Original articles

Reduced responsiveness of adenylate cyclase in alveolar macrophages from patients with asthma

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Alveolar macrophages from patients with asthma accumulated less cyclic adenosine monophosphate when these macrophages were exposed to isobutyl methylxanthine, salbutamol, or prostaglandin E_2, compared to cells from control subjects without asthma, and the degree of the hyporesponsiveness was related to the severity of asthma. In addition, a significantly lower adenylate cyclase activity was observed in crude membrane fractions of macrophages from the group with asthma in the presence of salbutamol and prostaglandin E_2. The refractoriness observed in patients with asthma is thus not accounted for by a specific β-adrenergic desensitization at the adenylate cyclase receptor level but should rather be explained by a cyclic adenosine monophosphate-dependent postreceptor mechanism. (J ALLERGY CLIN IMMUNOL 1991;88:322-8.)

Key words: *Adenylate cyclase, alveolar macrophages, asthma*

The mechanisms underlying airway hyperreactivity may involve a reduced β-adrenergic responsiveness of the airway smooth muscles, as suggested by Szentivanyi.[1] Desensitization of AC might account for the decreased β-adrenergic responsiveness in asthma, particularly since patients frequently undergo physiologic or pharmacologic adrenoceptor stimulations.

Multiple mechanisms participate in the regulation of the responsiveness of the receptor-coupled AC system, resulting in both amplification and desensitization. Studies performed in a wide variety of cells and tissues after in vitro exposure to agonists demonstrated the existence of two major pathways of AC desensitization involving the receptors and the nucleotide regulatory proteins. Desensitization characterized by a diminished response only to the desensitizing hormone has been termed "homologous" by contrast to "heterologous" desensitization, characterized by a reduced response to a wide spectrum of hormonal as well as to other activators that do not necessarily interact directly with the receptor.[2]

Abbreviations used

AC:	Adenylate cyclase
AM:	Alveolar macrophage
PGE_2:	Prostaglandin E_2
cAMP:	Cyclic adenosine monophosphate
BAL:	Bronchoalveolar lavage
EDTA:	Ethylenediaminetetraacetic acid
IBMX:	3-Isobutyl-1-methylxanthine
NaF:	Sodium fluoride
GTP:	Guanosine triphosphate

Studies concerning the β-adrenergic function in subjects with asthma generally evaluate the responses of circulating leukocytes to β-adrenergic agonists. It has been demonstrated that treatment with those agonists results in desensitization of the AC system through the loss of membrane receptors.[3-6] However, the mechanisms involved in this refractoriness are poorly defined, and it is not clear whether adrenergic therapy may have reduced the responsiveness of the AC system through homologous desensitization or whether other factors distal to the receptor may contribute. β-Adrenergic hyporesponsiveness has been

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confirmed in subjects with asthma taking no medication, as well as in other allergic individuals without asthma,[5, 7] and in actively sensitized guinea pigs,[8-10] suggesting that the refractoriness is a general defect of the atopic abnormality.

In this study, AMs from subjects with and without asthma were used to elucidate further the mechanisms involved in the hyporesponsiveness of the cAMP-generating system in subjects with asthma. The in vitro response to IBMX, a potent inhibitor of cAMP phosphodiesterase activity, alone or combined with PGE_2, or salbutamol was investigated in intact macrophages. In experiments with crude membrane fractions, the AC response was evaluated with fluoride ion (NaF) and guanine nucleotide (GTP) alone or combined with PGE_2 or salbutamol.

MATERIAL AND METHODS

Isolation of AMs

AMs were obtained by BAL from patients of both sexes undergoing diagnostic bronchoscopy. Patients with asthma were treated with oral theophylline derivatives (400 mg/day), sprays of salbutamol (300 to 600 μg/day), and steroids (beclomethasone, 750 to 1500 μg/day). Asthma was classified as mild, moderate, or severe according to the dosage of the drug treatment necessary for control of the disease. Patients with mild asthma were receiving intermittent medication; patients with moderate and severe asthma were receiving daily drug therapy with higher dosages for severe asthma. Patients without asthma included smokers and nonsmokers presenting a variety of lung diseases, such as sarcoidosis, tuberculosis, chronic bronchitis, and miscellaneous diseases (fibrosis, pulmonary embolism, pneumonia, or pulmonary abcess). They did not receive steroids or antiasthmatic drugs. No patient with asthma smoked.

Bronchoscopy was performed with a flexible fibroscopic instrument (Olympus Winter & IBE GmbH, Hamburg, Germany) under local anesthesia (5 to 10 ml of 2% lidocaine, Roger Bellon, Neuilly-sur-Seine, France). Adrenaline (0.5 mg) was instilled just before lavages to prevent bronchospasm. Usually, three aliquots of 0.9% saline (60 ml each) were injected into the bronchus at room temperature and retrieved by gentle aspiration. Cells were sedimented by centrifugation (475 *g*) for 10 minutes at room temperature, and the cell pellet was resuspended in a culture medium (RPMI 1640, Jacques Boy SA, Reims, France, supplemented with 10 mmol/L of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer, 3% fetal calf serum, 50 U/ml of penicillin and 50 μg/ml of streptomycin). An aliquot (0.5 ml) was removed for determining cell numbers and for differential counts. The remaining cell suspension was plated in 35 mm plastic Petri dishes (2 ml per dish containing approximately 0.5 to 1 × 10^6 cells; Falcon Plastics, Becton Dickinson Labware, Lincoln Park, N.J.) for 1 hour at 37° C in a humidified atmosphere (95% air and 5% CO_2). Nonadherent cells were discarded, and the remaining adherent cells were incubated for an additional hour in 1 ml of serum-free RPMI medium.

Determination of intracellular cAMP concentrations in intact cells

After removing the RPMI medium, cells were incubated for 3 minutes at 37° C in 0.5 ml of Tris HCl buffer (50 mmol/L, pH 7.4) containing 2 mmol/L of EDTA. When this is stated, IBMX (0.2 mmol/L, alone or combined with PGE_2 (0.28 to 2.8 μmol/L), or salbutamol (0.3 to 3 μmol/L) was added to the incubation medium. At the end of the incubation, the Petri dishes were placed for 5 minutes in a drying oven at 100° C, and the cAMP content was measured in the supernatants with a protein-binding method.[11] The monolayers were scraped and dissolved in 0.1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) for protein determination. Results are expressed as picomoles of cAMP produced per 10^6 cells per 3 minutes.

Determination of AC activity in membrane preparations from macrophages

Macrophages (0.5 to 1 × 10^6 cells) were suspended in 1 ml of Tris HCl buffer (50 mmol/L, pH 7.4) containing 2 mmol/L of EDTA and centrifuged for 2 minutes at 15,000 *g*. The supernatants were removed, and the pellets were stored at −80° C overnight. Thawed pellets were homogenized by 3-second sonication in 1 ml of homogenizing medium (250 mmol/L of sucrose, 50 mmol/L of Tris HCl, pH 7.4, 25 mmol/L of KCl, and 5 mmol/L of $MgCl_2$). The homogenate was centrifuged at 50,000 *g* for 30 minutes at 4° C, and the pellet was resuspended in 0.5 ml of standard buffer (50 mmol/L of Tris HCl, pH 7.4, 5 mmol/L of $MgCl_2$, 2 mmol/L of EGTA, and 0.2 mmol/L of IBMX). Freshly prepared membrane fractions maintained at 0 to 4° C were used for evaluating AC. The reaction was started by the addition of 40 μl of the enzyme preparation to 40 μl of standard buffer containing adenosine triphosphate (3.2 mmol/L), bovine serum albumin (1.6 mg/ml), and when this is stated, NaF (16 mmol/L), GTP (0.1 mmol/L), alone or in the presence of PGE_2 (28 μmol/L), or salbutamol (30 μmol/L). The incubations were performed for 30 minutes at 37° C in Eppendorff tubes (Eppendorf Netheler Hinz GmbH, Hamburg, Germany). The samples were boiled in a water bath for 3 minutes and centrifuged (15,000 *g*) to yield a supernatant in which cAMP was measured as described above. Results are expressed as picomoles of cAMP produced per milligram of protein per 30 minutes.

Additional methods

The cell content was estimated by protein analysis with bicinchoninic acid,[12] and the yield was approximately 5 × 10^6 cells per milligram of protein. Cells were counted with a Malassez's hemocytometer in 0.05% trypan blue, and cell differentiations of the diverse populations present in the BAL were performed in cytospin preparations (Hettich Universal, Tuttingen, Germany) after staining with the Diff-Quick stain (Merz & Dade AG [Baxter Dade AG] Duedingen, Switzerland) and by the nonspecific esterase test that

TABLE I. Bronchoalveolar cell populations in patients with and without asthma (nonsmokers and smokers)

Patients	Macrophages ($\times 10^6$)	Eosinophils ($\times 10^6$)	Lymphocytes ($\times 10^6$)	Neutrophils ($\times 10^6$)	Total ($\times 10^6$)
Without asthma					
Nonsmokers	7.0 ± 1.1	0.17 ± 0.04	2.2 ± 0.6	0.8 ± 0.35	10.2 ± 1.2
	69.6% ± 4.7(%)	1.6% ± 0.3 (%)	20.6% ± 4.0(%)	7.8% ± 3.2(%)	
Smokers	18.2 ± 5.0*	0.3 ± 0.1	2.7 ± 0.5	0.33 ± 0.06	21.2 ± 5.3
	80.5% ± 4.1(%)	1.6% ± 0.5(%)	15.9% ± 3.9(%)	2.3% ± 0.5(%)	
With asthma	2.8 ± 3.8†	0.49 ± 0.12*	0.85 ± 0.14*	0.23 ± 0.05	4.4 ± 0.5†
	62.3% ± 3.9(%)	9.8% ± 2.0(%)	19.7% ± 2.6(%)	6.5% ± 1.5(%)	

Results are the mean ± SEM from 30 patients without asthma (18 nonsmokers and 12 smokers) and 22 patients with asthma.
*$p < 0.05$.
†$p < 0.01$ versus nonsmoking patients without asthma.

TABLE II. Relationship between intracellular cAMP and disease severity in asthma

Drug agonist	Asthma		
	Mild (N = 4)	Moderate (N = 9)	Severe (N = 4)
None	12.9 ± 5.5	11.8 ± 2.3	3.9 ± 1
IBMX (0.2 mmol/L)	30.4 ± 14	17.1 ± 3.3	4.6 ± 0.9*
IBMX (0.2 mmol/L) plus PGE_2 (2.8 μmol/L)	40.7 ± 14	25.2 ± 3.6	4.6 ± 0.6†
IBMX (0.2 mmol/L) plus salbutamol (3μmol/L)	31.9 ± 12	21.8 ± 4.3	3.2 ± 0.7*

Results (mean ± SEM) are expressed as picomoles of cAMP per 10^6 cells.
*$p < 0.05$.
†$p < 0.01$ versus patients with moderate asthma.

distinguishes AMs from lymphocytes.[13] The presence of mast cells was examined with alcian blue staining (0.1% in 3% acetic acid). Cell viability was judged by the trypan blue exclusion test.

Statistics

Data were analyzed by two-way variance analysis and, if this were necessary, by the one-tailed Mann-Whitney U test or the Student's *t* test, according to the normality hypothesis. Results were expressed as the mean ± SEM.

RESULTS

Bronchoalveolar cell populations in patients with and without asthma

Both the proportion and the number of eosinophils were significantly increased in the bronchoalveolar cell population of the group with asthma (Table I). In addition, total cell numbers recovered from patients with asthma were significantly below the number of cells from patients without asthma, whether the subjects were smokers or nonsmokers. This decreased number was unrelated to significant changes in the volume of BAL fluid recovered (81.0 ± 9.1 ml in nonsmoking patients without asthma, 73.2 ± 7.5 ml in patients who smoked and without asthma, and 65.5 ± 7.3 ml in patients with asthma). The decreased cell recovery led to a decrease in the number of macrophages and of lymphocytes recovered in the group with asthma, but the proportion of those cells observed in the three groups was not significantly different. Finally, an increased number of cells was recovered in the BAL from the smoking group without asthma, resulting in an increased number of AMs (Table I).

Intracellular levels of cAMP in AMs from patients with and without asthma

A significant negative correlation between the basal cAMP content of AMs was observed with age in patients with and without asthma. In patients without asthma, aged 33 to 86 years, the correlation coefficient was -0.49 ($N = 18$; $p < 0.05$).

PGE_2 (2.8×10^{-10} to 2.8×10^{-5} mol/L) and salbutamol (3×10^{-8} to 3×10^{-5} mol/L) significantly increased the intracellular concentration of cAMP in

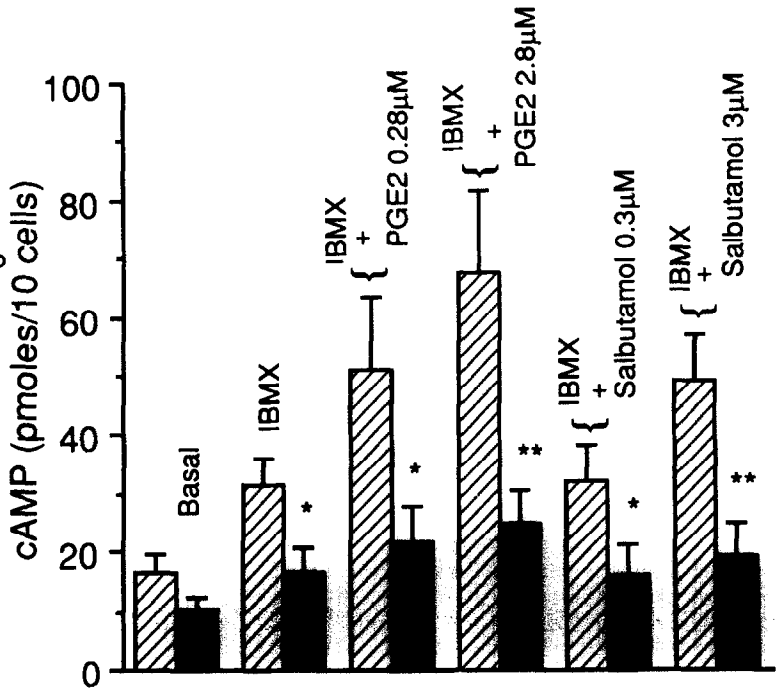

FIG. 1. Intracellular levels of cAMP in AMs from patients with and without asthma. Basal and stimulated levels of cAMP were measured in AMs from 13 patients without asthma (▨) and 17 patients with asthma (■) matched for age. The effect of IBMX (0.2 mmol/L) was evaluated alone and in the presence of PGE_2 (0.28 and 2.8 μmol/L) and salbutamol (0.3 and 3 μmol/L); $*p < 0.05$; $**p < 0.01$.

AMs from patients with and without asthma in a concentration-dependent manner. When patients of comparable age were studied, no significant differences in the basal intracellular concentration of cAMP were observed in AMs between patients with asthma (11 women and six men; 25 to 60 years of age; mean age, 41.3 ± 2.8 years) and patients without asthma (four women and nine men; 27 to 64 years of age; mean age, 47.5 ± 3.1 years). However, when AMs from both groups were exposed to IBMX (0.2 mmol/L), alone or combined with salbutamol (0.3 or 3 μmol/L), or PGE_2 (0.28 or 2.8 μmol/L), the increase of the cAMP concentration in cells from patients with asthma was markedly reduced compared to the increase in patients without asthma (Fig. 1). The group with asthma included seven patients taking no theophylline derivatives. The increase of the intracellular cAMP concentration measured in this group in the presence of IBMX, PGE_2, or salbutamol in the same conditions was not significantly different from the patients with asthma receiving medication and was also significantly lower compared to the group without asthma. However, the response to IBMX, PGE_2, and salbutamol in the patients with asthma more severely affected was also reduced much lower (Table II). The group without asthma included seven nonsmokers and six smokers, and negligible differences were observed in basal levels of cAMP as well as in IBMX, salbutamol, or PGE_2 responses between both groups.

AC activity in macrophages from patients with and without asthma

Like the intact cells, membrane preparations of macrophages from patients with asthma were less responsive to the stimulating effects of salbutamol (30 μmol/L) and PGE_2 (28 μmol/L) combined with GTP (0.1 mmol/L) in terms of AC activity, compared to membranes from patients without asthma (Fig. 2). Unlike the intact cells, the presence of IBMX (0.2 mmol/L) in the incubation medium induced a negligible increase in the AC activity, and no differences between the two groups were observed in the basal activity when IBMX was included in the medium. Exposure of membrane fractions to NaF (16 mmol/L in the absence of GTP) for 30 minutes induced a ninefold to tenfold elevation of cAMP levels and was less effective, although this was below the level of significance in stimulating AC in membranes from the group with asthma. Exposure to GTP alone (0.1 mmol/L) led to a twofold to threefold stimulation of AC and was equally effective in patients with and

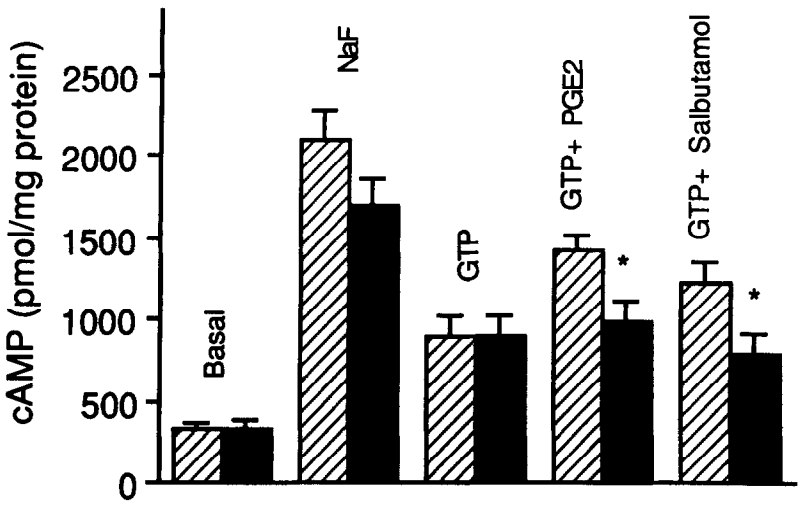

FIG. 2. AC activity in macrophages from patients with and without asthma. Basal and stimulated levels of AC activity were measured in crude membrane fractions of AMs from 11 patients without asthma (▨) and 13 patients with asthma (■). The effect of NaF (16 mmol/L) and GTP (0.1 mmol/L) alone or combined with PGE_2 (28 μmol/L) or salbutamol (30 μmol/L) was evaluated in both groups; $*p < 0.05$.

without asthma. No significant differences were observed between patients with asthma taking no theophylline derivatives and patients receiving such drugs.

DISCUSSION

In confirmation to findings by other investigators,[14, 15] our group of patients with asthma demonstrated an increased eosinophilia in the BAL, unaccompanied by changes in the neutrophil counts. In contrast, smokers without asthma had an increased proportion of AMs, which responded the same as AMs from control subjects to stimulations of AC and presented similar eosinophil counts. These characteristics indicate clearly that our control subjects and patients with asthma display the expected cell distribution.

In the present study, we found that, in analogy to the AMs from sensitized guinea pigs,[8] AMs from patients with asthma are significantly less sensitive to the cAMP-stimulating effect of PGE_2 and salbutamol. This finding in AMs is also observed in lymphocytes from patients with asthma that also became refractory to β-adrenergic agonists.[4, 5] However, the refractoriness now observed was not confined to β-adrenergic agonists, since the effect of PGE_2 was also reduced and cannot therefore be accounted for by homologous desensitization after β-adrenergic therapy. In fact, macrophages from patients with asthma failed to build up similar levels of intracellular cAMP as did macrophages from control subjects without asthma when they were exposed to the stimulating effect of agonists acting either beyond the receptor level, as the phosphodiesterase inhibitor, IBMX, or through different cell surface receptors, as salbutamol and PGE_2. The refractoriness observed was thus nonspecific and appeared to be dependent on the synthesis of cAMP. In analogy with our findings, a cAMP-dependent process of desensitization has been described in cultured cells[16, 17] and mammalian tissues,[18] and in most cases the refractoriness could be mimicked in vitro by incubation with cAMP analogs.

Different mechanisms may account for such desensitization. It is known that the levels of catecholamines influence the concentration of adrenoceptors in cells.[19] Their number decreases in vivo in aging humans,[20, 21] in which the levels of catecholamines are known to increase[21] and in vitro in the presence of increased concentrations of catecholamines.[22] The lower cAMP levels observed in this study in older individuals may relate to these increased levels of circulating catecholamines and to the resulting alteration in the AC activity.

It is, nevertheless, likely that neither the levels of circulating catecholamines, nor medication, nor patient selection can account completely for the observed hyporesponsiveness. In agreement with this hypothesis is the finding that untreated patients with asthma revealed a less pronounced, but still important, reduction in lymphocyte β-adrenoceptor number compared to that of normal subjects.[5] In addition, our findings suggest that the reduced responsiveness of patients with asthma was independent from exposure to theophylline, although this cAMP phosphodiesterase inhibitor causes desensitization of the β-adren-

ergic-dependent rise of cAMP synthesis in cultured cells.[17] However, a relationship between hyporesponsiveness and greater severity of disease was noted, in analogy with findings in lymphocyte β-adrenergic desensitization.[4, 5] Since severely affected patients were more medicated, it is possible that those differences are accounted for by therapy. Steroids have been demonstrated to increase β-adrenergic responsiveness, probably by increasing airway β-adrenoceptors.[19] However, this role appears particularly controversial, since our patients with asthma receiving higher doses of inhaled steroids have a more severe defect, in agreement with previous findings.[4]

An alternative explanation for these modifications may be related to the binding of immunoglobulins to the cell surface of macrophages, since an increased production of IgE by patients with asthma has been reported and since AMs become cytotoxic when they are exposed to the specific antigen.[23] Radioligand binding studies have demonstrated that an experimental sensitization procedure may induce modifications in the ratio of α/β-adrenoceptor-binding sites.[9] An increased proportion of α- and fewer β-adrenergic receptors were observed in membranes from guinea pig lung homogenates, providing a possible explanation for the reduced sensitivity to β-adrenergic agents in asthma that may involve immunoglobulin binding to lung cells. Although those immunoglobulin- or potential drug-induced changes at the receptor level might be important, our findings provide evidence to suggest that changes observed in patients with asthma cannot be explained only by specific β-adrenergic blockade.

The stimulating effect of GTP was clearly the same when cell membranes from patients with and without asthma were compared, and the effect of NaF was only slightly impaired in patients with asthma. Since the binding of GTP is believed to result in the dissociation of the α-subunit (GTP-binding protein), which combines to the catalytic unit,[2] these results suggest that the refractoriness involves an early event taking place before the activation of the catalytic unit by the α-subunit (GTP-binding protein). In addition, since the refractoriness was not present when membranes were treated with GTP or NaF, that is, in the absence of functional coupling between the receptor and the guanine nucleotide regulatory protein, it might involve modifications both at the receptor and at the guanine nucleotide regulatory protein levels without changes of the AC catalytic unit, as has been proposed for heterologous desensitization in cultured cells.[17]

In conclusion, our results demonstrate that AMs from patients with asthma are less responsive to the cAMP-increasing effects of PGE_2, salbutamol, and IBMX, compared to AMs of control subjects without asthma. This reduced sensitivity is not accounted for by desensitization of a specific receptor and may involve modifications at the guanine nucleotide regulatory protein level. Alterations of guanine nucleotide regulatory proteins are a likely explanation for the abnormal behavior of the cAMP-generating system of AMs from patients with asthma, a possibility now being explored. Potentially, the understanding of the mechanism of the macrophage abnormality may offer a new target for pharmacologic intervention.

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Monitoring human basophil activation via CD63 monoclonal antibody 435

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On activation of human basophilic granulocytes with anti-IgE or with the chemotactic peptide, formyl-methionyl-leucyl-phenylalanine, the expression of the CD63 antigen on the cell surface, detected by monoclonal antibody (MAb) 435, increased up to 100-fold. The kinetics of CD63 up regulation and histamine release were identical, and a strong correlation was found between percentage of MAb 435-binding basophils and extent of histamine release. Immunoelectronmicroscopy demonstrated that the epitope for MAb 435 in resting basophils is located on the basophilic granule membrane. After basophil activation, MAb 435 bound to the exterior of the plasma membrane. Experiments with various doses of anti-IgE demonstrated that the binding of MAb 435 to basophilic granulocytes follows an all-or-nothing–like response per cell. Basophils either do not bind the MAb at all, or they bind a maximal amount of the MAb. We also measured the up regulation of the CD11/CD18 leukocyte adhesion complex. Here, too, we noted an increase in cell-surface exposure of all subunits after activation. This increase was not as strong as increase found with MAb 435. Thus, MAb 435 is an interesting new tool for investigating the activation of human basophils, in addition to the measurement of mediator release. This MAb may be useful for the detection of basophil activation in vivo. (J ALLERGY CLIN IMMUNOL 1991;88:328-38.)

Key words: *Basophils, degranulation, activation, histamine release, CD63, CD18, CD11a,b,c, up regulation*

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