Interaction of Respiratory Syncytial Virus with Human Cord Blood Mononuclear Cells

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Respiratory syncytial virus (RSV) is the principal cause of severe lower respiratory tract infection in infants and young children. [1-3] Although antibody responses have been documented in children infected with RSV, cell mediated immunity appears to have a role in both the recovery from infection as well as pathogenesis of disease. [4,5] The increased susceptibility of children with congenital heart disease to RSV infection [6] can be explained on the basis of immunodeficiency in a significant proportion of these children. [7,8]

In vitro investigations of the interaction between RSV and mononuclear cells (MNCs) may offer some insight into host immune responses to RSV infection. However, not many studies have addressed this issue. [9-13] Moreover, these studies were performed with adult blood MNCs. Since RSV infections tend to be more severe in young infants [14] and cord blood MNCs have been shown to be more permissive to certain viruses [15], we examined the interaction between RSV and cord blood MNCs in the context of viral replication, lymphocyte malfunction and natural killer (NK) cell cytotoxicity.

SUMMARY This study on the interaction between respiratory syncytial virus (RSV) and human cord blood mononuclear cells shows that RSV replication can occur in neonatal macrophages. Although neonatal lymphocytes were not supportive of RSV replication, exposure to RSV resulted in significant inhibition of mitogen-induced transformation. Both adult and neonatal NK cell cytotoxicity were unaffected by exposure to RSV. These results suggest that RSV has preferential effects on human cord blood mononuclear cell subpopulations.

MATERIALS AND METHODS

Cell preparation

Heparinized cord blood samples were obtained from placentas at delivery. Mononuclear leukocytes were isolated from the heparinized blood by Ficoll-Hypaque sedimentation [16], washed twice and resuspended in RPMI 1640 medium (Commonwealth Serum Laboratories, Melbourne, Australia) for the experiments.

Culture medium

Cultures were maintained in RPMI 1640 medium with 1% glutamine aqueous penicillin G (100 units/ml) and streptomycin (100 μg/ml). The basal medium was further supplemented with 10% fetal calf serum (FCS).

Preparation of macrophage and lymphocyte cultures

Mononuclear leukocytes (5×10^6 cells/ml) were incubated in plastic dishes (Corning, NY, USA) for 2 hour at 37° C with basal medium supplemented with 20% autologous plasma. The adherent cells (predominantly macrophages) were vigorously washed 3 times to remove non-adherent cells. The non-adherent

cells (mainly lymphocytes) were washed 3 times and reincubated with 1 ml fresh medium in new plastic dishes for use in lymphocyte culture.

Virus infection

RSV (Long strain) was grown in HEp-2 cells to a titer of 10^6 TCID$_{50}$ per 0.02 ml. Stock virus was stored at -70° C until used. Uninfected HEp-2 cell fluid was processed similarly for use in mock infection of all experiments except immunofluorescence tests in this study. Virus at 100 TCID$_{50}$ was added to the macrophage and lymphocyte cultures for 2 hours at 37° C. The cultures were infected with virus after 1, 2 and 4 days of cultivation. After incubation, the cells were washed 3 times and then reincubated with 1 ml of fresh medium. This was designated day zero.

Viral infectivity assay

On day 1, 2, 4 and 7 after RSV infection, the supernatants from macrophage and lymphocyte cultures were assayed for viral infectivity, by the determination of 50% tissue culture infectious dose (TCID$_{50}$) per ml by the method of Karber as previously described.[17] Briefly, 0.02 ml of serial 10-fold dilutions was added to each of 4-6 replicated wells containing HEp-2 cells in 96 well microtiter plates. The cytopathic effect (CPE) was observed for 10 days after inoculation and 50% end points were determined.

Assay of lymphocyte transformation

The assay was performed in 96 well microtiter plates, as previously described.[19] On day 1 and 4 after virus infection, the lymphocytes were assayed for the response to phytohemagglutinin (PHA) in triplicate. The infected or uninfected cells were added to wells to give a final concentration of 5×10^5 cells/ml (1/10^5 cells/well) and incubated for 3 days with medium alone or medium containing PHA (Difco, Detroit, USA) at a concentration of 40 μg/ml in

the final volume of 0.2 ml/well. The cells were pulsed with 1 μCi per well of ^{3}H-thymidine (Amersham, Buckinghamshire, UK) 6 hours prior to harvesting. Cells were harvested by using a semiautomatic cells harvestor (Flow Laboratories, USA) and samples were assessed for ^{3}H-thymidine incorporation by liquid scintillation spectrophotometry (LKB, Uppsala, Sweden).

Immunofluorescence (IF) test

The HEp-2 and macrophage cultures grown on coverslips and lymphocyte cultures were infected with RSV. At 2, 24, 48 and 72 hours after RSV infection, the infected cultures were examined for viral antigen by immunofluorescence.[18] The cells were stained with bovine polyclonal antisera to RSV (Wellcome, Dartford, UK) at 1:20 dilution and fluorescein isothiocyanate coupled goat anti-bovine IgG at 1:30 dilution, washed three times and observed under a fluorescence microscope.

Assay of NK cell activity

Unfractionated mononuclear leukocytes prepared from 4 cord blood specimens and 4 healthy adult blood specimens were exposed to RSV for 2 hours as described above. The infected leukocytes were reincubated in fresh medium for 2 days ·and then tested for NK cell activity by cytotoxicity assay on K-562, a human erythroid myeloid cell line as target.[20] The K-562 cells were washed and resuspended in RPMI 1640 medium supplemented with 10% FCS at a concentration of 5×10^6 cells/ml. The 5/10^6 ml of cells were labelled with 100 μCi of Na$_2$ ^{51}CrO$_4$ at 37° C for 1 hour, then washed 3 times and adjusted to a concentration of 1×10^5 cells/ml. To each round bottom well of the microtiter plate was delivered 0.1 ml of ^{51}Cr-labelled target cells and 0.05 ml of

leukocytes (1×10^7 cells/ml) as effector cells with an effector : target ratio of 50:1. The microtiter plates were incubated for 4 hours and centrifuged at 4° C, and $800 \times g$ for 10 minutes. The supernatant was removed and the amount of ^{51}Cr released was determined in a gamma counter. All assays were conducted in triplicate. NK cell activity was expressed as percent ^{51}Cr release as follows :

$$\% \text{ Specific } ^{51}\text{Cr release } = \frac{\text{test release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Statistical analysis

Analysis of variance (ANOVA) was used to determine the statistical significance of observed differences.
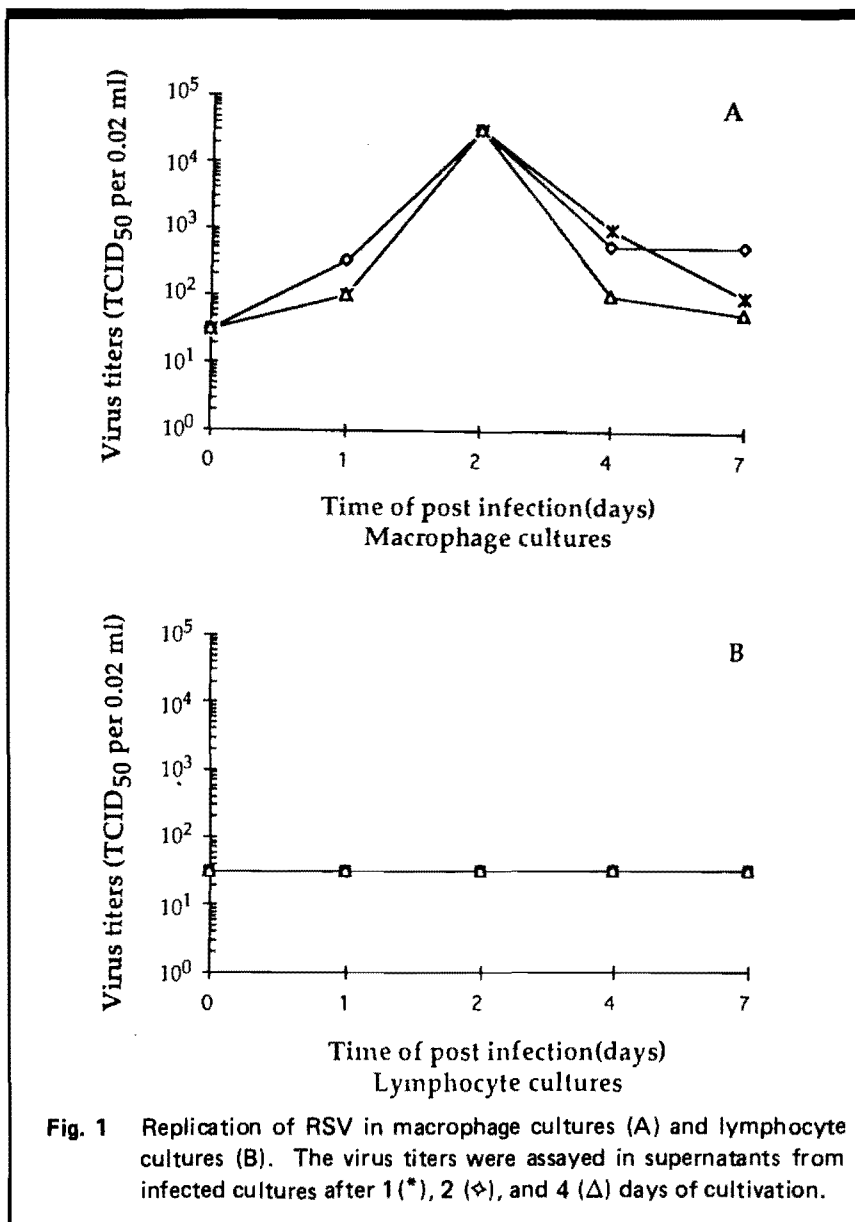
RESULTS

Replication of RSV in cord blood MNCs

Replication of RSV in macrophages and lymphocytes is shown in Fig. 1. The data demonstrated that RSV can replicate and produce infectious virus in macrophages. The virus titers from macrophage cultures as assayed in HEp-2 cell cultures increased from $10^{2.5}$ on day 0, to $10^{4.5}$, 10^3 and 10^2 TCID$_{50}$ per 0.02 ml after 2, 4 and 7 days of infection, respectively. The age of macrophage cultures (1, 2, and 4 days) did not directly affect replication of RSV (Fig. 1A). By contrast, lymphocytes did not support RSV replication. The data from Fig. 1B show that the virus titers from lymphocyte cultures did not increase throughout the experiments. The virus titers were undetectable from both uninfected macrophage and lymphocyte cultures in mock infections.

Lymphocyte transformation

The blastogenic responses of RSV-treated lymphocytes and uninfected (control) lymphocytes from three experiments are shown in Table 1. Lymphocytes exposed to RSV have significantly depressed responses

Fig. 1 Replication of RSV in macrophage cultures (A) and lymphocyte cultures (B). The virus titers were assayed in supernatants from infected cultures after 1(*), 2 (◇), and 4 (△) days of cultivation.

treated mononuclear leukocytes from cord blood (cord cells) and from adult blood (adult cells) were assayed for NK cell cytotoxicity. There was no significant difference between activities of infected cord cells and uninfected cord cells (73.5 ± 4.7 and 68.6 ± 4.9, p > 0.1). In addition, NK cell cytotoxicity of infected cord cells were similar to those of infected adult cells (75.3 ± 4.7 versus 74.9 ± 2.6, p > 0.1).

DISCUSSION

The interaction between human MNCs and RSV has been explored in only a small number of reports. Chonmaitree et al. [9] found that RSV is a poor inducer of interferon production. Roberts [12] showed that RSV had inhibitory effects on mitogen induced lymphocyte transformation. He also showed that RSV failed to induce interferon production in lymphocytes, while it could induce low levels of interferon secretion by macrophages. Domurat et al. [10] found that in vitro exposure of both lymphocytes and macrophages to RSV resulted in the expression of RSV antigens on the cell surface. They also found that RSV antigens could be detected in MNCs obtained from symptomatic children. Recently, Roberts et al. [13] reported that RSV can induced both the production of interleukin-1 and interleukin-1 inhibitors by human macrophages. Krilov et al. [11] examined the ability of RSV to proliferate in human monocytes-macrophages. They found that in vitro replication was greater in 2 to 4-day-old cultures than in 1-day-old cultures, but this could not be explained on the basis of interferon production.

The present study on cord blood MNCs show some similarities and differences with adult cells. The data clearly demonstrate the ability of RSV to replicate in neonatal macrophages as shown by the production of infectious viruses and expression of viral antigens. In contrast to adult lym-

to PHA stimulation as compared to control cells. One-day old lymphocytes infected with RSV for 1 day before PHA stimulation showed a response only 43.5% of that of the control. Cultures treated with PHA after 4 days of infection showed an even greater reduction in the response (11.6%). There was statistically significant inhibition of PHA stimulation by time of post-infection (p < 0.01), but not by age of culture (p > 0.1) as campared the infected lymphocyte cultures to the uninfected control cells.

Antigen expression in MNCs

RSV antigens were detectable in cord blood macrophages and HEp-2 cells at 24 hours after RSV infection (Table 2). However, the yield of RSV from macrophages was much less than that from HEp-2 cells (data not presented). By contrast, RSV antigens were undetectable on cord blood lymphocytes.

Effect of RSV on NK cell cytotoxicity

NK cell activity after RSV infected is shown in Table 3. RSV-

Table 1. RSV induced inhibition of mitogen stimulated lymphocyte transformation

Age of culture at infection (days)	Time of post—infection before PHA addition (days)	^{3}H-thymidine incorporation		
		Infected cells cpm*	Control cells cpm	Percent of response**
1	1	5,609 ± 1,992	12,890 ± 2,568	43.5
	4	4,131 ± 129	35,610 ± 1,454	11.6
2	1	6,048 ± 345	13,622 ± 545	44.4
	4	3,241 ± 133	22.501 ± 2,282	14.4
4	1	2,526 ± 288	12.825 ± 1,518	19.7
	4	1,320 ± 67	17,868 ± 1,886	7.4

*   The data represent mean (counts per minute) ± standard error from three experiments with triplicate determinations at 3 days after PHA were corrected by substracting with values of the corresponding cells incubated in medium without PHA.

**   Statistical analysis of ANOVA showed significant inhibition of ^{3}H—thymidine incorporation by RSV treated lymphocytes by time of post—infection ($p < 0.01$) but not age of culture ($p > 0.1$).

Table 2. Detection of antigen in RSV—infected cells by specific immunofluorescence

Time of post—infection (hours)	Presence of RSV antigen		
	Macrophages	Lymphocytes	HEp—2 cells
2	—	—	—
24	+	—	+
48	+	—	+
72	+	—	+

phocytes which seem to be susceptible to RSV infection as evidenced by antigen expression[10], neonatal lymphocytes exposed to RSV do not appear to support replication or express antigen. In addition, unlike adult macrophages where older cultures tend to be more supportive of RSV replication[11], neonatal macrophages were equally permissive at any age. Of interest to this discussion is the finding that measles virus replication does not increase in older cultures.[21]

Data from this study also show that like those of adult lymphocytes[16], the mitogenic responses of neonatal lymphocytes were significantly reduced in the presence of RSV. Furthermore, we found that long exposure of lymphocytes to RSV resulted in greater suppression of mitogen-induced lymphocyte transformation.

No previous study has examined the effects of RSV on NK cell cytotoxicity. Our data show that RSV has no discernible effect on NK cell cytotoxicity for either adult or neonatal NK cells. This finding suggests that this aspect of immune function may have a role in recovery from RSV infection.

In conclusion, our results indicate that neonatal MNCs differ to some extent from adult MNCs in regard to RSV infection. The data also show that RSV has preferential effects on human MNC subpopulations. Further studies on the *in vitro* interactions between RSV and immune cells may help to elucidate immune mechanisms in RSV infection which are not very well understood at present.

Table 3. Effect of RSV on NK cell cytotoxicity

Treatment	% Specific ^{51}Cr release	
	Cord cells	Adult cells
Infected cells	75.3 ± 4.7	74.9 ± 2.6
Uninfected cells	68.6 ± 4.9	70.3 ± 2.4

The data represent mean cpm ± standard error from 4 experiments, each performed in triplicare. There was no statistically significant difference between infected and uninfected cells (p > 0.1); neither was there diff—erences in NK cell cytotoxicity between cord blood and adult MNCs (p > 0.1).

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