CONCISE COMMUNICATION

Alterations in Apoptosis of Cord and Adult Peripheral Blood Mononuclear Cells Induced by In Vitro Infection with Respiratory Syncytial Virus

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Respiratory syncytial virus (RSV), a major cause of morbidity in children, results in severe lower respiratory tract infections. With an in vitro infection system of isolated cord or adult peripheral blood mononuclear cells, addition of virus to cell cultures resulted in significant reductions in cell deaths, as measured by 2 independent assays: quantitation of cells with subdiploid levels of DNA and cells with DNA strand breaks. Decreased cell death was observed in lymphocytes and monocytes of cord and adult samples, with more dramatic effects evident in cells from cord blood. This may be linked to the increased virulence observed in infants with RSV infection. These data suggest that RSV may be equipped with some mechanism to prevent apoptosis, which is a major component of the host defense system used to eliminate virally infected cells.

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Materials and Methods

Cell culture conditions. RSV (Long strain) was grown in HEp-2 cells cultured in Eagle MEM with 2% fetal calf serum and stored at ~70°C. The virus titer in a plaque-forming unit (pfu) assay on HEp-2 cells was 5–10×10^6 pfu/mL. PBMC were obtained by use of Ficoll-Paque (Pharmacia, Uppsala, Sweden) separation of heparinized whole blood obtained from healthy adult volunteers and umbilical cord specimens from normal newborns. Cell concentrations were adjusted to 2×10^5 cells in RPMI with 10% fetal calf serum in separate tubes and infected with 2×10^5 pfu of RSV or an equivalent volume of medium (mock).

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Changes induced in apoptosis % after culture with or without (mock) respiratory syncytial virus (RSV). Cord (n = 19) or adult (n = 7) peripheral blood mononuclear cells were assessed for apoptosis by quantitation of subdiploid (A0) peak detected by propidium iodide staining after 24 or 48 h of culture. Comparisons were made by paired Student’s t test or Wilcoxon signed rank test as appropriate. *Statistical significance between mock and RSV-exposed samples.

Measurement of apoptotic cell death by subdiploid DNA content. Apoptosis was quantitated by flow cytometry as reported elsewhere [13]. We harvested 10^6 cells and fixed them in 70% ethanol for 1 h at 4°C. The cells were washed and resuspended in 0.125 mL of Hanks’ balanced salt solution (HBSS) to which 0.125 mL of RNase (Sigma, St. Louis) solution (1 mg/mL in HBSS) was added followed by 0.25 mL of propidium iodide (Sigma) solution (100 mg/mL in HBSS).

Simultaneous quantitation of leukocytes and apoptotic cell death by presence of DNA strand breaks. In separate experiments, apoptosis was assessed by use of the TUNEL assay [14] in conjunction with immunophenotyping of monocyte and lymphocyte subsets. For these experiments, 2 x 10^6 cells were exposed to 2 x 10^6 pfu of RSV (MOI = 1) or an equivalent volume of media (mock). At 24- and 48-h time points, cells were counted, labeled with anti-CD14 monoclonal antibody (MAb) conjugated with allophycocyanin, and fixed with reagent (Permeafix; Ortho, Raritan, NJ); the TUNEL assay was done with fluorescein isothiocyanate (FITC)–labeled d-UTP (Phoenix Flow Systems, San Diego).

Quantification of monocyte percentage. We determined the percentage of monocytes by labeling cells with anti-CD45 antibody conjugated with FITC and anti-CD14 antibody conjugated with phycoerythrin (PE; Becton Dickinson, San Jose, CA) for 10 min at room temperature. After samples were initially gated on CD45 fluorescence to exclude red blood cells from the analysis, we determined the percentage of CD14 monocytes.

Immunophenotyping of lymphocyte subsets. In further experiments, specific PBMC subpopulations were quantitated by immunophenotyping at the time of isolation and after 24 and 48 h of culture in the presence or absence of RSV. Reagents utilized were anti-CD3 FITC, anti-CD16/56 PE, anti-CD19 peridinin chlorophyll protein (Becton Dickinson), and anti-CD14 allophycocyanin (Caltag Laboratories, Burlington, CA). Samples were incubated with MAb for 10 min at room temperature, washed, and fixed in Permeafix (Ortho) in order to eliminate residual red blood cells.

Statistical analyses. Populations of RSV and mock cells were compared within each experiment for percentage of each leukocyte subpopulation and percentage of apoptotic cells, by paired Student’s t test, Wilcoxon signed rank test, or Mann Whitney test as appropriate, by use of statistical software (SAS Institute, Cary, NC; Sigmastat, Jandel Scientific, San Rafael, CA).

Results

PBMC apoptosis after RSV exposure. The percentage of apoptotic cells in cord PBMC assessed by propidium iodide staining revealed a decrease in cells in the subdiploid (A0) peak in the RSV-exposed cells at 24 and 48 h compared with mock cells. This effect was observed in 18 of 19 experiments at 24 and/or 48 h. The median (25%–75%) percentages for apoptosis were 4% (3%–6%) and 7.5% (5%–15%) for RSV-exposed cells at 24 and 48 h versus 7.5% (5%–15%) and 15.5% (9%–22%) for the mock cells at these times (P<.001, Wilcoxon signed rank test at both time points; figure 1A, 1B). In 7 experiments with adult PBMC, the percentages of apoptosis were again lower at 24 and 48 h for the RSV-exposed cells, compared with those for the mock cells: 2% (1%–4%) in RSV-exposed cells.
versus 5% (2%–15%) in mock cells at 24 h and 4.5% (2%–7%) for RSV-exposed cells versus 11.5% (8%–12%) for mock cells at 48 h ($P = .02$ and .008, respectively) (figure 1C, 1D). In addition, when cord and adult cells were compared, there were higher percentages of apoptosis in cord cells regardless of virus exposure.

Simultaneous assessment of lymphocyte and monocyte apoptosis with TUNEL assay. A major limitation of the subdiploid assay for apoptosis is the inability to determine the phenotypic characteristics of the cells that are dying. Since we wanted to directly determine which leukocyte populations were affected by virus, we did additional experiments, using the TUNEL assay, subsequent to labeling with anti-CD14 MAb to differentiate monocytes from lymphocytes. These experiments demonstrated a similar decrease in apoptosis in RSV-exposed PBMC, compared with that in mock cells. For the cord cells ($n = 12$), the median percentages (25%–75%) for apoptosis were 10% (8%–12%) and 13% (9%–16%) for RSV-exposed cells at 24 h and 48 h versus 18% (12.5%–21.5%) and 16.5% (13.5%–19.5%) in the mock cells ($P = .003$ at 24 h; Wilcoxon signed rank test; figure 2A). For the adult cells ($n = 10$), the total apoptosis in the RSV-exposed cells was 5.5% (2%–11%) and 4.5% (3%–7%) versus 9% (6%–12%) and 10.5% (9%–12%) in the mock cells at the same time points ($P = .002$ at 48 h; figure 2B). Decreases in lymphocyte and monocyte apoptosis were also demonstrated in these experiments after RSV exposure.

**Effect of RSV coculture on monocytes and lymphocytes.** As assessed by flow cytometry, the percentage of monocytes in RSV-exposed cord blood PBMC was greater than that in mock cell populations at 24 and 48 h after infection in 20 of 25 experiments. The median (25%–75%) percentage of monocytes at 24 h was 18% (17%–23%) in RSV-exposed cells, compared with 10% (8%–13%) in mock-infected cells ($P < .0001$, Wilcoxon signed rank test). At 48 h after exposure, the median (25%–75%) was 17.5% (12%–19.5%) for RSV-exposed cells and 7.5% (5.5%–10.5%) for the mock cells ($P < .0002$). In 2 experiments,
serial 10-fold dilutions of RSV at the time of initial inoculation demonstrated loss of this effect at a 1:100–1:1000 dilution of RSV. In contrast to the results with cord blood, the percentage of monocytes in adult PBMC (n = 9) was the same in mock and RSV-exposed samples.

We next sought to determine if RSV affected cell death of a particular population of lymphocytes. Lymphocyte immunophenotyping studies did not reveal RSV-induced differences in the relative proportions of the subpopulations tested (B, T, and NK cells) in adult or cord PBMC, demonstrating that the reduction of cell death occurred equally in all lymphocyte subsets.

Discussion

Our results indicate that exposure of PBMC to RSV leads to increased survival of monocytes and lymphocytes for 24–48 h after virus exposure, when compared with uninfected cells. Although there were significant decreases in lymphocyte apoptosis detected after RSV exposure, there were no differences in the distribution of T, B, or NK cells between RSV-exposed and mock cells, suggesting a uniform effect of the virus in decreasing apoptosis among the lymphocytes. Increased percentages of monocytes in the RSV samples, compared with those that had been mock infected, indicated an effect on this population as well. In addition, differences were noted between the cord and adult cells. For mock- and RSV-exposed cells, the percentage of apoptotic cells was higher in the cord PBMC. Furthermore, although the decrease in apoptosis and increase in monocytes was seen in both cord and adult cells, it was detected earlier in cord PBMC, an observation that may have relevance to the severe disease this virus causes in infants.

It is interesting to speculate about the possible role of decreased apoptosis in response to RSV in disease pathogenesis. Immune responses to RSV may involve respiratory epithelial cells, granulocytes, eosinophils, lymphocytes, resident macrophages, and monocytes. The nature of the host responses to the virus have been hypothesized to play important roles in the pathogenesis of severe RSV disease (i.e., bronchiolitis), in the clearance of the virus, and in a child’s increased risk for subsequent episodes of wheezing or reactive airway disease following hospitalization for RSV infection in the first year of life. Several lines of evidence support a role for PBMC and/or res-
ident macrophages in orchestrating the host response to RSV, since these cells are observed in the lungs of children with severe bronchiolitis. In addition, RSV can infect monocytes and macrophages in vitro [3, 4]. Thus, one can envision a survival advantage for the virus if it can inhibit the death of the monocyte it has colonized for its own purposes. The decreased apoptosis observed in the lymphocyte pool is more difficult to explain and may involve inflammatory mediators or cytokines produced by monocytes, which have been implicated in the pathogenesis of RSV disease [5, 6]. Recent studies in mice showed that CD8 T lymphocytes are necessary for the development of RSV-induced airway hyperresponsiveness [15]. Activated T cells, destined for apoptosis but afforded a longer life span because of viral intervention, may be an important piece of the disease-causing process.

The modulation of apoptosis by RSV may be a critical determinant of the effectiveness of the host immune response to the virus. Further understanding of this interaction may be imperative for determining the mechanisms by which RSV orchestrates its effects. Such studies could include analyses of the molecular basis for the observed decrease in apoptosis in response to RSV and assessment of in vivo changes in PBMC in response to natural RSV infection.

References