Acquisition of Serum Antibodies to Specific Viral Glycoproteins of Parainfluenza Virus 3 in Children

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A radioimmunoprecipitation assay was used to study antibody responses to parainfluenza virus 3 glycoproteins in human sera. The method was not only more sensitive than the neutralization test for the detection of antibody but also provided semiquantitative assessments of the antibody response to both glycoproteins in a single assay system. Anti-hemagglutinin-neuraminidase titers were consistently higher than anti-fusion levels in the same serum specimen. Thirteen children were monitored serologically and virologically from birth until 12 months or more after their primary infection with parainfluenza virus 3. At 1 to 3 months after infection, a significant increase in the level of antibody to the hemagglutinin-neuraminidase protein developed in 12 children; of these, 9 showed rises in the level of fusion protein. In 11 of the children, antibody titers continued to rise and the geometric mean titers to the hemagglutinin-neuraminidase protein was highest in sera collected 8 to 10 months after primary infection. Reinfection as the reason for these progressive increases in antibody levels could only be confirmed for four of the children. Three other children had reinfections after the 10-month sera were obtained; in each instance the only antibody responses were to the fusion protein.

Parainfluenza viruses are important causes of acute respiratory disease in infancy and early childhood (4, 8). Of these, parainfluenza virus 3 is second only to respiratory syncytial virus as the cause of severe lower respiratory tract illness in the early months of life. Moreover, reinfection with this serotype appears to be a relatively common event (2-4, 8-10). In previous studies, host immunity associated with serum-neutralizing antibody was reported as being incomplete, although the severity of respiratory illness and duration of virus shedding in children was shown to be inversely related to the level of preinfection serum antibody (3, 9). It is now recognized that neutralization of viral infectivity can be mediated by antibodies to either of the two immunologically distinct envelope proteins of parainfluenza viruses. Antibody to the hemagglutinin-neuraminidase (HN) or fusion (F) glycoproteins can prevent the initiation of infection of susceptible cells, and anti-F (but not anti-HN) has been shown in vitro to prevent the spread of virus from cell to cell by fusion (5, 14, 19).

In view of the potential importance of F antibody in immunity, the characterization of the human antibody response to each envelope protein is needed. This report describes results of a survey of cord serum specimens for antibodies to HN and F and details the induction of such antibody populations in children who had been monitored longitudinally for 1 year or more after infection with parainfluenza virus 3. For these assessments, a radioimmunoprecipitation assay in which antibodies to both glycoproteins could be determined simultaneously was used.

MATERIALS AND METHODS

Study population. Cord sera were collected from infants delivered at the Jefferson Davis Hospital, Houston, Tex. A stratified sample of specimens with a range of titers of neutralization (Nt) antibody was selected for analysis by the immunoprecipitation (IP) assay.

Serum specimens from 13 children who had been monitored from birth in the Houston Family Study were tested for development of antibody responses to the glycoproteins of parainfluenza virus 3. These children were selected because they had virological or serological confirmation of primary infection as well as appropriate followup serum specimens. The surveillance protocol for the Houston Family Study has been described (7, 25). Briefly, enrollment of a family into the study was initiated with the birth of an infant, and a serum sample was collected at that time or shortly thereafter. Additional blood specimens were obtained from the infant at ca. 4-month intervals during the first year and at 6-month intervals thereafter. Routine home visits were made weekly during the winter season and biweekly during the remainder of the year to obtain nasal washings for virus culture from infants monitored from birth. In addition, nasal wash specimens were obtained from any member of the family developed a respiratory illness. All nasal wash specimens were tested for virus whether or not the donor was symptomatic.

Immunoprecipitation assay, gel electrophoresis, and fluorography. A previously described method (13, 15-17) with some minor modifications was used to determine antibody levels in the study sera. A monolayer of LLC-MK2 cells grown in a flask (150-cm² growth area) was inoculated with a dilution of prototype virus (HA-1) that produced cytopathic effects in 65 to 75% of the cell culture at 18 to 20 h postinfection. The cell monolayer was then washed and incubated with 30 ml of methionine-deficient Eagle minimal essential medium for 1 h before being pulsed for 4 h with 5 ml of the same medium containing 400 μ Ci of [³⁵S]methionine per ml. After removal of the medium, cells were lysed by the addition of 2 ml of RIPA buffer, pH 7.4 (0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.01 M Tris-hydrochloride [pH 7.4], 1 mM phenylmethylsulfonyl fluoride, 1 mM methionine, 196 Kallikrein International Units of Trasylol per ml), and centrifuged at 35,000 rpm for 35 min to remove insoluble materials. To reduce nonspecific binding of labeled proteins, 0.3 ml of

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normal goat serum was added to the resulting supernatant fluid. After 60 min at 4°C, the pellet from 1.2 ml of *Staphylococcus aureus* protein A suspension was added to remove goat immunoglobulins (The Enzyme Center, Inc., and Bethesda Research Laboratories, Inc.). The labeled antigen preparation was stored at -70°C and used within a 4-week period. Repeat assays performed with different batches of freshly prepared and stored material and a panel of six sera obtained from children in this study shows that the antigens were not limiting since labeled antigen cell lysates could be diluted from 4- to 16-fold without any reduction of HN or F antibody titers. Viral antigen preparation (25 µl), fourfold dilutions of test human serum dilution (50 µl), and RIPA buffer (75 µl) were incubated for 3 h at 4°C. After addition of protein A reagent, mixtures were kept on ice for 1 h with frequent agitation. Immune complexes were pelleted by centrifugation and washed successively in high-salt, low-salt and salt-free buffers (21). [³⁵S]methionine-labeled viral proteins were dissociated from the final pellet by boiling for 3 min in 70 µl of lysis buffer (3.8% glycerol, 3.8% sodium dodecyl sulfate, 1.9% dithiothreitol, 0.06 M Tris-hydrochloride [pH 6.8]). After centrifugation, supernatant fluids were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a discontinuous buffer system (15). The stacking and separating slab gels (1.5 mm thickness) consisted of 3.2 and 10% acrylamide, respectively. (The acrylamide/bisacrylamide ratio of the gels was 30:0.8%). On completion of electrophoresis, the gels were processed for fluorography by using dimethyl sulfoxide and 2,5-diphenyloxazole (1). After drying, the gels were exposed to Kodak XRP-5 X-Omat film at -70°C for 18 to 20 h. Cord sera representing a range of Nt antibody titers were always included in the same IP assay, and all serum specimens from an individual child were tested simultaneously. The reciprocal of the highest serum dilution precipitating a visible viral band was considered to be the antibody endpoint. A rise was considered significant when the titer increased fourfold. Titers are expressed as the reciprocal of the serum dilution and for geometric mean calculations a titer of <4 was classified as 2.

Antisera to parainfluenza virus 3 viral proteins raised in rabbits were used to identify these proteins in IP assays of human sera. Immunogen preparations were obtained by a purification method previously described for paramyxoviruses (22). The fraction containing the membrane proteins consisted mainly of two glycoproteins as determined by staining of gels with Coomassie blue and periodic acid-Schiff's reagents. Comparison of the migration rates of these two glycoproteins with those observed with proteins of known molecular weight indicated that they were of ca. 71,000 and 58,000 molecular weight, respectively. Antiserum to this preparation precipitated large amounts [³⁵S]methionine-labeled proteins of the same molecular weights. In addition, the antiserum precipitated a protein of 65,000 molecular weight that had not been observed in preparations of whole virus or in the purified protein fractions. The HN glycoprotein was specifically identified with the aid of two monoclonal antibodies possessing high hemagglutination-inhibition titers (generously provided by K. Van Wyke, National Institute of Allergy and Infectious Diseases, Bethesda, Md.). These antibodies precipitated a single protein of 71,000 molecular weight. Because the 65,000-molecular-weight protein was only precipitated when antibodies to the 58,000-molecular-weight protein were present we believe that they represent the uncleaved (F₀) and cleaved (F₁) forms of the fusion glycoprotein. These molecular weights are in close agreement with those of the putative F proteins of human and bovine 3 viruses (11, 24) and with known F

proteins of other paramyxoviruses (23). The nucleoprotein and matrix protein fractions in the purification scheme yielded nonglycosylated proteins of 67,000 and 42,000 molecular weight, respectively. Antiserum to the nucleoprotein precipitated a [³⁵S]methionine-labeled protein of the same molecular weight as the immunogen. The same result was also obtained with a monoclonal antibody to the nucleoprotein (generously supplied by W. C. Russell, Division of Virology, National Institute for Medical Research, Mill Hill, London). A satisfactory antiserum was not obtained with the matrix protein. A mixture of sera containing antibodies raised to the surface glycoproteins and nucleoprotein was included in each assay of human serum.

Neutralization test. Sera were assayed for Nt antibody by a previously described microneutralization procedure in which the serum-virus mixture remained in the cell culture until the test was read (18). The titer was the highest initial dilution of serum that prevented the development of any cytopathic effects in less than one-quarter of the tissue culture monolayer in at least one of two duplicate wells.

RESULTS

A fluorogram depicting the precipitation patterns of viral proteins by cord sera with increasing levels of neutralizing antibody is shown in Fig. 1, and the relative frequencies of antibody titers to the two viral glycoproteins in 54 cord sera according to the level of Nt antibody are summarized in Table 1. Antibody to the HN protein was identified in all specimens that were tested and increased in titer in parallel with the Nt antibody titer. Antibody to the F protein was detected in low titer in only 19% of sera with Nt antibody titers of <4 to 16, but the frequency of detection and the titer increased with increasing Nt titers. For each Nt antibody classification, the mean IP titers to the F protein were ca. 16-fold lower than those to the HN.

All sera collected from 13 children monitored from birth were tested for antibodies to the HN and F proteins for a period of at least 12 months after their primary infection. Incidence of primary infection with parainfluenza virus 3 was distributed approximately equally among the first, second, and third 6-month period of life of the members of the group. Cord sera were available from ten children, and these showed anti-HN, anti-F, and Nt antibody titers that ranged from levels of 64 to ≥1,024, 4 to 64, and 32 to 384, respectively. Testing of sera collected from five mothers at the time of birth of their children showed each to have an antibody profile similar to that of the cord serum.

The antibody response of one child to infection is shown in Table 2. Presumably little, if any, maternally transmitted antibody was still present at the time the infant was infected with parainfluenza virus at 3 months of age. The first virus-positive specimen was obtained during a routine visit when the child was well. Respiratory symptoms began 4 days later, and virus was isolated from a specimen collected on day 4 of the illness. The child recovered and was asymptomatic when the virus was isolated the third time. The latter isolation was preceded by an unsuccessful attempt at virus isolation at 3.9 months of age. An assessment of the serum specimen collected at 4.3 months by both assays showed the child to have titers of HN, F, and Nt antibody that were about the same as those present at birth, and these were considered to be responses to infection. In this child, significantly higher titers of antibody to HN and F proteins were observed in the 8- and 12-month sera, and Nt antibody titers at those times were also increased. Virus was not isolated from any of the 24 nasal wash specimens collected between

FIG. 1. Immunoprecipitation of [³⁵S]methionine-labeled parainfluenza 3 viral proteins by (A) positive control antiserum (rabbit anti-3) and by fourfold dilutions of cord sera with neutralizing antibody titers of (B) <4, (C) 16, (D) 64, and (E) 256.

4.3 and 12 months. After the child reached 12 months of age, the levels of antibody declined to a relatively constant plateau and remained unchanged for as long as sera were available for testing.

A summary of serological findings as determined by the IP assay for all 13 children is shown in Table 3. Antibody titers to the viral glycoproteins are presented in relation to the time interval between primary infection and the collection of subsequent sera. In 12 children the primary infection was detected by virus isolation and in one child it was based on an antibody response. At the time of primary infection, only four children had low levels of passively transferred HN antibody, and of these one also had F antibody. A comparison of antibody titers in preinfection (not shown) and the first postinfection sera obtained at 1 to 3 months after primary infection revealed that a high proportion of children developed significant increases in antibody titer to each viral protein. The levels of response to HN and F ranged from 64 to 1,024 and <4 to 64, respectively. Children with a high anti-HN titer tended to have a relatively high anti-F level ($r = +0.5143$, $P < 0.05$).

The titers in 11 of the 12 children who gave an antibody response to the HN protein in the first postinfection sera continued to increase during the next 6 to 8 months. The same pattern was also observed for eight of the nine children who previously had increases in anti-F titers. For all of these children, the peak titers occurred 5 to 10 months after the primary infection at levels at least fourfold higher than those

seen at 1 to 3 months. Only 4 of the 11 children showed any indication of exogenous reinfection with parainfluenza virus 3 by 10 months; 2 had positive virus specimens 3 to 4 months after the primary infection and 2 others had the same titers in the first and second postinfection serum specimens, but then the levels rose again significantly at 8 to 10 months. Three other children showed evidence of reinfection after the 10-month specimens were obtained. Virus was isolated in two of these three instances in which significant increases in the level of antibody to the F protein only were detected. In the seven children with evidence of reinfection, the preinfection geometric mean serum antibody titers to HN and F were 116 and 11, respectively. By the end of the study period, all 13 children had antibody to both viral glycoproteins.

Nt antibody levels were determined for most of the serum specimens (data not shown). The pattern of rise and fall in antibody titers closely paralleled those determined for the HN protein.

DISCUSSION

In the present study an IP assay was used to measure the presence of antibody to HN and F proteins of parainfluenza virus 3 in human sera. The IP method was a more sensitive procedure for antibody determinations than the Nt test that was used. Moreover, the former assay provided semi-quantitative measurements of antibody to both viral glycoproteins

TABLE 1. Incidence and level of antibody to viral glycoproteins in cord sera according to the level of neutralizing activity

Nt anti-body titer	No. of specimens	Incidence and magnitude of antibody titer to:			
		HN		F	
		No. (%)	Geometric mean titer ^a	No. (%)	Geometric mean titer ^a
<4-16	16	16 (100)	45	3 (19)	3
32-64	13	13 (100)	155	9 (69)	11
128-256	25	25 (100)	501	22 (88)	34

^a For geometric mean calculations a titer of <4 was assigned a value of 2.

TABLE 2. Antibody responses in the serum of a child after natural infection with parainfluenza virus 3

Age (mo) at time of serum collection ^a	Antibody titer by:		
	IP		NT
	HN	F	
Birth	64	4	32
4.3	64	4	16
8	256	16	32
12	≥1,024	64	128
14	256	16	64
20	64	4	16
27	64	4	16

^a Virus was isolated at 3.0, 3.2, and 4.3 months of age.

TABLE 3. Antibody titers to parainfluenza virus 3 glycoproteins in sera of children monitored longitudinally after primary infection

No. of children	Mean-time (mo) after primary infection (range)	Incidence (%) of significant increase in ^a :		Geometric mean titer ^b	
		HN	F	HN	F
13	1.5 (1-3)	12 (92)	9 (69)	135	13
10	5.2 (5-6)	6 (60)	3 (30)	223	14
13	8.5 (8-10)	7 (54)	6 (46)	485	19
13	12.2 (12-21)	0 (0)	3 (23)	230	20

^a An increase was considered significant if the titer between a pair of sera increased fourfold.

^b For the purpose of calculation, a titer of <4 was considered to be 2.

in the same test system. In an individual serum specimen, the level of antibody to the F protein rarely reached the same titer as that to the HN protein. This was seen only once in more than 100 sera that were evaluated. Several circumstances could have accounted for this result: antigenic stimulation was lower because the quantity of the F protein produced during infection was lower; the F protein was not as immunogenic as the HN protein; the specific activity of the labeled F protein was lower than that of the HN protein; and the anti-F antibody response was associated with the immunoglobulin G subclass 3 and not precipitated by protein A. It is unlikely that the last two considerations account for the disparity in antibody titers. Most proteins possess a relatively uniform percentage of methionine residues so that the specific activity should have been comparable (6), and the fact that the absolute level of immunoglobulin G subclass 3 is only ca. 8% of the total immunoglobulin G pool makes it unlikely that titers to the F protein would have been high in this subclass.

Despite the generally low anti-F titers, this study has also shown that measurement of F antibody provided serological confirmation of reinfection with parainfluenzavirus 3 in three children; this may not have been identified by conventional methods in the absence of virus isolation.

The data in this study were insufficient to identify the reason(s) for the progressive rises in antibody titers in seven of the children. In each instance, these were evident in successive sera that were collected within 2 to 6 months of each other. We cannot definitely ascertain whether the increases reflected undetected reinfection or prolonged immunogenic stimulation from the primary infection. With regard to the latter, prolonged or recurrent virus shedding has been previously documented in this population (7). Moreover, other studies have shown that virus shedding may occur for many months in the presence of hemagglutination-inhibition antibody (12, 20). As reported by others, isolation of parainfluenza virus 3 was greatly reduced in previously infected children (3). The demonstration of increases in antibody titer usually has an obvious diagnostic implication even in the absence of virus isolation. However, caution is necessary for establishing the time of infection with parainfluenza virus 3 by increases in antibody titer in the absence of virus isolation.

The degree of host immunity has been shown to be related to the level of Nt antibody (2, 9). In this study, only sera possessing relatively high levels of such antibody showed anti-F activity. Consequently it is tempting to speculate that antibody to F plays a significant role in preventing and moderating the severity of reinfection with parainfluenza virus 3. However, reinfection probably occurred in children who had F antibody titers of ca. 16.

Other studies are now in progress to obtain sera at more frequent intervals after infection so that the kinetics of the antibody response to HN and F and other viral proteins may be more precisely defined and their relative roles in the prevention of reinfection may be assessed.

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