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Activation of Complement by Cells Infected with Respiratory Syncytial Virus

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The ability of respiratory syncytial virus (RSV)-infected HEp-2 cells in culture to activate complement was investigated. After incubation of cells with various complement sources and buffer, binding of C3b to surfaces of infected cells was demonstrated by immunofluorescence with a double-staining technique. Nonsyncytial and syncytial (i.e., fused, multinucleated) cells were separately enumerated. Also, lysis of RSV-infected cells was assessed by lactic dehydrogenase release. In this system only RSV-infected cells stained for C3b, and they did so only after incubation with functionally active complement. Blocking of classical pathway activation with ethylenediaminetetraacetic acid diminished the number of infected nonsyncytial cells positively stained for C3b, but had no effect on staining of syncytial cells. Blocking of alternative pathway activation with either zymosan incubation or heat treatment decreased the number of both syncytial and nonsyncytial cells stained for C3b. Decreasing immunoglobulin concentration of the serum used as the complement source also decreased numbers of both cell types stained for C3b. Eliminating specific anti-RSV antibody diminished numbers of both cell types stained for C3b, but staining was not eliminated. Lastly, incubation with functionally active complement markedly increased lactic dehydrogenase release from infected cells. This study demonstrated that RSV-infected nonsyncytial and syncytial cells are able to activate complement by both classical and alternative pathways. Activation of complement by syncytial cells appears to be less dependent on the classical pathway than is activation by nonsyncytial cells, and activation by syncytial cells may require immunoglobulin but not specific antibody. These experiments suggest the possibility of complement activation during respiratory tract infection by RSV. Implications of this are discussed.

Respiratory syncytial virus (RSV) is a major cause of respiratory tract infection in children. It has been speculated that the severity of clinical symptoms is increased by immunological mechanisms (17), but specific enhancement of inflammation has not been demonstrated in primary RSV infection. The host response which results in resolution of RSV infection also is not completely understood, although secretory antibody (either immunoglobulin A [IgA] or IgG) has been implicated in diminished viral shedding (11).

The complement system in human serum is able to neutralize or enhance neutralization of certain viruses in vitro by mechanisms involving both classical and alternative pathways (2-5, 9, 13, 15). Complement components are present in respiratory tract secretions (8, 16). Recent work has suggested that complement is important in recovery from viral respiratory tract infection (6), and complement may potentiate immunopathological injury as well as decrease replication in viral infection (7).

These observations suggest the possibility that complement might be important in RSV infection. We investigated the ability of RSV to activate the complement system in vitro by using RSV-infected HEp-2 cells in culture and serum containing hemolytically active complement. Binding of C3b to surfaces of infected cells was demonstrated by immunofluorescence with a double-staining technique that has been used previously to identify antibody attached to infected cells in nasal washings (11). Since RSV infection causes single cells to fuse into multinucleated syncytia, we also addressed the difference in complement activation by nonsyncytial and syncytial cells. Finally, we looked at the influence of complement on lysis of RSV-in-

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fected cells as measured by lactic dehydrogenase (LDH) release.

MATERIALS AND METHODS

Protocol. Mycoplasma-free HEp-2 cells were grown in glass bottles and infected with the Long strain of RSV. After approximately 48 h, infected and control monolayers were dispersed with equal parts of 0.25% trypsin and 0.05 M ethylenediaminetetraacetic acid and then washed three times by centrifuging at 200 × *g*. Cells were finally suspended at a concentration of 10^4 to 10^5 cells per ml in Hanks balanced salt solution containing 2% fetal calf serum and 0.1% sodium azide. They were then aliquoted in 400-μl amounts into glass tubes and incubated with an equal amount of the complement source or buffer for 10 min at 37°C. Afterward, they were centrifuged at 400 × *g* at 4°C for 5 min. The cell pellets were washed three times with phosphate-buffered saline and resuspended, and 10-μl samples were placed on glass slides and allowed to air dry. These were then fixed in acetone at 4°C for 20 min for immunofluorescent staining.

Complement sources. Complement activation was tested by using normal human serum (NHS) which had low levels of IgG, IgA, and IgM anti-RSV antibody by fluorescent antibody staining. Serum from individual donors or pooled serum was used; this source of NHS did not affect the results. These sera contained hemolytic activity in the normal range found in our laboratory (120 to 150 CH_{50} units per ml). This was compared to the results of incubation of NHS and cells with 0.01 M ethylenediaminetetraacetic acid, which prevents the activation of both classical and alternative complement pathways. It also was compared to the results of incubation of cells with serum that had been heated at 56°C for 1 h.

Alternative pathway activity was assessed by the incubation of serum and cells with 0.01 M ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid and 0.01 M magnesium chloride. Classical pathway activity was assessed by the incubation of cells with serum which had been depleted of alternative pathway components by incubation with washed zymosan at 22°C for 30 min. It also was assessed by using NHS which had been heated at 50°C for 15 min. Both of these sera retained at least 75% of their hemolytically active complement as measured by a standard sheep erythrocyte assay (10), but lacked activity in a hemolytic assay of alternative pathway with rabbit erythrocytes and buffer containing ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid and magnesium chloride (14). These maneuvers did not alter serum immunoglobulin content or specific anti-RSV antibody titer.

The role of immunoglobulin was assessed in two ways. First, serum deficient in all immunoglobulin classes was incubated with cells. This serum was prepared by passing hypogammaglobulinemic serum that had no detectable IgA or IgM over a column of Sepharose CL-4B (Pharmacia) to which staphylococcal protein A had been coupled by cyanogen bromide activation. This reduced the concentration of IgG to 107 mg/dl as measured in a nephelometric assay; it had an IgG anti-RSV FA titer of 1:8. A second serum was prepared by serial absorption of NHS with RSV-infected HEp-2 cells in the cold. This serum had no IgG, IgA, or IgM antibody against RSV by fluorescent antibody staining after the fifth absorption. These sera retained at least 50% of their hemolytically active complement in the sheep erythrocyte assay; total immunoglobulin concentration was not affected.

All complement sources were incubated with both infected and noninfected HEp-2 cells. In addition, incubation of cells with phosphate-buffered saline was included as a negative control.

Evaluation of complement activation by immunofluorescent staining. Cells fixed to slides were incubated sequentially for 30 min at 37°C in a humidified chamber with rabbit anti-RSV antibody, rhodamine-conjugated goat anti-rabbit globulin (Nordic Immunologic Laboratories), and fluorescein isothiocyanate-conjugated goat anti-human C3 (Cappell Laboratories), with three washes in phosphate-buffered saline after each incubation. The slides then were air dried and examined with two interference filter systems with a Leitz Orthomat microscope equipped with epi-illumination. This allowed rhodamine- and fluorescein-stained cells to be viewed independently; in this way, C3b attached to RSV-infected cells could be assessed. Cells were enumerated as syncytial (that is, multinucleated) or nonsyncytial; cells clearly could be differentiated as one or the other (Fig. 1). The number of each type of cell which was rhodamine positive (RSV antigen) and fluorescein positive (C3b) was recorded. At least 300 cells were counted per experiment.

LDH release. LDH was measured by a method reported previously (14) in supernatants and cell pellets after incubation of infected and uninfected HEp2 cells for 1 h at 37°C with (i) NHS, (ii) serum that had been treated at 56°C for 1 h, and (iii) media (without fetal calf serum) alone. Samples were assayed in triplicate, and results from incubations with serum were corrected for its LDH content.

RESULTS

Examples of immunofluorescent staining patterns are shown in Fig. 1; the staining for RSV antigen, which was mainly in the cytoplasm, was distinct from the staining for C3b on the cell membrane. In this assay only RSV-infected HEp-2 cells stained for C3b, and they did so only after incubation with serum which had functional complement activity. The absence of staining after incubation in the presence of ethylenediaminetetraacetic acid or serum heated at 56°C indicates that this staining reflects generation of C3b and subsequent deposition on the cell membrane rather than passive uptake of C3 from the serum. It is possible that other C3 cleavage products contributed to positive staining. The number of cells which were RSV infected varied between experiments, but incubation with NHS consistently yielded C3b bound to about 80% of the infected nonsyncytial cells and 80 to 100% of the syncytial cells. We sepa-

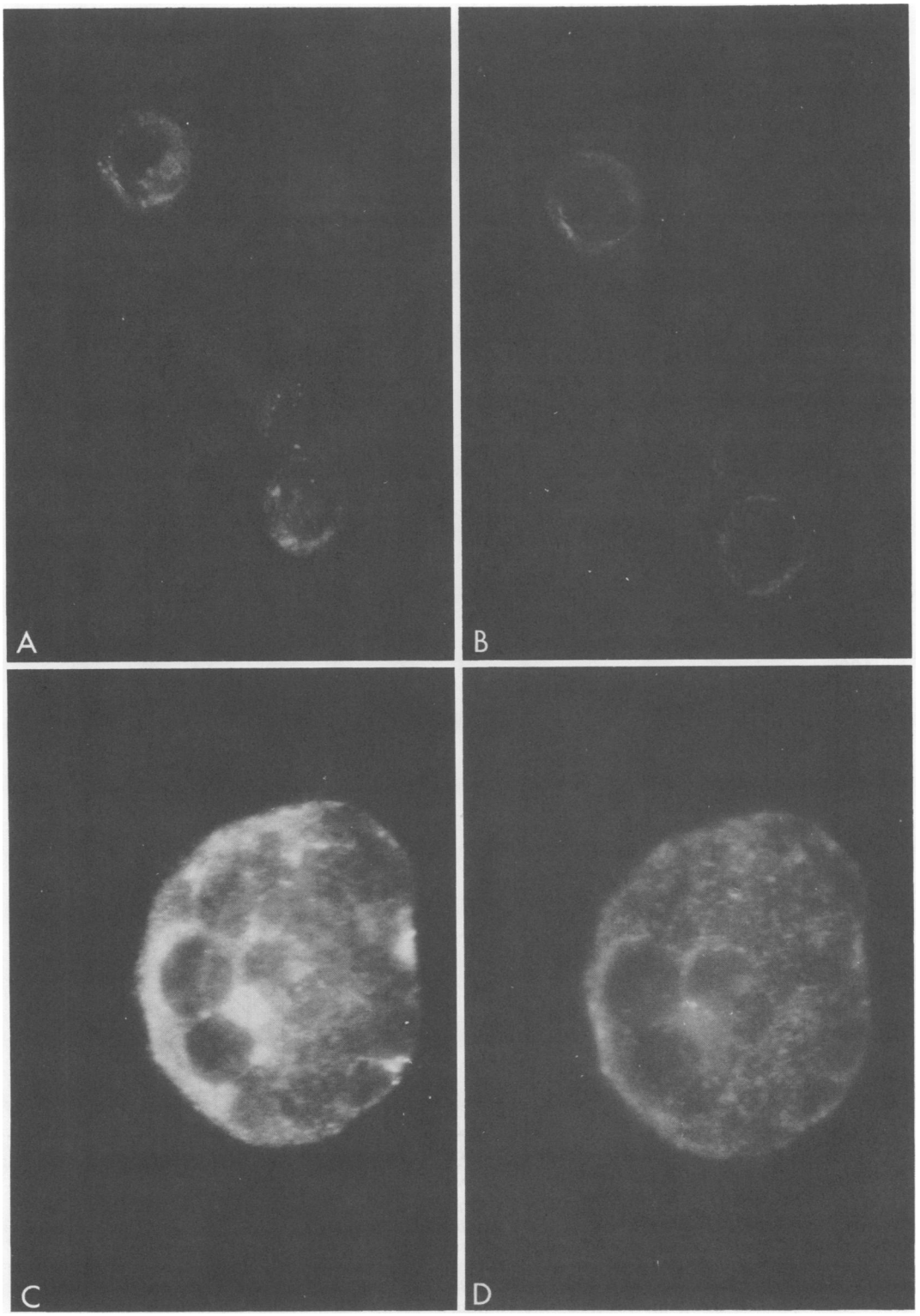

FIG. 1. *Positive staining of the same nonsyncytial cells for RSV (A) and C3b (B). Positive staining of the same syncytial cell for RSV (C) and C3b (D).*

rately evaluated nonsyncytial and syncytial cells, and results from use of the various complement sources or buffer are represented in Fig. 2.

Blocking classical pathway activation with ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid-treated NHS diminished, but did not eliminate, the number of nonsyncytial (but infected) cells positively stained for C3b when compared with results using NHS. It had no effect on cells which had formed syncytia. On the other hand, blocking alternative pathway activation by either zymosan incubation or heat treatment resulted in diminished numbers of both nonsyncytial and syncytial cells stained for C3b, but did not abolish staining on either.

In addressing the role of antibody in complement activation by RSV antigen, we found the following. Incubation with hypogammaglobulinemic serum which was deficient in both immunogloulin as well as specific antibody of the IgM and IgA classes resulted in diminished numbers of nonsyncytial as well as syncytial cells stained for C3b. Absorption with virus to eliminate specific antibody titer diminished C3b staining on infected nonsyncytial cells more than it did on syncytial cells (Fig. 3).

Incubation of infected cells with media alone resulted in 32 ± 5% ($\bar{x}$ ± standard error of the mean) release of LDH. Incubation with NHS resulted in 60 ± 3% LDH release, whereas incubation with the heat-inactivated serum produced only 30 ± 3% LDH release. Incubation of uninfected cells with each of these yielded uniformly low amounts of LDH in the supernatant (medium alone = 5 ± 2%; NHS = 3 ± 1%; heat-inactivated serum = 6 ± 2%).

DISCUSSION

This study demonstrated complement activation in vitro by RSV-infected human epithelial cells in tissue culture. Nonsyncytial cells appeared to be able to activate complement by either classical or alternative pathways, in that elimination of the activity of either pathway separately failed to eliminate binding of C3b to infected cells surfaces. Syncytial cells also appeared to be able to activate complement by either pathway for the same reason. However, this seemed to be less dependent on the classical pathway in that the percentage of syncytial cells positively stained for C3b was not changed by elimination of classical pathway activity with

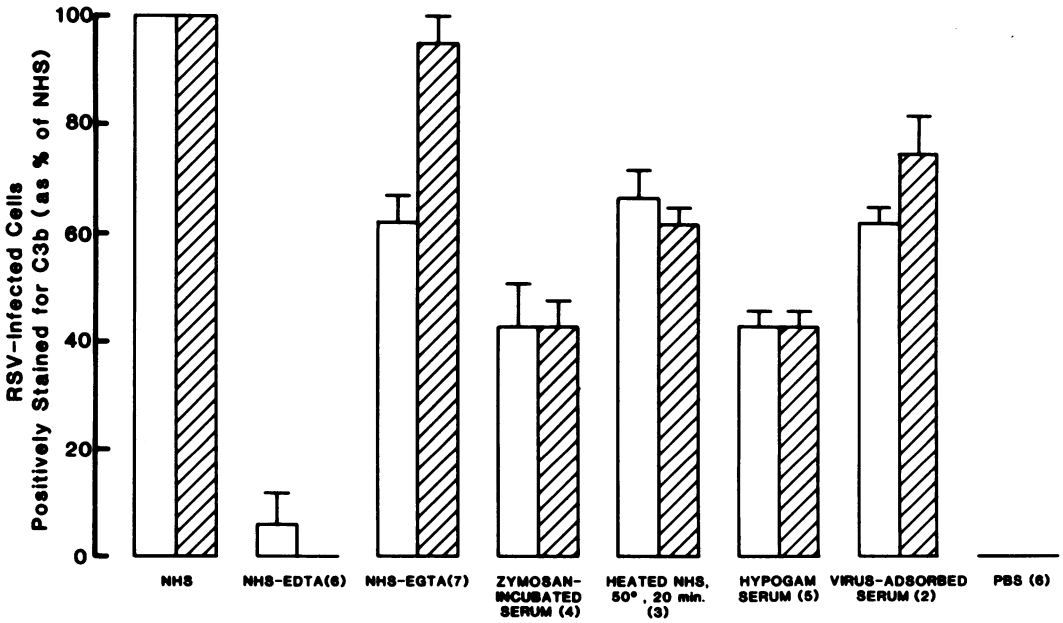

FIG. 2. *Binding of C3b to RSV-infected HEp-2 cells using different complement sources and buffer. Results are shown as mean plus standard error bar; number of experiments is in parentheses at the bottom. Nonsyncytial cell results are shown as the open bars, and syncytial cell results are shown as the cross-hatched bars. Since the percentage of nonsyncytial cells which were infected varied between experiments, but the percent of infected nonsyncytial cells which bound C3b after incubation with NHS was relatively constant, results are expressed using the latter as the standard (100%). PBS, Phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N-*tetraacetic acid.*

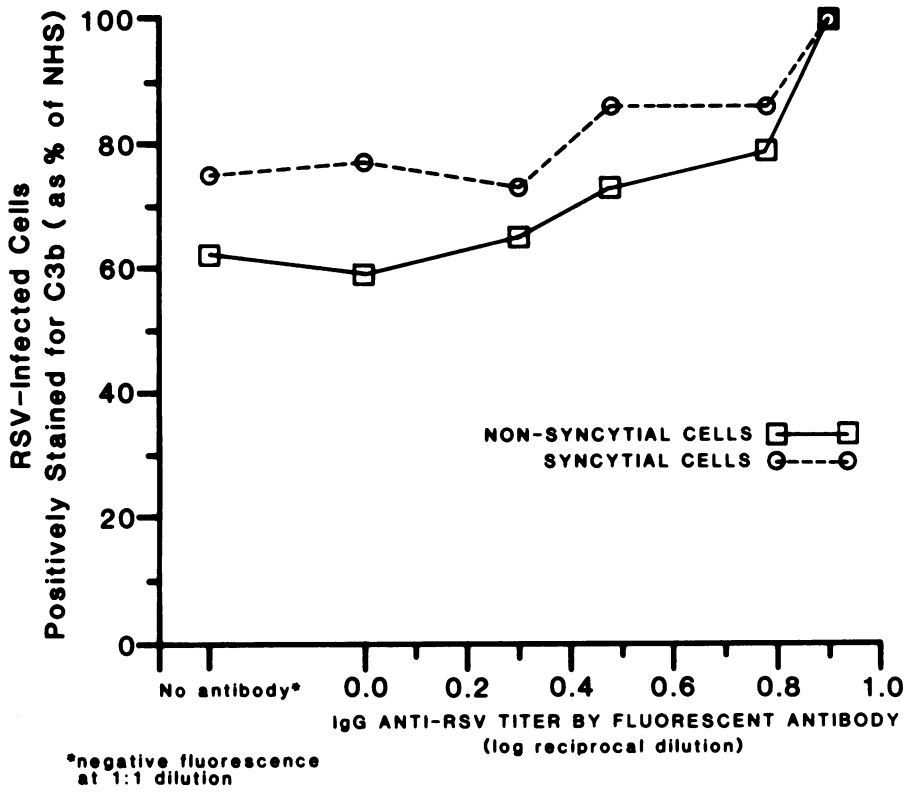

FIG. 3. *Effect of specific antibody titer on C3b binding to RSV-infected nonsyncytial and syncytial cells.*

ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid. Lastly, the alternative pathway appears not to require specific antibody for its activation, but may require immunoglobulin. This is concluded from the following observations: (i) the number of syncytial cells binding C3b was significantly decreased by maneuvers which affect alternative pathway activity but was not affected to the same extent by eliminating specific antibody, and (ii) the number of syncytial cells binding C3b was diminished by decreasing serum immunoglobulin concentration.

It is unlikely that a subpopulation of cells was responsible for the results; any maneuver which diminished the number of cells stained for C3b also diminished the intensity of staining on the remainder of the cells.

This assay system appears reasonable as a basis for making qualitative statements about complement activation by infected tissue culture cells using the different complement sources. The amount of complement activation is not quantitated, but infected versus noninfected cells and nonsyncytial versus syncytial cells may be assessed separately.

It is interesting that the virus-induced syncytia, which are a major cytopathic effect of paramyxoviruses, are able to activate complement under circumstances which diminish activation by the infected nonsyncytial cells. The multinucleated giant cells formed by cell fusion contain large amounts of viral proteins (12); this increased concentration of viral products may render the syncytial cells different in their ability to activate complement (16). Presumably, RSV antigen on the cell surface is primarily responsible for complement activation in our system. However, if there was any leakage of subcellular constituents during virus budding, cell fusion, or cell lysis, these constituents also might activate the alternative complement pathway (17). Also, in fusion the cells might express different molecules on their surfaces.

Measurement of LDH in the supernatants of virus-infected cells suggests that release of subcellular constituents might occur during the course of infection. The twofold increase in LDH release with the addition of hemolytically active complement is consistent with complement-mediated lysis of infected cells following C3b binding to their surfaces.

This study raises the possibility of complement activation during respiratory tract infec-

tion by RSV. Complement components have been demonstrated in respiratory tract secretions (8, 16). In addition, it is theoretically possible for serum complement proteins to appear in secretions during viral infections because of increased mucosal permeability. Generation of anaphylatoxins C3a and C5a during complement activation would result in increased vascular premeability and might potentiate this effect.

Complement activation might contribute to recovery from RSV infection through either direct or cell-mediated lysis of infected cells; our results here suggest lysis of RSV-infected HEp-2 cells by complement in vitro. Additionally, activation via the alternative pathway before specific antibody appears might be an important early mechanism of defense against infection by RSV. The importance of complement in animal models of viral respiratory tract infection has been shown previously (6). Anaphylatoxin action on mast cells would amplify the inflammatory response. On the other hand, in certain individuals, mediator release secondary to complement activation might play a role in the inflammation seen in primary or repeated RSV infection and might contribute to atopic symptoms as well. However, at this point the role of complement during RSV infection in vivo, either in potentiating immunopathological damage or in recovery, is speculative.

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