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

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Abstract

Background—Eosinophils infiltrate into intestinal tissue during necrotizing enterocolitis (NEC) and adult bowel diseases. We theorized that epithelial damage causes eosinophilic activation and recruitment during 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 the severity of NEC severity. We utilized a SPSS database for statistical analyses.

Results—Among 50 cases with NEC, Group 1 infants (n = 15) had eosinophilia at less than two days after onset; Group 2 had NEC but no persistent eosinophilia (n = 25). Control infants without NEC were matched for birth weight and gestational age (Group 3, n = 46). Group 4 included four 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 as high in Group 1 versus Group 2 ($p = .002$). Group 1 had a mean duration of eosinophilia of 8 days versus one day in Group 2 ($p < .001$). A Likert score of NEC severity was 3-fold higher in Group 1 versus Group 2 ($p < .001$). 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 high risk for adverse outcomes during NEC convalescence.

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Keywords

eosinophilia; necrotizing enterocolitis; cytokines; fibrosis; biomarker

Introduction

Infants requiring surgery for complications of necrotizing enterocolitis (NEC) have an excess of eosinophils in their intestinal specimens [1]. Gastrointestinal (GI) diseases with eosinophilic infiltrates occur at different intestinal locations during childhood and adult life [2]. Complications observed in adult eosinophilic GI disease parallel adverse outcomes during 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 predicts late medical and/or surgical complications. Immunopathology currently defines immune cell-, cytokine- and chemokine-related participation 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 that 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, respectively, increase bone marrow production of eosinophils and mediate eosinophil-associated chemotaxis to the intestinal compartment [9, 10]. While IL-33 helps restore damaged intestinal mucosa [5], IL-33 also increases transforming factor-beta (TGF- β) and other biomolecules that promote structural abnormalities like sub-epithelial 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. The aim of this research evaluates whether blood eosinophilia significantly identified future adverse outcomes during the course of NEC.

Methods

Study design and participants

The Health Science Institutional Review Board (IRB) for 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 (NICU) at MU Children's Hospital from January 2008 to December 2013. Our Vermont Oxford Neonatal Network (VON) database identified infants hospitalized with necrotizing enterocolitis (NEC). We identified NEC cases based on VON criteria. Control infants were born near the admission of infants who developed NEC. They had a similar birth weight and gestational age, but these infants did not have NEC during their hospital stay.

Procedures

Electronic medical records categorized clinical, nutritional, laboratory, radiographic, and surgical pathology of each NEC-related case. We divided cases of NEC into classic

presentations and infants who developed NEC <48 hours after a blood transfusion (TANEC) [3]. All infants with NEC and control infants had complete blood counts (CBC) 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 four groups, we defined persistent eosinophilia as $\geq 5\%$ of the total white blood cell count (WBC) for ≥ 5 consecutive days after the onset of NEC. We calculated the absolute eosinophil counts over the same interval. Table 1 shows how this analysis segregated infants into four groups. Lastly, our practice is to examine the feces of infants diagnosed with NEC for eosinophils to exclude eosinophilic colitis.

Among the four groups, we reviewed all entries made in the electronic medical record by physicians, nurses, the laboratory, and radiology for each case of confirmed NEC. We established a case of NEC for a preterm infant using VON criteria [12]. Briefly, a definition of NEC requires one or more of the following clinical signs: (a) bilious gastric aspirate or emesis, (b) abdominal distension, and (c) occult or gross blood in stool (fissure excluded), and one or more of three radiographic findings: (1) pneumatosis intestinalis, (2) hepatobiliary gas, and (3) pneumoperitoneum. We measured the severity of NEC-related complications during the hospital stay using a Likert scale (table 2).

Statistical analysis

Our sample size calculation used analysis of variance, four groups, a power of 0.80, an effect of 0.50, an alpha = 0.05, and 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, Version 22.0. Armonk, NY). Data analysis used descriptive statistics, ANOVA for parametric measures, and the Fisher's Exact, Mann-Whitney, and Kruskal-Wallis tests for non-parametric measures. A statistically significant difference was $p < 0.05$ level. To show clinical significance, we present results as the mean $\pm$ standard deviation (SD), receiver operator curves expressed as areas under the curve, and 95% confidence intervals.

Results

We excluded infants if their inclusion would have biased the findings. After reviewing GI pathology, we excluded two infants who had spontaneous intestinal perforation rather than NEC. In Group 1, we excluded two infants who had NEC but who died later of causes other than NEC. We excluded six infants 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 in groups 1 through 4. In Group 1, 12 of 17 infants (71%) had a classic NEC. Five of 17 infants (29%) in Group 1 had TANEC. No infants in groups 1 with prolonged blood eosinophilia had fecal eosinophilia or features consistent with eosinophilic colitis. In Group 2, 12 of 25 infants (48%) presented with classic NEC, while 13 of 25 (52%) had TANEC. Of six deaths in group 2 infants at <48 hour after the onset of NEC, the mean absolute eosinophil counts were 0.12×10^9 cells/L. In these six infants, small intestinal pathology described bloodless and necrotic bowel. This devitalized intestine probably produced low amounts of cytokines and chemokines. We

suggest this gut pathology explains the low absolute eosinophil counts in these dying babies of Group 2. Two infants in Group 2 had late deaths after the resolution of NEC. Group 3 infants did not have 5% blood eosinophilia for more than 2 days; however, four infants in Group 4 had bloodstream infection or pneumonia with 5% blood eosinophilia for 3 to 5 days. Among infants in groups 3 and 4, the neonates never had signs or symptoms of NEC and 5% eosinophilia that persisted for more than 5 days. None of the infants in groups 3 and 4 developed complications like those infants with NEC during hospitalization. Lastly, among the four groups, infants assigned to group 1 had the lowest birthweight and gestational age (table 3). We suggest birth weight and gestational age may be an additional risk factor for NEC-related complications besides that of elevated, persistent blood eosinophilia.

Discussion

Necrotizing enterocolitis is the major inflammatory GI disease and a leading cause of death and prolonged hospitalizations among preterm infants [3]. Major GI surgery for complications of NEC doubles the risk of death. Advances in neonatal care have improved the survival rate for smaller and more immature infants, but this advance 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 to no sequelae, a meaningful number of infants develop life-threatening complications after successful medical management. Schimpl et al. 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 [14]. Hepatic fibrosis and/or stricture occurred 8 times more often in Group 1 versus Group 2 (table 3). As shown in Figure 1, injured intestinal epithelia secretes 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 treatments 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, mongersen, targets ileal and colonic *SMAD7* and reduces fibrosis in Crohn's disease [18]. Furthermore, complementary medicine discovered halofuginone. This analog of febrifugine disrupts TGF- 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 hematologic profiles in late-onset sepsis and NEC among very preterm infants [21]. The difference in this study compared to past reports is a persistent duration of eosinophilia in the early stages of NEC. We theorized that NEC is a major stimulus for prolonged eosinophil production, activation, recruitment, and 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 intra-intestinal 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 require more investigation (fig. 1) [5, 10, 23]. Some investigators have suggested eosinophilia and bloody stools identify the onset of NEC presenting <48 hours after a blood transfusion [24], but TANEK in our infants did not necessarily have 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 sub-epithelial fibrosis. This finding may be responsible for 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 five days gave a precise identification of infants at risk for complications after the onset of NEC. We selected this biomarker for three 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 white blood cells. In fact, 31% of infants with NEC have neutropenia [25].

Limitations of the Study

This study has advantages and disadvantages. A single site was an advantage because a) there was a consistent application of policies and protocols among neonatologists and pediatric surgeons, b) laboratory measurements and reporting were quality controlled and dependable, and c) interpretations by pediatric radiologists were constant and reliable. Over five years, sufficient cases at one site 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 groups 1 through 4. Group 2 was a disadvantage because fewer infants had abdominal surgery. Hence, these infants had no hepatic biopsies, but group 2 verifies 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 multi-center investigation that examines persistent blood eosinophilia, serum cytokine and chemokine concentrations, and analyses of the fecal microbiome. Insight from this study will likely recognize microbiologic and immunologic biomarkers that will enhance prediction of complications during the course of NEC.

Conclusions

This investigation establishes persistent blood eosinophilia as a predictor of complications during the course of NEC. Previously, researchers had proposed eosinophilia as a white blood cell-related biomarker for the onset of NEC [24]. Moreover, fibrotic disease of the gut and liver are major causes of organ injury, and today physicians have a better understanding of the pathogenesis and prevention of fibrosis [26]. Blood eosinophilia may be an indicator of that pathology following the onset of NEC (fig.1). Health care 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 follow these infants for the complications described in Table 2.

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Fig. 1. Immune cells, cytokines, and chemokines participating in eosinophilic during necrotizing enterocolitis

Abbreviations: DAMPs – damage associated molecular patterns; IL – interleukin; ILC - innate lymphoid cell; TGF- β – transforming growth factor-beta; MMP-9 - matrix metalloproteinase-9; CTGF – connective tissue growth factor

Fig. 2. CONSORT Diagram of Preterm Infants in the Study Population

Abbreviations: SIP – spontaneous intestinal perforation; NEC – necrotizing enterocolitis

Table 1

Definition of Study Group Assignments

Preterm Infant Cohorts	GROUP DEFINITIONS
Group 1	Persistently elevated eosinophil counts after the onset of NEC (defined as $\geq 5\%$ of blood eosinophilia for ≥ 5 days)
Group 2	Infants with NEC and either low or normal eosinophil counts; no persistent blood eosinophilia
Group 3	Controls with normal eosinophil counts; no infection or NEC
Group 4 *	Controls with eosinophil counts $\geq 5\%$ for 3 - 5 days.

NEC is necrotizing enterocolitis.

* 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 necrotizing enterocolitis (NEC).

Scale	Definitions
0	Minimal side effects after the onset of NEC until hospital discharge; namely, delays in full enteral feeding caused by residuals and/or abdominal distension.
1	Prolonged (> 2 weeks) or significant (need for parenteral nutrition) feeding problems after NEC; bowel wall thickening on abdominal radiographs
2	Isolated intestinal stricture not needing surgery during convalescence after NEC
3	Need for Surgery for complications after NEC (bowel obstruction, perforation, obstructive or multiple strictures and/or adhesions, pneumoperitoneum)
4	Need for bowel removal and/or ostomy at surgery (non-viable gut, multiple strictures)
5	Death caused by complications of NEC before hospital discharge*

* Exclusions: We did not assign a Likert score to infants who died < 2 days after NEC onset to eliminate scoring bias. These infants did not survive long enough to have persistent blood eosinophilia. We also excluded infants dying from causes other than NEC.

Table 3

Findings in Infants with persistent eosinophilia and necrotizing enterocolitis (Group 1) compared to Infants with necrotizing enterocolitis (NEC) and no persistent eosinophilia (Group 2) and Controls (Groups 3 and 4)

FINDING	GROUP 1 (n = 15)	GROUP 2 (n = 25)	GROUP 3 (n = 46)	GROUP 4 (n = 4)	STATISTICS Comparison Grp 1 v. Grp 2	Area under the ROC, AUC 95% CI, p value
Birth Weight (g)	911 ± 422*	1340 ± 498	1099 ± 466	992 ± 293	$p = 0.006^{**}$	0.73 (0.57, 0.89) $p = .016$
Gestational Age (wks)	26.8 ± 2.9	29.2 ± 3.3	28.3 ± 3.4	26.9 ± 1.4	$p = 0.02^{**}$	0.70 (0.53, 0.87) $p = .04$
Absolute Eosinophil Count (× 10 ⁹ /L)	1.37 ± 0.79	0.65 ± 0.41	0.50 ± 0.35	1.44 ± 1.37	$p = 0.002^{**}$	0.80 (0.66, 0.95) $p = .002$
Percentage of Eosinophils/Total WBC	15 ± 7%	4 ± 2%	3 ± 2%	11 ± 7%	$p < 0.001^{**}$	0.97 (0.92, 1.00) $p < .001$
Mean Days of 5% Blood Eosinophilia	8 ± 2	1 ± 1	1 ± 1	4 ± 2	$p < 0.001^{**}$	0.93 (0.81, 1.0) $p < .001$
Late Bloody Stools (? mucosal ulcers)	17	8	0	0	$p = .002^{\#}$	0.82 (0.62, 1.0) $p = .01$
Likert Scale	3.1 ± 1.5	1.0 ± 1.4	N/A	N/A	$p < 0.001^{\ddagger}$	0.88 (0.78, 0.98) $p < .001$
Intestinal Strictures and/or Liver Fibrosis (n)	8	1	0	0	$p = 0.02^{\ddagger}$	0.50 (0.31, 0.69) $p = > .05$
TANEC and Bloody Stools (yes/no)	1/4	4/9	0	0	$p = 1.0^{\ddagger}$	0.50 (0.31, 0.69) $p = > .05$

Abbreviations: ROC – Receiver Operator Curve; AUC – Area Under Curve; CI – Confidence Interval; WBC – white blood cell count; N/A – Not Applicable; TANEC – transfusion-associated NEC.

* Mean ± standard deviation;

** p value from ANOVA,

$\#$ p value from Kruskal-Wallis,

$\ddagger$ p value from Mann-Whitney;

$\ddagger$ p value from Fisher Exact test.