Expression and Regulation of L-Selectin on Eosinophils from Human Adults and Neonates¹

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ABSTRACT. L-Selectin, previously known as LEC-CAM-1, LECAM-1, LAM1, and as the MEL-14, Leu-8, TQ1, and DREG-56 antigens, is a leukocyte membrane protein that participates in adhesion to endothelium. We studied its expression on eosinophils using flow cytometry and the MAbs Dreg-56 and Leu-8. Unstimulated peripheral blood eosinophils from healthy adults expressed about one third the level of L-selectin as neutrophils (mean $\pm$ SD specific fluorescence: 20.9 ± 3.2 versus 54.5 ± 8.4 , $p = 0.0001$, $n = 18$). After stimulation with A23187, L-selectin expression on eosinophils was rapidly lost. This was temporally correlated with increased expression of Mac-1 (CD11b/CD18); the kinetics on eosinophils and neutrophils were similar. Eosinophil expression of L-selectin decreased modestly after stimulation with platelet activating factor, but was minimally affected by N-formyl-methionyl-leucyl-phenylalanine, leukotriene B₄, or C5a compared with their effects on neutrophils. Eosinophils from cord blood of healthy neonates born at term expressed less L-selectin than adult eosinophils (10.4 ± 3.8 versus 19.4 ± 2.7 , $p = 0.0001$, $n = 9$); the relative reduction was the same as on cord blood neutrophils (36.4 ± 8.2 versus 55.5 ± 4.8 , $p = 0.0001$, $n = 9$). Relative to baseline expression, the responses of neonatal and adult cells to stimulation did not differ. We conclude that neonatal eosinophils have abnormalities in L-selectin expression similar to neonatal neutrophils and suggest that decreased expression of L-selectin and a diminished responsiveness to direct stimulation with chemotactic factors are possible mechanisms that may limit the exudation of eosinophils. (*Pediatr Res* 32: 465-471, 1992)

Abbreviations

FMLP, N-formyl-methionyl-leucyl-phenylalanine
LTB₄, leukotriene B₄
PAF, platelet activating factor
PE, phycoerythrin
HBSS, Hanks' balanced salt solution
FBS, fetal bovine serum

late eosinophil trafficking could lead to important advances in the prevention and treatment of asthma, acute lung injury, and other conditions in which eosinophil-mediated tissue injury may play an important role (1-3). The existence of abnormalities in eosinophil trafficking in the neonatal period is suggested by several longstanding clinical observations. Neonatal inflammatory exudates often contain an increased proportion of eosinophils (4-6), and an infiltrate of eosinophils is characteristic of erythema toxicum, a self-limited rash that occurs in as many as 70% of normal neonates (7). It is also well known that a majority of premature infants develop significant eosinophilia during the first several weeks of postnatal life (8, 9). We recently reported that, in fetuses with Rh disease studied at 20-26 wk gestation by *in utero* umbilical cord sampling, 60% of circulating granulocytes were eosinophils (10). Even in the healthy fetus, eosinophils average 10 to 20% of granulocytes at that stage of gestation (11). The mechanisms underlying these phenomena are not known.

Adhesion to endothelium via the specific interaction of cell-surface adhesion molecules is a crucial step in the process of leukocyte emigration from the bloodstream to the tissues (12, 13). Like neutrophils, eosinophils express the leukocyte integrins (CD18 family), and adhesion of eosinophils to endothelium via CD18-dependent mechanisms has been demonstrated (14-17). The importance of this family of adhesion receptors is exemplified by the profound defects of neutrophil exudation shown by patients with the severe form of the leukocyte adhesion deficiency syndrome, in which the CD18 molecules are congenitally absent (18). However, CD18-independent adhesion mechanisms also exist and may be of even greater importance for eosinophils than neutrophils, inasmuch as eosinophils of patients with the leukocyte adhesion deficiency syndrome are able to migrate into infected tissues (19). Like neutrophils, eosinophils bind to endothelial ELAM-1 (20-22). Unlike neutrophils, eosinophils express VLA-4, which binds to endothelial VCAM-1 (21-24). Another potential mechanism for CD18-independent adherence of eosinophils involves a leukocyte adhesion molecule recently designated L-selectin (25).

The selectins are a family of integral membrane proteins characterized by the presence of lectin-, epidermal growth factor, and complement-binding domains (25, 26). L-selectin was previously known as LEC-CAM-1, LECAM-1, LAM1, and as the MEL-14, Leu-8, TQ1, and Dreg-56 antigens (25, 27-31). It is involved in lymphocyte homing to peripheral lymph nodes and in the adhesion of neutrophils to inflamed endothelium (31, 32). Stimulation of neutrophils with chemotactic factors simultaneously produces a rapid decrease in expression of L-selectin together with an increase in expression of the leukocyte integrin Mac-1 (CD11b/CD18) (33, 34). Although closely coordinated, the mechanisms of these changes are different. The decrease in L-selectin is due to shedding from the cell surface of a fragment that is about 5 kD smaller than the cell-associated form (100 kD on neutrophils) (33, 35), whereas the increase in Mac-1 (CD11b/

Better understanding of the molecular mechanisms that regu-

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CD18) expression occurs by translocation of molecules from an intracellular pool to the plasma membrane (36). In addition to these changes in quantitative surface expression, cell stimulation also results in functional activation of these molecules (36, 37). The roles of these molecules in neutrophil adhesion appear to be distinct. It has been proposed that L-selectin is primarily involved in the initial adhesion of neutrophils to inflamed endothelium, especially under conditions of flow where CD18-dependent mechanisms are not significant, whereas Mac-1 is involved in subsequently strengthening the adhesion and in transendothelial migration (32). Shedding of L-selectin may be required for subsequent steps in migration to occur (38). Neonatal neutrophils have diminished expression of L-selectin compared to neutrophils from adults, and this correlates with diminished adhesion of neonatal neutrophils to IL-1-stimulated endothelium in conditions of flow (39).

The purpose of this study was to investigate the expression and regulation of L-selectin on eosinophils from adults and neonates. L-Selectin is known to be expressed on eosinophils from adults (27, 40), but regulation of its expression in response to stimulation has not been studied on either adult or neonatal eosinophils. Cell-specific responses to chemotactic factors, together with cell-specific patterns of expression of different types of adhesion molecules, may be an important mechanism for selective regulation of the emigration of different classes of leukocytes (34, 40). We recently reported dramatic differences between eosinophils and neutrophils in Mac-1 up-regulation in response to stimulation with FMLP, C5a, PAF, LTB₄ (41). We also showed that up-regulation of Mac-1 on neonatal eosinophils is significantly diminished compared to eosinophils from adults (41), similar to the defect previously demonstrated in neonatal neutrophils (10, 42, 43). We hypothesized that, if coordinate up-regulation of Mac-1 and down-regulation of L-selectin is a general feature of the regulation of the receptors on granulocytes, then changes in eosinophil L-selectin expression would follow the same pattern of responsiveness to different stimulating factors as shown for Mac-1. We also hypothesized that L-selectin expression on neonatal eosinophils would be diminished compared to eosinophils from adults.

MATERIALS AND METHODS

Subjects. Blood specimens were drawn from a peripheral vein of healthy adult volunteers or from the placental side of the clamped umbilical cord immediately after uncomplicated vaginal or cesarean deliveries of healthy infants at term gestation (38–41 wk). The specimens were obtained, with appropriate consent, in accordance with an approved institutional protocol.

Materials. The Dreg-56 antibody (IgG1 isotype) was produced and characterized as an anti-L-selectin antibody as previously described (31). PE-conjugated anti-Leu-8 (IgG2a) (anti-L-selectin), PE-conjugated anti-Leu-15 (IgG2a) (CD11b, anti-Mac-1 α -chain), FITC-conjugated anti-Leu-11a (IgG1) (CD16, anti-Fc γ R_{III}), and control MAb [unconjugated and FITC-conjugated mouse IgG1 (clone X40) and PE-conjugated mouse IgG2a (clone X39)] were obtained from Becton Dickinson (Mountain View, CA). Human Ig-adsorbed PE-conjugated goat anti-mouse F(ab')₂ antibodies (Tago, Inc., Burlingame, CA) were used for second-step labeling of Dreg-56 and its control. PE-conjugated beads from Flow Cytometry Standards Corporation (Research Triangle Park, NC) were used in each experiment as a fluorescence standard.

HBSS without bicarbonate or phenol red and PBS were obtained from GIBCO BRL (Grand Island, NY). HBSS was supplemented with 2% by volume heat-inactivated FBS (HBSS-FBS), adjusted to pH 7.35–7.40, and maintained sterile until use. HBSS-FBS without Ca and Mg was the buffer in all experiments except as specified. For eosinophil purification, the Dynal MPC1 magnetic particle concentrator and 4.5- μ m polystyrene magnetic

beads (Dynabeads M-450) coated with goat anti-mouse IgG were obtained from Robbins Scientific (Mountain View, CA).

Calcium ionophore A23187, FMLP, human recombinant C5a, PAF, LTB₄, zymosan, histamine, and the eosinophil chemotactic factor of anaphylaxis tetrapeptides Ala-Gly-Ser-Glu and Val-Gly-Ser-Glu were obtained from Sigma Chemical Co. (St. Louis, MO). Colloidal silica particles coated with polyvinyl pyrrolidone (Percoll) were purchased from Pharmacia (Piscataway, NJ).

Cell preparation. To minimize the possibility that L-selectin could be lost from the cell surface by inadvertent activation of the cells during *in vitro* procedures, EDTA-anticoagulated blood specimens were placed on ice immediately after collection, and all purification and labeling procedures were carried out at 0–4°C. For experiments using whole blood leukocytes, erythrocytes were removed by two cycles of hypotonic lysis and washing in HBSS-FBS, and the cell suspension was used with no further processing. For experiments using mixtures of eosinophils and neutrophils, two-layer discontinuous gradients of Percoll in PBS were prepared as described by Roberts and Gallin (44). Whole blood was diluted with isotonic PBS (1:1 for adult specimens or 1:2 for neonatal specimens with a total leukocyte count $\geq 12 \times 10^6$ /mL) and made slightly hypertonic by the addition of 10 μ L of 10 $\times$ -concentrated PBS per mL of the diluted blood. The cells were not exposed to FMLP. Up to 6 mL of cold, diluted blood was overlaid on gradients of 65% and 70% Percoll (3 mL in each layer) in a 15-mL conical tube and centrifuged at 4°C for 20 min at 250 $\times g$. The plasma layer and the cells at the upper interface were discarded, and the cells at the interface of the two Percoll layers were collected. If necessary, residual erythrocytes were removed by one cycle of hypotonic lysis after the cells were washed in HBSS-FBS to remove Percoll. For brevity, we refer to these preparations as granulocyte suspensions, but it should be noted that they do not include basophils, which remain with the mononuclear cells on Percoll gradients. Differential counts of Wright-stained smears of whole blood and of cytocentrifuge preparations of the whole blood leukocyte or granulocyte suspensions were made for each specimen.

Eosinophil purification. A modification of the procedure described by Hansel *et al.* (45) was used. Briefly, granulocytes prepared on a cold Percoll gradient as described above were resuspended in 200 μ L of buffer containing 0.1% Na azide and incubated with anti-Leu-11a (40 μ L per 10^6 cells) for 30 min on ice. The cells were then washed to remove unbound antibody, resuspended in a minimal volume of buffer (40 μ L for 10^6 cells) in a 1.5 mL microcentrifuge tube, mixed with an aliquot of goat anti-mouse IgG-coated magnetic beads (25 μ L containing 10^7 beads for 10^6 cells), and incubated at 4°C for 15 min with continuous rotation. The tube was then placed in a magnet (the Dynal MPC1) to retain the beads and bound cells, and the supernatant and unbound cells were removed by washing. After resuspension in 40 μ L of buffer, the unbound cells were incubated with a second aliquot of the magnetic beads and the separation was repeated. The purity of the preparation was verified by a differential count of a Wright-stained cytocentrifuge slide. Preparations containing >98% eosinophils were obtained routinely.

Cell stimulation. Incubation of the cells with stimulating factors was performed in a shaking water bath at 37°C. HBSS-FBS with Ca and Mg was used in this step. Parallel aliquots kept on ice (cold control) or incubated with buffer alone (37°C control) were included in each experiment. In the kinetics experiments, the cells were warmed for 10 min at 37°C before mixing (time zero) with solutions of the stimulating factor in buffer at 37°C. At intervals, aliquots were removed from the bath, mixed with cold buffer, and placed on ice.

Antibody labeling. HBSS-FBS (without Ca or Mg) with 0.1% Na azide was used in the remaining steps of the procedure. In each labeling step, the cells were resuspended in 0.1 mL of azide buffer, incubated for 30 min on ice with saturating amounts of antibody (as determined in preliminary experiments), and then

washed in 2 mL of azide buffer. Experiments using Dreg-56 required three labeling steps: incubation with Dreg-56 (or mouse IgG1 control MAb), then with PE-conjugated goat anti-mouse IgG F(ab')₂, and finally with FITC-conjugated anti-Leu-11a. For the PE-conjugated MAb (Leu-8, Leu-15, or mouse IgG2a control), labeling was done in a single step together with the FITC-conjugated anti-Leu-11a. After a final wash to remove unbound antibody, the cells were resuspended in 0.3 mL of azide buffer for analysis.

Flow cytometry and sorting. Analysis of eosinophils and neutrophils in mixed suspensions was performed on a FACScan flow cytometer (Becton Dickinson) as previously described (41). Briefly, red fluorescence (of PE-labeled antibody) was measured using linear amplification and expressed relative to the fluorescence of PE-conjugated beads measured in each experiment. Fluorescence of the PE-conjugated control (nonspecific background) was subtracted to give the net fluorescence corresponding to specific antibody binding. Corrections for spectral overlap (color compensation) were set using labeled and unlabeled neutrophils. Light-scatter gates were used to select the granulocyte region (neutrophils and eosinophils), excluding lymphocytes, monocytes, and basophils (46). Eosinophils (CD16-dim) and neutrophils (CD16-bright) were distinguished by their well-separated peaks on the CD16 (FITC-Leu11a) fluorescence distribution (41, 46). For eosinophils, the light-scatter gate was refined by back-gating of the CD16-dim granulocytes as described (41). Cell sorting was performed on a FACStar Plus (Becton Dickinson). Granulocytes or whole blood leukocyte suspensions were incubated with FITC-anti-Leu-11a, and the populations for sorting were defined as described above. The sorted cells were transferred to glass slides using a cytocentrifuge, then Wright-stained and counted.

Statistics. Mean fluorescence of individual distributions was calculated with the FACScan Research Software, version B (Becton Dickinson). Group statistics were calculated with StatView 512+ (Brainpower Inc., Calabasas, CA) on a Macintosh computer (Apple Computer, Cupertino, CA), and results were expressed as the group means $\pm$ SD of the individual distribution mean values. Statistical differences were assessed by repeated-measures analysis of variance and the paired *t* test.

RESULTS

Down-regulation of eosinophil L-selectin. Highly purified, unstimulated eosinophils were isolated from blood by Percoll gradient separation at 4°C of a mixture of neutrophils and eosinophils, followed by immunomagnetic removal of the neutrophils using a CD16 MAb (45). Typical fluorescence distributions of L-selectin on eosinophils purified by this method from peripheral blood of a healthy adult donor are shown in Figure 1. L-Selectin was expressed on more than 95% of unstimulated, cold eosinophils. Incubation of these cells for 20 min at 37°C in buffer had no effect on their expression of L-selectin, whereas incubation with 0.5 μ M A23187 resulted in a nearly complete loss of L-selectin expression. In contrast, A23187 produced a large increase in eosinophil Mac-1 expression. Incubation of eosinophils with 1 μ M FMLP produced a minimal decrease in their expression of L-selectin and a minimal increase in Mac-1 compared to the 37°C controls. These reciprocal changes in L-selectin and Mac-1 expression on eosinophils were similar to the behavior previously reported for neutrophils stimulated *in vitro* or *in vivo* (33, 38). The insensitivity of eosinophils to FMLP is consistent with the lack of a high-affinity receptor for this substance on eosinophils (44, 47, 48).

L-Selectin expression of unstimulated eosinophils. Because surface expression of L-selectin is affected by cell stimulation, we compared its expression on neutrophils and eosinophils using flow cytometry with a multiparameter gating method that allowed these cells to be distinguished in mixed leukocyte suspensions (41). This ensured that the two cell types were studied

Fig. 1. Representative fluorescence distributions of L-selectin and Mac-1 expression on eosinophils. Aliquots of eosinophils (>99%) purified from adult peripheral blood were kept on ice (cold control) or incubated at 37°C for 20 min in buffer with 1 μ M FMLP or with 0.5 μ M A23187, then labeled with PE-conjugated MAb to L-selectin (Leu-8) or the α -chain of Mac-1 (Leu-15) or with an isotype-specific control antibody. Each graph shows the relative number of cells at each fluorescence intensity on a 1024-channel logarithmic scale for 4500 total cells. The vertical dotted line indicates the cutoff for positivity defined by the control antibody distribution for each condition. The numbers in the upper right corners indicate the mean fluorescence channel and the percentage of positive cells for the distribution.

Fig. 2. L-Selectin expression of unstimulated neutrophils (solid symbols) and eosinophils (open symbols) from adult peripheral blood ($n = 18$). Whole blood leukocytes kept at 0–4°C were labeled with the Dreg-56 and anti-Leu-11a MAb and analyzed by flow cytometry with multiparameter gating as described in Materials and Methods. The squares and error bars indicate the mean $\pm$ SD for each cell type.

under identical conditions. The expression of L-selectin on unstimulated eosinophils and neutrophils from peripheral blood of healthy adult donors is shown in Figure 2. Eosinophils expressed significantly less L-selectin than neutrophils (20.9 ± 3.2 versus 54.5 ± 8.4 , $p = 0.0001$, $n = 18$), in specimens kept at 0–4°C. Because we had previously found that incubation in buffer for 20 min at 37°C resulted in a 200% increase in Mac-1 expression on neutrophils and a 60% increase in Mac-1 expression on eosinophils (41), we examined the effect of warming on L-selectin expression. A 20-min incubation at 37°C did not affect the expression of L-selectin on either cell type (eosinophils: 20.9 ± 3.1 , neutrophils: 54.5 ± 7.8 ; $n = 18$), and prolonging the incubation for as long as 60 min also had no effect.

Kinetics of L-selectin down-regulation. The time course of the changes in eosinophil and neutrophil L-selectin expression induced by stimulation with A23187 were compared in three

experiments (Fig. 3). For both cell types, there was little change in the first minute after introduction of A23187, then a rapid decrease to $35 \pm 13\%$ of the initial value for neutrophils and $54 \pm 9\%$ for eosinophils at 2 min. At 4 min, neutrophil L-selectin expression was $2.9 \pm 1.3\%$ of its initial value, compared to $17.6 \pm 9.3\%$ for eosinophils ($p = 0.05$), but the overall time courses did not differ significantly. In one experiment, Mac-1 expression was measured in parallel. The time courses of the increases in Mac-1 and the decreases in L-selectin were similar, except that there was no initial delay in the rise of Mac-1 expression.

Effects of chemotactic factors. The responses of eosinophils and neutrophils to increasing concentrations of A23187, PAF, FMLP, and zymosan-activated serum were compared (Fig. 4). Although A23187 stimulation at concentrations of 250 nM or more produced virtually complete losses of L-selectin expression on both cell types, the other substances had much more limited effects on eosinophils compared to neutrophils. PAF at 10- and 100-nM concentrations decreased eosinophil L-selectin expression to $76 \pm 27\%$ and $59 \pm 27\%$ of the control value, respectively ($p < 0.002$), compared to $34 \pm 13\%$ and $17 \pm 7\%$ for neutrophils ($p < 0.0001$). FMLP at 10 nM had no effect on eosinophils but produced a nearly complete loss of L-selectin expression from neutrophils. FMLP at 1 μ M reduced eosinophil L-selectin to 84%

Fig. 3. Kinetics of the changes in eosinophil and neutrophil expression of L-selectin after stimulation. At time zero, buffer containing A23187 at a final concentration of 250 nM (solid circles) or control buffer (open circles) was mixed with whole blood leukocytes at 37°C. At intervals, aliquots were removed from the 37°C bath, cooled by the addition of ice-cold buffer, then labeled with MAb (Dreg-56 or anti-Leu-15, plus anti-Leu-11a) and analyzed as described in Materials and Methods. The results are the mean $\pm$ SD for experiments with blood from three adults.

Fig. 4. Dose response of the decrease in L-selectin expression of neutrophils (solid circles) and eosinophils (open circles). Adult whole blood leukocyte suspensions were incubated at 37°C for 20 min with the indicated concentrations of the activating factors, then labeled with Dreg-56 and anti-Leu-11a MAb and analyzed as described in Materials and Methods. Results for A23187, PAF, and FMLP are the mean $\pm$ SD for nine experiments. The effect of zymosan-activated autologous serum is shown for one donor.

of the control value ($p = 0.02$). Similarly, zymosan-activated serum (Fig. 4), 100 nM recombinant C5a, or 100 μ M LTB₄ each had minimal effects on eosinophil L-selectin while reducing neutrophil L-selectin expression to less than 10% of the level on unstimulated controls. Incubation with PAF, LTB₄, or C5a for 60 min had no additional effect on either neutrophil or eosinophil L-selectin expression compared to a 20-min incubation (not shown). In other experiments, histamine (0.1 and 1.0 mM) and the eosinophil chemotactic peptides Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (10 nM to 100 μ M) had no effect on L-selectin expression of either eosinophils or neutrophils.

Neonatal eosinophils. The use of the CD16 gating method to distinguish eosinophils and neutrophils in cord blood is more demanding than for specimens from healthy adults, because cord blood sometimes contains a significant subpopulation of CD16-dim immature myeloid cells with distinctly larger forward light scatter than the eosinophils. We previously showed that this subpopulation was removed when granulocytes were separated by Percoll gradient centrifugation and that, for whole blood, CD16-gating combined with exclusion of this large light-scatter subpopulation gave results for eosinophil Mac-1 expression close to the values obtained for purified eosinophils (41). To confirm the validity of the gating procedure directly, we performed cell sorting experiments (Table 1). For whole blood suspensions from both cord blood and adult specimens, before and after stimulation with A23187, the CD16-dim granulocytes with light scatter in the eosinophil region were $\geq 93\%$ eosinophils and the CD16-bright granulocytes were $\geq 98\%$ neutrophils. When Percoll-purified granulocytes were sorted, $\geq 98\%$ of the CD16-dim cells were eosinophils and $>99\%$ of the CD16-bright cells were neutrophils. For three adult and four neonatal blood samples, we evaluated the possible effects of the cell preparation and analysis methods by comparing the values for L-selectin expression obtained using whole blood with those obtained using Percoll-separated granulocytes. For two adult and two neonatal specimens, these values were also compared with the results for eosinophils purified from the same blood samples. The cell preparation and analysis methods had no significant effects on the results for L-selectin fluorescence. The mean coefficient of variation between pairs of measurements for the same specimens was $\leq 5\%$ for neutrophils and $\leq 9\%$ for eosinophils.

L-selectin expression on unstimulated neutrophils and eosinophils kept at 0–4°C was compared for nine pairs of adult and cord blood specimens (Fig. 5). L-selectin expression was significantly less on neonatal compared to adult eosinophils (mean $\pm$ SD specific fluorescence 10.4 ± 3.8 versus 19.4 ± 2.7 , $p = 0.0001$) and on neonatal compared to adult neutrophils (36.4 ± 8.2 versus 55.5 ± 4.8 , $p = 0.0001$). For both adults and neonates, eosinophils expressed significantly less L-selectin than neutrophils ($p = 0.0001$), with a mean ratio of eosinophil to neutrophil L-selectin of $35 \pm 6\%$ for adults and $29 \pm 8\%$ for neonates.

It was previously noted that neonatal neutrophils stimulated with FMLP did not appear to down-regulate their expression of L-selectin to the same extent as adult neutrophils (39). In this study, L-selectin down-regulation on neonatal eosinophils and neutrophils stimulated with A23187, FMLP, and PAF was investigated. As with the adult cells, incubation in buffer at 37°C did not affect L-selectin expression on either neutrophils or eosinophils. A23187 and PAF produced large reductions in L-selectin on both cell types, whereas FMLP had a much greater effect on neutrophils. When L-selectin expression was expressed as a percentage of its level on the unstimulated controls for each cell (Fig. 6), there were no significant differences in the responses to stimulation of neonatal and adult eosinophils or in the responses of neonatal and adult neutrophils.

DISCUSSION

The regulation of eosinophil trafficking is likely to involve multiple mechanisms—some that promote the specific exudation

Table 1. Confirmation of analysis procedure for cord blood by cell sorting*

	CD16 dim granulocytes % Eosinophils	CD16 bright granulocytes % Neutrophils
Whole blood (0°)		
Adult	95, 99	99, 100
Neonatal	93, 93	100, 100
Whole blood (A23187)		
Adult	94, 97	98, 98
Neonatal	97	98
Granulocytes (0°)		
Adult	100	100
Neonatal	98	100

* Whole blood leukocytes or granulocytes from two adults and two neonates were kept cold (0–4°) or incubated with 500 nM A23187 for 20 min at 37°C, then labeled with FITC-conjugated anti-Leu-11a antibody (CD16). Granulocytes were identified by light-scatter gating and sorted into CD16-bright and CD16-dim subpopulations. For the whole-blood preparations, a refined light-scatter gate for the eosinophils was set as described in Materials and Methods. The table shows the number of eosinophils or neutrophils in 100-cell differential counts of Wright-stained slides of the two populations of sorted cells.

Fig. 5. Expression of L-selectin (mean ± SD) on unstimulated cord blood neutrophils and eosinophils. Whole blood leukocytes or granulocytes from nine pairs of adults and neonates kept at 0–4°C were labeled with Dreg-56 and anti-Leu-11a MAb and analyzed as described in Materials and Methods. *, $p = 0.0001$ between groups.

Fig. 6. Comparison of the responses of adult neutrophils (light shading), neonatal neutrophils (white), adult eosinophils (dark shading), and neonatal eosinophils (black) to incubation with activating factors. Whole blood leukocytes were kept cold or incubated for 20 min at 37°C in buffer (37° control) or with the indicated concentrations of A23187, PAF, and FMLP, then labeled with Dreg-56 and anti-Leu-11a MAb and analyzed as described in Materials and Methods. L-Selectin fluorescence was expressed as a percentage of the cold control value for each donor. The graph shows the results (mean ± SD) for five pairs of adults and neonates.

of eosinophils, and others that normally limit their involvement at sites of infection or inflammation. The results of this study suggest two possible mechanisms that may tend to limit eosinophil exudation: diminished quantitative expression of L-selectin on eosinophils compared to neutrophils and a diminished responsiveness of eosinophils to chemotactic factors.

We found that the baseline expression of L-selectin on unstimulated peripheral blood eosinophils from healthy adults was about one third the level expressed on neutrophils in the same

specimens. It was shown previously that under conditions of flow corresponding to the shear stress prevailing in postcapillary venules, neutrophil adherence to IL-1-stimulated endothelium was dependent on L-selectin and virtually independent of CD18-dependent mechanisms (32). Furthermore, there was a direct correlation between the quantitative expression of L-selectin and the number of adherent cells (32). It is possible, therefore, that the reduced expression of L-selectin on eosinophils may result in diminished endothelial adhesion of eosinophils compared to neutrophils in conditions where L-selectin-mediated adhesion may be important. At present, there is no information on the relative importance of different adhesion mechanisms for eosinophils under conditions of flow.

The second mechanism that may serve to limit exudation of eosinophils is their relative unresponsiveness to direct stimulation with chemotactic factors. We found that, although FMLP, C5a, PAF, and LTB₄ all induced large changes in neutrophil L-selectin expression, eosinophils in the same cell suspensions had a modest response to PAF and minimal or no responses to C5a, FMLP, and LTB₄. The magnitudes of the eosinophil L-selectin responses to these factors followed the pattern previously found for Mac-1 up-regulation on eosinophils (41). These results suggest that some additional priming or activation signal is required to initiate a response to C5a in eosinophils, because PAF and C5a are both potent chemotactic factors for eosinophils (49, 50). It should be noted that the processes involved in triggering eosinophil migration are probably complex and that, although the precise roles of these particular adhesion receptor responses are not known, they are likely to be coordinated with other mechanisms. The idea that the diminished L-selectin and Mac-1 responses to direct stimulation with chemotactic factors are important characteristics of eosinophils is therefore supported by the results of Giembycz *et al.* (51), who found that PAF was more effective than C5a, FMLP, and phorbol myristate acetate in stimulating release of thromboxane B₂ by eosinophils but far less effective than A23187. The mechanisms involved in eosinophil activation or lack of activation by specific chemotactic factors are not well understood, except that eosinophils lack a high-affinity receptor for FMLP (44, 47, 48). In addition, the eosinophil C5a receptor has been shown to be distinct from the neutrophil C5a receptor (52). Elucidation of the mechanisms responsible for the differing responses of neutrophils and eosinophils to chemotactic stimulation will be an important goal for future studies.

These studies demonstrate that down-regulation of L-selectin expression on human eosinophils was coordinated with Mac-1 up-regulation, as previously described for neutrophils (33, 34). There were two aspects to this coordination. First, the changes in Mac-1 and L-selectin were correlated in time, proceeding to

completion within 4 to 8 min, and the kinetics for eosinophils and neutrophils were similar. Second, the magnitude of the changes in eosinophil Mac-1 and L-selectin induced by a particular stimulus were similar, *i.e.* the stimulus triggered both processes to the same relative extent. However, some differences in the timing and initiation of the L-selectin and Mac-1 responses were observed. After exposure of the cells to A23187, Mac-1 expression began to increase immediately, whereas there was a 1-min lag before L-selectin decreased. In addition, Mac-1 continued to rise after the changes in L-selectin were complete. We also found that a 20-min incubation at 37°C in buffer produced no change in either eosinophil or neutrophil L-selectin, whereas Mac-1 increased significantly. This shows that the Mac-1 and L-selectin responses can be dissociated in part. Future studies of signal transduction pathways in neutrophils and eosinophils may be able to exploit this observation.

The cell sorting studies reported here and the demonstration of the lack of an effect of the cell preparation method on eosinophil and neutrophil expression of L-selectin add to the previous description of the use of CD16 to distinguish eosinophils and neutrophils in mixed suspensions of neonatal leukocytes (41). Back-gating of light scatter allowed us to distinguish eosinophils from the CD16-dim immature myeloid cells that were present in cord blood but not in adult peripheral blood. Alternatively, the complication introduced by this population could be avoided by partial purification on a simple two-layer Percoll gradient. For cell populations with a very large number of immature myeloid cells (*e.g.* bone marrow) or where extensive *in vivo* loss of CD16 on neutrophils may occur (53), the ability of light scatter to discriminate eosinophils from other CD16-dim cells would need to be specifically examined.

Newborn infants have an enhanced susceptibility to serious bacterial infections, which may be due in part to transient abnormalities of neutrophil function. Studies of neonatal animals have demonstrated diminished or delayed emigration of neutrophils into sites of inflammation, and numerous *in vitro* studies of human neutrophils have found defects in chemotactic functions (54, 55). Although the etiology of these changes is not fully understood, neonatal neutrophils have been shown to have specific abnormalities of adhesion and transendothelial migration related to L-selectin and Mac-1 (39, 55). The results for neonatal neutrophils in this study confirmed the previous finding of significantly reduced expression of L-selectin compared to adult neutrophils (39). However, in that study the magnitude of the reduction was greater, and stimulation with FMLP failed to induce a further decrease of L-selectin on neonatal neutrophils. We attribute these differences to the use in the previous study of neutrophils isolated at room temperature by dextran sedimentation and Ficoll-Hypaque centrifugation. In the present study, we found that neonatal neutrophils as well as neonatal eosinophils responded to FMLP and other factors to the same relative extent as the adult cells.

This study shows that neonatal eosinophils have decreased expression of L-selectin compared to peripheral blood eosinophils from normal adults. The ratio of neonatal to adult eosinophil L-selectin expression (54%) was similar in magnitude to the ratio for neonatal to adult neutrophils (66%). We previously demonstrated that maximum expression of Mac-1 on neonatal eosinophils in response to stimulation was 68% of that of adult eosinophils, compared to 67% for neonatal *versus* adult neutrophils (41). This remarkable parallelism suggests that a common mechanism may underlie the abnormal expression of these receptors on both eosinophils and neutrophils of neonates.

Furthermore, this parallelism raises the possibility that neonatal eosinophils may have Mac-1 and L-selectin-related abnormalities in adhesion and migration similar to those of neonatal neutrophils. For neonatal and adult neutrophils, expression of L-selectin correlated with adhesion of neutrophils to IL-1-stimulated endothelium under conditions of flow where CD18-dependent mechanisms were unimportant (39). It is not presently

known whether L-selectin-mediated adhesion has a similar importance for eosinophils under these conditions. It is also not known whether there is a direct relationship between the Mac-1-related abnormalities in adhesion and transendothelial migration of neonatal neutrophils and their abnormality of quantitative expression, because abnormalities of activation of Mac-1 are also thought to contribute (55).

Future study of neonatal eosinophils together with neonatal neutrophils may provide a useful physiologic model for investigating the specific functions of Mac-1, L-selectin, and other adhesion receptors. If eosinophils prove to have functional defects in Mac-1 and L-selectin similar to those of neonatal neutrophils (39, 55), the increased involvement of eosinophils in neonatal inflammatory exudates would require a significant enhancement of other adhesion systems, such as the VLA-4/VCAM-1 interaction (21–24), or other mechanisms entirely. We conclude that neonatal eosinophils have abnormalities of L-selectin expression similar to neonatal neutrophils and that decreased expression of L-selectin and a diminished responsiveness to chemotactic factor stimulation are possible mechanisms that may tend to limit the exudation of eosinophils. The adhesion and migration properties of neonatal eosinophils will be important areas for further study.

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