Expression of the Fusion Protein of Human Respiratory Syncytial Virus from Recombinant Vaccinia Virus Vectors and Protection of Vaccinated Mice

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Vaccinia virus (VV) recombinants were constructed that contained full-length cDNA copies of the fusion (F) protein gene of human respiratory syncytial (RS) virus. The F protein gene was placed next to the strong early-late VV 7.5-kilodalton promoter and was located within the VV thymidine kinase (*tk*) gene. Full-length recombinant transcripts that initiated at both the *tk* and the 7.5-kilodalton promoters accumulated in cells early in infection, and one or more of these transcripts was translated to yield a glycoprotein which comigrated with F₀, the fusion protein precursor. This precursor was processed by proteolytic cleavage to produce the two disulfide-linked subunits F₁ and F₂, which were both glycosylated and of the same electrophoretic mobility as authentic F₁ and F₂. Immunofluorescence studies demonstrated that the mature F protein was transported to and expressed on the surface of recombinant VV-infected cells. Inoculation of rabbits with a recombinant vector expressing F resulted in the production of antiserum specific for the RS virus F protein. This antiserum neutralized virus infectivity and was capable of preventing fusion in RS virus-infected cells. Mice were vaccinated with recombinants expressing the F protein. At 3 weeks postinoculation, these animals had serum antibody against RS virus F protein. At 5 days after intranasal challenge with RS virus, the lungs of the mice previously vaccinated with recombinants expressing F protein were free of detectable RS virus, whereas the lungs of unvaccinated mice contained 10^{4.2} PFU of virus per g.

Human respiratory syncytial (RS) virus is a major cause of serious pediatric lower respiratory tract infection. No effective vaccine against this agent is currently available, and previous attempts in which a formaldehyde-inactivated vaccine was tested resulted in excess morbidity and mortality (3, 12). The failure of this vaccine to protect and the fact that it exacerbated the disease underscores the need to understand in detail the role of each RS virus antigen in infection and in the immune response.

RS virus is an enveloped, negative-stranded RNA containing virus that shares many of the characteristics of the *Paramyxoviridae* and has been provisionally classified within that family (13). However, the virus has some distinctive features which are responsible for its further classification into the separate pneumovirus group. Among the dissimilarities is the lack of both a hemagglutinin and a neuraminidase activity (23), which, in other paramyxoviruses, reside in the larger of two envelope glycoproteins, HN (4). The larger envelope glycoprotein of RS virus, however, has no known enzymatic activities and has an unusual structure that includes extensive O-linked oligosaccharides (24, 33). However, RS virus resembles the other paramyxoviruses in its ability to cause fusion of the plasma membranes of infected cells, which results in the formation of large multinucleate syncytia, a characteristic cytopathic effect of RS virus infection. This property resides in the smaller surface glycoprotein, F, as shown by the observation that some monoclonal antibodies to the F protein block cell-to-cell fusion (30).

The F protein is synthesized as a 68- to 70-kilodalton precursor glycoprotein (68K to 70K protein) which is

proteolytically cleaved to yield two disulfide-linked subunits, F₁ (47K protein) and F₂ (20K protein) (9). Sequence analysis of cDNA clones of the F mRNA shows that the mRNA is 1,899 nucleotides in length and contains a single open reading frame encoding 574 amino acids (6, 8). The amino acid sequence of the F protein predicts an amino-terminal signal sequence, a hydrophobic carboxy-terminal anchor sequence, a site for proteolytic cleavage (residues 131 to 136) to generate the F₁ and F₂ subunits, and five potential acceptor sites for asparagine-linked carbohydrate side chains (6, 8). Neither the primary nucleotide nor the amino acid sequence of RS virus F protein has significant levels of homology with the sequences of other paramyxovirus F proteins. However, the overall structural features of the F protein described above resemble those of other paramyxovirus fusion proteins (6, 8, 20, 26).

Cell-to-cell fusion, with the resultant formation of syncytia, caused by the F protein is not only a characteristic cytopathic effect but also a potential mechanism of virus spreading in a manner which may evade neutralizing antibody. It has been shown that in other paramyxovirus systems, blockage of the fusion activity by antibody to the F protein is required in addition to neutralizing antibody in preventing dissemination of virus in cell culture (17).

Because human RS virus replicates poorly in cell cultures, it has been difficult to obtain sufficient quantities of individual viral proteins to analyze their roles in infection. In an effort to understand the role of each RS virus protein in infection and immunity, we have constructed recombinant vectors by using vaccinia viruses (VV) that express individual RS virus proteins (2, 27). VV is a useful eucaryotic vector because it can be used not only to express foreign genes at high levels in cell culture but also to immunize

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animals owing to its broad host range. Thus, recombinant VV vectors allow dissection of the role of individual viral proteins in infected cells as well as in the overall immune response. Furthermore, VV vectors have proven useful in determining the function of individual proteins in the cell-mediated immune response (34).

In the present work, we constructed recombinant VV vectors containing the RS virus F protein gene. We describe the analysis of F protein expression in infected cells and the ability of the recombinant vectors expressing F protein to protect the lungs of immunized mice against live RS virus challenge.

MATERIALS AND METHODS

Cells and viruses. Human RS virus (A2 strain) was propagated in HEp-2 cells as described previously (11). Wild-type (wt) (WR strain) and recombinant VV were propagated in HEp-2 or thymidine kinase negative (*tk*⁻) 143 cells as described previously (10).

Plasmid construction and propagation and DNA sequence determination. The original sources of the plasmids used in this work have been described previously (2, 5–7). All plasmids were propagated in *Escherichia coli* HB101, and cloning procedures were performed as described by Maniatis et al. (15). *Bal31* exonuclease treatment was used to remove GC tails from the inserts prior to construction of expression vectors (2). DNA sequences at the 5' and 3' termini of genes were determined by the chemical method (16) with DNA fragments end-labeled by reverse transcriptase.

Isolation of recombinant VV vectors. Recombinant VV were isolated as described in detail previously (2, 27). Two recombinant viruses (vF325 and vF317) that contained full-length cDNA copies of the F gene, in the positive orientation with respect to the VV promoter, but that differed in sequences preceding the start of the gene (see Results) were used in the work described here.

Analysis of protein expression. Proteins synthesized in infected cells were labeled with [³H]glucosamine or [³H]threonine and analyzed by electrophoresis in 10 or 12.5% sodium dodecyl sulfate-polyacrylamide gels either directly or following immune precipitation as described previously (33).

Analysis of RNA transcripts. RNA transcripts from recombinant VV-infected cells were analyzed by Northern blot hybridization with strand-specific RNA probes. HEp-2 cells were infected with RS virus at a multiplicity of infection (MOI) of 3 or with vF325 or vaccinia virus at a MOI of 10. Cycloheximide was added (100 µg/ml) to the wt- and recombinant VV-infected cells at the time of infection. At 22 h (RS infection) or 5.5 h (VV and recombinant vF325 infection) postinfection, the cells were lysed with Nonidet P-40 and the cytoplasmic RNA was isolated by phenol extraction and ethanol precipitation (11). The RNAs were resolved by electrophoresis on a 1.5% agarose-urea gel. The resolved RNA was transferred onto diazobenzylxymethyl papers as described previously (11). ³²P-labeled RNA probes were transcribed in vitro from recombinant plasmid vectors derived from pGEM-1 or pGEM-2 (Promega Biotech., Inc.) that contained cDNA inserts within the multiple cloning regions. RS virus F gene-specific RNA probes were transcribed from a plasmid that contained a 586-base *Pst*I fragment from RS virus F cDNA (6) inserted within the *Pst*I site of pGEM-1. VV *tk* gene-specific RNA probes were transcribed from plasmids containing DNA fragments from the *Hind*III J region of the VV genome. One of these

plasmids contained the 0.77-kilobase (kb) *Hind*III-to-*Eco*RI fragment, which includes the 5' half of the *tk* gene, inserted into pGEM-1 (cut with *Hind*III and *Eco*RI). The other plasmid contained the 1.05-kb *Eco*RI-to-*Xho*I fragment, which includes the 3' half of the *tk* gene, inserted into pGEM-2 (cut with *Eco*RI and *Sal*I; see Fig. 1). Strand-specific RNA probes were obtained by transcription directed by either SP6 (Bethesda Research Laboratories, Inc.) or T7 (U.S. Biochemicals) RNA polymerase. The blots were probed with the riboprobes specific for the RS virus F gene mRNA or VV sequences flanking the insert. The blots were then exposed to Kodak AR5 X-ray film at -70°C.

Indirect immunofluorescence. For intracellular staining, HEp-2 cells were grown as monolayers on multichambered slides (Miles Scientific). The monolayers were infected with RS virus, wt VV, recombinant vF325, or recombinant vF317 at a MOI of 10. At 22.5 h (RS-infected) or 4 h postinfection (VV and recombinant infected), the slides were washed twice in phosphate-buffered saline (PBS). The cells were permeabilized by being fixed for 5 min in a 1:1 mixture of methanol and acetone at -20°C and were then washed once in PBS. Five microliters of RS-specific antiserum (Burroughs Wellcome Co.) or monoclonal antibody specific for the RS virus F protein was added to each chamber, and the slides were incubated at 37°C for 30 min. The slides were then washed three times (2 min per wash) in PBS. Thirty microliters of fluorescein isothiocyanate-conjugated second antibody (anti-bovine and anti-mouse, respectively) was added to each chamber, and the slides were again incubated at 37°C for 30 min. The slides were washed as before and mounted with 40% glycerol in PBS containing 1% bovine serum albumin.

For surface staining, HEp-2 cells were infected with RS virus at a MOI of 0.5 or with VV recombinants vF325 or vF317 at a MOI of 5. After 18 h at 37°C, cells were removed with trypsin and resuspended in medium at a concentration of 10⁶ cells per ml. Fifty microliters of cell suspension was incubated with an equal volume of diluted monoclonal antibody to F protein for 60 min on ice. Cells were washed three times in PBS and suspended in PBS containing fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody. After 60 min on ice, the cells were washed three times in PBS and mounted on glass slides in 80% glycerol.

Production and characterization of antibody. Antibody production in rabbits or mice, titration by enzyme-linked immunosorbent assay (ELISA), and virus neutralization tests were performed as described previously (27).

Protection of mice. The ability of VV recombinants containing the F gene to protect animals was analyzed with BALB/c mice (21, 29). Details of vaccination, virus challenge, and plaque assay were described previously (27).

RESULTS

Recombinant genome structures. The construction and characterization of full-length cDNA clones of the F protein gene of human RS virus were described previously (6). VV recombinants containing these cDNAs were constructed and isolated by methods previously described (2, 27). The general genome structure of these recombinants is illustrated in Fig. 1. They all contained the RS virus F protein cDNAs in either positive or negative orientation relative to an adjacent strong VV promoter within the VV *tk* gene. In early constructions, a VV promoter derived from the *Hind*III F fragment of VV DNA (2, 27) was used, but much higher and more prolonged levels of expression were observed from

vectors that used the 7.5K VV promoter (2, 27), and so all the results to be described in this paper were derived from two such recombinants, vF317 and vF325. Recombinants vF317 and vF325 contained a full-length F cDNA having the complete F protein open reading frame in the positive orientation downstream of the 7.5K promoter; however, the two recombinants differed at the junctions between the VV and RS virus sequences. As determined by chemical sequencing of the recombinant plasmids used to construct the vectors (see Materials and Methods), vF325 contained 85 base pairs between the insertion site and the start of the F protein gene: 63 base pairs derived from pBR322 and 22 GC base pairs derived from the original cDNA cloning (6). In contrast, vF317 had the extra bases removed by *Bal31* exonuclease digestion (2, 15) and also lacked the first three noncoding nucleotides of the F protein gene.

Analysis of recombinant transcripts. The RNA transcripts that accumulated in cells infected with the recombinant viruses were resolved by agarose-urea gel electrophoresis and analyzed by Northern blot hybridization for size and direction of transcription by using strand-specific radioactive RNA probes. Three strand-specific probes were prepared (for details, see Materials and Methods). One was specific for mRNA-sense transcripts of the RS virus F protein gene. The other two were specific for VV transcripts derived by rightward transcription of either the 0.77-kb sequence between the left-hand boundary of the *HindIII* J fragment and the *EcoRI* site within the *tk* gene (upstream VV probe) or the 1.05-kb sequence between the *EcoRI* site within the *tk* gene and the following *XhoI* site (downstream VV probe; Fig. 1). These VV-specific probes contained sequences that hybridized to regions of the *tk* mRNA that preceded and followed the insertion site, respectively.

Autoradiographs of the Northern transfers are shown in Fig. 2A. Three major F-specific transcripts of about 1.9, 2.3, and 2.8 kb accumulated in cells infected with recombinant vF325 at early times postinfection. On the basis of their hybridization patterns with the VV-specific probes, these transcripts were assigned the map positions shown in Fig. 2B. For example, the 2.8-kb transcript hybridized to both the upstream and the downstream VV probe and was therefore probably derived from initiation at the *tk* promoter and termination at or near the end of the *tk* sequence. On the

FIG. 1. Genome structure of recombinants vF317 and vF325. The *HindIII* map of VV DNA is shown, with an expanded view of the 1.85-kb *HindIII*-*XhoI* subfragment of VV *HindIII*-J that contains the *tk* gene. The position and orientation of the transplanted promoter fragment and the inserted RS virus fusion protein cDNA are shown. Recombinants vF317 and vF325 contained a 0.28-kb promoter fragment derived from the VV 7.5K promoter gene (14). The orientation of the RS virus cDNA was positive with respect to the transplanted promoter.

FIG. 2. Analysis of RNA transcription. (A) Cytoplasmic RNA isolated from RS virus, VV, or recombinant vF325-infected HEP-2 cells by phenol extraction and ethanol precipitation was separated by electrophoresis on an agarose-urea gel, then blotted onto DBM paper. Riboprobes specific for VV *tk* mRNA or RS virus F mRNA were hybridized to the blots. ³²P-labeled RNA probes specific for RS F mRNA were synthesized from a plasmid DNA template by using SP6 RNA polymerase. ³²P-labeled RNA probes specific for VV sequence upstream or downstream of the F gene insertion site were synthesized from plasmid DNA templates by T7 RNA polymerase as described in Materials and Methods. Lanes: UN, uninfected; RS, RS virus infected; VVF, recombinant vF325 infected; VV, VV infected. (B) Schematic representation of the DNA region surrounding the VV *tk* gene and its corresponding early transcripts in wt VV DNA and in recombinant vF325. Schematic poly(A) tails indicate the 3' ends of the transcripts, and approximate transcript sizes are shown in kilobases. P represents the 7.5K promoter fragment.

other hand, the 2.3-kb transcript hybridized to the downstream but not the upstream VV probe and was thus attributed to initiation at the 7.5K promoter and termination at the end of the VV *tk* gene. The 1.9-kb transcript was indistinguishable in size from the authentic F mRNA made in RS virus-infected cells and was attributed to initiation at the 7.5K promoter and termination at or near the end of the RS virus sequences. It is not clear whether the poly(T) or poly(G) tracts or some other feature of the nucleotide sequences at the 3' recombinant junction signalled the termination of VV transcription at this site, but similar transcriptional behavior in other VV recombinants has been

FIG. 3. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of glycoproteins from RS virus-infected, recombinant-infected, and VV-infected cells. RS virus-infected cells were exposed to [3 H]glucosamine (100 μ Ci/ml) from 24 to 26 h postinfection. wt and recombinant VV-infected cells were exposed to [3 H]glucosamine (100 μ Ci/ml) from 2 to 4 h postinfection. The labeled proteins were analyzed by electrophoresis on a 12.5% polyacrylamide gel following immunoprecipitation with anti-RS antiserum (Burroughs Wellcome). A fluorogram of the dried gel is shown. The virtual absence of the RS virus G protein among the proteins from RS virus-infected cells is because the antiserum used failed to react with the G protein of this strain of RS virus (A2 strain). Lanes: UN, uninfected control; RS, RS virus infected; 325, recombinant vF325 infected; 317, recombinant vF317 infected; VV, VV infected.

shown to be due to the homopolymeric stretches (A. M. Q. King, unpublished observations). Two minor, F-specific transcripts of about 3.7 and 4.1 kb were also detected in vF325-infected cells and appear to result from occasional readthrough of the termination signal at the end of the *tk* gene, a phenomenon which also occurs at this site in wt VV. A very minor, negative-sense, F-specific transcript of about 0.54 kb was also detected in vF325-infected cells and was attributed to fortuitous initiation and termination of VV transcription within the F protein cDNA (data not shown).

Analysis of proteins made in recombinant-infected cells. The proteins synthesized in cells infected with vF317 and vF325 were examined by metabolic labeling with either [3 H]glucosamine or [3 H]threonine. RS virus-specific proteins were selectively immunoprecipitated with anti-RS virus antiserum and analyzed by electrophoresis in sodium dodecyl sulfate-polyacrylamide gels. Both RS virus and the recombinants directed the synthesis of three RS virus-specific glycoproteins in infected cells (Fig. 3). These three proteins comigrated respectively with the uncleaved F_0 polypeptide and the cleaved F_1 and F_2 subunits seen in RS virus-infected cells. The large amount of F_0 observed in Fig. 3 relative to its cleavage products was due to the use of a relatively short continuous labeling period without a chase.

Expressed F protein is transported to the cell surface.

Expression of the RS virus F protein in cells and its transport to the cell membrane were analyzed by indirect immunofluorescence of recombinant-infected permeabilized fixed cells and live cells. Immunofluorescence of permeabilized cells showed the intracellular distribution of the maturing F protein. F was seen predominantly in the perinuclear region of recombinant-infected cells (Fig. 4c and e). This distribution in recombinant-infected cells was similar to that shown in RS virus-infected cells probed with a monoclonal antibody against the F protein (Fig. 4a). Approximately 90 to 100% of cells infected with recombinant vF317 at a MOI of 10 expressed the F protein. Levels of immunofluorescence in vF317-infected cells reproducibly were higher (90 to 100%) than in cells infected with vF325 (40 to 50%), the construct with 85 base pairs between the promoter and the start of the F gene.

The F protein from recombinant-infected cells was transported to and expressed on the cell surface as shown by indirect immunofluorescence of live cells (Fig. 4g). Comparison of recombinant infected cells under fluorescence or phase-contrast optics showed that 90 to 100% of cells infected with recombinant vF317 expressed F on their surface (Fig. 4g and h).

Immunogenicity of F protein expressed by recombinant vectors. The ability of the F protein expressed from the recombinant vectors to stimulate an antibody response was tested by inoculating vF325 into rabbits. At 4 weeks postinoculation and at biweekly intervals thereafter, blood was collected and assayed by ELISA for serum antibody to RS virus. The 4-week-collected serum from animals immunized with recombinant vF325 gave ELISA titers of $10^{4.5}$. Peak antibody titers were reached by 4 to 6 weeks postinoculation (Fig. 5). A rabbit vaccinated with wt VV gave no RS virus-specific response (Fig. 5).

The serum produced by the rabbit immunized with recombinant vF325 was used to test the specificity of the immune response. Serum collected at 4 or 10 weeks postinoculation with vF325 specifically immunoprecipitated the RS virus F protein (data not shown; see Fig. 7).

This serum was tested for its ability to neutralize the infectivity of RS virus. The F protein-specific antibody collected at 6 or 10 weeks after immunization of rabbits with recombinant vF325 neutralized RS virus infectivity in the absence of complement. Fifty percent plaque reduction was achieved at a serum dilution of 1:1,024. This neutralization titer was significantly higher than that noted for antisera generated from rabbits immunized with VV recombinants expressing the other RS virus surface glycoprotein, G (27); however, it confirmed previous observations that antiserum to either the G or F surface glycoprotein was capable of neutralizing viral infectivity (19, 30, 31). The above data demonstrate that antibodies specific for RS virus F protein were produced in response to immunization with a recombinant expressing the F gene and that these antibodies could neutralize RS virus infectivity.

The neutralizing antibody produced in response to immunization with vF325 also inhibited fusion in RS virus-infected cells. HEp-2 cells infected with RS virus at a MOI of 0.02 were exposed at 4 h postinfection to sera drawn from a rabbit prior to or 10 weeks after immunization with vF325 or from a rabbit immunized with vG301, a recombinant expressing the RS virus G protein (2, 27), or were left untreated. Serum collected 10 weeks postimmunization with vF325 inhibited RS virus-induced cell fusion and the formation of multinucleate syncytia (Fig. 6d) compared with untreated controls (Fig. 6b). Serum collected prior to immunization or from an

FIG. 4. Intracellular localization and cell surface detection of RS virus F protein in HEp-2 cells infected with recombinant VV. HEp-2 cell monolayers on multichambered tissue culture slides (internal fluorescence) or 25-cm² flasks (surface fluorescence) were infected with RS virus, recombinant VV, or wt VV. Live cells in suspension (panels g and h) were stained for cell surface expression of RS viral antigens by indirect immunofluorescence with an F protein-specific monoclonal antibody as first antibody and fluorescein-conjugated rabbit anti-mouse immunoglobulin G antibody as the second antibody. Permeabilized cells in monolayers (panels a through f) were stained for intracellular RS viral antigens by using RS virus-specific (recombinants vF325 and vF317 and VV infected) or F monoclonal (RS virus infected) first antibody. (a) RS virus-infected cells; (b) wt VV-infected cells; (c) recombinant vF325-infected cells; (e and g) Recombinant vF317-infected cells; (d, f, and h) phase-contrast photographs of cells shown in panels c, e, and g, respectively.

FIG. 5. Antibody production in rabbits inoculated with recombinant vF325 or wt VV. Rabbits inoculated intradermally with recombinant vF325 or wt VV were bled at intervals following vaccination. Serum antibody titers against RS virus were measured by ELISA. Symbols: —, titers from vF325-immunized rabbit; ---, titers from wt VV-immunized rabbit.

animal immunized with vG301 and previously shown to produce neutralizing antibody to the G protein did not inhibit formation of syncytia (Fig. 6c and e). These data show that antibody to the F protein expressed from recombinants not only neutralizes virus but also inhibits fusion in previously infected cells. The failure of neutralizing antibody to G to inhibit syncytium formation shows that the inhibitory activity of the F serum is specific to the F antibody and is not due to an antibody reaction stimulated by the recombinant VV. These data show in the case of RS virus, as has been shown with other paramyxoviruses (17), that fusion inhibitory antibody, as well as neutralizing antibody, is necessary to block spread of infection. Equally importantly, however, these data show that the F protein produced from recombinant VV vectors stimulated the production of antibodies to the regions of the F protein involved in neutralization and fusion.

Despite the unambiguous evidence that F protein was correctly cleaved and presented on the surface of recombinant infected cells, it was not possible to document the formation of large, multinucleate syncytia such as those seen in RS virus-infected cells. At most, small groups of fused cells with 3 to 5 nuclei were visible. The probable explanation for this is not that the fusion protein expressed from recombinant VV-infected cells was unable to fuse cells but rather that the early cell rounding, which is a characteristic cytopathic effect of VV infection, caused separation of adjacent cells and interfered with the ability of the F protein expressed on the surface to cause syncytium formation.

Animal protection studies. Of several animal model systems available for human RS virus, we chose to use the inbred BALB/c mouse (21, 29). In the experiments reported here, we tested the ability of recombinant vectors expressing the RS virus F protein to protect animals against live virus challenge. Groups of 10 mice were inoculated intraperitoneally with 10^7 PFU of recombinant vF325 or vF317 or left unvaccinated as controls. The mice were challenged intranasally with $10^{4.2}$ PFU of RS virus 3 weeks after vaccination. Lungs were harvested 5 days after challenge, a time previously determined to be the peak time of RS virus replication. The lungs of animals that had been vaccinated with recombinant vF325 or vF317 were found to be free of detectable RS virus (Table 1). In contrast, unvaccinated animals had RS virus titers of $10^{4.2}$ PFU per g in the lung. Vaccination with

wild-type VV had no protective effect against RS virus challenge (27).

The mice also were bled at 3 weeks (prior to being challenged); all mice immunized with vF325 or vF317 had high serum antibody titers to RS virus. Serum titers of $10^{4.0}$ or $10^{4.3}$ were achieved in immunized animals; no antibody to RS virus was detectable in unvaccinated animals (Table 1). The specificity of the sera collected from mice was analyzed by immunoprecipitation. Serum from mice immunized with vF325 specifically immunoprecipitated F protein from RS virus-infected cell lysates (Fig. 7). These data demonstrate the ability of vaccination with recombinant vectors expressing the RS virus F protein to protect against RS virus challenge in mice.

DISCUSSION

The RS virus F protein was expressed from cDNA placed in recombinant VV vectors under the control of the VV 7.5K early-late promoter. RNA transcripts of the appropriate size and strand specificity to represent the entire F mRNA or the F mRNA plus readthrough into adjacent VV sequences were synthesized in amounts equal to or in excess of the amounts of F mRNA seen in RS virus-infected cells. The F-specific RNA transcripts were translated to yield F glycoprotein in a form indistinguishable in electrophoretic mobility, antigenicity, intracellular distribution, and cell surface expression from F protein in RS virus-infected cells. The F_0 precursor synthesized in recombinant vF325- and vF317-infected cells was processed to yield the F_1 and F_2 subunits which comigrated with RS virus F_1 and F_2 . In a previous report, a recombinant vF313 was shown to express the F_0 protein in a form that was not cleaved to the F_1 and F_2 subunits (G. Wertz, K. Young, K. Anderson, and L. A. Ball, in B. Mahy and D. Kolakofsky, ed., *Biology of Negative Strand Viruses*, in press). We have now determined that this property is specific to the cDNA clone used for the construction (data not shown) and, as this work shows, that recombinants vF317 and vF325 constructed from an independently derived clone code for an F_0 protein that is cleaved normally.

The maturation and expression of the mature F protein on the cell surface in the absence of any other RS virus proteins show that the F protein can be processed and transported independently of any other RS virus protein.

The poor replication of RS virus in cell culture has made difficult the isolation of quantities of individual proteins sufficient to analyze their roles in infection. We have chosen to express individual proteins of RS virus from recombinant VV vectors. This strategy allows us to examine expression, maturation, and function of the individual proteins in cell culture in the absence of other RS virus proteins. Additionally, since VV recombinants are nondefective and VV has a wide host range, it is possible to express individual proteins in animals and to study the immunological response to that protein and its potential role in protection against live virus.

TABLE 1. Protection of mice by intraperitoneal vaccination with recombinant vectors

Recombinant	Log ₁₀ ELISA titer ^a in serum	Log ₁₀ virus titer in lungs (PFU/g)
vF325	4.0 ± 0.4	<1.7
None	<1.5	4.2 ± 0.4
vF317	4.3 ± 0.2	<1.7
None	<1.5	4.2 ± 0.1

FIG. 6. Inhibition of RS virus-induced cell fusion by antibody to F protein expressed from recombinant vectors. HEP-2 cells were uninfected (a), infected with RS virus and untreated (b), or infected with RS virus and then treated at 4 h postinfection with serum from a rabbit prior to (c) or 10 weeks after (d) immunization with vF325 or with serum from a rabbit immunized with recombinant vG301 (e).

As shown above, immunization of rabbits or mice with recombinant VV expressing RS virus F protein resulted in the production of antibody specific for F protein. This antibody was capable of neutralizing virus infectivity and of blocking cell-to-cell fusion and syncytium formation induced by RS virus. These results indicate that the F protein is produced in biologically active form.

Intraperitoneal inoculation of mice with recombinant vF325 and vF317 resulted in production of high titers of antibody specific for the F protein 3 weeks postinoculation. At 5 days after intranasal challenge with live RS virus, animals vaccinated with vF317 or vF325 were free of detectable RS virus in the lungs. These results show that immuni-

zation with the F protein of RS virus alone is capable of conferring protection against lower respiratory tract disease in mice.

In previous work, we showed that immunization of mice with recombinants expressing the G protein of RS virus results in production of antibody which could neutralize virus (27). Furthermore, the lungs of mice immunized with the G-expressing recombinant vAG301 were free of detectable RS virus 5 days after intranasal challenge (27). These data indicate that immunization with the RS virus G protein alone can confer protection against RS virus in mice.

Despite the data derived in cell culture showing that both neutralizing antibody and fusion-inhibiting antibody are

FIG. 7. Analysis of sera from mice immunized with recombinant vF325 by immunoprecipitation. Sera collected from unvaccinated mice (lanes 3 and 4) or mice immunized with recombinant vF325 (lanes 1 and 2) were used to immunoprecipitate proteins from RS virus-infected cells in which proteins had been labeled by exposure to [3 H]threonine for 3 h (lanes 1 and 3) or 18 h (lanes 2 and 4). Immunoprecipitated proteins were subjected to electrophoresis on a 12.5% polyacrylamide gel.

needed to inhibit the spread of RS virus (see above and reference 17), the animal protection studies reported here and previously (27) indicate that immunization with recombinant virus expressing either G or F protein alone provides protection in mice against live virus challenge. Moreover, these studies are consistent with the report that prophylactic administration of monoclonal antibodies against either the G or the F protein can protect mice against live RS virus challenge (28, 32).

The RS virus used for challenge in these studies was the same strain (A_2) as that from which the cDNA clones had been constructed. However, it has become apparent recently that at least two major antigenic subtypes (A and B) of human RS virus exist (1, 18). Furthermore, available evidence indicates that the major difference in subtype specificity resides in the G protein (18). Experiments are in progress to test the protective response of immunization with subtype A_2 G or F proteins against challenge with the heterologous RS virus B subtype.

A further consideration comes from the observation that attempts to vaccinate with inactivated RS virus do not protect and result in enhanced disease on challenge (3, 12, 22). The reasons for this enhanced disease remain unclear. It is possible that the use of individual proteins as antigens for immunization avoids the problem of enhanced disease. Bronchiolitis causes detectable histological changes in the

lungs of mice infected with RS virus. It is therefore possible to investigate the ability of prior vaccination to enhance or diminish these lesions. Histological examination of lungs of animals vaccinated with recombinant VV vectors expressing individual antigens of RS virus is in progress.

In summary, the work reported here shows that a powerful protective effect against RS virus was conferred by immunization with VV vectors expressing the RS virus F protein. The extension of this approach to examine not only the protective effects but also the potentially harmful effects of immunization with this and other individual RS virus antigens may point the way to determination of the components that could constitute a safe and effective vaccine.

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