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Development of Antibody-Dependent Cell-Mediated Cytotoxicity in the Respiratory Tract After Natural Infection with Respiratory Syncytial Virus

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Antibody-dependent cell-mediated cytotoxicity (ADCC) was measured in nasopharyngeal secretions collected from 42 infants and young children at various intervals after primary or secondary infection with respiratory syncytial virus. ADCC was determined by specific immune release of ^{51}Cr from respiratory syncytial virus-infected HEp-2 cell culture monolayers, with lymphocytes from adult volunteers as effector cells. Specific ADCC responses in nasopharyngeal secretions after primary infection were observed as early as 3 days after the onset of clinical symptoms, and peak activity was observed 14 to 29 days after the onset of illness. ADCC responses after reinfection were significantly greater in both the acute and convalescent phases ($P < 0.05$) than were ADCC responses after primary infection. ADCC in secretions was mediated primarily by the immunoglobulin G isotype of respiratory syncytial virus antibody.

Respiratory syncytial virus (RSV) is a major cause of serious respiratory illness in the first few years of life (8). Repeated infections with RSV result in milder forms of illness (5), although the mechanism by which such immunity to serious illness is achieved remains unknown. Infection may occur in the presence of preexisting, transplacentally acquired maternal antibody in the serum (14) or in the presence of serum antibody acquired as a result of previous infection (1). It appears that, in general, serum antibody does not play a major role in protection against infection with RSV.

In studies of experimental infection with RSV, adults with high titers of antibody to RSV in the nasopharynx before experimental challenge exhibited decreased shedding of virus as compared with those with low titers of preexisting antibody (13). It is also known that secretory antibody of RSV appears earlier and persists for longer periods after secondary RSV infections than after primary infection (7). These data are somewhat suggestive of a role for secretory antibody in modifying the severity of illness on reinfection with RSV. However, the exact mechanism by which antibody results in the eradication of RSV is unknown. It has been clearly shown that the presence of secretory antibody to RSV in nasopharyngeal secretions (NPS) alone does not seem to be a sufficient condition for neutralization of virus, since both infectious virus and secretory immunoglobulin A (IgA) antibody to RSV may coexist in secretions (10). The addition of complement to in vitro neutralization assays enhances the neutralizing activity of serum RSV antibody, suggesting that fixation of complement to virus-antibody complexes in secretions may play some role in clearing the virus after natural infection (6). Another potential mechanism by which secretory antibody may contribute to the eradication of RSV is via antibody-dependent cell-mediated cytotoxicity (ADCC). Previous studies have demonstrated that RSV antibody in serum, colostrum, and NPS (2, 12, 15) may participate in cell-mediated cytotoxicity against RSV-infected cells in vitro. The present study was undertaken to delineate the temporal kinetics of the serum and secretory ADCC response after RSV infection. Second, an analysis was made of the effect of patient age at the onset of illness on the ADCC response and the effect of repeated infection with RSV on the ADCC response. Finally, a comparison of ADCC responsiveness was made among patients with different clinical forms of illness due to RSV.

MATERIALS AND METHODS

Study group. The subjects who served as the population base of this report are part of an ongoing, long-term study of immunology of respiratory illnesses in childhood. Participants in the larger study are examined at the time of each episode of respiratory illness, at which time samples of NPS and serum are obtained. Although these samples are obtained primarily for the purpose of identifying the agent causing the infection,

parents of subjects are informed at the time of enrollment in the study that samples may be used for further immunological tests. A signed statement of informed consent was obtained after the potential benefits and risks of the study were explained. Data in the present report were obtained from the study of samples of serum and NPS obtained from 42 infants, selected at random from the larger study, who were known to be undergoing primary RSV infection. Serum samples were obtained from 32 subjects at random intervals up to 100 days after the onset of illness due to primary RSV infection. In addition, 59 samples of NPS were available from the entire group of 42 subjects after primary RSV infection. No subject had more than two samples of NPS obtained for the purposes of this study. All patients were 1 to 12 months of age at the time of primary RSV infection. During the course of long-term follow-up, 16 patients in the initial group of 42 developed a second RSV infection. A total of 18 samples of NPS were available for study after the second infection.

In all cases, RSV infection was documented by identification of viral antigen in NPS by indirect immunofluorescence and by recovery of infectious virus in tissue culture, as previously described (17). Patients were examined by a single member of the study team, who reviewed the results of chest radiographs with a radiologist. Patients were diagnosed as having upper respiratory illness alone, pneumonia, or bronchiolitis on the basis of criteria previously described (17).

Processing of samples. Serum samples were stored at −20°C until analyzed. Samples of NPS were collected by gentle suctioning through polyethylene catheters inserted into the nasopharynx. No samples contained blood grossly. Aspirates were rinsed into mucus traps with 2.5 ml of Hanks balanced salt solution. The samples were centrifuged immediately, and the supernatants were dialyzed against distilled water, lyophilized, and reconstituted in buffer to 1-ml volumes before analysis.

ADCC assay. The assay for ADCC was carried out with minor modifications of methods previously described (15). Target cells consisting of human epithelial (HEp-2) tissue culture monolayers were infected with stock strains of RSV at a multiplicity of infection of 2 to 3 plaque-forming units per cell. Uninfected HEp-2 cell monolayers were used as controls. Infected and uninfected monolayers were labeled with 200 μCi of ^{51}Cr (New England Nuclear Corp., Boston, Mass.). After a period of 2 h, the cell monolayers were trypsinized, washed, and suspended in Eagle minimum essential medium with 2% chicken serum in a final concentration of 10^5 cells per ml. A 100-μl amount of cell suspension was placed in wells in flat-bottom microtiter plates (Linbro, New Haven, Conn.) along with 25 μl of serial twofold dilutions of patient serum or secretions.

Peripheral blood lymphocytes (effector cells) were separated by Ficoll-Hypaque centrifugation from 50 ml of peripheral blood obtained from adult volunteers. Macrophages were removed by adherence to glass petri dishes, and nonadherent cells were suspended in medium to 10^7 cells per ml; 100 μl of effector cells was added to each microtiter well containing target cells and serum or secretions (effector cell/target cell ratio, 100:1). Plates were centrifuged at 100 rpm for 10 min at 4°C and incubated for 16 h at 37°C. Plates were recentrifuged, and 100 μl of supernatant was aspirated and counted for radioactivity in a gamma scintillation counter (Packard Instrument Co., Downing Grove, Ill.).

Controls consisted of effector and target cells incubated in the presence of medium instead of serum or secretions, effector and target cells incubated in the presence of serum and secretions obtained from patients with infection due to viruses other than RSV, target cells incubated in the absence of effector cells (nonspecific isotope release), and target cells subjected to three cycles of freezing-thawing in distilled water (maximum release). Specific immune release (SIR) was calculated as follows:

$$\text{SIR (\%)} = \frac{\text{VLA}-\text{VLM}}{\text{M}-\text{VLM}} - \frac{\text{CLA}-\text{CLM}}{\text{M}-\text{CLM}} \times 100$$

where *VLA* is the counts per minute in wells containing infected target cells, effector cells, and serum or secretions; *VLM* is the counts per minute in wells containing infected target cells, effector cells, and medium; *CLA* is the counts per minute in wells containing uninfected target cells, effector cells, and serum or secretions (control); *CLM* is the counts per minute in wells containing uninfected target cells, effector cells, and medium (control); and *M* is the maximum isotope release.

Determinations of RSV antibody titers in serum and secretions. Titers of RSV antibody in IgG, IgM, and 7S IgA immunoglobulin classes in serum, and in IgG, IgM, and 11S IgA immunoglobulin classes in secretions, were determined by indirect immunofluorescence methods previously described (7, 18).

Absorption of antibody activity from NPS. RSV antibody activity was absorbed from samples of NPS by incubation of secretions for 1 h with heavy-chain specific goat anti-human IgG, IgM, or IgA (Meloy Laboratories, Springfield, Va.). The antisera were tested for immunological specificity to each isotype as described previously (18).

Determination of total secretory immunoglobulin concentrations. Concentrations of total IgG, IgM, and 11S IgA in secretions were determined in radial immunodiffusion plates (Behring Diagnostics, Somerville, N.J.).

Statistical analysis. Differences in SIR among study groups were tested for statistical significance by using the Student *t* test. Regression lines were drawn by using the method of least squares, and coefficients of correlation (*r* values) were compared to determine degrees of statistical significance.

RESULTS

ADCC response in serum to primary RSV infection. The ADCC response mediated by serum antibody after primary RSV infection is shown in Fig. 1. The geometric mean percent SIR as early as 1 to 4 days after the onset of illness was 20.8% and rose to a maximum level of 37% by 5 to 13 days after the onset of illness. The percent SIR fell thereafter to a level of 14.6% at ≥90 days after the onset of illness.

ADCC response in NPS to primary RSV infection. The ADCC response in NPS after primary

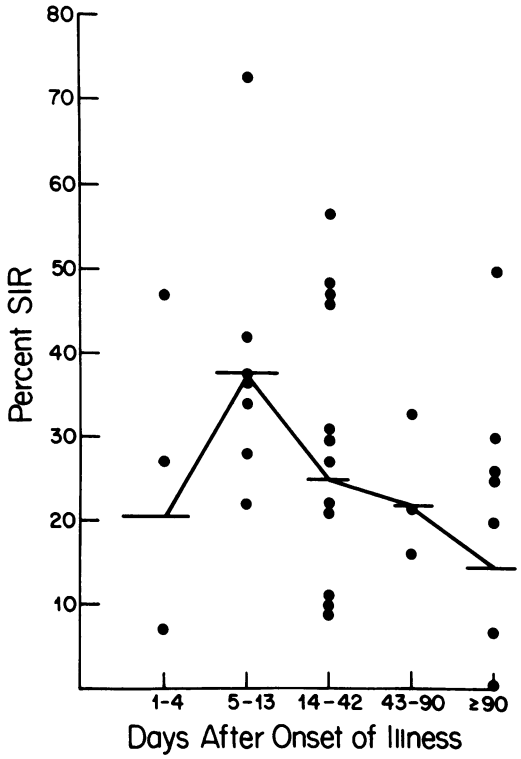

FIG. 1. Development of ADCC response in serum samples at intervals after primary infection with RSV. Solid line connects geometric mean percent SIR at each interval.

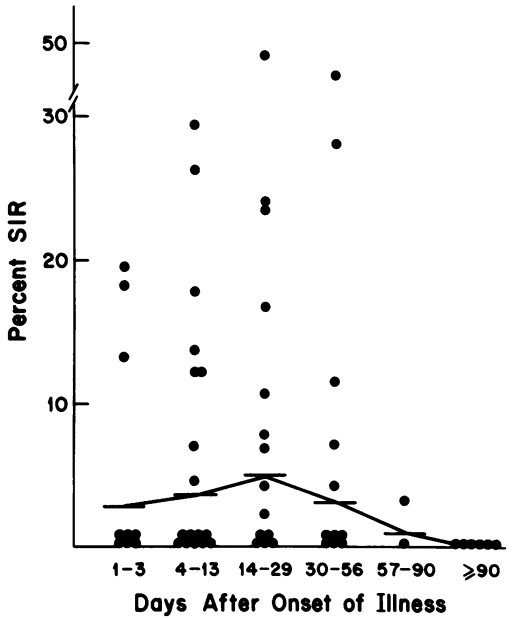

FIG. 2. Development of ADCC response in NPS after primary infection with RSV. Solid line connects geometric mean percent SIR after primary infection with RSV.

RSV infection is shown in Fig. 2. A detectable ADCC response was observed in samples from three of nine patients obtained as early as the first 3 days of illness. Both the geometric mean percent SIR and the percentage of patients exhibiting an ADCC response in their secretions reached maximal values from 14 to 29 days after the onset of illness. Thereafter, the degree of ADCC activity declined slowly, and by ≥90 days after the onset of illness, none of five samples had any detectable ADCC activity. All secretions from patients with infections due to agents other than RSV resulted in ADCC activity of less than 1% SIR in the current assay.

Relationship of form of illness to ADCC response in NPS. The ADCC responses in patients with various forms of clinical illness due to primary RSV infection are shown in Fig. 3. Results obtained in samples taken 1 to 7 and 14 to 90 days after the onset of illness were pooled to represent acute- and convalescent-phase responses. Results of 12 samples are not included in the figure either because the form of illness was not known with certainty or because the samples were obtained 8 to 13 days after the onset of illness. Geometric mean ADCC responses in the acute phase were not different among the three illness groups. The geometric mean convalescent-phase ADCC response in the group with bronchiolitis or asthma was more than twofold greater than ADCC responses at similar intervals in the upper respiratory illness or pneumonia groups. However, this difference

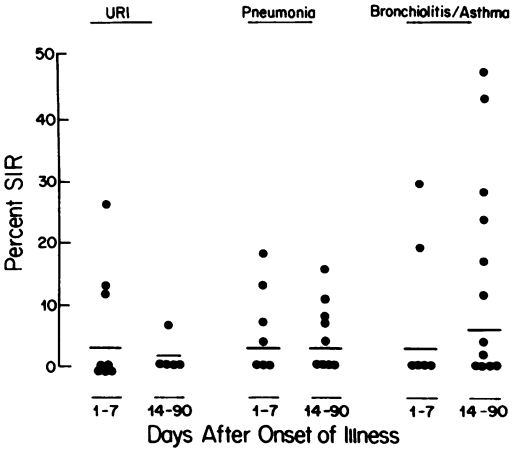

FIG. 3. ADCC response in secretions in acute- and convalescent-phase samples obtained from patients with various forms of illness caused by primary infection with RSV. Cross bars represent geometric mean percent SIR. URI, Upper respiratory illness.

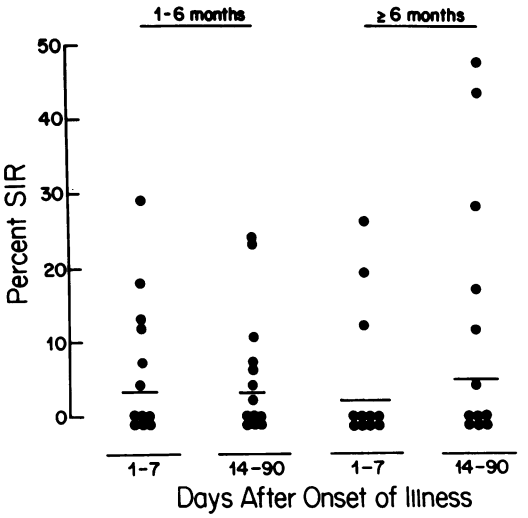

FIG. 4. ADCC response in secretions in acute- and convalescent-phase samples obtained from patients either less than 6 months of age or 6 to 12 months of age at the time of onset of RSV illness.

was not statistically significant ($t = 1.97$, $P > 0.05$).

Relationship of patient age to ADCC response in NPS. The relationship of the age of the patient at the time of primary infection to ADCC responsiveness in the acute and convalescent phases of illness is shown in Fig. 4. Results of 10 samples obtained 8 to 13 days after the onset of illness were not included. One convalescent-phase sample was deleted because the patient's exact age at the time of illness was not known to us. ADCC responses in the acute and convalescent phases were similar in patients less than 6 months of age at the onset of illness and in patients more than 6 months of age at the onset of illness.

ADCC response in NPS after reinfection with RSV. ADCC responses in secretions obtained from patients after secondary RSV infection were compared with those obtained after primary infection (Fig. 5). For purposes of statistical analysis, responses occurring 1 to 7 and 14 to 90 days after illness due to primary and secondary infection were compared. At each of these intervals, the magnitude of the ADCC response after secondary infection was significantly greater than that after primary infection (for each comparison, $t \geq 2.2$ and $P < 0.05$). Too few samples were available at >90 days after illness for a meaningful statistical comparison.

Relation of ADCC to immunoglobulin isotypes in NPS. Antibody titers to RSV in IgG, IgM, and 11S IgA immunoglobulin classes were determined in samples of NPS to find the correlation of ADCC activity with virus-specific antibody of each immunoglobulin isotype (Fig. 6). ADCC activity was most closely correlated with titers of RSV-IgG ($r = 0.45$, $P < 0.01$). ADCC activity was not correlated with titers of either RSV-IgM or RSV-IgA (for each comparison, $r \leq 0.22$ and $P > 0.10$).

An attempt was also made to determine the immunoglobulin isotype responsible for ADCC activity by adsorption of NPS specimens with goat anti-human IgG, IgM, or IgA. The reduction in SIR that would be achieved by incubation of NPS samples with anti-human globulins is shown in Fig. 7. ADCC activity could be completely abolished by incubation of NPS specimens with anti-human IgG. However, ADCC activity could also be reduced by 40% by incubation of NPS specimens with anti-human IgA and by less than 10% by incubation with anti-human IgM.

DISCUSSION

Previous studies in smaller numbers of patients with RSV infection have demonstrated the development of RSV-specific antibody in serum which is capable of mediating ADCC (2, 12, 15).

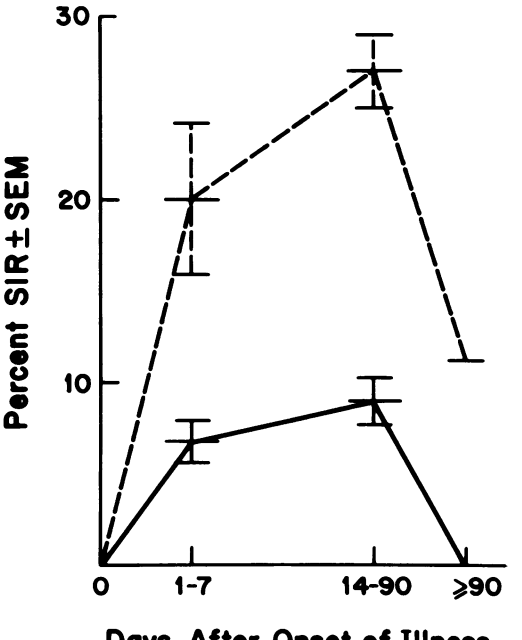

FIG. 5. Development of ADCC response in NPS after primary (solid lines) and secondary (broken lines) infection with RSV. Cross bars represent geometric mean values ± standard error. The percent SIR was significantly greater at 1 to 7 and 14 to 90 days after illness due to reinfection ($P < 0.05$) than was the corresponding percent SIR after primary infection with RSV. SEM, Standard error of the mean.

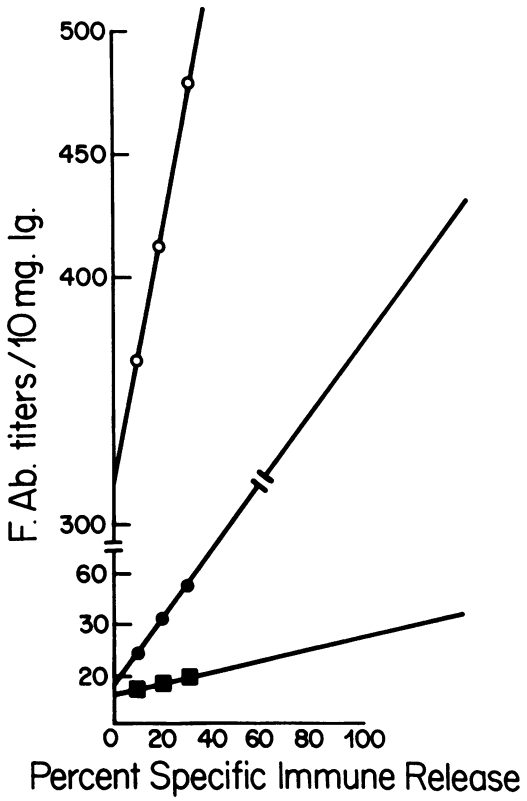

FIG. 6. Correlation of percent SIR in secretions with RSV-specific fluorescent-antibody (F. Ab.) titers in IgG, IgM, or secretory IgA immunoglobulin (Ig) classes. Regression lines for (○) secretory IgA, (●) IgG, and (■) IgM fluorescent-antibody titers. ADCC responses in NPS correlated significantly with IgG titers ($r = 0.45$, $P < 0.01$), but no significant correlation was observed with IgM or IgA titers.

The current study confirms these observations in a larger patient population. The combined data suggest that ADCC activity in serum reaches a maximum level between 1 and 2 weeks after the onset of illness and declines over the first few months after the onset of illness, but may persist at low levels for intervals of up to 90 days or more.

A recent study of 11 patients with RSV infection demonstrated ADCC activity in respiratory tract secretions which persisted for up to 9 days after the time of hospitalization with respiratory illness (2). The present study has demonstrated that peak ADCC activity in secretions occurs 2 to 4 weeks after the onset of illness and disappears from NPS by 90 days after the onset of illness. The magnitude of ADCC activity is not related to the form of clinical illness or the age of the patient at the time of primary infection. Of particular importance is the observation that repeated RSV infection results in the development of markedly enhanced ADCC activity in NPS.

In studies of the ADCC response in serum to RSV infection, Meguro and colleagues (12) found no difference in ADCC responsiveness related to the form of illness after RSV infection, a result similar to the findings of the present study. In contrast to the present study, in which increased ADCC activity with repeated RSV infection was observed, Meguro and colleagues found a decrease in ADCC response with repeated RSV infection. An explanation for this disparity is not apparent; however, it should be noted that previous studies have demonstrated increased fluorescent-antibody response to RSV in IgG, IgM, and IgA immunoglobulin classes in both serum and secretions after repeated RSV infections (7, 18). Therefore, ADCC activity should presumably increase as total virus-specific antibody increases with repeated exposure to RSV.

Quantities of secretions obtained in the present study were insufficient to allow separation of IgG, IgM, and IgG isotypes and subsequent determination of their relative contribution to ADCC activity. Previous studies (2, 12) have conclusively demonstrated that ADCC in serum against RSV-infected cells is mediated by antibody in the IgG immunoglobulin class. In a previous study of RSV-specific ADCC activity in NPS (2), ADCC activity was directly correlated with titers of both RSV-IgG and RSV-IgA antibody in secretions. In the present study, ADCC activity in secretions was correlated with titers of RSV-IgG antibody, but not RSV-secre-

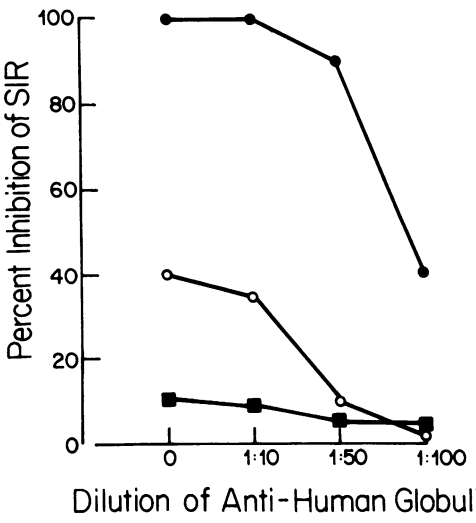

FIG. 7. ADCC response in NPS after incubation of samples with goat anti-human IgG, IgM, or IgA. ADCC activity after incubation with (●) anti-human IgG, (○) anti-human IgA, and (■) anti-human IgM.

tory IgA or RSV-IgM. ADCC activity could be totally abolished by adsorption of NPS specimens with anti-human IgG, but smaller reductions in ADCC activity could also be accomplished after absorption of NPS with anti-human IgA or anti-human IgM. The most likely explanation for these results is that IgG and IgA antibodies act cooperatively to mediate ADCC in secretions, with IgG being the isotype most responsible for mediating ADCC in both serum and secretions. Secretory IgA alone has been shown not to mediate ADCC reactions involving neutrophils, monocytes, or lymphocytes, but, when secretory IgA is added to in vitro assays, it does enhance IgG-directed leukocyte-mediated ADCC response (16). Interestingly, in the present study, ADCC activity in NPS did not increase with increasing patient age over the first year of life. Previous studies in our laboratory (7) have shown that secretory IgA antibody responses (but not IgG antibody responses) to RSV are greater in infants 6 months or more of age at the time of primary RSV infection than in younger infants. The latter observations therefore also suggest that IgG, and not IgA, is the principal mediator of the ADCC response in secretions.

The role of ADCC in recovery from RSV infection remains uncertain. RSV is almost exclusively a cell-associated virus (9), and it has been shown that RSV-infected airway epithelial cells are coated with IgG, IgM, and IgA antibody to RSV (11). Lymphocytes were observed in reasonable quantity in methylene blue-stained smears of secretions obtained from patients in this study (data not shown). Therefore, it seems logical to assume that lysis of antigen-bearing cells by means of ADCC might be an important mechanism in recovery from infection. RSV is apparently a poor inducer of interferon (4), which induces natural killer cell activity after infection (3). This may suggest that ADCC is a comparatively more important defense mechanism in RSV infection.

It is known that repeated infections with RSV result in progressively milder forms of respiratory illness (5). Although the exact mechanism by which this immunity is acquired remains undefined, previous studies indicate that secretory antibody responses to RSV in IgA, IgM, and IgG immunoglobulin classes are enhanced and more persistent after repeated RSV infection (7, 18), and the present study indicates that ADCC responsiveness also increases with repeated infection. The results of these studies may suggest a role for RSV antibody in NPS in modifying the severity of illness upon RSV infection. RSV-IgA antibody, being the predominant isotype in secretions after infection (7), presumably functions most importantly by neutralization of virus, whereas RSV-IgG antibody in the respiratory tract may function by mediating ADCC.

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