The Envelope Proteins from Purified Respiratory Syncytial Virus Protect Mice from Intranasal Virus Challenge (42871)

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Abstract. **A lyophilized subunit vaccine prepared from purified respiratory syncytial virus, which contained the envelope glycoproteins F and G and the nonglycosylated matrix protein VPM, was tested in SJL mice for its ability to protect the lungs of mice from intranasal viral challenge. Initially, the mice were injected subcutaneously with one, two, or three doses of 5 or 25 μg of vaccine in 50% complete Freund's adjuvant or with complete Freund's adjuvant or phosphate-buffered saline only. Although none of the mice produced neutralizing serum antibody, three doses of 25 μg elicited antibodies to F, G, and VPM. Despite the absence of detectable neutralizing antibodies, the lungs of 93% of the vaccinated mice were protected from intranasal viral challenge. Because the initial protocol did not elicit neutralizing antibodies and a few single-dose animals were not protected, a second vaccine trial was carried out. For these studies the priming dose was increased to 50 μg, which was followed, in half the vaccine recipients, by a second dose of 25 μg. Mice given the priming dose of vaccine produced antibody to G and showed no neutralizing activity, whereas the mice given two doses of vaccine produced antibodies to G, F, and VPM and also displayed neutralizing activity for respiratory syncytial virus. The lungs of 100% of the vaccine recipients in this trial were protected from intranasal challenge. Although the vaccine elicited antibody to VPM, this response did not correlate with protection. In addition, examination of the sera from unimmunized mice recovering from respiratory syncytial virus infection revealed a serum antibody profile similar to that noted for humans, lacking antibody to VPM. Thus, the data show that a combined glycoprotein subunit vaccine affords complete protection to viral challenge and offers an approach to develop a multivalent subunit vaccine.**

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Although respiratory syncytial (RS) virus infection is the major cause for hospitalization of infants with severe respiratory disease (1, 2), there is no vaccine for its prevention. An inactivated vaccine sensitized its recipients to natural infection instead of protecting them and all attempts to develop an attenuated virus vaccine have, so far, been unsuccessful (3–7).

The RS virion contains seven polypeptides, three associated with the nucleocapsid and four associated with the envelope. The nucleocapsid proteins include VPN, the major nucleocapsid protein; VP32, a phosphorylated protein; and L, the putative RNA polymerase. The envelope contains two glycoproteins; F (70,000) and G (90,000) as well as two nonglycosylated proteins; VPM (28,000), the membrane organizing or matrix protein and a 24,000–25,000 protein (8–10). The 70,000 glycoprotein, F, is a proteolytically processed, disulfide-linked fusion protein that separates into two polypeptides, F1 and F2, when reduced and electrophoresed on sodium dodecyl sulfate- polyacrylamide gels (11–14). The 90,000 glycoprotein, G, the attachment protein, is an unusual viral glycoprotein with oligosaccharides, mostly *O*-linked, contributing more than 50% to its M_r, and with an amino acid composition of 30.6% serine and threonine and 10% proline (15–18). Both F and G glycoproteins induce neutralizing antibodies in laboratory animals (14, 19).

The demonstration that the administration of RS virus convalescent serum, human immune globulin, or monoclonal antibodies to either one of the viral glycoproteins (F or G) would protect the lungs of experimental animals from infection after intranasal challenge suggested that a vaccine containing the viral glycopro-

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teins might prevent the lower respiratory tract disease caused by RS virus (20–23). Most recently, it has been demonstrated that the lungs of cotton rats and mice can be protected from intranasal challenge with RS virus by immunization with either F or G alone. In these studies, vaccines contained purified F or G or were vaccinia recombinants containing the gene for either F or G (24–29). Data from investigations employing F or G recombinant vaccinia vaccines have generally shown a greater degree of protection by F when compared directly with G recombinant vaccines (26, 29). In the one instance where animals were immunized with both F and G recombinant vaccinia vaccines, the lung virus titers were lower than those in animals receiving the individual vaccines (26).

Based on the findings described above and the fact that both F and G glycoproteins induce neutralizing antibody, it is possible that more than one glycoprotein should be included in a prospective subunit vaccine as has been the case for other experimental paramyxovirus vaccines (30, 31). The present investigation documents the protective capacity of a combined glycoprotein subunit vaccine containing soluble complexes of envelope glycoproteins F and G prepared from purified RS virus.

Materials and Methods

Virus and Cells. Respiratory syncytial virus strain Long was grown in HeLa cells in minimal essential medium-5% fetal bovine serum and assayed by plaquing on HeLa cells (32).

Virus Purification. Medium from RS virus-infected monolayers was clarified by centrifugation in a refrigerated IEC PRJ centrifuge and the virus in the supernatant purified by two cycles of centrifugation on discontinuous gradients of sucrose-Hanks' balanced salt solution (HBSS) followed by isopycnic banding in a linear 30–60% sucrose-HBSS gradient (33).

Preparation of Viral Antigens. For immunization, the antigens were prepared as glycoprotein complexes (micelles) free of lipid and detergent according to the method of Helenius and von Bonsdorff (34) and Simons *et al.* (35). Pellets containing 0.5–1.0 mg of purified virus were solubilized in 300 μl of 2% Triton X-100 in HBSS and then layered over 300 μl of 15% sucrose in TN (0.05 *M* Tris-HCl, pH 7.4, 0.1 *M* NaCl) containing 1% Triton X-100, which in turn was layered over a 12-ml 20–50% linear sucrose-TN gradient. Centrifugation was in an SW41 Ti rotor at 20°C for 22 hr at 40,000 rpm. The gradient was fractionated and the fractions containing the viral glycoproteins were determined by Western blot using 30- to 40-μl aliquots. These fractions were pooled and dialyzed against 0.005 *M* Tris-HCl, 0.01 *M* NaCl (pH 7.4) for 20 hr at 4°C. The protein content of the dialyzed antigen was determined by the Lowry procedure (36) and the antigen was concentrated by lyophilization (30).

Protocols for the Immunization of Mice. SJL mice were selected for this study because of their greater susceptibility to RS virus infection when compared with C57BL/6, BALB/c, and DBA strains (unpublished observations). SJL mice (The Jackson Laboratory; Sendai free) were 4 weeks old at the time of the first vaccine dose. Two trials were run with the vaccine. For all vaccinations, virus challenges, and bleedings, the mice were lightly anesthetized with ether.

Trial 1: to test the efficacy of the vaccine. The vaccine was resuspended in water and mixed with an equal volume of complete Freund's adjuvant (CFA) to give a final concentration of 25 μg or 5 μg of envelope proteins per 0.25 ml and injected subcutaneously.

SJL mice were separated into four groups of 15 mice. The mice in each group were injected at biweekly intervals, with a total of one, two, or three doses according to the following protocol: Group 1, 25 μg of envelope proteins/dose; Group 2, 5 μg of envelope proteins/dose; Group 3, 50% CFA in phosphate-buffered saline (PBS) (PBS = 0.85% NaCl, 0.02 *M* phosphate buffer, pH 7.4); and Group 4, PBS.

Before the first injection, mice were bled from the retroorbital sinus and the sera pooled. Two weeks after each injection, five mice from each group were bled for serum and then challenged intranasally with 5×10^5 plaque-forming units (PFU) of RS virus/mouse. Four days after challenge, the time for maximum virus growth (data not presented), the lungs were harvested for virus assay. The sera obtained from the mice before each challenge were pooled by group and these pools as well as the preimmunization pool were tested for antibodies to viral proteins and neutralizing activity against RS virus.

Trial 2: to test the effect of increasing the priming vaccine dose. The mice were separated into four groups, and some were bled, as described above, for the preimmunization serum pools. The vaccine was resuspended and mixed with an equal volume of CFA to yield a concentration of 50 μg or 25 μg/0.1 ml. The mice in each group were vaccinated subcutaneously as follows: Group 1—10 mice, 50 μg of envelope proteins, 1 dose; Group 2—10 mice, 50 μg of envelope proteins, 1 dose; 25 μg of envelope proteins, 1 dose; Group 3—20 mice, 50% CFA in PBS; and Group 4—20 mice, PBS.

Two weeks after the first injection, the mice in Group 1 were bled retroorbitally and their sera were pooled. They were then challenged and the lungs harvested as described below. Ten mice from each of the Groups 3 and 4 were also bled, challenged, and killed to serve as controls. Then, the remaining mice were given the second injection. Two weeks after the second injection, the mice in Group 2, as well as the remaining mice in Groups 3 and 4, were bled, challenged, and their lungs harvested.

Assay of Lung Homogenates from Challenged

Mice. Following sacrifice by an ether overdose, the lungs from each animal were removed, rinsed in cold HBSS, blotted with sterile filter paper, weighed, and stored at −70°C. For assay, lungs were thawed on crushed ice and placed in a homogenizer tube containing sufficient cold minimal essential medium-5% fetal bovine serum to make a 10% (w/v) suspension. The tube was placed in crushed ice and the lungs were homogenized with a motor-driven pestle. The homogenate was refrozen at −70°C, then thawed and resuspended with the motor-driven pestle in order to disrupt more cells. The suspension was then clarified by centrifugation for 10 min at 1000 rpm in a refrigerated IEC-PRJ centrifuge. The individual lung supernatants were assayed by plaquing on HeLa cell monolayers at 10^0, 10^{-1}, and 10^{-2} dilutions. A stock RS virus was titrated in each assay as a control for the sensitivity of the HeLa monolayers.

For the statistical analysis, the log of the virus yields from the lungs of all of the mice in each treatment group were pooled to obtain a mean virus yield for each group. For this analysis, lungs from which no virus was isolated were assigned a titer of $10^{1.4}$ PFU/g of lung, the lowest virus concentration detectable by the plaque assay. In each experiment, the mean virus titers in the lungs of each group were compared using Student's t test (P values of ≤ 0.05 were considered significant).

Assays for Neutralizing Antibody Responses to RS Virus. Each pool of mouse serum was inactivated at 56°C for 15 to 30 min. The serum pools were assayed at 1/10, 1/30, and 1/90 dilutions against 25,000 PFU of RS virus. After an incubation period of 2 hr at room temperature, the serum-virus mixtures were diluted 100-fold in cold HBSS and assayed for residual virus by plaque assay (37). The control consisted of virus in minimal essential medium-5% fetal bovine serum, and the 1/30 and 1/90 dilutions of mouse serum were supplemented with fetal bovine serum to bring the serum concentration to 5%. The end point of the assay was the reciprocal of the serum dilution that inactivated 50% of the virus (ND_{50}) and was calculated by interpolation (37).

Western Blots. Antigen was partially purified RS virus prepared by two cycles of centrifugation of clarified virus harvests through discontinuous sucrose gradients onto a 60% sucrose-HBSS cushion, first through 20% sucrose-HBSS and through 30% sucrose-HBSS (37).

The antigen mixture (40 μg) was separated by reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose was blocked with a 5% solution of skim milk powder, incubated with the pooled groups of mouse sera diluted 1/20 in the 5% skim milk powder solution and antibody binding detected with affinity purified ^{125}I-labeled antimouse γ-globulin, as previously described (37). All steps were standardized, i.e., electrophoresis conditions and time, blotting conditions and time, and after reassembly of the nitrocellulose sheet, the exposure and developing time for the film. An RS virus positive adult human serum, containing antibodies to all viral structural proteins except VP200, VPM, and F2 (37), was included as a marker in each run. The human serum was run at dilutions of 1/20 or 1/50 and antibodies visualized with ^{125} I-protein A.

Results

Demonstration of the Viral Proteins in the Vaccine. A Western blot with two batches of the reconstituted lyophilized viral vaccine and partially purified RS virus is presented in Figure 1. The reactivity evident with the control human serum indicates that both viral glycoproteins are present in the vaccine. Although not seen in Figure 1, the vaccine also contains VPM which is solubilized when RS virus is treated with Triton X-100 in isotonic or lower concentrations of salt (8).

The Antibody Response to Immunization. *Trial 1.* Two doses of vaccine, 25 μg and 5 μg, were examined. The antibodies produced to the viral proteins are shown in Figure 2. Mice that received 3 × 25-μg doses developed antibodies to the two glycoproteins and to VPM (Fig. 2, Lane 6), whereas mice receiving 3 × 5-μg doses developed much lower levels of antibodies to these components (Fig. 2, Lane 7). These data also suggest that the high vaccine dose may have elicited antibodies to VPN. However, no mice from this group developed serum neutralizing antibodies to RS virus.

Trial 2. Because the mice did not develop neutralizing antibody to RS virus and a few single-dose animals were not protected (see below) in the first trial, the priming dose of vaccine was increased to 50 μg. Group 1 received a single 50-μg dose and Group 2 received a second dose of 25 μg 2 weeks later. The antibodies produced to the viral proteins are shown in Figure 3. Only antibodies to G were produced after a single dose of 50 μg (Fig. 3, Lane 4), whereas antibodies to G, F (F1), VPM, and apparently VPN were produced following the booster dose of 25 μg. No neutralizing antibodies were detected in the Group 1 serum pool and the Group 2 serum pool had a neutralizing antibody titer of 30 ND_{50}/ml. Thus, increasing the priming dose followed by a boost with the higher dose (25 μg) employed in Trial 1 resulted in the production of neutralizing antibodies. None of the serum from control mice neutralized RS virus.

The Response to Intranasal Virus Challenge. The virus titers in the lungs from each treatment group in both vaccine trials are presented in Figure 4. In Trial 1 (Fig. 4A) the lungs from one mouse in each immunized group yielded virus and these mice were among those challenged after a single vaccine dose. The differences between the mean viral titers of each immunized group and the sham-immunized groups were significant ($P < 0.001$). The difference between the sham-immu-

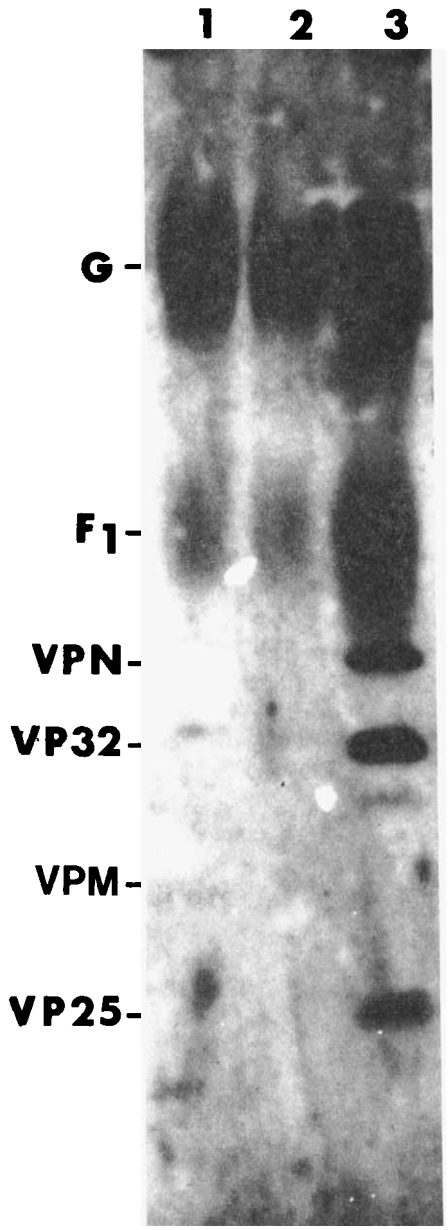

Figure 1. Western blot analysis of vaccine lots 3 and 4. Lane 1—vaccine lot 4, 21 μg; Lane 2—vaccine lot 3, 25 μg; Lane 3—partially purified RS virus, 40 μg. The blots were reacted with the adult human control serum (1/20) and then with ^{125}I-staphylococcus protein A.

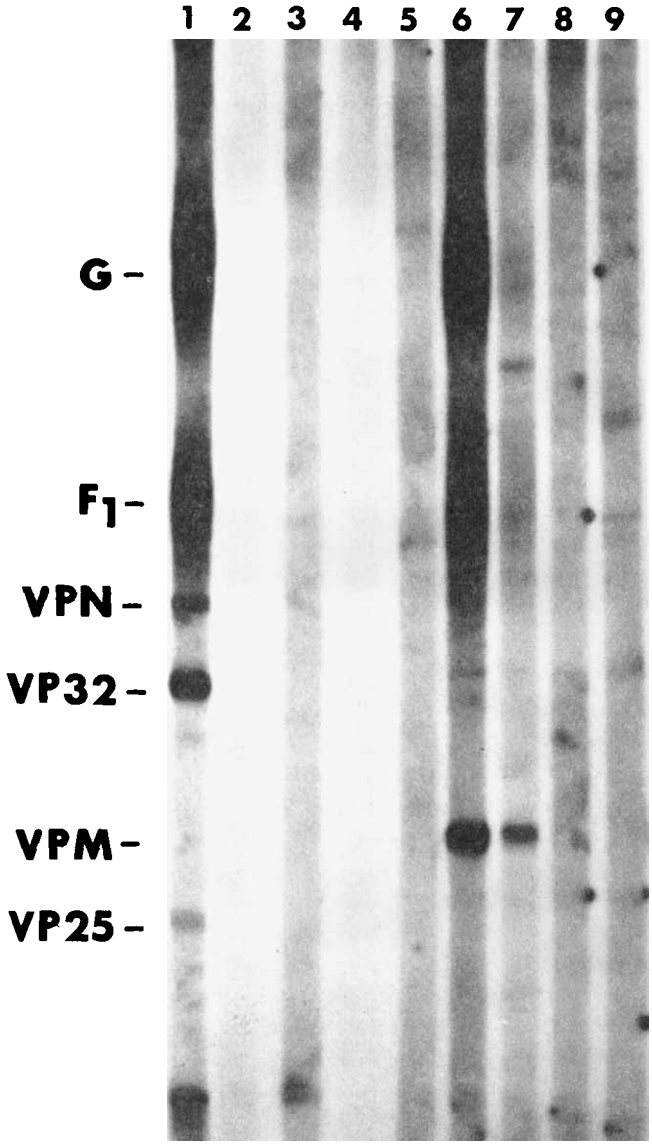

Figure 2. Western blot analysis of pooled sera from mice in vaccine Trial 1 immunized subcutaneously with a RS virus envelope protein vaccine or sham immunized. The antigen consisted of 40 μg/lane of partially purified RS virus. Lane 1—control human serum (1/50) reacted with ^{125}I-staphylococcus protein A; Lanes 2–9—mouse sera (1/20) reacted with ^{125}I-antimouse γ-globulin; Lanes 2–5—after one dose, Lane 2—25 μg of vaccine (Group 1); Lane 3—5 μg of vaccine (Group 2); Lane 4—CFA (Group 3); Lane 5—PBS (Group 4); Lanes 6–9—after three doses; Lane 6—25 μg of vaccine (Group 1); Lane 7—5 μg of vaccine (Group 2); Lane 8—CFA (Group 3); Lane 9—PBS (Group 4).

nized groups was not significant($P > 0.5$). In Trial 2 (Fig. 4B) no virus was found in lungs from any immunized mouse. Again, the differences between the immunized and sham-immunized groups were significant ($0.01 > P > 0.005$ for one or two doses of vaccine vs CFA and $P < 0.001$ for one or two doses of vaccine vs PBS). The difference between the sham-immunized groups was not significant ($0.2 > P > 0.1$). For statistical purposes the sham groups in Figure 4B were treated as a single population because there were no differences (within either group) between animals undergoing one or two sham immunizations. In comparing the infection rates from both trials following intranasal viral challenge, Trial 1 showed a 6.7% (2 of 30) rate for vaccine vs a 65.5% (17 of 26) rate for sham-immunized animals and Trial 2 showed a 0% (0 of 18) rate for vaccine vs a 63.2% (24 of 38) rate for sham-immunized animals.

The Antibody Response of Untreated SJL Mice to Intranasal Infection with RS Virus. The antibody response of mice following intranasal infection with RS virus was assessed in order to compare it with the antibody response obtained with the envelope protein

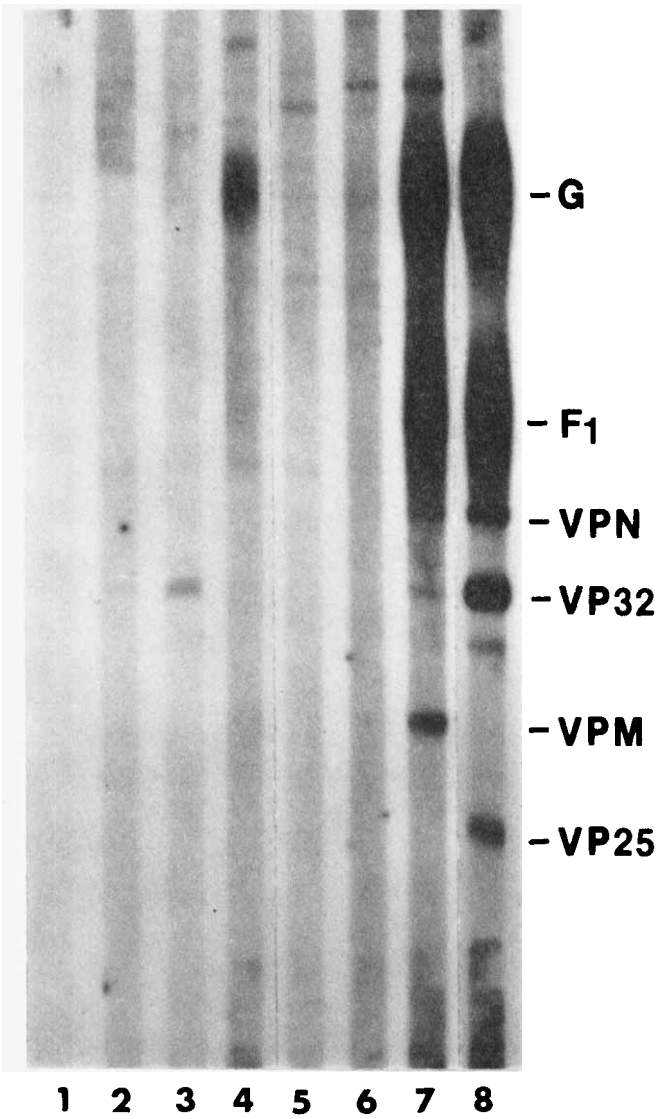

Figure 3. Western blot analysis of pooled sera from mice in vaccine Trial 2 immunized subcutaneously with a RS virus mixed envelope protein vaccine or sham immunized. Lanes 1–7—mouse sera (1/20); Lane 1—preimmunization; Lanes 2–4—after one dose, Lane 2—PBS (Group 4); Lane 3—CFA (Group 3); Lane 4—50 μg of vaccine (Group 1); Lanes 5–7—after two doses, Lane 5—PBS (Group 4); Lane 6—CFA (Group 3); Lane 7—50 μg and 25 μg of vaccine (Group 2); Lane 8—control human serum (1/50).

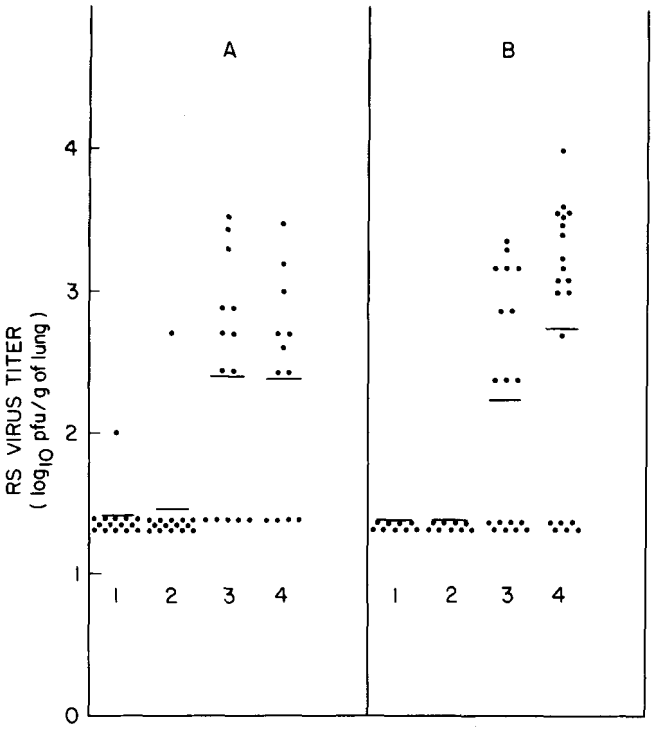

Figure 4. Growth of RS virus in the lungs of individual mice following intranasal challenge with 5×10^5 PFU of virus at 2 weeks after immunization with an envelope protein vaccine or sham-immunized with CFA or PBS. The lungs were grouped according to the immunization protocol and lungs with no detectable virus were assigned a titer of $10^{1.4}$ PFU/g of lung, the theoretical limit of detection of the assay. The bars represent the mean virus titers of each group. (A) Vaccine Trial 1. The mice were injected with one, two, or three doses of either (1) 25 μg (Group 1) or (2) 5 μg of vaccine (Group 2) in 50% CFA, (3) 50% CFA in PBS (Group 3), or (4) PBS alone (Group 4). (B) Vaccine Trial 2. Mice were given (1) one dose of 50 μg (Group 1) or (2) two doses, one of 50 μg and one of 25 μg (Group 2) of vaccine in 50% CFA, (3) 50% CFA in PBS (Group 3), or (4) PBS alone (Group 4).

vaccine. Sera that were collected 4–6 weeks after intranasal infection with SJL mice were pooled for Western blot and neutralization assays. This pool had a neutralization titer of 130 ND_{50}/ml. The Western blot is presented in Figure 5, Lane 2. Antibodies are present to F (F1) and G as well as to VPN, VP32, and VP25. The response to F and G resembles that achieved with the vaccine (Fig. 2, Lane 6; Fig. 3, Lane 7); however, in contrast to the response to the vaccine, mice that responded to RS virus infection did not produce antibodies to VPM.

Discussion

We have demonstrated that subcutaneous injection of detergent-free complexes of the envelope proteins of purified RS virus protects the lungs of mice from intranasal challenge with homologous virus. Our results, therefore, are generally comparable to those obtained by immunization with either purified F or G or the vaccinia virus recombinants containing the gene for either F or G (24–29, 38). However, in those studies where neutralizing antibody was measured, differences in correlation with protection have been noted. In one study, increased protection was elicited by immunization with an F recombinant, as opposed to the G recombinant, and was correlated with a higher serum neutralizing antibody titer induced by the F recombinant (26). In a very recent report utilizing purified F or G glycoproteins, protection against challenge occurred in a number of mice that did not display neutralizing antibodies (38). These later findings support our observations showing protection against intranasal challenge in vaccinated mice in the absence of detectable neutralizing antibodies. However, the absence of detectable viral neutralization does not rule out antibody involvement in the protection process since antibody to F and G or to G alone was present in most groups (Fig. 2,

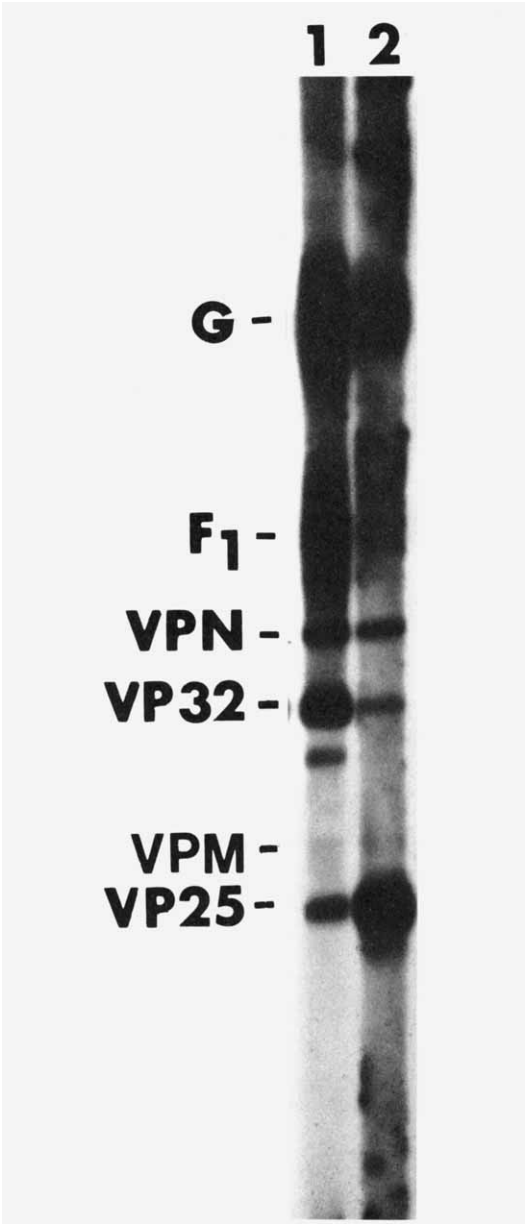

Figure 5. Western blot analysis of pooled sera collected from previously nonimmunized SJL mice 4–6 weeks after intranasal infection with RS virus. Lane 1—control human sera (1/20); Lane 2—mouse serum pool (1/20).

Lanes 6 and 7; Fig. 3, Lanes 4 and 7). Although our inability to induce neutralizing antibodies in some groups could reflect the denaturation of neutralizing epitopes during our purification procedure, increasing the priming dose to 50 μg yielded 100% protection against challenge and when followed by the second dose of 25 μg elicited the production of neutralizing antibodies. While our current studies do not directly rule out the possibility of denaturation, the latter observation coupled with reports showing that monoclonal antibodies to a number of currently known neutralization epitopes on F and G continue to be reactive after exposure to denaturing conditions (39, 40) suggest that our findings may reflect a concentration rather than a denaturation effect.

Rather unexpectedly, some mice apparently developed antibodies to the major nucleocapsid protein, VPN, which is not solubilized by Triton X-100 even in the presence of high salt. The presence of trace levels of VPN may result from the extreme fragility of the RS virus nucleocapsid which could result in the contamination of the vaccine with small quantities of short fragments of nucleocapsids (8).

As noted above, some SJL mice that are subcutaneously injected with the RS virus envelope protein vaccine develop antibodies to VPM. By contrast, no antibody to VPM was found in a screen of adult human sera or of sera from infants hospitalized with bronchiolitis (37). Since the antibody profile of mice (Fig. 5, Lane 2) and humans recovering from intranasal infection (37) lack antibody to VPM, the difference may relate to the antigen type (virus vs subunit vaccine) or the method of antigen delivery (infection vs subcutaneous injection). These data, the fact that others have achieved protection in animals without antibodies to VPM (25, 26), as well as our data showing a lack of correlation between protection and the presence of VPM antibodies (Fig. 2, Lanes 2 and 3; Fig. 3, Lane 4), support the premise that an antibody response to VPM is not an absolute requirement for protection.

The resistance in the lungs that we achieved in Trial 2 appears to be comparable to that achieved by intranasal infection (25, 26), although the level of neutralizing antibody produced is somewhat lower. Although we did not assess the growth of virus in the upper respiratory tract, it appears that none of the vaccines prevent growth of virus in the nasal turbinates (24–26), perhaps because protection of the upper respiratory tract requires the induction of local immunity.

With respect to designing a vaccine to RS virus, it is clear that other issues require resolution. One such issue involves the role of cellular immunity in the protection process. In both mice and humans, infection with RS virus induces a cytotoxic T cell response whose major targets in infected cells are the major nucleocapsid protein, VPN, and the fusion glycoprotein, F (41, 42). With the possible presence of VPN, both of the major target proteins known to elicit cell-mediated responses would have been present in the vaccine. Furthermore, with another paramyxovirus (simian virus 5), others have achieved protection with matrix protein and this protection appears to be mediated by cytotoxic T cells (43). Taken together with our observation that protection did occur in the absence of a detectable neutralizing antibody response, the possibility remains that cell-mediated immunity may have contributed to the effectiveness of our vaccine.

A second unresolved issue concerns the existence of RS virus subgroups. Two subgroups, A and B, have been demonstrated among the RS virus isolates from

different epidemics (44, 45). The major difference resides in the G proteins (46). Although the lungs of animals vaccinated with a vaccinia recombinant containing the RS virus F gene were protected against both homologous and heterologous intranasal challenge, the lungs of mice vaccinated with a RS virus G recombinant were protected from homologous challenge, but not from heterologous challenge (29). This raises the possibility that the G proteins of both subgroups should be included in any subunit vaccine. However, the significance of these subgroup differences in human susceptibility and disease is still undetermined.

Another consideration involves the relative concentrations of F and G in the vaccine. Since the limiting factor in the production of viable RS virus by infected cells is glycoprotein synthesis, it is likely that during infection the immune system is presented with F and G in the ratio in which they occur in the virion (47). It is even possible that most if not all of the F and G in infected cells are present in budding or budded virus, since more than 90% of the infectious virus produced is cell associated throughout the growth cycle and at least 90% of the infectivity as well as almost all of the viral glycoproteins are on the surface of the infected cell (12, 15, 32, 47). In the virion G is the major glycoprotein, which probably accounts for the higher anti-G titers found in human sera (37) and the earlier response of mice to G (Fig. 3). In our vaccine, the ratio of F to G may be similar to that found in the virion, since the vaccine was prepared from purified virus.

Although further investigations will be required to address the issues noted above and care must be exercised in extrapolating from animal models utilizing complete Freund's adjuvant directly to humans, the current studies provide evidence that a combined glycoprotein vaccine confers protection against viral challenge and offer one approach to the development of a protective multivalent subunit vaccine.

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1. Chanock RM, Parrott RH. Acute respiratory disease in infancy and childhood: Present understanding and prospects for prevention. Pediatrics **36**:21–39, 1965.
2. Gardner PS. Virus infections and respiratory disease of childhood. Arch Dis Child **43**:629–645, 1968.
3. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiological study of altered clinical reactivity to respiratory syncytial (RS virus) infection in children vaccinated with an inactivated RS virus vaccine. Am J Epidemiol **89**:405–421, 1969.
4. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccines. Am J Epidemiol **89**:422–434, 1969.
5. Wright PF, Schinozaki T, Fleet W, Sell SH, Thompson J, Karzon DT. Evaluation of a live, attenuated respiratory syncytial virus vaccine in infants. J Pediatr **88**:931–936, 1976.
6. Wright PF, Belshe RB, Kim HW, Van Voris L, Chanock RM. Administration of a highly attenuated live respiratory syncytial virus vaccine to adults and children. Infect Immun **37**:397–400, 1982.
7. Belshe RB, Van Voris LP, Mufson MA. Parenteral administration of live respiratory syncytial virus vaccine: Results of a field trial. J Infect Dis **145**:311–319, 1982.
8. Peeples M, Levine S. Respiratory syncytial virus polypeptides: Their location in the virion. Virology **95**:137–145, 1979.
9. Pringle CR, Shirodaria PV, Gimenez HB, Levine S. Antigen and polypeptide synthesis by temperature-sensitive mutants of respiratory syncytial virus. J Gen Virol **54**:173–183, 1981.
10. Huang YT, Collins PL, Wertz GW. Characterization of the 10 proteins of human respiratory syncytial virus: Identification of a fourth envelope-associated protein. Virus Res **2**:157–173, 1985.
11. Fernie BF, Gerin JL. Immunochemical identification of viral and nonviral proteins of the respiratory syncytial virus virion. Infect Immun **37**:245–249, 1982.
12. Gruber C, Levine S. Respiratory syncytial virus polypeptides: III. The envelope-associated proteins. J Gen Virol **64**:825–832, 1983.
13. Gruber C, Levine S. Respiratory syncytial virus polypeptides. V. The kinetics of glycoprotein synthesis. J Gen Virol **66**:1241–1247, 1985.
14. Walsh EE, Hruska J. Monoclonal antibodies to respiratory syncytial virus proteins: Identification of the fusion protein. J Virol **47**:171–177, 1983.
15. Gruber C, Levine S. Respiratory syncytial virus polypeptides. IV. The oligosaccharides of the glycoproteins. J Gen Virol **66**:417–432, 1985.
16. Fernie BF, Dapolito G, Cole PJ Jr, Gerin JL. Kinetics of synthesis of respiratory syncytial virus glycoproteins. J Gen Virol **66**:1983–1990, 1985.
17. Wertz GW, Collins PL, Huang Y, Gruber C, Levine S, Ball LA. Nucleotide sequence of the G protein of human respiratory syncytial virus reveals an unusual type of viral membrane protein. Proc Natl Acad Sci USA **82**:4075–4079.
18. Levine S, Klaiber-Franco R, Paradiso PR. Demonstration that glycoprotein G is the attachment protein of respiratory syncytial virus. J Gen Virol **68**:2521–2524, 1987.
19. Walsh EE, Schlesinger JJ, Brandriss MW. Purification and characterization of GP90, one of the envelope glycoproteins of respiratory syncytial virus. J Gen Virol **65**:761–767, 1984.
20. Prince GA, Horswood RL, Camargo E, Koenig D, Chanock RM. Mechanisms of immunity to respiratory syncytial virus in cotton rats. Infect Immun **42**:81–87, 1983.
21. Hemming VG, Prince GA, Horswood RL, London WT, Murphy BR, Walsh EE, Fischer GW, Weisman LE, Baron PA, Chanock RM. Studies of passive immunotherapy for infections of respiratory syncytial virus in the respiratory tract of a primate model. J Infect Dis **152**:1083–1087, 1985.
22. Walsh EE, Schlesinger JJ, Brandriss MW. Protection from respiratory syncytial virus infection in cotton rats by passive transfer of monoclonal antibodies. Infect Immun **43**:756–758, 1984.
23. Taylor G, Scott EJ, Bew M, Fernie BF, Cote PJ, Collins AP, Hughes M, Jebbett J. Monoclonal antibodies protect against respiratory syncytial virus infection in mice. Immunology **52**:137–142, 1984.
24. Walsh EE, Hall CB, Briselli M, Brandriss M, Schlesinger JJ. Immunization with glycoprotein subunits of respiratory syncytial virus to protect cotton rats against viral infection. J Infect Dis **155**:1198–1204, 1987.
25. Elango N, Prince GA, Murphy B, Venkatesen S, Chanock RM, Moss B. Resistance to human respiratory syncytial virus (RSV) infection induced by immunization of cotton rats with a recom-

binant vaccinia virus expressing the RSV G glycoprotein. Proc Natl Acad Sci USA **83**:1906–1910, 1986.
26. Olmsted RA, Elango N, Prince GA, Murphy BR, Johnson PR, Moss B, Chanock RM, Collins PL. Expression of the F glycoprotein of respiratory syncytial virus by a recombinant vaccinia virus: Comparison of the individual contributions of the F and G glycoproteins to host immunity. Proc Natl Acad Sci USA **83**:7462–7466, 1986.
27. Stott EJ, Ball LA, Young KK, Furze J, Wertz GW. Human respiratory syncytial virus glycoprotein G expressed from a recombinant vaccinia virus vector protects mice against live-virus challenge. J Virol **60**:607–613, 1986.
28. Wertz GW, Stott EJ, Young KKY, Anderson K, Ball LJ. Expression of the fusion protein of human respiratory syncytial virus from vaccinia virus recombinant vectors and protection of vaccinated mice. J Virol **61**:293–301, 1987.
29. Stott EG, Taylor G, Ball LA, Anderson K, Young KKY, King AMQ, Wertz GW. Immune and histopathological responses in animals vaccinated with recombinant vaccinia viruses that express individual genes of human respiratory syncytial virus. J Virol **61**:3855–3861, 1987.
30. Morein B, Sharp M, Sundquist B, Simons K. Protein subunit vaccines of parainfluenza type 3 virus: Immunogenic effects in lambs and mice. J Gen Virol **64**:1557–1569, 1983.
31. Morein B, Sundquist B, Hogland S, Dalsgaard K, Osterhaus A. Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses. Nature **308**:457–460, 1984.
32. Levine S, Hamilton R. Kinetics of respiratory syncytial virus growth cycle in HeLa cells. Arch Gesamte Virusforsch **28**:122–132, 1969.
33. Levine S. Polypeptides of respiratory syncytial virus. J Virol **21**:427–431, 1977.
34. Helenius A, von Bonsdorff C. Semliki Forest virus membrane proteins. Preparation and characterization of spike complexes soluble in detergent free medium. Biochim Biophys Acta **436**:895–899, 1976.
35. Simons K, Helenius A, Leonard K, Sarvas M, Gething MJ. Formation of protein micelles from amphiphilic membrane proteins. Proc Natl Acad Sci USA **75**:5306–5310, 1978.
36. Lowry CH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
37. Levine S, Dajani A, Klaiber-Franco R. The response of infants with bronchiolitis to the proteins of respiratory syncytial virus. J Gen Virol **69**:1229–1239, 1988.
38. Routledge EG, Willcocks MM, Samson ACR, Morgan L, Scott R, Anderson JJ, Toms GL. The purification of four respiratory syncytical virus proteins and their evaluation as protective agents against experimental infection in BALB/c mice. J Gen Virol **69**:293–303, 1988.
39. Samson ACR, Willcocks MM, Routledge EG, Morgan LA, Toms GL. A neutralizing monoclonal antibody to respiratory syncytial virus which binds to both F_1 and F_2 components of the fusion protein. J Gen Virol **67**:1479–1483, 1986.
40. Tsutsumi H, Flanagan TD, Ogra PL. Monoclonal antibodies to the large glycoproteins of respiratory syncytial virus: Possible evidence for several functional antigenic sites. J Gen Virol **68**:2161–2167, 1987.
41. Bangham CRM, Openshaw PJM, Ball LA, King AMQ, Wertz GW, Askonas BA. Human and murine cytotoxic T cells specific to respiratory syncytial virus recognize the viral nucleoprotein (N), but not the major glycoprotein (G) expressed by vaccinia virus recombinants. J Immunol **137**:3973–3977, 1986.
42. Pemberton RM, Cannon MJ, Openshaw PJM, Ball LA, Wertz GW, Askonas BA. Cytotoxic T cell specificity for respiratory syncytial virus proteins: Fusion protein is an important target antigen. J Gen Virol **68**:2177–2182, 1987.
43. Randall RE, Young DF, Southern JA. Immunization with solid matrix-antibody-antigen complexes containing surface or internal virus structural proteins protects mice from infection with paramyxovirus, simian virus 5. J Gen Virol **69**:2517–2526, 1988.
44. Anderson LJ, Hierholzer JC, Tsou C, Hendry RM, Fernie BF, Stone Y, McIntosh K. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. J Infect Dis **151**:626–633, 1985.
45. Mufson MA, Orvell C, Rafnar B, Norrby E. Two distinct subtypes of human respiratory syncytial virus. J Gen Virol **66**:2111–2124, 1985.
46. Johnson PR, Spriggs MK, Olmsted RA, Collins PL. The G glycoprotein of human respiratory syncytial viruses of subgroups A and B: Extensive sequence divergence between antigenically related proteins. Proc Natl Acad Sci USA **84**:5625–5629, 1987.
47. Peeples M, Levine S. Metabolic requirements for the maturation of respiratory syncytial virus. J Gen Virol **50**:81–88, 1980.