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The Effect of Immunotherapy on the *In Vitro* Productions of Histamine, Prostaglandin E2 and Leukotriene C4 in Asthmatic Children

Jiu-Yao Wang*, Kue-Hsiung Hsieh

SUMMARY **In order to elucidate the working mechanisms of immunotherapy (IT), the *in vitro* productions of histamine, prostaglandin E2 (PGE2) and leukotriene C4 (LTC4) were studied in 18 newly diagnosed and 20 hyposensitized (>2 yr) asthmatic children. All were sensitive to house dust and dust mites. (D. pteronyssinus). Ten age-matched normal children were included as control. Polymorphonuclear (PMNs) and mononuclear (MNCs) leukocytes were separated by density gradient centrifugation and dextran sedimentation. PMNs (2×10^7 cells/ml) and MNCs (2×10^7 cells/ml) were stimulated with mite allergen (10 μg/ml) and calcium ionophore A23187 (1 μg/ml) for 15 minutes. The plasma and culture supernatant (sup) histamine levels and sup PGE2 and LTC4 were measured by RIA. The results showed; 1) When compared to new patients, the treated patients had much lower plasma and sup histamine ($p < 0.001$), no matter whether PMNs and MNCs were stimulated with allergen or A23187 and the normals had the lowest histamine level among 3 groups; 2) LTC4 in A23187-stimulated sup was lower in treated patients ($p < 0.05$); 3) The PGE2 in allergen-stimulated sup was markedly increased in treated patients as compared to new patients ($p < 0.01$) and the PGE2 in sup of normals was also much higher than that of new patients.**

Thus, immunotherapy is able to reverse the abnormal secretory pattern of inflammatory mediators of allergic patients, and this change may account, partly, for its clinical effectiveness.

Bronchial asthma is characterized by episodic, variable airflow obstruction and increased responsiveness of the airways to various stimuli.[1] Although the mechanisms which trigger the asthmatic attacks are not completely understood, immunologically, the allergic reaction involves the interaction of specific allergens and IgE antibodies bound to receptors on mast cells and basophils.[2] The bridging of cell surface bound IgE molecules, and hence of IgE receptors, generates a signal that results in a complex sequence of biochemical events, causing cellular activation, arachidonic acid metabolism and mediator release.[3] Evidence exists that a number of types of cells including airways epithelial cells, polymorphonuclear leukocytes, macrophages, eosinophils and platelets,[4,5] and a variety of mediators including histamine, chemotactic factors, leukotrienes, prostaglandins, platelet activating factor and enzymes[6,7] are involved in the complex reaction which results in both acute and sustained bronchial inflammation in asthmatic patients.[8]

Immunotherapy (IT) or hyposensitization has been shown, since its introduction in 1911,[9] to be effective in treating some patients with respiratory allergy.[10,11] Studies on the working mechanisms of IT have focused almost completely on the alteration of immunologic status,[12-18] but only few on allergy mediators (histamine).[19,20] This project was conducted to study the *in vitro* production of histamine, prostaglandin E2 (PGE2) and leukotriene C4 (LTC4) in new asthmatic patients and in those after receiving immunotherapy for at least 2 years.

Departments of Pediatrics, College of Medicine, *National Chengkung University Hospital, Tainan and National Taiwan University Hospital, Taipei, Republic of China.

Correspondence : Kue-Hsiung Hsieh, M.D. Department of Pediatrics, National Taiwan University Hospital. No. 1. Chang-Te Street, Taipei, 10016, Taiwan. Republic of China.

MATERIALS AND METHODS

Study populations

The characteristics of the study populations are shown in Table 1. They were comparable with regard to sex and age, but the patients had much higher total serum IgE than did the normals. All patients were sensitive to only house dust and dust mites (Dermatophagoides pteronyssinus), in terms of a positive history, positive skin test and a 2+ or greater RAST. The normal subjects showed a negative skin test and RAST. The peak expiratory flow rate (mini-Wright peak flow meter, Airmed, England) at the time of blood samplings was comparable among 3 groups. IT was started with weekly injections and the allergen dose was increased as rapidly as possible until a maximal tolerated dose was reached (usually within six months). This dose was then maintained at four-week intervals. The treated patients were considered to be good responders as judged by the decreased frequency and shortened duration of attacks and diminished requirement for medications.

Table 1. Characteristics of study populations

Subjects	No. of case	Sex M	Sex F	Mean age (years)	Total serum IgE (mean ± SD, IU/ml)	PEFR (L/min)
Normals	10	6	4	12.1	123 ± 51	384 ± 42
New patients*	18	10	8	11.7	1185 ± 443	356 ± 59
Hyposensitized patients	20	14	6	11.4	1526 ± 555	368 ± 45

*Patients who were freshly diagnosed and had never received immunotherapy.

Purifications of mononuclear cells (MNCs) and polymorphonuclear leukocytes (PMNs)

Thirty mls of heparinized (1 U/ml) fresh blood were collected from newly diagnosed asthmatic children before IT, and from the treated asthmatic children after they had received IT for more than 2 years. No steroids had been given for at least 2 weeks and bronchodilators were withheld for at least 1 day before blood samplings. Peripheral blood mononuclear cells were isolated by the method of Boyum.[21] After washing with Hanks' balanced salt solution (HBSS, GIBCO) three times, the MNCs were suspended at a concentration of 2×10^6 cells/ml in HBSS. After removal of MNCs at the interface layer, the packed cells at the bottom of the centrifuge tube were mixed with an equal volume of HBSS and 6 percent dextran in normal saline (0.2 ml/ml of cell suspension) was added. After standing at room temperature for one hour, the PMN-rich supernatant was collected and the contaminated RBCs were lysed hypotonically. The PMNs were washed three times with HBSS and then adjusted to a concentration of 2×10^7 cells/ml. The purity of PMNs in the final cell suspension was more than 95 percent and the viability was over 96 percent by trypan blue exclusion test. The average percentage of basophils in PMNs suspension was 0.3 ± 0.3 percent for normals, 0.5 ± 0.3 percent for new patients and 0.6 ± 0.5 percent for treated patients. The figure in MNCs was 1.5 ± 0.8 percent for normals, 2.0 ± 1.6 percent for new patients and 1.8 ± 1.5 percent for treated patients. The basophils were stained by toluidine blue.

Preparation of crude mite allergen

The crude extract of the house dust mite (D. pteronyssiuns) was generously supplied by Torii & Co., Tokyo, Japan. The mite allergen was extracted according to the method of Nakada *et al.*[22] The protein content of the extract was determined by Bio-Rad protein assay kit (Richmond, CA). The same extract was used for skin testing, immunotherapy and *in vitro* culture experiments.

Calcium ionophore A23187 and allergen (house dust mite) stimulations of PMNs

The PMNs and MNCs suspensions were incubated alone or stimulated with calcium ionophore A23187 (final concentration 1 μg/ml. sigma Co.,) and house dust mite extract of various concentrations (0, 1, 5, 10 and 20 μg/ml), respectively. The cells were incubated for 5 to 30 min in a 37° C water bath. At the end of incubation, supernatants (sup) were collected by centrifugation at 1,500 rpm for 20 min at 4° C and stored at -70° C until testing. House dust mite extract at a concentration of 10 μg/ml and incubation for 15 min were found to be optimal for mediator release (data not shown).

Determinations of mediators in plasmas and supernatants

The histamine levels in plasmas and culture supernatants (sup) were measured by radioimmunoassay (RIA) kits (Immunotech, France).[23] PGE2 and LTC4 in sup were measured directly by using RIA kits (New England Nuclear, USA). No previous extract procedure was done for PGE2 and LTC4 as the *in vitro* experiments used only balanced salt solution (HBSS). The net amount of mediators produced *in vitro* was calculated by substracting the value of spontaneous release from that of stimulated cultures. The percent increment of mediator production of basophils in response to allergen stimulation was calculated by the following formula :

$$\text{Percent increment} = \frac{\text{mediator concentration in stimulated culture} - \text{spontaneous release in unstimulated culture}}{\text{spontaneous release in unstimulated culture}} \times 100$$

Statistics

Student's *t*-test was used for statistical analysis.

RESULTS

Fig. 1 shows that when compared to normal children, both newly diagnosed and treated asthmatic patients had higher plasma histamine concentrations than did normals (39.5 ± 12.5 *vs* 11.2 ± 4.6 nM, $p < 0.001$ for new patients and normals; and 23.9 ± 6.5 *vs* 11.2 ± 4.6 nM, $p < 0.05$ for treated patients and normals). Moreover, the plasma histamine level of treated patients was much lower than that of new patients (23.9 ± 6.5 *vs* 39.5 ± 12.3, $p < 0.01$). *In vitro* production of histamine by MNCs, and PMNs particularly, showed a patern very similar to that of plasma histamine, no matter whether stimulated with allergen or calcium ionophore A23187.

The effect of IT on leukotriene C4 (LTC4) production is depicted in Fig. 2. A23187-stimulated *in vitro* production of LTC4 by MNCs ($p < 0.05$) and PMNs ($p < 0.01$) was lower in treated patients than in normals and new patients. No difference was found when leukocytes were stimulated with allergen.

Fig. 3 demonstrates PGE2 production in the 3 studied groups. The *in vitro* production of PGE2 by MNCs ($p < 0.05$) and PMNs ($p < 0.01$) was higher in treated patients than in new patients when leukocytes were stimulated with allergen, but no difference was found when leukocytes were stimulated with A23187.

Fig. 4 shows the mediator release of basophils (contaminated in PMNs) in response to allergen stimulation in the 3 studied groups. The percent increment of histamine production decreased from 135.6 ± 66.6 percent in new patients to 54.5 ± 30.3 percent in treated patients ($p < 0.05$). To the contrary, the percent increment of PGE2 production increased from 154.3 ± 52.2 percent in

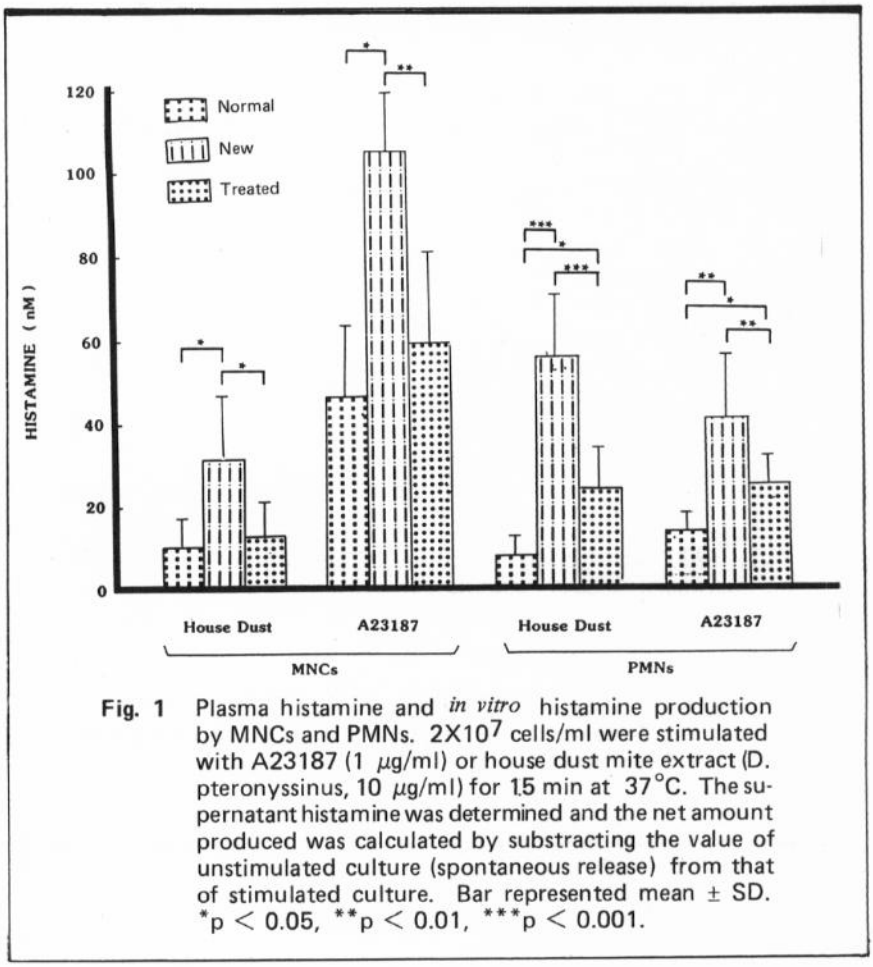

Fig. 1 Plasma histamine and *in vitro* histamine production by MNCs and PMNs. $2X10^7$ cells/ml were stimulated with A23187 (1 μg/ml) or house dust mite extract (D. pteronyssinus, 10 μg/ml) for 15 min at 37°C. The supernatant histamine was determined and the net amount produced was calculated by substracting the value of unstimulated culture (spontaneous release) from that of stimulated culture. Bar represented mean ± SD. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

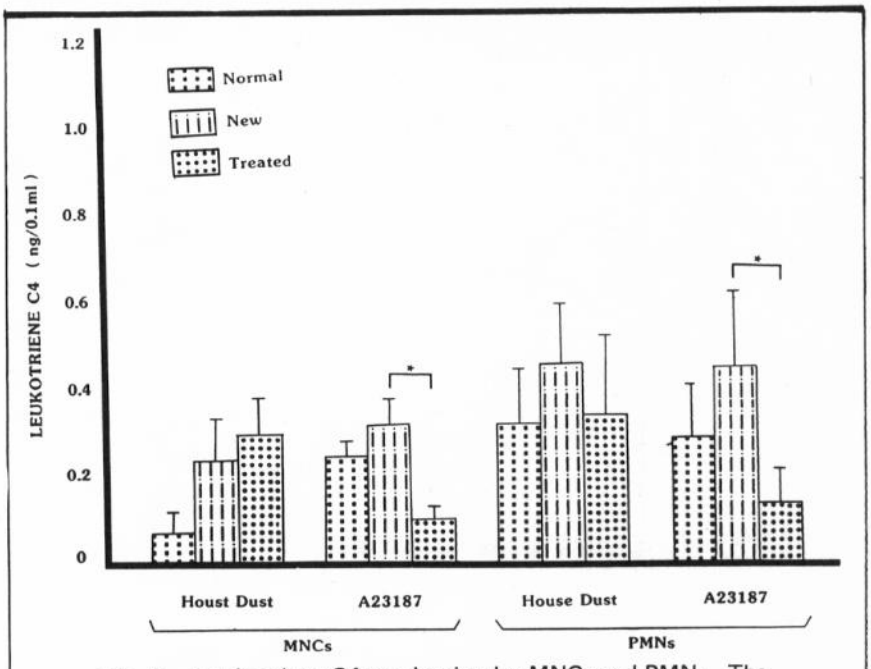

Fig. 2 Leukotriene C4 production by MNCs and PMNs. The experimental method was same as that described in Fig. 1. $^*p < 0.05$, $^{**}P < 0.01$.

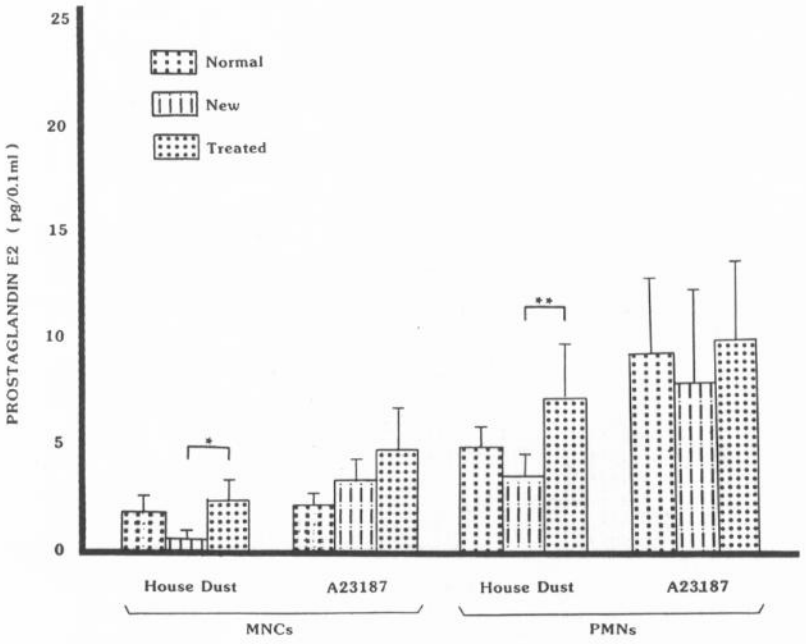

Fig. 3 Prostaglandin E2 production by MNCs and PMNs. The experimental method was same as that described in Fig. 1 $^{*}p < 0.05$, $^{**}p < 0.01$.

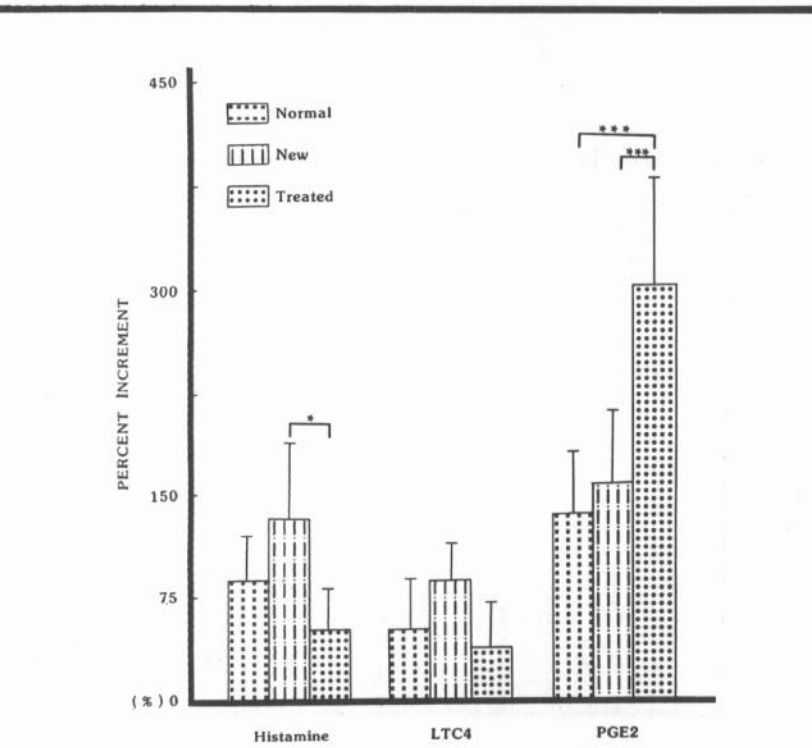

Fig. 4 The percent increment of mediator production by basophils (contaminated in PMNs) after allergen challenge. The method of calculation was detailed in text. $^{*}p < 0.05$, $^{***}p < 0.001$.

new patients to 300.1 ± 72.4 percent in treated patients ($p < 0.001$). No difference in percent increment of LTC4 production among the 3 studied groups was found.

DISCUSSION

Many studies have been conducted to explore the working mechanisms of IT in the past decades. Although it has been well known that basophil/mast cell-derived mediators play a major role in the pathogenesis of allergic disorders, most of those studies have focused on the alteration of immunological status.[12-18] Only a few investigators have studied the effect of immunotherapy on histamine release.[19,20] In this communication, the *in vitro* production of allergic mediators including histamine, LTC4 and PGE2, were studied in newly diagnosed and hyposensitized (> 2 yr) asthmatic children and age-matched controls.

Histamine, the major component of preformed mediators which are stored in the secretory granules of human mast cells and basophils, is released after IgE receptor bridging as well as by stimuli that directly degranulate these cells in the absence of IgE antibody or antigen.[24] Several reports had shown that plasma histamine of allergic patients was higher than that in normals,[25] especially in the symptomatic period.[26,27] Other investigators also reported that the dose response to antigen of untreated patients remained relatively constant and after immunotherapy, the cells became less reactive to antigen.[26] Our results were similar to those reports that plasma histamine and *in vitro* production of histamine, either stimulated with allergen or A23187, decreased after immunotherapy. The diminished production could be caused by a decrease in IgE antibody and the number of receptors for IgE, or by a decrease of preformed mediator within these cells after IT.[20]

The sulphidopeptide leukotrienes LTC4, LTD4 and LTE4, one

of groups of newly formed mediators after activation of mast cells/basophils, comprise the activity previously recognized as the slow-reacting substance of anaphylaxis (SRS-A).[7] The pharmacologic effect of leukotrienes on lung parenchyma are 200- to 1,000-fold more potent on a molar basis than that of histamine, and 30- to 1,000-fold greater in provoking bronchoconstriction in normal subjects.[28] The availability of specific radioimmunoassay has made possible the measurement of leukotrienes in biological fluids.[29] Moreover, those developments in turn lead to identification of the cells and nonspecific stimuli, such as calcium ionophore A23187, that result in the generation of leukotrienes *in vitro*.[30,31] However, there were only few studies demonstrating the production of these mediators *in vivo* or by specific antigen challenge *in vitro*.[32]. The results obtained in this study that leukocytes were able to release LTC4 after allergen and A23187 stimulations *in vitro* were consistent with previous reports.[32-34] The decreased production of LTC4 by A23187-stimulated MNCs ($p < 0.05$) and PMNs ($p < 0.01$) from hyposensitized patients suggests that IT may affect the metabolic pathway or secretion of LTC4 *via* non-specific stimulation. However, as *in vitro* production of LTC4 after allergen stimulation was not different among normals newly diagnosed and hyposensitized patients, the role of LTC4 in allergic disorders may need further investigation.

Lung tissue from all mammalian species so far tested has a propensity for synthesis and release of a wide array of prostaglandins (PGs) in response to immunological, chemical or mechanical stimualtion.[35-37] The possibility that PGs may be involved in the modulation of airways smooth muscle tone was first suggested by the observation that human bronchial smooth muscle contracts in the presence of PGF2 while PGE2 produce relaxation.[38-40] In the present study, there was increased production of PGE2 by PMNs ($p < 0.01$) and MNCs ($p < 0.05$) stimulated with allergen in treated patients as compared to newly diagnosed patients. The results of the present study add further evidence for the important role of PGE2 in maintaining normal bronchial smooth muscle tone,[41] and the effect of IT in restoring the capacity of PGE2 production in newly diagnosed and untreated asthmatic patients.

This study has several drawbacks. The new patients and hyposensitized patients are not the same individuals, and therefore it is not a longitudinal follow-up study. The results of mediator release are expressed as percent increment rather than percent release and therefore the present results may be not easily comparable with other reports. Nevertheless, the demonstration that the abnormal secretory pattern of inflammatory mediators of new patients can be reversed after immunotherapy may be used to partially explain its clinical effectiveness.

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