IMPROVEMENT OF CLINICAL RESPONSE IN ALLERGIC RHINITIS PATIENTS TREATED WITH AN ORAL IMMUNOSTIMULATING BACTERIAL LYSATE: *IN VIVO* IMMUNOLOGICAL EFFECTS

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Allergic rhinitis is known to be one of the most common chronic diseases in the industrialized world. According to the concept that allergic rhinitis patients generally suffer from an immune deficit, in order to stimulate specifically or aspecifically their immune system, immunomodulating agents from various sources, such as synthetic compounds, tissue extracts or a mixture of bacterial extracts, have been used. The aim of the present trial is to evaluate the efficacy of the treatment with an immunostimulating vaccine consisting of a polyvalent mechanical bacterial lysate (PMBL) in the prophylaxis of allergic rhinitis and subsequently to analyze its *in vivo* effects on immune responses. 41 allergic rhinitis patients were enrolled: 26 patients were randomly assigned to the group for PMBL sublingual treatment and 15 others to the group for placebo treatment. For all 26 patients blood samples were drawn just before (T_0) and after 3 months of PMBL treatment (T_3) to evaluate plasma IgE levels (total and allergen-specific) and the cytokine production involved in the allergic response (IL-4, IFN-γ). The results of our study indicate that PMBL is effective *in vivo* in the reduction or in the elimination of the symptoms in rhinitis subjects during the treatment period in comparison to a non-immunostimulating treatment. A significant and clinically relevant improvement was found in 61.5%, a stationary clinical response was registered in 38.4% and no negative side effects associated with the medication or worsening were recorded. At the end of a 3-month follow up period the clinical picture remained the same as that observed at T_3. PMBL treatment did not affect the serum IgE levels (either total or allergen-specific) and did not induce significant changes in IFN-γ concentration. In contrast, PMBL therapy may be accompanied, in some patients, by a potential immunomodulating activity by decreasing IL-4 cytokine expression.

In the last 20 years allergies have increased in countries with a relatively high standard of living (1). Residency in urban or industrial areas, changes in lifestyle and the falling incidence of microbial infections may be responsible for this increase to some extent. Prevalence estimates suggest, for example, that seasonal allergic rhinitis occurs in 10% and perennial allergic rhinitis in 10-20% of individuals living in industrialized countries, and for children the prevalence rates may be as high as 40% (2). Poorly controlled allergic rhinitis is also a major risk factor for sinus infections and may contribute to the development of asthma and to exacerbations in those subjects who already have

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the disease (3). Therefore, to slow down, or better to stop this trend, the prophylaxis of allergies is an important goal in clinical practice and in health cost control. Various strategies have been studied and tested in these subjects. In particular, according to the concept that generally these patients suffer from an immune deficit, in order to stimulate specifically or aspecifically their immune system, immunomodulating agents from various sources, such as synthetic compounds, tissue extracts or a mixture of bacterial extracts, have been used (3-5). Among the last preparations, a series of results show that the administration of a lysate obtained by chemical lysis of different bacterial strains can modulate cytokine synthesis and mast-cell degranulation or enhance the expression of adhesion molecules on normal human blood monocytes and granulocytes (5-7).

The aim of the present trial is to evaluate the efficacy of the treatment with an immunostimulating vaccine consisting of a polyvalent mechanical bacterial lysate (PMBL) in the prophylaxis of allergic rhinitis and subsequently to analyze its *in vivo* effects on immune responses.

MATERIALS AND METHODS

Bacterial lysate preparation

The immunostimulating vaccine [ISMIGEN – PMBL supplied by Zambon Italia SRL (Bresso/Milan, Italy)] is the product of mechanical lysis, through sonication, of 8 different bacterial strains (6 x 10^9 bacteria for each strain) selected among those most frequently responsible for respiratory tract infection, i.e. *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus viridans*, *Streptococcus pneumoniae* (6 different serotypes: TY1/EQ11, TY2/EQ22, TY3/EQ14, TY5/EQ15, TY8/EQ23, TY47/EQ24), *Klebsiella pneumoniae*, *Klebsiella ozaenae*, *Haemophylus influenzae* serotype B, *Moraxella catharralis*. The product is formulated in tablets containing 7 mg of freeze-dried lysate of 48 billion bacteria, and 43 mg of excipients (4). The placebo tablets were indistinguishable for taste and appearance from PMBL tablets.

Patients

From July 2004 to July 2005, volunteers with anamnesis of allergic rhinitis were recruited at the Physiopathology Department of the University of Turin, I ENT Division, Turin, Italy. Informed written consent was obtained for epicutaneous allergy testing with a panel of common seasonal and perennial inhalation allergens and, if the subject was enrolled in the study, for 2 blood samples.

All the patients were probed with epicutaneous allergy testing and were enrolled in the study only if the skin test was positive for at least one of the following allergens: mites, cats, dogs, mixed molds, grass or trees. The patients were enrolled according to the following inclusion/exclusion criteria. Inclusion criteria: vasomotor rhinitis, positive skin test result. Exclusion criteria: history of previous anti-allergy vaccination, immunomodulatory treatments, anti-allergic treatments, like anti-histamine drugs, anti-influenza or hepatitis B vaccination within 6 weeks before the enrolment, immunodeficiency, fever diseases.

41 eligible patients, 18 females (43.9 %) and 23 males (56.1 %), aged 5-78 years (mean age 29.3) were enrolled. After a clinical examination, the most common symptoms and signs reported from the population examined were: nasal blockage with rhinorrea, ocular symptoms, asthmatic symptoms, nasal or palate itching, headache. Each symptom was evaluated according to a 0-3 grading scale (0-absence of symptoms, 1-light symptoms, 2-moderate symptoms, 3-severe symptoms) and recorded (8).

Study design

Twenty-six patients [11 females (42.3%) and 15 males (57.7%) aged 7-76 years (mean age 28.9)] were randomly assigned to the group for PMBL treatment and fifteen others randomly to the group for placebo treatment [7 females (46.7%) and 8 males (53.3%), aged 5-78 years, (mean age 29.7)] (Table I). All 26 patients were treated by sublingual route with one tablet of immunostimulating lysate every morning for 10 days of each month of the 3 month period. The placebo group received placebo treatment using the previously described protocol.

For all patients, blood samples were drawn just before (T_0) and after (T_3) the treatment to evaluate plasma IgE levels (total and allergen-specific) and the cytokine production involved in the allergic response (IL-4, IFN-γ).

The clinical assessment of the treatment efficacy was made on a range of commonly accepted subjective and objective symptoms determined before, during and after treatment. At the last visit, the patients were asked some questions about any eventual subjective improvement in their allergic symptoms. All the volunteers were required not to take any allergy medicines during the entire study. At the end of the treatment period the patients of both groups were followed-up for three months without any immunostimulating treatment.

Isolation of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Hypaque (Pharmacia Fine Chemicals,

Uppsala, Sweden) gradient centrifugation from peripheral venous blood collected in sterile evacuated blood collecting tubes containing EDTA (0.47 mol/L). Monocytes were separated from other mononuclear cells by centrifugation on a discontinuous (46%) gradient of iso-osmotic (285 mOsmol) percoll (Pharmacia) and adhesion to plastic plates in RPMI 1640 (GIBCO, Grand Island, NY, USA) containing 100 UI/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 10% heat-inactivated fetal calf serum (FCS, Invitrogen Corporation, Milan, Italy). The cells were counted in a Bürker Chamber (Lancefield, Germany); the viability of PBMCs, assessed by trypan blue exclusion, was greater than 95%. PBMCs (1×10^6 cells/ml) were stimulated with the addition of 10 ng/ml phorbol 12-miyristate 13-acetate (PMA) and 500 ng/ml Ca-ionophore (Sigma Aldrich, Milan, Italy) and incubated for 18 h at 37°C in 5% CO_2 incubator (9).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted with an acid guanidinium thiocyanate-phenol-chloroform mixture using RNAzol (Amplimedical, Turin, Italy), according to the manufacturer's instructions. The cDNA synthesis was performed by reverse transcription in a mixture reaction containing 1 μg of total RNA, oligo (dT) primers, RT buffer, dNTPs, 4 U/μl RNAse-stop and 40 U/μl of MMLV-reverse transcriptase (Amplimedical). The reaction mixture was incubated at 37°C for 90 min and stopped at 95°C for 5 min. 5 μl aliquots of cDNA were amplified in a 50 μl reaction volume containing PCR buffer, dNTPs, primer and 0.2 U/μl of DNA Polymerase. The mixture was capped with 50 μl of sterile mineral oil (Amplimedical). To ensure that equivalent amounts of cDNA were used in each reaction, PCR was also performed for abelson gene from each sample, and the cDNA was adjusted to equivalent levels. PCR for IL-4 was performed using a thermal cycler (MJ research, Waltham, USA) with the following setting: denaturation at 94°C for 2 min, annealing at 55°C for 1 min and extension at 72°C for 1 min. Amplification of IFN-γ mRNA was performed with an annealing step at 60°C for 1 min and PCR for abelson gene with an annealing step at 65°C for 1 min. A sample (20 μl) of each PCR reaction was electrophoresed through a 4% agarose gel and visualized with ethidium bromide. Gels were photographed under UV light.

Quantitation of cytokine production (ELISA)

Cell culture supernatants were assayed for cytokines by ELISA according to the manufacturer's instructions. ELISA kits for hIFN-γ and hIL-4 were purchased from Amersham Biosciences Europe GmbH (Milan, Italy). According to the High Sensitivity (h)IL-4 ELISA System (code RPN2783) the minimum detectable dose of (h)IL-4 was determined to be 0.5 pg/ml.

Serum detection of immunoglobulin E (total and specific)

Sera were analyzed for total and specific IgE levels using the UniCAP Total IgE fluoroenzyme immunoassay and UniCAP Specific IgE fluoroenzyme immunoassay (Pharmacia). Anti-IgE or the allergen of interest, covalently coupled to ImmunoCAP, reacts with the total IgE or specific IgE in the patient serum specimen. After washing, enzyme-labelled antibodies against IgE were added to form a complex. After incubation, unbound enzyme-anti-IgE (total or specific) was washed away and the bound complex incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate was measured. The fluorescence is directly proportional to the concentration of IgE in the serum sample. To evaluate the test results, the responses for the patient samples were transformed to concentrations with the use of a calibration curve. Total IgE levels 0-100 kU/l were considered within the normal range.

Statistical analysis

The results were analyzed using descriptive statistics (mean values and standard deviations) and tested by unpaired *T*-Student test.

RESULTS

All 26 subjects enrolled in the study reported an anamnesis of allergic rhinitis and demonstrated positive skin test for common seasonal and perennial inhalation allergens. The patients characteristics are reported in Table I. Before PMBL therapy there was a 1-month period of observation for all patients, to assess the clinical picture. Symptoms at entry (T_0) are shown in Table II.

A clinical considerable improvement (Fig. 1) was observed in 16 of the 26 PMBL-treated patients (61.5 %). These results were evaluated by means of the 0-3 grading scale for the symptoms scores: especially in 14 patients (53.8%) we noticed a relevant decrease in nasal blockage and rhinorrea; ocular symptoms in 13 cases (50%) almost disappeared, and 10 patients (38.4%) had a large improvement in asthmatic symptoms. Improvement was not immediately observed but started almost 2-3 weeks or later after the beginning of treatment for all symptoms. A stationary clinical response was registered in 10 of 26 patients (38.4%). In no case negative side effects associated with the medication or worsening were recorded (Fig. 1). At the end of a 3-month follow up period, the clinical variables only were checked. The

clinical picture remained the same as that observed at T_3. In the placebo-treated group (Fig. 1), the symptoms improved in 1 of 15 patients (6.6%), were unmodified in 6 (40%) and worsened in 8 (53.4%).

The serum IgE levels (both total and allergen-specific) were not affected by PMBL treatment. In particular, before therapy (T_0) in 15 of 26 patients (57.7%) the total serum IgE levels were normal, whereas in 11 of 26 patients (42.3%) the levels were high (> 100 kU/l). After 3 months of therapy (T_3) negligible variations were detected (Table III).

PCR evaluation shows that at time T_0, 18 of 26 patients expressed IFN-γ and no IL-4, 4/26 expressed no cytokines, and 4/26 expressed both. After therapy (T_3) all subjects expressed IFN-γ and no IL-4 (Table IV). A comparison of the supernatant cytokine values recorded by ELISA assays before and after PMBL therapy showed no significant changes in IFN-γ concentration (Fig. 2) with values varying from 44.38 ± 6.7 pg/ml at T_0 to 29.30 ± 3 pg/ml after treatment (T_3), (P=0.12). At T_0 IL-4 concentration in PBMC supernatants, from patients positive for IL-4 mRNA expression by PCR, reached 1.78 ± 0.2 pg/ml; after T_3 treatment the IL-4 values decreased to 0.75 ± 0.2 pg/ml (P<0.05; Fig. 3). A negligible variation in the humoral values was observed in the placebo treated patients (data not shown).

DISCUSSION

PMBL is an immunostimulating vaccine containing a lyophilized extract of 8 different bacterial strains (6 x 10^9 bacteria for each strain) selected among those most frequently implicated in acute and chronic respiratory tract infections (RTIs). The beneficial therapeutic effect of PMBL for the treatment of the RTIs has been recently reported (4-5). PMBL exerts its immunomodulating properties by activating either different subsets of effector cells, i.e. B lymphocytes, CD4+, CD8+ T lymphocytes, as demonstrated by its capability of inducing in these cells the expression of the receptor for interleukin 2 (IL2-R), or modulating the production of regulatory cytokines. A marked increase in serum concentration of IgG, IgA and IgM induced by this vaccine has even been documented (4, 10). Furthermore, the therapeutic efficacy of PMBL was found to be superior to that of other bacterial extracts prepared by bacterial chemical lysis, since the mechanical lysis seems more suitable to preserve the surface antigenic structure of the bacteria (5). In addition, concerning the PMBL sublingual route of administration, it is known that in immunotherapy it is considered a route of administration particularly efficient for evoking a strong immune response (11).

The results of our study indicate that PMBL is even effective in the treatment of allergic rhinitis, known to be one of the most common chronic diseases in the industrialized world related to an immune defect (2). In fact, PMBL is effective in some subjects, both in the reduction and in the elimination of the symptoms during the treatment period in comparison to a non-immunostimulating treatment. Of the 26 PMBL-treated patients a significant and clinically relevant improvement was found in 16 (61.5 %), a stationary clinical response was registered in 10 (38.4%) and no negative side effects associated with the medication or worsening were recorded (Fig. 1). The clinical results of the study were evaluated both with subjective and objective methods: firstly, patients were asked to answer

Table I. *Allergic rhinitis patient characteristics.*

	PMBL group*	Placebo group
Number of patients	26	15
Mean age	28.9	29.7
Age range	7-76	5-78
Gender (M/F)	15/11	8/7

** PMBL: polyvalent mechanical bacterial lysate*

Table II. *Allergic rhinitis patient symptoms before PMBL or placebo treatment (T_0).*

	PMBL group*	Placebo group
Nasal Blockage	23/26 (88.46%)	12/15 (80%)
Rhinorrea	22/26 (84.62%)	10/15 (66.7%)
Ocular Symptoms	21/26 (80.77%)	11/15 (73.3%)
Asthmatic Symptoms	11/26 (42.31%)	6/15 (40%)
Nasal/Palate Itching	7/26 (26.92%)	3/15 (20%)
Headache	4/26 (15.38%)	1/15 (6.67%)

* *PMBL: polyvalent mechanical bacterial lysate*

standard questions about their allergic symptoms and their variation after the cure; then an ENT evaluation was conducted, by means of flexible fibroendoscopy and anterior rhinomanometry. In particular, we found an improvement in 14 patients (53.8%), considering the symptoms of nasal blockage and rhinorrea; in 13 (50%) the ocular symptoms decreased; in 10 cases (38.4%) the asthmatic symptoms improved relevantly (Fig. 1). These beneficial effects of PMBL therapy persisted in the follow-up period. The clinical data recorded during PMBL treatment probably correlate with its efficacy as both an immunostimolant agent and a vaccine capable of directly activating effector cells, hence inducing an innate protective immune response. On the other hand, in the placebo-treated group, the symptoms improved in only 1 of 15 patients (6.6%), were unmodified in 6 (40%) and even worsened in 8 (53.4%) (Fig. 1).

57.7% of our randomly selected patients with the clinical diagnosis of allergic rhinitis may be termed as non-IgE associated forms of allergic rhinitis patients since they had normal total and allergen-specific IgE levels, confirming what was noted by other authors in allergic atopic patients (12). In contrast, 42.3% of the subjects showed higher IgE levels (Table III): however, 3 months (T_3) PMBL therapy resulted in negligible variations in all 26 patients (Table III) underlying no influence upon IgE serum levels.

It is well known that mucosal tissues are defended by a local secretory immune and mucosal immune system, that may act in concert with the systemic immune system (13-15). The secretory immune system provides an integrated network linking mucosal sites and regulating trafficking of mucosally activated lymphocytes from induction to effector sites (13). T cells play a critical role in controlling immune responses and they are involved in the inflammatory processes characteristic of allergic diseases. Based on their cytokine production profile helper, T cells (T_h) are generally distinguished into two distinct subsets: T_h1 cells producing IL-2 and IFN-γ and T_h2 cells producing IL-4, IL-5, IL-6, IL-10, IL-13 (16-17). Several studies investigated the cytokine mRNA expression and protein production profiles (T_h1 versus T_h2) of T cell clones and of peripheral mononuclear cells of allergic patients (10, 16-19). These studies showed that allergic diseases are often associated with an increased expression of T_h2-like cytokine responses. IL-4 is essential for isotype switching toward IgE, while IL-5 together with IL-4 switches antibody response toward IgA (16-17). On the other hand, T_h1 cells have a down-regulatory role in the production of T_h2 cytokines by releasing IFN-γ. However, these studies showed conflicting conclusions. IFN-γ m-RNA expression and production were reported to be reduced in atopic subjects, whereas in others it was normal or elevated, compared to healthy controls. IL-4 production was usually, but not always, reported to be elevated (17, 19-20).

The possibility of achieving protection at mucosal surfaces is now obtained by means of new

Table III. *Total serum IgE levels (kU/l*) in 26 PMBL-treated allergic rhinitis patients recorded before (T_0) and after (T_3) treatment.*

PMBL group**	IgE (T_0)	IgE (T_3)	PMBL group**	IgE (T_0)	IgE (T_3)
1	118	125	**14**	136	143
2	35.7	38.9	**15**	31.5	42
3	154	144.3	**16**	76.1	63.5
4	162	159.9	**17**	45.6	38
5	44.8	40.5	**18**	9.2	10.4
6	44.2	51.5	**19**	117	132
7	9.74	12.5	**20**	16	12.1
8	310	298	**21**	198	202
9	324	315	**22**	23.4	31
10	42.8	41.1	**23**	35	42.3
11	9.96	10.5	**24**	166	144
12	34.3	31.8	**25**	117	112
13	50.1	55	**26**	113	115

* *Normal value ≤ 100 kU/l*
** *PMBL: polyvalent mechanical bacterial lysate*

therapeutic strategies (synthetic compounds, tissue extracts or a mixture of bacterial extracts). Preparations comprising bacterial lysates have in fact shown to provide humoral and cellular responses (13-14). Recent studies (17, 21) have shown that cytokine production is modified during anti-allergy treatments (histamine antagonist, Chinese herbs) and by immunotherapy; however the effect of therapy on cytokine production has been the subject of much debate and is still unclear. H2 antagonists have been shown to induce their effects by enhancing the amount of IFN-γ and by reducing IL-4 (17). Decrease in IL-4 serum level following immunotherapy with no significative increase in IFN-γ was reported (22). Conversely, MacDonald et al., studying cytokine release in patients on ragweed immunotherapy, showed a significant reduction in the production of IFN-γ, but no significant changes in IL-4 (23). Similar results were observed in patients with perennial allergic rhinitis after treatment with a mixed formula of Chinese herbs (21). In addition, Tanaka et al. (24) showed that immunotherapy suppresses both T_h1 and T_h2 responses by allergen stimulation.

In our study PMBL treatment was accompanied by only moderate changes in mRNA expression and production of IL-4 and IFN-γ. At time T_0, 18 of 26 patients expressed IFN-γ and no IL-4, 4 of 26 expressed no cytokines at all, and 4 of 26 expressed both. After therapy (T_3), all subjects expressed IFN-γ and no IL-4 (Table IV). The supernatant cytokine values determined before and after PMBL therapy showed no significant changes in IFN-γ concentration (Fig. 2). Of 26 patients, only four produced IL-4 (Table IV): after PMBL therapy a reduction in IL-4 concentration in PBMC supernatants was observed in these patients (Fig. 3). It must be underlined that these subjects synthesized high levels of IgE (Table III). Lanzilli et al. (15) have previously shown that *in vitro* PMBL stimulates cytokine production (IL-2, IL-12, IFN-γ) in both naive and PMBL-sensitized human lymphocytes, and increases IL-2R expression on different lymphocyte subsets (B, CD4+ and CD8+ T cell). Recent results from Lanzilli et al. (25) also demonstrate that *in vivo* PMBL administration induces the expansion of IgG memory B cells. Data so far obtained by other groups concern immunomodulatory activity of PMBL treatment in order to prevent recurrent upper respiratory tract infection (4-5).

In spite of the low number of enrolled patients, our data suggest that PMBL exerts a potential immunomodulating activity by decreasing IL-4 expression. These data need to be confirmed by further study including both a larger number of

Table IV. *Cytokine mRNA expression in 26 PMBL-treated allergic rhinitis patients recorded before (T_0) and after (T_3) treatment.*

	T_0			T_3		
PMBL group*	IL-4	IFN-γ	ABL**	IL-4	IFN-γ	ABL
1	-	-	+	-	+	+
2	-	-	+	-	+	+
3	-	+	+	-	+	+
4	+	+	+	-	+	+
5	-	+	+	-	+	+
6	-	+	+	-	+	+
7	-	+	+	-	+	+
8	-	+	+	-	+	+
9	+	+	+	-	+	+
10	-	-	+	-	+	+
11	-	+	+	-	+	+
12	+	+	+	-	+	+
13	-	+	+	-	+	+
14	+	+	+	-	+	+
15	-	+	+	-	+	+
16	-	+	+	-	+	+
17	-	+	+	-	+	+
18	-	+	+	-	+	+
19	-	+	+	-	+	+
20	-	+	+	-	+	+
21	-	+	+	-	+	+
22	-	+	+	-	+	+
23	-	-	+	-	+	+
24	-	+	+	-	+	+
25	-	+	+	-	+	+
26	-	+	+	-	+	+

* *PMBL: polyvalent mechanical bacterial lysate*
** *Abelson gene*

allergic rhinitis patients and subjects with higher IgE levels. Ours is the first study to investigate whether PMBL administration in allergic rhinitis patients might modulate cytokine pattern. Local immunomodulation of T cell response, rather than a peripheral response, may play a role in this form of PMBL treatment. Measurement of IL-4 and IFN-γ in nasal secretion could allow to detect local changes in the nasal mucosa. A stronger preactivation of the inflammatory cells might occur as part of the allergic nasal reaction as compared with peripheral cells. In agreement with this hypothesis, immunotherapy with a pollen allergoid, clinically effective on allergic rhinitis, influences cytokine production in the nose but does not modulate the peripheral blood T cell responses (26).

In conclusion, the present data show that the great improvement of clinical conditions observed in allergic rhinitis patients following a 3 month PMBL therapy, in some patients may be accompanied by a potential immunomodulating activity by decreasing IL-4 cytokine expression.

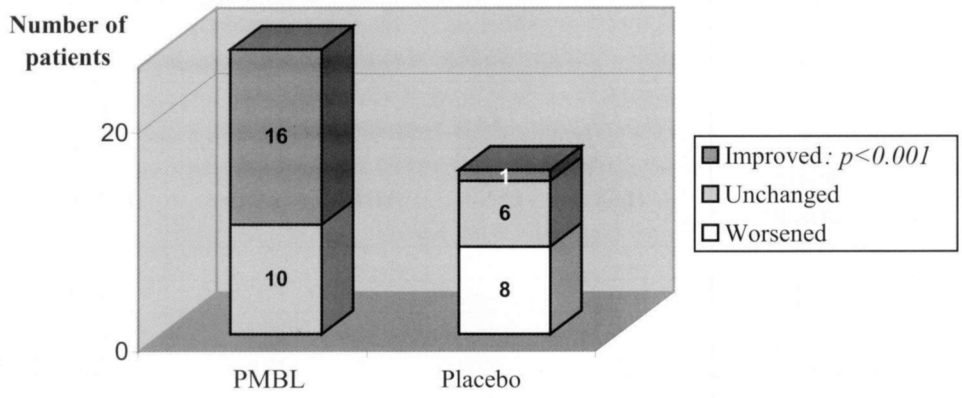

Fig. 1. *Allergic rhinitis patients' clinical evaluations after PMBL* treatment period versus placebo.*
** PMBL: polyvalent mechanical bacterial lysate*

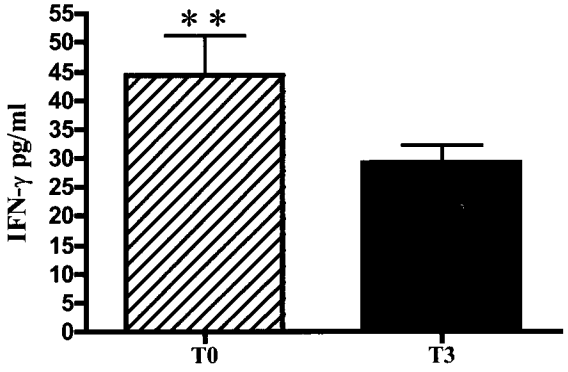

Fig. 2. *IFN-γ supernatant concentration measured by ELISA assay before (T_0) and after (T_3) PMBL* treatment in 26 allergic rhinitis patients.*

** PMBL: polyvalent mechanical bacterial lysate*
***P=0.12*

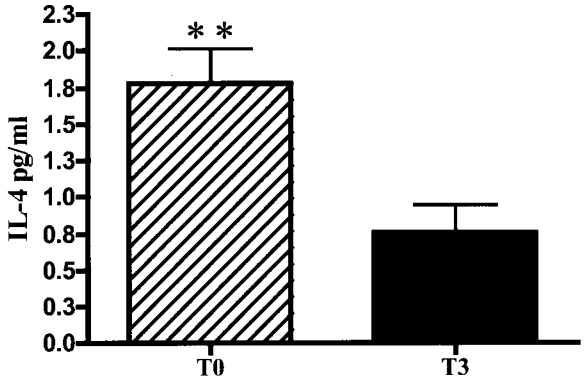

Fig. 3. *IL-4 supernatant concentration measured by ELISA assay before (T_0) and after (T_3) PMBL* treatment in allergic rhinitis patients positive for IL-4 mRNA expression by PCR (patients: 4, 9, 12, 14).*

** PMBL: polyvalent mechanical bacterial lysate*
*** P<0.05*

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