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Natural cytotoxicity in the neonate: high levels of lymphokine activated killer (LAK) activity

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SUMMARY

In 28 healthy full-term newborns the percentage of circulating cells expressing the Leu7 antigen, the marker of natural killer (NK) cells, was significantly lower than in healthy adults. However, newborns and adults did not differ with regard to the percentage of cells reacting with the Leulla, Leullc and TEC NK-1, monoclonal antibodies directed against the IgG Fc receptor of killer cells. Spontaneous NK activity of neonatal cells was profoundly reduced compared to the adult. In contrast, antibody dependent cellular cytotoxicity and NK-like activity generated in mixed lymphocyte cultures were similar in the two groups and lymphokine-activated killer cell (LAK) activity was high in the neonate. Natural killing is thought to play an important role in antiviral immunity since the neonate has a deficient capacity to deal with viral infections. Consequently, the present data indicate either that spontaneous NK is the most informative *in vitro* measure of newborn natural cytotoxicity *in vivo*, or, alternatively, that natural killing is not as important in antiviral immunity as previously suggested.

Keywords cord blood lymphocytes NK cell subsets natural cytotoxicity

INTRODUCTION

Human natural killer (NK) cytotoxicity develops gradually during intrauterine life (Uksila *et al.*, 1983) and low NK activity as well as antibody dependent cell cytotoxicity (ADCC) have been reported in the neonate by some groups (Abo, Cooper & Balch, 1982; Uksila, Lassila & Hirvonen, 1982; Uksila *et al.*, 1983; Kohl, 1983; Kohl, Loo & Gonik, 1984; Vitiello *et al.*, 1984; Nair, Schwartz & Menon, 1985). Also the frequency of cells with the surface HNK-1 antigen, known to be expressed by human cells with NK activity, is very low in the neonate (Abo *et al.*, 1982; Uksila *et al.*, 1982; Vitiello *et al.*, 1984).

Cells with cytotoxic activity are thought to play an important role in antiviral immunity (Herberman & Holden, 1978) so that an impaired activity might contribute to the high susceptibility of the neonate to viral infections.

In this report we investigated NK activity, ADCC and natural cytotoxic activity generated in mixed lymphocyte cultures (MLC) (NK-like activity) and after interleukin 2 (IL-2) stimulation (LAK activity) of cord blood mononuclear cells. We also studied the distribution of cord blood lymphocytes (CBL) reacting with monoclonal antibodies MoAb Leu 7 (HNK-1), Leu 11a (NKP15), Tec NK-1 (AB8.28) and Leu 11c (B73.1).

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MATERIALS AND METHODS

Cell preparation

Mononuclear cells from cord blood of full-term healthy newborns and from peripheral blood of healthy adults were isolated by Ficoll-Hypaque density gradient as previously described (Maccario *et al.*, 1983). The cells were resuspended in RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol (complete medium).

Target cells

Cell lines used as target cells in cytotoxic assays included: K562 (human erythroleukaemia), P815 (mouse mastocytoma), Daudi (derived from a patient with Burkitt's lymphoma), and HL60 (human promyelocytic leukaemia). Cell lines were maintained by serial passages in complete medium supplemented with 10% fetal calf serum (FCS).

Membrane immunofluorescence with monoclonal antibodies

Leu7, Leu11a, Leu11c, were purchased from Becton Dickinson, Tec NK-1 was purchased from Techno Genetics.

Mononuclear cells (3×105) were resuspended in 0·2 ml of PBS supplemented with 0·01% sodium azide and 0·5% bovine serum albumin (BSA) and were incubated with the optimal dilution of each antibody for 30 min in an ice bath. The cell pellet was then washed twice and resuspended in 0·03 ml of PBS

containing the second antibody, a fluorescein-labelled goat/mouse (Bionetics) diluted 1/20 (v/v). The suspension was incubated for 30 min in an ice bath. The cell pellet was washed again, resuspended in a small volume of glycerol-PBS and was counted using a Leitz Orthoplan microscope equipped for epifluorescence and phase contrast. Two hundred lymphocytes were counted. Monocytes were excluded from the count by morphological criteria.

NK assay

The cytotoxicity was assayed in U-shaped wells of microtitre plates. K562 line were used as the target. Target cells (2×10^6) were labelled with 100 μCi of sodium ^{51}chromate solution (New England Nuclear) for 60 min at 37°C in 0·2 ml of RPMI supplemented with 10% FCS. The cells were then washed four times and resuspended in complete medium supplemented with 10% FCS. Then 5×10^3 cells in 0·1 ml were added to each well. CBL or adult peripheral blood lymphocytes (a-PBL) were used as effector cells. Various concentrations of effector cells in 0·1 ml of complete medium supplemented with 10% FCS were added to the wells so as to achieve an effector:target ratio of 100:1, 30:1, 10:1. The plates were centrifuged for 4 min at 200 ***g*** and incubated for 4 h at 37°C. After incubation the plates were centrifuged for 8 min at 200 ***g*** and 100 μl of the supernatant from each well was collected and counted for 1 min in a gamma counter. Maximal release was determined by freezing and thawing the target cells. The percentage of isotope released was calculated by the formula:

$$\% \text{ release} = \frac{\text{experimental release} - \text{spontaneous release} \times 100}{\text{total release} - \text{spontaneous release}}$$

Spontaneous release from the target cells was always less than 15%. A lytic unit (LU) was defined as the number of cells required to produce 30% specific cytotoxicity using 5×10^3 labelled target cells. The cytotoxic activity of each sample was expressed as the number of LU/106 effector cells.

ADCC assay

P815 cells sensitized with rabbit IgG anti-P815 were used as target cells. ^{51}Cr-labelled P815 cells ($1–2 \times 10^6$) were incubated with anti-P815 antiserum (1:10^5 dilution) for 30 min at room temperature, washed twice and resuspended in complete medium supplemented with 10% FCS. ADCC assay was performed under the same conditions as described for NK assay.

Generation of lectin-free MLA-144 supernatants rich in IL-2 and assay for IL-2 activity

The gibbon lymphosarcoma cell line MLA 144 has been shown to produce IL-2 spontaneously (Rabin *et al.*, 1981). MLA 144 cell line is maintained by serial passage in complete medium supplemented with 1 mM Na-Pyruvate, 1% nonessential amino acids and 10% FCS. Lectin-free MLA 144 crude supernatants, rich in IL-2, were prepared culturing the cells at the concentration of 10^6/ml and harvesting the culture supernatants 2 days later. The supernatants were centrifuged at 100,000 ***g*** for 2 h, filtered (0·2 μm, Flow Lab.) and stored at −70°C.

IL-2 activity of the lectin-free MLA 144 was measured using an IL-2 dependent CTL line (CTLL) as indicator line. These cells were plated at 5×10^3 cells per well in flat-bottomed microplates and the supernatants were added in six serial doubling dilutions (1:2 to 1:64 final dilution) in duplicate samples.

Controls included CTLL incubated alone or with a purified human IL-2 standard (Welte *et al.*, 1984) kindly provided by Dr K. Welte (Memorial Sloan-Kettering Cancer Centre, New York). The cells were incubated for 24 h and 0·5 μCi of tritiated thymidine (^{3}HTdR) (2 Ci/mmol) was added during the last 6 h. The cells were then harvested and ^{3}HTdR incorporation was measured by the standard procedure. The data were analysed against the IL-2 standard and expressed in units per ml.

LAK activation and cytotoxic assay

CBL and a-PBL were cultured in 24-well plates at a concentration of 2×10^6/ml in 2 ml of complete medium supplemented with 10% human AB serum and 50% of lectin-free MLA 144 supernatant rich in IL-2 (100 U/ml). Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 7 days cells were recovered and cytotoxicity was measured against K562, Daudi, HL-60 and P815 target cells under the same conditions as described for NK assay.

Allogeneic mixed lymphocyte reaction (MLR) and NK-like activation

Responder lymphocytes from CBL and a-PBL were cultured in 24 well plates at the concentration of 10^6/ml in complete medium supplemented with 10% human AB serum; allogeneic human mononuclear cells, used as stimulator, were irradiated with 2000 R and added at the same concentration in a total volume of 2 ml. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 7 days cells were recovered and NK-like cytotoxicity was measured against HL-60, K562, Daudi and P815 target cells under the same conditions as described for NK assay. To measure cell proliferation generated in MLR, 10^5 responder cells were cultured in triplicate samples with 10^5 stimulator cells in U shaped wells of microtitre plates. After 6 days incubation the cultures were labelled for 21 h with 0·5 μCi/well ^{3}HTdR and were then harvested by the standard procedure.

Statistical analysis

Student's *t*-test was adopted for the analysis of unpaired samples. Values before $P < 0{\cdot}05$ were considered significant.

RESULTS

The results obtained from 28 newborns and 28 adult controls confirmed previous findings of a low percentage of Leu7$^+$ lymphocytes in cord blood; nevertheless the percentages of Leu11a$^+$, Leu11c$^+$ and TEC NK$^+$ cells were the same in the two groups (Table 1).

Spontaneous NK activity against K562 was significantly lower in CBL than in a-PBL (Fig. 1), while Daudi, HL60, and P815 targets were resistant to spontaneous NK activity of both a-PBL and CBL.

ADCC against the P815 cell line sensitized with rabbit IgG anti-P815 showed comparable results in CBL and a-PBL (Fig. 2).

In the MLC used to generate NK-like activity, all CBL and a-PBL proliferated in response to the allogeneic stimulus (data not shown); mean values of the NK-like activity generated in MLC were similar in CBL and a-PBL; nevertheless CBL showed a wide range of responses with some neonates displaying very

Table 1. Proportions of Leu 7$^+$ (HNK-1), Leu 11a$^+$ (NK15) Leu 11c$^+$ (B73.1) and TEC NK1 (AB8.28) in human cord blood (CBL) (28 subjects) and in adult peripheral blood (a-PBL) (28 subjects) mononuclear cells

	Leu7$^+$	Leu11a$^+$	Leu11c$^+$	TEC NK1$^+$
CBL ($n=28$)	4±2	24±6	19±11	22±13
a-PBL ($n=28$)	18±7	23±6	21±7	26±5
P	0·001	NS	NS	NS

Results are expressed as mean values ± s.d. of the percentage of positive cells. Number of subjects in parenthesis. P values were calculated by Student's t-test. NS, not significant.

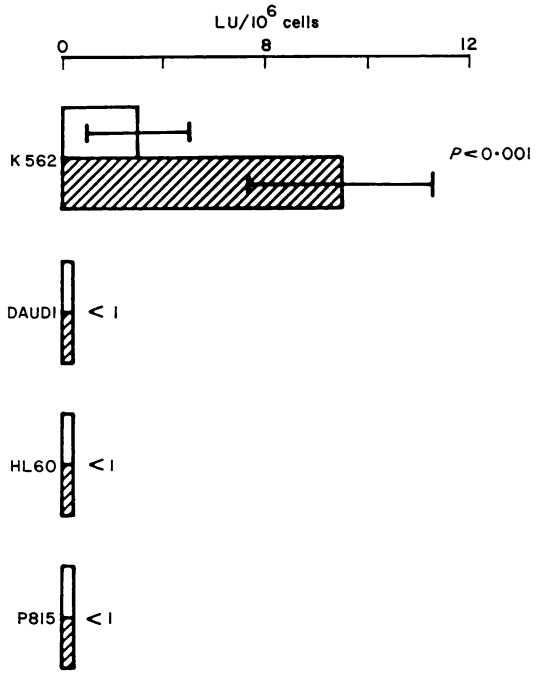

Fig. 1. Spontaneous NK activity produced by CBL (□, 28 subjects) and a-PBL (■, 28 subjects). Results are expressed as lytic units per million of effector cells (LU/10^{-6}). Mean ± 1s.d. P values by Student's t-test.

low levels of NK-like activity. Daudi and P815 target cells were resistant to NK-like activity (Fig. 3).

LAK activity was significantly higher in CBL than in a-PBL (Fig. 4) against both K562, HL60 and Daudi target cells, while the P815 cell line was resistant to LAK activity.

DISCUSSION

The results of this study show that spontaneous NK activity against K562 targets is significantly lower in CBL than in a-PBL, as previously described by several groups (Abo *et al.*, 1982; Uksila *et al.*, 1982; 1983; Kohl, 1983; Vitiello *et al.*, 1984; Kohl *et al.*, 1984; Nair *et al.*, 1985), but other natural cytotoxic functions are adequate in the neonate.

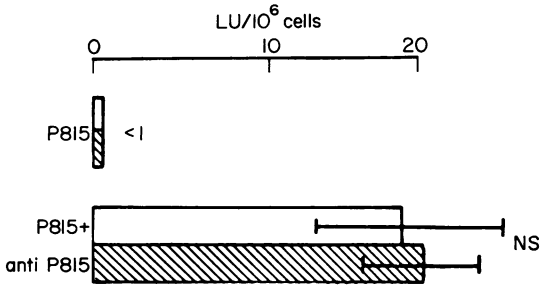

Fig. 2. Characterization of ADCC produced by CBL (□, 28 subjects) and a-PBL (■ 28 subjects). Results are expressed as lytic units per million of effector cells (LU/10^{-6}). Mean ± 1s.d. NS, not significant; P values by Student's t-test.

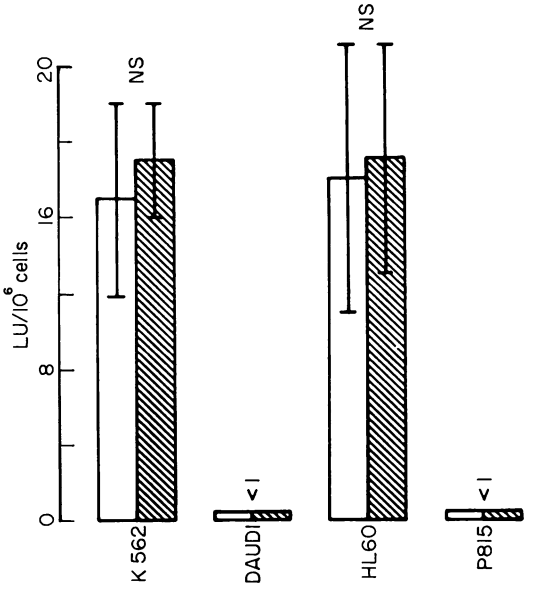

Fig. 3. Characterization of NK-like activity produced by CBL (□, 28 subjects) and a-PBL (▩ 28 subjects) activated by allogeneic cells in MLC. Results are expressed as lytic units per million of effector cells (LU/10^{-6}). Mean ± 1s.d. are reported, NS, not significant. P values by Student's t-test.

In fact ADCC and NK-like activity are comparable in CBL and a-PBL, while LAK activity is significantly higher in the neonate than in adult subjects. Our finding of normal ADCC in CBL is in contrast with the results of impaired activity reported by several authors (Kohl, 1983; Kohl *et al.*, 1984; Nair *et al.*, 1985). This discrepancy may result from differences in the target cells employed for the ADCC assay because most other studies were carried out with virus-infected targets. Neonatal cells have been reported to show a limited capacity for specific T cell-mediated lympholysis against semiallogeneic and allogeneic target cells and it has been hypothesized that neonatal cytotoxic T lymphocytes are immature or inhibited by suppressive factors (Jacoby, Olding & Oldstone, 1984). It is well known that responders cells generated in the MLC also show cytotoxicity against target cells not carrying the alloantigens of the stimu-

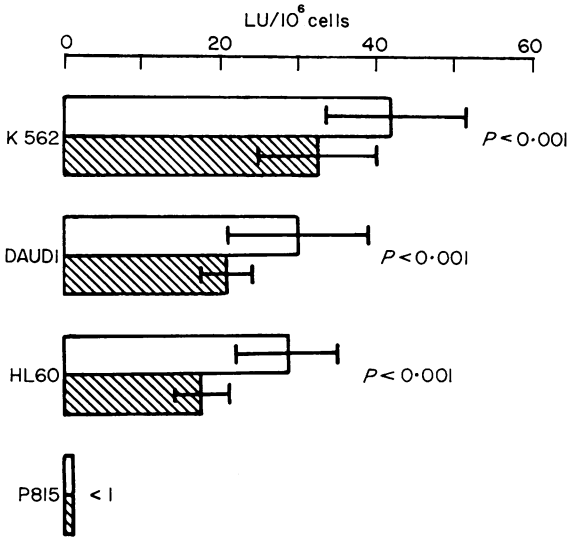

Fig. 4. Characterization of LAK activity produced by CBL (□, 28 subjects) and a-PBL (▨, 28 subjects). Results are expressed as lytic units per million of effector cells ($LU/10^{-6}$). Mean ± 1s.d. *P* values by Student's *t*-test.

lator cells and this cytotoxicity has been called NK-like activity (Lopez *et al.*, 1982; Seeley & Golub 1978; Poros & Klein 1979; Ortaldo *et al.*, 1979; Masucci, Klein & Argov, 1980). It has been demonstrated that both cytotoxic T lymphocytes (Moretta *et al.*, 1984) and NK-cells (Phillips, Le & Lanier, 1984) are able to mediate NK-like activity and in particular Phillips *et al.* (1984) have demonstrated that the majority of cells mediating NK-like activity express the Leu11 surface antigen and that the precursors of these NK cells are $Leu7^-$, $Leu11^+$. We found that NK-like activity generated in MLC is within the normal range in CBL from the majority of the neonates tested. However in agreement with Chin *et al.* (1986) who described lessened allogeneic cell-induced cytotoxicity in cord blood we also found same neonates with very low levels of NK-like activity.

Therefore in the present study the average NK-like activity of CBL was not significantly different from that of a-PBL as a result of the presence of 'high responder' as well as 'low responder' suggesting that neonates are quite heterogeneous in respect to the distribution of precursor cells mediating NK-like activity generated in MLC.

There is evidence that LAK activity represents a cytotoxic phenomenon distinct both from specific T-cell-mediated lympholysis and spontaneous NK activity or NK-like activity (Grimm *et al.*, 1982; 1983a,b; Grimm & Wilson, 1985; Itoh *et al.*, 1985; Rosenstein *et al.*, 1984). The characteristics of precursor cells generating LAK activity is still a matter of debate. Grimm *et al.* (1983) have shown that LAK precursors are $Leu7^-$, $T3^-$, $T8^-$ and $OKM1^-$, while LAK effectors cells express T3 and T8 antigens. Moretta *et al.* (1986) have reported that 1 out of 25 human blood T cells is a LAK precursor. However Ortaldo, Mason & Overton (1986) have demonstrated that several lymphocyte subsets including both T and NK cells, can mediate LAK activity: the cells mediating LAK activity are 10–50 times more frequent among NK cells than among T cells.

It has been suggested that LAK activity is of crucial importance in immune surveillance: the high activity in the neonate may be related to the importance of 'self-non-self' discrimination against the potential invasion by semiallogeneic maternal cells.

On the whole, our results suggest that in the neonate natural cytotoxic activity is probably adequate, at least under the conditions used in our laboratory tests, with the exception of spontaneous NK activity. As many viral infections acquired during the neonatal period have a severe clinical course or tend to become chronic, our present findings suggest either that natural cytotoxic mechanisms are on the whole of little importance in antiviral immunity or alternatively that only spontaneous NK activity against K562 is a good *in vitro* correlate of natural cytotoxicity *in vivo*.

Together with the finding that CBL include a low percentage of $HNK\text{-}1^+$ lymphocytes but an adult proportion of cells with Fc receptors (Malavasi *et al.*, 1986; Perussia *et al.*, 1983; Lanier *et al.*, 1983) measured with three different monoclonal antibody, our data suggest that the distribution of NK subsets in the neonate differs from that of the adult and that spontaneous NK activity is associated with the functional activity of a subset expressing the HNK-1 antigen (Abo & Balch, 1981; Abo *et al.*, 1982; Allavena & Ortaldo, 1984).

Other natural cytotoxic activities may be related to the presence of NK and NK-like subsets expressing the Fc receptor of NK cells but not the HNK-1 antigen.

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