Serum and Nasal-Wash Immunoglobulin G and A Antibody Response of Infants and Children to Respiratory Syncytial Virus F and G Glycoproteins Following Primary Infection

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An enzyme-linked immunosorbent assay (ELISA) with immunoaffinity-purified fusion (F) or attachment (G) glycoprotein was used to measure the serum and secretory immune responses of 18 infants and children, 4 to 21 months of age, who underwent primary infection with respiratory syncytial virus (RSV). Most of the 10 older individuals (9 to 21 months of age) developed moderate levels of serum and nasal-wash immunoglobulin A (IgA) and IgG F and G antibodies. These individuals developed a moderate level of serum or nasal-wash antibodies that neutralized virus infectivity. One of the eight younger individuals (4 to 8 months of age) failed to develop an F antibody response, while three failed to develop a G antibody response. The most notable difference in the responses of the two age groups involved the titer in convalescent sera of G, F, and neutralizing antibodies which were 8- to 10-fold lower in younger individuals. Most of the younger infants failed to develop a rise in serum or nasal-wash neutralizing antibody. It is possible that the presence of maternally derived antibody in the younger infants suppressed the immune response to RSV infection, and that this accounted, in part, for the low level of postinfection antibody titer in this group. This low level and the irregular response of the infants less than 8 months of age may contribute to the severity of their initial infection and may also be responsible, in part, for their failure to develop effective resistance to subsequent reinfection by RSV.

Respiratory syncytial virus (RSV), which belongs to the pneumovirus subgroup of the *Paramyxoviridae* family, is the leading cause of severe lower respiratory tract disease in infancy (14, 15, 23). RSV is a nonsegmented negative-strand RNA virus that codes for at least 10 distinct gene products (7). Two of these are virion-associated glycoproteins with molecular weights of approximately 70,000 and 90,000 which are referred to as the F (fusion) and G (large or attachment) glycoproteins, respectively (6, 8, 25a, 28, 31, 33a). The F protein is a typical paramyxovirus fusion glycoprotein with disulfide-linked F1 and F2 monomers, carboxy-terminal hydrophobic anchor domain, hydrophobic F1 amino terminus, and N-linked oligosaccharides composing about 30% of the molecular weight of the glycoprotein (2, 6, 8, 24). Immune sera raised to the purified F glycoprotein as well as F-specific monoclonal antibodies neutralize virus infectivity and prevent spread of virus from cell to cell (28, 29). Passive transfer of certain F-specific monoclonal antibodies protects animals against challenge infection with RSV (26, 30). The G glycoprotein is an unusual glycoprotein made up of up to 60% carbohydrate, the vast majority of which is O linked, presumably to the abundant serine or threonine residues of the protein (10, 25a, 27). Antibodies induced by purified G glycoprotein as well as certain G-specific monoclonal antibodies neutralize virus infectivity but, in contrast to F-specific antibodies, do not prevent syncytium formation (26, 29, 31). Passive transfer of certain G-specific monoclonal antibodies also protects animals from RSV infection (26, 30). Passive transfer of monoclonal antibodies to nonglycoprotein internal antigens does not protect animals from

infection (26, 30). These observations identify the F and G glycoproteins as antigens to which protective antibodies are produced.

Previously, the immune response following RSV infection has been measured by using complement fixation, neutralization, immune fluorescence, and enzyme-linked immunosorbent assays (ELISAs) (4, 17-19, 22, 23, 25, 33). However, these tests were not antigen specific, and it was thus not possible to quantitate the response to individual RSV proteins. Recently, immunoprecipitation of radiolabeled RSV proteins has been used to detect immune responses of children infected with RSV to specific RSV proteins (27, 32). By use of the radioimmunoprecipitation assay, antibodies to the RSV G protein were not detected in sera obtained from children 1 year of age recovering from RSV infection. It was suggested that young children respond poorly to the G protein, because it is so heavily glycosylated that the immune response to it might be more typical of that to a polysaccharide antigen than to a conventionally glycosylated protein (32).

The availability of highly purified F and G glycoproteins has permitted a reexamination of the response to the G and F protein, using a highly sensitive ELISA (21, 28, 31). We chose to measure the serum and nasal-wash immunoglobulin G (IgG) and IgA F and G antibody responses of 18 infants and young children undergoing primary infection with RSV. These children, who were outpatients in a pediatric clinic, had an illness that was not severe enough to require hospitalization but was typical of illness caused by this virus in most young individuals. Thus, their antibody responses should have been representative of the majority of primary RSV infections. The findings indicated that children 4 to 21

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TABLE 1. Purity of F and G antigens used in the ELISA

Monoclonal antibody clone no.	Specificity of monoclonal antibody	Titer of monoclonal antibody against antigen:		
		F	G	PIV3
661	F	1:1,640,000	≤1:25	≤1:25
13-1	F	1:400,000	≤1:25	≤1:25
111-2	G	≤1:25	1:400,000	1:100
91-4	G	≤1:25	1:400,000	≤1:15
25-2	N	≤1:25	≤1:25	≤1:25
6-3	N	≤1:25	≤1:25	≤1:25
170/7	PIV3	≤1:25	≤1:25	1:100,000

months of age produce moderate levels of ELISA antibodies in serum and nasal secretions to both G and F glycoproteins, but that the magnitude of the response of infants 4 to 8 months of age is significantly less than that of the older individuals 9 to 21 months of age.

MATERIALS AND METHODS

Collection of serum and nasal-wash specimens. The infants and young children were enrolled in the Vanderbilt Vaccine Clinic, where they had received comprehensive medical care since birth. These individuals did not have underlying diseases, and their nutritional status was considered normal. Regularly collected serum and nasal-wash specimens as well as viral cultures obtained at the time of each respiratory illness allowed the identification of each infection with RSV. A total of 18 individuals 4 to 21 months of age from whom RSV was isolated for the first time were included in this study. Each of these individuals was ill at the time RSV was isolated. Of the 18 individuals, 13 were febrile (temperature, $\geq 100^\circ\text{F}$) ($\geq 37.8^\circ\text{C}$), 7 had bronchiolitis, 1 had pneumonia, 14 had acute otitis media, and 17 had coryza. Serum and nasal-wash specimens were collected on the average of 2.5 months before and 2.8 months after isolation of RSV.

The methods for collection and concentration of nasal-wash specimens were described previously (11, 34). The amount of total IgA present in the concentrated nasal-wash specimens was determined by ELISA. A 100 μl sample of alpha-chain-specific goat anti-human IgA (Cappel Laboratories, Cochranville, Pa.) at a concentration of 20 $\mu\text{g}/\text{ml}$ in carbonate buffer (pH 9.8) was added to the wells of flat-bottom, Nunc microtiter plates (GIBCO Laboratories, Grand Island, N.Y.). The outermost row of wells on each plate was not used. After overnight incubation at room temperature, the plates were incubated at room temperature with 1% bovine serum albumin and then were washed three times with 0.02% Tween in phosphate-buffered saline. Purified human IgA (Cappel) was used as the standard. The human IgA and the concentrated nasal-wash specimens were diluted in a 0.05% Tween-1% bovine serum albumin-borate saline solution, and 0.1 ml was added to each well. The plates were incubated at room temperature overnight and subsequently washed three times before 0.1 ml of anti-IgA alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, Mo.) (1:2,000 in borate saline solution) was added to each well. The plates were incubated for 2 h at 37°C and washed three times, and then 0.1 ml of *p*-nitrophenyl phosphate disodium (Sigma) (1.0 mg/ml in diethanolamine buffer) was added to each well. After 15 min the optical density at 405 nm was measured for each well in a Titertek Multiscan spectrophotometer (Flow Laboratories, Inc., McLean, Va.),

TABLE 2. Serum antibody responses of children undergoing primary infection with RSV

Age at time of infection (mo)	No. tested	No. with antibody response	Response to F protein						Response to G protein					
			IgG			IgA			IgG			IgA		
			Reciprocal mean log ₂ titer $\pm$ SE	No. with $\geq$ four-fold rise	Mean log ₂ -fold rise ^a	Reciprocal mean log ₂ titer $\pm$ SE	No. with $\geq$ four-fold rise	Mean log ₂ -fold rise ^a	Reciprocal mean log ₂ titer $\pm$ SE	No. with $\geq$ four-fold rise	Mean log ₂ -fold rise ^a	Reciprocal mean log ₂ titer $\pm$ SE	No. with $\geq$ four-fold rise	Mean log ₂ -fold rise ^a
4-8	8	7	10.6 $\pm$ 0.6 ^b	4	2.0	4.1 $\pm$ 0.3	6	5.0	10.6 $\pm$ 0.6	2	2.0	3.3 $\pm$ 0.0	4	6.0
9-21	10	10	5.1 $\pm$ 0.8	10	9.1	3.3 $\pm$ 0.0	10	6.4	5.7 $\pm$ 1.1	10	8.0	3.5 $\pm$ 0.2	8	6.0

^a Mean log₂-fold rise was calculated for responders only.

^b The lowest dilution tested in ELISA was 1:40. Serum specimens without detectable ELISA antibody at 1:40 were assigned a titer of 1:10 for calculation of mean titers because the serial dilutions were fourfold.

TABLE 3. Serum neutralizing antibody responses of children undergoing primary infection with RSV

Age at time of infection (mo)	No. tested	Neutralizing antibody response ^a to strain:						Maximum titer (vs A2 or 18537) achieved in postinfection serum
		A2			18537			
		Preinfection	Postinfection	No. with $\geq$ fourfold rise	Preinfection	Postinfection	No. with $\geq$ fourfold rise	
4–8	8	5.8 $\pm$ 0.3	5.4 $\pm$ 0.5	0 ^b	6.2 $\pm$ 0.5	6.3 $\pm$ 0.6	2 ^c	6.5 $\pm$ 0.6 ^d
9–21	10	3.7 $\pm$ 0.2	9.7 $\pm$ 0.2	10 ^b	3.8 $\pm$ 0.3	6.9 $\pm$ 0.7	9 ^c	9.8 $\pm$ 0.2 ^d

^a Titers are reciprocal mean log₂ titers $\pm$ standard error. The lowest dilution of serum tested in the neutralization assay was 1:20. The titers of specimens with undetectable antibody activity were assigned a value of 1:11 in the 60% plaque inhibition assay.

^b Fisher's exact test, $P < 0.01$.

^c Fisher's exact test, $P < 0.03$.

^d Student's t test, $P < 0.001$.

and the concentration of IgA in the nasal specimens was determined from the curve of optical density versus concentration generated with the human IgA standard.

Measurement of immune response. The titer of neutralizing activity in serum and nasal-wash specimens was determined by 60% plaque reduction neutralization, using the A2 and 18537 strains of RSV and HEp-2 cells as previously described (19).

The ELISA used to detect IgG and IgA antibodies to the purified RSV F and G glycoproteins was performed as described previously for the influenza A virus hemagglutinin except that the RSV F or G glycoprotein was used as the antigen on the solid phase (21). IgM titers were not similarly determined because of the paucity of reagents available. The F and G glycoproteins of the Long strain of RSV, which were prepared by using immunoaffinity chromatography as previously described, were used at a concentration of 100 ng/ml (28, 31). The F and G glycoproteins were found to be free of other RSV or cellular proteins by polyacrylamide gel electrophoresis as previously described (28, 31). The assays were read after 1 h of incubation, except for the nasal-wash IgG assay, which was read at 2 h. This was done because of the small amount of color generated after 1 h of incubation.

An ELISA with monoclonal antibodies to RSV G, F, and nucleocapsid (N) protein was used to further assess the purity of the G and F glycoproteins as previously described (3). The monoclonal antibodies specific for the F, G, or N protein of RSV were kindly provided by Bruce Fernie of Georgetown University, Washington, D.C., and those specific for the parainfluenza virus type 3 (PIV3) hemagglutinin-neuraminidase glycoprotein were provided by Kathleen Coelingh of the National Institutes of Health. Dr. Coelingh also provided the PIV3 virus which was prepared and purified as previously described (5).

RESULTS

Purity of the F and G antigens. To determine if immunoreactive contaminating RSV proteins were present in the purified F and G preparations, monoclonal antibodies specific for RSV F, G, or N protein, as well as for PIV3 hemagglutinin-neuraminidase glycoprotein were tested in ELISAs against the purified RSV F and G proteins and against an irrelevant antigen, purified PIV3 virus (Table 1). There was no evidence that the F glycoprotein was contaminated with G glycoprotein or with an immunogenic RSV internal protein, the N protein (32). Similarly, the G glycoprotein was shown to be free of detectable F and N antigens (Table 1).

Response of infants and young children to primary RSV infection. The serum IgG and IgA G and F antibody re-

sponses to primary infection with RSV were determined (Table 2). Of the 18 individuals, 17 had a rise in serum antibody to the G or F glycoprotein or both. As expected, the infants 4 to 8 months of age had significant preinfection levels of serum IgG F and G antibodies, presumably of maternal origin. Seroreponses of these infants to F and G antigens were most often detected in the IgA isotype. The frequency of response of the younger individuals to either antigen were lower than that of the older individuals. When one examines the mean titer rise of those children with responses, it is clear that the magnitude of the serum IgA responses of infants 4 to 8 months of age is similar to that of the older individuals with respect to G antibody but is less for F antibody. The magnitude of the rise in serum IgG antibodies could not be accurately assessed because of the presence in the pre- and postinfection serum specimens of significant quantities of maternally derived antibodies (Table 2).

The serum neutralizing response was measured with the A2 and 18537 strains of RSV. RSV strains can be classified into two major neutralization groups by using postinfection animal sera. The prototype A2 and Long strains compose one major neutralization group, and the 18537 strain composes the second group (1). Since the isolates were not available from these infants and children, it was not possible to tell which strains they were infected with. However, the serum antibody responses were measured for both strains of virus, and therefore a response to either strain should have been detected (Table 3). Only 2 of the 8 young infants developed a response to either virus, whereas all 10 of the older individuals did ($P < 0.03$, Fisher's exact test). When the maximum postinfection titers (i.e., the highest titer to either the A2 or 18537 strains) were compared (Table 3), the titers of serum neutralizing antibody were 8- to 10-fold higher in the older individuals. The titers of G and F ELISA antibodies were also 8- to 10-fold higher.

Sixteen of the children developed nasal-wash G or F antibodies in the IgG or IgA isotype (Table 4). Although IgA antibody is the major isotype detected in nasal-wash specimens, virus-specific IgG antibodies are frequently detected in nasal-wash specimens following primary virus infection (13, 17, 20). The younger individuals had a lower frequency of IgA or IgG responses or both, but the magnitudes of the responses were comparable in both age groups. Whereas 16 of 18 individuals had rises in nasal-wash antibody titer to G or F glycoprotein, only 7 of 18 developed neutralizing antibody responses. The titers of postinfection IgA F and G and neutralizing antibodies to the A2 strain of the older individuals were higher than those of the 4- to 8-month-old infants.

TABLE 4. Nasal-wash antibody responses children undergoing primary infection with RSV

Age at time of infection (mo)	No. tested	No. with antibody response	Response to F protein							
			IgG				IgA			
			Reciprocal mean log ₂ titer ± SE		No. with ≥four-fold rise	Mean log ₂ -fold rise ^b	Reciprocal mean log ₂ titer ± SE		No. with ≥four-fold rise	Mean log ₂ -fold rise ^c
			Preinfection	Postinfection			Preinfection	Postinfection		
4-8	8	6	3.2 ± 1.2 ^c	5.4 ± 0.8	4	4.7	1.8 ± 0.5	6.2 ± 1.0	5	6.9
9-21	10	10	1.6 ± 0.4	7.2 ± 0.8	9	6.0	1.8 ± 0.5	7.3 ± 0.6	9	6.1

Correlation of serum and nasal-wash F, G, and neutralizing antibody titers. There was a statistically significant association between the magnitudes of the serum ELISA IgG F antibody and neutralizing antibody (A2 strain) titers ($r = +0.84$, $P < 0.001$), between ELISA IgG G antibody and neutralizing antibody titers ($r = +0.73$, $P < 0.001$), and between ELISA IgG F and ELISA IgG G antibody titers ($r = +0.90$, $P < 0.001$) when the pre- and postinfection serum specimens of the 18 children were analyzed. The relationship between the serum neutralizing antibody and ELISA F IgG antibody levels was determined, and a roughly linear relationship was observed between these two titers (Fig. 1). A similar pattern (data not presented) was observed as expected when ELISA G IgG antibody titer was plotted against neutralizing antibody titer, because the IgG G and F antibody titers were so highly correlated. Because both G and F glycoproteins can stimulate neutralizing antibodies, the relative contributions of G and F antibodies to virus-neutralizing activity could not be assessed. Of interest, two sera from young infants convalescent from RSV infection had titers of IgG F antibodies equal to or greater than 1:2,048 that

were not associated with neutralizing activity (Fig. 1). Neither of these infants had a rise in IgG antibody titer to the F or G glycoprotein, so it is not possible to determine if the activities measured represented maternal antibodies or IgG antibodies produced by the infants.

There was a strong association between IgA G and F antibody levels in nasal-wash specimens ($r = +0.81$, $P < 0.001$) that was similar to that observed for serum antibodies. Only 7 of the 36 nasal-wash specimens examined for neutralizing antibody exhibited significant activity. In each instance the specimen was collected during convalescence. Of these convalescent nasal-wash specimens, 4 had IgA F or G (or both) antibody titers of equal to or greater than 1:512. Only one convalescent specimen, which had a titer of 1:512, failed to exhibit neutralizing activity.

DISCUSSION

The two hallmarks of RSV infection in the first several years of life are early infection attended by serious disease and repeated infection with the development of immunity to serious lower respiratory tract disease after several reinfections (9, 12, 23). Second infections can be accompanied by severe lower respiratory tract disease, but by the third or fourth infection a relatively strong immunity to pneumonia and bronchiolitis develops (7, 11). The present study provides some information that bears on the central question of why first infection with RSV does not provide effective resistance to first reinfection and associated disease.

It was previously observed that infection in infants less than 8 months of age does not consistently induce an immunological response (4, 13, 16, 23, 25, 33). This analysis has been difficult to fully interpret because of the relative insensitivity of the assays employed and also because of the difficulty of demonstrating a rise in complement fixation or neutralization antibody in the presence of maternally derived antibody. By use of a sensitive ELISA assay which can detect IgA F and G antibody in the presence of maternally derived IgG antibody, rises in antibody to RSV F or G antigen or both were detected in 7 of 8 infants 4 to 8 months of age and in each of 10 older individuals 9 to 21 months of age. However, only 4 of 8 infants who were 8 months or less developed F and G antibodies in both serum and nasal-wash specimens, whereas 9 of 10 of the individual 9 months of age or older developed these responses. This indicated that although 87% of the young infants responded, their responses were not as consistent as the responses of the older individuals. Importantly, the titers of postinfection serum IgG F and G antibodies as well as of neutralizing antibodies of the older individuals exceeded those of the younger infants by 8- to 10-fold. Previous studies indicated that antibody responses to the G glycoprotein were not detected efficiently in young individuals undergoing first infection (27,

FIG. 1. Correlations between the serum ELISA IgG F antibody and neutralizing antibody titers. Symbols for serum specimens: ▲, postinfection, from 9- to 21-month-old children; △, preinfection, from 9- to 21-month-old children; ●, postinfection, from 4- to 8-month-old children; ○, preinfection, from 4- to 8-month-old children.

TABLE 4—Continued

Response to G protein						Neutralizing ^a antibody response					
IgG			IgA			Reciprocal mean log ₂ titer ± SE			No. with ≥four-fold rise		
Preinfection	Postinfection	Mean log ₂ -fold rise ^b	Preinfection	Postinfection	Mean log ₂ -fold rise ^b	Preinfection	Postinfection	Mean log ₂ -fold rise ^b	Preinfection	Postinfection	Mean log ₂ -fold rise ^b
1.9 ± 0.4	3.9 ± 0.9	3	2.0 ± 0.5	5.1 ± 1.1	4	5.3 ± 0.6	4.8 ± 1.1	2	2	2	2.8
1.6 ± 0.5	5.7 ± 0.8	8	2.1 ± 0.5	5.5 ± 0.9	7	5.1 ± 0.9	7.0 ± 0.8	5	5	5	4.4

^a A2 strain used to measure neutralizing antibody response.

^b Mean log₂-fold rise was calculated for responders only.

^c The nasal-wash titers were corrected to 10 mg% IgA. The lowest dilution tested in the ELISA was 1:2. Nasal-wash specimens without detectable ELISA antibody were assigned a titer of 1:0.5 for calculation of mean titers (see Table 2, footnote b). Similarly, the lowest dilution of nasal wash tested for the neutralization assay was 1:4, and the assigned value of specimens without detectable antibody was 1:2.

32). In the present study, we demonstrated a response to F antigen in 7 or 8 of the young infants and in each of 10 older infants and children. Similarly, a response to the G protein was detected in 5 of 8 of the young infants and in each of 10 of the older individuals. Thus, young infants do respond, but their responses of F and G glycoprotein-binding antibodies and of neutralizing antibodies are significantly diminished. In the study by Ward et al. (32), each of 10 infants 4 to 12 months of age failed to develop a radioimmunoprecipitation assay G antibody response, whereas in our study 9 of 12 children of that age range responded with ELISA G antibody. The greater sensitivity of the ELISA is a partial explanation for this difference. Thus, infants undergoing primary infection can clearly respond to the G protein despite its extensive glycosylation. Although the young infants (≤8 months) could respond to both G and F glycoproteins, it is reasonable to conclude that their inconsistent responses and their failure to achieve high titers of antibodies following infection likely play a role in their subsequent susceptibility to reinfection and associated disease. A possible explanation for this reduced response is that maternally derived anti-RSV glycoprotein antibody suppressed the immune response of the 4- to 8-month-old infants to RSV as previously suggested (23). We have recently shown that anti-RSV antibody passively transferred during the height of RSV infection in animals markedly suppressed the development of serum antibody response to the G and F glycoproteins (11a). Alternatively, the young age per se could account for the poor immune response. It will be important to dissect the effect of immaturity versus immunosuppression by maternal antibody in the diminished immunologic response of young infants to RSV infection.

The serum-neutralizing antibody responses of the infants and children were determined with viruses that would detect titer rises to the two major strains of RSV (1). Clearly, the decreased responses of the younger infants were largely strain independent since the maximum titers of neutralizing antibody to either strain achieved were approximately 10-fold higher in the older group. We have subsequently confirmed this observation in a larger group of 50 infants and children. Although differences can be detected between strains using monoclonal antibodies and postinfection animal sera (1), the virus strains are still closely related as indicated in the present study by the development of neutralizing antibody responses to both strains by 9 of the 10 older children. It is unlikely that the decreased ELISA antibody responses of the younger infants to the G and F glycoproteins resulted from using only the Long strain glycoprotein in

the ELISA. Since the G and F glycoproteins both stimulate neutralizing antibody (26, 31), it is reasonable to assume that by using the two prototype strains in the neutralization assay responses to the G and F glycoproteins of either strain would have been detected indirectly by a rise in neutralizing antibody titer. The lack of a neutralizing antibody response in 75% of the younger infants suggested that the response to both the G and F glycoproteins was reduced in this age group. This indeed was what the response to the purified glycoproteins indicated. Studies are in progress to specifically determine the relatedness of the G and F glycoproteins of the prototype strains.

These observations have relevance to the development of live virus vaccines effective against RSV. Because most of the severe RSV virus disease occurs in 2- to 6-month-old infants, it will be necessary to induce protective levels of immunity by 2 months of age (14). Since infection with wild-type virus does not induce neutralizing antibody efficiently in this age group, how can protection against disease be achieved? If attenuated vaccines are to be used, more than one dose will have to be administered within the first several months of life, and perhaps the immune response will need to be potentiated to achieve a protective level of immunity.

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