

Received: 2009.05.29
Accepted: 2010.01.29
Published: 2010.07.01

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

Adrenaline inhibits the immunological activation of human basophils at pharmacological and ultra-low doses

**Pier Francesco Mannaioni^{1ABCD}, Rosanna Mastroianni^{1ABCD},
Domenico Mastrangelo^{2ABCD}**

¹ Department of Preclinical and Clinical Pharmacology "Mario Aiazzi - Mancini", University of Florence, Florence, Italy

² Department of Biomedical Sciences, University of Siena, Siena, Italy

Source of support: Departmental sources

Background:

Adrenaline is known to inhibit the immunological activation of human basophils. The same effect can be obtained with ultra-low doses of histamine.

Material/Methods:

The present investigation was undertaken to verify the effects of ultra-low doses of adrenaline on the immunological activation of human basophils. For this purpose, purified human basophils were isolated from the blood of atopic donors and the response of the basophils to anti-IgE anti-serum was evaluated by measuring the release of histamine and the expressions of the activation markers CD63 and CD203c on the basophil surface.

Results:

The antigenic activation of basophils was significantly decreased by adrenaline at both pharmacological and ultra-low doses. The inhibiting effects of adrenaline were reversed by the beta blocker propranolol at either dose level.

Conclusions:

Although seemingly unexplainable by the currently accepted pharmacological principles, this phenomenon, already reported for histamine, deserves further investigation.

key words:

adrenaline • ultra low doses • basophil activation

Full-text PDF:

<http://www.medscimonit.com/fulltxt.php?ICID=880924>

Word count:

2461

Tables:

—

Figures:

6

References:

25

Author's address:

Domenico Mastrangelo, Department of Biomedical Sciences, University of Siena, Siena, Italy,
e-mail: mastrangelo@unisi.it

BACKGROUND

Allergic asthma and systemic anaphylaxis are triggered by IgE cross-bridging brought about by the antigen on the surface of tissue mast cells and circulating basophils, leading to a sequential release/neosynthesis of different allergic mediators, including histamine. For many decades, adrenaline has been a cornerstone in the treatment of systemic anaphylaxis and severe asthmatic episodes and its role has been confirmed by a body of experimental evidence showing its ability to reduce the response of immune-competent cells to the antigen. Adrenaline down-regulates histamine release and anaphylactic shock in the isolated lung of the guinea pig [1]. Moreover, the release of histamine from passively sensitized human chopped lung is maximally reduced by adrenaline and isoprenaline [2] at very low concentrations (10^{-11} , 10^{-10} M). Furthermore, in mast cells isolated from serous cavities of actively sensitized rats, 0.1–1 mM adrenaline significantly reduces the release of histamine due to antigen and compound 48/80 [3]. The inhibitory effect of adrenaline involves human basophils, which are of paramount importance in the second phase of the allergic response in an asthmatic attack [4]. In actively sensitized human basophils, high concentrations of adrenaline are required to inhibit the release of histamine [5]. Conceivably, the secretion of adrenaline exerts a negative feedback limiting the further release of allergic mediators in both systemic anaphylaxis and asthmatic attack.

Besides adrenaline, histamine has been shown to exert a feedback control on the spread of allergic reaction by decreasing the response to the allergenic challenge of other target cells, including tissue mast cells and blood basophils. In human basophils from allergic donors, 10^{-6} M histamine inhibits the release of histamine due to allergens, and this activity is blocked by burimamide but not by diphenhydramine, thus indicating the presence of H₂ receptors on basophils [6]. The antigen-induced IgE-mediated release of histamine from human basophils is blocked by extracellular histamine, presumably as a result of its ability to increase cyclic AMP levels, an effect which histamine shares with catecholamines [7]. Interestingly, this negative feedback is still preserved when histamine is over-diluted. In this regard, a European multicenter study recently showed that human basophil degranulation induced by anti-IgE is blocked by over-diluted histamine, as revealed by the optical counting of stained basophils [8].

Recent investigations using immune staining of basophils with specific activation markers, such as CD63 and CD203, and flow cytometry confirmed the results of previous reports [9] and further investigations showed that over-diluted histamine (10^{-28} – 10^{-30} M) significantly inhibits the anti-IgE-induced expression of CD-63 and the release of histamine from human basophils [10]. Since adrenaline also inhibits the antigen-stimulated release of histamine from mast cells and basophils at conventional concentrations, in the allergic response we hypothesized that it could, as histamine does, preserve this activity at ultra-low doses. To test this hypothesis, the present investigation comparing the effects of conventional and ultra-low doses of adrenaline on basophil activation was undertaken.

MATERIAL AND METHODS

Adrenaline dilutions

Adrenaline hydrochloride (21.9 g) was dissolved in 1 l of distilled water to a final concentration of 1 M and carefully stirred with a vortex for 30 s at full speed. This mother solution was then serially diluted 1/100 (v/v) up to 19 times and carefully stirred with a vortex at full speed for 30 s for each dilution to final theoretical molar concentrations (TMCs) ranging from 10^{-30} to 10^{-38} . Distilled water, as a control, was also prepared using the same procedure. Prior to use, the solutions were made isotonic by dilution 1/10 (v/v) with HEPES buffer (127 mM NaCl, 5 mM KCl, 20 mM HEPES, pH 7.4). All the steps were performed using disposable 20-ml polystyrene tubes and tips.

Measurement of basophil activation by flow cytometry

Human basophil activation *in vitro* follows the interaction of anti-IgE and reagenic (IgE) antibodies on the surface of basophils isolated from atopic donors and serves as a model of human allergy since the intensity of activation usually reflects the clinical severity of allergic diathesis [5]. For the human basophil activation test, atopic donors were enrolled in the study at the Transfusion Unit of Careggi General Hospital (Florence) after signing an informed consent form to participate in the study. Blood (400 ml) was collected from each individual in citrate solution. Fifty-ml aliquots were transferred to 50-ml tubes and centrifuged at 3500 rpm for 11 minutes at 20°C. The supernatant plasma was then carefully removed. The remaining blood was left at room temperature for 24 hours, the "buffy coat" was centrifuged at 900 rpm for 9 minutes at 20°C, and the platelet-rich supernatant was carefully removed. Thirty ml of the residual leukocyte-rich preparation was diluted 1:4 in HEPES buffer (127 mM NaCl, 5 mM KCl, 20 mM HEPES, 5 IU/ml heparin; pH 7.4) and aliquots of 10 ml were layered over 10 ml of Ficoll-Paque and centrifuged at room temperature at $420 \times g$. The basophil-rich Ficoll-Paque layer was then removed, washed, centrifuged ($200 \times g$, 10 min, 20°C) and re-suspended in HEPES buffer as above.

Aliquots of 250 μ l of cell suspensions were then incubated for 30 min at room temperature with the adrenaline dilutions and controls. To test the effect of beta-receptor antagonists, propranolol was co-incubated with adrenaline before adding anti-IgE, at the reported concentrations. Aliquots of 20 μ l were then incubated with anti-IgE for 30 min at 37°C.

For the measurement of basophil activation by flow cytometry, the basophil-rich suspensions were washed and labeled with 10 μ l of two fluorescent antibodies: fluorescein isothiocyanate (FITC) anti-IgE antibodies as a marker of IgE-bearing cells and phycoerythrin (PE)-conjugated antibodies – to the membrane – and granule-associated proteins CD63 and CD203c, respectively, as markers of allergenic activation. The fluorescent antibodies were incubated with the cells for 20 min at 4°C. The cells were then washed with buffer, centrifuged at $200 \times g$ for 10 min at room temperature, and re-suspended in buffer.

After the lysis of the residual erythrocytes, the leukocyte suspensions were analyzed with a flow cytometer (Coulter XL; Coulter Cytometry, Hialeah, FL, USA). Since the separation

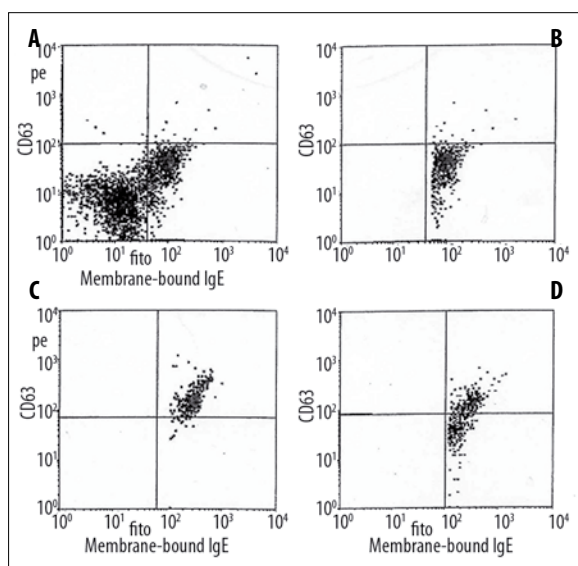


Figure 1. Flow cytometric analysis of human basophils: (A) percentage of activated basophils under basal conditions, (B) activated basophils after treatment with anti-IgE antibodies (1 µg/ml), (C) activated basophils after treatment with anti-IgE antibodies (1 µg/ml) and adrenaline at 10^{-30} TMC.

technique provides a leukocyte preparation with no more than 70% basophils, it was necessary to sort the basophils using the appropriate function in the flow cytometer (Figure 1). Basophils were recognized by their high expression of membrane-bound IgE, which results in a high signal related to FITC fluorescence (emission peak at 530 nm). The fluorescent signal of PE (emission peak at 575 nm) was used to discriminate between activated and non-activated cells. In the baseline inactivated state, human basophils showed no expression of CD63 and only a slight expression of CD203c, but on activation there was a significant increase in the expressions of both markers, as shown in Figure 1. Basophil activation was measured as the percentage of cells expressing CD63 and CD203c.

Measurement of basophil activation by histamine release

The histamine release assay used in this study is that proposed by Shore et al. [9] as modified by Kremzer and Wilson [11]. Histamine was measured in the supernatants and pellets of samples incubated with anti-IgE for 30 min at 37°C and in samples challenged with anti-IgE after 30 min of incubation with the adrenaline dilutions. For this purpose, O-phthalaldehyde was added directly to the sample supernatants after alkalization. The same procedure was used for the cells after extraction with 0.1 M HCl using the method proposed by Bergendorff and Uvnas [14]. Histamine release (supernatant histamine) was expressed as the percentage of the total histamine in the sample (cells plus supernatant). The spontaneous release of histamine, ranging between 1 and 5%, was subtracted from the reported values.

List of reagents and suppliers

Bovine serum albumin (BSA) and O-phthalaldehyde were from Boehringer (Germany), heparin from Parke-Davis

(Milano, Italy), and adrenaline hydrochloride and propranolol hydrochloride from Merck (Darmstadt, Germany). Anti-human IgE antibodies and anti-human IgE fluorescein isothiocyanate antibodies were from Sigma (Milano, Italy). Anti-human CD63 and CD203c phycoerythrin-conjugated antibodies were from Coulter (Milano, Italy).

Statistical analysis

All data were validated by calculating the coefficient of variations (CV) of the absolute basophil expressions of CD63 and CD203c and basal histamine levels in the absence of anti-IgE. The minimum percentage activation was set at 3.4 CV for the 0.1% level of risk. Only data exceeding this value were included in the analysis. Statistical analysis was performed using SPSS statistical software (release 11.0, Chicago, IL, USA). Comparisons of groups were done using Student's *t* test for unpaired values of Kruskal-Wallis *H* test. *p* values <0.05 were considered statistically significant.

RESULTS

Effects of ultra-diluted adrenaline on the antigenic release of histamine from human basophils

The human basophils of the atopic donors significantly reacted to antigenic stimulation. In six consecutive experiments using different atopic donors with serum IgE levels >500 ng/ml, exposure to antigen caused a 60% release of the total basophil histamine content. When the same antigenic stimulus was applied to cells which had been pre-incubated with adrenaline dilutions within the range of 10^{-28} to 10^{-36} , the antigenic release of histamine was significantly decreased. (Figure 2). No dose-effect relationship could be observed in the inhibiting action of adrenaline within the adrenaline dilutions reported in Figure 2. A comparable decrease in the release of histamine was reported by others [8] using ultra-high dilutions of histamine.

Flow cytometric evaluation of basophil activation

The expression of CD63 on the outer membrane of activated basophils can be detected by phycoerythrin (PE)-labeled monoclonal antibodies and measured by flow cytometry; it represents a marker of cellular exocytosis in response to antigenic stimuli (Figure 1). CD63 is a glycoprotein belonging to the tetraspanin family located within the granules of quiescent basophils and is expressed on the outer membrane only upon activation. In atopic donors, the challenge with anti-IgE induced the expression of CD63 in about 50% of circulating basophils, but after incubation of activated basophils with adrenaline concentrations ranging from 10^{-2} to 10^{-12} M, the anti-IgE-induced expression of CD63 was significantly reduced (Figure 3). More importantly, the same effect can be observed in cells pre-incubated with a wide range of ultra-high dilutions of adrenaline, within the range of 10^{-30} to 10^{-40} TMC (Figure 3), and the effect on CD63 expression is similar to that of ultra-high dilutions of histamine (Figure 4).

Other experiments indicated that pre-incubation with both pharmacological and ultra-high dilutions of adrenaline (10^{-6} M) decreased the anti-IgE-induced activation of basophils and this effect was reversed by propranolol (10^{-8} M) (Figure 5).

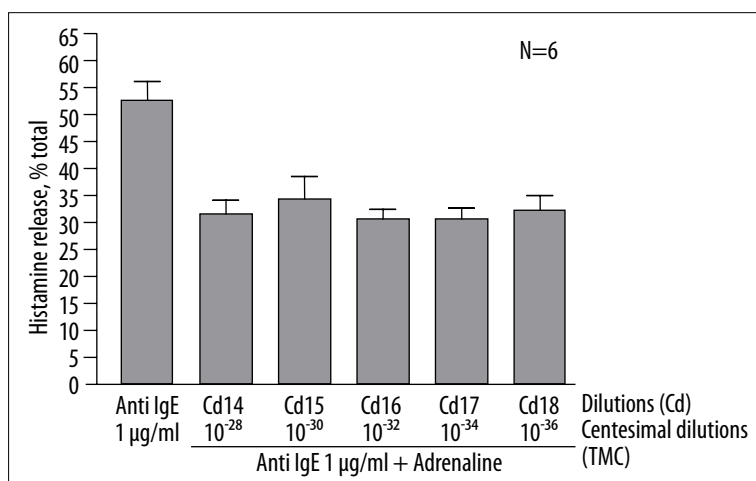


Figure 2. Effect of pre-incubation with adrenaline at ultramolecular dilutions on anti-IgE (1 µg/ml)-induced histamine release. Data are reported as means $\pm$ SEM for six experiments. Using the Kruskal-Wallis test, significant differences could be found with the control histamine release ($p=0.01$). Adrenaline dilutions are expressed as centesimal dilutions (Cd) and as the corresponding theoretical molar concentrations (TMC).

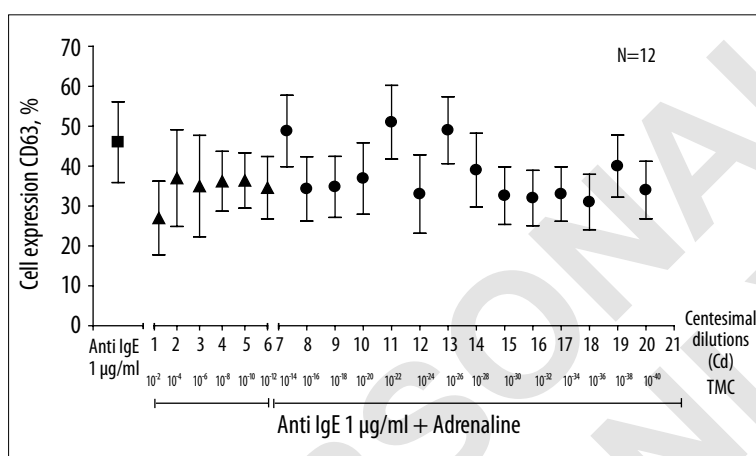


Figure 3. Basophil activation assessed by the percentage of cells expressing CD63 after incubation with anti-IgE (1 µg/ml) in control cells (■) and in cells pre-incubated with adrenaline at pharmacological concentrations (▲; 10⁻²/10⁻¹² M) and at ultramolecular dilutions (●; <10⁻¹² M). Adrenaline dilutions are expressed as centesimal (Hahnemannian) dilutions (Cd) and as the corresponding theoretical molar concentrations (TMC). The data reported here concern 12 experiments performed in duplicate. Using the Kruskal-Wallis test there were significant differences between adrenaline and control ($p\geq 0.001$).

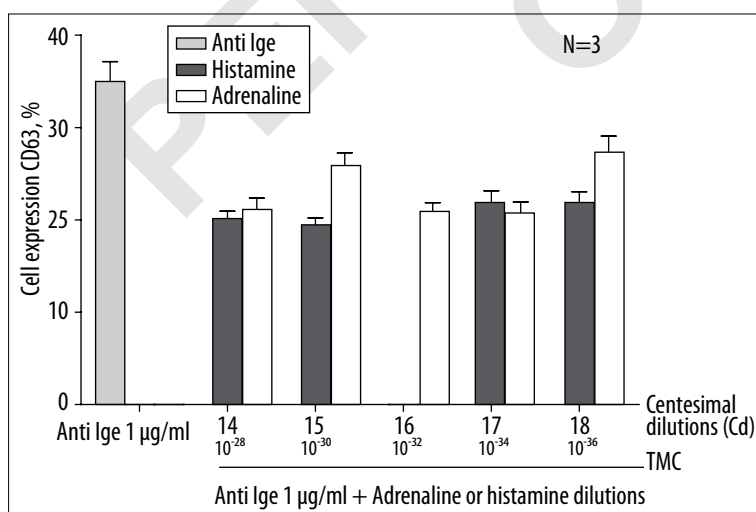


Figure 4. Basophil activation assessed by the percentage of cells expressing CD63 after incubation with anti-IgE (1 µg/ml) in control cells and in cells pre-incubated with ultra-molecular dilutions of histamine (striped bars) and of adrenaline (open bars). Centesimal dilutions (Cd) and theoretical molar concentrations (TMC) are given. The data reported here concern three experiments performed in duplicate. Using the Kruskal-Wallis test there were significant differences from control ($p\geq 0.001$). Data for histamine are taken from Belon et al. 2004 [8].

CD203c antigen, a glycosylated type II trans-membrane protein belonging to the family of ectonucleotide pyrophosphatase/phosphodiesterase enzymes, is located within the cytoplasmic granules and appears on the outer membrane only upon activation. According to current evidence, CD203c is more specific to basophils than CD63, which is also expressed

by platelets and macrophages [15–17]. Figure 6 shows a sharp increase in the percentage of cells expressing CD203c after antigen challenge. Pre-incubation with adrenaline at doses in the range of 10⁻⁸ to 10⁻¹² M blocks the expression of this activation marker and the block is still evident and significant at doses lower than 10⁻¹² M.

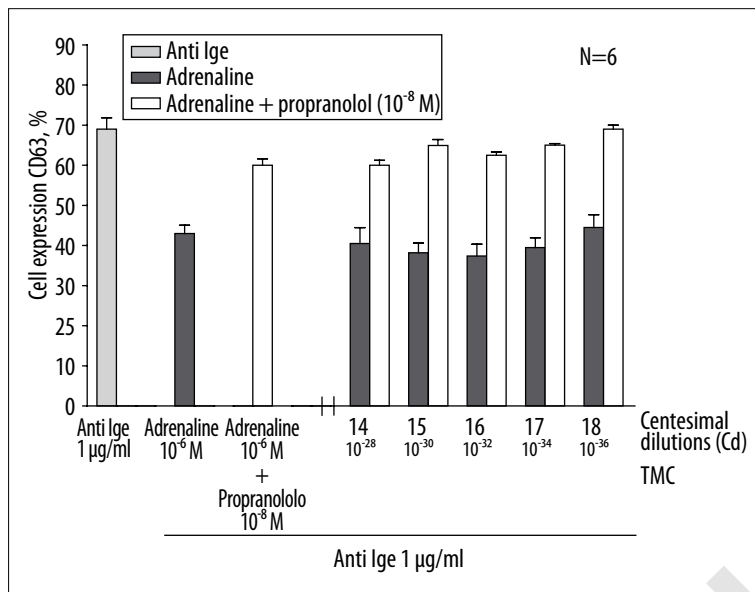


Figure 5. Effect of pre-incubation with the beta-receptor blocker propranolol (10^{-8} M) on inhibition of the antigenic activation of basophils induced by conventional concentrations (10^{-6} M) and by ultramolecular dilutions of adrenaline (beyond 10^{-14} M). Activation of basophils was assessed by the percentage of cells expressing CD63 after incubation with anti-IgE (1 µg/ml) in control cells and in cells pre-incubated with adrenaline at pharmacological concentrations and at ultramolecular dilutions in the absence and presence of propranolol. Centesimal dilutions (Cd) and theoretical molar concentrations (TMC) are given. $p > 0.001$ compared with anti-IgE alone using the Kruskal-Wallis test in six experiments performed in duplicate.

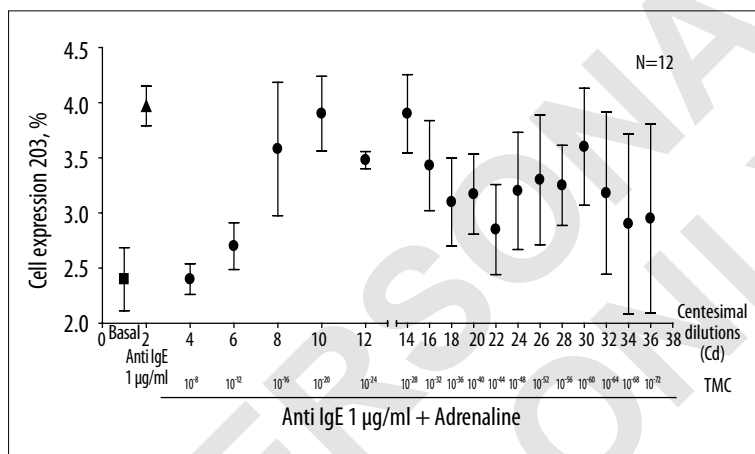


Figure 6. Basophil activation assessed by the increase in the percentage of cells expressing CD203c after incubation with anti-IgE (1 µg/ml) in control cells (■) and in cells pre-incubated with adrenaline at pharmacological concentrations (▲; 10^{-2} – 10^{-12} M) and at ultramolecular dilutions (●; $< 10^{-12}$ M). Centesimal dilutions (Cd) and theoretical molar concentrations (TMC) are given. Data are shown from 12 experiments performed in duplicate. Using the Kruskal-Wallis test there were significant differences from control ($p \geq 0.001$). The arrow is positioned at Avogadro's number.

DISCUSSION

Measurement of the expressions of CD63 and CD203c by flow cytometry on circulating basophil also known as the Basophil Activation Test (BAT) has been proposed as a novel *in vitro* test for allergy of the immediate type and is presently considered a very reliable *in vitro* test for monitoring wasp venom [11,15] and amoxicillin allergy [17]. In circulating basophils, over-expression of the activation markers CD63 and CD203c is functionally coupled with the release of histamine in response to anti-IgE antibodies and therefore the BAT is useful in evaluating the effects of substances known to modulate the allergic response [18,19]. By using the BAT we have shown that ultra-low doses of adrenaline significantly reduce the antigenically induced release of histamine by human basophils from atopic donors in a dilution-independent manner. More importantly, the experiments reported herein show that basophil activation in the BAT test is independent of the adrenaline dilution used and was always reversed by the beta blocker propranolol (Figure 5). Furthermore, the present experiments also shows that the inhibiting effect of ultra-low doses of adrenaline is comparable to that induced by ultra-low doses of histamine in the

same experimental setting [5,6,11]. Therefore it can be argued that under conditions such as stress, inflammation, anaphylaxis, and asthma, adrenaline and histamine could exert a negative feedback on basophil activation, both at pharmacological concentrations and at ultra-low doses.

The effects of pharmacological doses of adrenaline on basophils are due to the interaction of the molecule with beta-adrenoceptors on the cell membrane. The same does not apply, however, to the ultra-low doses used in the present investigation. Moreover, the inhibiting effect of ultra-low doses of adrenaline does not follow either the classical pattern of the dose-effect relationship or that of the hormetic dose-response curve [20]. Equally unexplainable seems to be the reversal of the inhibition induced by a beta-blocker in the case of ultra-low doses of adrenaline. A tentative explanation of the results of our investigation would be that the inhibiting effects of ultra-low doses of adrenaline on basophil activation could be produced in a still unknown receptor-independent way. Within this view it would be of interest to know whether ultra-low doses of adrenaline would actually increase the intracellular levels of cyclic 3–5-AMP in human basophils and whether the increase in cyclic 3–5 AMP

could be abrogated by propranolol through an alternative receptor-independent pathway.

The hormetic dose-response curve [19] has taught us that many drugs have a certain effect at pharmacological concentrations and a totally opposite effect at ultra-low doses. As an example, ultra-low doses of aspirin show pro-thrombotic properties and the ability to normalize platelet function in portal hypertension [21], ultra-low doses of morphine induce hyperalgesia in rats [22], and ultra-low doses of the narcotic antagonist naloxone (20 ng intrathechally) paradoxically enhance analgesia induced by morphine (2 µg intrathechally) in patients with severe back pain [23]. Moreover, as has been shown, an oral formulation that combines oxycodone (doses between 2.5 and 40 µg) and the narcotic antagonist naltrexone (1 µg) is superior to both oxycodone alone and placebo in the amount and quality of analgesia obtained in patients with severe osteoarthritic pain [24]. Finally, it has been shown that ultra-low doses of opioid receptor antagonist in conjunction with very low doses of cannabinoids may provide a potent strategy to modulate seizure susceptibility [25]. The above are only a few of the literature reports showing the effectiveness of ultra-low doses of drugs.

CONCLUSIONS

It is conceivable that ultra-low doses (ranging from ng to µg/kg), when distributed in an average body mass of about 70 kg, would reach the receptor area as ultra-high dilutions, thus implying the possibility that ultra-low doses may still be active at infinitesimal concentrations in selected individuals. Further investigations in this field are highly recommended.

REFERENCES:

1. Shild H: Histamine release and anaphylactic shock in isolated lungs of guinea-pig. *Q J Exp Physiol*, 1936; 26: 165–78
2. Assem ESK, Schild H: Inhibition by sympathomimetic amines of histamine release induced by antigen in passively sensitized human lung. *Nature*, 1969; 224: 1028–29
3. Johnson AR, Moran NC: Inhibition of the release of histamine from rat mast cells The effect of cold and adrenergic drugs on release of histamine by compound 48/80 and by Antigen. *J Pharmacol*, 1970; 175: 632–40
4. Elenkov IJ, Chrousos GP: Stress system organization, physiology and immunomodulation. *Neuroimmunomodulation*, 2006; 13: 257–67
5. Lichtenstein LM, Margolis S: Histamine release *in vitro*; inhibition by catecholamines and methylxanthines. *Science*, 1968; 161: 902–3
6. Lichtenstein LM, Gillespie E: Inhibition of histamine release by histamine controlled by H2 receptor. *Nature*, 1973; 244: 287–88
7. Lichtenstein LM, Gillespie E: The effect of the H1 and H2 antihistamines on “allergic” histamine release and its inhibition by histamine. *J Pharmacol*, 1975; 192: 441–50
8. Belon P et al: Inhibition of human basophil degranulation by successive histamine dilutions: Results of a European multi-centre trial. *Inflamm Res*, 1999; 48(Suppl.1): S17–18
9. Boumiza R et al: The basophil activation test by flow cytometry: recent developments in clinical studies, standardization and emerging perspectives. *Clin Mol Allergy*, 2005; 3: 1–8
10. Belon P et al: Histamine dilutions modulate basophil activation. *Inflamm Res*, 2004; 53: 181–88
11. Eberlein-Konig B et al: *In vitro* basophil activation using CD63 expression in patients with bee and wasp venom allergy. *J Invest. Allergol Clin Immunol*, 2006; 16: 5–10
12. Shore PA, Burkhalter A, Cohn VH: A method for the fluorimetric assay of histamine in tissues. *J Pharmacol*, 1959; 127: 182–86
13. Kremzer LT, Wilson IB: A procedure for the determination of histamine. *Biochem Biophys Acta*, 1961; 50: 364–67
14. Bergendorff A, Uvnas B: Storage of 5-hydroxytryptamine in rat mast cells. Evidence for an ionic binding to carboxyl group in granule heparin-protein complex. *Acta Physiol Scand*, 1972; 84: 320–21
15. Erdmann SM, Sachs B, Kwicien R et al: The basophil activation test in wasp venom allergy: sensitivity, specificity and monitoring specific immunotherapy. *Allergy*, 2004; 59: 1102–9
16. Buring HJ, Streble A, Valent P: The basophil-specific ectoenzyme E-NPP3 (CD203c) as a marker for cell activation and allergy diagnosis. *Intern Arch Allergy and Immunology*, 2004; 133: 317–29
17. Abuaf N, Rostane H, Rajoely B et al: Comparison of two basophil activation markers CD 63 and CD 203c in the diagnosis of amoxicillin allergy. *Clin Exp Allergy*, 2008; 38: 921–28
18. Yasnowsky KM, Dreskin SC, Efav B et al: Chronic urticaria sera increase basophil CD203c expression. *J All Clin Immunol*, 2006; 117(6): 1430–34
19. Sterba PM, Alexander V, Eckman JA et al: Relationship Between Allergen-Induced Basophil Histamine Release and CD203c Stimulation During Omalizumab Treatment. *J All Clin Immunol*, 2009; 123(2 Suppl.): S198
20. Calabresi EG: Hormesis: changing views on the dose-response. *Mutation Res* 2002; 511: 181–89
21. Eizayaga FX, Aguejoui O, Belon P, Doutremepuich C: Platelet aggregation in portal hypertension and its modification by low doses of aspirin. *Pathophysiol Haemost Thromb*, 2005; 34: 29–34
22. Fereidoni M, Javan M, Semnani S, Ahmadiani A: Chronic forced swim stress inhibits ultra-low doses induced hyperalgesia in rats. *Behav Pharmacol*, 2007; 18: 667–72
23. Hamann S, Sloan PA, Witt W: Low-dose intrathecal naloxone to enhance intrathecal morphine analgesia: a case report. *J Opioid Manag*, 2008; 4: 251–54
24. Leri F: Co-administration of opioid agonists and antagonists in addiction and pain medicine. *Expert Opin Pharmacother*, 2008; 9: 1387–96
25. Bahremand A, Shafaroodi H, Ghasemi M et al: The cannabinoid anticonvulsant effect on pentylenetetrazole-induced seizure is potentiated by ultra-low dose of naltrexone in mice. *Epilepsy Res*, 2008; 81: 44–51