INFECTION AND IMMUNITY, Feb. 1979, p. 276-281
0019-9567/79/02-0276/06$02.00/0
Vol. 23, No. 2

Cell-Free and Cell-Bound Antibody in Nasal Secretions from Infants with Respiratory Syncytial Virus Infection

KENNETH McINTOSH,* JOYCE McQUILLIN, AND PHILLIP S. GARDNER

Department of Pediatrics, University of Colorado Medical Center, Denver, Colorado, 80220[1] *and the Department of Virology, University of Newcastle-upon-Tyne, Newcastle-upon-Tyne, England*[2]

Received for publication 7 November 1978

Twenty-two infants under 9 months of age hospitalized with bronchiolitis or pneumonia due to respiratory syncytial virus (RSV) were serially sampled to determine the pattern of secretory antibody response. Using double labeling techniques, we found several types of immunoglobulin in secretions: cell-free antibody to RSV of the immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobublin M (IgM) classes; and immunoglobulins of all three classes bound to RSV-infected cells shed from the nasal epithelium (presumably cell-bound antibody to RSV). IgA attached to RSV-infected epithelial cells was almost always detected in the first available nasal sample (day 1 or 2 of hospitalization). In contrast, cell-free anti-RSV IgA first appeared an average of 3.5 days later at a time when virus antigen was disappearing from the secretion. IgG and IgM attached to RSV-infected cells appeared more irregularly. The titer of cell-free anti-RSV IgM was often higher than that of IgA early in the illness and declined as the infection resolved. Cell-free anti-RSV IgG was usually present earlier than IgA and rose during convalescence.

The secretory immunological response to viral respiratory infections in infants is probably related to the process of recovery (6) and to subsequent protection against reinfection (7) and may be involved in the pathogenesis of disease as well (4). We have recently used immunofluorescence techniques to measure the appearance of antibody in the nasal secretions (6) and immunoglobulin attached to respiratory syncytial virus (RSV)-infected nasal epithelial cells (presumably antibody) (3) in infants recovering from bronchiolitis or pneumonia due to RSV. The present communication describes the appearance of both cell-associated and cell-free nasal secretion antibody in a single group of infants hospitalized with RSV lower respiratory tract disease at the Royal Victoria Infirmary, Newcastle-upon-Tyne, England.

MATERIALS AND METHODS

Patients. Twenty-two infants under 9 months of age, admitted with bronchiolitis or pneumonia to the pediatric wards of the Royal Victoria Infirmary and found by rapid diagnostic techniques (2) to be shedding RSV, were included in the study. After informed consent had been obtained, nasopharyngeal secretions were collected daily or every other day by mechanical suction. Some infants were hospitalized for 1 week or more. Others returned 3 to 7 days after discharge for collection of a convalescent secretory specimen.

Nasopharyngeal samples. A 0.2- to 1.0-ml amount of nasopharyngeal secretion (NPS) was obtained and transported on ice to the laboratory. The samples were processed 10 to 40 min after collection. From a small portion, replicate slides were prepared for rapid immunofluorescence diagnosis (2). In brief, cellular elements were washed by centrifugation, spotted in 8-mm squares on microscope slides, dried, and fixed for 10 min in acetone at 4°C. They were then stained by the indirect method with rabbit anti-RSV serum and fluorescein-conjugated anti-rabbit globulin (Burroughs Wellcome, Beckenham, Kent). The remainder of the secretion was frozen at −40°C. Initial samples were, in addition, cultured in HEp-2, rhesus monkey kidney, and human fetal lung fibroblasts. In all, 99 secretions were collected from the 22 infants (mean, 4.5 per infant). From nine samples, only replicate slides were prepared. Only 6 of the remaining 90 samples contained visible blood; immunoglobulin and antibody levels in these were not appreciably different from those of comparable specimens not containing blood, and they were included in the analysis.

Frozen NPS were subsequently thawed, diluted with an equal volume of phosphate-buffered saline, shaken for 1 h at 4°C with glass beads, and centrifuged at 3,500 rpm for 2 h. The clear or slightly cloudy cell-free supernatant was then removed, divided in two aliquots, and stored at −40°C until testing.

Measurement of cell-free antibody. Immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) anti-RSV antibody was measured by a modification of the membrane antibody test of Scott et al. (8). Mycoplasma-free HEp-2 cells were grown in glass bottles and infected with the Long strain of RSV. After 2 to 4 days, infected and control monolayers were dispersed with a mixture of equal

parts of 0.25% trypsin and 1:5,000 ethylenediaminetetraacetic acid, and then washed three times by low-speed centrifugation. Cells were finally suspended in Hanks balanced salt solution containing 2% fetal calf serum and 0.1% sodium azide (Hanks-serum-azide). At the same time, serial twofold dilutions (in Hanks-serum-azide) of thawed NPS specimens (15 μl) were prepared in V-bottom microtiter plates. An equal volume of cell suspension (adjusted to yield about 8×10^4 cells per well) was added, and the mixture was incubated for 1 h at room temperature. The cells were washed three times by centrifugation in Hanks-serum-azide. A 15-μl amount of the appropriate monospecific fluoresceinated conjugate prepared against the heavy chains of IgG, IgA, or IgM (Burroughs Wellcome, tested for immunoglobulin class-specificity by immunoelectrophoresis) was added to the washed cell button and incubated at room temperature for 30 min. Three further washes in Hanks-serum-azide were performed, and the button was finally resuspended in 10 μl of washing fluid and placed on a slide. A cover slip was then placed on top, and the cells were examined with a 40× oil-immersion objective under incident light with a Vickers M41 fluorescence microscope. Typical membrane ring-fluorescence was graded from 1+ to 4+, and the titer of antibody was taken as the highest dilution showing a 1+ reaction. Uninfected cells demonstrated membrane fluorescence in three infants with IgG or IgM conjugates, and these were omitted from analysis. All readings were performed in random order and "blind" by a single observer.

Measurement of immunoglobulin bound to RSV-infected epithelial cells. The measurement of IgA, IgG, and IgM bound to RSV-infected epithelial cells in secretions has been previously described (3). In brief, washed epithelial cells from each nasal secretion specimen were spotted on slides and fixed as described above. All samples containing RSV antigen by preliminary immunofluorescence screening were further studied. To detect cell-bound immunoglobulins, acetone-fixed cell smears were incubated first with rabbit anti-RSV serum for 30 min at 37°C and then washed. Appropriate dilutions of rhodamine-conjugated goat anti-rabbit globulin (Nordic Immunological Laboratories) and mono-specific fluorescein-conjugated sheep anti-human IgA, IgG, or IgM were mixed and then incubated overnight at 4°C on individual spots. The slides were subsequently washed again, dried, and examined with two interference filter systems which allowed rhodamine- and fluorescein-stained cells to be detected independently. In this way, immunoglobulin attached to RSV-infected cells could be assessed. At least four infected cells were required in each square for double staining evaluation. All the conjugates used had been previously shown to lack antibody to RSV by the absence of fluorescence on infected tissue culture cells incubated with conjugate alone.

Total secretion immunoglobulins. Total IgA and IgG were measured on Oxford low-level plates (Searle Diagnostics) by using serum IgG and IgA standards supplied with the kits. Total IgM was not measured.

RESULTS

Appearance of cell-free antibody in NPS. Anti-RSV IgA was universally absent from cell-free secretions obtained on the first 2 days of hospitalization. Subsequently, such antibody was detected (at a dilution of 1:2 or greater) in 21 of the 22 infants (Fig. 1). The one child who

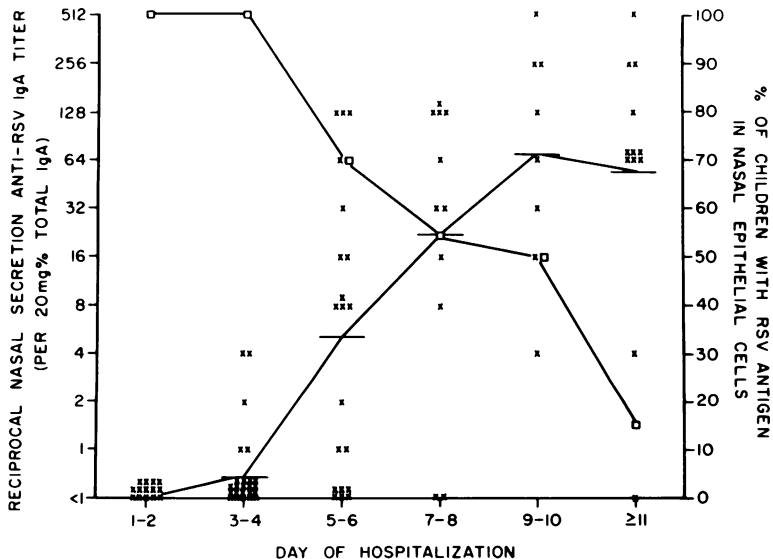

FIG. 1. *Appearance of cell-free anti-RSV IgA during hospitalization. Data from all 22 infants with RSV infection are shown. Individual titers (×) are shown as well as the geometric mean titer (———). Also shown is the percentage of infants for each 2-day interval with RSV-containing nasal epithelial cells (□).*

did not demonstrate a rise in anti-RSV IgA was followed for only 4 days in the hospital, and a specimen during convalescence was not obtained. The geometric mean (GM) antibody titer (per 20 mg of total IgA per 100 ml) began to rise on day 3 or 4 of hospitalization and reached a peak on day 9 or 10. The reduction in fluorescence-stainable RSV antigen in shed nasal epithelial cells mirrored the GM anti-RSV IgA titer (Fig. 1). In the majority of instances (14 of 22) free anti-RSV IgA appeared for the first time before RSV antigen disappeared. In the remainder, anti-RSV IgA was detected on the first day the shed cells were negative for viral antigen.

Anti-RSV IgG in cell-free secretions was often present, although at low titer, on the first 2 days of hospitalization (Fig. 2). This antibody also rose as the disease progressed and was universally present by day 5 or 6. A fourfold or greater rise in anti-RSV IgG in NPS specimens (per 10 mg of total IgG per 100 ml) was measured in 20 of the 21 infants in whose specimens control staining did not interfere with the reading.

Anti-RSV IgM was measurable at a dilution of 1:2 or greater in 16 of the 19 infants with satisfactory control readings. There was a tendency for IgM antibody in secretions to rise earlier than IgA, and this is shown in Fig. 3, where the ratio of IgA to IgM antibody in individual secretions is plotted. Moreover, in five infants the titer of IgM antibody appeared to rise and then fall whereas IgA and IgG rose and remained high.

Total IgA and IgG in cell-free secretions. The total immunoglobulin concentration in secretions (diluted 1:2, as described in Materials and Methods) is shown in relation to the progress of hospitalization in Fig. 4. Mean total IgG concentration did not vary significantly from that at admission (when secretions were usually copious) to convalescence (when secretions were often scanty). Mean total IgA concentration, on the other hand, was significantly higher ($P < 0.01$, two-tailed t test) during the first 8 days of hospitalization than later. The apparent rise in IgA concentration on day 7 or 8 is not statistically significant.

Cell-associated immunoglobulin in secretions and its relation to cell-free antibody. Fluorescence-stainable IgA was usually seen (19 of 22) coating at least 25% of RSV-containing nasal epithelial cells on the first specimen available (Fig. 5). In the three infants where it was not seen in the first available NPS, it appeared in the next. The average day of hospitalization during which IgA was seen bound to infected cells was 2.3, in contrast to that during which cell-free antibody first appeared, which was 3.5 days later.

In serial specimens, the percentage of RSV antigen-containing cells which were coated with IgA usually increased, often reaching 100% before infected cells disappeared entirely from the secretion. The most common pattern was an increase in the proportion of cells coated with immunoglobulin from 25 to 100% over the course of 3 to 5 days, with a concomitant decrease in the total number of virus-infected cells. Acute

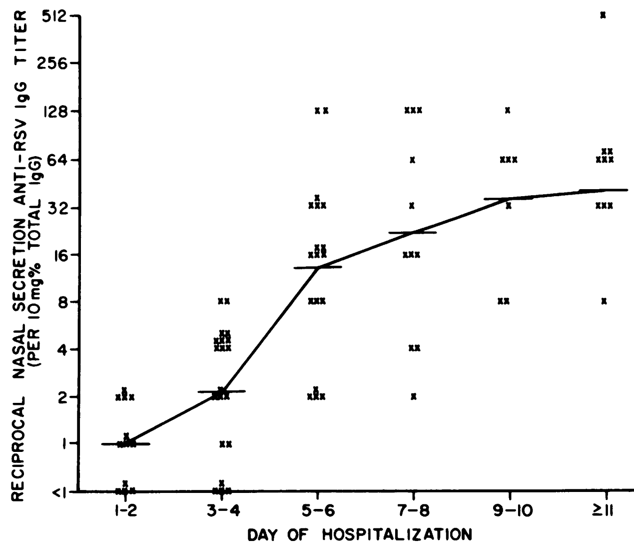

FIG. 2. *Appearance of cell-free anti-RSV IgG during hospitalization. Data from 21 infants with RSV infection are shown. Individual titers (×) and the geometric mean titer (———) are shown.*

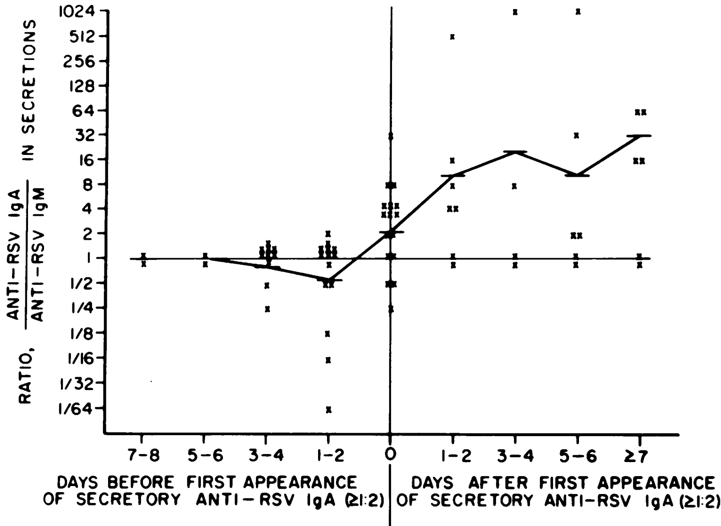

FIG. 3. *Ratio of cell-free anti-RSV IgA to anti-RSV IgM in individual secretions from 20 infants with RSV infection. Individual titers (×) and geometric mean titer (———) are shown. Timing is in relation to the appearance of cell-free anti-RSV IgA.*

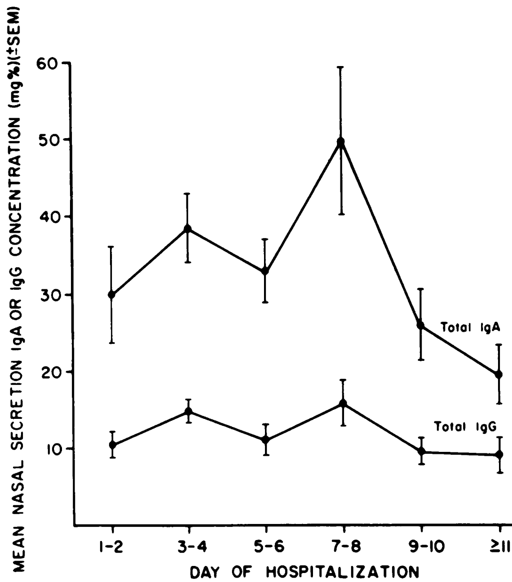

FIG. 4. *Total IgA or IgG concentration (milligrams per 100 ml) in nasal secretions of 22 infants with RSV infection (mean ± standard error of the mean [SEM]).*

samples contained as many as 30 infected cells per microscopic field, while convalescent samples often had as few as 3 to 4 cells per square. Cell-free anti-RSV IgA emerged either as soon as or immediately after 100% of the infected cells were coated with IgA.

IgG, on the other hand, rarely was present on cells in the first available specimen (3 of 22), and it did not appear at all (on 25% or more of cells) in nine infants. This was in marked contrast to cell-free anti-RSV IgG, which was universally present at some point in the course of disease or recovery. Likewise, in those infants in whom it was found, IgG appeared on cells later than it did free in the secretion (Fig. 6).

IgM appeared on infected cells somewhat later than IgA, but earlier and more consistently than IgG. Likewise, it preceded the appearance of

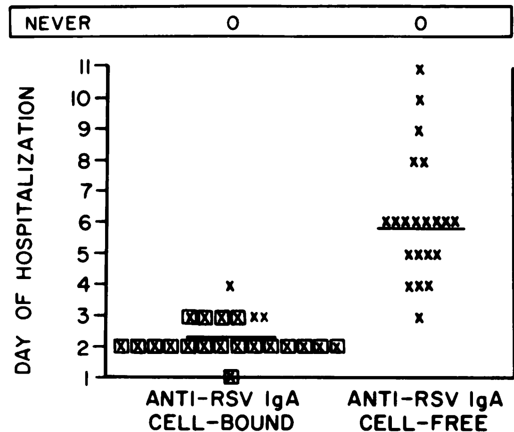

FIG. 5. *First appearance of anti-RSV IgA in nasal secretions. ×, The earliest nasal secretion in which immunoglobulin attached to infected cells (>25% of RSV-infected nasal epithelial cells) or cell-free antibody (≥1:2) was found. ⊠, An infant in whom antibody was found in the first available secretion. ———, The mean hospital day of first appearance of antibody.*

cell-free anti-RSV IgM, but only by an average of 1.5 days (Fig. 7).

Immunoglobulins were never seen coating epithelial cells not infected with RSV; that is, sticking non-specifically in amounts sufficient to detect by this method. Occasional very small uninfected cells were observed which stained with anti-human globulin. These were presumably plasma cells or B lymphocytes and were easily distinguished from epithelial cells by size and morphology.

DISCUSSION

Until recently, studies of the secretory immunological response in RSV infection have depended on measurement of antibody by the neutralization test (3, 9, 10). We showed, in a previous publication, that in secretions there was no correlation between antibody, as measured by indirect fluorescence, and neutralizing activity (6). In that study, only 60% of infants under 6 months of age responded to natural infection with a rise in secretion IgA antibody to RSV. Total secretory IgA levels were, however, low, owing to extensive dilution during preparation and storage of samples. In the present study 21 of 22 infants developed cell-free anti-RSV IgA, and it is likely that the one infant who failed to respond was simply not followed long enough. The mean total IgA concentration in our samples (diluted only by 1:2) was, however, high (35.1 mg/100 ml, using a serum IgA standard, which would distort the measurements toward a falsely low level).

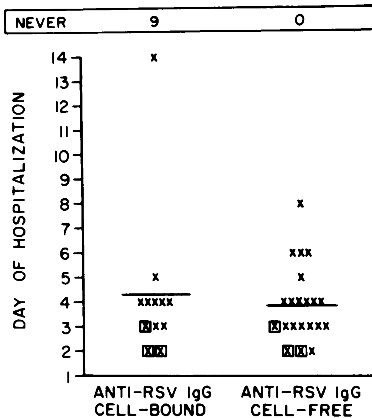

FIG. 6. First appearance of anti-RSV IgG in nasal secretions. Symbols are the same as in Fig. 5. In nine infants, IgG attached to infected epithelial cells never appeared.

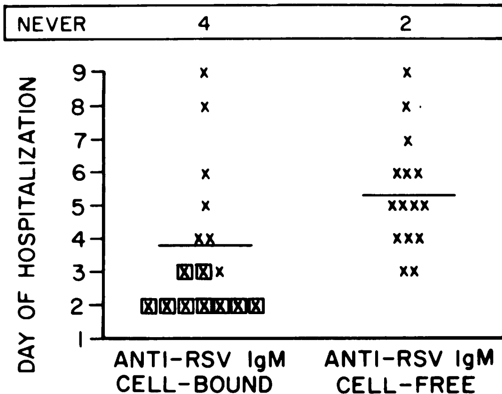

FIG. 7. First appearance of anti-RSV IgM in nasal secretions. Symbols are the same as in Fig. 5. IgM attached to infected epithelial cells was never detected in four infants. Cell-free antibody did not appear in two infants.

The most significant finding was the presence of IgA coating RSV-containing nasopharyngeal epithelial cells an average of 3.5 days before anti-RSV IgA appeared free in the secretions. IgM was also found to appear in much the same way. It seems likely that antibody, present at the mucosal surface, is first avidly absorbed by the large number of antigen-coated epithelial cells lining that surface and that it does not appear in the cell-free secretion until all available cell surface sites have been occupied and there is surplus antibody available. It is not clear, however, why early in the infection, only a small proportion of antigen-bearing cells should have immunoglobulin attached. Presumably, antibody is distributed so locally that some of the shed cells are not exposed. An alternative explanation is that some virus-containing cells are located beneath the epithelial surface and are not exposed to antibody in situ.

We feel that the immunoglobulins seen on infected nasal epithelial cells represented antibody to RSV antigens expressed on the cell surface for the following reasons. First, immunoglobulins were seen only on epithelial cells which contained RSV antigen as determined by rhodamine staining. Second, Fc receptors on the surface of RSV-infected cells have never been described. Indeed, these investigations provide evidence that they are not detectable by the technique we used: surface fluorescence on infected HEp-2 cells was totally absent when they were incubated with acute secretions. Third, our own studies of immunosuppressed leukemic children excreting RSV over prolonged periods have demonstrated an absence of immunoglobulin coating of infected nasal epithelial cells despite the presence of other immunoglobulins in nasal secretions.

The possible function of this cell-associated immunoglobulin is a matter for speculation. Presumably, immunoglobulin could function to neu-

tralize virus after it had budded from the cell surface. An alternative theory, more attractive because of the nonneutralizing nature of much of the antibody in our previous study (6), is that it functions to prevent the successful maturation of virus at the cell surface and thus allows the infected cell to be shed before progeny virus is released. Such a mechanism has been demonstrated in vitro for anti-neuraminidase and anti-hemagglutinin (1) antibody to influenza viruses.

More difficult to explain were our findings for IgG. Cell-free anti-RSV IgG was more prominent in early secretions than was IgA. Moreover, we often detected cell-free IgG antibody when there was no evidence for its cell-bound counterpart. We have no entirely satisfactory explanation for this. It is possible that it was purely an artifact of our test—that the membrane fluorescence test for anti-RSV IgG was extraordinarily sensitive and detected minute quantities of immunoglobulin which were not visible on cells. This seems unlikely because all tests depended on fluorescence and the same reagents at similar dilutions were used for both on- and off-cell antibody. Second, it is possible that the sampling procedure somehow induced the release of anti-viral IgG, presumably through trauma to the mucosa and weeping of serum antibody into the secretions. If this were true, it would be difficult to explain why antigen-bearing epithelial cells were not coated with antibody during the 30 to 60 min that the secretion was being transported and processed. High viscosity with absence of mixing might have impeded this reaction. Serum antibody measurements would have assisted in the solution of this problem, but ethical considerations prohibited frequent venous bleeding of these small infants. Finally, it is possible that anti-viral IgA selectively attached to infected nasal epithelial cells and thereby blocked attachment of IgG.

The development in many infants of cell-free secretion IgM before IgA and at higher titer early in the illness has not been described before. Once again, however, IgM was not seen attached to infected epithelial cells earlier than IgA, and this observation therefore remains unexplained.

Finally, the presence of immunoglobulin, presumably antibody, often of several classes, on virus-infected cells during much of the acute illness, and, in particular, the phase of disease requiring hospitalization for bronchiolitis or pneumonia, implies the possibility that mucosal antigen-antibody complexes might play some role in the pathogenesis of disease as well as in recovery. To our knowledge, immune complex disease on a mucosal surface has not been described, but this point requires further exploration.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service contract NO1-AI-22501 from the National Institute of Allergy and Infectious Diseases, and by Fogarty International Fellowship 1 F06 TW00098-01.

LITERATURE CITED

1. **Dowdle, W. R., J. C. Downie, and A. G. Laver.** 1974. Inhibition of virus release by antibodies to surface antigens of influenza viruses. J. Virol. **13**:269-275.
2. **Gardner, P. S., and J. McQuillin.** 1974. Rapid virus diagnosis: application of immunofluorescence. Butterworth, London.
3. **Gardner, P. S., and J. McQuillin.** 1978. The coating of respiratory syncytial (RS) virus-infected cells in the respiratory tract by immunoglobulins. J. Med. Virol. **2**: 165-173.
4. **Gardner, P. S., J. McQuillin, and S. D. M. Court.** 1970. Speculation on pathogenesis in death from respiratory syncytial virus infection. Br. Med. J. **1**:327-330.
5. **Kim, H. W., J. A. Bellanti, J. O. Arrobio, J. Mills, C. D. Brandt, R. M. Chanock, and R. H. Parrott.** 1969. Respiratory syncytial virus neutralizing activity in nasal secretions following natural infection. Proc. Soc. Exp. Biol. Med. **131**:658-661.
6. **McIntosh, K., H. B. Masters, I. Orr, R. K. Chao, and R. M. Barkin.** 1978. The immunologic response to respiratory syncytial virus infection in infants. J. Infect. Dis. **138**:24-32.
7. **Mills, J., J. E. Vankirk, P. F. Wright, and R. M. Chanock.** 1971. Experimental respiratory syncytial virus infection of adults. J. Immunol. **107**:123-130.
8. **Scott, R., M. O. DeLandazuri, P. S. Gardner, and J. J. T. Owen.** 1976. Detection of antibody to respiratory syncytial virus by membrane fluorescence. Clin. Exp. Immunol. **26**:78-85.
9. **Scott, R., and P. S. Gardner.** 1970. Respiratory syncytial virus neutralizing activity in nasopharyngeal secretions. J. Hyg. Camb. **68**:581-588.
10. **Scott, R., and P. S. Gardner.** 1974. The local antibody response to R.S. virus infection in the respiratory tract. J. Hyg. Camb. **72**:111-120.