Early Persistent Blood Eosinophilia in Necrotizing Enterocolitis Is a Predictor of Late Complications

Lila S. Wahidi, Jan Sherman, Mindy M. Miller, Habib Zaghouni, Michael P. Sherman

Department of Child Health, School of Medicine, Departments of Molecular Microbiology and Immunology and Neurology, and Sinclair School of Nursing, University of Missouri, Columbia, Mo., USA

Abstract

Background: Eosinophils infiltrate intestinal tissue during necrotizing enterocolitis (NEC) and adult bowel diseases. We theorized that epithelial damage causes eosinophilic activation and recruitment at NEC onset. Objective: We studied the relationship between persistent blood eosinophilia and medical or surgical complications during NEC. Methods: NEC cases and controls at MU Children’s Hospital (2008–2013) underwent review. A Likert scale measured NEC severity. We utilized an SPSS database for statistical analyses. Results: Of 50 NEC cases, infants in group 1 (n = 15) had eosinophilia <2 days after onset and those in group 2 (n = 25) had NEC but no persistent eosinophilia. Group 3 (n = 46) consisted of controls, i.e. infants without NEC matched for birth weight and gestational age and group 4 (n = 4) of preterm infants with infection and ≤5 days of eosinophilia. Hematologic assessment defined persistent eosinophilia as ≥5% eosinophils for ≥5 days after NEC onset. Absolute eosinophil counts were 2 times higher in group 1 than in group 2 (p = 0.002). The mean duration of eosinophilia was 8 days in group 1 versus 1 day in group 2 (p < 0.001). A Likert score of NEC severity was 3-fold higher in group 1 than in group 2 (p < 0.001). Compared to group 2, group 1 infants were 8 times more likely to have hepatic fibrosis or intestinal strictures. Conclusions: Early persistent blood eosinophilia is not currently a predictor of complications after the onset of NEC. This biomarker identifies immature infants at a high risk for adverse outcomes during NEC convalescence.

Introduction

Infants requiring surgery for the complications of necrotizing enterocolitis (NEC) have an excess of eosinophils in their intestinal specimens [1]. Gastrointestinal (GI) diseases with eosinophilic infiltrates occur at different locations during childhood and in adult life [2]. The complications observed in adult eosinophilic GI disease parallel the adverse outcomes of the clinical course of NEC. These complications include enteric motility disorders, feeding problems, fibrotic strictures, bowel adhesions and intestinal perforations, after an infant develops NEC [3, 4]. We hypothesized that persistent blood eosinophilia during NEC is a predictor of late medical and/or surgical compli-
cations. Immunopathology currently implicates the participation of immune cells, cytokines and chemokines in eosinophilic inflammation following epithelial damage of the intestinal mucosa (fig. 1). One of the key cytokines in GI immunity is interleukin (IL)-33 [5]. Upon injury, epithelia secrete IL-33 which interacts with mast cells and tissue macrophages [6, 7]. As a member of the IL-1 family, IL-33 signals via the ST2 receptor and induces T helper type 2 (Th2)-associated cytokine secretion [5, 8]. Th2 cell-secreted cytokines and chemokines, namely IL-5, IL-13 and eotaxin, increase bone marrow production of eosinophils and mediate eosinophil-associated chemotaxis to the intestinal compartment [9, 10]. IL-33 helps restore damaged intestinal mucosa [5], and also increases transforming growth factor β and other biomolecules that promote structural abnormalities like subepithelial fibrosis [5, 11]. The complications observed in eosinophilic esophagitis, Crohn’s disease and ulcerative colitis are consequences of this antecedent immunopathology. Despite the frequency of blood eosinophilia during NEC, this hematologic biomarker is not a current predictor of disease progression in neonatal NEC. This research study evaluates whether blood eosinophilia significantly identifies future adverse outcomes during the course of NEC.

**Methods**

**Study Design and Participants**

The Health Science Institutional Review Board of the University of Missouri approved this retrospective research study and waived written informed consent. The study subjects were preterm infants admitted to the neonatal intensive care unit at the University of Missouri Children’s Hospital from January 2008 to December 2013. Our Vermont Oxford Neonatal Network (VON) database identified infants hospitalized with NEC. We identified the NEC cases based on the VON criteria. Controls were infants who were born near to the time of admission of infants who developed NEC, and had a similar birth weight and gestational age to NEC infants, but no NEC during their hospital stay.

**Procedures**

Electronic medical records categorized the clinical, nutritional, laboratory, radiographic and surgical pathology data of each NEC-related case. NEC cases were divided into infants with a classic presentation and those who developed NEC <48 h after a blood transfusion (transfusion-associated NEC, TANEC) [3]. All infants with NEC and controls had their complete blood counts examined for blood eosinophil percentages and absolute eosinophil counts on consecutive days. Four groups of infants emerged from this analysis (table 1). Based on the hematologic review of these 4 groups, we defined persistent eosinophilia as ≥5% of the total white blood cell (WBC) count for ≥5 consecutive days after the onset of NEC. We calculated the absolute eosinophil counts

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**Fig. 1.** Immune cells, cytokines and chemokines that participate in eosinophilia during NEC. CTGF = Connective-tissue growth factor; DAMPs = damage-associated molecular patterns; ILC = innate lymphoid cell; MMP-9 = matrix metalloproteinase 9; TGF-β = transforming growth factor β.
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We then examined the feces of infants diagnosed as having NEC for eosinophils to rule out eosinophilic colitis. We reviewed all entries in the electronic medical records made by physicians, nurses, and from the laboratory and radiology for each case of confirmed NEC. We determined a case of NEC in preterm infants according to the VON criteria [12]. Briefly, a definition of NEC requires ≥1 of the following clinical signs: (1) bilious gastric aspirate or emesis, (2) abdominal distension and (3) occult or gross blood in stools (excluding fissures) and ≥1 of 3 radiographic findings, i.e. pneumatosis intestinalis, hepatobiliary gas and pneumoperitoneum. We measured the severity of NEC-related complications during the hospital stay on a Likert scale (table 2).

Statistical Analysis

Our sample size calculation used analysis of variance, 4 groups, a power of 0.80, an effect of 0.50 and α = 0.05, and it resulted in a minimal total sample size of 48 subjects [13]. Data storage and analyses used SPSS Software (IBM Corp., released 2013; IBM SPSS Statistics for Windows v22.0. Armonk, N.Y., USA). Data analysis made use of descriptive statistics, ANOVA for parametric measures and the Fisher exact test, the Mann-Whitney U test and the Kruskal-Wallis test for nonparametric measures. p < 0.05 represented a statistically significant difference. To show clinical significance, we present results as the mean ± standard deviation (SD), receiver operator curves expressed as areas under the curve and 95% confidence intervals.

Results

We excluded infants if their data would have biased the findings. After reviewing GI pathology, we excluded 2 infants who had spontaneous intestinal perforation rather than NEC. We excluded 2 in group 1 with NEC who died later from causes other than NEC. We excluded 6 in group 2 who died at ≤2 days after the onset of NEC and thus could not have had persistent eosinophilia. Figure 2 shows a CONSORT diagram for all infants in this study. Table 3 summarizes the findings for groups 1–4. In group 1, 12/17 (71%) had classic NEC and 5/17 (29%) had TANEC. None of the infants in group 1 with prolonged blood eosinophilia had fecal eosinophilia or features consistent with eosinophilic colitis. In group 2, 12/25 infants (48%) presented with classic NEC, while 13/25 (52%) had TANEC. The mean absolute eosinophil count of 6 infants in group 2 who died <48 h after the onset of NEC was 0.12 × 10^9 cells/l. The pathology of their small intestine involved a bloodless and necrotic bowel. This devitalized intestine likely produced low amounts of cytokines and chemokines. We suggest that this gut pathology explains the low absolute eosinophil counts in these babies. Two infants in group 2 died after the resolution of NEC. Group 3 infants did not have ≥5% blood eosinophilia for ≥2 days; however, 4 infants in group 4 had bloodstream infection or pneumonia with ≥5% blood eosinophilia for 3–5 days. Group 3 and 4 neonates had no signs or symptoms of NEC or 5% eosinophilia that persisted for >5 days, and none of them developed complications like the infants with NEC during hospitalization.

Table 1. Definition of study group assignments

<table>
<thead>
<tr>
<th>Preterm infants</th>
<th>Group definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>persistently elevated eosinophil counts after the onset of NEC (defined as ≥5% blood eosinophilia for ≥5 days)</td>
</tr>
<tr>
<td>Group 2</td>
<td>infants with NEC and either low or normal eosinophil counts; no persistent blood eosinophilia</td>
</tr>
<tr>
<td>Group 3</td>
<td>controls with normal eosinophil counts; no infection or NEC</td>
</tr>
<tr>
<td>Group 4</td>
<td>controls with eosinophil counts ≥5% for 3–5 days</td>
</tr>
</tbody>
</table>

1 Infants with blood eosinophilia elevated by a blood or focal infection with bacteria.

Table 2. Likert scale, measuring the severity of complications after onset and during the course of NEC

<table>
<thead>
<tr>
<th>Score</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>minimal side effects after the onset of NEC until hospital discharge, i.e. delays in full enteral feeding caused by residuals and/or abdominal distension</td>
</tr>
<tr>
<td>1</td>
<td>prolonged (≥2 weeks) or significant (need for parenteral nutrition) feeding problems after NEC; bowel-wall thickening on abdominal radiographs</td>
</tr>
<tr>
<td>2</td>
<td>isolated intestinal stricture not needing surgery during convalescence after NEC</td>
</tr>
<tr>
<td>3</td>
<td>need for surgery for complications after NEC (bowel obstruction, perforation, obstructive or multiple strictures and/or adhesions, pneumoperitoneum)</td>
</tr>
<tr>
<td>4</td>
<td>need for bowel removal and/or ostomy at surgery (nonviable gut, multiple strictures)</td>
</tr>
<tr>
<td>5</td>
<td>death caused by complications of NEC before hospital discharge¹</td>
</tr>
</tbody>
</table>

¹ We did not assign a Likert score to infants who died ≤2 days after NEC onset, in order to eliminate a scoring bias. These infants did not survive long enough to have persistent blood eosinophilia. We also excluded infants dying from causes other than NEC.
Lastly, the infants assigned to group 1 had the lowest birth weight and gestational age out of the 4 groups (table 3). We suggest that birth weight and gestational age may be additional risk factors for NEC-related complications besides elevated, persistent blood eosinophilia.

**Discussion**

NEC is the major inflammatory GI disease and a leading cause of death and prolonged hospitalizations of preterm infants [3]. Major GI surgery for the complications of NEC is a major cause of mortality and morbidity among these infants. Preterm infants are at increased risk of NEC, possibly due to their immature immune system and gastrointestinal (GI) tract [4]. A number of NEC risk factors have been identified, including birth weight and gestational age [5].

Table 3. Findings for infants with persistent eosinophilia and NEC (group 1) compared to infants with NEC and no persistent eosinophilia (group 2) and controls (groups 3 and 4)

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n = 15)</th>
<th>Group 2 (n = 25)</th>
<th>Group 3 (n = 46)</th>
<th>Group 4 (n = 4)</th>
<th>Statistical comparison group 1 vs. 2</th>
<th>Area under the ROC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Birth weight, g</td>
<td>911 ± 422</td>
<td>1,340 ± 498</td>
<td>1,099 ± 466</td>
<td>992 ± 293</td>
<td>p = 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73 (0.57 – 0.89)</td>
</tr>
<tr>
<td>Gestational age, weeks</td>
<td>26.8 ± 2.9</td>
<td>29.2 ± 3.3</td>
<td>28.3 ± 3.4</td>
<td>26.9 ± 1.4</td>
<td>p = 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70 (0.53 – 0.87)</td>
</tr>
<tr>
<td>Absolute eosinophil count, × 10&lt;sup&gt;9&lt;/sup&gt;/l</td>
<td>1.37 ± 0.79</td>
<td>0.65 ± 0.41</td>
<td>0.50 ± 0.35</td>
<td>1.44 ± 1.37</td>
<td>p = 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.80 (0.66 – 0.95)</td>
</tr>
<tr>
<td>Percentage of eosinophils/total WBC</td>
<td>15 ± 7</td>
<td>4 ± 2</td>
<td>3 ± 2</td>
<td>11 ± 7</td>
<td>p &lt; 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.97 (0.92 – 1.00)</td>
</tr>
<tr>
<td>Duration of ≥5% blood eosinophilia, days</td>
<td>8 ± 2</td>
<td>1 ± 1</td>
<td>1 ± 1</td>
<td>4 ± 2</td>
<td>p &lt; 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.93 (0.81 – 1.0)</td>
</tr>
<tr>
<td>Late bloody stools (mucosal ulcer?), n</td>
<td>17</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>p = 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.82 (0.62 – 1.0)</td>
</tr>
<tr>
<td>Likert scale score</td>
<td>3.1 ± 1.5</td>
<td>1.0 ± 1.4</td>
<td>n.a.</td>
<td>n.a.</td>
<td>p &lt; 0.001&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.88 (0.78 – 0.98)</td>
</tr>
<tr>
<td>Intestinal strictures and/or liver fibrosis, n</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>p = 0.02&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.50 (0.31 – 0.69)</td>
</tr>
<tr>
<td>TANEC and bloody stools, yes/no</td>
<td>1/4</td>
<td>4/9</td>
<td>0</td>
<td>0</td>
<td>p = 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.50 (0.31 – 0.69)</td>
</tr>
</tbody>
</table>

Values represent mean ± SD, unless otherwise indicated. CI = Confidence interval; n.a. = not applicable; ROC = receiver operating curve.

<sup>a</sup> ANOVA; <sup>b</sup> Kruskal-Wallis test; <sup>c</sup> Mann-Whitney U test; <sup>d</sup> Fisher exact test.
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of NEC doubles the risk of death. Advances in neonatal care have improved the survival rate for small and immature infants, but this advance has also increased the risk of acquiring NEC [3]. Group 1 infants with NEC and early persistent eosinophilia belong to a population of extremely immature infants (table 3). While many survivors of acute NEC suffer few/no sequelae, a meaningful number of infants develop life-threatening complications after successful medical management. Schimpl et al. [14] emphasized that intestinal strictures occur in 15–35% of infants recovering from NEC, and stricture formation increases the risk of bowel perforation, septicemia and death. Hepatic fibrosis and/or stricture occurred 8 times more often in group 1 than in group 2 (table 3). As shown in figure 1, injured intestinal epithelia secrete IL-33 that enters tissues and the portal circulation, acting as a stimulus for fibrogenesis [5, 11, 15]. Recent articles on cytokine-associated biomarkers during NEC are silent about IL-33 in the immunopathology of NEC [16, 17]. Clinical care makes it imperative to identify infants with NEC and persistent eosinophilia because treatment may reduce complications. In mice, anti-IL-33 antibody and soluble ST-2, the IL-33 receptor, inhibit the detrimental intestinal effects of IL-33. An oral SMAD7 antisense oligonucleotide, morgesen, targets ileal and colonic SMAD7 and reduces fibrosis in Crohn’s disease [18]. Furthermore, complementary medicine discovered halofuginone. This analog of febrifugine disrupts transforming growth factor β messaging, thereby blocking fibroblast-to-myofibroblast transformation and fibrosis [19]. Utilizing these treatments could prevent intestinal damage in infants that display high eosinophil counts during NEC.

Reviews of cellular biomarkers during NEC do not mention eosinophils as a predictor of unfavorable outcomes [16, 17]. Low-concentration, nonspecific eosinophilia is a frequent finding in preterm infants during hospitalization [20], and researchers have described blood eosinophilia in the hematologic profiles of late-onset sepsis and NEC in very preterm infants [21]. Our study differed from previous reports by demonstrating a persistent duration of eosinophilia in the early stages of NEC. We theorized that NEC is a major stimulus for prolonged eosinophil production, activation and recruitment as well as adverse inflammatory outcomes during NEC. Among the causes for dysmotility during NEC [3], eosinophilic granular secretions are a major irritant to motor ganglia in the bowel [22]. Interestingly, a major intraintestinal member of the cytokine network associated with blood eosinophilia and NEC is IL-13 [10, 23]. The interplay between IL-33, IL-4, IL-5 and IL-13 as mediators of fibrotic complications during NEC requires more investigation (fig. 1) [5, 10, 23]. Some investigators have suggested that eosinophilia and bloody stools can identify the onset of NEC presenting <48 h after a blood transfusion [24], but TANEC in our infants was not necessarily accompanied by early persistent eosinophilia and/or bloody stools (table 3). In surgical specimens, the intestinal mucosa of subjects with eosinophilia and NEC had ulcers possibly related to subepithelial fibrosis. This finding may be responsible for the bloody stools seen during convalescence from NEC (table 3). In our population of preterm infants, the percentage of eosinophils in the total WBC count and an eosinophilic elevation for 5 days gave a precise identification of the infants at risk for complications after the onset of NEC. We selected this biomarker for 3 reasons. First, current immunopathology fits our clinical observations (fig. 1). Second, caregivers can easily obtain the information on complete blood counts performed on consecutive days. Lastly, we observed that leukopenia influenced the absolute eosinophil count more often than the percentage of daily eosinophils/100 WBCs. In fact, 31% of infants with NEC have neutropenia [25].

**Limitations of the Study**

This study had advantages and disadvantages. A single institution was an advantage because (1) there was a consistent application of policies and protocols among neonatologists and pediatric surgeons, (2) laboratory measurements and reporting were quality controlled and dependable and (3) interpretations by pediatric radiologists were constant and reliable. Over 5 years, sufficient cases allowed us to meet sample-size calculations and have an appropriate control population. The number of subjects permitted a detailed clinical analysis of each subject in all 4 groups. In group 2, only a few infants had abdominal surgery, so no hepatic biopsies were performed; nevertheless, the results for this group verified that low-level eosinophilia and a low Likert score are predictors of fewer surgical interventions after NEC (table 3). Notably, a role for gut microbiota as an inducer of eosinophilic inflammation and fibrosis during NEC was largely unexplored [17]. Thus, we propose a multicenter investigation to examine persistent blood eosinophilia, concentrations of serum cytokines and chemokines and analyses of the fecal microbiome. Insights from this study will likely recognize microbiologic and immunologic biomarkers that will enhance the prediction of complications during the course of NEC.

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Conclusions

This investigation established persistent blood eosinophilia as a predictor of complications during the course of NEC. Previously, researchers had proposed eosinophilia as a WBC-related biomarker for the onset of NEC [24]. Moreover, fibrotic disease of the gut and liver are major causes of organ injury and physicians have a better understanding today of the pathogenesis and prevention of fibrosis [26]. Blood eosinophilia may be an indicator of the pathology that follows the onset of NEC (fig. 1). Healthcare professionals caring for infants who develop NEC should look for an early increase in blood eosinophils of ≥5% for ≥5 consecutive days. If preterm infants have NEC and early persistent eosinophilia, caregivers should monitor these infants for the complications described in table 2.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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