Respiratory Syncytial Virus Specific Serum Antibodies in Infants Under Six Months of Age: Limited Serological Response Upon Infection


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The decline of maternal respiratory syncytial virus (RSV) specific serum antibodies was studied in 45 children during the first 6 months of life, using a virus neutralization assay and competition ELISAs measuring fusion protein and glycoprotein specific antibodies. In all children RSV neutralizing antibodies were demonstrated at birth, with titers ranging from 33 to 1382. The calculated mean half life of these antibodies was 26 days. Furthermore, in a group of 38 children with suspected RSV infection, all younger than 6 months of age on admission, the diagnostic value of serological assays was evaluated. In 32 children RSV infection was confirmed by virus isolation, direct immune fluorescence and RT-PCR. In 7 patients of this group a significant titer rise in virus neutralization assay was demonstrated. Six additional RSV infected children could be identified by showing the presence of RSV-specific IgM or IgA serum antibodies or by showing an increase in fusion protein or glycoprotein specific antibodies. All serological tests together identified 13 (41%) of the 32 RSV infected patients. It is concluded that in children of this age group, which represent the majority of patients hospitalized with RSV infections, serological assays not only have a limited diagnostic value but are of limited value for sero-epidemiological studies. J. Med. Virol. 52:97–104, 1997.

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KEY WORDS: RSV; diagnosis; serology; respiratory infection; children

INTRODUCTION

In developed countries, respiratory syncytial virus (RSV) is the most important cause of severe respiratory tract infections in children under 2 years of age [Glezen et al., 1986; Kim et al., 1973]. Severe RSV infection related disease is predominantly seen in children between 6 weeks and 6 months of age, when most infants still have RSV specific maternal serum antibodies. Vaccination with a formalin inactivated candidate RSV vaccine, inducing high titers of RSV specific antibodies, gave rise to more severe clinical disease upon subsequent exposure to the virus [Chin et al., 1969; Murphy et al., 1986]. Therefore antibodies have been incriminated as a factor in more severe clinical outcome of the infection. The presence of RSV specific IgE has been suggested in particular to be a contributing factor in RSV bronchiolitis [Bui et al., 1987; Welliver et al., 1985]. On the other hand, there is evidence from both human and laboratory animal studies that high titered virus neutralizing (VN) antibodies may be protective against RSV infection [Groothuis et al., 1993; McIntosh, 1993; Prince et al., 1985; Silber et al., 1992].

Data are presented on the decline of RSV specific maternal serum antibodies in 45 children, and the development of specific serum antibodies upon infection with RSV in 32 children, all younger than 6 months of age. In this study, existing and newly developed assays were used to measure RSV specific antibodies of different classes, isotypes, and protein specificities.

PATIENTS AND METHODS

Patients and Sera

Group I: Serum samples collected during the years 1989–1991, from 45 healthy children participating in a hepatitis B vaccination trial, were used to study the decline of maternal RSV specific antibodies. Sera were taken at birth, at 3 months, and at 6 months. Children born in the months March through June were selected to minimize the chance of RSV infection during the first 6 months.

Group II: Serum samples from 38 children, all younger than 6 months of age, seen at Sophia’s Children Hospital during the period November 1993 to

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April 1994, with a respiratory infection suspected to be RSV, were collected in the acute stage of the disease and 3-4 weeks later. Demographic and clinical data of these children were collected on admission and at a control visit. They included gender, age, duration of pregnancy, presence of underlying disease (defined as congenital heart disease, bronchopulmonary dysplasia, or immune deficiency), feeding difficulties (defined as an increase of time required for feeding or a decrease of feeding volume), a positive history for apnea (defined as either a history of respiratory arrest with cyanosis or an observation of respiratory arrest for a period of more than 20 seconds and/or bradycardia with accompanying cyanosis in the pediatric emergency room or during hospital admission), the presence of retraction, respiratory rate, oxygen saturation (\(\text{SaO}_2\)) in room air (\(\text{pCO}_2\)), pH, and abnormalities on X ray (hyperinflation, consolidation, or atelectasis) as described by a radiologist, admission to an intensive care unit, and the need for artificial ventilation.

**Virus Preparation**

The A2 strain of human RSV (ATCC VR1302) was used for serological tests. The virus was passaged in HEp-2 cells and cultured until the majority of the monolayer exhibited cytopathic changes. The supernatant was cleared of cell debris by centrifugation for 10 minutes at 1000 G and subsequently aliquoted and frozen at −70°C until use for virus neutralization assays (VN). The same stock of RSV was used for all virus neutralization tests.

Virus for competition ELISAs and IgM and IgA capture ELISAs was partially purified by polyethylene glycol precipitation of the supernatant of infected cells and subsequently centrifuged for 2 hours at 25000 rpm through a discontinuous 30-60% (w/w) sucrose gradient in a Beckman SW28 rotor. The resulting opaque virus containing band was collected, aliquoted, and frozen at −70°C until use.

**Virus Isolation(VI) and Direct Immune Fluorescence Assay (DIFA)**

Nasopharyngeal washings were taken from the children of group II. The samples were diluted with 5 ml DMEM, homogenated, and centrifuged for 10 minutes at 1000 rpm. The supernatant was used for DIFA. The pellet was pipetted onto multispot slides, dried at room temperature, and fixed with acetone. These slides were used for DIFA as previously described [Rothbarth et al., 1988], using fluorescein isothiocyanate (FITC) labeled RSV specific monoclonal antibodies (DAKO, Ely, UK). For RSV subtyping, monoclonal antibodies against subtype A (MAB 92-11c, Chemicon, Temecula, USA) or subtype B (MAB 109-10B, Chemicon, Temecula, CA) were pipetted on the slides and incubated for 30 minutes at 37°C. After washing 3 times with phosphate buffered saline pH 7.2 (PBS), a FITC labeled anti mouse conjugate (DAKO, Ely, UK) was applied and incubated for 30 minutes at 37°C. After washing 3 times with PBS and once in distilled water, the slides were examined under a epifluorescence microscope using a 620 nm filter. Slides were scored positive when a typical granular fluorescence was observed in the cytoplasm of cells.

**Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)**

RT-PCR was carried out on nasopharyngeal washings of the children as described previously [van Milaan et al., 1994]. In short, total RNA of nasopharyngeal washings was extracted using guanidinium isothiocyanate and phenol/chloroform. RNA PCR was undertaken in 2 steps. For cDNA synthesis, 10 μl of the RNA template and 1μl (containing 10 pmol) cDNA primer were heated to 80°C for 2 minutes and put on ice. Then 14 μl of a cDNA reaction mixture was added, which made a solution containing 50 mM Tris-HCl pH 8.3, 37.5 mM KCl 3 mM MgCl₂, 10 mM DTT, 0.5 mM of each of the dNTPs (dTTP, dATP, dCTP, and dGTP), 1.6 U RNAsin (Promega, Leiden, the Netherlands), and 8 U molony murine leukemia virus (M-MLV) reverse transcriptase (Gibco-BRL, Breda, the Netherlands). The mixture was incubated at 42°C for 45 minutes, heated at 95°C, and put on ice. Then 75 μl of the PCR mix was added, resulting in a 100 μl PCR solution containing 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton-X-100, 0.2 μM of each of the dNTPs, 20 pmol cDNA primer, 20 pmol reverse primer, and 1 U of Taq polymerase (Promega, Leiden, the Netherlands). A PCR program containing 36 cycles of 3 steps (1 minute at 95°C, 1 minute at 52°C, 1 minute at 74°C) was used.

For analysis of the amplified products, 25 μl of the PCR products were electrophoresed in a 1.5% agarose gel. The gel was then denatured for 10 minutes in 0.4 N NaOH and the amplified products were transferred to nylon membrane using a semi-dry blotter (Biorad, CA) at 3 mA/cm² for Southern blot analysis. Southern blots were hybridized overnight using subtype A and B specific 32P labeled oligonucleotides.

**Virus Neutralization (VN) Assay**

50 μl serially diluted sera were incubated with 50 μl DMEM containing 100 TCID50 of RSV-A2 for one hour at 37°C in 96 well tissue culture microtiter plates (Costar Plastics, Badhoevedorp, the Netherlands). Control wells contained no serum (positive or virus growth control) or only DMEM (negative or cell control). HEp-2 cells were added to all wells and incubated for 3 days at 37°C 5% CO2. Subsequently the cells were washed once carefully with PBS and fixed in ethanol at −70°C for 30 minutes.

The expression of viral antigen on the HEp-2 cells was detected in an ELISA system; the plates were blocked with 100 μl 1% gelatin in PBS for 30 minutes and washed 5 times with PBS 0.05% tween 20 (PBST). 50 μl of an RSV fusion protein specific mouse monoclonal (MAB 92-11C, Chemicon, Temecula, CA) was added to the cells and incubated for 2 hours at 37°C. After washing five times, HRPO labeled rabbit anti-mouse
(DAKO, Ely, UK) was used as conjugate, incubated for one hour at 37°C, washed 5 times, and shaken dry. Then the substrate tetramethylbenzidine/H₂O₂ was added. The reaction was stopped after 10 minutes with 2 M H₂SO₄. The optical density (OD) was read at 450 nm. The percentage virus neutralization was calculated by the following formula:

\[
\frac{\text{experimental OD} - \text{cell control OD}}{\text{virus control OD} - \text{cell control OD}} \times 100\%
\]

The titer of the serum was defined as the reciprocal of the dilution, which gave 50% virus neutralization. A significant rise in titer to confirm infection was arbitrarily defined as greater than a threefold titer rise.

**Competition ELISA**

To measure antibodies against separate RSV membrane proteins, competition ELISAs for RSV fusion protein (comp-F ELISA) and glycoprotein (comp-G ELISA) were carried out essentially according to methods described previously for hantavirus serology [Groen et al., 1992]. Briefly, antigen was coated (2 ng/well) to 96 well ELISA plates (Costar Plastics, Badhoevedorp, The Netherlands) overnight at 4°C. 100 μl of a tenfold serum dilution in ELISA buffer (PBS with 3% NaCl, 0.1% BSA, 0.1% milk powder, 5% normal rabbit serum, and 1% fetal calf serum) were pipetted into the wells. For the negative control (0% inhibition), ELISA buffer containing no serum was used. For the positive control (100% inhibition), ELISA buffer containing no serum was used, and ELISA buffer containing no monoclonal antibody was used in the second step. After incubation for 2 hours at room temperature, half (50 μl) of the serum dilution was removed and replaced by 50 μl of a mouse monoclonal anti-fusion protein (133/1H, Chemicon MAB 858-1, Temecula, CA) or anti-glycoprotein (131/2G, Chemicon MAB 858-2, Temecula, CA) RSV dilution in ELISA buffer and incubated for one hour at 37°C. The specificity of these monoclonals has been described previously [Anderson et al., 1988]. Subsequently the plates were washed 3 times in PBST. HRPO labeled rabbit anti-mouse (DAKO, Ely, UK) was used as the conjugate and incubated for 1 hour at 37°C, washed 3 times, and shaken dry. Then 100 μl tetramethylbenzidine/ H₂O₂ substrate was added. The reaction was stopped after 10 minutes with 2 M H₂SO₄. The OD was spectrophotometrically read at 450 nm. Percentage inhibition was calculated by the formula:

\[
\frac{\text{OD}_{0\%\text{inhibition}} - \text{OD}_{100\%\text{inhibition}}}{\text{OD}_{0\%\text{inhibition}} - \text{OD}_{100\%\text{inhibition}}} = \%\text{inhibition}
\]

A significant rise in inhibition to confirm infection was arbitrarily defined as greater than a threefold rise in inhibition percentage.

Sera proved negative tested at a 1:10 dilution in this assay never reached inhibition levels above 10%.

**RSV Specific IgA and IgM Detection in Serum**

RSV specific antibodies of the IgA and IgM class were detected in sera by capture ELISA essentially as described previously for hantavirus serology [Groen et al., 1992]. Positive results were confirmed with an indirect immune fluorescence assay as previously described [Groen et al., 1989].

For capture ELISA, goat anti-human IgA or IgM was coated to 96 well ELISA plates overnight at 4°C. 100 μl of a hundredfold serum dilution, in ELISA buffer, was pipetted in the wells and incubated for 1 hour at 37°C. Subsequently the plates were washed 3 times in PBST. The optimal dilution of RSV antigen was pipetted in the wells and incubated for 1 hour at 37°C and the plates were washed 3 times in PBST. Subsequently 50 μl of biotinylated polyclonal goat anti-RSV (Chemicon, Temecula, CA) was added to the cells, incubated for 1 hour at 37°C, washed 3 times in PBST, and shaken dry. Streptavidin-HRPO (Amersham, Little Challont, UK) was used as conjugate, incubated for 30 minutes at room temperature, washed 3 times, and shaken dry. Then 100 μl tetramethylbenzidine/H₂O₂ substrate were added. The reaction was stopped after 10 minutes with 2 M H₂SO₄. OD was measured at 450 nm. An OD exceeding more than 2 times the OD of the negative control was considered positive.

For confirmation of positive results in an indirect immune fluorescence assay, cells infected with RSV were mixed with uninfected HEP-2 cells and spotted on 12 well multispot slides. The slides were dried at room temperature and the cells were fixed with ethanol at -70°C. Sera were pre-incubated with sheep anti-human Fc gamma globulin (RIVM, Bilthoven, The Netherlands). Pre-incubated sera (1:16 end dilution) were pipetted on the wells with RSV infected cells and incubated 2 hours at 37°C. Subsequently the slides were washed 3 times for 5 minutes in PBS and 1 minute in distilled water. Then FITC labeled anti-human IgM or IgA (DAKO, Ely, UK) was pipetted on the slides and incubated for 30 minutes at 37°C. After washing, the slides were examined in an epifluorescence microscope using a 620 nm filter. Results were considered positive when a characteristic granular fluorescence pattern in the cytoplasm of the cells was observed.

**Complement Fixation Assay (CF)**

The CF assay was carried out as described previously [Hawkes, 1979]. Briefly, serum was inactivated at 56°C for 30 minutes. A 2 log titration of the serum starting at 1:7 was made. The working dilution of first RSV antigen and then complement (both Bio-Withakker, Walkersville, MD) were added to each serum dilution. After incubation of 18 hours at 4°C, sheep red blood cells, sensitized with hemolysin (Bio-Withakker) were added and incubated for 1 hour at 37°C. After settling of the red blood cells, titers were read at 50% endpoint.
Statistical Analyses

For statistical analyses, geometric mean titers (GMT) were calculated for the VN assay. For the competition ELISAs, mean inhibition percentages were calculated. For comparison of mean rise in antibodies in infected and non-infected children, a 2-tailed student's t-test for paired samples was used. For comparing RSV specific titers upon admission with parameters of clinical severity a 2-tailed student's t-test for independent samples was used.

RESULTS
Decline of Maternal RSV Specific Antibodies

The decline of RSV specific maternal antibodies during the first 6 months of life was monitored in 45 children (group I) using the VN assay and competition ELISAs (Fig. 1). At birth, VN antibodies were present in the sera of all 45 children, with titers ranging from 33 to 1382 and a geometric mean titer of 301. Geometric mean titers at 3 and 6 months were 24 and 10, respectively. In the majority of sera taken at 6 months after birth, no VN antibodies could be demonstrated. Comparison of mean inhibition percentages measured in comp-F ELISA and comp-G ELISA at birth, at 3 months, and at 6 months showed a linear decline of 86%, 43%, and 21% in the comp-F ELISA and of 91%, 58%, and 21% in the comp G-ELISA, respectively. Two of the 45 children tested showed a significant titer rise in VN between 3 and 6 months after birth (from <10 to 48 and from 22 to 132, respectively), indicating that these infants had been infected with RSV during this period. In these paired sera a rise in inhibition percentage was found from 8.4% to 38.9% and from 43.0% to 63.7%, respectively, in the comp F-ELISA. However, no rise was observed in the comp-G ELISA. From the comparison of the VN serum antibody titers at birth with those found 3 months later, a mean half-life of maternally derived serum antibodies of 26 days was calculated.

Kinetics of RSV Specific Antibodies After Infection

Of the 38 infants with a suspected RSV infection (group II), 32 indeed proved to be infected with RSV. This was shown by VI, DIFA, and RT-PCR analyses. No other viral infections were found in any of the 38 infants. Individual titers and GMT of RSV infected and noninfected children are shown in Figure 2. Comparing the serum antibody titers measured in the VN assay upon admission and 3 to 4 weeks later showed a significant mean antibody titer rise measured in children with a confirmed infection (GMT1 = 51, GMT2 = 75; P = 0.01) and a decline in antibody titer in uninfected children (GMT1 = 48, GMT2 = 30; P = 0.06). The mean titer rise in infected children was significantly different from the mean titer decline of noninfected children (ΔT-infected = 30, ΔT-noninfected = -44; P = 0.04). No significant changes in mean inhibition levels were found between infected and uninfected children when using the comp-F and comp-G ELISA.
Antibodies to RSV in Infants Under 6 Months

The results of VI, DIFA, and RT-PCR assays were in complete agreement; in 32 patients an RSV infection was identified, of which 17 were of subtype A and 15 of subtype B, as shown with DIFA and RT-PCR. Analyses of paired sera in the VN assay allowed the identification of seven (22%) RSV infected infants by showing greater than a threefold titer rise. With the comp-F and comp-G ELISA, one (3%) and seven (22%) RSV infected infants could be identified, respectively. Detection of RSV specific IgM and IgA in single serum samples identified 1 (3%) and 5 (16%) RSV infected infants, respectively. With a CF assay, no RSV infected infants were detected. All serological tests together only identified 13 (41%) of the 32 RSV infected patients.

As shown in Figure 3, a greater than threefold rise in RSV specific serum antibody rise was predominantly found in RSV infected children older than 3 months.

**Comparison of VN Titer Upon Admission With Disease Severity**

In order to evaluate whether a relationship exists between the presence of RSV specific maternal antibodies on one hand and severity of RSV related disease on the other, antibody titers measured in the VN assay and inhibition percentages in the comp-F and comp-G ELISA were related to parameters of clinical severity (pCO₂, SaO₂, ICU admission, artificial ventilation).

Children with a higher pCO₂ upon admission had significantly higher titers in the VN assay (P = 0.05). A higher pCO₂ also correlated strongly with a younger age upon admission. No correlation could be found between SaO₂, ICU admission, or artificial ventilation and VN titers or percentage inhibition in the competition ELISAs (data not shown).

**DISCUSSION**

We have shown that in children under 6 months of age the diagnostic value of RSV serology is limited and by far inferior to the direct detection methods for RSV antigen or viral RNA. This may, at least in part, be caused by the relative inability of young infants to mount a specific antibody response upon infection. This is best illustrated by the virtual absence of RSV specific IgM, which is not vertically transmitted via the placenta. Furthermore, preexisting maternal antibodies may interfere with the antibody response upon infection and may also hinder the interpretation of serological results.

The detection of IgM, IgG, and IgA antibodies for the serodiagnosis of RSV in young infants is known to be relatively insensitive as a diagnostic tool. However, we investigated whether antibody recognition of different structural RSV proteins would be a useful parameter for the diagnosis of RSV infection. This is a well-established approach for other virus infections, such as HIV [Portera et al., 1990] and hantavirus [Groen et al., 1992].

All children in group I had detectable maternal an-
TABLE I. Summary of Diagnostic Analyses in 38 Children under 6 Months of Age Clinically Suspected of Having RSV Infection

<table>
<thead>
<tr>
<th>Patient</th>
<th>VI</th>
<th>DIFA subtype</th>
<th>RT-PCR subtype</th>
<th>VN</th>
<th>Competition ELISA(^a)</th>
<th>Capture ELISA</th>
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<td></td>
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<td></td>
<td>anti-F</td>
<td>IgM</td>
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<td>anti-G</td>
<td>IgA</td>
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VI = virus isolation, DIFA = direct immune fluorescence on cells of nasopharyngeal washings using subtype A and B specific monoclonal antibodies; RT-PCR = reverse transcriptase polymerase chain reaction using subtype A and B specific probes; VN = virus neutralization assay; anti-F = anti fusion protein antibodies; anti-G = anti glycoprotein antibodies; CF = complement fixation test.

\(^a\)A threefold rise in antibody level was considered positive in VN, CF, and anti-F and -G competition ELISA.

Antibodies at birth, which declined with a half-life of 26 days in the first months of life. This value is in agreement with normal half-life values of passively acquired antibodies, which is estimated to be 3 to 4 weeks [Morell et al., 1970]. The presence of RSV specific antibodies has been shown to correlate with protection against severe RSV infection in mice [Prince et al., 1985] and in children [Groothuis et al., 1993], although relatively high titers seemed to be required for protection. Studies in children have shown that the administration of high titered anti RSV immune globulines may protect young children from developing a severe RSV infection with the involvement of the lower respiratory tract [Meissner et al., 1993; Groothuis et al., 1993]. This allows speculation about the protective value of maternal antibodies, which may provide sufficient protection against severe disease development after birth. However, with a half-life of 26 days, antibody levels may be expected to drop relatively fast to unprotective levels. With the exception of pCO\(_2\) levels, which correlated with VN titers upon admission, none of the parameters of clinical severity correlated with antibody levels upon admission in group II. However, the correlation of pCO\(_2\) levels with VN titers may probably be explained by the higher pCO\(_2\) usually found in younger infants with RSV infection [Mulholland et al., 1990], at which age higher maternal antibody levels are also present. Thus no causal relationship between RSV specific antibody titers and severity of infection was detected in this study.

The discrepancy between the data generated by the VN assay and the competition ELISAs may be explained by the fact that the inhibition percentages of maternal antibodies found in noninfected children are relatively high, as compared to VN antibodies. Therefore maternal antibodies should be expected to cause more interference in competition ELISAs than in the VN assay. This would result in the absence of a demonstrable rise in inhibition percentages upon infection.

We used a 1:10 dilution of the serum sample in the competition ELISA, although we realized that such a low dilution might cause nonspecific binding. When the RSV competition ELISA was established, we tested several negative serum samples at a 1:10 dilution. None of these sera caused nonspecific reduction of the absorbance. Furthermore, the use of the principle of a competition ELISA with 1:10 diluted serum samples has been investigated extensively by us in several other systems, including infections with hantavirus [Groen et al., 1992], rabies virus [van der Heijden et al., 1993], and *Borrelia burgdorferi* [Rijpkema et al., 1994].

At different intervals after infection or reinfection, serum antibodies have different affinities for the respective epitopes. Especially in the acute phase of RSV infection, antibodies with low affinity may be present [Meurman et al., 1992]. In the competition ELISA the monoclonal antibodies which have a relative high affinity may interfere with the binding of the serum antibodies, especially when the whole sample is removed. We therefore removed only half of the sample and replaced it with 50 \(\mu\)l of the respective monoclonal antibody preparation.

Although the children with a proved subtype A infection showed more often a serological response in the assays used than children with a subtype B infection, the number of individuals studied is too small for us to conclude that this may have been due to the use of a subtype A strain in the assays.

Taken together, the data presented in this study show that VI, DIFA, and RT-PCR are more reliable tools for the diagnosis of RSV infections than serological methods. For practical reasons, the use of DIFA,
followed by confirmatory VI, is probably the best option at present for the rapid and accurate diagnosis of RSV infection.

The overall poor performance of the serological assays used indicates the limited diagnostic value of serology in these young children. Furthermore, it shows that serology cannot be used for sero-epidemiological studies, at least in the age group of children younger than 6 months, which comprises more than 50% of all patients hospitalized for RSV infection.

REFERENCES


