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Serum cytokine changes in Turkish children infected with *Giardia lamblia* with and without allergy: Effect of metronidazole treatment

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Abstract

The present report is a case control study aimed to determine the levels of cytokines and other parameters in the sera of allergy-complicated and uncomplicated giardiasic children before and after metronidazole treatment. The study included a total of 126 subjects; 52 giardiasic children, 34 allergy-complicated giardiasis (36.9%) and 34 healthy controls, as well as six cases of giardiasis simultaneously infected with other parasites or bacterial pathogens. Serum levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, soluble IL-2 receptor (sIL-2R), IL-6, IL-8, nitric oxide (NO), and C-reactive protein (CRP) were determined. TNF-α and sIL-2R levels significantly increased in giardiasic cases. IL-1β, IL-6, IL-8, CRP, and NO levels increased only in the cases associated with allergy. All increased variables significantly decreased following metronidazole treatment and returned to normal levels. Metronidazole-treated patients became 100% parasite free. In conclusion, increased TNF-α and sIL-2R may be involved in pathogenesis of non-allergic giardiasis and probably Th1 type immune response seems to be predominant and this response may be protective rather than causative of the disease. Activation of the immune system takes place in giardiasis. It is broader and more intense in allergy-complicated giardiasis than that of uncomplicated cases, most probably due to non-invasive character of *G. lamblia*. Enhanced IgE production pointed to Th2-type immune response and confirms its association with allergy.
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1. Introduction

Giardia lamblia is one of the most common intestinal parasites being endemic in Turkey (Balcioglu et al., 2003). The rate of infection varies in different parts

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of Turkey but as high as 50% was reported in the south eastern part (Balcioglu et al., 2003; Simsek et al., 2004; Ozturk et al., 2004). Children are more frequently affected than adults (Ali and Hill, 2003).

Excretory and secretory antigens released by the parasite are responsible for the immune responses (Jimenez et al., 2004). The immune response may be protective and it is mediated via both T-cells and IgA antibodies (Singer and Nash, 2000; Langford et al., 2002; McDonald, 2003). Of the T-cells, $CD4^+$ but not $CD8^+$ cells are responsible for cytokine production (Djamiatun and Faubert, 1998). Type and amount of cytokine responses may be affected by variability in duration and severity of infection as well as great antigenic variation of specific variant surface proteins of *G. lamblia* (Muller and Gottstein, 1998; Adam, 2001). IL-6 appears to be important in the early control of acute *G. lamblia* infection; IL-6-deficient mice are only able to control infection slowly (Bienz et al., 2003; Zhou et al., 2003). Proliferation of $CD4^+$ T-cells with cytokine response such as gamma interferon production was also addressed in giardiasis (Ebert, 1999). Interestingly, it was found that IFN-γ and IL-4 knockout mice can control giardia infections provided that Th2 cells are sufficient (Singer and Nash, 2000). Intensity of *G. muris* infections was increased in mice treated with an anti-IFN-γ antibody (Venkatesan et al., 1996).

It was demonstrated that 70% of symptomatic giardiasis was associated with allergy (Di Prisco et al., 1998). An increased incidence of urticaria and food allergies in giardiasis was suggested to be the consequence of an alteration in intestinal mucosal cells leading to absorption of foreign substances and development of allergy (Hardin et al., 1997; Di Prisco et al., 1998). Mast cell response was found to be important for protective immunity via increased IL-6 production as well as in allergy via degranulation and release of a variety of inflammatory mediators such as histamine, cytokines, and leukotrienes (Erlich et al., 1983; Venkatesan et al., 1997; Li et al., 2004). These mediators act on different tissues and cause allergic diseases (Yazdanbakhsh et al., 2002; Giacometti et al., 2003). Mice with mast cell suppression or deficiency were more prone to chronic or enhanced giardiasis (Erlich et al., 1983; Li et al., 2004). IgE has been shown to be important in immunity to parasitic infections particularly helminthiasis (Abbas and Lichtman, 2003). An elevation in total IgE levels was reported in giardiasis (Perez et al., 1994; Di Prisco et al., 1998). Giardia proteins may be responsible for such an increase since they induce IgE response (Jimenez et al., 2004).

Nitric oxide (NO) is an important bio-regulatory molecule. It is produced by a number of different cell types in response to cytokine stimulation and plays an important role in immunologically mediated protection against numerous parasites in vitro and in animal models (James, 1995). It may inhibit proliferation and differentiation of *G. lamblia* via its cytostatic action (Eckmann et al., 2000; McDonald, 2003).

CRP is an acute phase marker produced by hepatocytes in response to cytokines. It is used to detect and monitor the activity of a range of inflammation and infections (Vermeire et al., 2004). Increases in serum CRP and IL-6 levels were also observed in allergic reactions (Lin et al., 2001).

Although the role of cytokines in giardiasis has been widely investigated in animal models, there is a paucity of published studies that address the cytokine response in human cases. Moreover, there have been no studies on NO and CRP in human giardiasis yet. Thus, the present work was undertaken to study the levels of these variables in the sera of giardiasic children with and without allergy and to assess the effect of metronidazole therapy.

2. Materials and methods

2.1. Subjects

The study covered 126 subjects referred to the Turgut Ozal Medical Centre, Malatya, located in eastern Turkey. It comprised a total of 92 giardiasic children (44 males and 48 females) and 34 healthy controls. The ratios of males to females in patient and control groups were 0.91 and 0.79, respectively. The ages of the subjects with giardiasis ranged from 2.5 to 15 years (mean ± S.D.; 8.9 ± 5.3 years). Healthy children were between 4 and 14 years old (mean ± S.D.; 7.8 ± 2.7 years). The patients consulted with Pediatrics and Dermatology Clinics for gastrointestinal and dermatologic complaints. It included 52 giardiasic subjects having gastrointestinal symptoms, such as diarrhoea, abdominal pain, flatulence, nausea, and

occasional vomiting for 1–10 days. Thirty-four giardiasic children had a history of allergy. Of these, 25 had gastrointestinal symptoms for 3–15 days accompanied by cutaneous allergy, such as urticaria and sometimes low grade fever (Group I). Nine children had chronic urticaria (remitting and relapsing bouts of erythematous and pruriginous lesions) with intermittent gastrointestinal symptoms for more than one month and this group may be regarded as chronic giardiasis (Group II). Moreover, other six giardiasic children were suffering from other diseases, such as sore throat, gastritis, salmonellosis, brucellosis, and ascariasis. The healthy control group was from primary school and nursery. Decision was made whether control subjects were healthy according to the results of routine laboratory examinations (parasitological, biochemical and haematological) in addition to apparent healthy status with no history of current or recent illness.

Giardiasis was diagnosed by detecting cysts and/or trophozoites of *G. lamblia* in three consecutive stool examinations using wet mount with normal saline and Lugol's iodine and formalin–ether concentration methods. Flotation stool examination (saturated sodium chloride solution) was also made to confirm the presence of other intestinal parasites. All patients were successfully treated with metronidazole at a dose of 25 mg/kg/day tid for 10 days (Ali and Hill, 2003). Those with allergy were also treated with an additional antihistaminic drug, such as hydroxyzine hydrochloride at a dose depending on age to relieve allergic symptoms. Symptoms disappeared and the efficacy of the treatment was verified by microscopic examination of three consecutive stool specimens 1 month after treatment. All stools were found to be negative for *G. lamblia*. Permission was taken from the local Ethical Committee of the Medical School of Inonu University and parents of all subjects gave their informed consent to the study.

2.2. Cytokines

Serum levels of IL-Iβ, sIL-2R, IL-6, IL-8 and TNF-α were measured by means of a solid-phase two site chemiluminescent enzyme immunometric assay with the immulite automated analyzer (DPC IMMULITE, manufactured by EURO DPC Ltd. under a quality system approved by the British Standards Institute). The results were expressed in pg/ml except for sIL-2R (U/ml).

2.3. Total IgE

It was measured by ELISA method (Clone System, Biochem Immuno Sytems, Italia S.P.A., Italy) with the minimal detectable concentration of 5 IU/ml following the manufacturer's directions. According to kit guidelines, expected normal values of total IgE are between 0 and 52 IU/mL in children.

2.4. Total nitrites

NO is unstable and rapidly converted to nitrates and nitrites, therefore, it is necessary to determine both nitrite and nitrate concentrations in the samples as representatives for NO (Moshage et al., 1995). It is a common practice to use enzymatic or chemical reduction to convert all nitrates to nitrites in a sample and then measure total nitrite (Moshage et al., 1995). In the present study, serum samples were mixed with granular cadmium and shaken for 2 h for chemical reduction of nitrate to nitrite, and total nitrite was then measured by spectrophotometry using the Griess reagent. All chemicals and biochemicals used for NO determination were obtained from Sigma (St. Louis, MO, USA).

2.5. C-reactive protein

CRP levels were measured via automated analyzer system based on nephelometry (Boehring Nephelometer 100 Analyzer, Germany). They were expressed as milligram per liter.

2.6. Statistical analysis

Results were expressed as mean and standard deviation (mean ± S.D.). Comparison of means of variables showing normal distribution was calculated by parametric unpaired Student's *t*-test whereas statistical analysis of parameters not showing normal distribution was done by non-parametric Mann–Whitney *U*-test. Pearson's linear correlation test was used to assess correlation between parameters. The level of significance was set at $p < 0.05$ in all analyses. All analyses were performed using the SPSS 9.0 software.

3. Results

Of 92 giardiasic individuals, 34 cases were allergy-complicated giardiasis (36.9%). The cytokine values of giardiasic individuals were higher than normal upper limits and can be considered as elevated. The mean levels of cytokines, total IgE, CRP, and nitrate/nitrite were shown on Table 1. The uncomplicated giardiasic patients had significantly elevated serum levels of sIL-2R and TNF-α compared with healthy controls but levels of IL-1β, IL-6, IL-8, NO, and CRP were not significantly changed ($p < 0.05$).

In children with both giardiasis and allergy, the mean levels of cytokines, NO, and CRP increased (Table 1). Total IgE levels increased in sera of all allergic children and 38.5% of non-allergic cases and they were higher than controls. In two groups of allergy-associated giardiasis, there were no statistical differences between the parameters but the mean levels of sIL-2R, IL-6, CRP, and NO in those with chronic urticaria (Group II) were significantly lower than those of Group I (Table 2). Six children simultaneously affected by other infectious agents or parasites had markedly intense cytokine response and they were excluded.

No significant correlations were determined between parameters in giardiasic cases without allergy. Within allergy-complicated group, strong correlations between IL-6 and each of CRP and NO were seen ($r = 0.889$ and 0.854, $p < 0.001$ respectively). There were also positive correlations between the levels of CRP and each of IL-1β and NO ($r = 0.721$ and 0.728, $p < 0.001$, respectively). No correlation was demonstrated between cytokine production and total serum IgE levels in any group.

Table 2
Comparison of studied parameters (mean ± S.D.) in sera of two groups of allergy-complicated giardiasis

Parameters	Group I[a] ($n = 25$)	Group II[b] ($n = 9$)	p-value[c]
TNF-α (pg/mL)	42.9 ± 7.6	33.6 ± 7.5	>0.05
IL-1β (pg/mL)	16.4 ± 6.9	9.9 ± 3.4	>0.05
sL-2R (U/mL)	2244 ± 690	1665 ± 426	<0.01
IL-6 (pg/mL)	28.7 ± 14.4	17.8 ± 3.7	<0.05
IL-8 (pg/mL)	20.4 ± 11.1	23.2 ± 10.1	>0.05
CRP (mg/L)	20.9 ± 13.7	7.8 ± 3.3	<0.01
Total IgE (IU/mL)	143.4 ± 36.9	169.1 ± 27.5	>0.05
Total nitrite (μmol/L)	57.1 ± 9.5	38.7 ± 5.4	<0.01

[a] Having acute urticaria following gastrointestinal symptoms.
[b] Having chronic urticaria only.
[c] Mann–Whitney *U*-test.

Table 3
Normal limits and mean values of serum cytokine following metronidazole therapy

Cytokines	After therapy (mean ± S.D.)	Normal limits[a]
TNF-α (pg/mL)	9.1 ± 0.7	4.0–10.0
IL-1β (pg/mL)	<5.0	0.0–10
sL-2R (U/mL)	683 ± 136	200–1000
IL-6 (pg/mL)	8.62 ± 2.8	2.0–15
IL-8 (pg/mL)	12.9 ± 11.3	0.0–70

[a] According to kit values.

All subjects were treated with metronidazole and the cure rate was 100% with no adverse reaction. All the patients were free of *G. lamblia*. Elevated serum parameters declined to normal levels one month after the therapy (Table 3).

Table 1
Comparison of cytokines, nitrate/nitrite, total IgE, and CRP levels (mean ± S.D.) in the sera of giardiasic patients and controls

Parameter	Controls ($n = 34$)	Patients with giardiasis ($n = 52$)	P-value	Patients with allergy ($n = 34$)	P-value
TNF-α[a] (pg/mL)	8.6 ± 0.9	18.5 ± 5.7	$<10^{-3}$	40.4 ± 18.1	$<10^{-3}$
IL-1β[b] (pg/mL)	<5.0	<5.0	>0.05	15.2 ± 12.7	$<10^{-3}$
sL-2R[b] (U/mL)	631 ± 147	1501 ± 361	$<10^{-3}$	2090 ± 676	$<10^{-3}$
IL-6[b] (pg/mL)	6.7 ± 2.5	8.5 ± 2.2	>0.05	25.8 ± 13.3	$<10^{-3}$
IL-8[b] (pg/mL)	7.7 ± 2.3	10.6 ± 4.8	>0.05	21.19 ± 11.2	$<10^{-3}$
CRP[b] (mg/L)	<3.1	4.5 ± 0.3	>0.05	17.5 ± 13.9	<0.05
Total IgE[a] (IU/mL)	31.3 ± 12.28	83.4 ± 33.2	$<10^{-3}$	150.2 ± 36.1	$<10^{-3}$
Total nitrite[b] (μmol/L)	27.3 ± 3.7	29.7 ± 3.1	>0.05	52.2 ± 18.7	<0.05

[a] Student's *t*-test
[b] Mann–Whitney *U*-test

4. Discussion

We addressed human giardiasis since a few works were performed on human cases (Gottstein et al., 1991; Perez et al., 1994; Ebert, 1999). Much knowledge on the immune response has been originated from animal models infected with either G. *lamblia* or *G. muris* or in vitro studies involving the challenge of immune cells with trophozoites or cysts (Venkatesan et al., 1996; Djamiatun and Faubert, 1998; Stager et al., 1998). However, results obtained from such studies may not necessarily represent the conditions of natural human giardiasis. This is due to fact that laboratory conditions (medium, property of lymphoid tissues), strain of parasite, host susceptibility, the immune status and host strain, and existence of natural pathogens in laboratory animals (e.g. *G. muris* and others) may affect the results and cause problems in interpretation of the assays (Baker, 1998).

sIL-2R is a useful marker of in vivo immune system activation and its rate of release reflects T- and B-cells as well as macrophage activation (Rubin and Nelson, 1990). The present work confirms such activation since sIL-2R significantly increased despite non-invasiveness of the studied parasite (Josimovic-Alasevic et al., 1988). In addition, TNF-α also increased in giardiasic children without allergy. Although TNF-α is a pyrogen, no signs of fever were observed in these children. This may be due to the fact that concentration of these cytokines is not enough to induce pyrexia. Pro-inflammatory mediators, such as IL-1β and IL-6, and a chemokine, IL-8 that attracts neutrophils to the site of infection, were not elevated in this group (Table 1). These cytokines may have no influence on immunity to giardiasis.

A large number of parasites have been correlated with allergy but few data exist as regards to *G. lamblia* infection (Di Prisco et al., 1998; Giacometti et al., 2003). Our results confirmed the association of giardiasis with cutaneous allergic conditions since 36.9% of giardiasic cases had symptoms of allergy, such as itching and urticaria. Giardia antigens could contain allergen-like molecules enhancing the total and specific IgE production (Jimenez et al., 2004). Cross linking of IgE on mast cells and eosinophils through fcεR1 initiates activation and subsequent release of mediators, such as cytokines, histamine, and leukotrienes. These substances may have a role in clearing the parasite or even in development of allergy (Abbas and Lichtman, 2003; Li et al., 2004). Marked immune response in allergy-complicated giardiasis via the elevation of studied immune markers was observed in the present study (Table 1). It enhanced in group I more than the group with chronic urticaria since all parameters decreased in the latter group (Table 2). This clearly indicates that with the development of giardiasis, cytokine and other markers profile change. It may be due to decrease in the parasite burden as disease progresses to a chronic stage. Our findings may add evidence to the role of *G. lamblia* in inducing allergy and chronic urticaria. This may be in part due to allergic mechanisms induced by giardia antigens that function continuously for a long period and in part due to the enhancing sensitization towards food allergens in sequel to increased antigen penetration through damaged intestinal mucosa (Di Prisco et al., 1998). However, disappearance of urticaria symptoms occurred after specific therapy with metronidazole. Cytokine response may not only confine to giardiasis since certain cytokines are generally produced in considerable amount by basophils and mast cells in non parasitic allergic conditions (Bellanti, 1998; Ferrer et al., 2002; Yazdanbakhsh et al., 2002). High levels of total IgE were observed in giardiasis particularly in cases complicated with allergy. IgE elevation even in giardiasis without allergy suggests that Th2 cells respond in both conditions although the difference may be a matter of degree. To better elucidation, specific anti-giardia IgE should be investigated in future studies.

According to the literature review, the role of CRP has not yet been investigated in giardiasis. It did not change unless giardiasis was complicated with allergy. There were positive correlations between the levels of CRP and each of IL-1β and IL-6 ($r = 0.854$ and 0.889, $p < 0.001$). Thus, elevated serum CRP levels may be due to increased productions of IL-1β and IL-6 (Castell et al., 1989).

Cytokine profile was markedly different in six giardiasic children who simultaneously suffered from other diseases, such as brucellosis, typhoid fever, and ascariasis. These cases were excluded from the statistical evaluation since they might lead to misinterpretation. Finally this study demonstrated that metronidazole extensively used in our country to cure intestinal protozoal infections was still effective in giardiasis since all patients treated successfully.

In conclusion, the present study supports the involvement of Th1 cells (sIL-2R) in giardiasis without allergy. Marked activation of the immune system occurred in allergy-complicated giardiasis via elevation of all studied parameters demonstrating their association by complex regulating mechanisms with allergy and less likely to giardiasis itself. Elevated total IgE levels also suggest the role of Th2 cells. Metronidazole is still an important drug for treatment of giardiasis.

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