

Effects of Oral Bacterial Immunotherapy in Children with Atopic Eczema/Dermatitis Syndrome

A Pilot Study

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Abstract

Background: Atopic eczema/dermatitis syndrome (AEDS) is a chronic inflammatory skin disease, affecting 10–20% of children and 1–3% of adults. The purpose of this pilot study was to assess the clinical and anti-inflammatory effect of bacterial and ribosomal immunotherapy with Immucytal® (Pierre Fabre Médicament, France) in children with AEDS.

Methods: Seventeen children with allergic and non-allergic forms of AEDS (AAEDS and NAAEDS, respectively), graded moderate to severe (Severity Scoring of Atopic Dermatitis [SCORAD] index of >25), received ribosomal immunotherapy (Immucytal®) once daily according to the standard treatment regimen (4 consecutive days a week for 3 weeks, and then 4 consecutive days a month for 4 months). We assessed the clinical status of AEDS using the SCORAD index at baseline, and after 8 and 20 weeks of treatment. Furthermore, peripheral blood from patients was examined for the frequencies of CD4+ cells expressing interferon (IFN)- γ and interleukin (IL)-4 using flow cytometry.

Results: There was a progressive and significant clinical improvement of AEDS, confirmed by a reduction of the SCORAD index over time in both AEDS forms ($p < 0.01$). Pooled data from the two groups showed that the mean baseline index of 43 was reduced to 17 after treatment. Overall, these data indicate a marked improvement in total clinical severity of AEDS (–62%). Flow cytometry analysis showed that frequencies of the two CD4+ T cell subsets did not differ significantly from the beginning to the end of the study in both forms of AEDS. However, the percentage of CD4+ cells expressing IL-4 in children with AAEDS tended to decrease by the end of treatment with ribosomal immunotherapy. Clinical and laboratory data confirmed that immunotherapy was well tolerated.

Conclusions: The results of this pilot investigation suggest that ribosomal immunotherapy may be beneficial in the management of AEDS in children, and that this could be at least partially explained by a role in restoring the type 2 helper-T cell imbalance seen in allergic patients. Placebo-controlled, randomized clinical trials are recommended in order to confirm these findings.

Atopic eczema/dermatitis syndrome (AEDS) is a chronic inflammatory skin disease characterized by distressing itching that usually begins in early infancy.^[1] Recent epidemiological studies suggest a prevalence of AEDS in 10–20% of children and 1–3% of adults.^[2]

At least two forms of AEDS have been identified: an allergic form (AAEDS) characterized by immunoglobulin (Ig)E-sensitization and observed in about 70–85% of AEDS patients, and non-allergic AEDS (NAAEDS), a non-IgE-sensitized form with an approximate frequency of 15–30%.^[3]

Immune responses, mostly mediated by type 2 helper-T cells (T_H2) that stimulate increased production of interleukin (IL)-4, IL-5 and IL-13 cytokines, play an important role in the pathogenesis of AAEDS.^[1,4] T_H1 cell response, primed by microbial exposure and mediated by the production of interferon (IFN)- γ and IL-2, can counteract that of T_H2 . Such observations led to the so-called 'hygiene hypothesis' which proposed that a lower exposure to infections in early childhood may predispose children to an increased allergic response.^[5] However, this hypothesis was disproved recently, when a large case-control study of 307 children with AEDS and 297 healthy controls showed that there is no evidence that exposure to infections in infancy contributes to a protective effect.^[6]

In recent years, research into immunological triggers has focused on the role of oro-intestinal and atopic skin bacterial colonization in AEDS patients.^[7] Evidence suggests that a wide range of microorganisms are etiological factors, as are agents responsible for sustained disease activity or resistance to therapy via modulation of the immune response. In terms of therapeutic options, immunotherapy may therefore play a key role in the prevention of AEDS.

Bacterial immunostimulants have a two-fold action, namely as 'vaccines' and as 'immunostimulants'. The first is dependent upon a specific immune response (antibody and cell-mediated) and the second is a non-specific response (stimulation of phagocytes and cytokine production: IL-1, IL-6, and IL-8).^[8,9] The end result is immediate and delayed long-term protection. Repeat administration of bacterial immunostimulants is effective in promoting differentiation of T_H1 cells, though the mechanism of action is not yet fully understood.^[10] Therefore, immunostimulants may be beneficial in the treatment of allergic disorders.

Immucytal®¹ (other international trade names are Ribomunil, Biomunil, and Ribovac) is a ribosomal immunostimulant with activities affecting both the innate and acquired immune system.^[11] The drug is composed of ribosomal fractions (rRNA) from *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *S. pyogenes* group A, *Haemophilus influenzae* and of a membrane fraction from *K. pneumoniae*. It may be possible that an oral supplementation with this drug can achieve significant immunological effects in the context of the control of allergic disease.

There is a lack of available data on the use of immunotherapy for AEDS. Therefore, the objective of this pilot study was to evaluate the effects of repeat oral doses of Immucytal® (Pierre Fabre Médicament, France) on the clinical course of AEDS in children.

Patients and Methods

Patients

Between April 2003 and March 2004, 17 of 133 children (72 males and 61 females; median age 9.9 years [range 4.7–15.7]) with moderate-severe AEDS, consecutively referred to our Unit of Immunology and Allergology (Department of Pediatrics, University of Bari), were enrolled in the study. All patients received a topical treatment with emollients and moistening lotions. We excluded all children who: (i) had received antihistamines or oral/topical steroids within the previous 4 weeks; (ii) had a positive skin prick test for food allergens and who were undertaking a specific dietary program; and (iii) had a chronic disease, other than AEDS, and/or immunodeficiency, active infections, or were receiving antibacterials.

Among the 106 excluded children, 60 were receiving an oral and/or topical steroid at the beginning of the study, 30 had positive skin prick tests for food allergens, 14 had an active infection and were receiving antibacterials, and two were affected by IgA deficiency.

AEDS was diagnosed in the presence of typical features described by Hanifin and Rajka.^[12] Patients were selected according to disease severity as graded by the Severity Scoring of Atopic Dermatitis (SCORAD) index, and classified as having moderate eczema if the score was 25–50 points, or severe eczema if the score was >50 points.^[13]

Nine children were affected by AAEDS, classified by the presence of at least one positive skin prick test response to aero-allergens and/or high IgE levels (>100 kU/L) and/or a medical history of asthma or allergic disease; the remaining eight patients had NAAEDS. At enrollment, all children underwent skin prick tests to common airborne and food allergens.

Informed consent was obtained from parents before any study procedures. The study was approved by the local Ethics Committee and conducted following the Declaration of Helsinki and Good Clinical Practice guidelines.

Study Design

The study design planned a 20-week treatment period with three outpatient visits: at baseline (T0), during the study treatment at 8 weeks (T8), and after 20 weeks (T20).

All children were treated with ribosomal immunotherapy (Immucytal® Pierre Fabre Médicament, France). The immunotherapy was administered orally as one sachet/dose per day (0.525mg of active ingredient) during the morning according to the following

1 The use of trade names is for product identification purposes only and does not imply endorsement.

schedule: one daily dose for 4 consecutive days a week for the first 3 weeks, then one daily dose for 4 consecutive days a month for the remaining 4 months.

At each study visit, blood specimens were drawn by venipuncture, a complete physical examination was carried out, and the clinical efficacy of immunostimulant therapy was assessed with the SCORAD index. This takes into account data on the extent and severity of skin lesions (erythema, edema, crusts, excoriations, lichenification, and dryness) over the affected body surface.^[14]

The physicians who examined the patients and the person who performed the laboratory test were blinded for the enrolment of the subjects into the study and for the classification of the patient into the AAEDS or the NAAEDS group.

All children were able to successfully complete the study procedure, which was well accepted. After the administration of Immucytal®, none presented clinical signs of intolerance as reported by the parents during each scheduled visit.

Total IgE and Eosinophils Determination

Total IgE was measured using the Pharmacia CAP System (Uppsala, Sweden): anti-IgE was covalently coupled to ImmunoCAP and reacted with the total IgE in the patient's serum. After a washing process, enzyme-labeled antibodies against IgE were added to form a complex with the patient's IgE. Then the samples were incubated, unbound enzyme-anti-IgE was washed away, and samples were incubated with a developing agent. After stopping the reaction, the fluorescence of the eluate was measured. To evaluate the results, the responses for the patient's samples were converted to IgE concentration using a calibration curve. The assay was calibrated against the WHO Standard for IgE, with a range of 2 ± 2000 kU/L (normal values ≤ 100 kU/L).

Blood eosinophil counts were performed with a Fuchs-Rosenthal counter, after blood dilution in phloxine solution Becton Dickinson Unopette (Becton Dickinson, CA, USA), according to the manufacturer instructions. An eosinophil count $>400/\text{mm}^3$ was considered to be elevated.

Flow Cytometric Assay

Flow cytometric assessment of peripheral blood CD4+ T cell phenotypes was performed. To identify CD4+ cells we used multiparametric analysis. Briefly, 500 μL of whole blood was stimulated for 4 hours at 37°C with medium containing phorbol myristate acetate, ionomycin and brefeldin A (Sigma, MO, USA). Activated cells were subsequently aliquoted for staining with fluorescein isothiocyanate-conjugated surface antigen-specific CD4 antibody and peridinin chlorophyll protein-conjugated anti-CD3 (Becton Dickinson, CA, USA) for 15 minutes at room

temperature. Subsequently, FACS Lysing Solution (Becton Dickinson, CA, USA) was added. After a further short incubation, samples were washed and resuspended in 500 μL of FACS Permeabilizing Solution (Becton Dickinson, CA, USA) for 10 minutes at room temperature. Two aliquots of each sample were then washed and incubated separately with phycoerythrin-conjugated anticytokine (IFN γ and IL-4) antibodies (Becton Dickinson, CA, USA) for 30 minutes. After staining, sample tubes were washed again and resuspended in 1% paraformaldehyde prior to analysis on a FACScan (Becton Dickinson, CA, USA) flow cytometer using the Cell Quest program. Control samples were used to verify the activation (by staining for the activation marker CD69 without Brefeldin A) and membrane permeabilization (staining for CD69 after permeabilization). The results were expressed as the percentage of CD4+ lymphocytes producing the two cytokines.

Statistical Analysis

The statistical analysis was performed using the Number Cruncher Statistical System 6.0.1 program. Absolute values of the SCORAD index and CD4+ T cell subsets at different study visits were subjected to ANOVA for repeated measures, using the general linear model (GLM) procedure of statistical analysis system (SAS®) package (version 8.2, Milan, Italy); all available data were analyzed for each parameter. Repeated measures ANOVA is robust to violations of the assumptions associated with an ordinary analysis of variance, extended to the matrix case: multivariate normality, homogeneity of covariance matrices, and independence. We checked for sphericity using the Mauchly's sphericity test, which examines the form of the common covariance matrix. The Kruskal-Wallis multiple-comparison z-value test was used for comparison between the groups. The non-parametric Mann-Whitney test was used for comparison of continuous data. A p-value of <0.05 was considered significant. Most data were represented as mean values + standard error of mean (SEM) or median with a range interval.

Results

All 17 patients (6 males and 11 females; median age 6.1 years [range 2–12 years]) completed the treatment course.

Intensity of AEDS signs at admission was rated moderate to severe. The mean baseline value of the SCORAD index was 42.9 (41.4 in the NAAEDS group and 43.3 in AAEDS group) with values ranging from 25 to 65. On admission, no significant difference was found between the two diagnostic groups with regards to severity of AEDS signs. General characteristics of enrolled patients are summarized in table I.

Table I. Patient characteristics at baseline

	NAAEDS (n = 8)	AAEDS (n = 9)	p-Value
Male/female, n	2/6	4/5	0.6
Mean age, years (range)	6.3 (3.0–8.9)	5.9 (2.1–12.3)	0.8
Median disease duration, years (range) ^a	3.9 (1.0–6.0)	5.2 (1.8–12.0)	0.2
Mean total IgE (kU/L) ± SEM (range)	85 ± 15 (4–100)	890 ± 397.9 (60–3371)	0.01
Mean eosinophils (n/mm ³) ± SEM (range)	358 ± 114.7 (150–950)	306 ± 76.1 (130–850)	0.4

a Expressed as mean years after diagnosis.

AAEDS = allergic atopic eczema/dermatitis syndrome; **IgE** = immunoglobulin E; **NAAEDS** = non-allergic atopic eczema/dermatitis syndrome; **SEM** = standard error of mean.

At week 8 we observed a significant overall reduction of the mean SCORAD index compared with pre-treatment values ($p < 0.005$; table II). However, the SCORAD index only decreased significantly in the NAAEDS group ($p < 0.015$), not in the AAEDS group ($p < 0.11$). Upon treatment completion at week 20, the mean SCORAD index decreased by 58% in the NAAEDS group ($p < 0.01$) and by 66% in the AAEDS group ($p < 0.008$; table II). Data pooled from the two groups showed a mean reduction from baseline of 62% ($p < 0.001$). Figure 1 shows the course of SCORAD index values during the study period; a Kruskal-Wallis multiple comparison test showed a significant reduction of the SCORAD index across time ($p < 0.003$ and $p < 0.007$ for AAEDS and NAAEDS, respectively). The progressive and marked fall of the SCORAD index was indicative of a reduction in overall clinical severity of AEDS signs and symptoms during

immunotherapy in both patient groups. No patients received a restricted diet or received medical treatment with antihistamines or steroids, both of which could affect symptoms and eczema severity.

The percentages of peripheral blood CD4+ T lymphocytes producing IFN γ and IL-4 tended to differ between the two forms of AEDS (table II, figure 2, and figure 3). Overall, after 20 weeks of ribosomal immunotherapy, we observed a decrease in the mean percentage values of the two CD4+ T cell subsets (parallel in both NAAEDS and AAEDS). Although the decrease did not reach statistical significance, it is interesting to note that the decrease of CD4+ cells expressing IL-4 in children with AAEDS from baseline to 20 weeks approached statistical significance (3.3 vs 1.9; $p = 0.07$).

Table II. SCORAD index, percentage of CD4+ cells producing IFN γ and IL-4 at entry (T0) and at week 8 (T8) and week 20 (T20) of ribosomal immunotherapy in children with AAEDS and NAAEDS

Patient group	T0	T8	T20	p-Value for T0 vs T8	p-Value for T0 vs T20
SCORAD index^a					
All patients	42.8 (25–65)	28.2 (8–55)	15 (5–55)	0.005	<0.001
AAEDS	43.3 (25–65)	30.7 (13–55)	16 (5–28)	0.11	<0.001
NAAEDS	41.4 (25–55)	25.4 (8–50)	13.5 (10–55)	0.015	0.01
CD4+/IFNγ, %^b					
All patients	6 (2–27)	4 (2–14)	4 (2–13)	0.4	0.2
AAEDS	7 (4–13)	4 (2–14)	4 (3–10)	0.1	0.1
NAAEDS	4.7 (2–27)	6.5 (3–11)	3 (2–13)	0.7	0.9
CD4+/IL-4, %^b					
All patients	3 (1.5–8)	2.1 (1–7)	2 (1–5.9)	0.4	0.2
AAEDS	3 (4–13)	3.3 (1–7)	1.9 (1–5.9)	0.7	0.07
NAAEDS	2 (1–5)	2 (1–3.3)	2.1 (1–3)	0.6	0.6

a Data are reported as median (range).

b Data are expressed as percentage of CD4+ cells expressing IFN γ or IL-4.

AAEDS = allergic atopic eczema/dermatitis syndrome; **IFN** = interferon; **IL** = interleukin; **NAAEDS** = non-allergic atopic eczema/dermatitis syndrome; **SCORAD** = Severity Scoring of Atopic Dermatitis.

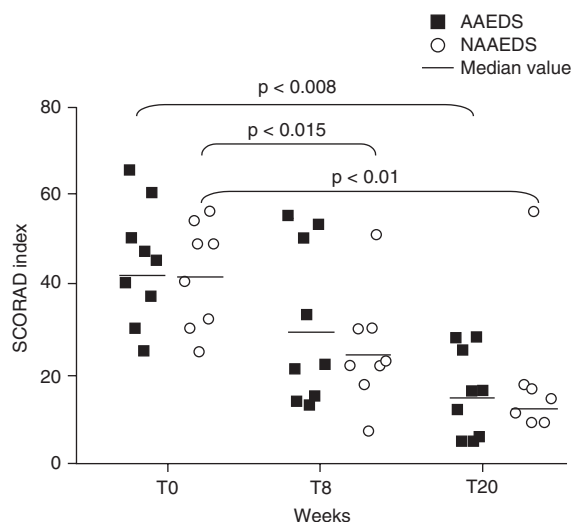


Fig. 1. Severity Scoring of Atopic Dermatitis (SCORAD) index during a 20-week study period in children with AAEDS and NAAEDS receiving treatment with ribosomal immunotherapy. In all patients there was a progressive decline of the SCORAD index both at week 8 (T8) and week 20 (T20). **AAEDS** = allergic atopic eczema/dermatitis syndrome; **NAAEDS** = non-allergic atopic eczema/dermatitis syndrome; **T0** = baseline.

At baseline, the eosinophil count and the IgE total levels were correlated with cytokine expression in CD4⁺ T cells for all 17 patients, using the Spearman test. The IgE total serum levels correlated with CD4⁺/IL-4 expression significantly ($r = 0.54$, $p = 0.03$). No correlation was found between eosinophils and CD4⁺/IL-4 expression ($r = -0.45$, $p = 0.07$), or between eosinophils or total IgE and IFN expression by CD4⁺ cells ($r = -0.257$, $p = 0.32$).

There were no adverse laboratory or clinical effects in any of the participating patients.

Discussion

In this pilot study, ribosomal immunotherapy was found to be effective in the treatment of AEDS as shown by the reduction of the SCORAD index. We evaluated the influence of this therapy on clinical outcome in 17 children with moderate to severe AEDS, with either NAAEDS (eight patients) or AAEDS (nine patients). A progressive and steady reduction in SCORAD index values indicating an improvement in patients' overall clinical condition was recorded at all observation times during immunotherapy ($p < 0.01$). In particular, at week 8 we observed a significant overall reduction of the mean SCORAD index compared with pre-treatment values (table II). However, compared with baseline, after 8 weeks of treatment the SCORAD index only decreased significantly in the NAAEDS group, not in the AAEDS group (table II). This is probably related to the limited number of patients. Upon study completion the reduction in severity of signs was 58% in

children with NAAEDS and 66% in those with AAEDS. In the absence of a control group, the statistically significant reduction of signs of AEDS after immunotherapy allows only for the hypothesis that the improvement observed in the clinical condition was not a chance variation in the values of the clinical index.

On all accounts it seems that the AAEDS group needed more time to get an improvement of eczema severity compared with the NAAEDS group, but at 20 weeks the effect of the ribosomal immunotherapy seemed stronger in the AAEDS group (table II). However, the improvement observed in this study with immunotherapy appears clinically meaningful and does warrant further investigation.

Patients did not received restrictive diets for 'offending' foods, nor receive antihistamine, antibacterial, or steroid treatment that could affect the results of the immunotherapy treatment. Thus, the clinical improvements observed in our patients can be ascribed to the ribosomal immunotherapy. However, the influence of the weather, particularly during summer, on the natural course of the disease must be taken into account. Thus, placebo-controlled, randomized clinical trials are warranted.

Children with allergies show a Th2 polarization characterized by high levels of IL-4 and low levels of IFN γ .^[15] As Th2 cytokines have a pivotal role in the development of allergic inflammation, there is accumulating evidence that the Th1-related cytokines exert a crucial role in reversing ongoing allergic reaction.^[16]

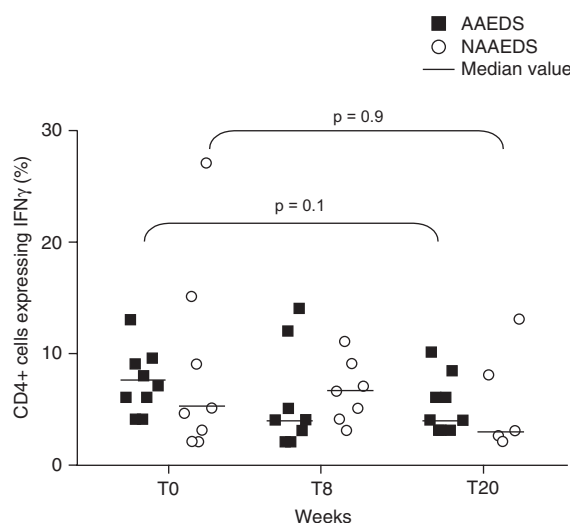


Fig. 2. Percentage of CD4⁺ cells expressing IFN γ in children with AAEDS and NAAEDS receiving treatment with ribosomal immunotherapy over 20 weeks. **AAEDS** = allergic atopic eczema/dermatitis syndrome; **IFN** = interferon; **NAAEDS** = non-allergic atopic eczema/dermatitis syndrome; **T0** = baseline; **T8** = week 8; **T20** = week 20.

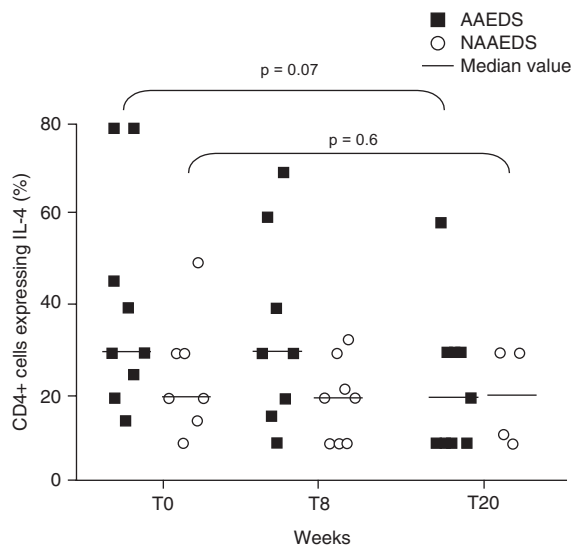


Fig. 3. Percentage of CD4+ cells expressing IL-4 in children with AAEDS and NAAEDS receiving treatment with ribosomal immunotherapy over 20 weeks. Horizontal bars represent median values. **AAEDS** = allergic atopic eczema/dermatitis syndrome; **IL** = interleukin; **NAAEDS** = non-allergic atopic eczema/dermatitis syndrome; **T0** = baseline; **T8** = week 8; **T20** = week 20.

To investigate whether ribosomal immunotherapy could reverse the $T_H2 : T_H1$ ratio in children with AEDS, we measured the expression by CD4+ lymphocytes of IL-4 and IFN γ (T_H2 and T_H1 cytokines, respectively). Cytokines can be assessed by several methods; our method was based on the fact that the detection of T_H1/T_H2 cytokine profiles by flow cytometry is a practical and mechanistically relevant assay that enables the production of cytokines to be studied at the single cell level, using limited whole blood volumes.^[17] Thus, this technique is a more reliable tool for the identification of the cellular source of the cytokine compared with the measurement of cytokine productions in peripheral blood mononuclear cells by enzyme-linked immunosorbent assay.

We found that CD4+/IFN γ expression does not increase significantly after 20 weeks of immunotherapy in our study population, while CD4+/IL-4 production slightly decreased at different study intervals in the AAEDS group. This result is particularly important since the same trend is not observed in the NAAEDS group, and it may partially explain the strongest improvement of clinical signs observed in the AAEDS group. Moreover, we may assume that a longer period of treatment might produce significant changes in cytokine patterns and perhaps the small size of our cohort might have affected the final result.

A possible immunological explanation of the clinical effect might involve cytokines different from those evaluated in the study. For example, the production of IL-1, a mediator of cellular interactions in the immune system,^[18] has been observed to be potentially stimulated by bacterial immunotherapy.^[9,14,19] Experi-

mental investigations have shown that *in vivo* administration of ribosomal extracts can stimulate the production of pro-inflammatory cytokines, among them IL-1,^[8,20] and stimulate major epidermal cell lines (keratinocytes, Langerhans' cells, melanocytes) that are then able to release additional IL-1.^[21] IL-1 plays a role not only along the inflammation pathway but also during the repair and tissue remodeling processes, all sustained by the innate and acquired immunological systems working synergistically.^[22]

However, IL-1 has never been reported to be involved in atopic dermatitis pathogenesis. Also, chronic lesions in the skin show a T_H1 -like pattern with increased levels of IL-12 and IFN γ .^[23] Thus, further studies are needed to investigate the effect of immunotherapy in modulating other patterns of cytokines.

Conclusion

The results of this pilot study suggest that oral ribosomal immunotherapy (Immunocytal®) may be used as a preventive treatment in children with AEDS. In our study design we decided to exclude patients who were receiving anti-inflammatory drugs to verify that the effects on the immune system were due specifically to Immunocytal® activity. However, larger placebo-controlled clinical trials, including a study design comparing current therapy with current therapy plus immunotherapy, are warranted to confirm these findings.

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Luigia Brunetti and Ruggiero Francavilla contributed equally to the work.

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