The Effect of Stimuli on Basophil-Mediated Atopic Responses During Asthmatic Lying-In Women and in Newborns

Ling Yang,^{1*} Yin-Shi Guo,^{2*} Jin-Qi Jiang,^{3*} Xue-Jun Guo,⁴ Yi-Ping Xu,² Ye Tian,⁵ Ying Xiong,⁶ and Li Han¹

Morbidity from allergic diseases is increasing. Basophils play a critical role in systemic anaphylaxis and chronic allergic inflammation. The prenatal environment must be regarded as a possible early risk factor for allergic diseases in children. Our objective was to determine if basophils harvested from neonates genetically predisposed to atopic disease had different levels of CD63 expression and IL-4 release properties in response to various stimuli (peptidoglycan, *Dermatophagoides farinae*, hyperosmotic mannitol). Blood samples were collected from 16 asthmatic and 18 healthy women and their newborns. Peripheral blood basophil histamine was measured using the human basophil degranulation test (HBDT), whereas activation was assessed by flow cytometric measurement of CD63 expression on the cord blood basophil surface. IL-4 levels were quantified by ELISA following allergen stimulation. The basophil degranulation index (DI) in granulocytes harvested from the peripheral blood of asthmatic women was assessed following stimulation with peptidoglycan (PGN), *Dermatophagoides farinae* (Df) extract, or hyperosmotic mannitol. The DI was significantly higher in atopic women than in healthy controls. Upregulation of CD63 on the cord blood basophil surface was also detected in response to these stimuli. Basophils purified from the cord blood of neonates born to atopic mothers produced more IL-4 compared to basophils purified from the controls. These data suggested that various stimuli play a role in augmenting allergic reactions via modulation of activated basophil cytokine secretion. It may require new methods to stabilize the basophils in allergic diseases.

Introduction

IN GENERAL THE PREVALENCE OF AND MORBIDITY from allergic diseases are increasing. Study on the pathogenesis of allergic diseases is a hot research topic. Recently basophil-mediated processes and signaling pathways have been utilized as indicators of allergic inflammation since the basophils serve as primary allergic triggering cells. It has been widely reported that basophils readily produce Th2 cytokines (e.g., IL-4), which provides new insights into the possible role basophils may play in atopic disease progression and/or immune responses.^(1–4) Some data support a primary role for basophils in the recruitment of additional inflammatory cells rather than as effectors of IgE-mediated chronic allergic inflammation (IgE-CAI)⁽²⁾ since basophils are primary sources of IL-4 production^(5,6) and activated

basophils release IL-4 earlier than T cells driving Th0 to Th2 differentiation.⁽⁷⁾

After birth, the immune system usually shifts from Th2-polarized responses to Th1-mediated responses. If IL-4 levels were elevated during pregnancy (or right after birth), the elevated IL-4 would interfere with the development of Th1-mediated responses during infancy, thereby facilitating the elicitation of allergic responses.⁽⁸⁾

The umbilical cord blood has not been directly stimulated by an outside antigen, thus making it a perfect sample for the study of the pathogenesis of allergic diseases. This study focused on determining whether umbilical cord blood-derived basophils could respond to various stimuli (a microbial component—peptidoglycan; an allergen—*Dermatophagoides farinae*; the hyperosmolar state—hyperosmotic mannitol) and release IL-4 as a means of promoting the

¹Geriatrics Department, ⁴Respiratory Department, ⁶Obstetrical Department, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

²The Center for Allergic Diseases Control and Prevention, Respiratory Department, Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

³Department of Emergency and Critical Care Medicine, Shanghai Thoracic Diseases Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

⁵Department of Cell Biology, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

*These authors contributed equally to this work.

differentiation of Th0 to Th2 and the development of allergic diseases.

Materials and Methods

Reagents

Dextran and Ficoll were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). CD45-PerCP and CD63-FITC (peridinin chlorophyll protein complex) were obtained from BD Biosciences (San Jose, CA). CD203c-PE (phycoerythrin) was obtained from Milteny Biotec (Bergisch Gladbach, Germany). IL-4 levels were determined using an ELISA kit (human high sensitivity IL-4 ELISA kit, Bender MedSystems, Vienna, Austria). The skin prick test kit was purchased from Allergopharma (Allergopharma, Reinbek, Germany). PGN was purchased from Sigma (Buchs, Switzerland).

Subjects

Atopic patients. Sixteen asthmatic mothers (average age 29 ± 2 years, 39 ± 2 weeks of gestation), whose asthma symptoms were declining, were enrolled in this study. Asthma diagnosis was consistent with parameters established by the 2006 Guidance for Branchial Asthma.⁽⁹⁾ Medical history and skin tests showed respiratory sensitization (allergic response to *Dermatophagoides farinae* [Df]). Enrolled patients did not suffer an acute episode nor did they receive systemic corticosteroids, immunotherapy, H1-antagonists, or mast cell stabilizers during pregnancy. The children's biological fathers did not have a history of atopic disease.

Non-atopic controls. Eighteen non-atopic, post-partum mothers (average age 30 ± 2 years, 39 ± 1 weeks of gestation) were enrolled in the study. Mothers and the newborns' respective fathers did not have a family history of asthma, atopic disorder, or other chronic diseases. No complications were reported during pregnancy. Placental cord blood was obtained from controls as well as from atopic mothers.

After obtaining informed consent and after approval from the Ethical Review Board at the Xinhua Hospital affiliated with the Shanghai Jiaotong University School of Medicine, 60–100 mL placental cord blood and 3 mL peripheral venous blood were collected from enrolled mothers.

Study design

Basophil degranulation assay. Three mL peripheral blood were diluted with PBS/EDTA (phosphate-buffered saline/ethylenediaminetetraacetic) to 6 mL. The 6 mL diluted blood was layered over Ficoll-Hypaque (6 mL, 1.085 g/mL) and was then centrifuged at 400 g for 30 min at 25°C. The cells

were harvested and washed twice with PH7.4 HEPES-ACM buffer and subsequently were incubated with PGN (100 µg/mL), Df (10 µg/mL), 9% hyperosmotic mannitol, or ACM buffer in a 37°C water bath for 30 min respectively. The basophil degranulation index (DI) was determined by staining cells with Alcian blue to visualize granule release. DI can be computed as follows: $DI = (\text{no. of basophils in blank control group} - \text{no. of basophils in stimulation group}) / \text{no. of basophils in blank control group} \times 100\%$.

Basophil purification. Heparinized cord blood was mixed 5:1 with 6% dextran and incubated for 30 min at 37°C to destroy remaining erythrocytes. The cells were washed twice with 50 mL of PBS (pH 7.2) and 10 mM EDTA and then diluted in PBS/EDTA to 4 mL.⁽¹⁰⁾ The 4 mL cells were layered over the Ficoll-Hypaque (4 mL 1.065 g/mL) and then centrifuged at 400 g for 30 min at 25°C. Basophils were concentrated at the 1.065 g/mL Ficoll interface, harvested, and washed twice with 50 mL of PBS/EDTA.

Granulocytes (from the cord blood) were pre-incubated with PBS/EDTA/0.5% bovine serum albumin (BSA) for 20 min at 4°C. Thereafter cells were suspended in PBS/EDTA/0.5% BSA at a concentration of $10^7/80 \mu\text{L}$ and incubated with 20 µL of FcR Blocking Reagent and 10 µL PE-labeled CD203c and 10 µL PerCP-labeled anti-CD45 per 10^7 cells for 10 min at 4°C with gentle shaking. The cells were then washed with PBS/EDTA/0.5% BSA. Basophils were sorted based on CD203c⁺CD45^{int+} staining.

Percent purity and recovery were determined by Alcian blue staining of cells both before and after sorting.⁽¹¹⁾ Viability was assessed using Trypan blue exclusion.

Culture conditions. Purified umbilical cord blood-derived basophils were washed with HEPES-ACM buffer and equilibrated at 37°C, 5% CO₂ for 15 min. The cells were then incubated with PGN, Df, 9% hyperosmotic mannitol, or ACM buffer for an additional 8 h, as described above. Each group contained approximately 100,000 cells.

Expression of CD63 on activated umbilical cord blood-derived basophils. Stimulation was stopped by adding 10 µL of 10 mM EDTA at RT for 5 min; cells were then stained with 10 µL of CD63-FITC antibody and incubated for 15 min at RT. Cells were washed and measured on a Fluorescence Activated Cell Sorting (FACS) Caliber machine (BD Pharmingen, San Diego, CA). Data for CD63 expression were calculated as percentage of the maximal value of mean fluorescence intensity (MFI) of staining with anti-human CD63 MAb.

TABLE 1. BASOPHIL DI FOLLOWING STIMULATION WITH DF, PGN, OR 9% HYPEROSMOTIC MANNITOL

	Control group	PGN group	Df group	9% Hyperosmotic mannitol group
Asthma group	18.41 ± 5.28	38.12 ± 6.41 ^{a,b}	39.25 ± 7.25 ^{a,b}	41.31 ± 7.39 ^{a,b}
Healthy group	16.89 ± 4.15	18.96 ± 5.39	19.54 ± 6.01	20.72 ± 6.05

Data are expressed as the mean percent ± SD. Cultures with PGN, Df, or 9% hyperosmotic mannitol increased the basophil DI in asthmatic group, respectively, suggesting that these stimuli enhanced cytokine releases from basophils isolated from asthmatic mothers compared to healthy group ($p < 0.01$).

^a $p < 0.01$ versus other conditions in each group using ANOVA analysis.

^b $p < 0.01$ versus the healthy group using Student *t* test.

FIG. 1. Flow cytometric analysis of purified basophils. Purified basophils were examined by flow cytometry using (A) low SSC of (B) CD203-PE- and CD45-Percp intermediate-stained cells (indicated by blue stain in the P5 box).

IL-4 assays. Cell-free supernatants were harvested from 8 h cultures. IL-4 levels were quantified by ELISA as described by the manufacturer.⁽¹²⁾

Statistics

Student *t* test, ANOVA, and Pearson correlation analyses were carried out using SPSS 17.0 software to assess statistically significant differences between the treatment groups.

Results

Peripheral blood basophil stimulation

The DI was assessed following incubation of peripheral blood basophils with various stimuli. PGN, Df, and 9% hyperosmotic mannitol enhanced cytokines release from baso-

phils isolated from asthmatic mothers compared to healthy controls ($p < 0.01$; Table 1).

Assessment of cord blood basophil purity following isolation

Basophil concentrations between cord blood and the peripheral circulation are different since cord blood contains a proportion of immature basophils with underdeveloped granules.⁽¹³⁾ Therefore characterizing the purity of the harvested basophils is particularly important. Reimer and colleagues demonstrated that CD203c expression was detectable early during basophil development.⁽¹⁴⁾ This marker was a good alternative to identify basophil precursors.⁽¹⁴⁾ In addition, CD45 is expressed on basophils but not on mast cells. These basophil-restricted markers allowed for the positive selection and purification of umbilical cord blood-derived basophils using low-side scatter (SSC) of CD203c-PE⁺ and intermediate CD45-Percp⁺ stained cells (Fig. 1). Basophil integrity was further assessed by staining purified cells with Alcian blue (Fig. 2). Purity of basophils by FCM with CD203c⁺CD45^{int} markers was $93.08 \pm 5.01\%$ in the asthma group and $92.82 \pm 4.89\%$ in the healthy group.

CD63 expression

CD63, also known as lysosome-associated membrane protein-3, is a member of the tetraspanin family of membrane-associated molecules⁽¹⁵⁾ and is a well-established component of the late endosomal and lysosomal membranes.⁽¹⁵⁾ Up-regulation of CD63 on the surface of stimulated granulocytes coincides with mediator release.^(15,16) Therefore, in clinical studies CD63 is used as basophil activation marker to estimate the reactivity of a patient's blood basophils to pollen allergens by flow cytometry.^(17,18)

Cord blood basophil response towards the different agonists was examined following changes in the mean fluorescence intensity (MFI) associated to specific membrane marker fluorochrome and by evaluating dot plots of the acquired events. Following PGN, Df, and 9% hyperosmotic mannitol

FIG. 2. Alcian blue staining. Basophil purity and integrity were assessed by staining harvested cells with Alcian blue. This analysis revealed the presence of significant numbers of Alcian blue positive stains Live cells, red arrow; magnification x400.

TABLE 2. MFI OF CD63 IN CORD BLOOD BASOPHILS AFTER STIMULATION WITH Df, PGN, OR 9% HYPEROSMOTIC MANNITOL

	Control group	PGN group	Df group	9% Hyperosmotic mannitol group
Asthma group	11.88 ± 3.13	199.85 ± 42.33 ^{a,b}	215.59 ± 47.15 ^{a,b}	233.65 ± 51.25 ^{a,b}
Healthy group	8.15 ± 2.87	10.67 ± 3.02	11.33 ± 3.26	12.98 ± 3.11

Data are expressed as mean percent ± SD of observations per group.

^a $p < 0.01$ versus other conditions in each group using ANOVA analysis.

^b $p < 0.01$ versus the healthy group using Student *t* test.

stimulation, CD63 expression was significantly upregulated in cord blood basophils from neonates born to asthmatic mothers compared with expression levels observed in neonates born to non-atopic mothers ($p < 0.01$; Table 2 and Fig. 3).

IL-4 production following PGN, Df, and 9% hyperosmotic mannitol stimulation

The IL-4 levels produced by cord blood basophils from neonates born to atopic mothers were significantly ($p < 0.01$) elevated compared to the levels detected for basophils isolated from neonates born to non-atopic mothers following PGN, Df, and 9% hyperosmotic mannitol stimulation (Table 3).

FIG. 3. Characterization of CD63 expression. Cord blood basophils were isolated from one atopic mother and stimulated with PGN. After 8 h, cells were incubated with a FITC-labeled anti-CD63 and staining was assessed by flow cytometry. The green line represents cells from one neonate born to an asthmatic mother before PGN stimulation and the red line represents cells from the neonate after PGN stimulation.

A significantly positive correlation was observed between the DI of peripheral blood basophils from asthmatic mothers and cord blood-derived IL-4 following stimulation with Df ($R = 0.61$, $p < 0.05$; Fig. 4A), PGN ($R = 0.62$, $p < 0.05$; Fig. 4B) or 9% hyperosmotic mannitol ($R = 0.63$, $p < 0.05$; Fig. 4C).

Discussion

Genetic and environmental factors are recognized as etiologic factors in the development of allergic airway disease. The role of genetic and environmental factors in the pathogenesis of allergic airway inflammation is complex and has not yet been elucidated.⁽¹⁹⁾ Paralleling observations that Th2 responses were intricately involved in the progression of atopic diseases have been reported. Recent data have revealed a role for basophils not only in the effector response, but also in the initiation of the inflammatory response via the production of IL-4, a key cytokine associated with the polarization of Th2 type responses,^(7,20) suggesting that basophils are key players in allergic inflammation and potential therapeutic targets.⁽²⁾ In our study, basophils purified from the cord blood of neonates born to atopic mothers produced more IL-4 compared to basophils purified from neonates born to non-atopic controls following various environmental stimuli.

Role of bacterial infection or intestinal flora on pathogenesis of asthma

Respiratory infections are the most common precipitating factors of acute asthma. A large number of asthma attacks among adults and children are precipitated by a respiratory infection that exacerbates asthma symptoms. Infection of the respiratory tract has emerged as an important clinical issue in stable patients with chronic asthma.⁽²¹⁾ Recently, Bisgaard and colleagues found a significant association between bacterial infection of the airways and acute wheezy episodes in young children.⁽²²⁾ Therefore, bacterial infections may trigger many asthma attacks in children. Our experimental results may expound on the mechanism. In this report, we studied basophil DI following PGN (a principal component of bacterial cell walls)

TABLE 3. IL-4 SUPERNATANT LEVELS AFTER STIMULATION WITH Df, PGN, OR 9% HYPEROSMOTIC MANNITOL

	Control group	PGN group	Df group	9% Hyperosmotic mannitol group
Asthma group	1.79 ± 0.42	3.36 ± 0.39 ^{a,b}	3.27 ± 0.40 ^{a,b}	3.56 ± 0.47 ^{a,b}
Healthy group	1.65 ± 0.37	1.85 ± 0.50	1.78 ± 0.48	1.92 ± 0.52

Data are expressed as mean percent ± SD of observations per group (pg/mL).

^a $p < 0.01$ versus other conditions in each group using ANOVA analysis.

^b $p < 0.01$ versus the healthy group using Student *t* test.

FIG. 4. Assessment of the IL-4 response. Cord blood basophils from 16 neonates born to asthmatic mothers were stimulated with (A) Df, (B) PGN, or (C) 9% hyperosmotic mannitol, and the IL-4 supernatant levels were correlated to the DI. Each point on the graph represents the IL-4/DI response for an individual patient.

stimulation and demonstrated that PGN-mediated signaling significantly elevated basophil degranulation in atopic patients compared to that in healthy controls.

Sepp and colleagues studied the association between changes in the intestinal flora and atopic disease in 1-year-old babies in Estonia (low incidence of atopic diseases) and Sweden (high incidence of atopic diseases), indicating that intestinal bacteria played a key role in the development of hereditary allergy.⁽²³⁾ Lactobacilli and eubacteria could be isolated from Estonian infants while *Clostridia* species were more common in Swedish infants.⁽²³⁾ The PGN used in our study was extracted from *Bacillus subtilis*, suggesting that intestinal microbial populations could interact (activate) basophils (especially in genetically predisposed infants) to release cytokines (e.g., IL-4) associated with the progression of allergic diseases. Cord blood basophils from atopic mothers produced significantly higher levels of IL-4 following stimulation with PGN compared to IL-4 levels produced by cord blood basophils isolated from non-atopic controls. A significantly positive correlation was established between the basophil degranulation of peripheral blood basophils from asthmatic mothers and cord blood-derived IL-4 following stimulation with PGN, presuming a microbial component plays a role in mediating atopic disease.⁽²⁴⁾

Allergic factors

Environment allergens play a significant role in the onset of disease and persistence of symptoms. Dust mite is one of the major sources of indoor allergens in temperate humid areas. Exposure to dust mite is an important risk factor for sensitization and subsequent development of allergic disorders in genetically susceptible patients.⁽²⁵⁾ A larger prenatal exposure to house dust mite allergens was related to higher total IgE levels directly after birth, indicating that allergen exposure in the prenatal period can influence the fetal immune response.⁽²⁶⁾ In our study, exposure to Df activated cord blood basophils to release IL-4. Our data suggest that prenatal or infantile dust mite exposure might influence developing immune responses on cord blood and clinical outcome in early life.

Hypertonic state

Most often, a hyperosmolar state develops when a person has an illness, such as a severe case of the flu or other infection or a heart attack. It has been suggested that hyperosmolarity may be one of the stimuli that provoke exercise-induced asthma and rhinitis. In our study, a hyperosmolar state (9% hyperosmotic mannitol) not only induced peripheral blood basophil degranulation in atopic patients but also activated cord blood basophils to release IL-4. The elevated IL-4 would interfere with the development of Th1-mediated responses during infancy, thereby facilitating the elicitation of allergic responses.⁽⁸⁾

The discovery of CD63 as a basophil activation antigen constituted the start of the development of a flow cytometric technique to analyze and quantify allergen-specific *in vitro* activation of peripheral blood basophils.⁽²⁰⁾ However, few studies have described the expression of CD63 on cord blood. Data presented in this report demonstrated that cord blood basophils isolated from neonates born to atopic mothers had elevated CD63 expression levels following stimulation with

PGN, Df, or hyperosmotic mannitol. We can deduce that these stimuli could activate the cord blood basophils.

The genetic propensity to put an individual at risk of developing allergic disease is modulated by exposure to environmental factors. A significantly positive correlation was observed between the DI of peripheral blood basophils from asthmatic mothers and cord blood-derived IL-4 following stimulation with Df, PGN, or 9% hyperosmotic mannitol. The choice of life style, and the ability to control it with respect to modulating the risk to develop allergic disease, requires individualized advice and proper information. This is where preventive measures can help. We want to develop preventive measures to reduce allergic sensitization, give advice to reduce allergen exposure and induce symptom reduction, and provide clues to the management of an existing allergy. The prenatal environment must therefore be regarded as one of the possible early risk factors for allergic diseases in children. Control of environmental allergens and irritants is essential for pregnant women to prevent asthma in their newborns. The real extent of the importance of prenatal environment stimuli exposure must be further explored in future studies. The link between basophil activation in pregnant women and their newborns will be the direction of future research and may require new methods to stabilize the basophils in allergic diseases.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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Address correspondence to:

Dr. Xue-jun Guo

Respiratory Department

Xinhua Hospital

Shanghai Jiao Tong University School of Medicine

1665 Kongjiang Road

Shanghai 200092

China

E-mail: guoxjz@yahoo.com.cn

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