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Cyclic AMP-Phosphodiesterase Activity and Histamine Release in Cord Blood Leukocyte Preparations

J. C. McMILLAN, N. S. HESKEL and J. M. HANIFIN

Department of Dermatology, Oregon Health Sciences University, Portland, Oregon 97201, USA

Atopic dermatitis, allergic rhinitis and asthma are a common group of diseases with a familial predisposition. At present there is no suitable predictive or diagnostic marker. Adults with atopic dermatitis or allergic respiratory disease have elevated mononuclear leukocyte cAMP-phosphodiesterase activity. This activity correlates closely with histamine release from basophils. We investigated newborn leukocyte phosphodiesterase activity and histamine release in umbilical cord blood. Phosphodiesterase activity was significantly elevated in cord blood leukocytes of 81 children with a positive history of atopy in first degree relatives, compared to 33 children with a negative history ($p<0.025$). In contrast to adults there was no correlation between phosphodiesterase activity and histamine release. Our studies suggest that elevated phosphodiesterase activity is a primary, genetically linked defect. Fetal basophils would appear to possess cytophilic IgE since they are capable of immunologically stimulated histamine release even without passive, in vitro IgE sensitization. In addition, there are functional differences between adult and cord blood basophils. Longitudinal studies may determine if elevated phosphodiesterase is predictive of atopic states.

Atopic dermatitis (AD) is one of the most common skin disorders of childhood. It is rarely seen before the age of six weeks (1) and is exceptional in the neonate. The reason for this delay in clinical manifestations is unknown but may be attributable to such factors as low IgE synthesis, low histamine content in basophils and mast cells, immature immune mechanisms or to lack of exposure to environmental antigens. It is also significant that coordinated scratching does not occur before the age of two months.

The incidence of atopic dermatitis in the general population ranges from 0.25 to 20% (2). A national screening survey in the United States showed a prevalence of 19/1000 among children and 7/1000 overall (3). A closed community survey in Bristol, England, recorded a prevalence of 3.1% among children under five years of age (4). In Sweden, a study reported an incidence of 6% in children with non-atopic parents rising to 12% with one atopic parent and 33% with two atopic parents (5). Atopic states have a familial predisposition but the mechanisms of genetic transmission have not yet been resolved. Children born to atopic parents clearly have a higher risk of being atopic compared to those with non-atopic parents. The lack of a specific diagnostic marker prevents prediction of which children will develop atopic diseases and also leads to inaccuracies is genetic studies.

Pruritus and erythema, both features of AD, are provoked by introduction of histamine into the skin. Following intramuscular administration of histamine, atopic patients developed erythema and increased skin temperature at sites of predilection of the dermatitis (6). Atopic dermatitis patients have elevated skin histamine concentrations, even in uninvolved skin (7). Basophil numbers are not increased in AD. However, several studies have revealed increased in vitro histamine release from atopic leukocytes in response to anti-IgE (8, 9) and concanavalin A (Con A) (10, 11) stimulation. This abnormality appears to be independent of basophil bound IgE and total serum IgE levels (12–14), suggesting that increased histamine release is modulated by cyclic nucleotides.

As early as 1936, studies showed that high concentrations of epinephrine, an alpha stimulator, inhibited histamine release from actively sensitized guinea pig lung (15).

Szentivanyi postulated that in atopy there is reduced function of the beta-adrenergic system due to receptor blockade, with resultant dysfunction of cAMP leading to an increase of inflammatory mediators such as histamine (16). Further studies showed reduced leukocyte cAMP response to histamine (17) and prostaglandin E_1 (18) stimulation, suggesting that the defect was not confined to the beta-adrenergic receptor. Increased histamine release in association with sub-normal levels of cellular cAMP may be due to enhanced nucleotide breakdown rather than depressed generation. Time course studies showed reciprocal decreases of cAMP responsiveness as phosphodiesterase levels increased during histamine desensitization (19). Cyclic AMP-phosphodiesterase (PDE) activity is significantly elevated in mononuclear leukocytes of adults with AD and allergic respiratory disease (20). Further studies indicated good correlation between leukocyte histamine release and PDE activity in both adult atopics and controls (11).

In this study we investigated cord blood leukocyte PDE activity. We also measured immunologically stimulated histamine release from cord leukocytes and assessed for correlation with PDE activity.

MATERIALS AND METHODS

Materials were purchased from the following sources: Hypaque-Ficoll (Isopaque), Pharmacia, Piscataway, NJ; 5′-nucleotidase, HEPES, Human Serum Albumin (Fraction V) and Bovine Serum Albumin (Fraction V), Sigma Chemical Co., St. Louis, MO; (^{3}H) cAMP (37 ci/mml) and Aquasol 2, New England Nuclear, Boston, MA; AG1-X2 resin (200-400 mesh) and Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, Richmond, CA; Gey's Balanced Salt Solution (GBSS), Gibco, Grand Island, NY; Sterile Water for Injection USP and hydroxyethyl starch solution (VOLEX). McGaw Laboratories, Irvine, CA; Goat anti-human IgE antibody (Lot number 041301), Tago, Burlingame, CA.

Subjects

We collected 114 random umbilical cord blood specimens from newborns with uncomplicated term deliveries. In addition, we studied leukocytes from 16 expectant mothers during the third trimester of pregnancy and from 14 expectant fathers. The parents of all newborns were interviewed to determine if they had an atopic background. All subjects had received no systemic medication for at least 72 hours and consumed no methylxanthine-containing beverages for at least 12 hours prior to studies.

Leukocyte preparation

(*a*) *Hypaque-Ficoll gradient centrifugation.* Coded venous blood samples from adults and umbilical cord blood from newborns were anticoagulated with 10 u/ml of heparin. Mononuclear leukocytes (MNL) were isolated on standard Hypaque-Ficoll (specific gravity 1.077) gradients and centrifuged at 400 g for 30 min. Umbilical cord blood MNL were recentrifuged on "heavy" Hypaque-Ficoll (specific gravity 1.119) to remove nucleated erythrocytes. Monocytes were quantitated by Giemsa stain, latex bead ingestion and esterase staining. Cells were washed, resuspended in GBSS and frozen at −70°C. Cell viability was monitored by trypan blue exclusion.

(*b*) *Hydroxyethyl starch sedimentation.* Leukocyte suspensions from umbilical cord blood anticoagulated with 10 u/ml of heparin, were prepared by a method modified from Thueson et al. (21). Cord blood was mixed in an approximately 1 : 1 ratio with 5 ml of 0.1 M EDTA, pH 7.2 and 10 ml hydroxyethyl starch solution. The syringe was tumbled gently, then allowed to stand 30-45 min at room temperature until a clear interface was evident between erythrocytes and supernatant. The leukocyte-rich supernatant was transferred to a sterile polypropylene 50 ml centrifuge tube (Corning no. 25330), brought to 50 ml with Buffer A (see below) and centrifuged at 300 g for 8 min at 4°C. Cell pellets were washed twice in Buffer A. Cells were resuspended in GBSS for measurement of PDE activity or in Buffer A (10^7/ml) for measurement of histamine release.

Leukocyte histamine release buffer preparation

Two buffer solutions (A and B) were prepared on the day of each experiment from a (10×) stock solution of HEPES buffered saline, which was prepared each week and stored at 4°C. Buffer A contained HEPES (10 mM), NaCl (137 mM), KCl (5 mM) at pH 7.4, to which was added human serum

albumin (0.3 mg/ml). Buffer B was identical to Buffer A except that it also contained calcium (2 mM) and magnesium (1 mM). Buffers were prepared and stored in sterile glass containers which were reserved for this purpose and rinsed in detergent-free sterile water. The above preacautions minimized spontaneous release.

IgE sensitization

Cell suspensions (10^7/ml) in Buffer A were divided into two equal portions. To one portion was added 50 µl IgE serum (3600 u/ml) per ml of suspension and to the other was added a similar volume of Buffer A. Both suspensions were then incubated at 37°C for 45 min followed by centrifugation at 300 g for 8 min. The cell pellets were washed twice in Buffer A (300 g for 8 min at 4°C) and the cells resuspended in Buffer B at a concentration of 10^7/ml, immediately prior to histamine release studies.

Histamine release reaction

A 5 µg/ml solution of goat anti-human IgE antibody was stored at −20°C in 0.5 ml aliquots until required. One hundred µl of anti-IgE was added to duplicate 900 µl aliquots of sensitized or unsensitized cells in plastic falcon no. 3052 tubes in an ice water bath. Cell suspensions were then incubated at 37°C for 30 min in a shaker bath. The final cell concentration was 9×10^6/ml and anti-IgE concentration 0.5 µg/ml. Total histamine release was determined by boiling 900 µl aliquots of cells with buffer for 5 min. Spontaneous release was determined by incubating cells with buffer for 30 min at 37°C. The reaction was terminated by centrifuging at 800 g for 8 min at 4°C. The supernatants were stored at −70°C for histamine determination.

Histamine determination

Concentrations of histamine in supernatants were determined by the automated fluorimetric method of Siraganian (22). The sensitivity of this method is 1–2 ng/ml. For each assay, a standard curve was generated with histamine solutions of known concentrations.

cAMP-Phosphodiesterase determination

PDE activity was assayed by a two-stage procedure using a modification of the method of Thompson et al. (23). ^{3}H-cAMP was hydrolyzed by PDE to ^{3}H-5′-AMP which, in turn, was enzymatically converted by snake venom 5′-nucleotidase to ^{3}H-adenosine. This was quantitated in a liquid scintillation counter after unreacted ^{3}H-nucleotides were absorbed by anion exchange resin.

Prior to assay 1 ml aliquots of cells in GBSS were freeze-fractured three times and then homogenized using a polytron PP 10–32 (Brinkman Inst., Westbury, NY). The assay mixture (0.4 ml) contained 1.0 µM cAMP, 20000 cpm ^{3}H-cAMP and 0.2 ml sample in 40 mM Tris-chloride buffer (pH 8.0) containing 3.74 mM beta-mercaptoethanol and 40 mM magnesium chloride. After incubation (30°C for 10 min) the reaction was terminated by snap freezing in ethanol dry ice and the mixture was boiled for 1 min. Purified 5′-nucleotidase (0.45 units) was added to the mixtures which were incubated at 30°C for 10 min and then cooled to 4°C. One ml anion exchange resin was then added to each tube which was vortexed and allowed to stand for at least 30 min. The reaction mixture was then transferred to plastic scintillation vials and 10 ml Aquasol 2 was added. Enzyme activity was expressed as pmol cAMP hydrolyzed per minute per 10^8 cells (''units'') or per mg protein.

Bio-rad protein assay

From a 20 µg/ml stock solution of bovine serum albumin in GBSS, standards ranging from 1–20 µg/ml were prepared. Dye reagent concentrate 0.2 ml was added to duplicate 0.8 ml aliquots of standards, GBSS blanks and unknowns (20 µl sample and 780 µl GBSS). Specimens were then vortexed. Optical density was measured after 10 min at a wavelength of 595 nM. Protein concentrations were read from the standard curve.

Basophil percentage and histamine content

The percentage of basophils was determined using the Alcian Blue staining technique described by Gilbert & Ornstein (24). The histamine content per basophil was determined by dividing the total histamine content of the final 1.0 ml cell reaction by the number of basophils.

Statistical analysis

Statistical analysis utilized Student's *t*-test and Mann-Whitney U-test.

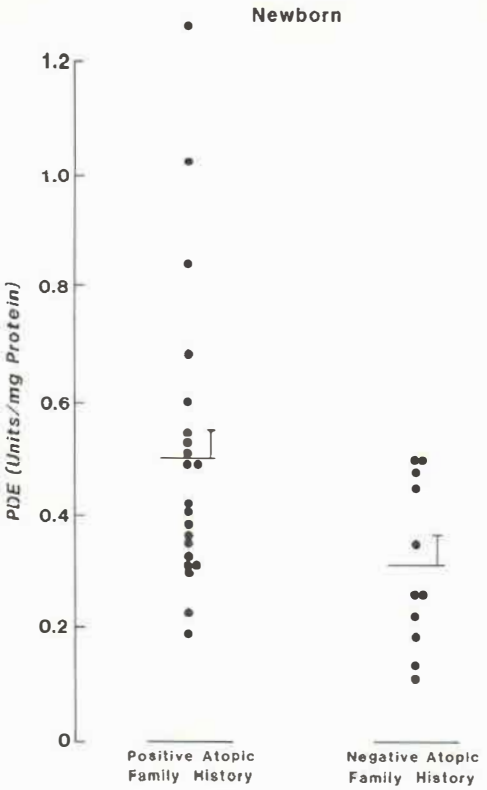

Fig. 1. PDE activity in total leukocyte preparations from cord blood in 11 newborns with no family history of atopy and 21 newborns with a positive family history of atopy. Bar represents mean ± SEM.

RESULTS

Phosphodiesterase activity

Fig. 1 shows cAMP PDE activity in *total* leukocyte preparations from cord blood in 11 newborns with no family history of atopy and 21 newborns with a positive family history of atopy. Mean PDE activity in newborns with a negative atopic family history was 0.314±0.044 units/mg, compared to a mean of 0.506±0.058 units/mg ($p<0.025$) in newborns with a positive family history.

Table I shows cAMP PDE activity in cord blood *MNL* preparations from newborns with one atopic parent, two atopic parents or two non-atopic parents. Newborns with either one or two atopic parents had mean PDE activities which were significantly greater than

Table I. *Phosphodiesterase activity in cord blood mononuclear leukocytes*

	Mean units ± SEM/ 10^8 cells	*p*
Both parents normal (22)	1.9±0.2	
Both parents atopic (18)	4.2±0.7	<0.005
One parent atopic (42)	3.2±0.3	<0.005
Father atopic (only) (16)	3.6±0.5	<0.025
Mother atopic (only) (26)	3.4±0.4	<0.005

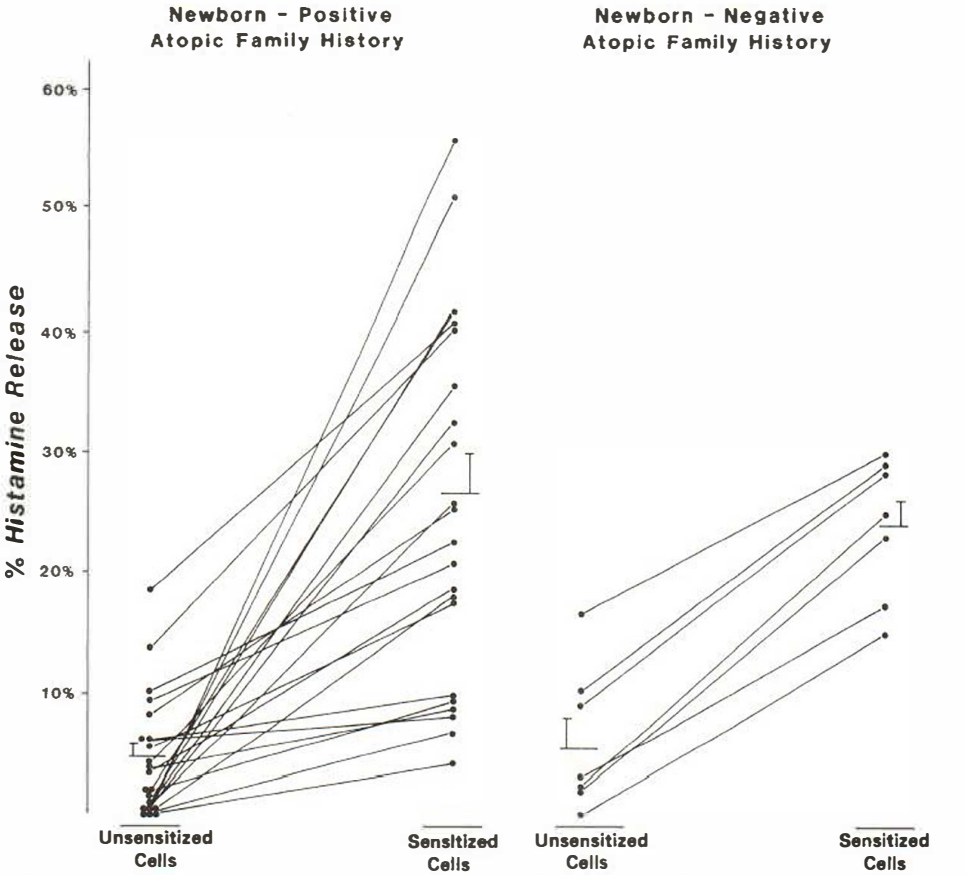

Fig. 2. Histamine release from cord blood leukocytes in 21 newborns with a positive family history of atopy and 7 newborns with a negative family history of atopy. Release is shown from cells passively sensitive with IgE in vitro, as well as unsensitized cells.

those with non-atopic parents ($p<0.005$) but did not differ from each other. Also shown in Table I is a comparison of PDE activity in newborns with atopic mothers and non-atopic fathers to atopic fathers and non-atopic mothers. Both groups had mean PDE activities which were significantly greater than the mean values from newborns with non-atopic parents ($p<0.025$) but did not differ significantly from each other.

Histamine release

Samples with a spontaneous release greater than 10% of total were discarded. Mean spontaneous release was 3.8±0.33%. All values given refer to net histamine release. Fig. 2 shows histamine release from both IgE sensitized and unsensitized cells in 29 cord leukocyte preparations. Unsensitized cells from babies with no family history of atopy released a mean of 5.7±2.4% ($n=7$), compared to a mean of 4.7±1.1% ($n=22$) for those with an atopic family history. Sensitized cells from babies with non-atopic family histories released a mean of 24.1±2.1% compared to a mean of 26.5±3.2% for those with an atopic family history. Differences between the two groups were not significant in either sensitized or unsensitized cells.

PDE activity/histamine release correlations

There was no correlation between PDE activity and histamine release in either total means or in high and low histamine releasing cells.

Basophil percentages and histamine content per basophil

We compared percentages of basophils in leukocyte preparations and found no significant difference between means of newborns with a non-atopic family history (0.83±0.16%, n=7) and newborns with an atopic family history (0.94±0.95%, =19). Likewise, we found no difference in basophil histamine content with mean values of 0.61±0.17 picograms per cell and 0.52±0.08 picograms per cell for the two groups, respectively.

DISCUSSION

Certain technical aspects in the methodology require further comment. Hypaque-Ficoll gradient preparation of cord blood resulted in monocyte-rich leukocyte suspensions. Unfortunately, cell yields were insufficient to enable both PDE activity and histamine release measurements to be made. We were, therefore, forced to use total leukocyte suspensions prepared by hydroxyethyl starch sedimentation. Given the large number of cells obtained by this method, it was felt that cell counts were less reliable. Using protein micro-assay techniques, it was possible to express PDE activity in units per milligram of protein rather than units per 10^8 cells.

We have demonstrated that PDE activity in umbilical cord blood leukocytes is significantly elevated in newborns who have a positive history of atopy in first degree relatives compared to those with a negative family history. Previous studies have shown that normal leukocytes may be desensitized by micromolar concentrations of prostaglandin E_1 or histamine (25). This results in significantly lower cAMP levels secondary to increased PDE activity (20), suggesting that elevated PDE activity in newborns could result from transplacental desensitization of fetal leukocytes by maternal inflammatory mediators. Our findings that there were no significant differences in cord blood leukocyte PDE activity in newborns with atopic fathers compared to newborns with atopic mothers argues against this as a possible explanation. However, our histamine release studies indicate the presence of cytophilic IgE on fetal basophils. Conceivably, in utero exposure of sensitized basophils and mast cells to anti-IgE antibodies, or to maternally transferred antigens could be primary events leading to histamine-stimulated increases in PDE. Elevated PDE activity in atopic parents agrees with previous investigations demonstrating elevated PDE activity in non-pregnant adults with AD or allergic respiratory disease (11, 20) and supports our selection procedure.

The human conceptus is capable of synthesizing IgE from as early as the 11th week in utero (26) but levels are very low in neonates (27, 28). Our results suggest that histamine release from cord blood leukocytes is limited by IgE levels as in vitro passive sensitization

Table II. *Phosphodiesterase activity in adult mononuclear leukocytes*

	Mean units ± SEM	p
Normal parents (15)	2.6±0.2	
Atopic parents (15)	6.0±0.7	<0.01

considerably enhances anti-IgE stimulated histamine release, whereas histamine release in adults appears to be independent of IgE levels, bound or unbound.

The mean histamine content per basophil is decreased in cord leukocytes, agreeing with previous studies (29). Although this level is considerably lower than in adults (29), no significant difference was noted between babies with a positive family history of atopy compared to those with a negative history.

In contrast to the good correlation between PDE activity and Con A stimulated histamine release in adult leukocytes, we found no such correlation in our anti-IgE stimulated cord blood leukocytes. As histamine release from passively sensitized cord leukocytes also fails to correlate with PDE activity, this argues against low IgE levels as a possible explanation. Studies indicate that the mode of action of Con A is the bridging of adjacent cytophilic IgE molecules (30–32). Con A stimulated histamine release also correlates closely with anti-IgE stimulated release in adult leukocytes (31). Studies in our laboratory have demonstrated that Con A, in doses causing maximal stimulation in adult leukocytes, fails to release significant histamine from cord leukocytes even after they have been passively sensitized (33). Classification of histamine releasing cells in cord blood has presented difficulty in that these cells possess features attributable to mast cells as well as basophils (34). These functional and morphologic differences may partly explain why manifestations of atopic disease rarely present during the neonatal period, although it is likely that other factors such as environmental influences also play an important role in the development of clinical features.

Elevation of PDE activity in cord blood leukocytes before the development of clinical manifestations of atopy suggests that increased PDE activity plays a primary role in the pathogenesis of atopic dermatitis. The lack of correlation between PDE activity and histamine release in neonates would tend to suggest that elevated PDE activity is a primary, genetically linked defect rather than secondary to in vivo desensitization by inflammatory mediators such as histamine and prostaglandin E_1. Longitudinal studies may determine if elevated PDE in newborns predicts AD, allergic rhinitis or asthma.

Our studies on mixed cell preparations have assumed that our observations apply to small subsets of cells. In mast cells, studies on mixed cell preparations demonstrating the inhibitory effects of cAMP on histamine release, have been confirmed using purified cells (35), suggesting that these assumptions are correct. Our results, however, await confirmation using pure subset cell populations.

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J. C. McMillan, Department of Dermatology, Oregon Health Sciences University, Portland, Oregon 97201, USA.