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Role of Individual Glycoproteins of Human Parainfluenza Virus Type 3 in the Induction of a Protective Immune Response

RANJIT RAY,[1]* BRENDA J. GLAZE,[1] AND RICHARD W. COMPANS[2]

Molecular Engineering Associates, Inc., 2126 7th Avenue South, Birmingham, Alabama 35233,[1] and Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294[2]

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Affinity-purified hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins of human parainfluenza virus type 3 (PI3 virus) were used to investigate their role in the induction of a protective immune response following immunization of hamsters. The efficacy of immunization with the glycoprotein antigens was tested by challenge infection. Results of virus recovery from lungs and trachea demonstrated that although immunization with HN or F alone induced an antibody response to the respective glycoproteins, it did not provide a significant level of protection. However, immunization with a mixture of both purified glycoproteins induced higher virus-neutralizing activity in bronchial lavages and afforded complete protection from challenge infection. Similarly, incomplete protection was observed after passive transfer of monospecific rabbit antibody to the purified HN or F in baby hamsters. On the other hand, passive transfer of a mixture of antibodies to HN and F conferred a higher level of protection. Thus, the presence of antibody to both glycoproteins of P13 virus may be essential for protective immunity.

Human parainfluenza virus type 3 (PI3 virus) causes mild to severe respiratory tract infection in infants (1, 2, 4, 5, 10). PI3 virus is an enveloped RNA virus and possesses two glycoproteins, hemagglutinin-neuraminidase (HN) and fusion (F), at its external surface. These two glycoproteins are known to be responsible for initiation and progress of the infection process (6, 16, 17). We have demonstrated that subcutaneous immunization of hamsters with two glycoproteins can confer protection from challenge infection with prototype live virus (14). The importance of antibodies to the HN and F glycoproteins for prevention of infection by parainfluenza viruses has already been documented (3, 9). These studies with simian virus 5 (SV5) have shown that antibodies specific for the HN glycoprotein were effective only in preventing spread of infection in a system where little cell fusion occurs and in which spread of virus is facilitated through released progeny virions. On the other hand, antibody to the F glycoprotein was capable of completely preventing the spread of infection in cells susceptible to virus-induced fusion as well as in cells resistant to fusion. Serological studies with naturally infected children have demonstrated that the appearance of antibodies to both the HN and F glycoproteins of PI3 virus correlates with protection from infection (8). We have recently reported the purification of the two PI3 virus glycoproteins by immunoaffinity chromatography with specific monoclonal antibodies (15). The study presented here was aimed at determining the contribution of the individual glycoproteins in induction of a protective immune response against challenge infection with live PI3 virus.

MATERIALS AND METHODS

Virus and cells. Human PI3 virus (strain 47885) was obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, Md.). African green monkey kidney (LLC-MK_2) and Vero cells were collected from the American Type Culture Collection (Rockville, Md.). LLC-MK_2 cells were routinely used for virus growth, and Vero cells were used in plaque assays for determination of virus titers or virus neutralization tests (14).

Purified virus glycoproteins. Virus envelope glycoproteins HN and F were purified from PI3 virus-infected LLC-MK_2 cell lysates by affinity chromatography with specific monoclonal antibodies by the procedure described recently (15). The purified glycoproteins were used for reconstitution into phosphatidyl choline vesicles for subsequent immunization of experimental animals.

Immunization and challenge infection of hamsters. Four-week-old hamsters were procured from Charles River Breeding Laboratories (Wilmington, Mass.) and immunized with either of the two virus envelope glycoproteins alone or mixed together by the subcutaneous or intranasal route as described (14). Immunizations were offered four times with the same quantity of glycoproteins at weekly intervals. Four weeks after the last immunization, animals were infected intranasally with 10^5 PFU of live virus. All animals were sacrificed on day 3 following challenge infection. Serum and bronchial lavage samples were collected for determination of the antibody response to virus glycoproteins. Lungs and trachea were collected together, suspended in Dulbecco medium containing 1% bovine serum albumin, frozen quickly, and stored at −70°C for subsequent use.

Protection by passive transfer of antibodies. Monospecific rabbit antiserum to affinity-purified HN or F was mixed with an equal volume of 50% saturated ammonium sulfate. The precipitated immunoglobulin portion was suspended and dialyzed extensively against phosphate-buffered saline, pH 7.2. The enriched immunoglobulin was sterilized by membrane filtration and injected intraperitoneally into baby hamsters 24 h prior to challenge infection with PI3 virus. Hamsters were sacrificed on day 3 postinfection, and trachea and lungs were collected together and homogenized for determination of virus titers by plaque assay.

Immunofluorescence with lung tissue. A portion of lung tissue was collected after the animals were sacrificed and washed with phosphate-buffered saline, pH 7.2. Tissues were fixed with cold ethanol-acetic acid (95:5, vol/vol) and frozen at −76°C. Frozen tissue sections (4 μm thick) were

* Corresponding author.

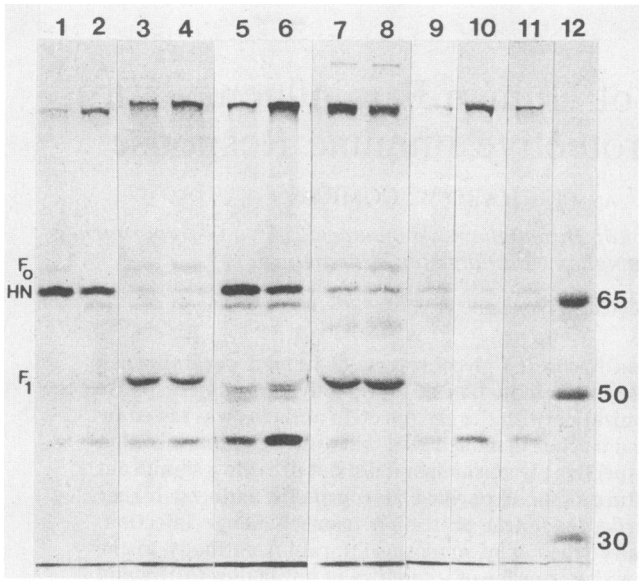

FIG. 1. Immunoprecipitation of [^{35}S]methionine-labeled PI3 virus-infected LLC-MK$_2$ cell lysate with representative bronchial lavage and serum samples from hamsters. Immunoprecipitates with bronchial lavages (lanes 1 and 2) and sera (lanes 5 and 6) from hamsters intranasally immunized with 20 μg of purified HN were analyzed on 7.5% acrylamide gels. Similar analyses were performed with the bronchial lavages (lanes 3 and 4) and sera (lanes 7 and 8) from hamsters immunized intranasally with 20 μg of purified F. Immunoprecipitates appearing with bronchial lavages (lanes 9 and 10) and sera (lane 11) from unimmunized control animals are also shown. Polypeptides of vesicular stomatitis virus are shown in lane 12 as molecular weight markers (in thousands).

cut with a Tissue-Tek II cryostat and examined by immunofluorescence with polyclonal mouse antiserum to PI3 virus and goat anti-mouse immunoglobulin conjugated with fluorescein isothiocyanate (Southern Biotechnology Associates, Inc., Birmingham, Ala.).

Other techniques. Immunoprecipitation, virus neutralization, and enzyme-linked immunosorbent assay (ELISA) were performed by procedures already described (14).

RESULTS

Antibody response following immunization with virus envelope glycoproteins. Results of immunoprecipitation by hamster serum and bronchial lavage samples indicated that intranasal immunization with the glycoproteins induced both a systemic and local antibody response (Fig. 1). Immunization with either HN or F resulted in the appearance of specific antibodies to the respective glycoprotein in both serum and bronchial lavage samples. Antibodies to both HN and F were likewise detected after immunization with a mixture of the glycoproteins (not shown). The antisera and bronchial lavage samples from each group of animals immunized with the same antigens were also tested for antibody response against whole PI3 virus antigens by ELISA and virus neutralization assay (Table 1). Animals immunized intranasally with purified HN or F or the glycoprotein mixture showed similar ELISA titers in sera. However, there were eightfold-higher ELISA and virus neutralization titers in bronchial lavage samples from animals immunized with a mixture of these two glycoproteins.

TABLE 2. Passive transfer and protection experiment with monospecific rabbit antisera to virus envelope glycoproteins in baby hamsters[a]

Antibody offered intraperitoneally	Volume of immunoglobulin offered (ml)[b]	Virus recovery (PFU/g) from lungs after challenge
Anti-HN	0.4	4.6×10^4
Anti-F	0.4	1.4×10^4
Anti-HN + anti-F	0.2 + 0.2	1.8×10^3
None		3.3×10^5

[a] Each hamster group consisted of 10 animals, and virus recovery is expressed as geometric mean titers.
[b] Equivalent to half of the serum volume.

Protective role of the surface glycoproteins. Results of virus recovery from the lungs and trachea of immunized and unimmunized control animals after challenge infection are shown in Table 1. Animals immunized intranasally with either the HN or F glycoprotein showed about a 10-fold reduction in virus titers compared with an unimmunized control group of hamsters. Subcutaneous immunization with the same quantity of the glycoprotein had very little inhibitory effect (less than 10-fold) on virus replication in the respiratory tract of hamsters. However, intranasal immunization with the mixture of both glycoproteins induced significant resistance to challenge infection. None of the lungs or tracheas collected from this group of immunized animals showed any detectable virus in plaque assays. Cryosections of lungs of these animals and infected control animals were examined for the presence of viral antigens by indirect immunofluorescence. Lung cells from infected control ani-

TABLE 1. Antibody response and virus recovery following immunization and challenge infection of hamsters

Route of immunization	Purified glycoprotein[a] and dose (μg)	Antibody response[b]				Virus recovery (PFU/g) from lungs and trachea after challenge[c]
		Immunoglobulin response		Neutralization	Bronchial lavage	
		Serum	Bronchial lavage	Titer serum		
Unimmunized		<200	<80	<20	<4	2.5×10^5
Subcutaneous	HN (20)	3,200	80	20	4	1.3×10^5
Subcutaneous	F (20)	6,400	80	80	8	7.1×10^4
Intranasal	HN (20)	1,600	80	20	8	2.3×10^4
Intranasal	F (20)	3,200	80	20	8	2×10^4
Intranasal	HN (10) + F (10)	3,200	640	320	64	<10

[a] The combined glycoproteins and those tested separately were from the same preparation. Each dose was delivered four times at 1-week intervals.
[b] Results expressed as the response in ELISA and neutralization of virus infectivity with pooled serum and bronchial lavage samples from each group of animals. Actual titers in bronchial lavages are estimated to be about 5- to 10-fold higher because of dilution in the collection process.
[c] Each hamster group consisted of six animals, and virus recovery is expressed as geometric mean titers.

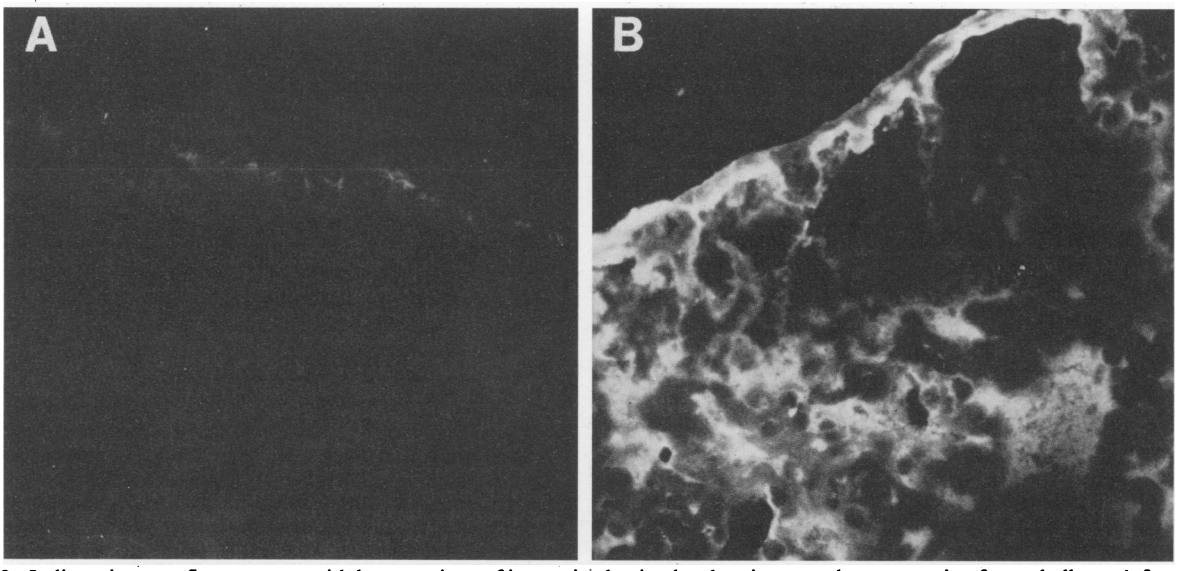

FIG. 2. Indirect immunofluorescence with lung sections of immunized animals, showing complete protection from challenge infection (A), and unimmunized infected animals, showing bright fluorescence (B). Magnification, 1,400×.

mals showed bright immunofluorescence, whereas lungs collected from hamsters showing protection to challenge infection contained little or no viral antigen (Fig. 2).

Protective role of monospecific rabbit antibodies to HN and F following passive transfer. Initially, a large group of baby hamsters (7 days old) were infected intranasally with 10^3 PFU of PI3 virus to study its replication pattern at different times after infection. Four hamsters from the group were sacrificed at 24-h intervals, and their lungs and tracheal homogenates were tested separately to determine the virus titer (Fig. 3). Lungs from infected baby hamsters showed similar virus titers up to day 6. On the other hand, virus titers in tracheas could not be detected until day 2, after which similar virus titers were observed up to day 6. Thus, PI3 virus showed a more prolonged replication pattern in baby hamsters than in young adult (4-week-old) hamsters (14). Different doses of virus inoculum (10^1 to 10^6 PFU) were also tested to determine the minimum infective dose in baby hamsters. Similar virus titers were recovered from animals infected with 10^3 to 10^6 PFU of infective virus. We could not detect any virus in the lungs of baby hamsters infected with 10^2 PFU of virus.

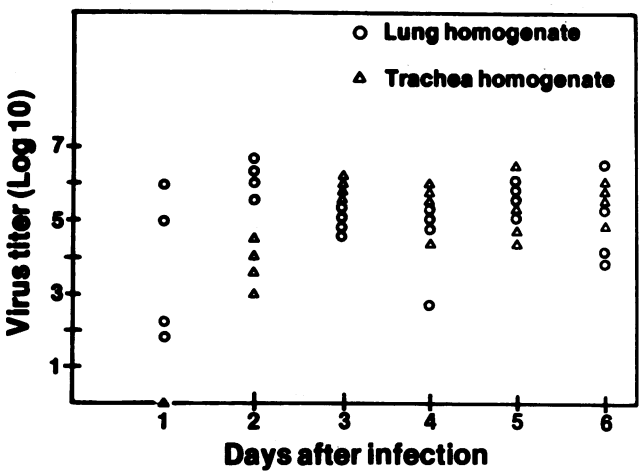

FIG. 3. Infection pattern of PI3 virus in baby hamsters. Hamsters (1 week old) were infected with 10^3 PFU of virus, and four animals were sacrificed at 24-h intervals for determining virus titer in lungs and tracheal homogenates by plaque assay on Vero cell monolayers.

Passive transfer of rabbit antibody to either HN or F in baby hamsters conferred weak protection from challenge infection (Table 2). A 7-fold reduction in virus titer with rabbit anti-HN and about 25-fold reduction with rabbit anti-F antibodies were observed compared with the control infected group of animals. However, passive transfer of a mixture of an equal volume of these two antisera resulted in about a 200-fold reduction in virus titer following challenge infection with live virions. Inoculation of higher quantities of rabbit immunoglobulin did not show any significant further change in virus recovery.

DISCUSSION

Two potentially important surface glycoproteins, HN and F, were purified from parainfluenza virus-infected cell lysates by immunoaffinity chromatography. The purified glycoproteins were shown to retain their biological activities as tested with monospecific rabbit antiserum by hemagglutination inhibition, virus neutralization, and inhibition of cell fusion activities (15). Monospecific antisera to HN or F demonstrated similar reactivities with the respective purified proteins and disrupted virus in ELISAs. On the other hand, some monoclonal antibodies to HN and F failed to show reactivity against these purified glycoproteins (our unpublished results; B. Murphy, personal communication). The absence of reactivity with some monoclonal antibodies may be due to extensive aggregation of the purified glycoproteins, particularly HN, in an aqueous environment or to partial denaturation of the glycoproteins. Similar aggregation of purified glycoproteins of paramyxovirus SV5 has been reported previously (16). To avoid aggregation and to increase immunogenicity, we reconstituted these purified glycoproteins separately into phosphatidyl choline vesicles, in which F was incorporated more efficiently than HN (15). In the present study, glycoprotein-lipid vesicles were used for

determining the role of individual glycoproteins in induction of a protective immune response in hamsters. The results presented here suggest that immunization with HN or F elicited similar antibody responses to the respective glycoproteins, as tested by ELISA or immunoprecipitation. However, titers of virus-neutralizing antibody were higher in serum and bronchial lavage samples from animals immunized with a mixture of the two glycoproteins. Challenge infection of these immunized animals demonstrated that neither of these two virus envelope glycoproteins alone induced a significant level of protection when administered subcutaneously or intranasally in hamsters. On the other hand, animals immunized with both glycoproteins showed complete protection, as none of the animals from this group showed any detectable virus in lung and tracheal homogenates following challenge infection with PI3 virus. These animals also showed little or no evidence of virus when lung sections were examined by immunofluorescence.

In recent studies with the pneumovirus respiratory syncytial virus with vaccinia virus recombinants expressing G and F or affinity-purified glycoproteins, it was demonstrated that either of the envelope glycoproteins can induce a high level of protection to challenge infection (12, 18–20). The G glycoprotein of respiratory syncytial virus was reported to induce a less complete protective response to challenge by homologous virus and provided less complete cross-subgroup protection. On the other hand, the F glycoprotein had a predominant role in conferring immunity to challenge infection by both homologous and heterologous virus strains (7). Evidence was also obtained that affinity-purified F glycoprotein of canine distemper virus may suffice as an immunogen against the disease (11). In another recent report, a vaccinia virus recombinant expressing F of SV5 was shown to induce higher levels of neutralizing antibody than did a vaccinia virus-HN recombinant. However, animals inoculated with vaccinia virus-HN were better protected from challenge infection with SV5. It was suggested that for SV5 there may be a qualitative difference in the in vivo properties of the neutralizing antibodies induced by the vaccinia virus-F and -HN viruses (13). Our results following immunization with either of the P13 virus glycoproteins alone or in combination showed similar antibody responses in serum and bronchial lavage samples, although there was a significant difference in resistance to infection. A moderate level of protection was also observed following passive transfer of antibodies to both glycoproteins. The effect of combined antibodies appeared to be more than additive, as the reduction of virus titer was about 10- to 25-fold higher than that obtained by passive transfer of antibody directed to either the HN or F glycoprotein alone.

The possibility of partial denaturation of the two glycoprotein antigens during the isolation procedure cannot be eliminated. However, consideration was given to this possibility, and the glycoprotein antigens used for combined immunization were from the same preparations used for immunization with the individual glycoproteins. The complete protection observed following immunization with the mixture of HN and F is correlated with higher levels of antibodies to viral glycoproteins in bronchial lavage samples than were observed after immunization with each glycoprotein alone. These results suggest that HN and F may act synergistically to elicit a protective immune response against parainfluenza virus infection. It is possible that the glycoproteins have immunomodulating properties, and further detailed studies with paramyxovirus glycoproteins may lead to better understanding of the mechanisms of protection.

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ADDENDUM

After submission of this article, a report (M. K. Spriggs, B. R. Murphy, G. A. Prince, R. A. Olmsted, and P. L. Collins, J. Virol. **61**:3416–3423, 1987) was published indicating that a vaccinia virus-HN recombinant induces a substantially better protective immune response in cotton rats than does a vaccinia virus-F recombinant. Similar results were also observed with a baculovirus-HN recombinant (K. V. Coelingh, B. R. Murphy, P. L. Collins, A. M. Lebacqi-Verheyden, and J. F. Battey, Virology **160**:465–472, 1987).

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