Polymorphonuclear Leukocyte Heterogeneity in Neonates and Adults

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We have used a mouse monoclonal antibody (31D8) to determine whether differences in neutrophil (PMN) subpopulations might help explain decreased PMN chemotaxis in neonates compared with that of adults. 31D8 has been shown to bind heterogeneously to adult PMNs. Approximately 80% of the PMNs that strongly bind 31D8 (31D8 "bright") are the same cells that depolarize and migrate chemotactically when stimulated with the chemoattractant N-formyl-methionyl-leucylphenylalanine, while the 20% that weakly bind 31D8 fail to similarly respond. All neonatal PMNs bound 31D8 heterogeneously. There was a smaller population of 31D8 "bright" cells in neonates at birth (76% ± 6%, n = 45) compared with that of neonates at three to 15 days of age (82% ± 5%, n = 10, P < 0.002) and both were smaller than that of adults (88% ± 4%, n = 45, P < 0.001 and P < 0.001). Neonatal cord PMNs, which traversed a micropore filter in a modified Boyden chemotaxis chamber in the presence of a chemoattractant, had an increased percentage of 31D8 "bright" cells (89% ± 7%) than did PMNs which remained above the filter (82% ± 7%, n = 10, P = 0.034). PMN chemotaxis was less in neonates at birth (32.7 ± 4.5 μm) than at three to six days of age (36.8 ± 11.3 μm) and both were decreased compared with that of adults (69.1 ± 12.4 μm, P < 0.001 and P < 0.001). These findings indicate that decreased PMN chemotaxis in neonates may be due in part to a smaller PMN subpopulation of highly motile cells.

METHODS

Study population. Blood was obtained from the fetal side of placentas (neonatal cord blood) within five minutes of birth, and from healthy, term neonates three to 15 days old. In each case blood was simultaneously drawn from healthy adult volunteers on no medication. Placentas were obtained from healthy women delivered vaginally or by repeat cesarean section who had local anaesthesia, no complications of pregnancy, labor, and delivery, and whose babies were in good health. Blood from older neonates was obtained at the time of heel stick blood sampling for phenylketonuria, a test performed on all newborn infants. All blood samples were immediately mixed with preservative free sodium heparin (50 U/mL blood) and brought to the laboratory for analysis. Informed consent to sample blood was obtained in accordance with the Hartford Hospital Human Investigation Committee guidelines.

Preparation and 31D8 labeling of PMNs. Whole blood was purified using 6% Hetastarch (McGaw Laboratories, Irvine, Calif) that yielded a population of cells comprised primarily of PMNs and monocytes as previously described.5 In some cases the cell population was further purified by Ficoll-hypaque separation yielding a preparation of greater than 90% pure PMNs. Labeling of PMNs with 31D8 antibody was performed as described previously.14,15 In brief, separated PMNs were washed twice in PBS-BSA, counted, mixed with 31D8 antibody, and allowed to incubate on ice for 45 minutes. The PMNs were then washed three times in PBS-BSA and incubated for 30 minutes on ice with fluorescein isothiocyanate labeled goat-antimouse IgG. The labeled PMNs were washed three more times in PBS-BSA and stored on ice, in the dark, and in the absence of Ca2+ or Mg2+ until use. Separated and labeled cells remained alive as shown by trypan blue exclusion. The following terminology is used in the manuscript for each study group. "adult PMNs" refer to PMNs isolated from the venous blood of adults, "neonatal cord PMNs" refer to PMNs isolated from the cord blood of newborn infants, and "older neonatal PMNs" refer to PMNs isolated from the venous blood of three- to 15-day-old infants.

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Supported in part by Hartford Hospital Research Free Funds and NIH Grants #AI 19768 and #CA 08341.

Submitted July 26, 1985; accepted Feb 28, 1986.

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0006-4971/86/6707-0032$03.00/0
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Analysis by flow cytometry. Flow microfluorometry was performed using an EPICS C (Coulter, Inc, Hialeah, Fla) with an argon laser emitting 500 mw at 488 n. Forward and 90° light scatter were used to identify the PMN population from lymphocytes and monocytes. The fluorescence intensity distribution of 10,000 PMNs was determined. The data was presented as the number of cells on the vertical axis and log green fluorescence on the horizontal axis divided into a three decade log scale consisting of 255 channels. In some experiments cells were sorted according to fluorescence intensity for further analysis. Gain settings for the photomultiplier tubes for 90° light scatter, forward light scatter, and green fluorescence were gain 5, PMT 400; gain 5; and gain 5, PMT 1300, respectively, and were constant for neonates and adults in all experiments.

PMN locomotory assay. PMN locomotory response was determined using transparent acrylic modified Boyden chambers (AhlcO Corp., Southington, Conn) and nitrocellulose filters (140 zm pore size, Nuclepore, Pleasanton, Calif). The assay procedures have been described in detail elsewhere.5,6 Cells were incubated for one hour with either f-met-leu-phe 10μg or 3% zymosan activated serum in Medium 199. The results of the nitrocellulose filter assay were assessed using the microsectioning technique which describes the average distance migrated by the cells (locomotion index or LI).7 PMNs incubated with the polycarbonate filter were separately harvested from the upper and lower compartments of the Boyden chambers, labeled with 31D8, and analyzed on the flow cytometer.

Statistical analysis. Significance of differences between groups were evaluated using the Student’s t test. A P value of less than 0.05 was considered significant.

RESULTS

31D8 binding to adult and neonatal PMNs. The 31D8 antibody bound to neonatal cord PMNs, older neonatal PMNs, and adult PMNs in a heterogeneous manner. The majority of PMNs strongly bound 31D8 (31D8 “bright”) and generated a highly fluorescent peak. The remaining PMNs weakly bound 31D8 (31D8 “dull”) and generated a contiguous, smaller, less fluorescent area to the left of the major peak. Three typical neonatal cord PMN and adult PMN curves are shown in Fig 1. The 31D8 bright peaks are similar. The 31D8 dull areas are more variable including a distinct peak (1A) or a “shoulder” to the left of the 31D8 bright peak that varies in size (1B–F). PMNs from adults are more concentrated in the bright areas than PMNs from neonates. The adult bright peak in 1D has only a very slight left shoulder and while the other adult curves (1E, F) have larger dull shoulders, they are smaller than those of most neonates. In order to quantitate the size of the bright and dull areas, it was assumed that the 31D8 bright population generated a symmetric curve and that any other cells were part of the dull population.13 Using the flow cytometer, the area of the entire curve was determined and the area of the symmetric curve about the major peak (bright curve) subtracted from the total area to determine the size of the dull area.

Significant differences in 31D8 antibody binding to neonatal cord PMNs, older neonatal PMNs, and adult PMNs were noted with this analysis as shown in Table 1. Neonates at birth had a smaller population of 31D8 bright cells (76% ± 6%) than neonates three to 15 days of age (82 ± 5%, P < 0.01), and both had a smaller population of 31D8 bright cells compared with that of adults (88% ± 4%, P < 0.001, and P < 0.001). The mean skew and kurtosis of the curves

Fig 1. 31D8 antibody binds PMNs heterogeneously. The illustrations are representative curves of 31D8 antibody binding to PMNs in neonates (1A–C) and adults (1D–F). In 1A there is a major peak consisting of 31D8 bright PMNs and a minor peak of 31D8 dull PMNs. In 1D–F there is a single major peak of 31D8 bright PMNs with a “shoulder” or “tail” to the left of the major peak consisting of 31D8 dull PMNs. The horizontal axis depicts fluorescence intensity with cells to the left of 1 exhibiting autofluorescence, 1–2 one log fluorescence, and 2–3 two log fluorescence. The vertical axis depicts the number of PMNs. The percentage of 31D8 bright cells are as follows: 1A: 65%, 1B: 69%, 1C: 77%, 1D: 91%, 1E: 87%, 1F: 86%.
were also determined as another method to compare neonatal and adult 31D8 binding. The neonatal fluorescence curves had a decreased mean skew and kurtosis compared with those of adults (Table 1). There was no significant difference in 31D8 bright populations in females or males in adults (89% ± 3%, and 88% ± 4%, respectively), neonates at birth (77% ± 5%, and 75% ± 6%, respectively), or neonates three to 15 days of age (84% ± 3% and 81% ± 5%, respectively).

Differences in 31D8 fluorescence intensity between adult PMNs, neonatal cord PMNs, and older neonatal PMNs were not due to cell size, as determined by comparing forward light scatter on the flow cytometer for the 31D8 bright and dull subpopulations in the three groups. There was also no difference in the location of antibody binding to neonatal cord PMNs, older neonatal PMNs, and adult PMNs. In all cases there was uniform distribution of fluorescently tagged 31D8 around the periphery of the PMNs. PMN activation with f-met-leu-phe for 60 minutes at 37 °C did not affect the bright/dull cell ratio. There was no difference in the percentage of bright cells in PMNs incubated in Medium 199 or f-met-leu-phe from four neonates at birth (79% ± 5% and 81% ± 6%, respectively) or from four adults (90% ± 3% and 89% ± 4%, respectively).

Because of the possibility that the differences observed in 31D8 binding between neonates and adults were due to an increased percentage of immature PMNs (bands and metamyelocytes) in neonates, we labeled PMNs from three neonates (cord blood) and three adults with 31D8, sorted them into 31D8 bright and dull subpopulations, and analyzed the subpopulations for percentage of mature and immature PMNs. There was no significant difference in the percentage of immature PMNs in 31D8 bright and dull subpopulations for neonates (16% ± 3%, and 21% ± 11%, respectively), adults (13% ± 7% and 17% ± 11%, respectively), or the total group of neonates and adults (15% ± 5% and 19% ± 10%, respectively).

Correlation of 31D8 binding and PMN chemotaxis. Neonatal cord PMNs from four individuals were incubated in Boyden chambers, collected above and below poly-carbonate filters after migration in response to 3% zymosan activated serum, and labeled with 31D8. PMNs that traversed the filter had a log greater fluorescence intensity than PMNs that remained above the filter (82% ± 7%, P = 0.034). These observations indicate that a motile PMN subpopulation in neonates expresses a larger amount of 31D8 antigen on its surface than a less motile subpopulation of cells.

The difference in 31D8 expression in the "fast" PMNs that traversed the filter and "slow" PMNs that did not traverse the filter was not due to cell activation during passage through the filter since the percentage of bright cells was not affected by preincubation with f-met-leu-phe as mentioned previously. Furthermore, studies of Seligmann et al showed that when PMNs are prelabeled with 31D8 and placed in the Boyden chamber, the population of cells that traverse the micropore filter into the bottom chamber have a higher percentage of 31D8 bright cells than those that remain in the upper compartment. This data shows that possible alteration in 31D8 expression by contact with the filter or exposure to greater concentrations of f-met-leu-phe does not account for differences in 31D8 expression between the "fast" and "slow" PMNs.

PMN chemotaxis using f-met-leu-phe and 31D8 antibody binding were simultaneously tested in five neonates at birth and adults. The mean chemotactic and 31D8 binding values for neonates were 28.8 ± 4.6 μm and 76.8% ± 6.2%,
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respectively, and for adults 52.7 ± 9.7 μm, (P < 0.001) and 88.8% ± 3.9%, (P < 0.001), respectively. PMN chemotaxis using 3% zymosan activated normal serum was studied in six other neonates at birth and again at three to six days of age and in 12 healthy adults. Mean chemotactic (L150) values were 32.7 ± 4.5 μm, 36.8 ± 11.3 μm, and 69.1 ± 12.4 μm (P < 0.001 and P < 0.001, respectively). Thus, the magnitude of both the PMN chemotactic response and 31D8 antibody binding were smallest in neonates at birth, increased in neonates three to 15 days of age, and greatest in adults.

DISCUSSION

The results of this study demonstrate a clear difference in 31D8 monoclonal antibody binding to PMNs between neonates and adults. There was heterogeneous binding to PMNs of both groups, but neonates had a smaller percentage of cells that strongly bound 31D8 (31D8 bright) compared with that of adults. The percentage of 31D8 bright cells in neonates three to 15 days of age was intermediate between that of neonates at birth and adults. Several possible explanations for these differences were examined. The location of antibody binding sites on PMNs from neonates and adults was similar with uniform distribution of fluorescently tagged 31D8 antibody over the cell surface. There was also no difference in cell size of the 31D8 bright and dull subpopulations in neonates and adults as determined by similar forward light scatter on the flow cytometer. There were no differences in eosinophil counts between neonates and adults. A higher eosinophil count in neonates could have accounted for the difference in 31D8 antibody binding since eosinophils weakly bind 31D8. Finally, although there was a greater percentage of immature PMNs (bands and metamyelocytes) in neonates than in adults, immature PMNs were equally distributed in the 31D8 bright and dull subpopulations. It is possible that 31D8 dull cells are less differentiated than 31D8 bright cells and that light microscopy is not sensitive enough to detect subtle PMN maturational changes. This is unlikely, however, because 31D8 expression is not a late maturational event in PMN development, but appears at the level of the myelocyte or metamyelocyte in the bone marrow. Furthermore, a recent study by Gallin et al of 31D8 antibody binding to PMNs of patients with chronic myelogenous leukemia suggests that 31D8 bright or dull cells may reflect true PMN subpopulations that originate from distinct stem cells.

The cause for the difference in 31D8 binding in healthy neonates and adults is unclear. Since 31D8 binding is not affected by cell activation, changes in 31D8 binding must be due to increased production or destruction of 31D8 subpopulations. It is probably not due to differences in circulating estrogens or progesterone since unpublished observations by us of 31D8 PMN binding in seven pregnant women showed no difference in 31D8 binding during pregnancy and postpartum compared with nonpregnant control subjects. Neonates have a marked neutrophilia at birth that persists for 48 to 72 hours. Recent studies in neonatal rats indicate that the size, number of cell divisions, and rate of production of PMNs is different in neonates than in adults. These differences might account for PMN 31D8 subpopulation differences between neonates and adults.

The PMN antigenic heterogeneity detected by 31D8 antibody binding correlates with functional heterogeneity and may help explain observed functional differences between neonates and adults. Neonatal cord PMNs that traversed polycarbonate micropore filters into the lower compartment of Boyden chambers expressed a greater amount of 31D8 antigen on their surface and had a higher percentage of bright cells compared with PMNs that remained above the filters. Seligmann et al also found that PMNs that traversed micropore filters had a significantly higher percentage of 31D8 bright cells when the cells were labeled with 31D8 before placement in the Boyden chambers. This indicated that up-regulation of 31D8 antigen was not a sufficient explanation for the increased percentage of 31D8 bright cells among the PMNs that traversed the micropore filter. Furthermore, we noted a progressive increase in PMN chemotaxis and 31D8 antibody binding in neonates studied at birth, again at three to 15 days of age and in adults. Although there is insufficient evidence to determine the quantitative contribution of 31D8 subpopulation changes to alterations in chemotactic responses, these results suggest that decreased PMN chemotaxis in neonates may be due in some part to the smaller percentage of highly motile 31D8 bright PMNs found in neonates compared with that of adults.

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