution. The minimum level of detectability was determined by using serial 10-fold dilutions of homogenates in a 500- μ l inoculation volume. Detectable virus in a single well receiving the lowest (10^0) dilution of a 10% (wt/vol) homogenate permitted a minimum level of detectability of $10^{1.3}$ TCID₅₀ per g of tissue. No detectable virus in either well of the lowest dilution of homogenate represents a value $\leq 10^{0.8}$ TCID₅₀ per g of tissue.

RESULTS

Expression of the F and HN glycoproteins by VV recombinants. To examine the intracellular expression of PIV3 glycoproteins by the VV recombinants, CV-1 cells were infected with vaccinia-F or vaccinia-HN, and radiolabeled cell lysates were prepared and analyzed by immunoprecipitation with anti-PIV3 hyperimmune cotton rat serum (Fig. 1).

Polyacrylamide gel electrophoresis under reducing conditions (Fig. 1, lanes d through h) identified the F₀ precursor ($M_r \sim 60,000$) and the F₁ subunit ($M_r \sim 51,000$), but did not resolve the F₂ subunit ($M_r \sim 9,000$). Under nonreducing conditions (Fig. 1, lanes a through c), the F protein expressed by both vaccinia-F and PIV3 migrated as two closely

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immunoprecipitated proteins synthesized by vaccinia-F, vaccinia-HN, or PIV3. CV-1 cells were infected with recombinant VVs or PIV3 and incubated with [35 S]methionine or [3 H]glucosamine (see Materials and Methods). All lysates were clarified and incubated with anti-PIV3 hyperimmune cotton rat (α PIV3) serum. Immunoprecipitates were analyzed by 10% polyacrylamide gel electrophoresis under nonreducing (lanes a through c) or reducing (lanes d through k) conditions. Lanes: a and d, 35 S-labeled proteins from VV vSC-14-infected cells; b and e, 35 S-labeled proteins from PIV3-infected cells; c and f, 35 S-labeled proteins from vaccinia-F-infected cells; g, [3 H]glucosamine-labeled proteins from vaccinia-F-infected cells; h and j, [3 H]glucosamine-labeled proteins from VV vSC-14-infected cells; i, [3 H]glucosamine-labeled proteins from vaccinia-HN-infected cells; k, [3 H]glucosamine-labeled proteins from PIV3-infected cells.

spaced bands (M_r ~60,000 to 63,000). These were interpreted as representing F_0 and one or more electrophoretic species of disulfide-linked F_1 and F_2 (designated $F_{1,2}$), although the bands were not identified individually. This indirectly confirmed the presence of the F_2 subunit and showed that the F protein expressed by vaccinia-F was very similar to that of PIV3 on the basis of both proteolytic processing and disulfide linkage. Furthermore, the F_0 and F_1 proteins expressed by vaccinia-F were glycosylated (Fig. 1, lane g) and on this basis were indistinguishable from the F_0 and F_1 proteins expressed by PIV3 (data not shown).

The HN proteins synthesized by vaccinia-HN (Fig. 1, lane i) and PIV3 (lane k) were indistinguishable by electrophoretic mobility under reducing (Fig. 1) and nonreducing (data not shown) conditions. Consistent with previous reports (43, 46), nonreduced HN protein migrated as a monomer with a slightly increased electrophoretic mobility, indicative of intramolecular disulfide bonds. The HN protein expressed by vaccinia-HN and PIV3 shared this feature of secondary structure. This is in contrast to the HN proteins of other paramyxoviruses such as Sendai virus and Newcastle disease virus, which exist as disulfide-linked multimers under nonreducing conditions (20, 39).

The HN proteins expressed by vaccinia-HN-infected cells were assayed for biological activity by incubating infected HEP-2 monolayers with guinea pig erythrocytes at 4°C. Cells infected with vaccinia-HN (1 PFU per cell) exhibited extensive hemadsorption when assayed at 16 h postinfection (Fig. 2B), whereas monolayers infected in parallel with the parental VV showed no hemadsorption (Fig. 2A). The neuraminidase activity of the expressed HN proteins was demonstrated indirectly by the elution of bound erythrocytes when vaccinia-HN-infected, hemadsorbed monolayers were incubated at 37°C for 30 min (Fig. 2C). Elution of erythrocytes at 37°C was presumed to be due to the neuraminidase activity of the expressed HN protein, although we cannot exclude the possibility that the cells contained an endogenous neuraminidase activity.

The expression of F and HN glycoproteins at the surface of recombinant infected cells was examined by indirect immunofluorescent staining of nonpermeabilized (Formalin-fixed) cells (Fig. 3). The level of expression of PIV3 antigen in cells infected with vaccinia-F (Fig. 3B) and vaccinia-HN (Fig. 3C) was equal to or exceeded that of PIV3 infected cells (Fig. 3A). Under these conditions, syncytium formation specific to vaccinia-F was not clearly demonstrated because of the rapid cytopathic effect of VV in CV-1 cells, consistent with previous results (27).

Serologic response to infection by vaccinia-F and vaccinia-HN. Cotton rats were inoculated i.d. with vaccinia-F, vaccinia-HN, or both to determine the level and nature of serum antibodies induced by the individual glycoproteins. In cotton rats, these conditions of inoculation did not result in dermal lesions with either the parental strain WR VV or the recombinant VVs. However, such inoculations induced high titers of VV-specific serum antibodies (R. A. Olmsted, B. R.

FIG. 2. Demonstration of biological activity by VV-expressed HN protein. HEP-2 monolayers were infected (1 PFU per cell) with vaccinia-HN (B and C) or parental WR VV (A) and incubated at 4°C with guinea pig erythrocytes 16 h postinfection. After photography, the hemadsorbed monolayers were incubated at 37°C for 30 min to demonstrate elution of the erythrocytes (C), suggesting that the expressed HN protein possessed a functional neuraminidase.

FIG. 3. Indirect cell surface immunofluorescence of VV recombinant infected cells. CV-1 cells were grown on glass cover slips and infected (1 PFU per cell) with PIV3 (A), vaccinia-F (B), vaccinia-HN (C), or VV vSC-14 (D). At 24 h postinfection, cells were fixed in 3% Formalin in phosphate-buffered saline for 15 min at room temperature. Fixed cells were incubated for 30 min at 37°C with anti-PIV3 hyperimmune cotton rat serum, washed, incubated for 30 min at 37° with a rabbit anti-cotton rat serum, washed, and incubated for 30 min with a goat anti-rabbit immunoglobulin G fluorescein-conjugated antibody.

Murphy, P. L. Collins, M. Buller, and B. Moss, unpublished data) and high titers of serum antibodies specific to the expressed foreign protein (27) (Table 1), indicating that an active VV infection had followed the inoculation. For com-

parison, other animals were inoculated i.n. with PIV3, which results in a self-limiting, asymptomatic respiratory tract infection that confers complete protection against reinfection (G. A. Prince, manuscript in preparation). Animals were bled 28 days postinoculation, and the sera were assayed for PIV3 antibodies by plaque reduction, hemagglutination inhibition (HI) assay, and ELISA with purified virus as the antigen (Table 1).

Sera from animals receiving both VV recombinants exhibited an ELISA titer equal to that of sera from PIV3-infected animals, whereas animals that received vaccinia-HN or vaccinia-F alone had titers that were 1.5- and 4-fold lower, respectively. Because the antigen for ELISA was whole virus, the titer of sera from PIV3-infected animals would represent reactivity with multiple viral antigens. Animals which received the VV control vSC-14 also exhibited a slight rise in PIV3-reactive ELISA titer when compared with preimmunization sera of this group (Table 1). This response might be due to nonspecific immune stimulation by VV or to a low level of cross-reactivity by VV-specific antibodies.

Infection with vaccinia-HN induced levels of PIV3 specific serum neutralizing antibodies that were equal to that induced by infection with PIV3. In contrast, vaccinia-F infection induced threefold lower neutralizing antibodies than did vaccinia-HN or PIV3 infection. Animals which received both vaccinia-F and vaccinia-HN had titers of serum neutralizing antibodies that were not significantly

TABLE 1. Serum antibody response of cotton rats to PIV3 infection or recombinant VV inoculation^a

Immunization (route)	Dose (PFU)	No. of animals	Reciprocal geometric mean serum antibody titer		
			ELISA ^b	Neutralization ^c	HI ^d
PIV3 (i.n.)	10 ⁶	18	7,525	1,326	145
Vaccinia-F (i.d.) plus vaccinia-HN (i.d.)	10 ⁸ , 10 ⁸	17	8,018	1,069	67
Vaccinia-HN (i.d.)	10 ⁸	15	4,889	1,391	122
Vaccinia-F (i.d.)	10 ⁸	15	1,940	459	≤2
VV vSC-14 (i.d.) ^e	10 ⁸	17	501	<11	≤2

^a Serum was collected from all groups 28 days postimmunization.

^b Purified PIV3 virions were used as the solid-phase antigen. The ELISA titer in VV vSC-14 control group was concluded to be nonspecific binding of VV-induced antibodies (see text). The mean preimmunization ELISA titer of control group was 1:127.

^c Measured by 60% plaque reduction neutralization assay.

^d Dilution that inhibits erythrocyte agglutination by four agglutinating units of virus.

^e VV recombinant expressing hepatitis B virus surface antigen.

TABLE 2. Response of cotton rats to PIV3 challenge^a

Immunization (route)	Dose (PFU)	No. of animals	Recovery of virus from respiratory tract tissues					
			Nasal turbinates			Lungs		
			% With detectable virus	Mean titer $\pm$ SEM ($\log_{10}$ TCID ₅₀ /g) ^b	Reduction in titer ($\log_{10}$) ^c	% With detectable virus	Mean titer $\pm$ SEM ($\log_{10}$ TCID ₅₀ /g) ^b	Reduction in titer ($\log_{10}$) ^c
PIV3 (i.n.)	10 ⁶	12	0	$\leq 0.8 \pm 0.0^d$	≥ 3.9	0	$\leq 0.8 \pm 0.0^d$	≥ 3.4
Vaccinia-HN (i.d.)	10 ⁸	11	36	1.2 ± 0.2	3.5	9	1.0 ± 0.2^d	3.2
Vaccinia-F (i.d.)	10 ⁸	11	100	3.9 ± 0.2^e	0.8	18	0.9 ± 0.1^d	3.3
VV vSC-14 (i.d.)	10 ⁸	11	100	4.7 ± 0.2		100	4.2 ± 0.2	

^a Animals were challenged 28 days postimmunization with 10^{5.0} PFU of PIV3 i.n. Animals were sacrificed 4 days later.

^b Minimum level of detectability was 10^{1.3} TCID₅₀/g (see Materials and Methods).

^c Compared with VV vSC-14 control group.

^d $P < 0.001$ compared with VV vSC-14 control group by Student's *t* test.

^e $P < 0.02$ compared with VV vSC-14 control group by Student's *t* test.

different from animals receiving vaccinia-HN alone or PIV3 by i.n. infection.

The glycoprotein specificity of the serum antibodies also was examined for the ability to inhibit hemagglutination by purified PIV3. Inoculation with vaccinia-HN induced HI titers which were similar to those induced by PIV3 infection (Table 1). Vaccinia-F or vSC-14-specific sera exhibited essentially no detectable HI activity. Sera from animals receiving both recombinants exhibited an HI titer that was twofold lower than that seen for sera from PIV3- or vaccinia-HN-infected animals, perhaps reflecting a slightly decreased immune response to the HN protein when the VV recombinants were administered together.

Protective efficacy of vaccinia-F and vaccinia-HN. To test the ability of the VV recombinants to confer protective immunity, the groups of cotton rats described above were challenged with 10^{5.0} PFU of PIV3 i.n. 28 days postimmunization. Four days later, animals from each group were randomly chosen and sacrificed, and the levels of virus replication in the nose and lungs were measured (Table 2). Control animals inoculated with vSC-14 (a negative control) supported the replication of 4.2 $\log_{10}$ units of virus per g of lung tissue and 4.7 $\log_{10}$ units of virus per g of nasal turbinate (Table 2), levels comparable to those of unvaccinated animals (data not shown). Animals vaccinated i.n. with PIV3 showed no detectable virus growth in either the LRT or URT. Animals receiving vaccinia-HN showed nearly complete protection of the LRT and greatly reduced (3,000-fold) virus replication in the URT. Animals receiving vaccinia-F also showed nearly complete protection of the LRT, but had less protection (sixfold reduction in virus titer) of the URT. The level of PIV3 replication in cotton rats receiving both vaccinia-F and vaccinia-HN recombinants was similar to that in animals which received vaccinia-HN alone (data not shown). Thus, under the conditions of this study, the protection conferred by vaccinia-HN exceeded that conferred by vaccinia-F.

DISCUSSION

Recombinant VV were constructed which carry the complete coding sequences for either the PIV3 F or HN gene under the control of the early-late P7.5 VV promoter. These recombinants expressed HN and F proteins in cell culture which appeared to be authentic with respect to electrophoretic mobility, proteolytic processing, glycosylation, antigenic specificity, and, in the case of the HN protein, biological activity. The proteins were expressed at the surface of

recombinant-infected cells, as well as intracellularly, in an abundance equal to or greater than that found in PIV3-infected cells.

Immunization of cotton rats with the VV recombinants established that each of the two PIV3 glycoproteins administered individually can induce a vigorous immune response that provides substantial protection against PIV3 infection. A single i.d. inoculation of cotton rats with vaccinia-F or vaccinia-HN induced high levels of PIV3-specific serum antibodies. Vaccinia-HN was found to stimulate a 2.5-fold higher titer of PIV3-specific serum antibodies (as measured by ELISA) and a 3-fold higher neutralizing antibody titer (as measured by 60% plaque reduction of PIV3) than did vaccinia-F. The LRTs of both groups of cotton rats were almost completely protected against challenge with PIV3. The URTs of immunized cotton rats, however, differed in the level of immunity induced by the two recombinant viruses.

This incomplete protection in the URT permitted a comparison of the relative abilities of vaccinia-HN and vaccinia-F to restrict replication of PIV3 in vivo. Animals receiving vaccinia-F exhibited a 6-fold reduction of virus growth in the URT compared with control animals, whereas the URT of the vaccinia-HN group exhibited a greater than 3,000-fold reduction in detectable virus. Thus, although vaccinia-HN induced only a 3-fold greater level of serum neutralizing antibodies than did vaccinia-F, it conferred a 500-fold greater level of protection in the URT. This suggested that immune mechanisms in addition to serum neutralizing antibodies were involved in the protective immunity. These additional mechanisms could include cellular immune responses as well as the stimulation of antibodies that are protective in vivo but do not neutralize viral infectivity in vitro, as have been described for influenza A and Sindbis viruses (16, 23, 37, 42). The observation that neither VV recombinant conferred complete protection in the URT was not unexpected because the i.d. route of administration presumably would not have stimulated local immune responses in the respiratory tract (29, 30).

Studies of influenza virus infections in humans have shown that a direct correlation exists between the level of virus replication in the URT and manifestation of clinical symptoms. Although this correlation has not been shown conclusively for PIV3, by analogy with influenza virus a reduction of 3,000-fold in virus replication in the URT as observed in animals immunized with vaccinia-HN would be expected to significantly diminish illness. Additionally, for RSV, serious disease is associated with viral replication in the LRT, and a similar situation probably exists for PIV3.

The high level of protection of the LRT conferred by both the VV recombinants would be expected to block serious viral disease, whereas the level of protection of the URT afforded by vaccinia-HN would permit reduced levels of PIV3 replication, thereby boosting immunity in the respiratory tract without the development of serious illness.

Recently, the expression and protective efficacy of VV recombinants containing the fusion (F) glycoprotein and putative attachment glycoprotein (G) of RSV have been described for the cotton rat model system (9, 27). The RSV F protein is analogous in structure and function to the PIV3 F protein, and the two proteins share moderate amino acid sequence homology (41). The RSV G protein is thought to be the attachment protein and therefore would be analogous to the PIV3 HN protein, but it lacks hemagglutinin and neuraminidase activities and does not share amino acid sequence homology with the PIV3 HN protein. In the cotton rat system, RSV vaccinia-F elicited a sixfold higher titer of neutralizing antibodies than did RSV vaccinia-G and provided greater resistance to replication in both the lungs and noses of immunized animals (27). Thus, for RSV, the F protein appeared to be significantly more immunogenic and protective than the G protein. In contrast, in the present study, recombinant VV expressing the HN protein, the PIV3 counterpart to the G protein, induced a moderately higher level of serum neutralizing antibodies and a much higher level of protection in the URT than did recombinant VV expressing the PIV3 F protein. One possible explanation for the apparent lower immunogenicity of RSV G might be its high carbohydrate content, most of which is likely present in multiple, small, O-linked side chains that might interfere with immune recognition (34, 47).

In tissue culture, monospecific antiserum against the F or HN proteins of simian virus 5 were effective individually in neutralizing the infectivity of released virus, but only anti-F antibodies were effective in preventing viral spread by cell-to-cell fusion (21, 22). As is the case for simian virus 5, rapid and extensive cell-to-cell fusion is a prominent cytopathic effect of PIV3 in tissue culture. These observations suggested that immunity against the HN protein alone might be less protective than immunity against F. However, for PIV3, the observed result (Table 2) was that immunization with either VV recombinant alone was highly protective, and that vaccinia-HN appeared to be more rather than less protective than vaccinia-F.

Attempts at immunoprophylaxis in humans for parainfluenza viruses as a group have met with discouraging results. Field trials with Formalin-inactivated RSV or measles vaccine failed to demonstrate protection against disease (11, 44). Paradoxically, LRT disease of RSV vaccinees and febrile illness and rash in measles virus vaccinees actually was enhanced over that observed in unvaccinated persons during subsequent natural infection (10, 15, 17). Thus, the possibility of disease enhancement after vaccination against parainfluenza viruses is a realistic and serious concern. Recently, RSV disease enhancement in human vaccinees was correlated with an unbalanced immune response, in which vaccinees developed a high titer of circulating antibodies that bound viral protein efficiently (as measured by ELISA) but failed to neutralize viral infectivity effectively (as measured by plaque reduction) (24). In contrast, the serum antibodies induced by the PIV3 vaccinia-F and vaccinia-HN recombinants was balanced with regard to ELISA, HI, and neutralizing titers relative to antibodies induced by viral infection. However, it will be important to resolve the possibility of disease enhancement by direct examination of pulmonary

pathology in immunized, challenged animals. This will also resolve the question of whether antibodies against HN in the absence of antibodies against F can induce disease enhancement (21).

PIV3 and RSV disease is most severe in the very young (2 to 8 months of age). Thus, recipients for any PIV3 or RSV vaccine will have significant levels of maternally derived antibody. Because the general population is no longer exposed or generally immune to variola virus or VV (14, 38), maternal antibody should lack anti-VV activity. Thus, VV has a unique advantage as a vector for immunoprophylaxis of the very young. Maternal antibody might also suppress the immune response to the expressed PIV3 or RSV antigens, a potential problem for any parenterally administered vaccine. The use of a replicating agent such as VV recombinants might overcome this effect. The major obstacle to the use of recombinant VV in humans is the occasional occurrence of postvaccination complications (1). It remains to be seen whether further developments, such as the reduction in virulence associated with the TK⁻ phenotype of VV recombinants (2), can reduce these hazards to an acceptable level. Alternately, the use of other recombinant viral vectors, such as adenovirus (7), could be investigated. The VV recombinants described here represent useful reagents for further analysis of host immunity to PIV3 and for testing the general usefulness of live recombinant viruses as vaccines.

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