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Immunohistochemical Demonstration of Blood Group Antigen Expression in Intestinal Endothelium Links Blood Type and Necrotizing Enterocolitis

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Abstract

Objectives: To determine the presence of A and B blood group antigens on vascular endothelial cells of intestinal tissue and compare tissue resected for necrotizing enterocolitis (NEC) with tissues resected for non-NEC pathologies (spontaneous intestinal perforation (SIP), intussusception, Hirschsprung disease, intestinal atresia, etc.) in an effort to implicate blood group antigen expression on bowel endothelium as a mechanism of bowel injury in NEC via a humoral immune-mediated inflammatory response.

Methods :Intestinal tissue from 21 patients with NEC and 23 non-NEC patients (5 of which were SIP) was stained with monoclonal antibodies against blood group antigens A and B. Vascular endothelial lined spaces were examined for expression of these blood group antigens and graded as 0 (no staining) to 3 (marked staining).

Results: Control group birth gestational age (GA) ranged from 26 to 40 weeks (Mdn=36.4-37.0). Both NEC and SIP groups had birth GA ranging from 24 to 37 weeks (Mdn=29.3 and Mdn=27.6, respectively). Overall, A and B blood group antigens were appropriately expressed on the endothelium of all intestinal tissue regardless of the presence of NEC. The A antigen appeared to stain more intensely than the B antigen in most tissue, except for the NEC sample from an AB blood type patient in which A and B antigens stained equally intense (grade 3). Multivariate regression analysis confirms the significantly inverse relationship between gestational age and NEC, but a significant relationship could not be established between blood group or IHC scoring of the blood group antigen expression and NEC.

Conclusions: Blood group antigens, A more than B or AB together, may increase the risk of a neonate to develop NEC in the presence of passively or actively transferred isoagglutinins.

Keywords: Necrotizing enterocolitis; Immunohistochemistry; Blood group antigen; Intestine; Isoagglutinin

Introduction

Necrotizing enterocolitis (NEC) and spontaneous intestinal perforation (SIP) are two of the most common gastrointestinal emergencies in neonates, primarily affecting the extremely premature. NEC is associated with significant morbidity and mortality, 20-40% mortality overall, but approaches 100% mortality in the most severe cases [1]. Despite the poor outcomes of this disease, the pathogenesis of NEC remains unknown. Multiple factors may increase the risk for a neonate to develop NEC including prematurity with intestinal immaturity, maternal chorioamnionitis, hypoxic-ischemic events, abnormal microbial gut colonization, and history of blood transfusions. The pathogenesis of NEC has therefore been hypothesized to be multifactorial, including ischemia, infection, inflammation, endothelial injury, and immune dysfunction [2]. Ultimately, the resulting intestinal injury causes bacterial infiltration and necrosis.

Clinical and radiographic signs of NEC, such as bloody stools, thrombocytopenia, intestinal pneumatosis and portal venous air, are used to diagnose and stage its severity according to the modified Bell staging criteria [3]. There are also clear histological changes associated with NEC including coagulative necrosis, bacterial overgrowth, and cellular inflammation [1].The immunohistochemical (IHC) properties of NEC, however, have yet to be determined.

Spontaneous intestinal perforation is similar to NEC as it is another gastrointestinal emergency that affects extremely premature neonates, but this is the only commonality these diseases share. In contrast to NEC, SIP occurs earlier in life and before feeding, a known inciting factor for NEC. Histologically, the intestinal mucosa in SIP is relatively healthy compared to the necrosis seen in NEC. The inflammation and ischemia that is the hallmark of NEC is not present in SIP. Unlike NEC, the pathogenesis of SIP is better described and includes association with extreme prematurity, early postnatal steroids, early use of indomethacin, and infection involving *Candida* and *Staphylococcus epidermis*. Immunogenicity, however, is not a known trigger of SIP, further differentiating it from NEC [4]. This particular difference makes comparing SIP and NEC patients, who have similar demographics but different pathogenesis, favorable.

In adults, the presence of A and B blood group antigens have been associated with increased risk of thrombotic disease. Individuals with AB phenotype had a 2.7-fold risk of venous thrombosis when compared to the O allele. Comparing the O allele with the A allele and B allele, the

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odds ratios were 1.79 and 1.82, respectively [5]. A previous study by Thomson et al. [6], showed that neonatal blood type, independent of Rh antigen status, is associated with high mortality from NEC, suggesting that thrombosis or a humoral immune response may be contributing pathogenic factors. They found that neonates with the AB blood group were at significantly higher risk of mortality from NEC compared to neonates with other blood groups (HR 2.87; 95% CI 1.40 to 6.589; P = 0.003). Analogous to maternal IgG antibodies crossing the placenta and causing ABO hemolytic disease of the newborn, it is hypothesized that maternal or passively transfused blood group isoagglutinins are an inciting factor for NEC, initiating an immune reaction, and consequently causing inflammation within the intestinal wall.

The purpose of this study is to evaluate if there is expression of blood group antigens on the intestinal endothelium of neonates, which is currently unknown unlike in adults [7,8]. In an effort to implicate a humoral immune-mediated inflammatory response as a mechanism of intestinal injury in NEC, we compared intestinal tissue from patients who had NEC to those who did not have NEC, including SIP patients. We hypothesize that the IHC properties of NEC will be different than that of control and SIP tissue.

Materials and Methods

Case selection

All cases of necrotizing enterocolitis from 2000 to 2013 were retrospectively identified from the surgical pathology files of the Department of Pathology at Loyola University Health System. H & E stained slides from each case were reviewed and formalin fixed paraffin embedded tissue blocks were obtained. Tissue blocks from 21 unique patients containing both viable mucosal tissue and evidence of necrotizing enterocolitis were chosen for study (n=34). In order to ensure tissue was viable enough for stain uptake, multiple blocks were selected per patient. Likewise, tissue blocks from 23 unique patients diagnosed with pediatric cases of Hirschsprung disease, atresia, intussusception and intestine resected for gastroschisis as well as patients who underwent appendectomy served as controls (n=25). Data was collected for each patient case including blood type, gestational age (GA) at birth, post-menstrual age (PMA) at time of surgery, surgical pathology diagnosis, and location of resected intestinal tissue.

Immunohistochemical studies

Formalin fixed paraffin-embedded tissue sections cut at 4 μm were deparaffinized and endogenous peroxidase was inactivated. Sections were stained with mouse monoclonal antibodies raised against blood group A antigen (Santa Cruz Biotechnology, Inc., clone Z2A) and blood group B antigen (Santa Cruz Biotechnology, Inc., clone Z5H-2) at a 1:100 dilution, and CD34 (Leica Biosystems, clone QBEnd/10) using a prediluted solution from the manufacturer. Staining procedures were performed on a Leica Bond III instrument.

Immunohistochemical analysis

The staining pattern of all the IHC stains was evaluated by a blinded surgical pathologist (SY) with special interest in gastrointestinal pathology. A visual semiquantitative grading scale was applied to assess the immunoreactivity of mucosal and submucosal vessels. Grading was scored as 0 (no staining), 1 (faint/mild staining), 2 (moderate staining), 3 (marked staining) of the endothelial nuclei. Immunohistochemical staining for CD34 morphologically confirmed the vascular structures evaluated in the mucosa and submucosa.

Statistical analysis

Calculations of the median gestational age were performed using Microsoft Excel. A multivariate logistic regression determined the odds ratio of gestational age, total IHC score (to incorporate the variability of staining strength into a single metric), and blood group (AB *vs.* A/B/O) in relationship to NEC.

Results

In total, the database chart review search found 21 patients who had resections for NEC (NEC group), 5 resected for SIP (SIP group), and 18 for non-NEC cases (control group). All patients with multiple stained blocks had no difference in stain uptake between blocks. The characteristics and antigen staining intensity of the control group, SIP control group, and NEC group are detailed in Tables 1-3, respectively. Term birth gestation is considered 37 to 40 weeks. The control group GA age at birth ranged from 26 weeks to 40 weeks (Mdn=36.4-37.0). Both the NEC and SIP groups had GA at birth ranging from 24 weeks to 37 weeks (Mdn=29.3 and Mdn=27.6, respectively). PMA was limited

Patient	Neonatal Blood Type	GA (wks) At Birth	PMA (wks) at Time of Surgery	Tissue Resected	Anti-A Staining Grade	Anti-B Staining Grade
1	A+	37	37	Meckel's SB/Appendix	3	0
2	A+	37.5	39	Rectum	3	0
3	A-	39.5	41	Rectum	3	0
4	B+	38	40	Rectum	0	3
5	B+	38.6	38	Rectum	0	1
6	B+	term	49-52	Ileum/ICV	0	2
7	AB	39.5	42	Distal rectum/sigmoid	3	2-Jan
8	AB	40	41	Ileum/Appendix/Cecum	3	0
9	AB	term	53-56	Rectum	2	1
10	O+	26	28	SB/Appendix/Colon	0	0
11	O+	32.5	35	SB/Colon	0	0
12	O+	33.4	33	SB	0	0
13	O+	34	37	Rectum	0	0
14	O+	35.6	51	Colostomy	0	0
15	O+	36.1	36	Jejunum	0	0
16	O+	36.6	36	SB	0	0
17	O+	40	40	SB	0	0
18	O+	Term	53-56	Appendix	0	0

GA: Gestational age; PMA: Post-menstrual age; SB: Small bowel; ICV: Ileal cecal valve

Table 1: Resected Non-NEC Intestinal Tissue (i.e. Control Group).

Patient	Neonatal Blood Type	GA (wks) At Birth	PMA (wks) At Time Of Surgery	Tissue Resected	Anti-A Staining Grade	Anti-B Staining Grade
1	A+	24.4	31	Ileum	3	0
2	B+	24.6	32	SB	0	2
3	AB+	37	37	SB	3	1
4	O+	24.6	27	SB/Appendix	0	0-Jan
5	O+	28	29	Ileum	0	0
GA: Gestational age; PMA: Post-menstrual age; SB: Small bowel						

Table 2: Resected SIP Intestinal Tissue (i.e. SIP Group).

Patient	Neonatal Blood Type	GA (wks) At Birth	PMA (wks) At Time Of Surgery	Tissue Resected	Anti-A Staining Grade	Anti-B Staining Grade
1	A+	25.6	30	SB/Colon	3	0
2	A+	26	32	Colon	3	0
3	A+	27.3	32	SB	3	0
4	A+	36.4	40	SB	3	0
5	A-	35.4	36	Colon	3	0
6	A-	38	38	SB	3	0
7	B+	24.1	32	SB	0	1-Jan
8	B+	26.2	30	SB	0	2
9	B+	31	42	Colon/Cecum	0	2
10	B+	32	35	SB	0	2
11	B+	32.3	39	Colon/Appendix/SB	0	1–2
12	AB	27	31	SB	3	3
13	O+	24	30	SB	0	0
14	O+	24.1	27	Ileum/Necrotic	0	0
15	O+	25.5	32	Transverse colon	0	0
16	O+	26.1	31	SB/Appendix	0	0
17	O+	26.3	34	Terminal ileum/Colon	0	0
18	O+	32	34	SB/Ileum	0	0
19	O+	33.5	35	SB	0	0
20	O+	37	39	Jejunum	0	0
21	O-	27	39	SB/Ileum	0	0
GA: Gestational age; PMA: Post-menstrual age; SB: Small bowel						

Table 3: Resected NEC Intestinal Tissue (i.e. NEC Group).

by documentation of GA at birth and date of surgery. Estimated PMA at time of surgery ranged from 30-42 weeks (Mdn=34) in the NEC group, 27-37 weeks (Mdn=31.0) in the SIP group, and 28-56 (Mdn=39.5) weeks in controls.

In the control group, A antigen was expressed on intestinal mucosal and submucosal endothelium of A blood type tissue (Figure 1) and B antigen on B blood type tissue (Figure 2). AB blood type tissue expressed both A and B antigens as shown in Figure 3. O blood type tissue did not express either A or B antigen, as expected (Figure 4). Antigens were expressed regardless of birth GA or PMA at time of surgery. Of note, for the majority of specimens, A antigen appeared to stain more intensely than B antigen (staining grade of 3 for A antigen versus grades 1 or 2 for B antigen).

The SIP group had findings identical to that of the controls for all blood groups and antigen stains.

Overall, IHC staining for the NEC group was similar to both groups (Figures 5-7). However, there was one notable difference in which specimens from the single NEC AB blood type case revealed equally intense staining of both A and B antigens (grade 3 for both) (Figure 8), whereas in both the control and SIP groups, blood type AB specimens had variable staining intensity between the A antigen (grade 3) and B antigen (grade 0 to 2).

Despite the increased intensity of A and B antigens in the single NEC AB blood type case, multivariate logistic regression could not confirm a relationship between NEC and IHC score (OR=1.22, P=0.474) or AB blood group (OR=0.44, P=0.662). In contrast, the expected inverse relationship between gestational age and NEC was confirmed (OR=0.80, P =0.02).

Discussion

Immunohistochemical staining for blood group A and B antigens in our retrospective study revealed their presence on the intestinal vascular endothelial cells of neonates as early as 30 weeks PMA, regardless of the clinical or pathologic diagnosis. Interestingly, A and B antigen staining of SIP tissue resembled control tissue in all blood types, especially given the differing range of PMAs at time of surgery (Mdn=31.0 in the SIP group versus Mdn=39.5 in the control group). Additionally, the similar age range of the SIP group with the NEC group (Mdn=31 versus Mdn=34, respectively) provided this study with a more favorable comparison.

Throughout the study, there appears to be more intense staining of A antigen compared to B antigen amongst the control and SIP groups, as well as in most of the NEC group. The only exception was that of the single NEC AB blood group case in which both A and B antigen had an equally intense staining of grade 3. Though IHC staining of A and B antigens and their intensity may not necessarily correlate with immunogenicity or to clinical outcome, the unique results of the NEC

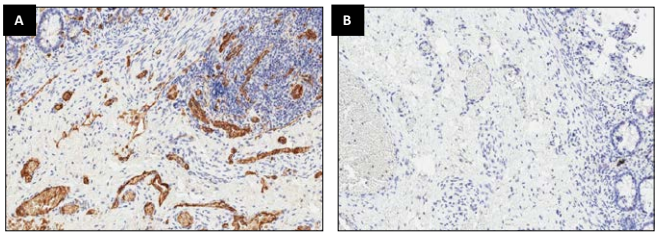

Figure 1: Immunohistochemical staining of control blood group A tissue (A-B). A, strong staining of intestinal endothelium with anti-A antibody; B, negative staining of intestinal endothelium with Anti-B antibody.

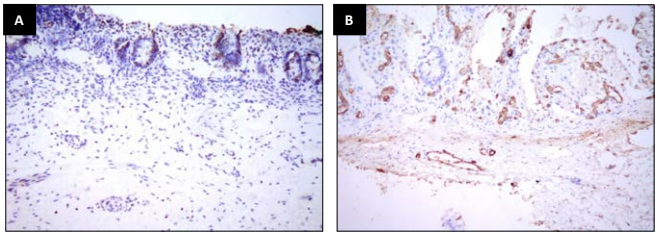

Figure 2: Immunohistochemical staining of control blood group B tissue (A-B). A, negative staining of intestinal endothelium with anti-A antibody; B, moderate staining of intestinal endothelium with Anti-B antibody.

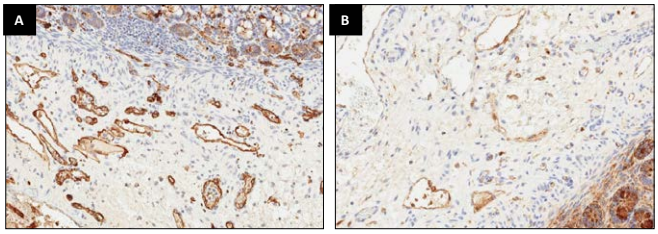

Figure 3: Immunohistochemical staining of control blood group AB tissue (A-B). A, strong staining of intestinal endothelium with anti-A antibody; B, moderate staining of intestinal endothelium with Anti-B antibody.

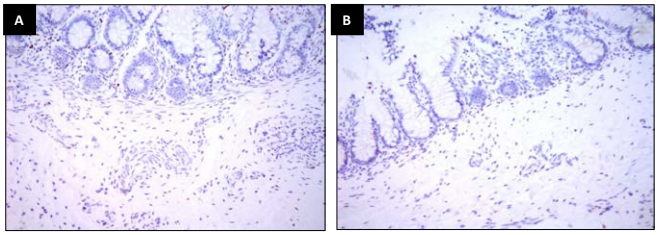

Figure 4: Immunohistochemical staining of control blood group O tissue (A-B). A, negative staining of intestinal endothelium with anti-A antibody; B, negative staining of intestinal endothelium with Anti-B antibody.

AB blood group case suggests an underlying pathogenic link to NEC in this patient.

Expression of the A and B antigens on intestinal vascular endothelium may predispose vascular injury via circulating isoagglutinins. Mothers with A blood type make anti-B IgG and mothers with B blood type make anti-A IgG. O blood type mothers can make anti-A, anti-B and anti-A,B with an IgG isotype. These immunoglobulins can cross the placenta and distribute within the bloodstream of the neonate. Such passive transfer of isoagglutinins could theoretically trigger an immune response to the A and B antigens in the intestinal vascular endothelium of the neonate and initiate the inflammatory cascade.

Active transfer of isoagglutinins also occurs via packed red blood cell (RBC) transfusions. Standard neonatal intensive care unit practice is to transfuse cytomegalovirus-seronegative, leukoreduced, and irradiated group O, Rhesus-negative RBC units. A neonate receives aliquots of a dedicated Group O units in attempt to minimize donor exposure. This source contains a residual amount of isoagglutinins in the supernatant that could similarly trigger an immune response.

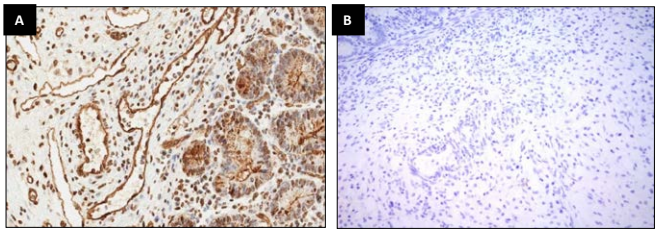

Figure 5: Immunohistochemical staining of NEC blood group A tissue (A-B). A, strong staining of intestinal endothelium with anti-A antibody; B, negative staining of intestinal endothelium with Anti-B antibody.

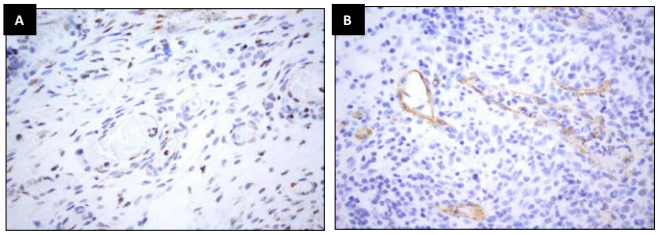

Figure 6: Immunohistochemical staining of NEC blood group B tissue (A-B). A, negative staining of intestinal endothelium with anti-A antibody; B, moderate staining of intestinal endothelium with Anti-B antibody.

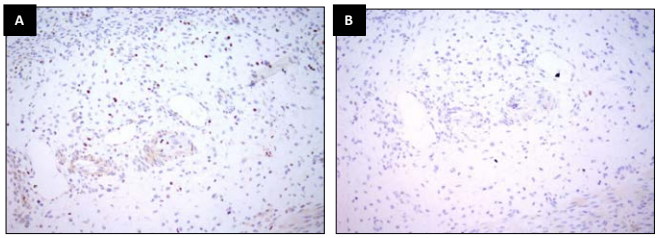

Figure 7: Immunohistochemical staining of NEC blood group O tissue (A-B). A, negative staining of intestinal endothelium with anti-A antibody; B, negative staining of intestinal endothelium with Anti-B antibody.

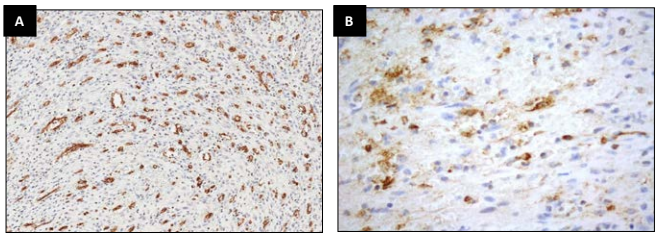

Figure 8: Immunohistochemical staining of NEC blood group AB tissue (A-B). A, strong staining of intestinal endothelium with anti-A antibody; B, strong staining of intestinal endothelium with Anti-B antibody.

Therefore, if intensity of staining does in fact correlate with increased immunogenicity, then this may explain the previous finding by Thomson, et al. where infants who had AB blood type were more likely to die from NEC. The increased mortality may be due to circulating isoagglutinins from active or passive transfer. The increased staining pattern of the A and B antigens in our AB blood type NEC case supports this hypothesis.

Blau et al. [9] coined the term TRAGI for transfusion-related acute gut injury after finding on a repeat cohort study that 25% of 36 cases of NEC were associated with transfusion of RBCs within 48 hours. Similar findings were found by El-Dib et al. [10] in the 48 to 72 hours after transfusions. At the same time that Thomson et al. published their findings, Boral et al. [11] compared the outcomes between blood group O and non-group O premature neonates receiving red cell transfusion. Overall they found no significant difference in adverse outcomes, including NEC, between the two groups despite using standard transfusion practices of giving group O RBCs. They, however, only studied neonates receiving transfusions instead of comparing all NEC cases as Thomson et al. did.

Further plans currently in process for this study is to stain these tissues for the presence of C4d, a complement split product that is known to remain on tissue after an antibody-mediated rejection in solid organ transplant patients [12,13]. It could therefore be used as a biomarker to support the activation of the classical complement pathway by an antigen-antibody mediated reaction within the same intestinal vascular endothelium where blood group antigens are expressed. We would expect there to be presence of C4d in the NEC tissues but negative staining in both the control and SIP tissues.

The obvious limitation of this study includes the small number of tissue samples examined. Given that the incidence of NEC in our unit is only 7% and that intestinal resection for NEC is not a common step in the management of the disease, the availability of tissue with all blood types, especially the least common AB blood type, is limited. The small sample size does not allow for meaningful comparisons of the amount of IHC staining for blood group antigens between blood groups. This is further borne out by the insignificant and conflicting odds ratios between the IHC score and blood group versus NEC by multivariate logistic regression. A larger, collaborative, follow-up study that also includes analysis for C4d could help further establish the relationship between NEC and blood group antigen expression. An additional limitation is that the gradient staining intensities between A and B antigen noted throughout the study may be due to varying affinities of the anti-A and anti-B antibodies to the antigens rather than indicating a quantitative difference. Consequently, conclusions cannot be drawn regarding the staining for blood group antigens in the intestine and its possible correlation with disease severity.

The underlying pathophysiology of NEC is multifactorial and uncertain. The establishment of IHC staining in the vascular endothelium of the intestine in premature infants with NEC, SIP, and other diseases, is unique and original. This is the first step in answering the question "Does an immune response in the vascular endothelium of the intestine contribute to the development of the clinical entity, necrotizing enterocolitis?" The next step will be to study larger numbers of infants, through collaboration, to further establish the expression of blood group antigen, and evaluate for the presence of complement activation through CD4d staining of bowel specimens in patients with NEC, SIP, and other diseases requiring bowel resection. The final step will be to correlate antigen expression, complement activation and disease severity in these vulnerable infants.

If our study with C4d staining confirms an antibody-mediated reaction in the NEC tissues, it would validate the hypothesis that blood group antigen presentation on intestinal vascular endothelial cells is the nidus for a humoral immune-mediated inflammatory response as a mechanism of intestinal injury in NEC. This outcome could stimulate a change of practice such that neonates would only receive ABO-specific blood transfusions, as suggested by the findings in Thomson et al. [6]. At the least, close observation of premature AB blood type neonates for NEC may be warranted.

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