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INVOLVEMENT OF FcεRII/CD23 AND L-ARGININE DEPENDENT PATHWAY IN IgE-MEDIATED ACTIVATION OF HUMAN EOSINOPHILS

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SUMMARY: Eosinophils display various receptors for immunoglobulin E (IgE) including the high affinity receptor for IgE (FcεRI), CD23 (FcεRII), and Mac-2/εBP. We attempted here to clarify the role of these receptors in IgE-mediated activation of eosinophils from normal human bone marrow cultures. Pretreatment of eosinophils with IL-4 is required for IgE/anti-IgE-mediated stimulation of TNF-α and peroxydes production. TNF-α release from eosinophils was also induced following ligation of CD23 and to a lesser extent with anti-Mac-2, while FcεRI-ligation had no effect. IgE/anti-IgE effect dramatically decreased when eosinophils were pretreated with Fab fragments of CD23-mAb. In addition, this effect could also be reversed by inhibiting CD23-dependent nitric oxide pathway by N^G-monomethyl-L-arginine. Nitric oxide chemical donor, SIN-1, induced TNF-α release from eosinophils. CD23 and nitric oxide pathway are thus involved in IgE-mediated stimulation of normodense human eosinophils.

In various human immune responses, IgE levels are elevated and correlate with an enhancement of *in situ* infiltration by hemopoietic effector cells and the release of multiple mediators from these cells (1-5). IgE and antigen directly activate the secretion of various cytokines from mast cells following cross-linking of the high affinity receptor, FcεRI (1-3). Two other IgE-binding cell surface structures, which are part of the lectin like receptor family, have also been identified: FcεRII/CD23 and Mac-2 (CPB35, εBP) (5-8). These antigens are expressed by a variety of hematopoietic cells, including eosinophils and monocytes/macrophages

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(6,7). We have recently shown that, in normal human monocytes, CD23-ligation by IgE/antigen mediates activation of nitric oxide pathway and is necessary for the stimulation of TNF-α, IL-6, H_2O_2 and tromboxane B2 release by these cells (9). While CD23 and Mac-2 were first incriminated in IgE/antigen dependent eosinophil activation (5), recent data showed the involvement of FcεRI in this response (10). We thus attempted to clarify the role of each above receptors in IgE-mediated activation of normal human eosinophils.

Human peripheral blood eosinophils are heterogeneous populations as defined by density gradient sedimentation and functional response in vitro. The eosinophils that are obtained from blood of healthy donors have been designated as normodense, non-activated eosinophils (d>1,082 g/ml) (11) in comparison with activated hypodense cells obtained from hypereosinophilic patients (12). Eosinophil can be isolated from peripheral blood by gradient density separation and subsequent negative selection of contaminating cells (13). These methods however lead to limited numbers of these cells. Alternative techniques have been developed allowing to obtain higher number of eosinophils using stem cell differentiation and proliferation with various growth factors, such as interleukin-3 or interleukin-5 (14,15). Early $CD34^+$ hematopoietic cell cultures with IL-3 permitted to obtain eosinophils with various densities, including normodense, non-activated cells (16). Activated hypodense eosinophils from hypereosinophilic patients express FcεRI (10), while FcεRI expression by normodense, non-activated eosinophils remains to be established. We thus used bone marrow-derived non-activated eosinophils to clarify the role of IgE/antigen on these cells and the receptors ligated by these immune complexes.

Materials and Methods

Culture medium and reagents. Bone marrow progenitor cell cultures were performed in Dulbecco's Modified Eagle Medium supplemented with L-glutamine, penicillin, streptomycin, and 10% FCS (all from Gibco Laboratories, Grand Island, NY). Additional culture reagents are rhIL-3 and rhIL-4 (Immugenex, Los Angeles, CA), human IgE (Stallergene, Paris, France); goat anti-human-IgE (Nordic Immunology, Tilburg, The Netherland), N^G-monomethyl-L-arginine (NMMA, Sigma, St. Louis, MO), is a competitive inhibitor of the conversion of L-arginine to L-citrulline by nitric oxide synthase; or SIN-1 (6-morpholino-sydnonimine, Glaxo, Paris, France), FITC-conjugated CD23-mAb (clone 25), CD19-mAb (clone BC3, Immunotech, Marseille Lumigny, France); CD23-mAb (clone 135, both IgG and Fab) (9); anti-Mac-2 mAb (Boehringer Mannheim, Meylan, France); and anti-FcεRI (clone 15-1, gift from Dr. J.P. Kinet, NIH, Bethesda, MD)(10). Isotonic Percoll solutions were made by dilution of a 100% stock solution in non supplemented DMEM (Percoll, Pharmacia, St Quentin en Yvelines, France).

Bone marrow cultures and eosinophil purification. Human bone marrow-derived mononuclear cells were obtained from trocanter marrow fragments of healthy volunteers undergoing hip replacement following centrifugation on Ficoll gradient. $CD34^+$ cells (>95%) were then purified by positive immunomagnetic selection using anti-CD34 coated magnetic beads (Dynal, Compiègne, France) and cultured with rhIL-3 (10 ng/ml) for 3 weeks as described (16). Cell suspensions (containing more than 60% eosinophils) were then collected, washed and centrifuged over a dicontinuous Percoll gradient (1,080 and 1,100 g/ml). Cells with density higher than 1,080 g/ml were collected (>95% eosinophils). These cells have low if any CD23 expression, but acquire CD23 (40-70%) following 24-48 hour treatment with 10ng/ml IL-4. Cells were then incubated 1h with IgE (10µg/ml), washed and recultured with anti-IgE (30µg/ml). Eosinophils were also treated with CD23-mAb (clone 135, 20µg/ml, IgG or Fab fragments) as well as isotype-matched control, CD19-mAb. Some of these cultures were also supplemented with 1mM NMMA or SIN-1 (17), respectively inhibitor and agonist of nitric oxide pathway (18-20), for 1h before eosinophil activation with IgE/anti-IgE. The optimal concentrations of these reagents were determined in preliminary studies (9,21). Following 48h incubation, cell supernatants were collected for TNF-α and NO_2^- measurement and the cells were analysed for their oxidative burst.

Mediator assays. TNF-α was quantified in cell supernatants by ELISA as recommended by the manufacturer (Genzyme, Cambridge, MA). The production of superoxide anions by eosinophils was measured through the reduction of ferricytochrome C as described in details elsewhere (9). To assess the amount of NO produced, cell supernatants were assayed for the stable end products of NO synthase (NOS) pathway, the NO_2^- using modified Griess reaction (22). Results were analysed and compared using Student *t* test for paired data.

Results

IgE-mediated activation of proinflammatory mediator production by human eosinophil. Normodense eosinophils were isolated from normal human bone marrow cultures and assayed for their ability to secrete inflammatory mediators following activation by IgE and anti-IgE. These cells had low if any response to IgE/anti-IgE stimulation in this respect (Table 1). However, and as recently shown for normal human monocytes (9), treatment of eosinophils for 24-48 hours with IL-4 (10ng/ml) enables them to be activated by IgE/anti-IgE, evidenced through a significant release of TNF-α and increased oxidative burst (Table 1, $p<0.0001$). IgE or anti-IgE alone had no effect on above cell functions.

Ligation of various IgE-receptors and TNF-α release. As IL-4 treated eosinophils had significant responses to IgE/anti-IgE, these cells were selected in order to assay the role of various IgE receptors on these cells. As with IgE/anti-IgE, ligation of CD23 or Mac-2 antigens by

Table 1 Stimulation of human normal eosinophils-derived TNF-α and oxidative burst following 48h treatment with IgE/anti-IgE

Cells Cultured With	TNF-α (pg/ml)		$O^{\circ}_2^-$ (nmol/10^6 cells)	
	None	*+ IL-4*	*None*	*+ IL-4*
none	15±2	17±3	0.8±0.2	0.9±0.3
IgE	NT*	18±1	0.9±0.1	0.7±0.2
anti-IgE	NT*	20±3	0.7±0.3	1.0±0.1
IgE→anti-IgE	35±12	361±43	1.4±0.4	7.6±1.2

Eosinophils were incubated (10^6/ml) with/without 10ng/ml IL-4 for 48 hours, washed and reincubated with IgE (1μg/ml) for one hour, washed and cultured with anti-IgE (30μg/ml). Cell supernatants were collected 48 hours later and assayed for TNF-α, while the cells were analyzed for peroxydes levels. Results are mean±SD values from three distinct eosinophil preparations, each done in triplicates. *NT: not tested.

appropriate mAb, stimulated TNF-α secretion from eosinophils, while ligation of FcεRI had no such effect (Figure 1). In control experiments, the anti-FceRI induced the release of 23% histamine from human basophils. Furthermore, we failed (not shown) to detect messenger RNA for FcεRI in these cells by specific PCR (10). CD23-ligation induced significantly higher TNF-α levels than Mac-2-ligation ($p<0.02$). This asked whether CD23 is the major functional IgE receptor on human normodense eosinophils. The cells were thus pretreated with Fab fragments of CD23-mAb (CD23-Fab, clone 135)(19) prior IgE activation. The use of Fab fragments allows the occupancy of surface CD23 antigen and prevents IgE-binding (23) and the

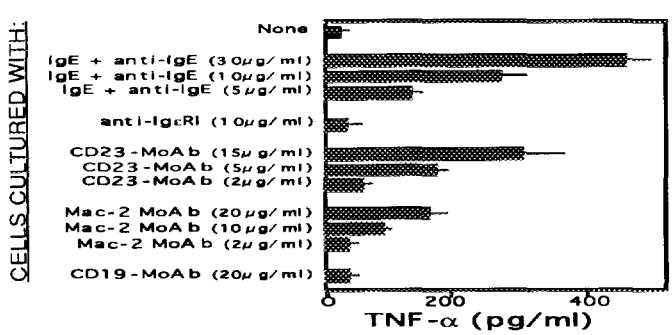

Figure 1. Induction of TNF-α release from human eosinophils following ligation of various IgE receptors. IL-4-treated eosinophils were treated with IgE/anti-IgE (as in Table 1), anti-FcεRI, CD23-mAb, CD19-mAb, or anti-Mac-2 mAb. Cell supernatants were collected 48 hours later and assayed for TNF-α levels. Mean±SD from two distinct eosinophils preparations, each done in triplicates.

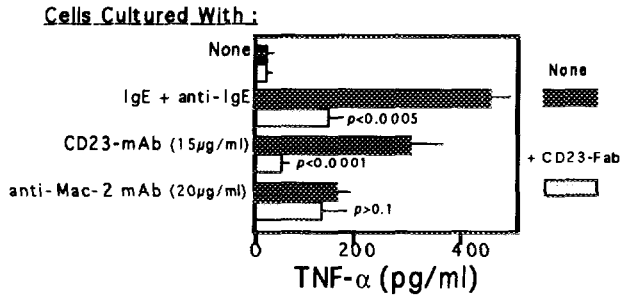

Figure 2. Involvement of CD23/FcεRII in IgE-mediated stimulation of human normodense eosinophils. The cells were incubated with or without CD23-Fab (20µg/ml) for one hour, washed and cultured with IgE/anti-IgE, CD23-mAb, or anti-Mac-2 mAb. TNF-α was quantified in 48 hours cell supernatants (mean±SD from triplicate cultures). *p* values compared CD23-Fab treated to untreated cells.

cross-linkage of this molecule. Figure 2 indicates that the addition of CD23-Fab significantly decreased eosinophil responses to IgE/anti-IgE and CD23-mAb, while this did not significantly affect Mac-2 mediated responses. The addition of anti-FcεRI mAb or isotype matched control (CD19-mAb) had no effect on IgE/anti-IgE mediated cell activation (not shown). These data demonstrate the involvement of CD23 in IgE-mediated activation of human normodense eosinophils.

Role of CD23-linked nitric oxide pathway in IgE-mediated activation of human eosinophils. Ligation of the b isoform of CD23 in human monocytes or keratinocytes induced a rapid accumulation of cyclic nucleotides, due in part to the activation of nitric oxide pathway (9,21). Eosinophils also express CD23b isoform, which led us to assay the role of NO in TNF-α production by IgE/anti-IgE treated eosinophils. These cells were therefore treated with N^G-monomethyl-L-arginine (NMMA), an inhibitor of the NO pathway (18-20), before the addition of IgE/anti-IgE or CD23-mAb. NMMA significantly inhibited the production of TNF-α in these culture conditions (Table 2). NMMA effect was itself antagonized by the addition of L- but not D-arginine to the cultures (data not shown). These data were further confirmed by the enhancement of TNF-α levels following the addition to eosinophil cultures of SIN-1 (17), a chemical NO donor (Table 2). Finally, TNF-α production corroborated with NO_2^- levels detected in the same cell supernatants (Table 2).

Discussion

The present study indicates that IgE/anti-IgE directly stimulate NO-dependent cell activation in human eosinophils following

Table 2. Involvement of L-arginine dependent pathway in CD23-mediated activation of human eosinophils

Cells Cultured With:	TNF-α (pg/ml)	NO_2^- (μM)
None	12±3	<2
NMMA	11±2	<2
IgE/anti-IgE	324±23	44±10
IgE/anti-IgE + NMMA	124±41	12±4
CD23-mAb	184±22	39±12
CD23-mAb + NMMA	61±11	6±2
SIN-1	257±35	>60

The cells were treated with 1mM N^G-monomethyl-L-arginine (NMMA) for 1h, before being supplemented with IgE/anti-IgE, CD23-mAb (15μg/ml), or SIN-1 (1mM) and incubated for additional 48h. TNF-α was measured thereafter (Mean±SD from three different eosinophil preparations).

FcεRII/CD23 ligation. This 45 kD glycoprotein belongs to the C-type lectin family and is expressed by a subset of B cells, monocytes/macrophages, platelets, follicular dendritic cells, keratinocytes, epidermal Langerhans cells, eosinophils and some T lymphocytes (6-7). In addition to IgE binding site, CD23 was shown to display other functional domains, including a cytokine-like site, a lectin region and a binding site for CD21 antigen (7,23,24). The transcription and surface expression of human b isoform of CD23, undetected in mice, are directly induced by IL-4 in a variety of human hemopoietic cells (7).

Recently, we have shown that ligation of CD23 directly mobilizes NO pathway in human monocytes as well as epithelial keratinocytes (9,21). The present work further extends this property to human normodense eosinophils. The ability of NMMA to inhibit the stimulatory effects of IgE/anti-IgE, the induction of NO_2^- formation, and TNF-α production by chemical NO donor demonstrate the involvement of NO in human eosinophil activation. However, CD23 expression by this eosinophil subset required cell activation by IL-4, an important early mediator in immune response as it induces both CD23 and its biologic ligand, the IgE (25). Simultaneous *in vivo* increase of $CD23^+$ eosinophils, serum IgE levels, and IL-4 is often observed in allergic patients (4,5,25,26). Subsequent ligation of CD23 by IgE/antigen or other potential ligands could thus stimulate the release of pro-inflammatory cytokines from eosinophils and other $CD23^+$ cells. *In situ* investigations are now in progress to show NO synthase presence and activation in $CD23^+$ eosinophils during allergic reactions and parasitic infections.

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