Differences in Participation of Innate and Adaptive Immunity to Respiratory Syncytial Virus in Adults and Neonates

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Innate and adaptive immune responses to respiratory syncytial virus (RSV) in neonates were assessed by cord blood mononuclear cell (MC) cytokine expression and proliferation and these responses were compared with those from adult peripheral blood MCs. In adult cells, inactivated and live virus invoked cytokines reflecting both innate and adaptive immunity (interleukin [IL]–6, interferon [IFN]–γ, IL-2, tumor necrosis factor [TNF]–α, and IL-10). Low levels of IL-4 were detected, although only with inactivated virus. In contrast, in neonatal cells, inactivated virus invoked large levels of the innate immune cytokines IL-6, TNF-α, and IL-10 and reduced levels of IFN-γ and IL-12 but no adaptive cytokines. Live virus induced fewer innate (IL-6, IL-10, and IFN-γ) and no adaptive immune cytokines. RSV-induced proliferation was absent in neonatal MCs, although positive in adult MCs. Thus, exposure to RSV does not appear to occur before birth, and adaptive immune insufficiency or greater innate responses may account for early life RSV-induced illnesses.

Respiratory syncytial virus (RSV) is the principal cause of severe lower respiratory tract infection in infants and young children [1]. Infection rates are ∼70% in the first year of life, and all children are infected by age 3 [2]. Disease manifestations vary markedly among individuals and may include pneumonia, bronchiolitis, or mild upper airway congestion alone. Morbidity is highest during the first 6 months of life [3]. In older children and adults, symptoms are mild and are usually restricted to the upper respiratory tract.

Variability in the development of a protective immune response to this virus may account for the very large interindividual differences in disease phenotype that accompany exposure in infants. Information regarding the dynamics of the development of an immune response to RSV in early life is sketchy. The development of detectable antibody responses to RSV is evident in many children within the first year of life and in most children before age 3 [4]. Maternal antibody provided passively to the fetus is present through the first few months of life when prevalence of the more-severe forms of RSV illness is greatest, thus casting doubt on a major protective role for antibody-mediated immunity [5]. Innate immunity to RSV also has been reported, as demonstrated by increased levels of tumor necrosis factor (TNF)–α, interleukin (IL)–1β, IL-6, and IL-8 in nasal washings of infants during active infection [6]. Perhaps the greatest lack of clarity concerns the presence, temporal nature, and degree of variability of cell-mediated immunity, with possibilities of an overzealous immune response [7, 8], immune deviation [9, 10], or transient early life immune deficiency [11], all of which have been offered as suggestions to account for the variability in disease severity.

To clarify the role of adaptive immune development to RSV, it is necessary to determine the timing of the initial exposure to RSV and address especially the possibility of in utero exposure. Monocyte-derived macrophages obtained from cord blood have been reported...
to produce IL-6 and TNF-α after exposure to live but not inactivated virus [12], thus suggesting that the capacity to produce an innate immune response is present at birth. Recently, proliferative responses and interferon (IFN)–γ expression were detected to RSV-infected, UV light–inactivated cells in one-third of infants exposed to an RSV season at the appropriate time of gestation [13]. However, whether this response is specific for RSV was not clearly shown.

As an initial phase of studies establishing the course of development of the immune response to RSV in early life, we report here a comparison of the responses to RSV stimulation of mononuclear cells (MCs) obtained from the umbilical cord blood of neonates and the peripheral blood of healthy adult volunteers, both randomly selected. We reasoned that the immune response is likely to be different depending on the state of the virus (inactivated or live) that initially induces immunity and that information as to which is the more prominent form of the virus (inactivated or live) that initially induces immunity is likely to be different depending on the state of the virus (inactivated or live) that initially induces immunity and that information as to which is the more prominent form of the virus (inactivated or live) that initially induces immunity is likely to be different depending on the state of the virus (inactivated or live) that initially induces immunity. We thus examined responses to in vitro challenge of MCs with both inactivated and live virus. We asked whether MCs at birth exhibited adaptive and/or innate immunity to RSV by assessing cytokine expression and proliferation. We sought to determine how the neonate response (if it occurred) differed from the response in adults (i.e., after many exposures to this antigen), to identify any evidence for adaptive immunity to RSV developing before birth.

SUBJECTS, MATERIALS, AND METHODS

Cord and adult blood MC isolation. Cord blood was obtained from 26 randomly selected healthy neonates after scheduled caesarian section births by umbilical vein puncture in heparinized syringes (Becton Dickinson). For adult blood, 7 mL was layered onto 4 mL of lymphocyte separation medium (LSM; ICN Biomedicals) and centrifuged; the pellet was resuspended in RPMI 1640 medium supplemented with 1-glutamine, Pen/Strep, and 5% fetal calf serum (all from Life Technologies), as recommended by ATCC. After 36 h of infection (indicative of 80%–90% cytopathic effect), HEp-2 cells were disrupted with sterile beads, and the culture fluid was centrifuged at 500 g for 10 min and preserved at −70°C. The RSV titer was determined in a standard plaque-forming unit assay [15] for the live and (subsequently) inactivated frozen aliquots. The titer of virus for the 2 aliquots was 1 × 10^5 pfu/mL and 0.7 × 10^4 pfu/mL. The 0.7 × 10^4 pfu/mL aliquot was inactivated by UV light irradiation (253.7 nm) for 20 min at a distance of 10 cm from the UV light source. Complete inactivation of virus was confirmed by the absence of plaque formation of UV-treated RSV preparation (data not shown). Uninfected HEp-2 cell culture fluid was processed similarly for use as controls.

MC culture and stimulation. MCs were counted and re-suspended to a concentration of 2 × 10^4 cells/mL in RPMI 1640, and 2-mL cultures were stimulated with either live RSV A2 strain 5 × 10^3 pfu or UV-inactivated RSV A2 strain 3.5 × 10^3 pfu (final concentrations). Initial dose-response data showed that inactivated RSV induced the same cytokine responses over the range 10^3–10^6 pfu. Unstimulated cells and cells stimulated with 100 μL of uninfected HEp-2 cell supernatant cultures served as negative controls, and cells stimulated with the mitogens concanavalin A (ConA; 10 μg/mL) and PMA (10 ng/mL) (final concentration) served as positive controls.

Infectivity assessment. To verify that MCs were infected with live RSV (as has been reported elsewhere [16]), adult and cord blood MCs stimulated with live RSV were assessed at 48 h for viral proteins on the cell surface by flow cytometry; we used anti-RSV protein antibody provided by Chemicon International. Each sample had appropriate isotope subtracted. Similar to the report of Midulla et al. [16], live virus yielded a mean ± SD of 17.0% ± 4.4%, which was significantly greater than the inactivated virus control (5.6% ± 5.1%; n = 6; P = 0.048). Assessment of lymphocyte staining revealed no difference between live RSV (7.0% ± 5.5%) and inactivated RSV (3.5% ± 3.6%) (data not shown).

Cytokine assays. Supernatants from the stimulated and
control cell cultures were collected on days 1, 3, and 5. The supernatants were assayed for these 9 cytokines: IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, and IL-13. They were assessed by ELISA by means of commercially available kits (R&D Systems) according to the manufacturer’s instructions. The IL-12 kit measured the p70 subunit of IL-12. The sensitivities of the cytokine ELISAs are as follows: IL-2, <31.2 pg/mL; IFN-γ, <15.6 pg/mL; IL-4, <0.25 pg/mL; IL-13, <62.5 pg/mL; IL-5, <7.8 pg/mL; IL-10, <7.8 pg/mL; IL-12, <0.31 pg/mL; IL-6, <31.2 pg/mL; and TNF-α, <4.4 pg/mL.

**Proliferation assay.** Cord blood and peripheral blood MCs (2–4 × 10^6 cells per well) were cultured in 96-well microtiter plates. MCs were cultured with ConA (10 ng/mL), UV-inactivated RSV (1.75 and 3.5 × 10^3 pfu), and uninfected HEp-2 supernatant controls for 5 days. Proliferation assays were performed by means of commercially available kits (Cell Proliferation ELISA Biotrak System, version 2, Amersham Biosciences) according to manufacturer’s instructions.

**Statistical analysis.** Cytokine levels in supernatants of stimulated adult MCs have been previously found to be log normally distributed [17]; thus, values here have been analyzed in this manner. Differences from control were assessed by Student’s paired t test, and differences between 2 groups were assessed by unpaired t test. P < .05 was considered to be significant.

## RESULTS

**Adult MC responses to RSV.** Cytokine levels in supernatants from adult blood MCs (n = 10) stimulated with inactivated RSV on days 3 and 5 are shown in figure 1. The response on day 5 included detectable levels of the following cytokines in decreasing rank order: IL-6 > IFN-γ > IL-2 > TNF-α > IL-10 > IL-4 (figure 1). Cytokines that were not detectable included IL-5, IL-12, and IL-13. The variation among individual subjects in the quantity of any one cytokine released (as indicated by error bars) was much more limited than the variation in expression among the different cytokines, for which mean values were distributed over 4 logs. Varying the time of incubation revealed that significant maximal cytokine levels were reached already at day 2 (data not shown) for all cytokines except for IL-2 (reached at day 3) and IL-10 (reached only at day 5). RSV concentrations of 1.75 and 3.5 × 10^3 pfu as the stimulant gave comparable results, whereas stimulation with 3.5 × 10^4 pfu resulted in lower levels of cytokines (data not shown). Stability of the responses over time within individuals was examined in cells obtained from blood samples drawn 3 weeks apart, and these second samples yielded no significant differences in mean level in the stimulated cells for any cytokine (data not shown).

The response in adult blood MCs (n = 10) to live RSV provided a similar pattern of cytokine secretion on days 3 and 5 (figure 2). Compared with inactivated virus, no IL-4, less IL-2, and more IFN-γ were expressed, and IL-10 levels reached significance at an earlier time point after stimulation (by day 3). The relative order of cytokine expression was still maintained at IL-6 > IFN-γ > IL-2 > TNF-α > IL-10. Cytokines that were not detectable included IL-4, IL-5, IL-12, and IL-13.

**Neonate MC responses to RSV.** Cytokine levels in supernatants from cord blood MCs (n = 14) stimulated with inactivated RSV on days 3 and 5 are shown in figure 3. The responses on day 5 included detectable levels of the following cytokines in decreasing rank order: IL-6 > TNF-α > IL-10 > IFN-γ > IL-12 (figure 3). The responses were evident on day 1 (data not shown) and were essentially maintained on days 3
and 5 for all cytokines except IFN-γ, which was undetectable on day 1. No IL-2, IL-4, IL-5, or IL-13 was detectable at any of the 3 time points after stimulation. The innate immune cytokines IL-12, IL-6, and TNF-α were significantly greater in neonates than in adults. The adaptive immune cytokines IL-2 and IL-4 that were expressed by adult cells were undetectable in neonatal cells.

The response in cord blood MCs (n = 12) to live RSV yielded several differences from that to inactivated RSV (figure 4). First, the response on days 1 (data not shown) and 3 consisted only of IL-6. On day 5, detectable levels of IL-6, IL-10, and IFN-γ were produced, although IL-6 and IL-10 levels were significantly reduced, compared with the inactivated virus response. No IL-2, IL-4, IL-5, IL-13, TNF-α, or IL-12 was detectable at any of the 3 time points after stimulation. Compared with inactivated virus, the overall cytokine expression was greatly decreased in cord blood MCs in response to live RSV.

**Adult and neonate MC responses to mitogen.** Cytokine levels in supernatants from the same adult (n = 10) and cord (n = 14) blood MCs stimulated with mitogen ConA/PMA for 24 h are shown in figure 5. The cytokine responses in adult cells to mitogen were greater than those induced by RSV stimulation for all cytokines and were expressed in a different rank order: IL-2 ∼ IFN-γ > TNF-α > IL-6 > IL-10 > IL-13 > IL-5 > IL-4. No IL-12 was detectable. Cord blood cell responses to mitogen were also different from those to the virus. Cord blood MCs produced greater amounts of IL-2, IFN-γ, IL-4, IL-13, and TNF-α to mitogen than to viral stimulation (either live or inactivated). IL-6, IL-10, and IL-12 were produced in lesser amounts in cord blood MCs stimulated with mitogen, compared with inactivated virus, but were essentially equivalent between mitogen and live viral stimulations. The rank order for mitogen-stimulated cord blood MCs was IL-2 > TNF-α > IL-6 > IFN-γ > IL-13 > IL-10 > IL-4. Levels of IL-5 and IL-12 were undetectable. When comparing the cytokine response in adult versus cord blood MCs to mitogen, the following observations were made: levels of IFN-γ, IL-4, IL-5, IL-10, and IL-13 were significantly greater in adults than in neonates, whereas IL-2, IL-6, and TNF-α levels were comparable.

**DISCUSSION**

Given that the respiratory illnesses to RSV are most devastating in the first 6 months of life [3], this study sought to establish the nature of the immune response to RSV at birth as an indicator of the in vivo status of the immune cells before direct exposure to RSV from the environment. Subsequent immune responses of the infants at first encounter with RSV were likely to contribute to the severity of illness through 3 possibilities:

**Adult and neonate proliferative responses to RSV.** Proliferation to inactivated RSV was assessed in neonatal MCs (n = 5) and compared with peripheral blood MCs (n = 5) from healthy adults (figure 6). Mitogen and HEp-2 supernatants were used as stimulants for positive and negative controls, respectively. No detectable proliferation to RSV was observed in neonatal cells, although positive proliferative responses to ConA/PMA were observed in neonatal cells (∼3-fold increase over unstimulated controls). Fetal age was ∼22 weeks at the time of the RSV season that occurred during the gestational period for each of these 5 infants. Positive proliferative responses to both RSV and ConA/PMA were seen in adult cells (4-5- and 9–12-fold increase over unstimulated controls, respectively).
Figure 5. Comparison of adult and neonate cytokine responses to mitogen. Adult (n = 10) and cord (n = 14) blood mononuclear cells (MCs) (4 × 10^5 cells in 2 mL cultures) were stimulated in vitro with concanavalin A (ConA; 10 μg/mL) and PMA (10 ng/mL) (final concentration). Supernatants were collected on day 1 and assayed for cytokines by ELISA.

*P < .05, vs. control (t test); #P < .05, adult response vs. neonate response (t test). IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

Figure 6. Adult and neonate proliferative responses to respiratory syncytial virus (RSV) and mitogen. Adult ( ) and neonate ( ) blood mononuclear cells (MCs) (cells per well) were cultured in vitro with concanavalin A (ConA; 10 μg/mL) and PMA (10 ng/mL), UV-inactivated RSV ( and pfu) and uninfected HEp-2 cells for 5 days, and proliferation assays were performed. *P < .05, vs. unstimulated control (t test).

An overzealous innate immune response, an immune insufficient adaptive response, or an adaptive response polarized in a Th2 direction. We also assessed the response to RSV in adult peripheral blood cells to provide an indication of the nature of a fully developed immune response to RSV in multiply exposed humans.

The present study was designed to (1) identify the developed immune status to RSV in adults, (2) identify any indication of previous exposure in infants, and (3) predict the likely immune contribution to the severity of illness in newborns. Thus, cytokine response patterns were assigned to 3 categories: innate (IL-6, TNF-α, IL-12, IFN-γ, and IL-10), T helper type 1/T cytotoxic type 1 (Th1/Tc1) (IL-2 and IFN-γ), and Th2/T cytotoxic type 2 (Th2/Tc2) (IL-4, IL-5, IL-10, and IL-13) immune responses. The immune response to inactivated RSV in adult cells included production of both innate and adaptive immune cytokines. The extremely low levels of IL-4, the lack of IL-5 and IL-13, and the high levels of IFN-γ and IL-2 support categorizing the adaptive portion of the response as predominantly a Th1/Tc1-type immune response. The significant, although low, expression of IL-4 suggests the possibility of a small Th2 or Tc2 response but also includes the possibility of natural killer cell participation.

Live RSV stimulation in adults yielded responses that were very similar to those observed with inactivated virus, as shown by a similar rank order of cytokine expression. One difference was the complete lack of detectable IL-4 with live RSV, essentially excluding even a small role for Tc2 or Th2 cells. Overall, these data support a scenario in which both innate and adaptive immunity are part of the immune response to RSV in adults, with the latter being primarily a Th1/Tc1 type of response.

An emerging concept purports that pathogens that induce Th1 responses are those that invoke large production of IL-12, whereas production of IL-10 and/or IL-4 is inhibitory to Th1 and facilitatory for Th2 responses [18–20]. Studies with some viruses, however, have demonstrated Th1 responses that are independent of IL-12 [21]. As seen here, RSV can be placed among the latter group of viruses. Future studies will determine whether IFN-α/β and/or IL-18 [22] might be involved.

Stimulation of cord blood MCs resulted in several differences from the adult response. The cord blood cells responded vigorously to inactivated RSV with a response that appears to be only innate in that no IL-2, IL-4, IL-5, or IL-13 could be detected, whereas large amounts of IL-6, TNF-α, and IL-10 and moderate amounts of IFN-γ were produced. Although IFN-γ and IL-10 are not uniquely produced from innate immune cells, the simultaneous lack of IL-2 production strongly suggests that the IFN-γ and IL-10 from the cord blood cells are part of the innate immune response and likely have come from non–T cell sources. Of interest, in the cord blood cells, a small but significant amount of IL-12 was induced, and thus we cannot clearly conclude that IFN-γ production is independent of IL-12 in the cord blood responses. Except for IFN-γ, cord blood innate cytokines in response to inactivated RSV were produced in amounts substantially greater than those from adult cells, including IL-10, which was increased ~3-fold, and TNF-α and IL-6, which were increased 14–30 times.

The immune response to live RSV in the cord blood cells.
was similar to inactivated RSV with respect to only being innate in nature. However, several differences from the inactivated virus response were noted. With live virus, TNF-α did not reach detectability. The IFN-γ and IL-10 responses were much slower in developing, reaching significance (and levels equivalent to those with inactivated virus) only at day 5. IL-6 was greatly reduced (by 10-fold) in level, compared with inactivated virus. As a result of these differences, the innate immune response of neonates to live virus was not greater than that of the adult MCs. Thus, the possibility of an overly vigorous innate response is not supported, nor is a Th2-deviated response. On the basis of these data, if the in vivo response is driven primarily by live virus, an adaptive immune insufficiency remains the most likely setting for severe responses to RSV seen in early life RSV exposure. If inactivated virus plays the predominant role, an overly vigorous innate response may also participate. We cannot, of course, completely exclude any possibility of development of transient immune deviation toward Th2 at the time of a subsequent RSV exposure. However, neither the newborn nor the adult responses assessed provide any support for such a response. Longitudinal follow-up studies in infants through their early exposures to RSV are needed.

The greatly decreased response to live virus in cord blood MCs suggests that live virus invokes the innate response via less efficient pathways or actively suppresses innate cytokine stimulation induced by inactivated virus. No similar suppressive effect on the innate immune response was significant in adult cells. Although our results differ from those of Matsuda et al. [12], who reported IL-6 and TNF-α from cord blood monocyte-derived macrophages were produced by live but not inactivated RSV, other studies have shown that live RSV can induce suppressive factors, including IL-1 inhibitor activity [23] and IFN-γ [24], that decrease the overall immune response. Although the possibility that RSV enhances host cell death might be suggested for smaller responses, this notion is not in keeping with the findings of Krilov et al. [25], who have shown that RSV actually decreased cell death in adult and cord blood MCs, compared with controls. We do not have an explanation for the discrepancy with Matsuda et al. [12], other than the possibility that macrophages alone respond differently than monocytes in the context of cord blood.

To determine whether the lack of an adaptive response to RSV in cord blood cells was indicative of a general incapacity to produce T cell cytokines, mitogen stimulation was performed and was found to induce cytokine production for all cytokines except IL-12 (also absent in adult cells) and IL-5. Such a capacity of cord blood cells to produce many adaptive immune cytokines thus strengthens our conclusion that the response to RSV in cord blood cells is solely innate and signifies that exposure before birth appears not to have occurred.

Studies have shown that fetal MCs are capable of proliferating in response to mitogenic and allergenic stimuli from ∼22 weeks’ gestation [26], and when we were doing these studies, Legg et al. [13] reported that antenatal sensitization is associated with proliferation and IFN-γ production in those infants >22 weeks’ gestation during the RSV season. At that point, we determined that most of the neonates in our study had not been older than 22 weeks during the RSV season that occurred during their gestation. We then tested a group of cord blood MCs from infants whose gestation had been >22 weeks during the RSV season. Proliferative responses to RSV were not detected in any of these neonatal MCs, whereas adult peripheral blood MCs showed significant proliferative responses. Thus, we found no evidence of adaptive immunity to RSV before birth. This difference from the results of Legg et al. [13] may be due to the stimulus they used, which was not RSV but whole cells that had been infected with RSV and then UV inactivated. Thus, the response in that study may be due to one or more cell-surface antigens, rather than to RSV per se.

Establishing the basic nature of the infant immune system status in response to RSV is also important in future development of vaccines against RSV disease. Attempts to enhance human immunization against RSV have not met with success. In the 1960s, a formalin-inactivated vaccine tested in infants led to disease enhancement during subsequent RSV infection [27], which may have been Th2 mediated. Thus, the need to clarify the nature of immune response to RSV in the infant in relation to host contribution to illness manifestations is further emphasized.

In summary, although cord blood MCs were shown to be capable of producing cytokines indicative of adaptive immunity by mitogen stimulation, it is clear that stimulation with both live and inactivated RSV leads to only innate responses, and, thus, our results provide no evidence for in utero sensitization. Adaptive, as well as innate, immune responses were evident among the adult cells for both inactivated and live virus. Our study provides the working hypothesis that humans are born with a capacity to mount an innate immune response to RSV and supports the concept that development of adaptive immunity occurs after birth and appears to be biased toward Th1/Tc1 polarization.

References