The Purification of Four Respiratory Syncytial Virus Proteins and Their Evaluation as Protective Agents against Experimental Infection in BALB/c Mice

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SUMMARY

The fusion (F) glycoprotein, large glyco- (G) protein, phospho- (P) protein and 22K protein of respiratory syncytial (RS) virus A2 strain were purified by a combination of immunoaffinity adsorption and preparative SDS-PAGE. All four proteins elicited serum antibody in mice after repeated inoculation in adjuvant, although the magnitude of the response as measured by ELISA varied from mouse to mouse. The F protein generated neutralizing antibodies in only 50% of the mice determined to be seropositive by ELISA. The G protein also induced neutralizing antibodies but in this instance neutralization tests and ELISA titres were more closely correlated. No neutralizing activity was detected in mice immunized with the P or 22K proteins although all produced antibody detectable by ELISA. Mice immunized with either the F or the G protein were found to be protected against subsequent RS virus challenge, whether they had developed neutralizing antibody or not. Mice inoculated with the P or 22K proteins were not protected.

INTRODUCTION

The pneumovirus respiratory syncytial (RS) virus is a member of the Paramyxoviridae (Kingsbury et al., 1978) and is the major cause of lower respiratory tract infection in infants (Glezen & Denny, 1973). Attempts to produce live attenuated vaccines have so far proved unsuccessful (Hall, 1980; Wright et al., 1982). A killed vaccine prepared by formalin inactivation of virus was also unsatisfactory, probably because the inactivation process altered immunogenic epitopes on the virion surface (Murphy et al., 1986b). Gene cloning techniques may soon allow the production of native virion proteins with vaccine potential in a non-infectious, pure form. This approach to vaccination could succeed if the virion proteins capable of inducing a protective response can be identified and shown to retain protective immunogenicity when purified free from other viral and host cell components.

The RS virion consists of an internal nucleocapsid containing negative sense RNA surrounded by an envelope derived from the host cell membrane (Stott & Taylor, 1985). Three proteins, the nucleocapsid (N) protein, the phospho- (P) protein and the large (L) protein are associated with the nucleocapsid and four proteins, the matrix (M) protein, the 22K protein, the large glyco- (G) protein and the fusion (F) glycoprotein are associated with the membrane. An additional protein (1A) with a molecular weight of 9500 is associated with the virion and the virus also codes for two non-structural proteins of Mr 11000 (1B) and 14000 (1C; Huang et al., 1985). The G and F proteins are thought to be responsible for viral attachment and membrane fusion respectively (Walsh et al., 1984a; Walsh & Hruska, 1983). They are both glycosylated (Fernie et al., 1985) and are expressed on the surface of infected cells along with the 22K protein.
(Routledge et al., 1987b). These surface proteins are most likely to play the leading role in RS virus disease due to their accessibility to the immune system. This view is supported by the protective effect of anti-G and -F protein monoclonal antibodies (MAbs) in cotton rats and mice (Walsh et al., 1984b; Taylor et al., 1983) and by the detection of antibodies to F and G proteins in naturally infected human infants (Murphy et al., 1986a; Ward et al., 1983; Vainionpaa et al., 1985).

In this paper we describe the purification of the membrane-associated F, G and 22K proteins and the internal P protein, followed by their evaluation as immunogens for protecting mice against experimental RS virus infection.

**METHODS**

**Virus and cell culture.** The A2 strain of RS virus was provided by Dr E. J. Stott of the Institute for Research on Animal Diseases, Compton, U.K. HeLa cells were cultured in Flow Autopow medium supplemented with 10% (v/v) heat-inactivated bovine foetal calf serum (HIFCS), antibiotics and glutamine, and maintained in TC medium 199 (Wellcome) supplemented with 2% (v/v) HIFCS, antibiotics and glutamine.

**Immunofaflinity purification of RS virus proteins.** Confluent roller bottle cultures of HeLa cells infected with RS virus at an m.o.i. of 0:01 p.f.u./cell were harvested when cytopathic effect was extensive. The cells from each bottle were scraped into the medium, collected by centrifugation at 4°C, rinsed once with 100 ml of Tris–saline (0:01 M-Tris–HCl, 0:15 M-NaCl pH 7:4) and made soluble in 50 ml of RIPA buffer [Tris–saline containing 0:1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100 pH 7:4] at 37°C for 30 min. Lysates were clarified (100000 g, 4°C, 3 h) and pooled. Anti-RS virus F protein MAb 1A12 immunoglobulin (37-5 mg: Routledge et al., 1986) purified from ascitic fluid by ammonium sulphate precipitation and coupled to Sepharose 4B beads (Routledge et al., 1985) was rolled with 1:5 1 of cell lysate containing 0:6 mg/ml protein, at 4°C for 24 h. The MAb beads were collected by passing the lysate–bead mixture through a sintered glass filter. The beads were rinsed three times with 100 ml volumes of RIPA buffer containing 20% (v/v) glycerol (pH 7:4) and twice with 100 ml volumes of Tris–saline containing 20% (v/v) glycerol and 1% (w/v) sodium cholate (pH 7:4). Adsorbed RS virus protein was eluted by rolling the beads for 15 min in 15 ml of Tris-saline containing 1% (w/v) sodium cholate and 6 M-NaSCN (pH 7:4), at 4°C. The eluates were collected by centrifugation (3000 g, 5 min) and immediately dialysed against two 1 1 volumes of Tris–saline for 24 h at 4°C.

The G, 22K and P proteins were successively extracted from the cell lysate in the manner described above using anti-RS virus MAbs 3F4, 1C1 and 2B9 respectively (Routledge et al., 1986, 1987a, b).

**Preparative SDS–PAGE.** Immunoaffinity-purified RS virus proteins in non-reducing SDS–PAGE sample buffer containing 0:1% (w/v) rather than 2% (w/v) SDS were electrophoresed without pre-boiling on polyacrylamide gels (Laemmli, 1970) in a column gel continuous elution apparatus (Bethesda Research Laboratories) at 6 mA, 4°C. The composition of the gels was varied as follows depending upon the protein to be electrophoresed; F protein, 2 cm 10% resolving gel and a 3 cm 4% stacking gel; G protein, 8 cm 6% resolving gel and a 2 cm 4% stacking gel; 22K and P proteins, 8 cm 10% resolving gel and a 2 cm 4% stacking gel. Fractions were collected at 13 min intervals at a buffer flow rate of 0.18 ml/min beginning when the dye front eluted from the gel. Fractions containing RS virus proteins were identified by SDS–PAGE under reducing conditions, followed by Western blotting (F, 22K and P proteins) or silver staining (G protein) (Moosai et al., 1986, 1987b). These surface proteins are most likely to play the leading role in RS

**Western blotting.** Western blot analysis of immunoaffinity-purified proteins was performed as described by Samson et al. (1986). Protein preparations run on Laemmli gels under reducing conditions were transferred to cellulose nitrate paper (Burnette, 1981) which was subsequently stained with anti-RS virus MAbs for the F, G and 22K proteins (MAbs, 1E3, 3F4, 2B9 and 1C1 respectively) using an immunoperoxidase system.
Western blotting was also used to investigate the antigenic specificity of antibody in mouse blood. The method differed slightly from that described above. Antigens consisting of RS virus A2 strain-infected HeLa cells or uninfected HeLa cells were solubilized in non-reducing Laemmli sample buffer containing 0.1% (w/v) SDS (20 ml per 4 oz bottle culture of cells) at 37 °C for 30 min. After clarification for 2 min at 10000 in an MSE microfuge, 2 ml of infected or control lysate was loaded into an elongated well spanning the width of a slab gel. Electrophoresis and blotting were performed as usual. Longitudinal strips 4 mm wide cut from the infected and control antigen blots were immersed in 2 ml of mouse blood diluted 1:50 in PBS-Tween 20 containing 1% (w/v) bovine serum albumin (BSA) and 10% HIFCS, and incubated for 2 h at 37 °C. After rinsing the strips with PBS-Tween 20, staining was completed using the immunoperoxidase system described above.

Immunization and challenge of mice. Groups of 14 to 24 female BALB/c mice (3 months old) were inoculated with 100 μl aliquots of immunoaffinity- and SDS–PAGE-purified F, G, P or 22K protein (approximately 0.2 to 2.7 μg ml) at 2-weekly intervals. Immunizations were performed alternately via the footpad (in Freund's incomplete adjuvant) and subcutaneous (complete adjuvant) routes. Four or eight inoculations were given depending upon the speed of seroconversion. Negative control mice received an equivalent number of inoculations with Tris-saline in the appropriate adjuvant. Positive control mice received a single intranasal inoculation of 2 × 10^4 p.f.u. of RS virus A2 strain in 100 μl of tissue culture maintenance medium. Mice were bled from the orbital sinus before the first inoculation and approximately 2 weeks after the last. Blood samples were assayed by ELISA and (post-inoculation bleeds only) Western blotting for RS virus-specific antibodies. Four weeks after the final inoculation, mice were bled by cardiac puncture, the lungs removed and homogenized in tissue culture maintenance medium supplemented with 2% (v/v) BSA, and the homogenates assayed for the presence of RS virus using an immunofluorescent infectious focus assay. Prior to homogenization, tissue for the preparation of frozen sections was taken from the lungs of selected mice. Three lung sections per mouse were fixed in acetone and stained for the presence of RS virus antigen by the indirect immunofluorescence technique using rabbit anti-RS virus serum (Gardner & McQuillin, 1980).

Virus-neutralizing activity in mouse sera. Neutralization tests on heat-inactivated (56 °C, 45 min) mouse sera were carried out in microtitre plates as previously described (Toms et al., 1980) with the following changes. HeLa cell monolayers inoculated with virus–serum dilution mixtures were overlaid with cell growth medium containing 0.2% (w/v) agarose (Miles Laboratories) and incubated at 37 °C for 48 h. After fixation for 10 min at 4 °C in 75% (v/v) acetone, 25% (v/v) PBS, the cell monolayers were air-dried. Infectious foci were stained using the pool of anti-RS virus MAbs and the immunoperoxidase method described for Western blotting, except that the antibodies and conjugates were used at dilutions of 1:500. Neutralization titres were expressed as the reciprocal of the highest serum dilution to give a 60% or greater reduction in the number of infectious foci.

Infectious virus in mouse lung tissue. Lung homogenates were diluted in two-fold steps in cell maintenance medium and assayed for the presence of infectious virus on microtray monolayers of HeLa cells as described above, with the following differences. Rabbit anti-mouse fluorescein isothiocyanate (FITC) conjugate was used in place of the peroxidase conjugate, and after rinsing off the conjugate with PBS-Tween 20, the cells were rinsed a further two times with distilled water. Infectious centres were counted under a fluorescence microscope fitted with 4× and 20× objective lenses.

RESULTS

Imunoaffinity purification of RS virus proteins

The immunoaffinity-purified protein preparations were analysed by SDS–PAGE under reducing conditions followed by Western blotting and immunoperoxidase staining with a pool of MAbs against the F, G, P and 22K proteins (Fig. 1 a). Two major bands with \( M_r \) of 52000 and 20000 were detected in the F protein preparation, representing the F1 and possibly the F2 polypeptides. Two minor bands of 30000 and 14000 were also seen. The 22K protein preparation contained a major band of 22K protein migrating with an \( M_r \) of 23000 in our gel system. Three other bands with \( M_r \) of 24000, 21000 and 17000 were also detected, agreeing with an earlier report (Routledge et al., 1987a), although in this instance sample overloading resulted in poor resolution of the 24000 and 21000 bands. The P protein preparation contained a major band of P protein at 34000 along with a ladder of minor lower \( M_r \) polypeptides ranging in size from 33000 to 19000. No G protein could be detected in the immunoaffinity-purified G protein preparation by blotting despite confirmation of its presence by ELISA using the same detector antibody (Table 1) and by silver staining (Fig. 1 b). Staining of an identical blot with antibody diluent demonstrated that none of the bands described above resulted from non-specific interactions (not shown). Sequential staining with individual MAbs indicated that there was no cross-contamination of the four preparations with the F, P and 22K proteins (not shown).
Fig. 1. SDS–PAGE of immunoaffinity-purified RS virus F protein (lanes 1), G protein (lanes 2), 22K protein (lanes 3) and P protein (lanes 4) performed under reducing conditions (approx. 3 to 8 μg protein loaded per lane). (a) Western blot stained with a pool of anti-RS virus MAbs IE3, 3F4, IC1 and 2B9 (specific for the F, G, 22K and P proteins, respectively). (b) Silver-stained polyacrylamide gel. M, mol. wt. markers (phosphorylase a, BSA, alcohol dehydrogenase, chymotrypsinogen a; Mr 91 000, 67 500, 41 000 and 25 100 respectively; numbers indicate Mr x 10^-3. Intermediate tracks contain PAGE sample buffer only and demonstrate the presence of a set of artefactual bands (X) migrating just below 67 000 in all tracks. These bands are seen in all silver-stained reducing Laemmli gels run in our laboratory and their origin is unknown.

Table 1. **ELISA titres and protein concentrations of immunoaffinity-purified RS virus proteins before and after further purification by preparative SDS–PAGE**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Before PAGE</th>
<th>After PAGE</th>
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<tr>
<td></td>
<td>ELISA titre</td>
<td>Concentration (μg/ml)</td>
</tr>
<tr>
<td>F</td>
<td>512</td>
<td>115</td>
</tr>
<tr>
<td>G</td>
<td>1024</td>
<td>42</td>
</tr>
<tr>
<td>22K</td>
<td>256</td>
<td>76</td>
</tr>
<tr>
<td>P</td>
<td>1024</td>
<td>62</td>
</tr>
</tbody>
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* Protein concentrations of the PAGE-purified protein samples were too low to be determined accurately by Hartree’s method. An approximate value was obtained by a comparison with a titration of immunoaffinity-purified protein of known concentration analysed by SDS–PAGE and silver staining.

Although no evidence of cross-contamination of the four preparations with other RS virus proteins was revealed by blotting, analysis of the preparations by ELISA (not shown) and by silver staining (Fig. 1b) indicated that this was not the case. By ELISA, the F and G protein preparations were cross-contaminated with G and F respectively. A moderate level of direct binding of conjugate to test antigen in the ELISA also suggested that all four preparations contained low levels of mouse immunoglobulin, presumably originating from the MAb beads used in the purification procedure. Silver staining revealed a strong band of Mr, 42 000, probably
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The N protein, in the P protein preparation. In addition, many other bands with $M_r$ spanning the entire fractionation range of the polyacrylamide gel were seen in all four preparations. It is likely that these represented contaminating polypeptides originating from the host cell or culture medium. These preparations were thus considered insufficiently pure for use as antigens and were purified further.

Preparative SDS–PAGE of immunoaffinity-purified proteins

Eluates from MAb-coated Sepharose beads were further purified by preparative SDS–PAGE. Due to the different sizes of the four proteins it was necessary to use several different gel compositions to achieve an acceptable compromise between elution time and separation from unwanted contaminants. The larger proteins (F and G) eluted more slowly than the smaller proteins and, as the buffer flow rate across the bottom of the gel was constant the larger proteins were eluted in a larger volume of buffer. The relative dilution factors for F and G were approximately 1/80 whereas for P and 22K they were approximately 1/8.

Under the non-denaturing conditions used in preparative SDS–PAGE the F protein migrated as two bands. Approximately 70%, as determined by scintillation counting of radiolabelled F protein (not shown), migrated with an $M_r$ of approximately 200000. The remaining 30% migrated as a band of 70000 with a smear of protein extending down to approximately 35000. The 200000 band was collected for immunization studies.

After dialysis the protein concentrations in the pooled fractions were determined. The protein content proved too low to be determined accurately by the method of Hartree (1972) but an approximate value was obtained by comparison with a titration of immunoaffinity-purified protein of known concentration analysed by SDS–PAGE and silver staining. The purified preparations were assayed by ELISA (Table 1). Assay titres had fallen commensurate with the reduction in protein concentration suggesting that little activity had been lost. Our inability to measure protein concentrations directly, however, precludes any conclusions from these data regarding the degree of purification achieved.

Protein preparations were analysed by PAGE followed by silver staining in parallel with the original immunoaffinity eluates diluted to give an approximately equivalent intensity of staining of the major bands (Fig. 2). A marked improvement in the purity of the P and 22K proteins was
Fig. 3. BALB/c mice were inoculated intranasally with live RS virus (A2) or were immunized with Tris-saline (T/S) or immunoaffinity-PAGE-purified F protein (F), P protein (P), 22K protein (22K) or G protein (G) as described in the text. (a) Post-immunization blood samples taken from the mice were assayed by ELISA for the presence of anti-RS virus protein antibody. Immunoaffinity-purified F (○), G (□), P (●) and 22K (▲) proteins were used separately as capture antigens. Each point indicates the blood titre of a single mouse except for those below 40, where the number of mice involved is given in parentheses. Pre-immunization blood samples were assayed in parallel with those above and all gave titres < 40 against all four capture antigens (not shown). (b) Post-immunization blood samples taken from the mice were tested for their ability to neutralize live RS virus in a plaque reduction assay. Each point represents the titre of a single mouse. (c) Quantity of infectious RS virus recovered from the lungs (p.f.u./g lung tissue) of the immunized mice 4 days after they were challenged intranasally with live RS virus.
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Fig. 4. Western blots of RS virus-infected (lanes 1 to 8) and uninfected (lanes 1’ to 8’) HeLa cell lysates electrophoresed on SDS–polyacrylamide gels under non-denaturing conditions (as described for preparative PAGE in Methods) were cut into strips and stained with blood taken from a BALB/c mouse either inoculated intranasally with infectious RS virus (lane 1) or immunized with Tris–saline (lane 2) or immunoaffinity-PAGE-purified RS virus F (lane 3), P (lane 4), 22K (lane 5) or G (lane 8) protein. Additional strips were stained with antibody diluent (lane 6) or the pool of anti-RS virus MAbs described in Fig. 1 (lane 7).

Evident. The preparative gel electrophoresis appeared to have effected a further change of the 22K protein which was resolved in gel eluates as a 23000 to 24000 doublet. No major contaminants were observed on the silver-stained gels of F and G protein preparations after SDS–PAGE. However, dilution of the immunoaffinity eluates rendered most of the contaminating bands too faint to be visualized and so the degree of purification could not be assessed. The F protein 200000 fraction harvested by preparative gel electrophoresis proved to contain only the 52000 F1 band. There was no sign of an F2 molecule with an expected Mr of approximately 20000 which would be present in equimolar amounts to F1 if the 200000 band comprised aggregates of the F protein spike.

Immunogenicity of the purified proteins

All four proteins were immunogenic in mice, although the magnitude of the serum response varied from mouse to mouse (Fig. 3) and 30% of mice inoculated with the G protein remained seronegative by ELISA after eight inoculations (Fig. 3a).

By Western blotting and ELISA the only antibodies detected in the mice inoculated with the purified protein preparations were directed against the particular protein administered. An example of a blot from each group of mice is illustrated in Fig. 4. The intensity of staining in the blots tended to mimic the antibody ELISA titre of the mouse blood concerned, thus the sera from those mice inoculated with the G protein which did not seroconvert did not react in Western blots. Negative control mice inoculated with Tris–saline produced no RS virus-specific antibodies detectable by ELISA or blotting. Positive control mice which received a single intranasal inoculation of infectious RS virus produced anti-F protein and anti-P protein antibodies which were detected in Western blots. This technique also indicated that anti-N protein antibodies may also have been induced. Antibodies to the F but not G, P or 22K proteins were detectable by ELISA, but, if positive control mice received a second intranasal inoculation
prior to serological testing antibodies specific for the F, G and P proteins could be detected both by blotting and ELISA (not shown). None of the sera from any group of mice reacted with Western blots of uninfected HeLa cells.

Immunization with the P and 22K proteins did not elicit significant amounts of neutralizing antibody to RS virus (Fig. 3b). Neutralizing antibodies were detected in 11 of 14 positive control mice and in eight of 15 F protein-inoculated mice. In both cases there was no relationship between the level of neutralizing antibody and the F protein-specific ELISA titre, and in several cases high ELISA titres were detected where neutralizing antibody was absent. However, in no case was neutralizing antibody detected in the absence of an ELISA titre. Neutralizing activity was detected in 12 of 16 G protein-inoculated mice and in this instance the correlation between the neutralization and ELISA G protein-specific titres was good. Of the six G protein mice with ELISA titres of <40, two had neutralization titres of 4, and four had neutralization titres of <4.

**Challenge of immunized mice with infectious RS virus**

Four days after challenge by intranasal inoculation, lung tissue was homogenized and assayed for virus. Infectious RS virus was recovered at a mean titre of $10^{3.8}$ p.f.u./g lung tissue from 23 of 24 mice immunized with Tris–saline (Fig. 3c). There was no significant difference between this and the quantity of virus recovered from the mice immunized with P protein (recovery from 14 of 15 mice) or 22K protein (recovery from 13 of 14 mice) (Student's t test; $P > 0.05$). No virus was recovered from those mice which had experienced a prior RS virus infection, 13 of 15 mice immunized with the F protein or 15 of 16 mice immunized with the G protein.

Frozen sections cut from the lungs of selected mice (i.e. from all groups but those immunized with the P and 22K proteins) were stained by immunofluorescence using polyclonal rabbit anti-RS virus serum. RS virus antigen was detected in seven of eight mice treated with Tris–saline, in none of seven treated with F protein, in three of 16 treated with G protein and in none of eight mice which had suffered a previous RS virus infection.

**DISCUSSION**

The multiple bands visualized in 22K and F immunoaffinity-purified protein preparations when analysed by Western blotting were largely those present in unpurified infected cell lysates (Routledge *et al.*, 1987a; Samson *et al.*, 1986). There was no evidence of further fragmentation of the eluted proteins during adsorption and elution from MAb-coated Sepharose. The 22K protein, however, seems to have suffered conformational changes most likely due to disulphide–sulphydryl interchange reactions. The eluted protein migrated with an apparent Mr of 16 000 in non-reducing SDS–polyacrylamide gels after treatment with iodoacetamide to block any further rearrangements of disulphide bonds during electrophoresis (not shown). It thus corresponds to the ‘b’ form of the 22K protein previously demonstrated to accumulate when cell lysates were exposed to oxidizing agents unless free sulphydryl groups were blocked (Routledge *et al.*, 1987a).

The ladder of minor P protein-related bands bears a strong resemblance to the V8 protease digest pattern of the P protein reported by Huang *et al.* (1985) and is similar to a series of polypeptides we have detected in MAb immunoprecipitates of P protein from the Long strain of RS virus (Routledge *et al.*, 1985). It is unlikely, however, that the minor bands detected are the result of proteolytic breakdown of the P strain occurring during purification as the preparative SDS–PAGE-purified P protein is stable in the presence of an uninfected HeLa cell RIPA lysate over 24 h at 37 °C (not shown). However it is possible that they have arisen due to proteolysis occurring prior to cell solubilization.

The identity of the two major bands with Mr, 52 000 and 20 000 in the F protein preparation which stain with the single MAb 1E3 remains unresolved. We have previously argued that, if these bands represent the F₁ and F₂ moieties of the F protein, both F protein subunits must share the 1E3 epitope (Samson *et al.*, 1986). Alternatively the 20 000 band may represent a cleavage product of the F₁ as described by Norrby *et al.* (1986). A further analysis of the identity of these bands is underway.

The G protein had clearly sustained some damage during the purification procedure. Whilst the epitope recognized by MAb 3F4 in crude cell lysates is stable and reactive following Western
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blotting (Fig. 4), this MAb did not stain immunoaffinity eluates although the epitope was still functional in ELISA and the presence of the protein was clearly demonstrable by silver staining of bands resolved by PAGE. Despite the damage, this partially purified preparation was immunogenic in both mice and rabbits (data not shown).

The ELISA revealed that adsorption and elution from MAbS failed to separate the two glycoproteins of the virus completely. In addition, silver staining of proteins resolved by PAGE revealed considerable contamination of each preparation, probably with both viral and non-viral proteins. The P and N proteins of RS virus are known to be strongly associated and may coprecipitate as protein aggregates during immunoaffinity purification procedures (Gimenez et al., 1984; Routledge et al., 1985). Clarifying the cell lysate at high g force prior to precipitation alleviated this problem to some extent, but silver staining still revealed a considerable quantity of N protein in the P protein preparation. Although not obvious by silver staining, the presence of small quantities of M protein was also inferred from preliminary immunoaffinity purification of radiolabelled cell lysates (not shown). These observations indicated that the immunoaffinity step alone cannot guarantee that the RS virus proteins destined for use in immunization experiments are free of other viral or host cell components.

Preparative SDS–PAGE, in which molecules are separated according to size, was chosen to purify the proteins further. Gels were run under non-denaturing conditions in an attempt to limit damage to significant epitopes. Even so, following SDS–PAGE changes were observed in the 22K protein which migrated as a doublet of bands in reducing gels. The 200000 M₉ band of F protein when isolated contained no F₂ polypeptide visible on silver staining. Whether this indicates that the 200000 M₉ band is an aggregate of F₁ alone, that the F₂ was lost during preparative SDS–PAGE or that the F₂ is not revealed by silver staining post-purification remains to be determined. All four proteins retained their ability to react with MAbS in ELISA.

The increase in purity attained after this second step could not be accurately ascertained as protein concentrations in gel eluates were too low to be determined. Comparison of silver-stained gels afforded clear qualitative evidence of an improvement in purity for the 22K and P proteins. Pilot experiments with crude cell lysates in which the separation characteristics of the gels used for the F and G proteins were defined suggested that the technique would similarly effect considerable purification of these proteins too. Although no major contaminants were observed on the silver-stained gels, the dilution factor introduced during preparative SDS–PAGE for the F and G proteins was itself sufficient to account for the disappearance of contaminants. Attempts to concentrate the eluates were unsuccessful, probably due to the loss of protein through non-specific adsorption during handling. The material inoculated into mice was therefore unconcentrated preparative SDS–PAGE eluate. Each preparation contained a single RS virus protein with no major contaminating bands demonstrable by silver staining.

All of the purified proteins proved immunogenic for mice following repeated inoculations in Freund’s adjuvant. ELISA and Western blot analysis indicated that in all four cases the only antibodies produced were directed against the immunizing protein. The multiple bands in infected cell lysates stained with antibodies from the blood of mice inoculated with the 22K protein or P protein correspond to those stained by MAbS and are thus alternative forms, precursors or breakdown products of each protein.

The variability in the magnitude of the antibody response as measured by ELISA reveals individual variations in the immune systems of the mice. The lack of correlation between the ELISA titre and the level of neutralizing antibody in mice immunized with F protein supports this view, suggesting that individual mice respond with different relative efficiencies to different epitopes on the protein. The very low protein concentration of the inoculum no doubt accentuated this variability and necessitated multiple inoculations to produce measurable immune responses. The total dose of each protein administered to each mouse in four or eight inoculations was only that quantity of SDS–PAGE-purified protein present in 0.15 to 2.5 μg of the cruder affinity-purified material.

Prior exposure to infectious RS virus or immunization with the purified F and G proteins prevented virus replication in the lungs of mice on subsequent challenge. The lack of virus antigen in lung sections demonstrable by immunofluorescent staining confirms that failure to
isolate virus does not result from neutralization of virus by antibody released during lung homogenization. The purified F and G proteins therefore, despite any changes brought about by the purification process, were effective as agents to protect mice against subsequent RS virus infection.

The mechanism of this protective effect is open to question and the results obtained with the F protein, where in six instances mice with high titres of non-neutralizing antibody were protected, suggest that some factor other than virus neutralization may be involved. Recent work with cotton rats and human infants has suggested that immunization which stimulates non-neutralizing antibody may contribute to a pulmonary Arthus reaction on subsequent infection which increases the severity of disease (Murphy et al., 1986b). No evidence of such a reaction was seen here and, although detailed histopathological examination was not attempted, at four days after infection there was little virus left in the lung to act as a focus for such a reaction.

Immunization with the P and 22K proteins did not protect mice against RS virus infection despite high ELISA antibody titres. The 22K protein was structurally altered in the purification process and it is possible that important epitopes may have been masked. However, our recent observation that the 22K protein appears on the infected cell surface only late in the virus replicative cycle (Routledge et al., 1987b) suggests that antibody to this protein alone would be ineffective in halting RS virus infection. There was no evidence of damage to the purified P protein but the possibility of epitope destruction leading to a deficient antibody response cannot be excluded.

We have established that the F₁ polypeptide and the G protein of RS virus in pure form as judged by PAGE gels stained with silver are capable of inducing virus-neutralizing antibodies and of protecting the host against subsequent challenge with live virus. This confirms previous work indicating that anti-G and anti-F protein antibodies are important in immunity to RS virus in the mouse (Taylor et al., 1983). Isolated viral glycoproteins may thus be considered as candidates for vaccines against RS virus and the search for a plentiful safe source of purified protein is underway.

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REFERENCES


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