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Heterogeneity of Fc Receptor Expression in Chemotaxis and Adherence of Neonatal Neutrophils

KIYOKAZU MASUDA, YO KINOSHITA, AND YOHNOSUKE KOBAYASHI

Department of Pediatrics, Kansai Medical University, Osaka 570, Japan

ABSTRACT. Chemotaxis and adherence to polymorphonuclear neutrophils (PMN) subpopulations from cord blood of 13 healthy neonates and blood of 16 healthy adults as control subjects were determined using the rosette-forming procedure, the modified Boyden method, and ^{51}Cr adherence assay. The percentage of rosette-forming neutrophils (RFN) (Fc receptor expression) of cord PMN (35 ± 8%) was significantly lower than that of adult PMN (60 ± 4%, $p < 0.01$). Differences in chemotaxis of PMN subpopulations between cord and adult PMN after stimulation by their own endotoxin-activated plasma were as follows: (i) unfractionated adult PMN (A) ($n = 10$) *versus* adult RFN (B) ($n = 5$) *versus* adult nonRFN (C) ($n = 5$); (A) < (B), $p < 0.01$ and (A) > (C), $p < 0.01$; (ii) unfractionated cord PMN (a) ($n = 5$) *versus* cord RFN (b) ($n = 5$) *versus* cord non-RFN (c) ($n = 5$); (a) < (b), $p < 0.01$ and (a) *versus* (c), NS; (iii) (A) > (a), $p < 0.01$; (iv) (B) > (b), $p < 0.05$ and (C) *versus* (c), NS. Similarly, differences in adherence were as follows: (i) (A) ($n = 13$) *versus* (B) ($n = 4$), NS and (A) > (C) ($n = 4$), $p < 0.01$; (ii) (a) ($n = 4$) *versus* (b) ($n = 4$), NS and (a) > (c) ($n = 4$), $p < 0.01$; (iii) (A) > (a), $p < 0.05$; (iv) (B) *versus* (b), NS and (C) *versus* (c), NS. These results suggest that differences in chemotaxis and adherence between cord and adult PMN may relate in part to differences of PMN subpopulations. (*Pediatr Res* 25:6–10, 1989)

Abbreviations

RFN, rosette-forming neutrophils
non-RFN, nonrosette-forming neutrophils
PMN, polymorphonuclear neutrophils
EAP, endotoxin-activated plasma
LPS, lipopolysaccharide
RBC, red blood cells
EA, erythrocytes sensitized with antibody

Decreased PMN chemotaxis and adherence in neonates are an important cause for increased susceptibility of neonates to overwhelming bacterial infections. Although the abnormalities of both the cellular and humoral immune systems have been demonstrated (3, 5–9), the mechanism of decreased chemotaxis and adherence is not well understood. The cinevideo assay (10) and Zigmond's method (11) prove that from the point of chemotaxis neutrophils have at least slowly and fast moving cells. In other words it is assumed that neutrophils that have so far been considered to be homogeneous cells have two heterogeneous subpopulations (12–14). Wong *et al.* (15) first investigated the method to examine the Fc receptor, forming EA rosettes which were assayed with rabbit antisheep RBC IgG.

Developing a modified method with rabbit antihuman RBC IgG, Klempner *et al.* (16) separated neutrophils into RFN and non-RFN populations and found that the RFN were functionally superior to the non-RFN. Using this method, we studied Fc receptor expression in PMN chemotaxis and adherence in neonates and adults. We noted a significantly lower Fc receptor expression and lower chemotactic and adherent response in neonates than in adults. Our results suggest that decreased PMN chemotaxis and adherence in neonates may relate in part to differences of PMN subpopulations.

MATERIALS AND METHODS

Blood samples. Blood (5–10 ml) was collected with 20 U/ml of heparin from the placental end of the cut umbilical cords of 13 healthy neonates (seven males and six females, body wt 2700–3500 g, gestational age 38–41 wk, APGAR score 8–10) at normal delivery. They were in good health after the APGAR score had been obtained and were cared for in the Department of Obstetrics and Gynecology of Kansai Medical University Hospital. Informed consent was obtained from mothers. For control studies, 5–10 ml of venous blood was obtained from 16 healthy adult donors.

Isolation of neutrophils. Neutrophils were isolated by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density centrifugation followed by dextran sedimentation described by Böyum (17). Residual erythrocytes were hypotonically lysed with distilled water. Finally, cells were suspended in 2 ml of RPMI 1640 (pH 7.30, GIBCO, Santa Clara, CA) at 1×10^7 cells/ml. The viability was more than 96%, as judged by trypan blue exclusion, and the purity was more than 97%.

Preparation of EAP. Escherichia coli LPS (Sigma) was placed into a 40 μg/20 μl/tube and stored at −80°C until needed. In the assay, 0.4 ml of plasma from cord or adult blood was added to a tube containing LPS and it was incubated for 30 min at 37°C. The plasma was then inactivated at 56°C in a water bath for 30 min. In addition, 1.6 ml of McCoy's 5A media (GIBCO) with 10% FCS (Sigma) was added to make a final dilution of LPS of 20 μg/ml.

Preparation of EA. Erythrocytes were obtained from healthy adult donors (blood type O) and stored in Alsever's solution in the cold. RBC were washed three times in PBS (0.163 M, pH 7.30) and adjusted to contain 1×10^9 RBC/ml in RPMI 1640. Cells were counted by a microcell counter CC-130 A (Toa Medical Electronics Corporation, Japan). Rabbit antihuman

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Correspondence and reprint requests to Kiyokazu Masuda, Department of Pediatrics, Kansai Medical University, Fumizonocho 1, Moriguchi, Osaka 570, Japan.
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RBC IgG was obtained from Cappel Laboratories (Cochranville, PA) (Lot 21403). The agglutinating titer of this IgG preparation as determined in microtiter plates was 1:400. A 2 ml RBC suspension (1×10^9/ml in RPMI 1640) and IgG preparation diluted in RPMI 1640 were mixed and incubated at 37°C for 30 min. After incubation, EA were washed twice in cold RPMI 1640 and adjusted to contain 4×10^8 RBC/ml in fresh RPMI 1640.

Preparation of EA rosettes. A 2-ml neutrophil suspension, 0.5 ml of salt-poor human albumin (25%) (Alpha Therapeutic Corporation, Los Angeles, CA) and 2.5 ml of EA were placed into a siliconized 10-ml glass tube. The cell suspension was centrifuged at 60 × g at 20°C for 10 min. The supernatant was discarded and the pellet was incubated for 15 min at 20°C and gently resuspended in RPMI 1640. A small aliquot was removed for counting the percentage of RFN, *i.e.* neutrophils with three or more attached RBC. At least 250 cells were counted to determine the percentage of RFN.

Separation of RFN and non-RFN. After resuspension of neutrophil-EA, a 4-ml aliquot of the suspension was placed over Ficoll-Conray discontinuous density gradient in a siliconized 15-ml glass tube. Gradients were prepared as previously described by Wong *et al.* (15). Solution I contained 11.2 g Ficoll (mol wt 400,000, Sigma) and 20 ml of Conray (meglumine iothalamate 600 mg/ml, Mallinckrodt Inc., St. Louis, MO) in 100 ml distilled water. Solution II contained 7.2 g Ficoll and 20 ml of Conray in 100 ml distilled water. A total of 3 ml of solution II was layered over 3 ml of solution I. Cell suspensions were then layered over solution II and centrifuged at 1500 × g for 25 min at 4°C. The non-RFN were at the interface between the two Ficoll-Conray solutions, and the more dense RFN were in the cell pellet. Each fraction was collected by aspiration and washed twice in RPMI 1640. EA were removed from RFN by hypotonic lysis with distilled water.

Chemotaxis assay. Neutrophil chemotactic assay was determined using the acrylic blind well chemotaxis chamber (Neuro Probe, Inc., Cabin John, MD). The assay procedure was the modified Boyden method (1). Each cell suspension was adjusted to contain 1×10^6/ml in McCoy's 5A media with 10% FCS. The upper compartment of a chemotaxis chamber received 200 μl of the suspension, and 200 μl of EAP as a chemoattractant or 200 μl of McCoy's 5A media with 10% FCS as a negative control were placed into the lower compartment. The compartments were separated by a 3-μm pore-sized polycarbonate filter with a 13-mm diameter (Nuclepore Co., Pleasanton, CA). After incubation for 45 min at 37°C, saline was added by a Pasteur pipette into each of the upper compartments in triplicate chambers, and the fluid was aspirated. The filters were removed with a forceps and placed upside down on a glass slide and rinsed in consecutive ethanol solutions (80, 70, 50%) and stained with Wright's stain and hematoxylin. Each filter was scanned on high power field (×1000), and neutrophils that had migrated completely through the filter in 10 representative fields were counted. Chemotaxis was determined by the means of triplicate samples.

Adherence assay. Glass beads, 4-mm diameter (Sohgo-Rikagaku Glass Co., Japan), were prepared by washing in distilled water and drying overnight at 37°C. Thirty-five beads were placed into each 5-ml plastic syringe, and the tip was sealed with a parafilm (American Can Co., CT). Then, 1 ml of ^{51}Cr-labeled neutrophils (4) at a concentration of 1×10^6 cells/ml and 100 μl of EAP or 100 μl McCoy's 5A media with 10% FCS as a negative control was layered over the beads, and the syringe was closed with a piston, then incubated with rotation at 20 rpm at 37°C for 60 min. After incubation, the piston was removed, and 2 ml of McCoy's media were added to each syringe, and the parafilm on each outlet was perforated by a heated needle. The elution fluid was collected into a 12 × 75 mm polystylen tube (Falcon Labware, Becton, Dickinson & Co., Oxnard, CA), and beads were collected into another tube. The sample was triplicated and counted in a gamma counter (Packard Instrument Co., Downers Grove, IL) to determine the relative percentage of adherence of neutrophils, calculated as follows:

$$\frac{\text{count of the beads (cpm)}}{\text{count of the beads + elution fluid (cpm)}} \times 100 = \text{percentage of adherence}$$

Under the same conditions, the adherence value of stimulated PMN of healthy adults by EAP was 67.7 ± 5.8%. Therefore, the reproducibility of this adherence assay was less than 6%.

Statistical analysis. Statistical analysis was carried out using the Student's *t* test for comparison of means for paired samples and analysis of variance in conjunction with Scheffés test for comparisons of multiple means. A p value of < 0.05 was considered significant in all cases. Data were reported as means ± SD.

RESULTS

Determination of optimal concentration of rabbit antihuman RBC IgG and EA/neutrophil ratio for rosette formation. When EA were prepared with dilutions of rabbit antihuman RBC IgG between 1:400 and 1:1200, the percentage of RFN increased with dilutions of antibody and reached the maximum at 50% (1:800) of agglutinating titer. When the EA/neutrophil ratio was in a range of 10–100 at this concentration of antibody, the percentage of RFN peaked at 50 EA/neutrophil. Consequently, the combination of diluted antibody at 1:800 and 50 EA/neutrophil was considered to be optimal in this study.

Percentage of RFN. The percentage of RFN of cord and adult blood samples is compared in Figure 1. The cord blood values ranged from 23–52% with a mean of 35%. The adults ranged from 50–70% with a mean of 60%. The cord blood values were significantly lower than the adult values ($p < 0.01$, Student's *t* test) when the means were compared.

RFN were examined to determine whether there is a difference in the number of attached RBC between segmented and band neutrophils. For this purpose, small aliquots of the suspension and acridine orange (Daiichi Pure Chemicals Co., Japan) diluted

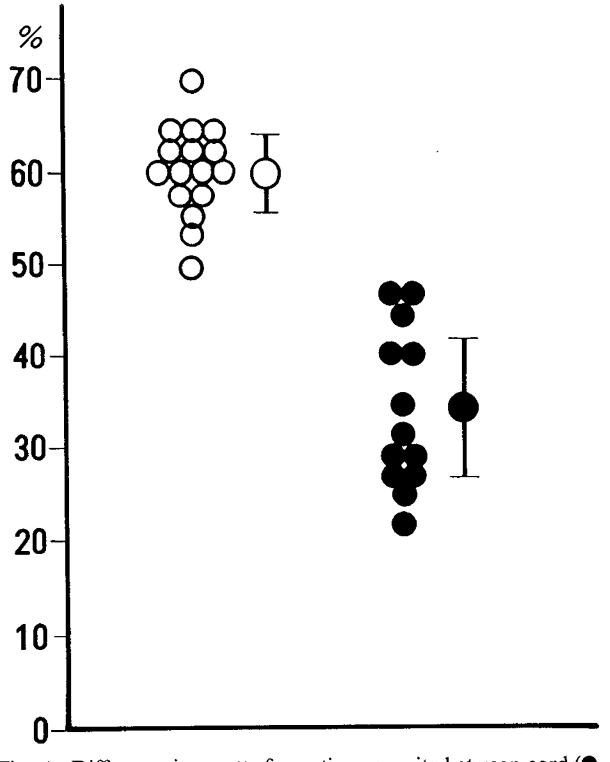

Fig. 1. Difference in rosette formation capacity between cord (●, n = 13) and adult (○, n = 16) PMN. Results are expressed as mean ± 1 SD. Significant difference ($p < 0.01$) was noted between the two groups.

1:10,000 in PBS were gently mixed on a glass slide and covered with a glass coverslip. Then the slide was scanned on a fluorescent microscope with UV illumination filtered to 520 nm, and at least 100 RFN were counted to determine the number of attached RBC (results are shown in Table 1). Statistical differences were noted as indicated in the footnote to Table 1.

Chemotaxis. As shown in Figure 2, differences in chemotaxis in PMN subpopulations between cord and adult PMN were as follows: (i) unfractionated adult PMN (A) (32 ± 2 migrated cells), adult RFN (B) (52 ± 4), adult non-RFN (C) (20 ± 1), (ii) unfractionated cord PMN (a) (23 ± 2), cord RFN (b) (43 ± 2), cord non-RFN (c) (19 ± 1).

Adherence. As shown in Figure 3, differences in adherence of PMN subpopulations were as follows: (i) unfractionated adult PMN (A) (68.3 ± 6.6%), adult RFN (B) (69.5 ± 9.7%), adult non-RFN (C) (24.4 ± 0.7%), (ii) unfractionated cord PMN (a) (54.9 ± 9.8%), cord RFN (b) (53.8 ± 3.2%), cord non-RFN (c) (12.8 ± 6.8%).

Table 1. *Differences in rosette formation capacity of segmented and band neutrophils between cord and adult PMN*

Neutrophils \ Number of attached RBCs		3	4	5	6	7<
Adult blood	Segmented	8 ± 6* (A)	14 ± 1 (B)	13 ± 2 (C)	13 ± 4 (D)	32 ± 1 (E)
	Band	2 ± 1 (F)	4 ± 1 (G)	5 ± 1 (H)	5 ± 1 (I)	6 ± 1 (J)
Cord blood	Segmented	19 ± 2 (a)	21 ± 4 (b)	9 ± 1 (c)	6 ± 2 (d)	14 ± 5 (e)
	Band	4 ± 1 (f)	7 ± 2 (g)	5 ± 3 (h)	5 ± 2 (i)	10 ± 2 (j)

* Percent mean ± 1 SD. $p < 0.01$: (E) > (e); (D)+(E) > (d)+(e). $p < 0.02$: (A) < (a); (A)+(B) < (a)+(b); (F)+(G) < (f)+(g). $p < 0.05$: (B) < (b); (C) > (c); (D) > (d); (F) < (f); (J) < (j); (I)+(J) < (i)+(j). N.S. (G) *versus* (g); (H) *versus* (h); (I) *versus* (i).

DISCUSSION

The mechanism of decreased PMN chemotaxis and adherence in neonates is not well understood. Miller (1, 2) suggests that the cell is rigid and lacks the capability of deforming in response to chemotactic stimulation and that decreased PMN chemotaxis is a main cause of increased susceptibility of neonates to overwhelming bacterial infections. We reported previously that, using ^{51}Cr-labeling assay (4), PMN chemotaxis and adherence in neonates were significantly decreased when compared to those of adults. Our present study further indicate that decreased PMN chemotaxis and adherence in neonates may relate in part to differences to PMN subpopulations, *i.e.* RFN and non-RFN representing Fc receptor expression.

The percentage of RFN was significantly lower in cord blood than in adult. By contrast, Pross *et al.* (18) reported that the average percentage of cord and adult RFN was identical. It is thought that the discrepancy may lie in the difference between sheep and human erythrocytes, *i.e.* probes necessary for rosette formation, and other technical procedures. In this respect, Whited *et al.* (19) noted that many kinds of cells which were rosette negative with the human erythrocyte formed rosettes with the sheep erythrocytes. Based on this observation, they considered that the human erythrocyte appeared to be a more sensitive probe for studying PMN subpopulations than the sheep erythrocyte and that the difference of rosette formation from probe cells was perhaps due to some characteristics of probe cells unrelated to their Fc receptor.

The present chemotactic assay showed that cord RFN were more chemotactic than unfractionated cord PMN and cord non-RFN, respectively. From these results, we also confirmed that neonatal neutrophils consist of two different populations and further concluded that the average percentages of cord and adult RFN are entirely different. Therefore, differences in rosette-forming capacity between cord and adult PMN using human erythrocytes may account for differences in chemotaxis so far observed between these two kinds of PMN. Furthermore, comparison of the rosette-forming capacity of segmented and band neutrophils between cord and adult PMN showed that the rosette-forming capacity of segmented neutrophils of cord PMN was significantly lower than that of adult PMN. This observation

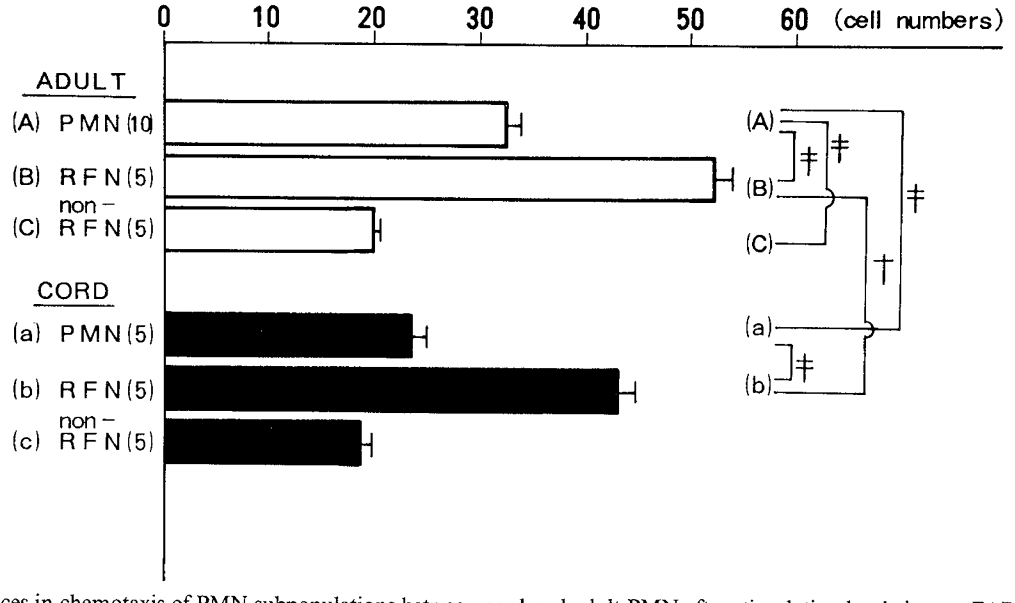

Fig. 2. Differences in chemotaxis of PMN subpopulations between cord and adult PMN after stimulation by their own EAP. Abscissa indicates the number of migrated cells ± 1 SD. Numbers in parentheses indicate numbers of specimens studied. †, $p < 0.05$; ‡, $p < 0.01$.

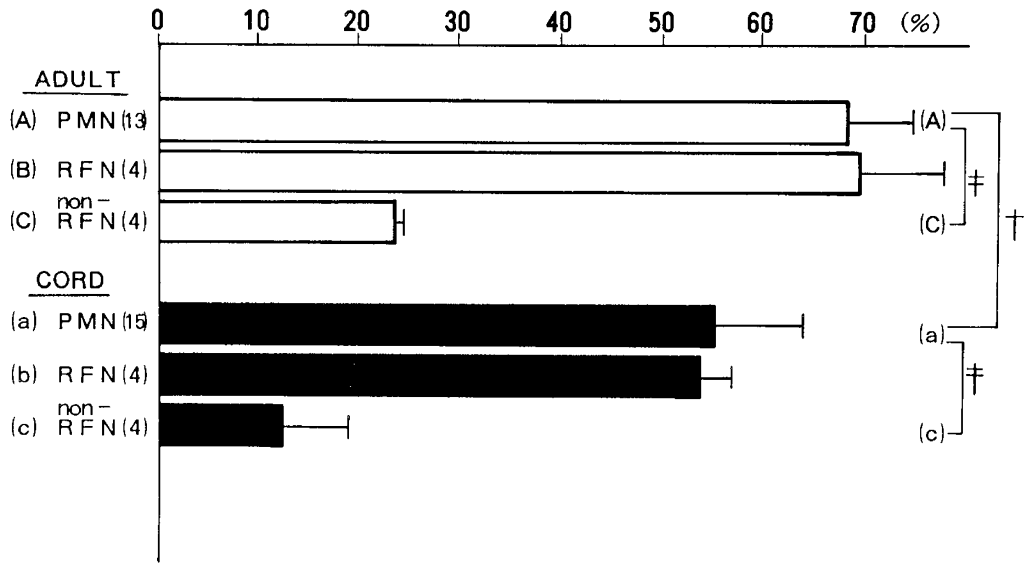

Fig. 3. Differences in adherence of PMN subpopulations between cord and adult PMN after stimulation by their own EAP. The abscissa indicates the percentage of adherence ± 1 SD. The numbers in parentheses indicate numbers of specimens studied. †, $p < 0.05$; ‡, $p < 0.01$.

appears to reflect the low capacity of rosette formation of cord PMN. Boner *et al.* (12) demonstrated that the band form from both cord and adult PMN had a less mean migration than the segmented form. Based on this observation, they considered that an increase in chemotactic response during neutrophil maturation may partially contribute to the smaller total chemotactic response of cord PMN compared to adult PMN because cord PMN had more band forms (31%) than adult PMN (8%). This explanation may be plausible, but we would rather think that a decreased PMN chemotaxis is perhaps due in part to the significantly low capacity of rosette formation of segmented neutrophils which occupy the majority of PMN. In support of this speculation, Krause *et al.* (20) pointed out with a mouse MAb (31D8) that there was a greater percentage of immature PMN (bands and metamyelocytes) in neonates than in adults, yet immature PMN were equally distributed in the 31D8 bright (more motile cells) and dull (less motile cells) subpopulations. According to Lewis *et al.* (21), although the segmented and band neutrophils of adult PMN similarly reached optimal EA rosettes, segmented neutrophils were more chemotactic than band neutrophils. However, we could not delineate the relationship between rosette forming capacity and functional capacity of cord PMN.

In the first line of host defense mechanism, the role of PMN adherence is important not only in PMN to the vascular endothelium but also in preliminary anchoring of the cell for forward chemotaxis. There are few reports in regard to PMN adherence in neonates; furthermore, there is a discrepancy of results which appears to be due to the difference of methodologies. Krause and coworkers (22, 23) found that neutrophil adherence of neonates was decreased when compared with that of adults, whereas Rao *et al.* (24) reported that it was significantly increased. However, Fontan *et al.* (25) noted that PMN adherence was similar in both. Adherence assay with ^{51}Cr glass beads yielded similar results to those of Krause and coworkers (22, 23) and showed reproducibility of less than 6%. With this sufficiently sensitive method, significant differences in adherence of PMN subpopulations between cord and adult PMN were noted between RFN and non-RFN. This observation suggests that differences of adherence are due at least to differences between RFN and non-RFN, as was observed in PMN chemotaxis.

In summary, our present study has shown that neonatal neutrophils have two heterogeneous subpopulations, indicating that PMN chemotaxis and adherence are characterized by the difference of rosette-forming capacity. Although the origin of PMN subpopulations is still unknown as Gallin (13) pointed out, it seems likely that differences of PMN subpopulations may be responsible in part for the cause of decreased PMN chemotaxis and adherence in neonates. Further examination into the heterogeneity of Fc receptor expression appears to provide more information about PMN defects in neonates.

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