Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 1906–1910, March 1986
Medical Sciences

Resistance to human respiratory syncytial virus (RSV) infection induced by immunization of cotton rats with a recombinant vaccinia virus expressing the RSV G glycoprotein

(paramyxovirus/expression vector/vaccination/neutralizing antibody/protective immunity)

NARAYANASAMY ELANGO*, GREGORY A. PRINCE†, BRIAN R. MURPHY†, SUNDARARAJAN VENKATESAN†‡, ROBERT M. CHANOCK†, AND BERNARD MOSS*

*Laboratory of Viral Diseases and †Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Contributed by Robert M. Chanock, November 15, 1985

ABSTRACT **A cDNA copy of the G glycoprotein gene of human respiratory syncytial virus (RSV) was placed under control of a vaccinia virus promoter and inserted into the thymidine kinase locus of the vaccinia virus genome. The recombinant vaccinia virus retained infectivity and expressed a 93-kDa protein that migrated with the authentic RSV G glycoprotein upon polyacrylamide gel electrophoresis. Glycosylation of the expressed protein and transport to the cell surface were demonstrated in the absence of other RSV proteins. Cotton rats that were inoculated intradermally with the infectious recombinant virus produced serum antibody to the G glycoprotein that neutralized RSV *in vitro*. Furthermore, the vaccinated animals were resistant to lower respiratory tract infection upon intranasal inoculation with RSV and had reduced titers of RSV in the nose.**

Human respiratory syncytial virus (RSV) is the most important cause of lower respiratory tract infection in infants and young children (1). It is unfortunate, therefore, that attempts to develop satisfactory inactivated or attenuated vaccines have not been successful (2, 3). Further research is needed to determine the humoral and cell-mediated immune responses to individual viral antigens that are necessary for effective immunoprophylaxis. The envelope of RSV, a member of the family Paramyxoviridae, contains two glycoproteins (4, 5). The larger one, designated G, has a molecular mass of about 90 kDa and is thought to be needed for virus adsorption. The smaller one, designated F because of its probable role in membrane fusion (6), consists of a 70-kDa protein that is proteolytically processed into 48- and 22-kDa products (7, 8). Passive immunization of cotton rats (9) and mice (10) with monoclonal antibodies to either glycoprotein provides protection against lower respiratory tract infection with RSV. Additional studies to delineate the roles of the glycoproteins in protective immunization have been hampered by the difficulty in obtaining sufficient amounts of purified materials. The recent cloning of cDNA copies of the mRNAs for both glycoproteins (11–14), however, opens the possibility of using expression vectors to produce the 90- and 70-kDa proteins.

Vaccinia virus provides a novel eukaryotic vector system (15, 16) that should be particularly suitable for studies with RSV. Surface antigen genes from a variety of DNA and RNA viruses (17–24) have been expressed by recombinant vaccinia viruses. When properly engineered, the proteins were synthesized, processed, and transported to the plasma membrane. In most cases, experimental animals were shown to produce neutralizing antibodies and were protected against a challenge with the corresponding virus. Evidence for priming of a cytotoxic T-cell response also has been obtained with recombinant vaccinia viruses that express influenza virus genes (25, 26). We now describe a recombinant vaccinia virus that expresses the RSV G glycoprotein and protectively immunizes cotton rats against lower respiratory tract infection with RSV.

MATERIALS AND METHODS

Viruses and Cells. Vaccinia virus (WR strain) was grown in HeLa (human) cells and purified from cytoplasmic extracts by sucrose gradient centrifugation. RSV (strain A2) was propagated on HEp-2 (human) cell monolayers.

Isolation of DNA. DNA was isolated from purified vaccinia virus as described (27). Plasmid DNA was prepared by using a modified alkaline sodium dodecyl sulfate ($NaDodSO_4$) procedure (28). Lysozyme was omitted from the lysis step and the DNA was purified from RNA by passage through a Sepharose-4B column. DNA fragments were purified from agarose gels by electroblotting onto DEAE-paper (29).

Oligonucleotide-Directed Mutagenesis. A cDNA copy of an mRNA containing the complete coding sequence for the RSV G glycoprotein was inserted into the *Bam*HI site of the replicative form of M13mp18 phage (30). After the single-stranded phage DNA had been isolated, the 5′ poly(dG) tail plus portions of the noncoding sequence and the 3′ poly(dC) tail were removed by mutagenesis (31) using oligonucleotide I [d(ACTCTAGAGGATCCGCAAACATGTCCAA)] and oligonucleotide II [d(TTACTTAAAAAAAAGGATCCCCGGGTAC)], respectively.

Isolation of Recombinant Vaccinia Virus. CV-1 cells infected with wild-type vaccinia virus were transfected with calcium phosphate-precipitated plasmid DNA as described (32). Thymidine kinase-negative (TK^-) recombinants were isolated by plaque assay on human 143 TK^- cells in the presence of 5-bromo-2′-deoxyuridine (BrdUrd) at 25 μg/ml. For detection of blue plaques, 300 μg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) per ml was used in the agarose overlay (33). After two successive plaque purifications, virus was amplified by infecting HeLa cell monolayers and then large stocks were prepared in HeLa cell suspension cultures.

Protein Analysis. CV-1 monkey cell monolayers of approximately 3×10^6 cells were infected with wild-type or

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: RSV, respiratory syncytial virus; BrdUrd, 5-bromo-2′-deoxyuridine; i.n., intranasal; i.d., intradermal; pfu, plaque-forming unit(s); X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; TK, thymidine kinase.

‡Present address: Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892.

recombinant vaccinia virus at 30 plaque-forming units (pfu) per cell. After 2 hr, cells were resuspended in 5 ml of medium without radioactive precursors or in 2.5 ml of medium with 500 μCi of D-[6-^{3}H]glucosamine (27 Ci/mmol, Amersham; 1 Ci = 37 GBq). After overnight incubation, cells were washed and then lysed with RIPA buffer (50 mM Tris·HCl, pH 7.2/150 mM sodium chloride/1% Triton X-100/0.1% NaDodSO$_4$/1% sodium deoxycholate/1 unit of aprotinin per ml). Polyacrylamide gel electrophoresis and blot analysis of unlabeled proteins were carried out as described (34), using rabbit anti-human RSV serum except that Tween-20 was used as a blocking agent instead of bovine serum albumin (35). The extract containing glucosamine-labeled proteins was first incubated with normal rabbit serum followed by fixed staphylococcal cells (IgGsorb, The Enzyme Center, Boston). After centrifugation, the supernatant was incubated with rabbit anti-human RSV serum and IgGsorb. The bound proteins were eluted, analyzed by electrophoresis through a 10% polyacrylamide gel, and detected by autoradiography.

Immunofluorescence. Approximately 1×10^5 CV-1 cells in monolayer were infected with wild-type or recombinant vaccinia virus at 1 pfu per cell. After 2 hr, the inoculum was removed and cells were incubated with medium for 24 hr. The monolayer was then washed three times with phosphate-buffered saline (PBS)/0.5% bovine serum albumin (BSA) and fixed with 3.7% (wt/wt) formaldehyde in PBS/0.5% BSA for 30 min. Fixed cells were incubated with monoclonal antibody to the RSV G glycoprotein in PBS/0.5% BSA for 30 min, washed three times with PBS/0.5% BSA, and then incubated for 30 min with the fluorescein conjugate of goat antibodies to mouse IgG. The unbound second antibody was removed by washing three times with PBS/0.5% BSA. The cells were air dried, mounted, and photographed.

Immunization of Animals. Cotton rats (*Sigmodon hispidus*) from the Veterinary Resources Branch, Division of Research Services, National Institutes of Health, were immunized at 4 weeks of age with 10^4 pfu of RSV by the intranasal (i.n.) or intradermal (i.d.) route or with $10^{8.5}$ pfu of a recombinant vaccinia virus containing the gene for the RSV G glycoprotein or influenza A H2 glycoprotein (19) by the i.d. route.

Response to Immunization. Serum RSV-neutralizing antibody response was measured by 60% plaque reduction in HEp-2 cell cultures, while specific responses to the RSV G and F glycoproteins were measured by ELISA using immunoaffinity-purified RSV glycoproteins (kindly provided by E. E. Walsh) as antigens (1, 36). Resistance of immunized cotton rats to RSV infection was evaluated by challenging animals 21 days after immunization with 10^4 pfu of RS by the i.n. route. Four days after i.n. challenge, animals were sacrificed by CO_2 inhalation, their nasal tissues (including turbinates) and lungs were homogenized, and the titer of RSV was determined by plaque assay on HEp-2 cells (37).

RESULTS

Construction of Recombinant Vaccinia Virus. Our objective was to insert the entire coding sequence of the RSV G glycoprotein into vaccinia virus so that the authentic protein would be synthesized. To ensure efficient expression, homopolymer tails that had been added during the initial cDNA cloning (12) as well as a segment of RSV DNA containing an out-of-frame ATG near the 5′ end of the cDNA were removed. These alterations were accomplished, as outlined in Fig. 1, by double oligonucleotide mutagenesis in an M13 single-stranded DNA phage vector. The cDNA was then excised from the replicative form of the phage DNA and blunt-end ligated into the unique *Sma* I site of the plasmid coexpression vector pSC11 (33). The important features of pSC11 include: the *P*7.5 promoter, which has early and late vaccinia virus transcriptional regulatory signals; a unique

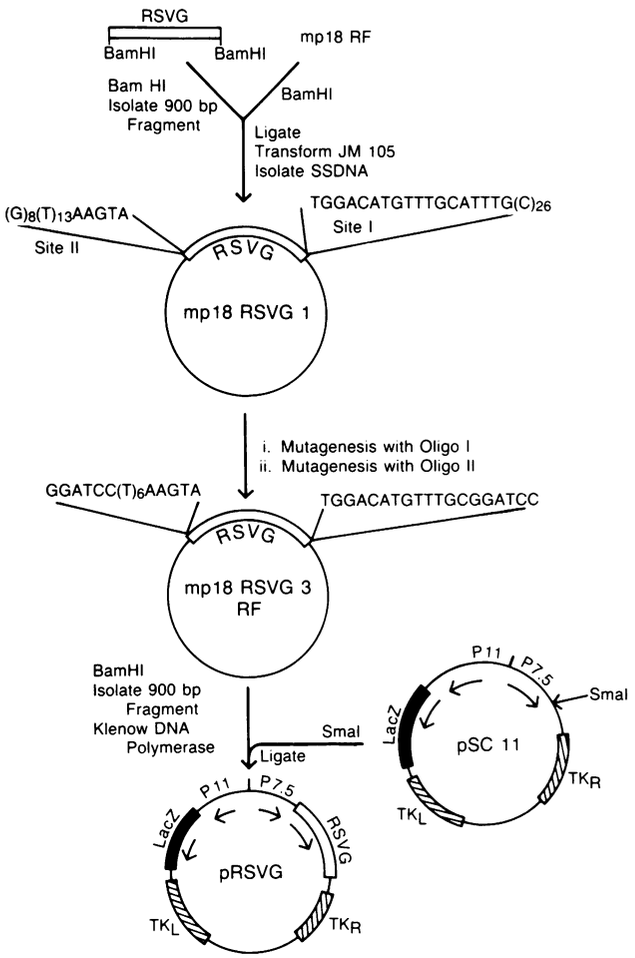

FIG. 1. Construction of recombinant plasmids. *Bam*HI linkers were added to a cDNA copy of the RSV G glycoprotein gene, which was then inserted into the replicative form (RF) of M13mp18 DNA to produce the recombinant mp18 RSVG1. The latter was mutagenized successively with oligodeoxynucleotide I at site I and oligodeoxynucleotide II at site II to form mp18 RSVG3 lacking the homopolymer G and C sequences. The *Bam*HI fragment from the replicative form of mp18 RSVG3 was blunt ended and inserted into the *Sma* I site of the coexpression vector pSC11 to produce pRSVG. Open bar, RSV G gene; line, *P*7.5 and *P*11 vaccinia transcriptional regulatory signals; filled bar, *E. coli lacZ* gene; hatched bar, left ($_L$) and right ($_R$) segments of vaccinia virus TK gene. bp, Base pair; SSDNA, single-stranded DNA.

Sma I site located just downstream of the *P*7.5 promoter for insertion of a foreign gene; the *Escherichia coli* β-galactosidase gene controlled by the *P*11 promoter, which has vaccinia virus late transcriptional regulatory signals; and vaccinia virus TK gene sequences flanking all of the above to direct homologous recombination into the TK locus of the vaccinia virus genome. The plasmid pRSVG (Fig. 1) contains the coding sequence for the RSV G glycoprotein oriented correctly with respect to the *P*7.5 promoter in pSC11. In this construction, the first ATG codon that lies downstream of the RNA start site is the one that naturally begins the open reading frame.

Because of the large size of the vaccinia virus genome, insertion of foreign DNA was accomplished by homologous recombination. CV-1 cells were infected with wild-type vaccinia virus and then were transfected with pRSVG. Serial dilutions of progeny virus were then applied to monolayers of TK$^-$ cells in the presence of BrdUrd to select TK$^-$ recombinant virus plaques. The latter were then distinguished from spontaneous TK$^-$ mutants by addition of X-Gal to the agar overlay. Plaques that stained blue, due to expression of

β-galactosidase, were picked and then plaque purified a second time prior to preparation of virus stocks.

Expression of RSV G Glycoprotein. The recombinant vaccinia virus was conveniently identified on the basis of its expression of β-galactosidase. Since the plasmid used for transfection also contained the gene encoding RSV G glycoprotein under control of a vaccinia virus promoter, the latter protein also should be synthesized. To verify this and to characterize the expressed protein, CV-1 cells were infected with the recombinant virus and cell extracts were analyzed by polyacrylamide gel electrophoresis. The resolved proteins were transferred to nitrocellulose and the membrane was incubated successively with rabbit anti-RSV serum and ^{125}I-labeled staphylococcal protein A. Autoradiography revealed a broad major protein band of about 93 kDa and a minor one of about 60 kDa (Fig. 2*A*). These bands were not detected in extracts of CV-1 cells infected with wild-type vaccinia virus (Fig. 2*A*) or uninfected CV-1 cells (data not shown). Furthermore, the 93-kDa protein migrated with authentic G glycoprotein from extracts of HEp-2 cells infected with RSV (Fig. 2*A*).

The size and somewhat diffuse nature of the major 93-kDa band suggested that it was glycosylated. Also, since the primary polypeptide was predicted to be only 32.5 kDa (11, 12), we suspected that the 60-kDa band was a less highly glycosylated form of the G protein. To confirm that the two forms of RSV G synthesized by the recombinant vaccinia virus were glycosylated, infected cells were labeled with [^{3}H]glucosamine and the cytoplasmic proteins that bound specifically to rabbit anti-RSV serum were resolved by polyacrylamide gel electrophoresis. Again, polypeptides of 93 and 60 kDa were detected in extracts of cells that were infected with recombinant virus but not wild-type vaccinia virus (Fig. 2*B*). Several [^{3}H]glucosamine-labeled proteins were immunopurified from RSV-infected HEp-2 cells. By comparison with previous studies (7, 8), we identified the 93-, 48-, and 22-kDa bands as G glycoprotein and the F_1 and F_2 products of the F glycoprotein, respectively. The 75-kDa band is probably uncleaved F glycoprotein, although it could also represent an incompletely glycosylated form of the G glycoprotein. The significant result is the similar migrations of authentic and recombinant-derived 93-kDa G glycoprotein molecules.

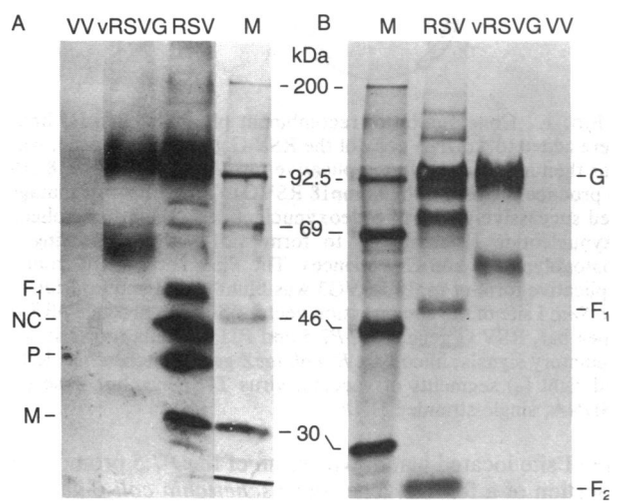

FIG. 2. Characterization of the RSV G protein synthesized by recombinant vaccinia virus. (*A*) Protein blot analysis. CV-1 cell monolayers were infected with wild-type or recombinant vaccinia virus and HEp-2 cells were infected with RSV. After 16 hr, the cells were lysed and the proteins were dissociated with mercaptoethanol and $NaDodSO_4$ and resolved by electrophoresis in a 10% polyacrylamide gel. The proteins were transferred to nitrocellulose paper by electroblotting and then incubated successively with rabbit anti-RSV serum and ^{125}I-labeled protein A. An autoradiograph is shown. (*B*) Immunoprecipitation. CV-1 cell monolayers were infected with wild-type or recombinant vaccinia virus and HEp-2 cells were infected with RSV and labeled with [^{3}H]glucosamine for 16 hr. The cells were lysed and the proteins were incubated with rabbit anti-RSV serum. The immune complexes were adsorbed to staphylococcal A cells and the bound proteins were resolved on a 10% polyacrylamide gel and autoradiographed. VV, extract of vaccinia virus-infected CV-1 cells; vRSVG, extract of recombinant vaccinia virus-infected CV-1 cells; RSV, extract of RSV-infected HEp-2 cells; and M, size markers. Positions of RSV viral proteins are indicated on the right and left.

Cell Surface Immunofluorescence. During a productive RSV infection, the G glycoprotein is transported to the plasma membrane, where virus budding occurs. Indirect immunofluorescence was carried out to determine whether the G glycoprotein made by recombinant vaccinia virus was transported to the cell surface. CV-1 cells that had been infected with wild-type or recombinant vaccinia virus were fixed with formaldehyde to prevent permeabilization and then incubated with monoclonal antibody to RSV G glycoprotein. After incubation with a fluorescein-conjugated second antibody, strong immunofluorescence was obtained only with the recombinant virus-infected cells (Fig. 3). Negative results also were obtained with uninfected CV-1 cells (not shown).

Serologic Response. Cotton rats that were inoculated intradermally with purified live vaccinia–RSV G recombinant virus developed a high level of serum antibodies that bound to the G glycoprotein (Table 1). This response, which was

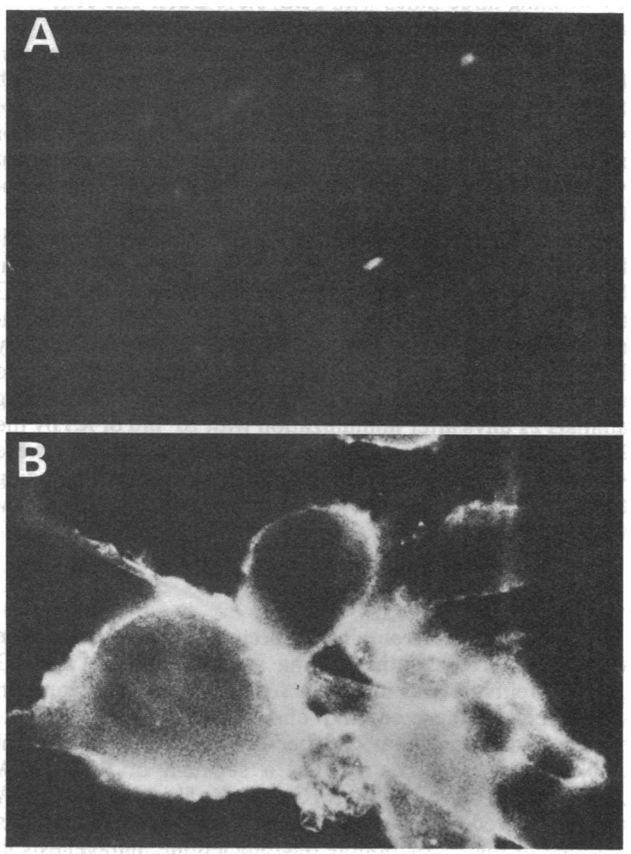

FIG. 3. Immunofluorescence. CV-1 cell monolayers were infected for 24 hr with wild-type or recombinant vaccinia virus, fixed with formaldehyde, and then incubated successively with monoclonal anti-RSV G and goat anti-mouse IgG-fluorescein conjugate. (*A*) Control vaccinia virus-infected cells. (*B*) Recombinant vaccinia virus-infected cells.

Table 1. Immunization with vaccinia–RSV G glycoprotein recombinant virus induces resistance to RSV in cotton rats

Immunization	*n*	Mean reciprocal titer of serum antibody		Response to i.n. inoculation of RSV		
				Mean titer‡ of virus, $\log_{10}$(pfu per g)		% of cotton rats free of detectable virus in lungs
		Neutralization*	Anti-G†	Nose	Lungs	
RSV i.n.	8	1622	951	<2.0	<2.0	100
RSV i.d.	8	355	806	3.17 ± 0.28§	2.34 ± 0.30§	62
Vaccinia–RSV G i.d.	17	372	4175	3.33 ± 0.23§	2.11 ± 0.08§	88
Vaccinia–influenza A H2 i.d.	11	26	116	4.5 ± 0.14	3.93 ± 0.26	9
None	13	<20	44	4.70 ± 0.15	4.59 ± 0.09	0

*Neutralization of RSV was determined by 60% plaque reduction.
†Determined by ELISA using purified RSV G glycoprotein as antigen.
‡Titer ± SEM. Level of detectability was $10^{2.0}$.
§$P < 0.001$ compared to control group by *t* test.

measured by ELISA, was 4- to 5-fold greater than that observed after infection induced by i.n. or i.d. inoculation of RSV. The levels of G antibodies measured in the sera of unimmunized cotton rats or cotton rats immunized with a vaccinia–influenza A H2 recombinant virus (19) represents base-line values for this sensitive ELISA. As anticipated, the RSV-infected cotton rats also developed serum ELISA antibodies for the F glycoprotein of RSV, whereas the animals that were infected with the vaccinia–RSV G recombinant virus did not (data not shown).

Animals that received an i.d. inoculation with the vaccinia–RSV G recombinant virus developed a moderately high level of serum neutralizing antibodies. This level was similar to that observed after i.d. inoculation of RSV but was less than one-fourth of that induced by RSV infection of the respiratory tract (Table 1).

Resistance to RSV Infection. i.d. inoculation of cotton rats with the vaccinia–RSV G recombinant virus induced almost complete resistance to lower respiratory tract infection with RSV 3 weeks later. Only 2 out of 17 animals had measurable RSV in the lungs when tested on the fourth day after challenge, and in those cases the titers were at the lowest detectable level (Table 1). This degree of resistance to lower respiratory tract infection was similar to that observed after immunization with live RSV.

The route of immunization with RSV is most important with regard to protection against upper respiratory tract infection with RSV. Thus, the titer of RSV in the nose after a challenge is lower if cotton rats have been immunized intranasally rather than intradermally (Table 1). Similarly, i.d. immunization with the vaccinia-RSV G recombinant virus provided only partial protection against upper respiratory tract infection.

DISCUSSION

The G glycoprotein of RSV is an integral membrane protein with unusual structural features. Its molecular mass deduced from nucleotide sequence analysis is only 32,588 Da (11, 12), yet it has an electrophoretic mobility that is appropriate for a protein of about 90,000 Da (4, 5). This apparent discrepancy has been attributed to extensive O-linked and more limited N-linked glycosylation (7, 11, 12). Our use of a vaccinia virus vector to express the 298 amino acid open reading frame supported this interpretation. The immunologically reactive product incorporated [^{3}H]glucosamine and migrated with authentic RSV G glycoprotein. A minor glycosylated species of 60 kDa, however, was unique to the recombinant virus-infected cells. Since this protein also was made by a second recombinant vaccinia virus that was constructed with an independently isolated cDNA, we do not believe that it was caused by a mutational artifact of cloning. A 45-kDa precursor of the G glycoprotein has been detected in RSV-infected HeLa cells (7, 8). It seems likely, therefore, that the 60-kDa protein represents an incompletely glycosylated intermediate that accumulates in vaccinia virus-infected CV-1 cells.

The deduced amino acid sequence of the RSV G protein revealed the absence of typical hydrophobic signal and anchor sequences (11, 12). Instead, both transfer and anchor functions have been attributed to a hydrophobic domain located between amino acids 38 and 66. Nevertheless, the possibility that a specific interaction with other RSV proteins, such as glycoprotein F, is required for membrane attachment was not ruled out. The present finding, that the RSV G protein can be detected on the surface of recombinant vaccinia virus-infected cells in the absence of other RSV proteins supports the contention that it is an independently transported membrane protein. Although not common, an internal transport and anchor sequence is also found in influenza virus neuraminidase (38) and some other membrane proteins (39). Expression of the RSV G glycoprotein in a vaccinia virus or other eukaryotic vector should make it possible to use *in vitro* mutagenesis techniques to define the parts of the protein that are important for membrane insertion and function.

The wide host range of vaccinia virus and the retention of infectivity by TK$^-$ recombinants make it possible to test the immunological response in a variety of experimental animals. The cotton rat was of particular interest since it has been developed as a model for experimental RSV infection (40). Although neither severe illness nor death is a consequence of i.n. inoculation, RSV grows to relatively high titers in both upper and lower respiratory tract tissues. Initial experiments indicated that cotton rats could be vaccinated intradermally with recombinant vaccinia virus and that the level of antibody to the G glycoprotein was even higher than that made after RSV infection. The level of neutralizing antibody obtained was in the range considered to be highly protective (37) and was similar to that produced when RSV was administered by the same route but less than that achieved by i.n. inoculation of RSV. An important distinction, however, is that the neutralizing antibody produced by animals vaccinated with the recombinant virus was specifically directed to the G glycoprotein, whereas animals vaccinated with RSV could have neutralizing antibody to both F and G glycoproteins.

After a single i.d. vaccination with recombinant vaccinia virus, the cotton rats were resistant to lower respiratory tract infection due to an i.n. challenge with RSV. However, i.d. immunization with either recombinant vaccinia virus or RSV did not completely prevent i.n. replication of RSV. Since local immunity produced by i.n. inoculation with RSV completely prevents both upper and lower respiratory tract infection, the next step is to intranasally vaccinate with recombinant vaccinia virus. Previous studies with a recombinant vaccinia virus that expresses the influenza virus hemagglutinin demonstrated that i.d. vaccination protected

against lower respiratory tract infection with influenza virus and accelerated recovery from upper respiratory tract infection but that i.n. vaccination prevented infection at both locations (41). In a clinical situation, resistance to upper respiratory tract infection might not be necessary to prevent severe disease. Moreover, a subsequent mild i.n. infection could serve to boost immunity. If prevention of spread of RSV is desired, however, resistance to both upper and lower respiratory tract infections would be desirable.

The role, if any, of cell-mediated immunity to RSV in experimental or natural infections is uncertain. It is known that passive administration of antibody to cotton rats can confer resistance to lower respiratory tract infections (9, 37, 42) and that the incidence or severity of disease in young infants is inversely correlated with their maternally derived antibody titer (43–45). Nevertheless, it seems likely that both humoral and cell-mediated immunity are important for recovery from RSV infection, since the virus was secreted persistently from children with either combined humoral or only cellular immune defects (46). The presence of RSV-specific memory cytotoxic T cells has been demonstrated in spleens of mice primed intranasally with RSV (47). It remains to be determined whether vaccinia virus recombinants that express the G glycoprotein stimulate cell-mediated immunity in mice. Also, construction of recombinant vaccinia viruses that express other RSV proteins should allow us to determine their roles in protective immunity.

We thank N. Cooper and R. L. Horswood for technical assistance, D. M. Lambert for rabbit hyperimmune anti-RSV serum, E. Norrby for monoclonal antibody B109, S. Chakrabarti for the plasmid vector, E. E. Walsh for purified RSV glycoproteins, and M. Rust for typing the manuscript.

1. Parrott, R. H., Kim, H. W., Arrobio, J. O., Hodes, D. S., Murphy, B. R., Brandt, C. D., Carmago, E. & Chanock, R. M. (1973) *Am. J. Epidemiol.* **98,** 298–300.
2. Kim, H. W., Canchola, J. G., Brandt, C. D., Pyles, G., Chanock, R. M., Jensen, K. & Parrott, R. H. (1969) *Am. J. Epidemiol.* **89,** 422–434.
3. Belshe, R. B., Van Vordis, L. P. & Mufson, M. A. (1982) *J. Infect. Dis.* **145,** 311–319.
4. Peeples, M. & Levine, S. (1979) *Virology* **95,** 137–145.
5. Fernie, B. F. & Gerin, J. L. (1982) *Infect. Immun.* **37,** 243–249.
6. Walsh, E. E. & Hruska, J. F. (1983) *J. Virol.* **47,** 171–177.
7. Gruber, C. & Levine, S. (1985) *J. Gen. Virol.* **66,** 1241–1247.
8. Fernie, B. F., Dapolito, G., Cote, P. J., Jr., & Gerin, J. L. (1985) *J. Gen. Virol.* **66,** 1983–1990.
9. Walsh, E. E., Schlesinger, J. J. & Brandriss, M. W. (1984) *Infect. Immun.* **43,** 756–758.
10. Taylor, G., Stott, E. J., Bew, M., Fernie, B. F., Cote, P. J., Collins, A. P., Hughes, M. & Jebbett, J. (1984) *Immunology* **52,** 137–142.
11. Wertz, G. W., Collins, P. L., Huang, Y., Gruber, C., Levine, S. & Ball, L. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4075–4079.
12. Satake, M., Coligan, J. E., Elango, N., Norrby, E. & Venkatesan, S. (1985) *Nucleic Acids Res.* **13,** 7795–7812.
13. Collins, P. L., Huang, Y. T. & Wertz, G. W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7683–7687.
14. Elango, N., Satake, M., Coligan, J. E., Norrby, E., Camargo, E. & Venkatesan, S. (1985) *Nucleic Acids Res.* **13,** 1559–1574.
15. Panicali, D. & Paoletti, E. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4927–4931.
16. Mackett, M., Smith, G. L. & Moss, B. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7415–7419.
17. Smith, G. L., Mackett, M. & Moss, B. (1983) *Nature (London)* **302,** 490–495.
18. Panicali, D., Davis, S. W., Weinberg, R. L. & Paoletti, E. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5364–5368.
19. Smith, G. L., Murphy, B. R. & Moss, B. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 7155–7159.
20. Paoletti, E., Lipinskas, B. R., Samsonoff, C., Mercer, S. & Panicali, D. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 193–197.
21. Moss, B., Smith, G. L., Gerin, J. L. & Purcell, R. H. (1984) *Nature (London)* **311,** 67–69.
22. Wiktor, T. J., Mcfarlan, R. I., Reagan, K. J., Dietzschold, B., Curtis, P. J., Wunner, N. H., Kieny, M. P., Lathe, R., Lecocq, J.-P., Mackett, M., Moss, B. & Koprowski, H. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7194–7198.
23. Mackett, M., Yilma, T. Y., Rose, J. A. & Moss, B. (1985) *Science* **227,** 433–435.
24. Cremer, K. J., Mackett, M., Wohlenberg, C., Notkins, A. L. & Moss, B. (1985) *Science* **228,** 737–739.
25. Bennink, J. R., Yewdell, J. W., Smith, G. L., Moller, C. & Moss, B. (1984) *Nature (London)* **311,** 578–579.
26. Yewdell, J. W., Bennink, J. R., Smith, G. L. & Moss, B. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1785–1789.
27. Garon, C. F., Barbosa, E. & Moss, B. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 4863–4867.
28. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7,** 1513–1523.
29. Winberg, G. & Hammarskjöld, M.-L. (1980) *Nucleic Acids Res.* **8,** 253–264.
30. Messing, J. & Vieira, J. (1982) *Gene* **19,** 269–276.
31. Zoller, M. J. & Smith, M. (1984) *DNA* **3,** 479–488.
32. Weir, J. P., Bajszar, G. & Moss, B. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1210–1214.
33. Chakrabarti, S., Brechling, K. & Moss, B. (1985) *Mol. Cell. Biol.* **5,** 3403–3409.
34. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
35. Batteiger, R., Newhall, W. J. V. & Jones, R. B. (1982) *J. Immun. Methods* **55,** 297–307.
36. Hemming, V. G., Prince, G. A., Horswood, R. L., London, W. T., Murphy, B. R., Walsh, E. E., Fischer, G. W., Weisman, L. E., Baron, P. A. & Chanock, R. M. (1985) *J. Infect. Dis.* **152,** 1083–1087.
37. Prince, G. A., Horswood, R. L. & Chanock, R. M. (1985) *J. Virol.* **55,** 517–520.
38. Fields, S., Winters, G. & Brownlee, G. G. (1981) *Nature (London)* **290,** 213–217.
39. Wickner, W. T. & Lodish, H. F. (1985) *Science* **230,** 400–407.
40. Prince, G. A., Jenson, A. B., Horswood, R. L., Camargo, E. & Chanock, R. M. (1978) *Am. J. Pathol.* **93,** 771–792.
41. Small, P. A., Jr., Smith, G. L. & Moss, B. (1985) in *Vaccines 85*, eds. Lerner, R. A., Chanock, R. M. & Brown, F. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 175–176.
42. Prince, G. A., Horswood, R. L., Camargo, E., Koenig, D. & Chanock, R. M. (1983) *Infect. Immun.* **42,** 81–87.
43. Lamprecht, C. L., Krause, H. E. & Mufson, M. A. (1976) *J. Infect. Dis.* **134,** 211–217.
44. Glezen, W. P., Pardes, A., Allison, J. E., Taber, L. H. & Frank, A. L. (1981) *J. Pediatr.* **98,** 708–715.
45. Ogilvie, M. M., Vathenen, A. S., Radford, M., Codd, J. & Key, S. (1981) *J. Med. Virol.* **7,** 263–271.
46. Fishaut, M., Tubergen, D. & McIntosh, K. (1979) *Pediatr. Res.* **13,** 447.
47. Bangham, R. M., Cannon, M. J., Karzon, D. T. & Askonas, B. A. (1985) *J. Virol.* **56,** 55–59.