NITRIC OXIDE AND IFN-γ PLASMA LEVELS IN PATIENTS WITH ATOPIC DERMATITIS

Vesna Milićić1, Dejan Baskić2, Nemanja Zdravković2 and Nebojša Arsenijević2
1Department of Dermatovenerology, Faculty of Medicine, University of Kragujevac, 2Department of Microbiology and Immunology, Faculty of Medicine, University of Kragujevac

KONCENTRACIJE AZOT MONOKSIDA I IFN-γ U PLAZMI PACIJENATA SA ATOPIJKIM DERMATITISOM

Vesna Milićić1, Dejan Baskić2, Nemanja Zdravković2 and Nebojša Arsenijević2
1Katedra za Dermatovenerologiju, Medicinski fakultet, Univerzitet u Kragujevcu, 2Katedra za mikrobiologiju i imunologiju, Medicinski fakultet, Univerzitet u Kragujevcu


ABSTRACT

The underlying mechanisms of skin inflammation in atopic dermatitis (AD) are not completely understood but inflammatory cell activation and dysregulated cytokine production appear to play a critical role in pathogenesis of AD. Inducible nitric oxide synthase (iNOS) is expressed by dermal endothelial cells and perivascular inflammatory cells in the atopic skin lesion, suggesting the involvement of nitric oxide (NO) in the skin inflammation of AD. Among the proinflammatory cytokines interferon-gamma (IFN-γ) is the most efficient inducer of NO production. The purpose of the study was to examine IFN-γ and NO plasma levels in patients with AD. We have also measured NO production by monocytic (MN) and polymorphonuclear (PMN) leukocytes in cells culture systems. Seventeen patients with atopic dermatitis and ten healthy volunteers were included in this study. NO plasma levels of patients with AD were significantly increased (p=0.001) as compared to nonatopic controls. No significant difference in NO levels in MN cells cultures of AD patients and nonatopic controls was observed (p=0.083). NO levels in PMN cells cultures of AD patients were significantly higher (p=0.011). IFN-γ plasma concentration in AD patients was significantly increased as compared to nonatopic controls (p=0.005). Our results suggest that PMN leukocytes in AD patients could be source of increased NO plasma levels in patients with AD. As our patients have lasting eczematous skin lesions, our results also lend support to the two-phase-model for the pathogenesis of AD were in a second phase expression of Th-1 cytokines, such as IFN-γ, predominates.

Key words: atopic dermatitis, nitric oxide, interferon-gamma.

INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease of unknown aetiology, characterized by typically distributed eczematous skin lesions with lichenification, pruritic excoriations, dry skin and a susceptibility to skin infections (1). A complex interrelationship of genetic, environmental, skin barrier, pharmacological, psychological and immunological factors plays an important part in the pathogenesis of the disease (2). The mechanisms involved in inflammation in AD are not completely clear, but inflammatory cell activation and dysregulated cytokine production appear to play critical roles in the pathogenesis of AD (1).

Controversies still exist regarding the role of the Th2 and Th1 immune system in the pathogenesis of AD (3-8). Skin lesions in AD are characterized by hypertrophy of the dermis and epidermis and infiltration by T cells, suggesting the possible role of cytokines in these processes (9). The exact role of IFN-γ in AD is still unclear. IFN-γ is a T-cell cytokine and a key mediator of Th1 cytokine expression (10). IFN-γ is known as a potent immunoregulator and has wide-ranging effects on the immune system. It is a key cytokine in the pathogenesis of AD (11). IFN-γ is not only a key mediator of Th1 immune response but also a potent inducer of NO production (12). NO plays a crucial role in the pathogenesis of AD (13).

Our study aimed to examine IFN-γ and NO plasma levels in patients with AD. We have also measured NO production by monocytic (MN) and polymorphonuclear (PMN) leukocytes in cells culture systems. Seventeen patients with atopic dermatitis and ten healthy volunteers were included in this study. NO plasma levels of patients with AD were significantly increased (p=0.001) as compared to nonatopic controls. No significant difference in NO levels in MN cells cultures of AD patients and nonatopic controls was observed (p=0.083). NO levels in PMN cells cultures of AD patients were significantly higher (p=0.011). IFN-γ plasma concentration in AD patients was significantly increased as compared to nonatopic controls (p=0.005). Our results suggest that PMN leukocytes in AD patients could be source of increased NO plasma levels in patients with AD. As our patients have lasting eczematous skin lesions, our results also lend support to the two-phase-model for the pathogenesis of AD were in a second phase expression of Th-1 cytokines, such as IFN-γ, predominates.
cells, monocyte-macrophages and eosinophils. Acute skin lesions exhibit increased levels of IL-4 and IL-5 mRNA and protein, suggesting preferential accumulation of Th2 cells (3). Chronic eczematous AD skin lesions contain increased levels of IFN-g mRNA and protein, alone or in combination with IL-4 (9-11), suggesting a switch from an initial Th2 response to a mixed Th1 plus Th2 response. A switch in time from a Th2 to a mixed Th1 plus Th2 response is also observed when patch tests with house dust mite allergen are performed in patients with AD (11). Initially, IL-4 predominates over IFN-g at the site of antigen application, but later the situation is reversed and IFN-g predominates over IL-4 (12). These studies indicate that both Th-cell subsets contribute to the pathogenesis of this disease and suggest that expression of Th1-like and Th2-like cytokines in AD is not mutually exclusive.

The proinflammatory cytokines interferon-gamma (IFN-g), tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) are involved in induction of inducible nitric oxide synthase (iNOS) and production of nitric oxide (NO). iNOS is expressed by dermal endothelial cells and perivascular inflammatory cells in the atopic skin lesion, suggesting the involvement of nitric oxide in the skin inflammation of AD (13). Among the proinflammatory cytokines, IFN-g is the most efficient inducer of NO production (14).

In our study, we examined IFN-g and NO plasma levels in patients with AD. We also measured NO production by mononuclear (MN) and polymorphonuclear (PMN) leucocytes in a cell culture system.

MATERIALS AND METHODS

Patients and controls

Seventeen patients with AD (8 male and 9 female; aged 5 to 21, mean 12.76±4.74), were included in this study. The diagnosis was based on the criteria of Hanifin & Rajka (19). AD was stable, without recent flare-up; none of the patients was treated with immunosuppressive drugs. AD was graded according to SCORAD (20). The mean SCORAD index was 32.35+14.73. The control group consisted of ten healthy volunteers (6 male and 4 female, aged 6 to 21, mean 13.5±4.45) with negative personal history of atopy.

METHODS

A specimen of peripheral venous blood was collected in the morning. Nitric oxide and IFN-γ levels in the plasma samples were measured. After centrifugation, one part of plasma was used for nitrite extraction and the second part was stored immediately at -20°C until analysis. At the same time, MN and PMN cell cultures were prepared. After 24 h of incubation, culture supernatants were collected and stored at -20°C until use.

NO DETERMINATION

Before testing, the plasma samples were deproteinized by using acid solution. In 1500 μl tubes, 100 μl of 3 M perchloric acid, 400 μl of 20 mM EDTA and 200 μl of plasma were added. Extracts were incubated on ice for 20 minutes, with occasional mixing, and then centrifuged at 1500 rpm for 5 minutes. The supernatants were removed into other tubes and 120 μl 2 M potassium-carbonate was added to neutralize the extracts. The neutralized extracts were stored at -20°C until testing. Immediately before use, extracts were defrosted and centrifuged in order to reduce the presence of potassium-perchlorate particles.

Nitrite (NO2-) is a stable product of NO metabolism that reacts with Griess reagent to create a pink colour. Plasma nitrite levels were measured by spectrophotometric assay as described by Miranda et al. (21). We also used this assay to measure nitrite levels in MN and PMN cell culture supernatants. Griess reagent was prepared just before the experiment by mixing equal amounts of stocks: 2% (w/v) sulfanilamide dissolved in 5% HCl and 0.1% (w/v) aqueous solution of N-1-naphthyl-ethylene-diamine-dihydrochloride (N-NEDA). Nitrite solutions in H2O (10 mM) were prepared fresh daily. The experiment was performed at room temperature. The nitrite standard solution was serially diluted (100-1.6 μl) in a 96-well, flat-bottomed, polystyrene microtiter plate in final volume of 100 μl. After loading the plate with plasma samples (100 μl), Griess reagent was added to each well. Distilled water and Griess reagent were used as the standard blank. The absorbance was measured at 540 nm (Multiplate reader 230S, Organon) following 30 minutes of incubation. Nitrite concentration was determined by using Xia software for data analysis, based on the standard curve that was obtained by linear regression absorbance values for each standard (reduced for blank values). Results were expressed as nanomoles per millilitre (nmol/ml).

MN AND PMN CELL CULTURE PREPARATION

MN and PMN leucocytes were obtained from venous peripheral blood according to a widely accepted method by Boym (22). We prepared MN (1x106/ml) and PMN (2x106/ml) cell cultures, and incubated them for 24 h in RPMI 1640 medium with 200U penicillin and 200 mg/ml streptomycin, at 37°C in an atmosphere of 5% CO2. After incubation was finished, supernatants were collected and stored at -20°C until use. Just before use, supernatants were defrosted and centrifuged in order to remove any residual cells.
NO DETERMINATION IN CELL CULTURES

NO production in MN (1x10^6/ml) and PMN (2x10^6/ml) cell cultures was measured indirectly by measuring NO concentration in culture supernatant. NO levels were measured by quantifying nitrite concentrations as described previously, based on a standard curve (RPMI 1640 medium was used as standard blank instead of distilled water).

IFN-γ DETERMINATION

We measured the levels of IFN-γ in the plasma of patients with AD and in controls, using a commercial enzyme-linked immunosorbent assay (HUMAN IFN-γ Elisa kit II BD Biosciences, Pharmingen, San Diego, CA, USA), according to the manufacturer’s instructions. Results were expressed as pg/ml.

STATISTICAL ANALYSIS

All values are expressed as mean±standard deviation (X±SD) and median. Commercial SPSS (Statistical Package for the Social Sciences) version 11.0 was used for statistical analysis. Normal distribution of data was tested by using the Kolmogorov-Smirnov test. Statistical evaluation was performed with the nonparametric Mann-Whitney U-test and Kruskal-Wallis test for unpaired data and Student's t-test for paired data. A P value of<0.05 was considered to be significant and highly significant when <0.01.

RESULTS

NO plasma levels (Fig.1)

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Figure 1. NO plasma levels

No significant difference in NO levels was observed between MN cultures of AD patients and nonatopic controls (Mann Whitney U test; p=0.083). The mean concentration of NO in MN cultures of AD patients was 1.82±2.61, median=0 (more than 50% of all measured values were 0). The same parameter in MN cultures of healthy controls was too low to be measured.

The NO levels in PMN cultures of AD patients were significantly higher as compared to healthy controls (Mann Whitney U test; p=0.011) The mean concentration was 2.88±2.97 and median 2.49, while the same
parameters in PMN cultures of healthy controls were too low to be measured.

IFN-γ plasma levels (Fig. 4)

![IFN-γ plasma levels](image)

There was a significant difference between IFN-γ plasma concentration in AD patients and in healthy controls (t-test: \( p = 0.005 \)). IFN-γ plasma concentrations in AD patients (21.77 ± 4.85) were significantly increased as compared to nonatopic controls (16.56 ± 1.61).

**DISCUSSION**

Although the mechanisms involved in inflammation in AD are not completely clear, inflammatory T-cell activation and dysregulated cytokine production appear to play a critical role in pathogenesis of AD (1). As T-cells are potent producers of a large variety of cytokines, several studies have been performed to investigate the cytokine pattern in AD (4-11). These studies indicate that both Th2- and Th1-type cytokines contribute to the pathogenesis of skin inflammation. Grewe et al. (3,10) demonstrated in situ expression of Th1-like (IFN-γ) and Th2-like (IL-4) cytokines in lesional AD skin, indicating that eczematous skin lesions are not the result of exclusive expression of either Th1-like or Th2-like cytokines. They proposed a two-phase-model for the pathogenesis of AD. Development of AD skin lesions results from sequential activation of Th cells: in an early phase, Th2-like cytokines are crucial for initiation of atopic eczema, and in a second phase, expression of Th1-like cytokines (such as IFN-γ) predominates. The predominance of IFN-γ-producing T-cells is responsible for the chronicity of AD lesions and determines the severity of disease.

Few published studies examine levels of IFN-γ in serum. Aleksza et al. (15) measured the levels of circulating cytokines (IFN-γ, IL-4, IL-10 and IL-13) in serum of AD patients and healthy controls. The levels of all cytokines were elevated in patients with AD, but significant differences was found only for IL-10 and IL-13. Niwa (16) assessed cytokine levels in both plasma and serum from the patients with AD and healthy volunteers and found that IL-2, IL-5, IL-10 and IFN-γ were significantly elevated in the plasma from AD patients, but not in their serum.

In our study, IFN-γ plasma concentration in AD patients was significantly increased as compared to nonatopic controls. According to the two-phase-model for the pathogenesis of AD, a predominance of IFN-γ-producing T cells is responsible for the chronicity and maintenance of eczematous skin lesions. As our patients have lasting skin lesions, our results also lend support to the two-phase-model for the pathogenesis of AD, in which a second phase involves predominate expression of Th-1 cytokines, such as IFN-γ.

IFN-γ is the most efficient inducer of NO production (14). IFN-γ plasma levels directly determine NO plasma levels, as well as ex vivo NO production by leucocytes. NO has been found to be important in a number of different physiological processes. NO plays an important role in the initiation and progression of atopic diseases such as asthma, hay fever and atopic dermatitis (24,25). Of particular relevance to the skin and atopic dermatitis are the roles of NO in vasodilatation, inflammation, and immunomodulation, as well as oxidative damage to cells and tissues (13).

Taniuchi et al. (17) showed increased NO metabolite levels in serum of children, aged 0.4-8 years with AD. These authors also showed a correlation between serum nitrate (NO3-) levels and skin lesion severity. Guzik et al. (18) undertook a similar study with adults, aged 18-47 years with AD, but could not confirm the observation of Taniuchi. They postulated that the difference between their observations and findings by Taniuchi et al. could be explained by the fact that the area of affected skin relative to total skin surface and body weight is smaller in adults with AD as compared to children with AD. Tsukahara et al. (23, 24) measured urinary concentrations of nitrite/nitrate in children with exacerbation of AD. They did not find significant differences in those parameters between AD patients and healthy controls. Their results suggest that endogenous NO synthesis in children with exacerbation of AD is similar to that in healthy controls.

Our patients were 5 to 21 years old and blood samples for analysis were taken during relative clinical remission (no significant vasodilation, erythema or oedema). NO plasma levels in our patients were significantly increased as compared to healthy controls. The results suggest that NO plasma concentrations in patients older than 8 years in clinical remission are increased as compared to healthy controls.
comparisons of our results with those obtained in similar studies.

NO levels in MN cultures of AD patients included in our study were very low and no significant difference was observed between NO levels in MN cultures of AD patients as compared to nonatopic controls. Those results suggest that mononuclear leucocytes do not represent the cellular source of increased NO plasma levels in patients with AD. NO levels in PMN cultures of AD patients were low but significantly higher as compared to healthy controls. Our results suggest that PMN in AD patients represent a source of increased NO plasma levels in patients with AD. Other sources such as endothelial cells, keratinocytes, Langerhans cells of the affected area, and their contribution to increased NO plasma levels, should not be overlooked.

At present, there is no treatment directed at the underlying cause of AD. A better understanding of the mechanisms that underlie AD is therefore critical for the design of new and more effective treatments for this common disease. Our results indicate that use of NO pathway modulators might be a potentially useful strategy for the treatment of AD. Also, in further research we plan to examine AD lesional skin for cytokine expression during remission and exacerbation of AD.

**ABBREVIATIONS:**

AD – Atopic dermatitis,
IFN-$\gamma$ - Interferon-gamma,
IL – Interleukin,
MN – Mononuclear,
NO – Nitric oxide,
PMN – Polymorphonuclear.

**REFERENCES**

