Appearance of Complement Components and Immunoglobulins on Nasopharyngeal Epithelial Cells Following Naturally Acquired Infection With Respiratory Syncytial Virus

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Nasopharyngeal epithelial cells (NPEC) were collected from 144 infants and children with respiratory syncytial virus (RSV) infection, and were analyzed by fluorescent antibody techniques for the presence of cell-bound complement (C'_3), IgA, IgG, and IgM class of immunoglobulins (Ig), and respiratory syncytial virus antigen. Viral antigen was present on the surface of NPEC in 100% of samples obtained in the first 3 days of illness. The percentage of patients positive for RSV antigen declined steadily, so that no patient still expressed viral antigen on NPEC by 57 days after the onset of illness. Cell-bound IgA, IgG, and IgM could be detected in most of the samples tested in the first 13 days after the onset of illness. Subsequently, the frequency of detection of cell-bound Ig gradually declined. Only 8–33% of patients tested 57 days after the onset of illness expressed IgA, IgG, or IgM on NPEC. About 45% of samples tested in the first 8 weeks after the onset of illness exhibited complement binding to NPEC. The percentage of subjects showing cell-bound C'_3 reached a maximum at 8–13 says after the onset of illness, while cell-bound C'_3 could not be detected in any of the samples collected 57–90 days after the onset of illness. Although cell-bound C'_3 was generally present in association with cell-bound Ig on NPEC, in a small percentage (4.6%) of patients cell-bound C'_3 could be detected in the absence of any cell-bound Ig. Cell-bound C'_3 and IgA, IgG, and IgM were present with equal frequency in patients with all forms of clinical disease caused by RSV, and in patients less than or greater than 6 months of age at the onset of illness.

INTRODUCTION

The deposition of IgG, IgA, IgM, and IgE class of immunoglobulins on nasopharyngeal epithelial cells has been observed in patients with acute respiratory syncytial virus (RSV) infection [Gardner and McQuillin, 1978, McIntosh et al, 1979, Welliver et al, 1980]. These immunoglobulins appear to bind specifically to RSV antigen on the surface of these cells. Such binding of immunoglobulins to the antigen-bearing cells could potentially play a role in the eradication of infecting virus by complement-mediated cytolysis or by antibody-dependent cell-mediated cytotoxicity.

Activation of complement components has been demonstrated in vitro by cells infected with several different viruses [Grewal et al, 1980, Hirsh et al, 1980a, Okada et al, 1979]. Activation of complement components can lead to neutralization and or lysis of intact virion [Cooper et al, 1976, Mills and Cooper, 1978]. The present studies were undertaken to learn whether deposition of immunoglobulins on respiratory epithelial cells is associated with the deposition of complement as well. Moreover an attempt was made to learn if deposition of complement or immunoglobulins on NPEC is significantly different in patients with various forms of illness caused by RSV infection.

MATERIALS AND METHODS
Study Group

The study group consisted of 144 infants and children less than 2 years of age, who had documented infection with RSV. All these patients are enrolled in an ongoing study of respiratory diseases in childhood. Twenty-four patients were diagnosed as having upper respiratory tract illness only, 28 patients as having pneumonia without wheezing, and 92 patients as having bronchiolitis or asthma on the basis of criteria previously described [Welliver et al, 1979]. The aims and objectives of the study were explained to the parents of the patients, and a signed statement of informed consent was obtained at the time of enrollment. RSV infection was documented by identification of viral antigen on NPEC by indirect immunofluorescence [Kaul et al, 1978], and by isolation of virus in HEp_2 tissue culture monolayers [Lennette and Schmidt, 1969].

Detection of RSV Antigen on Nasopharyngeal Epithelial Cells

Samples of NPS were obtained directly by suctioning into a polyethylene catheter, were rinsed into mucous traps using Hanks balanced salt solution (HBSS), and were transported on ice to the laboratory. One hundred and ninety-two NPS samples were collected sequentially from the patient population up to 90 days after the onset of illness caused by RSV infection. The samples were immediately centrifuged at 4°C, the epithelial sediment was washed three times with PBS, and the cells were spotted on microscope slides. The slides were air dried and later fixed in acetone at 4°C for 10 min. The slides were stained for RSV antigen by the method previously described [Kaul et al, 1978]. Briefly, the slides were incubated with diluted bovine RSV antiserum (Burroughs Welcome, Beckenham, England) at 37°C for 30 min. The slides were washed and later incubated with fluorescein-labeled rabbit antibovine serum (Burroughs Welcome, Beckenham, England). The slides were washed and examined under a fluorescence microscope.

Detection of Cell-Bound Immunoglobulins

Various cell-bound immunoglobulins were detected on RSV-infected respiratory epithelial cells by the method previously described [Gardner and McQuillan, 1978, Welliver et al, 1980]. Briefly, NPEC fixed on glass slides were incubated with appropriately diluted (1:5) bovine anti-RSV serum (Burroughs Welcome, Beckenham, England) at 37°C for 30 min. The slides were washed and subsequently incubated at 37°C for 30 min with a mixture of rhodamine-labeled rabbit antibovine serum (Capell Laboratories, Cochranville, PA) and fluorescein-labeled monospecific goat antihuman IgG, IgM, IgA (Meloy Laboratories, Springfield, VA). The slides were washed and examined with two interference filter systems which allowed rhodamine-stained viral antigen and fluorescein-stained immunoglobulins to be detected independently [Welliver et al, 1980].

Detection of Cell-Bound C′$_3$

Cell bound C′$_3$ was detected on the RSV-infected nasopharyngeal cells by the method described previously. Briefly, NPEC fixed on glass slides were incubated with appropriately diluted bovine anti-RSV serum at 37°C for 30 min. The slides were washed with PBS and were incubated at 37°C for 30 min with a mixture of rhodamine-labeled rabbit antibovine serum and fluorescein-labeled antihuman C′$_3$ (Meloy Laboratories, Springfield, VA). The slides were washed and examined under a fluorescence microscope as described previously.

Various controls and appropriate blocking experiments were employed in the study to rule out nonspecific fluorescence. RSV-infected HEp-2 tissue culture cells failed to show fluorescence when incubated with conjugated antisera to human globulins. Similarly NPEC from patients with infections due to agents other than RSV failed to show RSV antigen when tested under the present system. These cells from patients with other viral infections did show the presence of cell-bound immunoglobulins in some cases, as would be expected. However, the few samples tested failed to show fixation of C′$_3$.

RESULTS

Appearance of Viral Antigen and Fixation of C′$_3$ on NPEC

The frequency of expression of viral antigen and deposition of C′$_3$ on the surface on NPEC at different intervals after the onset of RSV illness is shown in Figure 1. Viral antigen was present on the surface on NPEC in 100% (16/16) of samples obtained in the first 3 days of illness. The percentage of patients positive for RSV declined steadily so that no patients still expressed viral antigen in NPEC by 57 days after the onset of illness. Cell-bound C′$_3$ was detected in 58–77% of patients tested in first 13 days after the onset of illness. The percentage of patients positive for cell-bound C′$_3$ fell steadily thereafter, so that all patients were negative for cell-bound C′$_3$ by 57 days after the onset of illness.

Appearance of Various Immunoglobulins on NPEC

The temporal kinetics of the development of IgA, IgG, and IgM immunoglobulins bound to NPEC is shown in Figure 2. Cell-bound IgA was present in 83.1% (59/71) of samples taken from patients in the first 7 days after the onset of illness. The
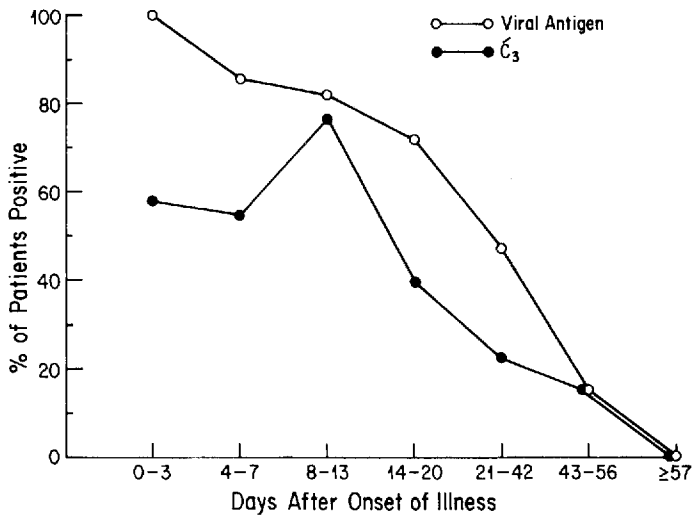
Fig. 1. Kinetics of the development of cell-bound C'_3 and viral antigen in nasopharyngeal epithelial cells at intervals after the onset of illness due to RSV infection.
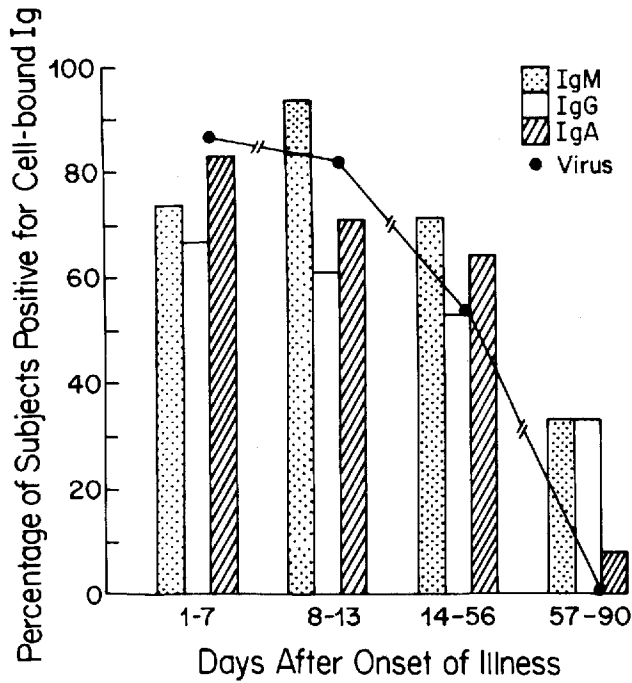
Fig. 2. Percentage of subjects positive for various immunoglobulins in NPEC at various intervals after the onset of illness due to RSV.

number of patients showing cell-bound IgA gradually declined, and by $\geq$ 57 days after the onset of illness, cell-bound IgA could be detected only in 8.3% (1/12) of the samples tested. The percentage of patients in whom cell-bound IgG could be detected varied between 61.3 and 67.1% in the first 13 days after the onset of illness. Eight weeks or more after the onset of illness 33.3% (4/12) patients had IgG bound to NPEC. Roughly 73% of the patients tested in the first 7 days after the onset of illness had cell-bound IgM on NPEC. The percentage of patients positive for IgM peaked (93.5%) 8-13 days after the onset of illness. The number of subjects having cell-bound IgM gradually declined, and by 57 days after the onset of illness, cell-bound IgM was detected in only 33% (4/12) of the samples tested. In the early phase of the infection only a small percentage of the RSV antigen-bearing cells had immunoglobulins attached. The number of RSV antigen-positive cells having fixed immunoglobulins increased gradually over the study period.

Relationship of Form of Illness to Appearance of Cell-Bound Immunoglobulins

The percentage of subjects showing various cell-bound immunoglobulins is analyzed by the form of clinical illness in Figure 3. Results obtained 1-7 days and 14-56 days after the onset of illness were pooled to represent the acute and convalescent phases. Cell-bound IgA was present in 55.6% (5/9) of the patients with URI in the first 7 days after the onset of illness, and in 83-89% of the patients with pneumonia and bronchiolitis in the first 7 days after the onset of illness. However, these differences were not statistically significant. Similarly, the differences observed in the frequency of appearance of NPEC-bound IgG or IgM in various clinical forms of the disease were not significant.

Relationship of Patient Age to Appearance of Cell-Bound Immunoglobulins

The correlation of cell-bound antibody classes to the age of the patients at the time of onset of the illness is shown in Figure 4. The data were again pooled into 1-7 days, and 14-56 days after the onset of illness. The appearance of cell-bound IgA, IgG and IgM isotypes were consistently greater in patients more than 6 months of age than in patients who were less than 6 months of age at the time of infection with RSV. However, these differences were not statistically significant.

Relationship of Appearance of Cell-Bound C'_3 to Form of Clinical Illness

The relationship of the appearance of cell bound C'_3 to the form of clinical disease observed is shown in Figure 5. Cell-bound C'_3 could be detected in 55% (11/20) of the patients with bronchiolitis in the first 5 days after the onset of illness. From 6 to 13 days after the onset of illness 80% (24/30) patients with bronchiolitis had cell-bound C'_3 on NPEC. In contrast, 50% or less of the patients with URI or pneumonia had cell-bound C'_3 present in NPEC in the first 13 days after the onset of illness. The frequency of appearance of cell-bound C'_3 in various forms of clinical disease was similar 31 days after the onset of RSV illness and did not differ in a statistically significant fashion among patients in the various illness groups at any point in the course of illness.
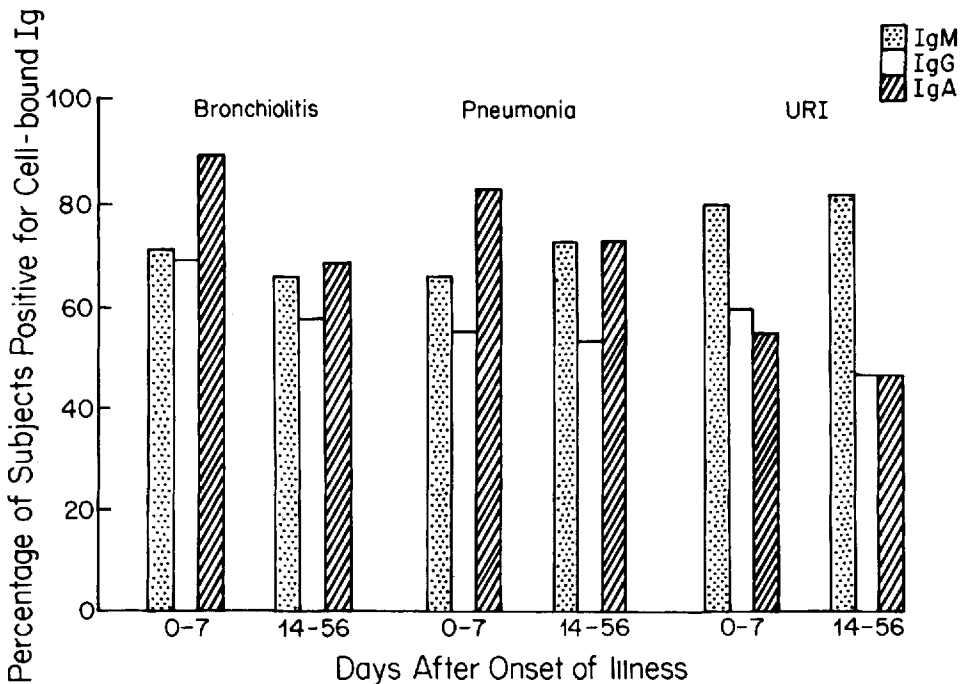
Fig. 3. Appearance of immunoglobulins on NPEC from patients with various forms of illness due to RSV.

Correlation of Presence of Cell-Bound C′₃ with that of Various Immunoglobulins and of Viral Antigen in NPEC

The correlation of the appearance of cell-bound C'_3 to that of various cell bound immunoglobulin classes and presence of viral antigen is shown in Table I. Cell-bound C'_3 was detected in association with presence of viral antigen in 87.7% of patients in first 8 weeks after the onset of RSV illness. Cell-bound C'_3 was most commonly (85.5%) associated with the presence of cell-bound IgM on NPEC, whereas IgG was simultaneously detected in 61.3% and IgA in 80.3% of the samples tested. Cell-associated C'_3 was usually present simultaneously with one of the immunoglobulins in NPEC, but in a small percentage (4.6%) of patients cell-bound C'_3 was detected in the absence of any cell-bound immunoglobulins. Moreover, in 4.6% (3/61) of patients cell-bound C'_3 was detected in association with IgA only, an immunoglobulin isotype which does not activate complement components by the classical pathway.

DISCUSSION

Presence of cell-bound IgA, IgG and IgM isotypes have been observed on nasopharyngeal epithelial cells of patients with acute RSV disease [Gardner and McQuillin, 1978, McIntosh et al, 1979]. In the present study no significant differences were observed in the relative frequency of the deposition of various cell-bound immunoglobulins in various forms of clinical disease caused by RSV, or in various age
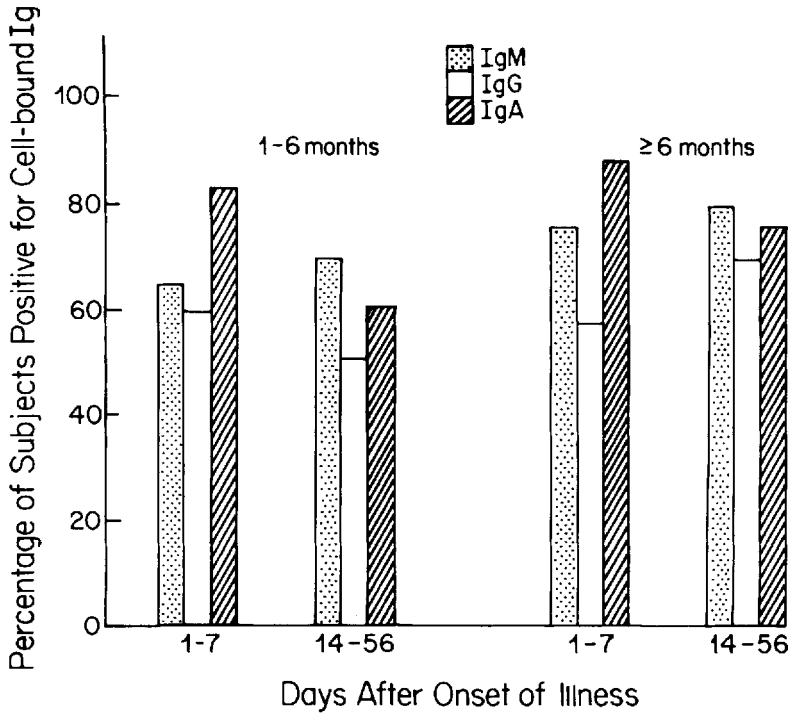
Fig. 4. Percentage of patients positive for various cell-bound immunoglobulins who were <6 or ≥6 months at the onset of illness due to RSV.
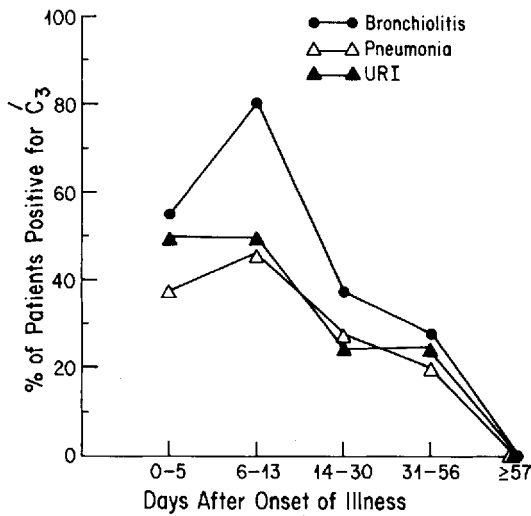

Fig. 5. Appearance of cell-bound C'_3 on nasopharyngeal epithelial cells in various forms of clinical diseases. (●——●), Bronchiolitis and asthma; (△——△), pneumonia; (▲——▲), upper respiratory tract infection.

TABLE I. Correlation of Various Cell-Bound Ig Classes to Cell-Bound C'7 and Viral Shedding in NPEC

NPEC positive for	Percentage of NPEC specimens simultaneously positive for				
	RSV	C'$_3$	IgM	IgG	IgA
RSV	x	56.3%	81.6%	67.2%	79.7%
C'$_3$	87.7%	x	85.5%	61.3%	80.3%
IgM	76.1%	54.4%	x	70.1%	78.9%
IgG	81.9%	53.5%	90.7%	x	85.6%
IgA	79.2%	50.5%	83.6%	71.1%	x

groups at the time of infection with RSV. However, in a previous study from our laboratory [Kaul et al, 1981b] we have reported that cell-free secretory-IgA levels are significantly higher in patients more than 6 months of age than in patients less than 6 months of age at the time of infection with RSV. These differences can be explained on the basis that the technique employed in the present study measures either the presence or absence of cell-bound immunoglobulins on the nasopharyngeal epithelial cells, and cannot measure the level of antibody free in secretions after RSV infection.

Based on the present evidence we cannot conclusively say that the immunoglobulins on NPEC are in fact RSV specific. However, immunoglobulin could be detected essentially only on RSV-infected cells, as determined by double-labeling techniques. Immunofluorescence was blocked if slides were preincubated with RSV antigen. Moreover, to our knowledge, Fc receptors on RSV-infected cells have not been described. Based on this evidence we presume that the various immunoglobulin classes detected on NPEC represent RSV-specific antibody.

It should be noted that samples of NPEC from patients infected with viruses other than RSV did, as one would expect, show the presence of cell-bound immunoglobulins on surfaces of a few cells. These immunoglobulins were presumably directed agains antigens of the virus responsible for the infection. In the small number of samples tested, no cell-bound C'$_3$ could be detected. The absence of C'$_3$ might have been due to obtaining samples very early in the illness before C'$_3$ was fixed, or to the possibility that RSV-infected cells may fix C'$_3$ more avidly than cells infected with other viral agents. Finally, a larger number of samples might have revealed the presence of C'$_3$ on NPEC from patients with non-RSV infection.

The protective role of cell-associated antibody can be presumed from in vitro studies with influenza virus and its specific antibody [Dowdle et al, 1974]. It has been shown that antihemagglutinin and antineuraminidase antibodies were equally effective in reducing the influenza virus titers in culture fluids. In addition both antisera prevented in vitro spread of the influenza virus. The binding of immunoglobulins to the antigen-bearing cells could play an important role in cytolysis of infected cells and elimination of viral antigen by the mechanism of antibody-dependent cell-mediated cytotoxicity. Alternatively antigen-antibody complexes could activate complement pathways and lead to cytolysis of the antigen-bearing cells.

Secretory antibody is believed to play a significant role in recovery from RSV infection [Chanock et al, 1970]. However, the development of local antibody in the respiratory tract may not be sufficient condition for neutralization of RSV, since RSV can be isolated from respiratory secretions in the presence of cell-free antibody

(McIntosh et al, 1978]. Fixation of complement either by cell-free or cell-bound antibody may be an important factor for neutralization of RSV in the respiratory tract, since addition of complement to in vitro neutralization assays enhances the capacity of the serum antibody to neutralize RSV [Kaul et al, 1981a].

Limited histopathological studies have failed to reveal the deposition of complement in the lung parenchyma in RSV infection [Chanock et al, 1970, Gardner et al, 1970]. However, in the present study deposition of complement on nasopharyngeal epithelial cells was demonstrated in about 46% of the samples tested in the first 8 weeks after the onset of RSV illness. Cell-bound C'_3 was usually present simultaneously with one of the immunoglobulins on NPEC, but in a small percentage (4.6%) of patients cell-bound C'_3 was detected in the absence of any cell-bound immunoglobulins. Moreover, in 4.6% of patients, cell-bound C'_3 was detected in association with IgA only, an immunoglobulin isotype which does not activate complement components by classical pathways. These findings suggest in vivo activation of the complement cascade either by antigen-antibody complexes or by RSV alone. In vitro studies by Grewal et al [1980] have shown that infectious bovine rhinotracheitis virus-infected tissue culture cells can activate the alternate complement pathway in the absence of any antiviral antibody. In a similar fashion, Sindibis virus grown in tissue culture cells could activate both classical and alternate pathways of complement in a variety of sera [Hirsh et al, 1980a]. Activation of complement by the alternate pathway can occur in C4-deficient guinea pig sera by Sendai virus-infected tissue culture cells in the absence of any detectable antiviral antibody [Okada et al, 1979]. In earlier studies by Cooper et al [1976] coranaviruses have been shown to activate the classical C' pathway by direct interaction of C1q with cornavirus. The activation of complement in such a system leads to complete lysis of the coronavirus. Similarly, vesicular stomatitis virus can directly attach to C1q in the serum and thus activate the classical complement pathway, which leads to complete neutralization of the virus [Mills and Cooper, 1978.].

The complement system in humans plays an important role in host defense against infection [Frank, 1979]. Various studies with experimental animals have clearly shown the importance of the complement system for host defense against viral infections. Hicks et al [1978] have shown that mice decomplemented with cobra venom factor had prolonged viral infection, and such mice had an increased morbidity and mortality compared to control, nondecomplemented mice. Similarly increased viral growth has been reported in the brains of decomplemented mice after subcutaneous inoculation of Sindbis virus [Hirsh et al, 1980b].

There are a variety of cells in the human body with C_3b receptors, including neutrophils, macrophages, B lymphocytes, and perhaps cytotoxic killer cells [Frank, 1979]. Thus deposition of C'_3 to the RSV-infected cells observed in the present study might be assumed to play a role in reducing the viral antigenic mass especially in the early phase of the infection, when RSV antibody is either absent or present in low titers [McIntosh et al, 1978]. Neutrophils and macrophages can play an important role in the early phase of infection either by phagocytosis or by neutrophil-mediated cytotoxicity. Such complement-dependent neutrophil-mediated cytolysis of herpes virus-infected cells has been demonstrated [Grewal et al, 1980].

In summary both cell-bound secretory antibody and cell-bound complement components may, independently or acting in concert, facilitate clearance of RSV from the respiratory tract of infected patients. Moreover, the fixation of C'_3 and, presumably, subsequent activation of complement cascades does not seem to play a role in the pathogenesis of RSV infection.

ACKNOWLEDGMENTS

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