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Evaluation of Immunoglobulin E-Specific Antibodies and Viral Antigens in Nasopharyngeal Secretions of Children with Respiratory Syncytial Virus Infections

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Enzyme immunoassays were developed to detect the presence of specific immunoglobulin E (IgE) antibodies and respiratory syncytial (RS) virus structural proteins in nasopharyngeal secretions in order to improve the knowledge on some aspects of the pathogenesis of severe acute lower respiratory tract infections caused by RS virus. These assays were used to analyze clinical specimens from children with RS virus-associated infections (bronchiolitis and pneumonia), and the findings were correlated with the patients' clinical symptoms. The results indicate the presence of specific IgE against the two external glycoproteins (G and F) and the absence of detectable IgE levels for the internal viral antigens. There was a correlation between the levels of IgE-specific antibodies and the amount of viral protein F in the secretions, indicating that the IgE response against the viral glycoproteins might be related to the antigen load. In addition, a correlation was found between higher levels of both viral protein F-specific IgE and F antigen with higher respiratory rates in children with pneumonia. These findings may be relevant because they suggest an association between the virus load and the immune response in the pathogenesis of RS virus infections.

Human respiratory syncytial (RS) virus is the leading cause of severe lower respiratory tract disease in children throughout the world (7, 9, 14). The respiratory pathology associated with RS virus infection may be partially due to the immunological response to the virus (1, 12). Support for the immunopathogenic hypothesis emerged from early trials of an inactivated RS virus vaccine that caused immunological sensitization that lead to exacerbation of lower respiratory tract disease after subsequent infections (8, 10).

The idea that immunoglobulin E (IgE) may be involved in the pathogenesis of RS virus infections was supported by the findings of Welliver et al. (16), who reported high levels of RS virus-specific IgE in infants with severe hypoxia during the acute phase of disease. Recently, the same group of investigators reported an IgE response against each of the two external glycoproteins of RS virus (glycoproteins G and F) after primary infections (15), although they did not correlate their findings with the clinical parameters of their patients.

The aim of the present work was to study the IgE immune response to RS virus antigens in nasopharyngeal secretions (NPSs) and to correlate the findings with the clinical features of the patients. For this purpose, we developed an anti-RS virus-specific IgE capture immunoassay. The test was used to study a group of children with lower respiratory tract infections. The findings were correlated with the levels of RS virus antigen shedding and the clinical presentations of the patients.

MATERIALS AND METHODS

Patients. Children aged less than 5 years who were hospitalized with bronchiolitis or pneumonia between 1988 and 1990 were recruited for the study. Pneumonia and bronchiolitis were defined on clinical and radiological grounds (5).

Collection and handling of specimens. NPSs were obtained at the time of admission from both nostrils with a polyethylene suction tube attached to a mucus trap. The mucus remaining in the tube was washed out with 1.5 ml of phosphate-buffered saline (PBS) supplemented with 0.5% gelatin (6). The secretions were homogenized by pipetting, and a sample was frozen at −70°C for IgE detection and antigen quantification. The rest of the specimen was processed for virus isolation and immunofluorescence as described previously (6). Cases were considered positive for RS virus when the virus was isolated from NPSs and/or viral antigens were detected in nasopharyngeal cells.

Antibodies. Monoclonal antibodies (MAbs) to G, F, N, P, M, and M2 proteins (MAbs 63G, 52F, 79N, 67P, 29M, and 37M2, respectively) were provided by J. A. Melero (Instituto Carlos III, Majadahonda, Spain), as was a polyclonal antiserum raised in rabbits inoculated with purified RS virus Long strain (2).

Purification of virus antigens. Proteins F, P, M, and NP were purified from lysates of RS virus (Long strain)-infected HEp-2 cells by immunoaffinity chromatography as described previously (2). Briefly, MAbs were purified from ascitic fluids by protein A-Sepharose chromatography and bound to CNBr-activated Sepharose following the manufacturer's instructions. HEp-2-infected cells were suspended in lysate buffer (10 mM Tris-HCl [pH 7.6], 140 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 5 mM EDTA) and applied to the immunoaffinity columns, and the retained proteins were eluted with 0.1 M glycine (pH 2.5).

The shed form of the G glycoprotein was purified from cultures as follows. Supernatants of Long-infected HEp-2 cells were collected when the cytopathic effect was complete and were cleared from the cell debris and viral particles by

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centrifugation. The Gs protein was then precipitated from the supernatant with 60% saturated ammonium sulfate and was purified by immunoaffinity chromatography as described above.

The purity of the antigen preparations was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and enzyme-linked immunosorbent assay (ELISA) with a panel of MAbs for the different viral proteins. The concentration of purified preparations was measured by the method of Lowry et al. (11).

Determination of RS virus-specific IgE. Polyvinyl chloride plates (Flow) were coated with 50 μl (per well) of rabbit anti-human IgE antibody, specific for epsilon chains (DAKOPATS), diluted 1:1,500 in PBS. After 1 h at 37°C, the plates were saturated with 200 μl of PBS containing 1% bovine serum albumin for 30 min at room temperature.

NPSs were cleared of cells and mucus by low-speed centrifugation, diluted 1:5 in PBS plus 0.1% bovine serum albumin, added to the plates (50 μl/well), and incubated at 4°C overnight. At the end of the incubation period, plates were thoroughly washed, 50 μl of the purified viral proteins (G, F, P, NP, and M) was added to the corresponding wells, and the plates were incubated for 1 h at 37°C. After further washing, 50 μl of the corresponding MAbs for each viral antigen was added and the incubation was continued for 1 h at 37°C. The bound antibodies were detected with biotinylated sheep anti-mouse immunoglobulin and peroxidase-streptavidin following the manufacturer's instructions (Amersham). The reaction was developed with 0.02% *o*-phenylenediamine (OPD) in citrate buffer (pH 5.0) containing 0.02% hydrogen, and the reaction was stopped with 1 N sulfuric acid.

The A_{492} values of the plates were read in a multiwell spectrophotometer. Testing of each NPS with all reagents except the corresponding viral antigen was included as a control. A specimen was considered reactive for specific IgE when its absorbance was 2.1 times the observed value for the respective control.

Additional controls were done with some of the reactive samples by blocking the reaction with a purified myeloma IgE (a gift of Abelló Laboratories) that was added to the wells prior to the addition of NPSs or by adsorption of IgE from clinical samples with anti-IgE-coated beads.

Quantification of F glycoprotein in NPSs. A double-sandwich test was developed for F glycoprotein quantification. Polystyrene plates (Greiner Mikrotiter F) were coated with rabbit immunoglobulins (partially purified by salting out with 50% ammonium sulfate) from animals immunized with the RS virus Long strain. A total of 50 μl was used to coat the wells (2 h at 37°C), and the plates were blocked with 200 μl of PBS plus 1% bovine serum albumin per well for 30 min at room temperature. Undiluted NPSs (50 μl) were added to the wells, and the plates were incubated for 18 to 20 h at 4°C. At the end of the incubation period, plates were washed and 50 μl of MAb 52F was added, and the mixture was incubated for 1 h. After further washing, the bound antibody was detected with biotinylated anti-mouse immunoglobulin and peroxidase-streptavidin (Amersham) by following the manufacturer's instructions. The reaction was developed with OPD and was stopped with 1 N sulfuric acid, and the A_{492} was measured in a multiwell spectrophotometer.

A control was run for each specimen with an irrelevant MAb (MAb 1BC11) instead of the specific MAb 52F. A plot of the optical density versus the concentration of purified glycoprotein F was generated for each run to determine the concentration of glycoprotein F in the NPSs by interpolation of the observed optical densities.

TABLE 1. Antibody response to RS virus antigens in the IgE isotype in children with acute lower tract respiratory infections[a]

RSV protein	No. of IgE-specific reactive cases/no. of specimens tested (% reactive cases)			
	Bronchiolitis cases		Pneumonia cases	
	RSV infection	Non-RSV infection	RSV infection	Non-RSV infection
G	14/32 (44)	0/16	8/22 (37)	3/25 (12)
F	8/32 (25)	0/16	12/22 (54)	7/25 (28)
M	0/32	0/16	0/22	0/25
NP	0/32	0/16	0/22	0/25
P	0/32	0/16	0/22	0/25

[a] RS virus infection was diagnosed by virus isolation and/or positive immunofluorescence for viral antigens in nasopharyngeal cells.

Statistics. The Student *t* test was used to compare means, and the Pearson chi-square test was used to analyze the differences between rates.

RESULTS

Patients. A total of 54 patients with proved RS virus infections were included in the study (32 patients had bronchiolitis and 22 patients had pneumonia). The mean age of the patients suffering bronchiolitis was 4.3 months, and that of patients with pneumonia was 11.2 months. None of the patients had had previous episodes of bronchiolitis or pneumonia.

A control group (16 children with bronchiolitis and 25 children with pneumonia) not associated with RS virus infection was included in the study. The control group was recruited at the same time and from the same population as the RS virus-associated cases, and the mean ages of the control group were 4.6 and 9.0 months for bronchiolitis and pneumonia cases, respectively.

RS virus-specific IgE response. Because of the lack of reference reagents to control the specificity of the test, a high cutoff (2.1 times the optical density of negative controls) was selected in order to overcome unspecific background readings. In our hands, the selected cutoff represents at least 3 standard deviations from the mean reading of the control level without the specific viral antigen.

Eleven samples were further tested to confirm the specificity of the test. The addition of purified myeloma IgE to the reaction wells prior to testing the positive NPSs produced a reduction in the optical density of the reactions, which ranged between 20 and 50%. Similar results were recorded when NPSs were incubated with polystyrene beads coated with anti-human IgE prior to testing.

The results obtained with clinical specimens (Table 1) indicate the presence of measurable amounts of IgE antibodies directed against glycoproteins G and F in NPSs, whereas no evidence of a response to the internal viral components was observed. Among patients with RS virus-associated bronchiolitis, 44% had detectable levels of IgE specific to glycoprotein G and 25% showed reactivity to glycoprotein F. None of the specimens from patients with bronchiolitis not associated with RS virus reacted positively in the test. Among the specimens from children with RS virus-associated pneumonia, reactivities for IgE-specific antibodies against glycoproteins G and F were 37 and 54%, respectively, compared with reactivities of 12 and 28%, respectively, observed in specimens from the control group.

TABLE 2. Antibody response to RS virus glycoproteins G and F in IgE isotype according to year of recruitment of the patients[a]

RSV protein	No. (%) positive		
	1988 (n = 26)	1989 (n = 16)	1990 (n = 12)
G	18 (69)[b]	5 (31)[b]	0
F	8 (31)	9 (56)	2 (17)

[a] Patients with lower respiratory tract infection associated with RS virus (virus isolation and/or positive immunofluorescence for RS virus antigens).
[b] $P < 0.05$ (Pearson chi-square test).

Influence of epidemic season. The distributions of IgE-reactive cases during three successive epidemics are given in Table 2. Yearly significant differences ($P < 0.05$) were observed for glycoprotein G (69% reactive cases in 1988, 31% in 1989, and none in 1990), while the differences were not significant for glycoprotein F (31% reactive cases in 1988, 50% in 1989, and 17% in 1990).

Age and IgE response. The childrens' ages had a significant effect on the IgE response for glycoprotein G. Children aged 1 to 6 months showed 17% reactivity compared with 50% reactivity in patients older than 7 months ($P < 0.05$). No differences were observed for glycoprotein F reactivity (39 and 36% reactivities, respectively) for children under and over 6 months old.

Virus shedding and IgE response. A significant positive correlation was found between the level of RS virus glycoprotein F shedding and the presence of IgE-specific antibodies for glycoproteins G and F (Table 3). All children (100%) with more than 200 ng of glycoprotein F per ml in their respiratory secretions had specific IgE antibodies. In contrast, only 37.5% of the children with less than 10 ng of glycoprotein F antigen per ml in their secretions tested positive in the assay.

Clinical features and IgE response in pneumonia cases. Some clinical features were recorded and analyzed jointly with specific IgE levels in respiratory secretions (Table 4). Statistically significant ($P < 0.05$) higher respiratory rates were observed in patients with specific IgE in their secretions (mean, 71.1 respirations per min [rpm]) than in those without detectable IgE (mean, 57.5 rpm). The significance level was higher when respiratory rates were analyzed in patients aged less than 1 year (mean respiratory rates, 79.5 versus 69.3 rpm [$P < 0.01$]).

The differences observed for the other recorded clinical features were not significant.

A similar analysis was made to compare the patients' clinical features with the presence of glycoprotein G-specific IgE (data not shown), but the differences were not statistically significant.

Clinical features and IgE response in bronchiolitis cases. A trend for respiratory rates similar to that observed in pneumonia cases was found in bronchiolitis cases, but the differences between children with and without IgE-specific responses were not significant (mean rates, 79.5 and 69.3 rpm, respectively).

Clinical features and F antigen shedding. Table 5 shows the correlation among glycoprotein F antigen shedding in NPSs and some selected clinical features that have usually been used as severity markers. In patients with pneumonia, a significant ($P < 0.05$) positive correlation was found between levels of glycoprotein F antigen in NPSs and respiratory rates. Among infants aged 1 to 6 months, shorter periods of evolution prior to hospitalization were associated with those patients who shed the higher levels of glycoprotein F antigen. Although more prolonged times of hospitalization were associated with children who shed the highest levels of glycoprotein F antigen, the observed differences were not significant.

TABLE 3. Antibody response to RS virus glycoprotein G and/or F in the IgE isotype and shedding of glycoprotein F in NPSs of children with RS virus infection[a]

Protein F shedding (ng/ml)	IgE response for glycoprotein G and/or F antigens		
	No. positive	No. negative	% Positive
<10	6	10	37.5[b]
10–200	22	9	71.0[b]
>200	3	0	100.0

[a] Patients with lower respiratory tract infection associated with RS virus (virus isolation and/or positive immunofluorescence for RS virus antigens). Fifty children were examined, but data for four of the 54 children enrolled in the study are missing.
[b] $P < 0.05$ (Pearson chi-square test).

DISCUSSION

The results presented here confirmed those presented in a previous report by Welliver et al. (15) on the presence of specific IgE for glycoproteins G and F in the respiratory secretions from children suffering RS virus infections. Our findings were obtained using a design for the ELISA different from the one used by Welliver et al. (15). The double-sandwich IgE-specific assay described by the authors was designed to avoid the drawbacks of previously reported procedures, in which plates coated with purified antigens were used as the solid phase for the reaction. The need for purified antigens is difficult to achieve for some viral proteins that usually copurify because of their intrinsic affinities. Copurification has been reported with N, NP, and M proteins, and in fact, our purified preparations of these antigens were only enriched preparations of the corresponding protein.

Another aspect of concern has been the possibility of modifications produced in antigen epitopes during the process of adsorption onto the plates.

Although purified or enriched antigens were selected for use in the investigation described here, our preliminary data suggested that virus purified by ultracentrifugation and disrupted with detergents can be used instead of glycoprotein F at least for the ELISA, avoiding the use of purification by immunoaffinity purification that could modify some antigen epitopes.

Although we did not extensively compare the sensitivity of our ELISA with that of the procedure in which plates coated directly with the antigens were used, our preliminary data showed that the former procedure had a higher sensitivity, probably because the amplification of the signal by the piling up of reactive reagents in the different steps of the procedure or because the capture design of the test avoids the interference of other immunoglobulins that exist in NPSs. It is difficult to compare the percentages of reactivity for the specific IgE observed by Welliver et al. (15) with our results because of differences in the procedure, dilution of the NPSs, and the ages of the children. We cannot even be sure that our patients were suffering their first infection with RS virus because of the lack of follow-up since birth.

TABLE 4. Clinical features of patients with RS virus-associated pneumonia by antibody response in the IgE isotype to glycoprotein F[a]

Patient group	Age (mo)	Days of evolution prior to hospitalization	Days of hospitalization	Respiratory rate	Wheezing (% positive)	Cyanosis (no. of patients)
With anti-glycoprotein F IgE	7.9 ± 7.5 (8)[b]	3.5 ± 1.0 (8)	7.0 ± 5.6 (8)	71.1 ± 14.0 (8)	37 (8)	12 (8)
Without anti-glycoprotein F IgE	13.5 ± 11.7 (12)	4.0 ± 2.2 (12)	6.4 ± 2.8 (12)	57.5 ± 10.6 (11)	40 (10)	10 (10)
P	>0.05[c]	>0.05[c]	>0.05[c]	<0.05[c]	>0.05[d]	>0.05[d]

[a] RS virus infection was diagnosed by virus isolation and/or positive immunofluorescence for viral antigens or nasopharyngeal cells.
[b] Values are means ± standard deviations. Numbers in parentheses are number of patients.
[c] By the Student *t* test.
[d] By the Pearson chi-square test.

Nevertheless, it should be pointed out that we found 41% of IgE anti-glycoprotein G-specific reactivities, while Welliver et al. (15) reported no reactivity during the first week of clinical evolution.

Some non-RS virus pneumonia cases enrolled as controls showed specific IgE that probably represented previous exposure to the virus. The persistence of a detectable IgE response in NPSs has not yet been established, nor has the effect of successive antigenic stimulation by reinfections. The absence of measurable specific IgE in the control group of children with bronchiolitis not associated with RS virus could be explained by the fact that young infants had less of a chance of becoming sensitized.

Antigenic variation should also be considered when evaluating immune responses, especially those against G glycoprotein, since extensive variation has been observed among RS virus strains of the same antigenic subgroup for that antigen (2). In fact, the differences in the number of reactive cases for anti-G glycoprotein-specific IgE observed in the three successive epidemics that we analyzed might reflect the antigenic variations of the circulating strains. The use of recently isolated strains in future studies should be considered, at least for investigation of the anti-G glycoprotein response. In the case of glycoprotein F, less extensive variation has been described and our results agree with that observation.

A clear-cut effect of age on the anti-G glycoprotein-specific IgE response was found, as opposed to the response to F glycoprotein. Low levels of serum antibodies against G glycoprotein have been reported in infants (13), and it has been postulated that the existence of high titers of maternal-derived antibodies interferes with the immune responses of the infants. Nevertheless, it is not possible to exclude the effect of maturation of the immune system as the children grow up.

TABLE 5. Clinical features and glycoprotein F shedding in NPSs of patients with RS virus-associated pneumonia[a]

Age group and glycoprotein F concn (ng/ml)	Respiratory rate	Days of evolution prior to hospitalization	Days of hospitalization
1–48 mo			
<10	61.4 ± 12.3[b]	5.3 ± 3.8	10.9 ± 6.0 (10)[c]
10–200	61.7 ± 16.5	2.5 ± 0.5	8.5 ± 1.6 (4)
>200	76.5 ± 9.6[b]	2.5 ± 0.5	14 ± 14.6 (4)
1–6 mo			
<10	65.7 ± 12.6	5.8 ± 4.4[b]	9.6 ± 6.7 (7)
10–200	70.3 ± 8.2	2.5 ± 0.5[b]	8.0 ± 1.4 (4)
>200	75.3 ± 10.9	1.7 ± 0.5[b]	18 ± 14.8 (3)

[a] RS virus infection was diagnosed by virus isolation and/or positive immunofluorescence for viral antigens in nasopharyngeal cells. Values are means ± standard deviations.
[b] $P < 0.05$ (Student *t* test).
[c] Numbers in parentheses are numbers of patients.

The observation of higher levels of specific IgE antibodies in patients shedding large amounts of glycoprotein F antigen in NPSs pointed to the importance of the antigenic load in the development of an immune response and should be considered in the design of safe vaccines, since unwanted effects such as hypersensitivity reactions should be avoided.

The lack of a detectable IgE response to the internal proteins of RS virus, in contrast to the response to external glycoproteins, needs further investigation in order to establish whether the nature of the antigens, the way that they are presented at the virions, or an insufficient antigenic load could explain the lack of a response. Even modification of the antigens during immunoaffinity purification should be considered to explain the absence of a response, although the antigens were readily recognized by their corresponding MAbs.

The close correlation observed between respiratory rates and the existence of anti-F glycoprotein-specific IgE pointed to a role of hypersensitivity in RS virus pathogenesis. Previous studies (4, 16) in which whole virus was used as the antigen reported an association between the IgE response with wheezing and hypoxia. Our observations relating the IgE immune response to the antigenic load point to a more complex interrelation of variables in the pathogenesis of RS virus infections.

The observation that a rapid evolution from the time of onset of signs to the hospitalization of the patients closely correlates with the shedding of glycoprotein F and that there is a trend toward longer hospitalization in patients excreting the largest quantity of antigens pointed to the importance of the level of virus replication in the pathogenesis of RS virus infections. A previous study (3) based on virus infectivity titrations established a relationship between virus shedding and severity of RS virus infections.

No definitive conclusion as to the relative importance of the different variables that are involved in the pathogenesis of RS virus infections can be drawn because of the small number of patients included in our study, but a complex pattern of interrelations should be considered.

The absence of a correlation between wheezing and the presence of specific IgE in NPSs observed in our study differs from the observations described in previous reports (4, 16). Although it is difficult to explain the reasons for such a difference, our finding pointed to the complexity of the mechanism involved in the pathogenesis of RS virus infections in children.

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