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Neonatal Eosinophils Possess Efficient Eotaxin/IL-5– and *N*-Formyl-Methionyl-Leucyl-Phenylalanine–Induced Transmigration *In Vitro*

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ABSTRACT

The majority of premature infants develop eosinophilia and abnormalities in eosinophil trafficking during the first period of postnatal life. We therefore thought to assess the ability of neonatal eosinophils to transmigrate *in vitro* toward chemotactic stimuli mimic either bacterial infections, or to allergic inflammation in vivo, and to compare the results with eosinophils in adults. We used an *in vitro* transmigration method and the chemotactic stimuli *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or a combination of IL-5 and eotaxin. The expression of the adhesion-promoting molecule CD11b and the fMLP receptor were assessed by flow cytometry. Both the fMLP- and IL5/eotaxin-induced eosinophil transmigration capacity was significantly more efficient in neonates than in adults ($p < 0.0001$ and $p < 0.0002$, respectively). The fMLP-induced up-regulation of CD11b on eosinophils was significantly ($p < 0.0003$) higher in neonates compared with that in adults. We also assessed a significant ($p < 0.0001$) higher expression of the fMLP receptor on resting eosinophils in neonates compared with that in adults. The integrated impact of increased transmigration capacity, fMLP receptor expression, and CD11b expression on eosinophil by bacterial peptide fMLP suggests that neonatal eosinophils possess the potential to play an alternative role compared with eosinophils in adults. **(*Pediatr Res* 58: 138–142, 2005)**

Abbreviations

ECP, eosinophilic cationic protein
fMLP, *N*-formyl-methionyl-leucyl-phenylalanine
MPO, myeloperoxidase

Eosinophils are bone marrow–derived, terminally differentiated granulocytes that in adults are mostly associated with helminthic infections and allergic diseases. Cord blood contains more mature eosinophils as well as more progenitor cells than adult peripheral blood; thus, neonates seem to have a high capacity to produce high eosinophil counts (1,2). It is also widely known that premature infants develop eosinophilia during the first weeks of postnatal life (3,4). Notwithstanding these observations, our knowledge of the potential physiologic role of the eosinophil in the neonate in the absence of overt disease remains poor.

The existence of abnormalities in eosinophil trafficking in the neonatal period is suggested by several clinical observations. Neonatal exudates from different compartments often contain an increased proportion of eosinophils (1,2). Moreover, eosinophils in the neonatal period have been claimed to participate in the inflammatory process in bronchopulmonary dysplasia (5,6), as well as in infants with acute wheezing episodes (7). Eosinophilia in the neonatal period has also been linked to nonparasitic infections, and a physiologic role for the eosinophil during the overt colonization of skin and mucosal surfaces that occurs during this period has been suggested (8).

Given that eosinophilia and abnormal eosinophil trafficking occur in the neonatal period without any of the obvious signs of disease commonly attributed to the action of eosinophils in adults, we hypothesized that the role of the eosinophil in the innate immunity of the neonate differs from its role in adults. To test this hypothesis, we studied the *in vitro* transmigration capacity of neonatal and adult eosinophils, in response to the bacteria-related peptide fMLP, as well as chemoattractants related to allergic inflammation, namely IL-5 and eotaxin. Moreover, we assessed the ability of eosinophils to up-regulate CD11b upon fMLP stimulation and the surface expression of the fMLP receptor.

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METHODS

Blood samples. Blood was collected from the placenta side of the umbilical cord immediately after uncomplicated vaginal deliveries ($n = 10$). All infants were full term (mean: 40 wk; range: 38–42 wk), with a mean birth weight of 3584 g (range: 3249–3750) and mean Apgar scores of 8, 9, and 10 at 1, 5, and 10 min, respectively.

Blood from 10 healthy nonallergic blood donors was collected, aged between 18 and 65 y. All samples were collected in citrate tubes (Vacutainer, 5 mL, with 500 μL of 0.129 M Citrate-Na; Becton-Dickinson, Plymouth, UK).

Preparation of blood leukocytes. Erythrocytes were specifically hemolyzed by adding 3 mL of NH_4Cl-EDTA [154 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA (pH 7.2)] to 150 μL of blood. The suspensions were incubated for 5 min at 15°C and centrifuged at 300 × g for 5 min at 4°C (9). The leukocyte preparations were washed in 2 mL of 4°C 0.15 M PBS (pH 7.4) supplemented with 0.1 mM EDTA and 0.02% NaN_3 (PBS-EDTA). The leukocyte pellets were kept on ice until further treatments.

In vitro *leukocyte activation.* Leukocyte preparations were resuspended in HEPES-buffered RPMI 1640 medium (GIBCO, Paisley, UK), in the presence of the following stimuli: recombinant human (rh) IL-5 (Prepro Tech Inc., Rocky Hill, NJ; 100 ng/mL), rh eotaxin (Prepro Tech Inc.; 100 ng/mL) and fMLP (Sigma Chemical Co. St. Louis, MO; 5×10^{-7} M). The selected IL-5 and fMLP concentrations and time periods for *in vitro* stimulation were based on three pilot data and previous reported effect on CD11b expression in human eosinophils and neutrophils (10). Incubation conditions were designed as follows: eotaxin, 60 min in buffer only followed by 15 min in buffer supplemented with eotaxin; IL-5, 75 min in buffer supplemented with IL-5; IL-5/eotaxin, 60 min in buffer supplemented with IL-5, followed by addition of eotaxin for another 15 min; and fMLP, 15 min in buffer supplemented with fMLP. All incubations were done at 37°C. Leukocytes that were incubated for 75 min in buffer alone at 4°C or 37°C were run in parallel. The leukocytes were subsequently washed with 2 mL of PBS-EDTA (300 × g, 5 min) and kept on ice until immunostained.

Table 1. *Total count and percentage of leukocytes in neonates and adults*

	Total count (10^9/L)		%	
	Neonate	Adult	Neonate	Adult
Leukocyte	15.1 ± 4.5*	7.5 ± 4	100 ± 30	100 ± 48
Neutrophils	7.7 ± 3.0	4.3 ± 3.5	51 ± 21	57 ± 40
Lymphocytes	4.8 ± 7.0	2.6 ± 1.1	32 ± 45	35 ± 15
Monocytes	1.5 ± 3.2	0.3 ± 0.5	10 ± 19	4 ± 7
Eosinophils	0.8 ± 1.3†	0.1 ± 0.02	5 ± 8	1 ± 0.2

Results are based on seven experiments.
* $p < 0.01$ *vs* total count of leukocytes in adults.
† $p < 0.01$ *vs* total count of eosinophils in adults.

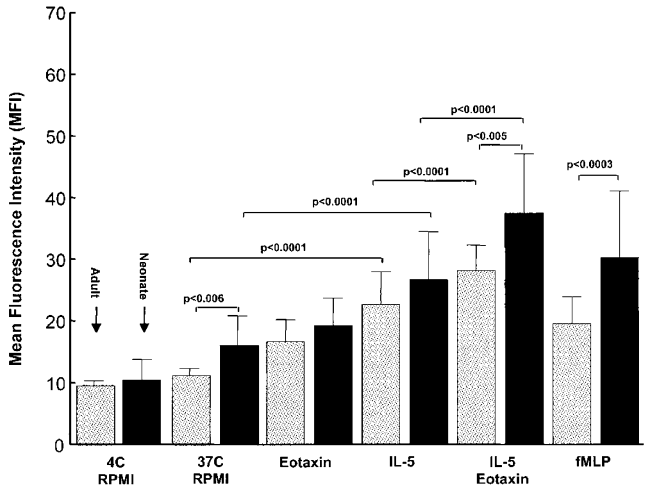

Figure 1. Expression of CD11b on eosinophils that were treated with RPMI alone at 4°C and 37°C, eotaxin alone, IL-5 alone, IL-5+eotaxin, and fMLP alone. Results, expressed as MFI units, are presented as means and SEM and are based on 10 separate experiments.

Immunostaining and flow cytometry. Leukocytes were incubated with phycoerythrin-conjugated MAb directed against CD11b (DAKO A/S, Glostrup, Denmark) at a final concentration of 50 μg/mL for 30 min on ice and thereafter were washed once in 3 mL of 4°C PBS-EDTA (300 × g, 6 min). For assessing the expression of the fMLP receptor, a FITC-conjugated formylated peptide (FITC-FNLPNTL; 5×10^{-7} M final concentration; Molecular Probes, Eugene, OR) was added to leukocytes for 30 min at 4°C. FITC-conjugated fMLP were mixed with unconjugated fMLP (1:1000) and used as a control and were run in parallel to define the cut-off for positive staining (11).

For enabling simultaneous analysis of eosinophils and neutrophils, leukocytes were treated according to the FOG method (12,13). The eosinophil population was confirmed by staining $CD9^+/CD16^-$, as previously reported (14). The final preparations of mixed leukocytes were analyzed and counted in an EPICS XL (Beckman Coulter, Inc., Hialeah, FL) flow cytometer.

Transmigration assay. The transmigration assay was performed as recently described using 24-well microchemotaxis chamber with a polyethylene terephthalate–treated membrane (Becton Dickinson), which has a high pore density membrane (2.0×10^6 pores/cm^2, 3.0 μm in diameter) for maximum permeability. Insert filters were coated with human fibronectin (20 μg/mL; Sigma Chemical Co.) (15). Briefly, for fMLP-induced eosinophil and neutrophil transmigration, Percoll-separated (Pharmacia & Upjohn, Uppsala, Sweden) granulocytes were resuspended in RPMI alone and added to the upper chamber. Buffer supplemented with fMLP (5×10^{-7} M; Sigma Chemical Co.) was added to the lower chamber. For IL-5/eotaxin-induced eosinophil transmigration, granulocytes were resuspended in RPMI supplemented with IL-5 (100 ng/mL; Prepro Tech Inc.) and added to the upper chamber. Buffer supplemented with eotaxin (100 ng/mL; Prepro Tech Inc.) was added to the lower chamber. Cells that transmigrated into the lower chamber ("transmigrated cells") were, together with the original cell suspension ("total cells"), subjected to analysis of either myeloperoxidase (MPO; competitive RIA, Pharmacia & Upjohn) or eosinophil cationic protein (ECP; ECP-CAP-FEIA, Pharmacia & Upjohn) as markers for respective neutrophils and eosinophils. A ratio between transmigrated cells and total cells was calculated for each experimental setting and expressed as percentage of transmigrated cells ("transmigration index"), as previously described (15). The transmigration assay was run for 240 min, and the transmigration index was calculated at stated time points.

Cell recovery, assessed by ECP and MPO levels, were >95%, and cell viability, judged by Trypan blue exclusion, was >95% in all experimental settings. As reported for adult cells (15), we found good correlation between cell counts that were measured by flow cytometry and cell counts that were measured by ECP/MPO levels [neutrophils ($r = 84$) and eosinophils ($r = 91$), with 95% confidence bands; eosinophils ($r = 89$), with 95% confidence bands).

Statistical analysis. Results are given as mean ± SEM. Comparison was made by using ANOVA and t test. Interactions between groups have been calculated. As *post hoc* analysis, the Tukey-test was used.

RESULTS

Absolute number of the eosinophils and neutrophils in neonates and adults. The total number of leukocytes in neonates was twice as high as in adults (15.1 ± 4.5 *versus* 7.5 ± 4.0 10^9/L; $p < 0.001$). The eosinophil count was also significantly higher in neonates compared with in adults (0.8 ± 1.3 *versus* 0.1 ± 0.02 10^9/L; $p < 0.001$; Table 1).

Expression of cell surface adhesion molecule CD11b on eosinophils in neonates and adults. The expression of CD11b was significantly higher in eosinophils that were preincubated with IL-5 than in eosinophils that were preincubated with RPMI alone in both adults and neonates (Fig. 1). Addition of eotaxin (0.1 μg/mL) further increased the expression of CD11b on IL-5 preincubated eosinophils ($p < 0.0001$ in both neonates and adults). Significant difference between adult and neonate eosinophils with respect to CD11b expression was observed, when the cells were incubated at 37°C with RPMI (11.3 ± 0.9 *versus* 17.9 ± 7.0 MFI units; $p < 0.006$), with IL-5/eotaxin (26.5 ± 4.7 *versus* 38.2 ± 13.8 MFI units; $p < 0.005$), or with

fMLP (18.9 ± 6.4 *versus* 29.9 ± 15.8 MFI units; $p < 0.0003$; Fig. 1).

Kinetics of eosinophil transmigration in neonates and adults with fMLP or IL-5/eotaxin. The chemotactic response of neonatal eosinophils toward fMLP was obvious. The transmigration reached a plateau after 60 min of incubation, which was significantly higher compared with 30 min of incubation ($p < 0.02$; Fig. 2). There was spontaneous transmigration of neonatal eosinophils, which started after 120 min of incubation. We also tested the transmigration capacity toward a combination of IL-5 and eotaxin. The transmigration started at 30 min and reached a plateau at 240 min. Transmigration of eosinophils from healthy blood donors started at 120 min and reached a plateau at 240 min (Fig. 3). Neonatal eosinophils possessed a significantly higher transmigration capacity toward IL-5/eotaxin than adult eosinophils at all time points ($p < 0.0002$ to $p < 0.002$). To validate our assay, we also analyzed the transmigration of IL-5–primed neutrophil toward eotaxin. We did not find any increase of transmigration capacity of IL-5–primed neutrophil toward eotaxin.

Kinetics of neutrophil transmigration. A gradual increase in both neonate and adult neutrophil transmigration was observed when fMLP was used stimuli and reached a plateau at 60 and 120 min, respectively (Fig. 4). The transmigration of neutrophils at 60 min was significantly higher in neonates compared with adults ($p < 0.005$), but at 120 min, the transmigration of adult neutrophils was significantly higher than neonatal neutrophils ($p < 0.005$). We also noted a spontaneous transmigration in neonates with medium only, which was continuously increased and reached a plateau at 240 min.

Expression of fMLP receptor on eosinophils and neutrophils in neonates and adults. The surface expression of fMLP receptor on eosinophils was significantly higher in neonates compared with only adults, judged by both MFI values and positive labeled cells [130 ± 5.0 *versus* 23.9 ± 3.1 MFI units ($p < 0.0001$) and 98.1 ± 0.4 *versus* 0.70 ± 0.1 positive cells ($p < 0.0001$); Table 2]. On neutrophils, the fMLP receptor was more abundantly expressed in adults than in neonates (Fig. 5).

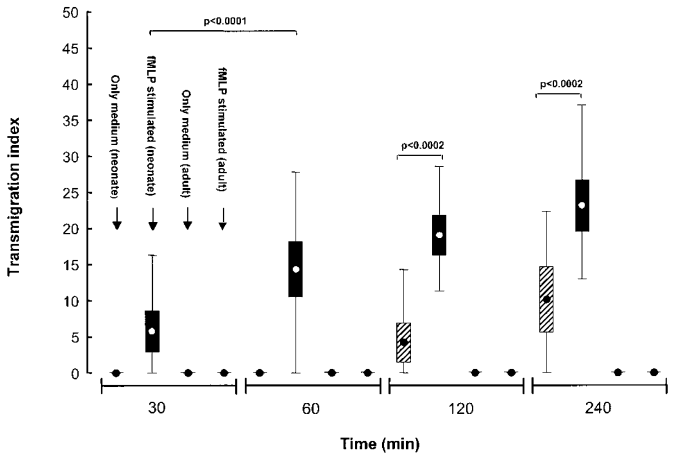

Figure 2. The effect of fMLP on *in vitro* transmigration of neonate and adult eosinophils. Results, expressed as transmigration index, are presented as mean and SEM and are based on 10 separate experiments.

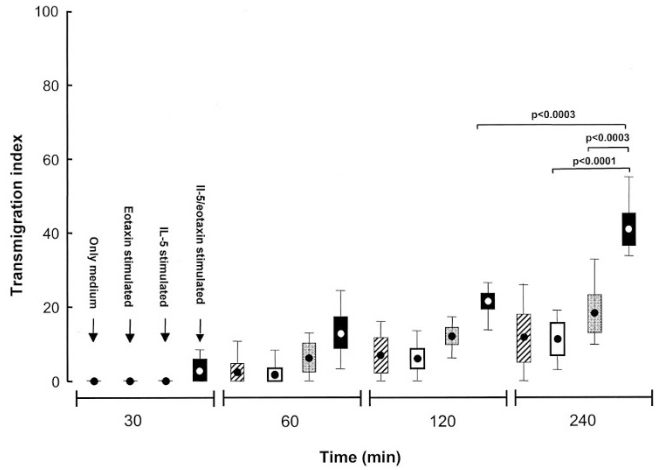

Figure 3. The time course of neonatal eosinophil transmigration to fibronectin-coated wells. Results, expressed as transmigration index, are presented as mean and SEM and are based on seven separate experiments.

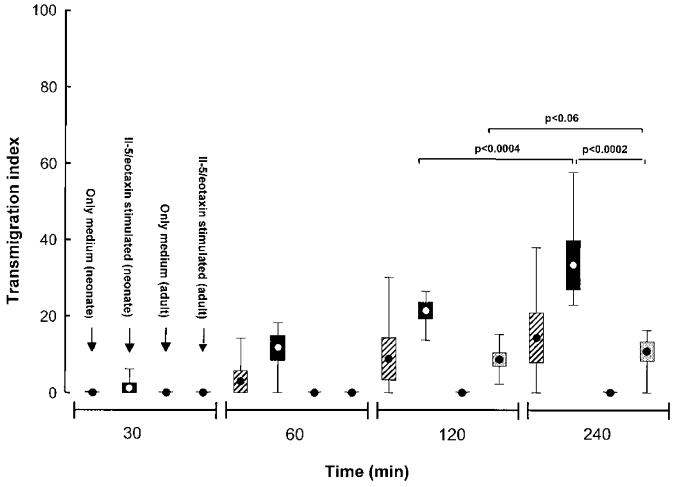

Figure 4. The effect of IL-5 and eotaxin on *in vitro* transmigration of neonate and adult eosinophils. Results, expressed as transmigration index, are presented as mean and SEM and are based on 10 separate experiments.

Table 2. *Surface expression of fMLP receptor on resting eosinophils and neutrophils*

	Eosinophils		Neutrophils	
	Positive cells	MFI	Positive cells	MFI
Neonate	98.1 ± 0.4*	130 ± 5.0†	87.9 ± 0.8	25.2 ± 0.8
Adult	0.70 ± 0.1	23.9 ± 3.1	99.6 ± 0.1	64.4 ± 0.4‡

Results are based on five experiments.

* $p < 0.0001$ *vs* positive eosinophils, which express fMLP-R on surface (adult).

† $p < 0.0001$ *vs* MFI of surface expression fMLP-R on eosinophils (adult).

‡ $p < 0.05$ *vs* MFI of surface expression of fMLP-R on resting granulocytes (neonatal).

DISCUSSION

Our main finding is that eosinophils in neonates are endowed with a profound responsiveness toward the bacteria-related peptide fMLP. This feature is elucidated by pronounced fMLP-induced transmigration capacity, CD11b up-regulation, and a significant expression of the fMLP receptor. These data provide plausible mechanisms for reported abnormalities in eosin-

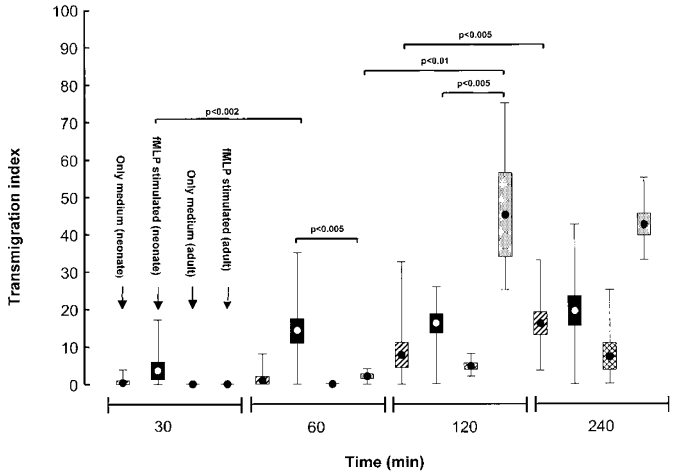

Figure 5. The effect of fMLP on *in vitro* transmigration of neonatal and adult neutrophils. Results, expressed as transmigration index, are presented as mean and SEM and are based on 10 separate experiments.

ophil trafficking in neonates and suggest that neonatal eosinophils may play an alternative role in innate immunity, compared with eosinophils in adults.

Despite the well-known transient eosinophilia that occurs in the neonatal period, our knowledge about the physiologic role of eosinophils in early life is limited. Data from several studies indicate that the eosinophil might be involved in inflammatory processes, unrelated to parasitic infections and allergic diseases, such as bronchopulmonary dysplasia, acute wheezing, and Rh disease of the newborn (5–7,16,17), and a physiologic role for the eosinophil during the overt colonization of skin and mucosal surfaces that occurs in early life has been proposed (8).

To exert these additional actions, we hypothesized that eosinophils in the neonatal period might be able to respond to stimuli that are not traditionally linked to the action of eosinophils. We selected the bacteria-related peptide fMLP. Among all of the chemoattractants so far discovered, the *N*-formyl peptide is unique. It is one of the first identified leukocyte chemoattractants, and the prototype, fMLP, contains only three amino acids and is the smallest chemotactic peptide. The fMLP peptide is also one of the most potent peptide chemoattractants, being able to induce all major phagocytic function (18).

In this study, we demonstrate that neonatal eosinophils have an efficient capacity to transmigrate toward the bacteria-related peptide fMLP. On the contrary, fMLP did not induce any significant transmigration of adult eosinophils. It is a matter of debate to which extent mature eosinophils in adults respond to fMLP, but several data support the view that fMLP has the ability to mount a cellular response in mature human eosinophils, exemplified by increased metabolic activity, as well as mobilization of intracellular stored adhesion molecules (10,19).

To further strengthen our finding that fMLP might show a pronounced response in neonatal eosinophils, we assessed the expression of the fMLP receptor in resting eosinophils. Indeed, we found a significant expression of the receptor in neonatal eosinophils. With the same method, only traces of the receptor were found in adult eosinophils. The receptor for fMLP is so far the only receptor that recognizes an exogenous chemoattractant, the bacterially derived *N*-formyl peptides. Neonatal neutrophils have been suggested to express normal numbers of fully functional fMLP receptors (20,21). Because of the well-recognized feature of recirculation, we selected an established method, in which the fMLP receptor was assessed in resting cells (11). The question of whether the fMLP receptor in neonatal eosinophils, besides being abundantly expressed on the surface, is endowed with additional specific characteristics is not addressed in the present study and merits further studies to be delineated.

Eotaxin has been claimed to be the most potent chemokine to selectively induce chemotaxis and infiltration of eosinophils to lung tissue after an allergen provocation in animal models (22–24). It has also been suggested that a relationship exists between IL-5 and eotaxin in regulating blood and tissue eosinophilia (25). Others and we have previously demonstrated that eotaxin is involved in the quantitative up-regulation of CD11b and increases the adhesion properties and transmigration of human eosinophils, acting synergistically in these respects (15,26). In line with these studies, the present study shows also the up-regulation of CD11b and efficient transmigration capacity of eosinophils, which was more pronounced in neonates compared with adults. Our data suggest a plausible generalization in eosinophil responsiveness in the neonatal period.

Accumulated data indicate that recruitment of eosinophils and neutrophils is a multistep process, and the combined action of multiple chemoattractants at different stages of inflammation regulates both the magnitude and the nature of transmigrating cells, thereby characterizing the cellular profile of a given inflammatory process (18). Several adhesion pathways have been attributed to eosinophil adhesion and transmigration. The integrins constitute an adhesion family that is deeply involved in different aspects of eosinophil function, and the αM subunit, also designated CD11b, has been claimed to be essential for both transmigration and degranulation (27,28). In this study, we demonstrate that neonatal eosinophils have an improved capacity to up-regulate CD11b, compared with eosinophils in adults. This contrasts with data from Smith *et al.* (29), who reported no significant differences in this respect between adults and neonates. The reason for this discrepancy is not known but might be technically related. For example, Smith *et al.* chose EDTA as anticoagulants, as opposed to citrate in our study. We recently reported that, in our hands, citrate is preferable because of its lesser impact on several eosinophil functional assays (13). However, both in the study by Smith *et al.* and in the present study, significantly lower up-regulation was noted in eosinophils than in neutrophils, an observation also previously reported (10). However, because we have not identified the relative contribution of the respective adhesion pathway in the transmigration assay used, the physiologic bearing of this finding on the transmigration data is only speculative. In contrast to neutrophils, eosinophils transmigrate to inflammatory sites in patients with leukocyte adhesion deficiency syndrome, indicating that eosinophils can use CD18-independent pathways for transmigration *in vivo* (30).

The defect in adherence and chemotaxis of neonatal neutrophils has been attributed mainly to altered adhesion molecule

expression as a prime cause of impaired neutrophil function (31–39). Our data agree with earlier findings that the fMLP-induced transmigration capacity is impaired in neonatal neutrophils. However, an additional finding was the increased spontaneous transmigration in neonates. The physiologic significance of this finding is not clear but might indicate that random neutrophil transmigration could be increased in neonates, maybe as a consequence of priming (40). This hypothesis is in line with recently reported data on an existing acute-phase reaction in neonates with increased levels of proinflammatory cytokines, even in the absence of overt infection (41).

CONCLUSION

To summarize, eosinophils in the neonatal period possess an enhanced responsiveness against the bacteria-related peptide fMLP, judged as enhanced transmigration capacity and CD11b up-regulation, presumably as a result of the ability to express the fMLP receptor. We therefore suggest that eosinophils in the neonatal period represent a first line of cellular defense that might be triggered by bacterial antigenic stimulation initiated by the early colonization of the mucosa surfaces. These data provide new insights into the mechanisms involved in the innate immune defense and point to a potential relevance of the eosinophil in the inflammatory process in early life.

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