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Possible Roles of LFA-1 and Fcγ Receptors on the Functional Immaturities of Cord Blood Polymorphonuclear Leukocytes

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The functional immaturity of PMNs is one of the major causes of overwhelming sepsis in newborns. In this study, we observed functions and surface markers of PMNs to investigate what causes the functional immaturity of PMNs in newborns. As results, the percentage of EA rosette forming PMNs (58.5 ±15.5%) and the chemotactic movement (0.14±0.09 mm) of cord blood PMNs were significantly lower than those of adult peripheral blood PMNs (70.8±9.9%, 0.60±0.34 mm). Cord blood PMNs showed decreased glass adherence and ADCC activity. The expression of FcγRII or FcγRIII was a little lower than those of adult peripheral blood PMNs, but the expression of FcγRI (43.1±26.8%) was significantly higher than that of adult peripheral blood PMNs (3.2±1.8%). There was a significant difference in LFA-1 expression between EA rosette forming PMNs (92.9±9.1%) and EA rosette non-forming PMNs (25.6±22.6%). From these results, it is assumed that neonatal PMNs may consist of heterogeneous populations. And the relatively high percentage of EA rosette non-forming PMNs which express a low level of LFA-1 may be responsible for the functional immaturity of cord blood PMNs.

Key Words: Cord blood PMNs, rosette forming cells, Fcγ receptors, LFA-1

Bacterial infection is one of the major causes of morbidity and mortality in newborn infants. Polymorphonuclear leukocytes (PMNs) are the important cellular elements which defend against bacterial invasion in the primary defence, and the transfusion of PMNs diminishes the mortality rate in neonatal infection (Stegano *et al.* 1985). But the causes of the immunological immaturity of PMNs in neonates are not clearly identified yet.

The chemotaxis to N-formyl-methionyl-leucyl-phenylalanine (fMLP) and glass adherence were reported to be low in PMNs from neonatal blood (Krause, 1986). The percentage of erythrocyte-antibody (EA) rosette forming PMNs was low in neonates (Klempner and Gallin, 1978).

The IgG Fc (Fcγ) receptor is a member of the immunoglobulin supergene family, and three different kinds of human Fcγ receptors, FcγRI, FcγRII, and FcγRIII, are identified. These receptors mediate phagocytosis (Lewis *et al.* 1986; Ravetch *et al.* 1986) and the removal of immune complexes (Anderson *et al.* 1986; Ravetch *et al.* 1986) by PMNs. Leukocyte function associated antigen-1 (LFA-1) is one of the adhesion molecules which is necessary for PMNs for chemotaxis (Marlin *et al.* 1986; Jutila, 1989), random migration (Springer and Anderson, 1986), and adhesion to vascular endothelial cells (Forsyth and Levinsky, 1989; Lo *et al.* 1989[a,b]; Smith *et al.* 1989).

It has already been suggested that PMNs are

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not a homogeneous population, and may be classified into several subpopulations according to phagocytosis (Seligmann *et al.* 1985), glass adherence (Krause *et al.* 1986), chemotaxis (Krause *et al.* 1986), motility (Krause *et al.* 1989), and the expression of the Fcγ receptor on the cell surface (Masuda *et al.* 1989) which mediate antibody dependent cellular cytotoxicity (ADCC).

To evaluate functions of PMNs in neonates, we investigated chemotaxis, glass adherence, EA-rosette formation, and ADCC in cord blood PMNs. And surface markers involved in inflammatory responses, the expression of Fcγ receptors and LFA-1, were observed.

MATERIALS AND METHODS

Separation of PMNs

Cord blood (36 cases) was obtained from the umbilical vein of placenta after normal delivery at Cha Womens' Hospital from 1990 to 1991. Adults peripheral blood (27 cases) was obtained from healthy persons aged from 20 to 35 years. Sodium heparin (20 U/ml blood) was used as an anticoagulant.

PMNs and red blood cells were separated by Ficoll/Hypaque (Phamacia, Piscataway, NJ) density gradient (Boyum, 1968) from mononuclear cells. PMNs were separated from red blood cells by gradient with 5% dextran (Mwt 250,000, Sigma Chemical Co., St. Louis, MA). PMNs were washed with RPMI 1640 and residual red blood cells were depleted by hypotonic lysis (Shen *et al.* 1987).

Assay for EA rosette formation

The EA rosette formation was done as previously described (Klempner & Gallin, 1978). In brief, hemolysin (Gibco, Grand Island, NY) diluted to 1 : 400 was added to the same volume of sheep erythrocytes suspension (1×10^9 cells/ml) and incubated at 37℃. After 30 min, EA was washed twice with cold RPMI 1640 and the EA number was adjusted to 4×10^8 cells/ml. Then, PMNs (1×10^6 cells/100 μl) and EA cells (4×10^6 cells/100 μl) were mixed and incubated for 15 minutes at room temperature and the supernatant was discarded after centrifugation at $60\times g$ for 10 minutes. Cells were resuspended in PBS containing 0.2% bovine serum albumin. More than 200 PMNs were counted under 400× magnification. The cells to which three or more erythrocytes were attached were counted as EA rosette forming cells.

To separate EA rosette forming PMNs and EA rosette non-forming PMNs, EA-PMNs suspensions were placed over Ficoll-Conray (Mallinckrodt Canada Inc., Pointe Claire, Canada) discontinuous density gradient solution in a 15 ml tube. The Ficoll-Conray discontinuous gradient solution consisted of solution I and II. Solution I contained 11.2 g Ficoll (Mwt 400,000, Sigma Chemical Co., St. Louis, MA) and 20 ml of Conray (Maglumine iothalamate, 600 mg/ml, Mallinckrodt Co., St. Louis, MO) in 100 ml of distilled water. Solution II contained 7.2 g Ficoll and 20 ml of Conray in 100 ml of distilled water. Three ml of solution II was layered over 3 ml of solution I. Cell suspensions were then layered over solution II and centrifuged at $1,500\times g$ for 20 minutes at 4℃. The EA rosette nonforming cells were at the interface between the two Ficoll-Conray solutions and the EA rosette forming cells were at the bottom. Each fraction was collected and washed twice with RPMI 1640. EA were removed from rosette forming PMNs by hypotonic lysis.

Assay for chemotaxis

Chemotaxis was done by the under-agarose method (Nelson *et al.* 1975). Briefly, two percent of melting agarose was mixed with same volume of 2× concentrated PRMI 1640 containing 4 mM L-glutamine and 0.5% (wt/vol) gelatin. Wells with diameter 2.4 mm were punched off from the agarose and 10 μl of PMNs (2×10^7 cells /ml) was put into center wells, and 10 μl of 10^{-7} M fMLP (Mwt 437.6, Lot No. 48F-5805, Sigma Chemical Co., St. Louis, MA) was put into the peripheral wells. After incubation for 2 hrs in a 37℃, 5% CO_2 incubator, cells were fixed with 3 ml of 0.3% glutaraldehyde (Merck, Munchen, Germany) and stained with 2% Giemsa solution (Fluka, Buchs, Germany). The migration distances were measured by an image anlyzer with program of VIDS (Analytical Measuring Systems Ltd., Pampisford, England). The PMNs migration distance was calculated as follows.

Migration distance=
distance to fMLP−
distance to the opposite side to fMLP

Assay for ADCC

ADCC was done as previously described (Shen *et al.* 1987). Briefly, 100 μl of ^{51}Cr sodium chromate (1mCi/ml, New England Nuclear, Boston, MA) labeled target L1210 cells (1×10^4 cells/well), 50 μl of PMNs effector cells, and 50 μl of anti-L1210 antibody (1:50 diluted from heatin-activated serum of NZW rabbit immunized with L1210 cells) were mixed. The various ratios of effector cell to target cell (12.5:1, 25:1 and 50:1) were tested. After 4 hrs of incubation, 100 μl of supernatant was removed for estimation of ^{51}Cr release. Percent cytotoxicity was calculated as follows.

$$\%\ ADCC = \frac{\text{release by PMNs and antibody} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Assay for glass adherence

Twenty μl of PMNs supension (1×10^6 cells/ml) was dropped onto a ring slide and incubated in a 37℃, CO_2 incubator for 45 min. After incubation, the slide was gently washed twice with warm RPMI 1640, and stained with 2% Giemsa solution. The control slide was not washed. PMNs were counted on five different fields (under 400× magnification) (Krause *et al.* 1982). The percentage of PMNs adherence was calculated as follows.

$$\%\ \text{adherence} = \frac{\text{No. of PMNs on the washed slide}}{\text{No. of PMNs on the unwashed slide}} \times 100$$

Assay for Fcγ receptors and LFA-1

PMNs were washed three times with PBS and adjusted to $1\sim2\times10^6$ cells/ml in a microfuge tube, and mixed with 20 μl of 10% human AB serum.

For LFA-1 assay, 20 μl of anti-LFA-1 monoclonal antibody (DAKO, Glostrup, Denmark) diluted to 9.4 μg/ml was added. The mixture was incubated for 1 hr at 4℃ and washed with cold PBS. Four μl of FITC-labeled goat antimouse immunoglobulin (Becton Dickinson, Mountain View, CA) was added and incubated for 1 hr at 4℃. After washing three times with cold PBS and resuspending in 1 ml of PBS, cytofluorograph analysis was done by FACStar (Becton Dickinson, Mountain View, CA). For Fcγ receptor analysis, anti-FcγRI 32.2 (Anderson *et al.* 1986), anti-FcγRII IV.3 (Looney *et al.* 1986), and anti-FcγRIII 3G8 (Fleit *et al.* 1982) monoclonal antibody (Medarex Inc., West Lenanon, NH) diluted to 20 μg/ml were used. The purity of PMNs was 98% or more.

Statistics

Differences of neonatal cord PMNs from normal adult PMNs in EA rosette formation, chemotaxis, ADCC, glass adherence and the expression of LFA-1 and Fcγ receptor were compared by using Student's two-tail t-test.

RESULTS

EA rosette formation

The mean percentage of EA-rosette forming cells in cord blood PMNs (58.5±15.8%) was significantly lower than that in normal adult peripheral blood PMNs (70.8±9.9%) ($p=0.015$). Moreover seven cases out of 19 cases (36.8%) in cord blood PMNs were below the lower limit which is determined by substracting 2 SD from the mean percentage of adult peripheral blood PMNs (Fig. 1).

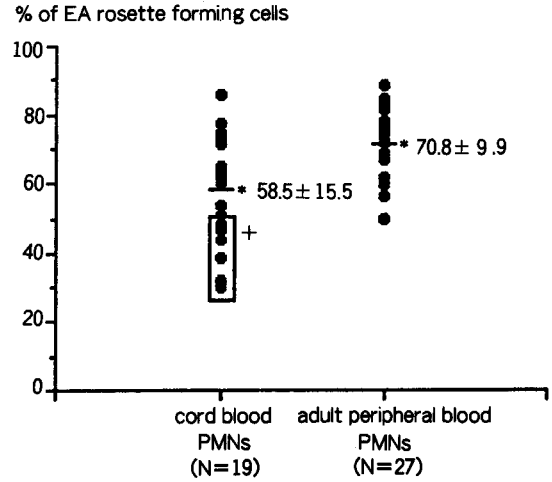

Fig. 1. *Percentage of EA rosette forming PMNs from cord blood or adult peripheral blood. All values are expressed as mean±SD. *Each bar indicates the mean percentage of EA rosette forming PMNs. +Bar means the low limit of 2 SD form the mean percentage of EA rosette forming PMNs from adult peripheral blood.*

Table 1. Chemotactic ability of PMNs from cord blood or adult peripheral blood

PMNs	No.	Migration distance (mm)
Cord blood	31	0.14±0.09*
Adult peripheral blood	17	0.60±0.34

Assay for chemotaxis was done by under agarose assay with fMLP as a chemoattractant. Mean migration distance was measured by the image analyzer and are expressed as mean±SD.
*p=0.001

Table 2. Glass adherence of PMNs from cord blood or adult peripheral blood

RMNs	No.	Adherence (%)
Cord blood	19	79.8±16.3*
Adult peripheral blood	11	91.9±18.9

Assay for adherence was done by incubating PMNs in ring slides at a 37℃ CO_2 incubator for 45 min. PMNs were counted under 400×magnification. Adherences are expressed as mean±SD.
*p=0.069

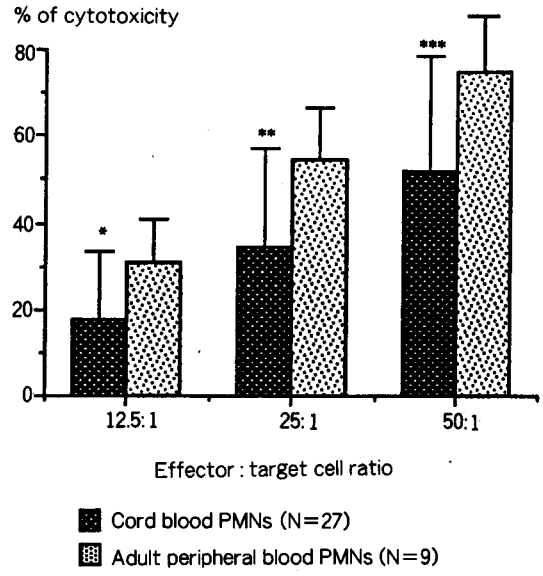

*Fig. 2. ADCC activity by PMNs from cord blood or adult peripheral blood. ADCC was measured by 4-hour ^{51}Cr release assay using anti-L1210 cell antibody and L1210 cells as target cells. *p=0.04, **p=0.01, ***p=0.02*

Chemotaxis

The migration distance of cord blood PMNs (0.14±0.09 mm) was significantly lower than that of adult peripheral blood PMNs (0.60±0.34 mm) (p=0.001) (Table 1).

Glass adherence

The mean percentage of glass adherence of cord blood PMNs (79.8±16.3%) was lower than that of adult peripheral blood PMNs (91.9±18.9%) (p=0.069) (Table 2).

ADCC

ADCC of cord blood PMNs was significantly lower than that of adult peripheral blood PMNs. The mean percentage of ADCC by cord blood PMNs were 20.7±17.5%, 34.7±21.6%, and 52.2±25.8% for the effector: target cell ratios of 12.5:1, 25:1, and 50:1, respectively. The mean percentage of ADCC by the adult peripheral blood PMNs were 34.2±18.4%, 54.3±18.3%, and 74.9±14.9% (p=0.04, p=0.01, p=0.02, respectively) (Fig. 2).

Assay for Fcγ receptor expression

The mean percentage of FcγRI positive cells was higher in cord blood PMNs (43.1±26.8%) than that of adult peripheral blood PMNs (3.2±1.8%). The mean percentage of FcγRII (84.6±16.2%) and FcγRIII (86.1±14.7%) positive cells were lower in cord blood PMNs than those in adult peripheral blood PMNs (90.3±7.9%, 91.0±6.0%, respectively) (Table 3).

Assay for LFA-1 expression

The mean percentage of LFA-1 positive cells was 86.9±11.9% in cord blood PMNs and 90.0±5.8% in adult peripheral blood PMNs (p=0.1) (Fig. 3) which shows no significant difference. But LFA-1 expression in six cases out of 36 cord blood PMNs (16.7%) were below the lower limit which is determined by substracting 2 SD from the mean percentage of adult peripheral blood PMNs.

After cord blood PMNs were separated into EA rosette forming and EA rosette non-form-

Table 3. Expression of Fcγ receptor of PMNs from cord blood or adult peripheral blood

PMNs	No.	Fcγ receptor expression (%)		
		huFcγI	huFcγII	huFcγIII
Cord blood	33	43.1±26.8*	84.6±16.2**	86.1±14.7***
Adult peripheral blood	21	3.2± 1.8	90.3± 7.9	91.0± 6.0

Fcγ receptor expression was measured by FACStar using anti-Fcγ receptor monoclonal antibody as the primary antibody and FITC-conjugated anti-mouse immunoglobulin as the secondary antibody. Fcγ receptor expressions are expressed as mean±SD.

*p=0.000, **p=0.332, ***p=0.281

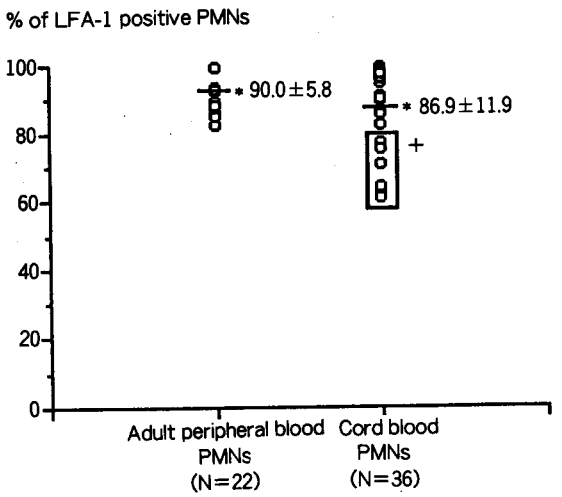

Fig. 3. *LFA-1 expression of PMNs from cord blood or adult peripheral blood. *Each bar indicates the mean percentage of LFA-1 positive PMNs. + Bar means the low limit of 2 SD from the mean percentage of LFA-1 positive PMNs from adult peripheral blood.*

Table 4. Expression of LFA-1 in EA rosette forming PMNs and EA rosette non-forming PMNs from cord blood

	No.	LFA-1 expression(%)
EA rosette forming PMNs	10	92.9± 9.1*
EA rosette non-forming PMNs	14	25.6±22.6

LFA-1 expression was measured by FACStar using anti- LFA-1 monoclonal antibody as the primary antibody and FITC-conjugated anti-mouse immunoglobulin as the secondary antibody. LFA−1 expressions are expressed as mean±SD.

*p=0.000

ing cells, the expression of LFA-1 was studied. The mean percentage of LFA-1 positive cells in EA rosette forming PMNs (92.9±9.1%) was significantly higher than that in EA rosette non-forming PMNs (25.6±22.6%) (p=0.000) (Table 4).

DISCUSSION

The functions of PMNs in neonates were reported to be lower compared to those of adult peripheral blood PMNs in chemotaxis, adherence to glass and EA rosette formation (Krause *et al.* 1982, 1986; Masuda *et al.* 1989). But there is no clear evidence that functional immaturity of PMNs in neonates is responsible for the susceptibility of bacterial infections in newborns.

In this study, we observed EA rosette formation, chemotaxis, glass adherence, and ADCC activity by cord blood PMNs. As results, all of them showed a significantly lower level in cord blood PMNs compared to those in adult peripheral blood PMNs. The results suggest definitely functional immaturity in cord blood PMNs.

And surface markers mediating immune functions in PMNs were studied to find out the association with this functional immaturity.

FcγRI, FcγRII and FcγRIII belong to the immunoglobulin supergene family (Unkeless *et al.* 1988). FcγRI is a high affinity receptor

known as CD64 which is expressed on monocytes and macrophages. It is expressed relatively less on PMNs than on monocytes (Guyre *et al.* 1983; Perussia *et al.* 1984; Maluish *et al.* 1988; Petroni *et al.* 1988). FcγRII known as CDw32 is classified as FcγRIIa, FcγRIIa′, and FcγRIIb (Ravetch and Anderson, 1990). FcγRIIa and FcγIIa′ are expressed on PMNs, monocytes, and macrophages with low affinity, and mediate phagocytosis and ADCC. FcγRIII is known as CD16, and classified as FcγRIII1 and FcγRIII2, and the FcγRIII1 is expressed on PMNs with low affinity (Ravetch and Anderson, 1990).

In this study, the numbers of FcγRII and FcγRIII positive PMNs were a little lower in cord blood compared to those in adult peripheral blood PMNs. But the number of FcγRI positive PMNs in cord blood PMNs was significantly higher than in that of adult peripheral blood PMNs although the fluorescence intensity of FcγRI was weaker than FcγRI and FcγRIII generally (data not shown). For FcγRI, it has been reported that the expression is increased in PMNs from patients with group A β-hemolytic streptococci infection (Kimura *et al.* 1987) because streptococci induces IFN-γ which increases FcγRI expression on PMNs, while the number of FcγRII and FcγRIII remain nearly unchanged. Thus one must consider the possibility that some factors from the mother such as IFN-γ might influence neonatal PMNs to show the high level of FcγRI in this study. And if there is no evidence for such factors influencing FcγRI expression, it should be studied why cord blood PMNs show a low percentage of EA rosette forming cells and why neonates are susceptible to bacterial infections in spite of the high expression of FcγRI.

LFA-1 mediates cell adhesion to vascular endothelial cells (Mentzer *et al.* 1986; Forsyth and Levinsky, 1989; Lo *et al.* 1989[a,b]; Smith *et al.* 1989), migration (Springer and Anderson, 1986), and ADCC (Hildreth *et al.* 1983; Kohl *et al.* 1984) in PMNs. We observed that there was no significant difference in the mean percentage of LFA-1 expression in PMNs from cord blood compared to that from adult peripheral blood. But interestingly, LFA-1 expression in six cord blood PMNs were below the lower limit which was determined by subtracting 2 SD from the mean percentage of adult peripheral blood PMNs. After cord blood PMNs were separated into two groups by EA rosette formation, we found that EA rosette forming PMNs showed a higher percentage of LFA-1 positive cells (92.9 ±9.1%) compared to EA rosette non-forming PMNs (25.6±22.6%). It suggests that EA rosette non-forming PMNs which show the low level of LFA-1 or no LFA-1 may be responsible for functional immaturity in cord blood PMNs. And the lower expression of FcγRII and LFA-1 in EA rosette non-forming PMNs which possessed high proportion of cord blood PMNs might be the causes of the low immunity in neonates. And these results showed that cord blood PMNs might be consisted of heterogeneous populations.

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