The Immune Modulatory Effect of Allergen Specific Immunotherapy in Treated Asthmatics

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ABSTRACT

Apoptosis is programmed cell death without induction of an inflammatory response. It is mediated by Fas—a cell surface protein which is expressed on activated lymphocytes. Interaction with its counterpart—the FasL induces the apoptosis of Fas bearing cells. The mechanism underlying successful immunotherapy has not been identified. The aim of the present work was to investigate whether immunotherapy affect Fas expression on T lymphocytes in asthmatic patients and to investigate its potential ability to shift the Th1/Th2 balance of immune response to allergic reaction in asthmatic airway. The study was conducted on 30 asthmatic subjects and 10 healthy control subjects. The asthmatic patients were treated with immunotherapy for more than one year. Blood samples were collected at basal time (before treatment) and one year after therapy (the end of the building up phase). The percentage of positive T cells expressing Fas on its surface was determined using flow cytometric analysis technique. The expression of Fas on asthmatic patients was significantly higher than in control subjects which decreases after immunotherapy but showing no evidence of apoptosis, levels of IgE, IL-4 were decreased significantly after treatment, also, level of IFN-γ was increased significantly. Conclusion: although high percentage of the Fas expressed in studied asthmatic group but with no clear evidence of apoptosis, may be a non concomitant increase in FasL which interfere with the apoptotic process in such asthmatics and might be a contributing factor in asthma pathogenesis. Thus, the lack of parallel increase of FasL to the increased Fas could explain the impaired apoptosis of the T-lymphocytes. It could be concluded that immunotherapy have a role in skewing the cytokine profile in asthma and maintain the balance between Th1/Th2.

INTRODUCTION

Allergen specific immunotherapy (SIT) is nowadays the only causal treatment of IgE-dependent allergic diseases, related to type 1 allergy, such as allergic rhinitis, rhinoconjunctivitis, atopic asthma and sting-induced anaphylaxis. Despite the fact that injection immunotherapy was introduced by L.Noon early in 1911, it still provokes numerous controversies. Conventional SIT is based on subcutaneous injection of gradually increasing doses of allergen vaccine. The optimal period of SIT duration is
not established and the patients with clinical improvement are recommended to continue therapy for 3-5 years\(^\text{(1,2)}\). The purpose of SIT is the decrease or withdrawal of clinical symptoms related to exposure to an allergen or to restrain the natural development of the disease. High efficacy of SIT measured by scale of clinical symptoms and consumption of drugs was observed in subjects sensitive to grass pollen\(^\text{(1,3)}\). No correlation was found so far between clinical improvement after SIT and many parameters of an immune response.

Unacquaintance with the precise mechanism of action of SIT causes the lack of standard parameters of its monitoring and prognosis\(^\text{(1,2,4,5)}\).

It is known that CD4+ T cells have a key role in initiation and regulation of the course of an allergic reaction, also, by an influence on the synthesis of IgE and various cytokines affecting the function of other cells\(^\text{(6,7)}\). Regarding the profiles of produced cytokines CD4+ cells, were divided into subpopulations: Th1 (IL-2, IFN-\(\gamma\)) and Th2 (GM-CSF, IL-4, IL-5, IL-9, IL-10, IL-13). Shifting of balance in synthesis of cytokines IL-4 and IFN-\(\gamma\), acting antagonistically on the synthesis of IgE in favour of IL-4 is observed in atopy.

T lymphocytes through T cell receptor (TCR) have the ability of allergen recognition. Stimulation of TCR dependent on concentration, time, frequency of interactions, functional status of cell, participation of antigen-presenting or costimulating molecules may lead to proliferation or apoptosis\(^\text{(8)}\).

T cells have at least two apoptotic pathways: active death, which is antigen driven, and passive death, which occurs at the end of an immune response and is due to lymphokine withdrawal or other mechanisms\(^\text{(9)}\). These two forms of death are molecularly distinct because the cell surface molecule Fas/Apo-1(CD95), a 45-kDa protein belonging to the tumor necrosis factor (TNF) receptor family\(^\text{(10)}\), and TNFs themselves are major participants in active, but not passive death. The cross-linking of Fas receptor with its recently identified ligand (Fas ligand) leads to the induction of apoptosis in lymphocytes\(^\text{(11)}\) and provides a mechanism for the removal of antigen–activated T cells\(^\text{(12, 13)}\). This leads to resolution of the inflammatory response, protecting the host against the detrimental effects that would ensue if cell disintegration or necrosis were to occur. Spontaneous remission of respiratory allergic disease invariably following withdrawal of allergen\(^\text{(14)}\) underlies the mechanism passive death. So, apoptosis is a normal and physiologic process that regulates the safe and efficient removal of functionally exhausted inflammatory cells\(^\text{(15)}\).

Spinozzi\(^\text{(16)}\) showed that Fas mRNA and its surface receptor (CD95 /Apo-1) are defectively expressed on the pulmonary T lymphocytes of patients with allergic asthma, a condition that impairs in vivo activation-induced programmed cell death that defect is not inherited. They show the presence of Fas receptor on the surface of peripheral blood T-lymphocytes from patients with asthma and the upregulation of Fas
receptor expression on pulmonary T cells after in vitro stimulation with anti-CD3 and interleukin-2. On the contrary, incubation with IL-4 down modulated surface Fas expression, indicating that the local presence of large amounts of that cytokine may explain the observed in vivo findings.

Pulmonary allergen–specific T lymphocytes have the characteristics of chronically activated T cells, but, lacking the Fas receptor on their surface, they are resistant to in vitro, perhaps in vivo, delivered apoptotic signals. In fact pulmonary T lymphocytes are phenotypically and functionally different from those circulating in the blood stream. It is therefore possible that an environmental, factor acting at the level of the mucosal surface, such as inhaled allergens, air pollutants, common respiratory viruses, and a specific cytokine could negatively influence the ability of mucosal T-lymphocytes to express Fas mRNA and Fas receptor on their surfaces after in vivo allergen activation. Hypoexpression of Fas mRNA and Fas receptor by T cell, with subsequent impairment of active death in patients with allergic asthma, may be the molecular basis for the development and persistence of inflammatory infiltrate in the mucosa of the respiratory tract. However, from a clinical point of view, remission of disease depends on allergen avoidance or the administration of drugs capable of inducing the death of lymphocytes and eosinophils. Further therapeutic strategies should consider the molecular characteristics of pulmonary T lymphocytes and their role in the pathogenesis of bronchial asthma.

The aim of the present work was to investigate whether immunotherapy affect Fas expression on T lymphocytes in asthmatic patients and to investigate its potential ability to shift the Th1/Th2 balance of immune response to allergic reaction in asthmatic airway.

SUBJECTS & METHODS

The study was performed on 30 asthmatic patients, their ages ranged from 5-60, with a mean age of 29.3±15.67 years (10 males, 20 females) & 10 control subjects. Venous blood sample was collected into a test tube containing an anticoagulant (3 ml of blood was collected on ethylene diamine tetraacetic acid EDTA) for assessment of apoptosis of lymphocytes by flowcytometry, also, 2 ml of venous blood sample was taken into plain tube left to clot & serum stored at -20°C for determination of cytokines before and after treatment with immunotherapy over a period of one year.

Determination of CD95: (According to the protocol provided by B.D)
Direct labeling of lymphocytes by purified monoclonal mouse antibody conjugated fluorescene isothiocyanate isomer.

The antibody is intended for use in flowcytometry to demonstrate the APO-1/Fas antigen which is a 40-50 kDa cell membrane protein functioning as a mediator of apoptosis.

About 100 μl of blood and 10 μl of FITC conjugated-monoclonal
mouse Anti-Human CD95, Fas-clone Dx2 were vortexed and incubated in dark at room temperature for 30 minutes, Diluted using lysing solution. Immediately after incubation, tubes were centrifuged at 300xg for 5 minutes at room temperature, then washed using phosphate buffered saline (PBS), diluted using sheath fluid then analyzed by flowcytometry. **Determination of serum immunoglobulin E**: This was done by using N Latex IgE mono determination kit supplied by Dade Behring Marburg GmbH (Germany) According to the method described by kapmeyer (1987)

\[(22)\]

polystyrene particles coated with specific antibodies to human IgE are agglutinated when mixed with samples containing human IgE. The intensity of the scattered light, measured using the Nephelometer, depends on the concentration of IgE in the sample.

**Determination of Interleukin 4 (IL-4) in serum**: IL-4 was measured using monoclonal 'sandwich' enzyme immunoassay (EIA) kit supplied by CytImmune Science, Inc.’s CytElisa TM kits. IL4 was measured with a sensitivity of 0.195 ng/ml in a standard range of 0-200ng/ml. Mouse monoclonal antibodies generated against human IFN-γ are used to capture human IFN-γ in a sample. Simultaneously, rabbit anti-human IFN-γ polyclonal antibodies detect IFN-γ in the sample. The assay is visualized using goat anti-rabbit alkaline phosphatase conjugate and an ensuing chromogenic substrate reaction. The amount of IFN-γ detected in each samples is compared to an IFN-γ standard curve which demonstrates a direct relationship between Optical Density (O.D.) and cytokine concentration: i.e. the higher the O.D. the higher the cytokine concentration in the sample.

**Statistical analysis**

"Paired t" test for comparison of means of the same group of patient's follow up (before and after treatment).

- Correlation studies using "Pearson 2-tailed" correlation in which at the 0.01 level correlations is highly significant while at 0.05 levels, it is significant

- Correlation plots using regression linear equation.

- Data were graphically represented using "spss" analysis program version 12 and "Microsoft Excel xp" statistical program.
RESULTS

Asthmatic patients before treatment show significantly higher expression of CD95 on T lymphocytes than in control with respective mean values of 29.71±9.27, 14.65±3.63 (p <0.001) as shown in Fig.1. after treatment the immunotherapy show deceased level of expression of CD95 on T lymphocytes surface with mean value of 27.94±8.19 which is still significantly higher than in control but CD95 lymph before and after treatment show non statistical significance difference as t=0.852 and p=0.401 (>0.05).

![Mean values of CD95 lymph before and after therapy compared with control group](image1)

Fig.1: The comparison between mean values of CD95 lymph before and after treatment show non significance difference as t=0.852 & p=0.401 (>0.05).

![Correlation study between CD95 and duration of treatment in asthmatic patients](image2)

Fig.2: Correlation study between CD95 and duration of treatment in asthmatic patients

As shown in figure 2, there is highly significant negative correlation between CD95 and duration of treatment in asthmatic group as r=-0.852, p= 0.0001 (<0.001)
In Fig.3, Asthmatic patients before treatment showed significantly higher mean levels of IgE than in control with respective mean values of 534.57±304.98, 97.4±21.7 (p <0.001). After treatment the immunotherapy showed deceased level of IgE with mean values of 369.33±299.71 which is still significantly higher than in control and in asthmatics before treatment (p<0.001).

Fig.3: The comparison between mean values of IgE, before and after treatment show highly significant difference (p <0.001)

![Image](image1.png)

Fig.4: correlation between IgE and duration of treatment in asthmatic patients.

As shown in figure 4, there is highly significant negative correlation between IgE and duration of treatment in asthmatic group r=-0.689, (p<0.001).

In Fig.5, Asthmatic patients before treatment showed significantly higher mean levels of IL-4 than in control with respective mean values of 14.56±6.74, 0.87±0.24 showing highly statistical significant difference as p=0.0001 (<0.001). After treatment the immunotherapy show deceased level of IL-4 with mean values of
11.53±5.0 which is still significantly higher than in control also there is high statistically significant deference in IL-4 level before and after treatment as (p<0.001)

Fig. 5: The comparison between mean values of IL-4 before and after treatment show highly significant difference as t= 5.288 and (p<0.001)

As shown in figure 6, there is highly significant negative correlation between IL-4 and duration of treatment in asthmatic group as r=-0.739, (p <0.001). Comparing the levels of IFN-γ before and after treatment in asthmatic patients Fig.7, it was observed that increased levels with respective mean values of 184.33±158.89, 466.37±340.87 showing highly statistical significant difference as (p<0.001). Comparing the levels of IFN-γ before and after treatment with control subjects showed that there was highly significant difference with t=−6.635 and (p<0.001)
Mean values of IFN-gamma before and after treatment compared with control group

![Graph showing mean values of IFN-gamma before and after treatment compared with control group.](image)

Fig. 7: The comparison between mean values of IFN-γ before and after treatment show highly significant difference (p<0.001)

Correlation between duration of treatment and IFN in asthmatic patients

![Graph showing correlation between duration of treatment and IFN-γ in asthmatic patients.](image)

Fig. 8: Correlation between IFN-γ and duration of treatment in asthmatic group

As shown in figure 8, there is highly significant positive correlation between IFN-γ and duration of treatment in asthmatic group as \( r = -0.894 \), (p<0.001).

DISCUSSION

T-lymphocytes as one of the principal immunoregulatory cells and inflammatory effector cells play an important role in orchestrating the allergic mucosal immune response\(^{23}\). Delayed elimination of activated T cells through apoptosis at the inflammatory site might impair the resolution of the inflammatory process and contributes to the persistence of the clinical symptoms\(^{24}\).

Study of apoptosis in the peripheral blood lymphocytes of the asthmatic patients pre and after immunotherapy was performed in the current study using flow cytometry the present results showed lymphocyte apoptosis in peripheral blood of asthmatic and control groups. The percentage of T lymphocytes expressing Fas on its surface is higher
in asthmatic patients than in treated asthmatics which is higher than in control. There was highly significant difference between the asthmatic and control, also, between treated asthmatic and control subjects but no significant difference before and after immunotherapy in the asthmatic group.

Druilhe et al (25) found that the expression of Fas was significantly increased in the epithelium of untreated asthmatics, possibly as a consequence of its activation secondary to the ongoing inflammatory process. The intensity of Fas expression, however, failed to correlate with apoptotic death, suggesting that the number of apoptotic cells in the epithelium might be underestimated because of their detachment in the bronchial lumen, or that Fas antigen might not be functional. A positive correlation between Fas and proliferating cell nuclear antigen (PCNA) expression was noted, indicating that Fas activation might be involved in epithelium proliferation rather than in apoptotic death. This paradoxical hypothesis is reminiscent of a recent report showing that Fas triggering resulted in human fibroblast proliferation (26).

The possibility of accumulation and thus delayed T lymphocytes apoptosis in asthmatics although the percentage of the T- lymphocytes expressing Fas is higher in asthmatic than in control (suggesting defect in apoptosis mechanism via Fas pathway) could be explained as follow:

1. The affinities or avidities of various agonists for Fas may determine their spectrum of target cells. Recently, Fadeel et al. (27) reported that various anti-Fas mAbs that recognize the same epitope exhibit different effects on Fas-mediated apoptosis, and that those mAbs with a moderate affinity killed Fas-expressing cells, while high affinity Abs were antagonistic and low affinity Abs showed no biological effect.

2. Guchouico et al. (28) found a marked reduction in airway epithelial cell FasL mRNA and protein expression during allergen-induced airway inflammation. The submucosal immune cell accumulation occurred despite FasL expression by some of the mononuclear cells located within the inflammatory aggregates, thus the down regulation of expression of airway epithelial Fas-L may be a contributing factor to the evolution of persistent airway inflammation.

3. Functional activity of FasL, and presence of RNA for Fas and FasL suggest that although rapid AICD in Th1 cells is mediated by the Fas/FasL pathway, the resistance of Th2 cells cannot simply be explained by the absence or low levels of Fas or FasL, and that some additional intrinsic differences is responsible for their failure to commit suicide. Since FAP-1 has been implicated in blocking the Fas/FasL mediated death pathway, the observed difference in FAP-1, expression by Th2 cells is an obvious candidate for a difference which might be partly or wholly responsible for the unequal death of the Th1 and Th2 effector population (29).
Recent studies have shown that there are very few activated apoptotic T-lymphocytes in the bronchial mucosa of asthmatic patients\textsuperscript{(29)} and that mitogen stimulated peripheral blood T-cells of asthmatic patients express Fas on their surface but do not undergo apoptosis after its ligation\textsuperscript{(30)}. De Rose et al.\textsuperscript{(31)}, found that Fas–agonistic mAb did not inhibit allergen induced T-cell proliferation. Together, these results suggest that the resistance of activated T cells from asthmatic patients to Fas–induced apoptosis reflects impaired transduction of the Fas signal and that IFN-$\gamma$ restores the Fas/FasL apoptotic pathway, up-regulating Fas and FasL on the T cell surface.

Wang et al.\textsuperscript{(32)}, examined the influence of house dust immunotherapy on in vitro IL-4 and IFN-$\gamma$ production by peripheral blood mononuclear cells in house dust sensitive asthmatic patients. Allergen immunotherapy in house dust sensitive asthmatic patients can significantly decrease IL-4 production from peripheral mononuclear cells. The production levels of IL-4 in patients without treatment had higher levels than those in patients with hyposensitization. Such therapy, also, have some effect on promotion of IFN-$\gamma$ production in asthmatic patients. Wang\textsuperscript{(32)} concluded that immunotherapy with house dust may have the potential ability to shift the Th1/Th2 balance of immune response to allergic reaction in the asthmatic airway. The current results are in harmony with those of Wang\textsuperscript{(32)}. Thus, during clinically effective immunotherapy a shift in cytokine dominance from IL-4, IL-5 predominant has been observed at high allergen concentration.

Rebordae, et al.\textsuperscript{(33)}, evaluated the effect of one year immunotherapy on cytokines profiles T1 and T2 of peripheral blood lymphocytes in atopic patients, they concluded that expression of IL4 & IL5 in T cells, characteristically increase in atopic patients, was significantly lower in the immunotherapy group and similar in the non atopic control group. The levels of IFN-$\gamma$ did not differ between the studied groups but the ratio IFN-$\gamma$/IL-4 produced by CD4+ T lymphocytes increased significantly in the patients receiving immunotherapy. The current results show a highly significant decrease in IL-4 in asthmatic patients after treatment with immune therapy which is significantly correlated to the duration of treatment. Also there was a highly significant increase in the levels of IFN-$\gamma$ which also correlated with the duration of the immunotherapy. The ratio of IFN-$\gamma$/IL-4 was significantly higher after treatment, thus reveling Th1 response to immunotherapy.

IFN-$\gamma$ assay is a useful and sensitive marker to monitor allergen-specific immunotherapy efficacy the measurement of this cytokine at basal time (before treatment) and one year after therapy (the end of the building up phase) is significant, also other parameters are significant. It could be concluded that immunotherapy have a role in skewing the cytokine profile in asthma and maintain the balance between Th1/Th2 cytokines, thus Allergen specific immunotherapy has proven to be clinically effective in the treatment of asthmatic patients.
REFERENCES


تأثير الأمصال الخاصة بالعلاج المناعي في تدفق المناعة في الحالات المصابة بالربو الشعبي

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أجريت هذه الدراسة في محاولة لإقياس بعض العوامل التي تؤثر على علاج المرضى في مركز مرضي مرضي. ويتم تدفق المناعة في الحالات المصابة بالربو الشعبي. مثلاً، فإن مرضي مرضي يمكن تطبيقه على حالات مرضي. لذلك، فإن هذه الدراسة تم اختيار 30 حالة مرضي بناءً على التاريخ المرضي و الفحص الطبي. ونسبة المرضى تراوحت أعمارهم بين 20-60 عامًا. و 10 أشخاص تراوحت أعمارهم بين 40-48 عامًا.

لمحفر هذا البحوث أجرى البحث بعد أخذ التاريخ المرضي الكامل والفحص الكنسي عليه كما يلي:

١. صورة مكملة

٢. عينة دم أخذت في بيوتية اختبار على مادة حافظة للتحلز لدراسةها باستخدام جهاز التدفق الخفيف

٣. قياس إنترلوتين ٤ في الدم

٤. قياس إنترلوتين ٥ في الدم

٥. قياس الإنترلوتين ٦

و قد أظهرت نتائج هذا البحث و ارتفاع ملحوظ في نسبة مستقبلات الفاس في حالات المرضي الشعبي مفرغة بجمعية الأسماك و قد أعربت هذه الزيادة إلى محاولة الجراح المناعي من اتخاذ أجراءاته الدوائية و حالات مرضي الفاس على أنها من أجل تحسينها للدخول في عملية العلاج الخفيف و لكن قد يفسر الفحص في عملية العلاج الخفيف إلى نفس نسبة مزدوجة من الفاس للية، و بلاحظ أن نسبة الفاس تقل بعد العلاج المناعي في حالات مرضي.

كما ظهر ارتفاع ملحوظ في كمية الإنترفيرون ٤ في مرضى الربو الشعبي بالمباركة في عينة المرضي. و قد اقتلت هذه النسبة بشكل واضح بعد العلاج المناعي مما جعل الربو المناعي يلعب دورًا مهمًا و مركزياً.

في نهاية مسقبلات الالتهاب في مرضى الربو الشعبي، و كا لوح ارتفاع ملحوظ في كمية الإنترفيرون ٤ في الدم لمرضي الربو الشعبي بالمقارنة بمجموعة الأصحاء و قد أختفت هذه النسبة بشكل واضح و ملحوظ عند العلاج المناعي.

كما تم دراسة الإنترفيرون ٤ في الدم لمرضي الربو الشعبي، و كذلك تم دراسة الألفايبرين ٤ في الدم لمرضي الربو الشعبي و جدارية بما أن أرتفعت بشكل ملحوظ عند العلاج المناعي، و بهذا يمكن أن نستثمر أن العلاج المناعي يعمل من خلال تدخل استجابة الحالات المناعية لمواد المناعية للحساسية و كذلك تحويل الألما الحساسية للحالات المناعية، و ٤ إلزامات الإنترفيرون ٤ و ٤ حساسية خفض على الحساسية المناعية و تقليل الإنترفيرون ٤ و ٤ ينجم عنه من إزالة الإنترفيرون ٤، و بذلك يلعب العلاج المناعي دورًا هاماً في إنهاء مسقبلات الالتهاب في مرضى الربو الشعبي.