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Pro and Anti-inflammatory Cytokine Production in CD4+ T Lymphocytes in Children with Asthma and Allergic Rhinitis Exposed to the Monocyte Locomotion Inhibitory Factor (MLIF)

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ABSTRACT

Entamoeba histolytica produces, in axenic culture, the monocytes locomotion inhibitory factor (MLIF), a oligopeptide with selective anti-inflammatory properties. We evaluated the effect of MLIF on the expression of pro- and anti-inflammatory cytokines in CD4+ T lymphocytes from children with asthma and allergic rhinitis.

Twelve children with severe asthma, 12 children with allergic rhinitis and 6 healthy controls were recruited for this study between May and December 2016. CD4+ T cells were cultured for 24 h at 37°C, 5% CO_2 in the presence of MLIF, 1-phorbol 12-myristate 13-acetate (PMA), MLIF+PMA or RPMI. Interleukin-10 (IL-10), IL-4, interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) expression levels were measured in the supernatants of T-cell cultures using the enzyme-linked immunosorbent assay (ELISA).

Pro- and anti-inflammatory cytokines were inhibited by MLIF (IFN-γ p=0.0036, TNF-α p<0.001, IL-4 p=0.0082) in asthmatic patients, however IFN-γ was not significantly inhibited (NS) in patients with allergic rhinitis when compared to the RPMI group. In CD4+ T cells treated with PMA+MLIF, the expression levels of IFN-γ, TNF-α and IL-4 were strongly inhibited (p<0.001, p<0.001 and p<0.0094), compared to PMA treatment alone, for both, rhinitis and asthma. IL-10 expression was not affected by MLIF in neither of the two diseases.

We conclude that MLIF alters the pro/anti-inflammatory balance and induces inhibition of IL-4, IFN-γ and TNF-α, but does not affect IL-10.

Keywords: Asthma; Inflammation; Lymphocytes; Monocytes locomotion inhibitory factor; Rhinitis

INTRODUCTION

Asthma and allergic rhinitis (AR) are the most common allergic manifestation, representing a major health problem. The prevalence of allergic diseases has increased worldwide in the last three decades.[1] Despite

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advances in the understanding of allergic inflammation mechanisms, the symptoms of the disease cannot generally be completely controlled with current therapies.[2-4]

The majority of respiratory diseases including asthma and rhinitis present an inflammatory process, which implies regulated and ordered activation of inflammatory cells that release cytokines, among other mediators of inflammation, which amplify the recruitment of immune cells into the airways. This may lead to abnormal regulation lymphocytes, which immediately alters the expression of different cytokines such as IL-4, IL-6, TNF-α, IL-13 and IFN-γ. This results in aggravation of inflammation in the airways.[5,6]

The monocytes locomotion inhibitory factor (MLIF) is a natural pentapeptide (Met-Glu-Cys-Asn-Ser) with selective anti-inflammatory properties. This peptide inhibits locomotion of human peripheral blood mononuclear phagocytes (PBMP*).*[7] MLIF is also able to inhibit the *in vitro* production of IL-1β, IL-2, IFN- γ, IL-5, and IL-6 cytokines without affecting the production of IL-10 in cultured human T lymphocytes from healthy volunteers.[8,9] The effect of MLIF in allergic respiratory diseases is still unknown. The aim of this study was to evaluate the pro and anti-inflammatory cytokine production in CD4+ T lymphocytes in children with asthma and allergic rhinitis exposed to the monocyte locomotion inhibitory factor (MLIF).

PATIENTS AND METHODS

Asthma was diagnosed according to the Global Strategy for Asthma Management and Prevention, GINA 2008.[10] All patients tested positive for at least one allergen, including fungi (such as *Aspergillus fumigatus*), domestic dust mites (*Dermatophagoides pteronyssinus*), and pollen (including grasses such as oats) in allergic skin prick tests (Allergic Kit, Alerquim, Mexico City, Mexico). Only patients with severe asthma were included in this study.

At study admittance, all patients were subjected to the forced expiratory volume in 1 s (FEV1) test after inhalation of 400 μg albuterol (salbutamol) and had shown an increased measurement of ≥ 12% and ≥ 200 ml. None of the subjects were under treatment with corticosteroids or anti-leukotrienes. The study was conducted in accordance with the recommendations of the Declaration of Helsinki and the Ethics Committee of the Mexican Institute of Social Security in Mexico City (approval number 088-2016). Patients with asthma and allergic rhinitis were recruited for the study between May and December 2016 at the Family Medicine Unit No. 7 of the Mexican Institute of Social Security IMSS, Mexico City. Informed written consent was obtained from parents or tutors of all children and control subjects.

Cell Purification

A 10 ml sample of heparin blood was diluted 1:3 with 1:1 v/v phosphate buffer (PBS; 0.15 M), placed on a Ficoll-Hypaque gradient (Sigma Chemical Co., St. Louis MO,USA) in sterile 10 ml tubes, (Falcon Oxnard, CA, USA) and centrifuged at 400 x g for 30 min at 22°C to recover interface cells.[11] CD4+ T lymphocytes were purified using a Negative Separation Kit (MACS Reagents Kit Isolation, Human Cell T CD4+, Milteny Biotec, Inc., Auburn, CA, USA). Mononuclear cells (15 x 10^6) were obtained from the Ficoll-Hypaque gradient. From these, 1 x 10^7 cells were placed in sterile tubes with 80 μL of buffer (PBS-albumin-EDTA) plus 20 μL of CD8, CD11b, CD16, CD19, CD36, CD56, CD123, TCRα/δ CD235a and glycophorin A (MACS). The mixture was incubated for 10 min at 4°C. Subsequently, the cells were magnetically labeled with 20 μL Anti-Biotin MicroBeads MACS and incubated for 15 min at 4°C. The sample was then run on a MACS LS/VS + column with a VS+plus adapter placed on a Midi Macs separator to remove labeled cells. The lymphocytes obtained were 95% pure. The viability of the cells always exceeded 95%, as determined by trypan blue dye exclusion (Sigma).

Lymphocytes (5 x 10^5) were incubated in 24-well plates for 24 h in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL L-glutamine, 100 U/mL streptomycin, 2 mg/100 mL gentamicin and 1 mM sodium pyruvate (Gibco Laboratories, Grand Island, NY, USA). Lymphocytes were cultured at 37°C, in humid atmosphere, with 5% CO_2, then stimulated with: 1) RPMI medium alone; 2) RPMI medium with MLIF (100 μg/mL); 3) RPMI medium with 50 ng/mL 1-phorbol 12-myristate 13-acetate (PMA) (Sigma, Chemical Co., St. Louis MO) or 4) RPMI medium with PMA+MLIF. The optimal concentrations of MLIF and PMA were determined from dose-response curves. MLIF was obtained from American Peptide Co.

(Sunnyvale, CA, USA) with 96% purity.

Cell culture supernatants were analyzed for a set of pro- and anti-inflammatory cytokines (IL-4, IL-10, TNF-α and IFN-γ) using the ELISA method (Beckman Coulter Immunotech, Marseille, France) following the protocols suggested by the manufacturer.

Statistical Analysis

Results were expressed as the mean±standard deviation (SD). The unpaired Student's t-test was used to compare the differences between the groups. Statistical analysis was performed with SPSS v.17.0 for Windows (SPSS Inc., Chicago, IL., USA); $p<0.05$ were considered statistically significant.

RESULTS

This study included 30 children with a mean age of 7.3 years (range: 1-16 years). The control group included 6 children (20%) with a mean age of 7 years (age range: 6-15 years). The experimental group included 12 children (40%) with severe asthma with a mean of age 8 years and 12 children (40%) with allergic rhinitis with a mean age of 7 years (age range: 1-16 years). The proportion of male to female was approximately 2:1, with a total of 19 male and 11 female. None of the participants had medication prior to sampling.

Table 1 shows the results in the levels of expression of pro-anti-inflammatory cytokines obtained in CD4 + T lymphocytes of patients with asthma and allergic rhinitis. The expression of pro-inflammatory cytokines TNF-α and IFN-γ, treated with MLIF, showed a significant inhibition compared to the production of the cells in RPMI medium (0.051 (p=0.0036) and 0.053 (p<0.001) vs. 0.068 and 0.085, respectively), in patients with asthma In contrast, the MLIF did not significantly inhibit (NS) the IFN-γ in patients with allergic rhinitis.

The values found in the production of anti-inflammatory cytokines exposed to PMA were the highest (asthma: 0.118, 0.088, 1.78, 0.083 pg/mL and AR: 0.090, 0.118, 2.517 and 0.085 pg/mL) than those compared with the other treatments. Of all the cytokines studied, IL-4 was the most vigorously expressed (2,517 pg / mL) due to PMA effect, in AR (Table 1).

In the PMA+MLIF treatments the production of the cytokines TNF-α, IFN-γ were significantly inhibited (p<0.001), in both asthma and AR groups. With the same treatment (PMA+MLIF) inhibited the production of the anti-inflammatory cytokine IL-4 in asthma (p=0.0094), and it was vigorously inhibited (p<0.001) in patients with AR, when compared with

Table 1. The monocyte locomotion inhibitory factor (MLIF)-induced inhibition of TNF-α, IFN-γ and IL-4 cytokines in vitro in CD4+ T cells from asthma and allergic rhinitis patients

Treatment	TNF-α (pg/mL)	IFN-γ (pg/mL)	IL-4 (pg/mL)	IL-10 (pg/mL)
Control group	0.023±0.018	0.027±0.004	1.02±0.03	0.064±0.002
RPMI				
Asthma	0.068±0.014	0.085±0.010	1.12±0.24	0.073±0.005
Rhinitis	0.063±0.007	0.070±0.010	1.421±0.19	0.075±0.007
MLIF				
Asthma	0.051±0.007[2]	0.053±0.007[1]	1.38±0.13[3]	0.074±0.007 ns
Rhinitis	0.049±0.006[1]	0.051±0.005[5]	1.698±0.60[6]	0.075±0.002 ns
PMA				
Asthma	0.118±0.022	0.088±0.009	1.78±0.29	0.083±0.016
Rhinitis	0.090±0.007	0.118±0.008	2.517±0.62	0.085±0.003
PMA+MLIF				
Asthma	0.069±0.005[1]	0.069±0.018[1]	1.72±0.14[4]	0.081±0.008 ns
Rhinitis	0.065±0.006[1]	0.090±0.012[1]	1.418±0.28[1]	0.081±0.007 ns

Data are expressed as the mean ± standard deviation of six experiments by duplicate. RPMI (RPMI-1640), MLIF (monocyte locomotion inhibitory factor), PMA (1-phorbol-12 myristate-13 acetate), TNF-α (tumor necrosis factor), IFN-γ (interferon), IL (interleukin); ns, not significant, p values for asthmatic patients [1]p<0.001, [2]p=0.0036, [3]p=0.0082, [4]p=0.0094), p values for patients with allergic rhinitis [1]p<0.001, [5]p=0.0016, [6]p=0.0264. Control, n=6; asthmatic and rhinitis n=24

PMA alone. In contrast, IL-10 expression was not significantly altered (0.081 pg/mL; p<ns) in patients studied with this treatment (Table 1).

DISCUSSION

Asthma and allergic rhinitis are characterized by mucosal inflammation, which is hypothesized to be responsible for many of the pathophysiological features of these diseases. CD4+ T lymphocytes are particularly abundant in such sites of inflammation. Activation of lymphocytes is a complex yet finely regulated cascade of events that results in the expression of cytokine receptors, and the production and secretion of cytokines, which eventually leads to divergent immune responses.

MLIF has powerful anti-inflammatory properties; this peptide inhibits the locomotion of normal human peripheral blood mononuclear cells, but has no effect on neutrophil polymorphonuclear cells.[7] In addition, MLIF decreases VLA-4 adhesion molecule expression in monocytes and VCAM-1 adhesion molecule expression in vascular epithelium.[12] The selective actions of MLIF upon a variety of cell types have suggested that MLIF disrupts the pro- and anti-inflammatory networks of organisms.[7,13]

The main finding of this study was that MLIF can inhibit pro-inflammatory cytokines TNF-α and IFN-γ in asthma, whereas IFN-γ was not significantly inhibited by effect of MLIF in patients with allergic rhinitis. This inhibition was even more evident when cells were treated with PMA+MLIF in asthma and allergic rhinitis T CD4+ lymphocytes.

Surprisingly we found that MLIF significantly inhibits IL-4 expression, which was not what was expected, since it had been shown that MLIF does not alter significantly the anti-inflammatory or Th2 cytokines.[8]

Not all cytokines promote allergic airway disease. Anti-inflammatory cytokines such as IL-10 and TGF-β help control some symptoms of the disease. It has been shown that IL-4 at high concentrations does not alleviate these diseases, but it does increase B-cell antigen presentation.[14] Furthermore, overexpression of IL-4 can lead to lymphocytic inflammation and eosinophilic but does not lead to airway hyperreactivity.

We have shown that MLIF has an inhibitory effect on the expression of IL-4 in patients with asthma and allergic rhinitis, which may lead to alleviation of symptoms of the disease. We also found that IL-10 expression was not altered in these cells. IL-10 may have a beneficial effect on airway remodeling, as it has been shown to reduce type I collagen synthesis and vascular smooth muscle proliferation.[15,16]

The exact molecular mechanism affected by MLIF has not been fully elucidated. From previous experiments, it has been shown that MLIF acts through surface receptors that contain mannose, and increases cAMP without simultaneously decreasing cGMP expression.[17,18]

MLIF had a marked effect on both the pro- and anti-inflammatory balance (Th1/Th2). This significantly reduced IL-4 and IFN-γ expression. We hypothesize that this novel anti-inflammatory that somehow inhibits the mechanisms responsible for characteristic inflammation of these pathologies may be considered as a possible treatment for allergic respiratory diseases.

Among the limitations of this study, the most important is the relatively small sample size. However, collection of samples in a pediatric population is always problematic, especially when samples are obtained from asymptomatic controls. A limitation directly related to small sample size is the inability to determine the potential influence of allergen sensitization and exposure to cytokine levels. More studies are needed to shed more light on the functionality of MLIF in asthma and allergic rhinitis leading to more effective therapies.

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