Original Article

The levels of cytokines IL-4, IL-10, IL-12p40, IFN-γ during acute toxoplasmosis

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Summary:

Background: The different roles of cytokines IL-4, IL-10, IL-12p40, IFN-γ during acute toxoplasmosis disease in pregnant women.

Objective: Evaluate the role of cytokines IL-4, IL-10, IL-12p40, IFN-γ during the first trimester of pregnant women with acute toxoplasmosis.

Patients and Methods: Two hundred-seventy pregnant women (in the first trimester-12 gestational weeks) admitted to AL-Yarmouk teaching hospital suspected with toxoplasmosis, between (1-5-2010 and 1-12-2010), their diagnosis was made by using ELISA test for anti toxoplasma IgM antibodies and for measurements of cytokines levels IL-4, IL-10, IL-12p40 and IFN-γ.

Results: Based on the serum level of IgM antibodies against Toxoplasma gondii 60 patients showed a significant increase of IgM antibodies level from total pregnant women suspected with toxoplasmosis. Most infections between age groups (20-24) years and (30-34) years. The mean serum level of IL-4 and IL-10 showed significantly decreased level in patients with acute toxoplasmosis, while the other two cytokines IL-12p40 and IFN-γ showed significantly increased mean serum level in the same patients.

Conclusions: The study of the immune response to acute infection with Toxoplasma gondii has consistently provided new insights into the importance of both the humoral immune response as antibody production, which includes IgM, and IgG in addition to cell-mediated immunity and the role of cytokines in the control of this intracellular pathogen, such effects can be explain in this study by decreased serum levels of Th2 cytokines production such as IL-4, IL-10 and increased serum level of IL-12p40 and IFN-γ could contribute to spontaneous abortion during the first trimester of pregnant women with acute toxoplasmosis.

Keywords: Toxoplasma gondii, IgM, IL-4, IL-10, IL-12p40, IFN-γ.

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Introduction:

Toxoplasma gondii is an obligate intacellular protozoan parasite occurring worldwide in humans and animals (1). Under normal conditions, this infection is largely asymptomatic, but it is an important cause of clinical disease in fetuses, infants and immunocompromised individuals such as patients with AIDS, resulting in the development of toxoplasmic encephalitis (2). T. gondii is also an important cause of ocular disease in both immunosuppressed and immunocompetent individuals (3). The host immune response against T. gondii can be an innate acute response or an antigen–specific cell-mediated immune response, cell mediated immunity is essential for protection against T. gondii (4). Gamma interferon (IFN-γ)-dependent cell mediated immunity plays the major role in resistance to toxoplasmic encephalitis (5). IFN-γ is likely produced predominantly by CD4+Tcells, which are the major source of the production of IFN-γ and TNF-α during systemic infection with T. gondii (6), in addition it is produced by natural killer (NK) cells (7). IFN-γ signals through signal transducer and activator of transcription (STAT) to activate a variety of antimicrobial effector mechanism, including the upregulation of inducible nitric oxide synthase (iNOS). Reactive oxygen intermediates (ROI) produced by iNOS were intitially thought to be the hosts primary means of controlling parasite replication (8). IFN-γ production in response to T.gondii was largely IL-12-dependent in both immunodeficient and immunocompetent individuals (9). Macrophages, dendritic cells and neutrophils were all identified as sources of IL-12 during toxoplasmosis (10), these different cell types become activated and produce IL-12 following the engagement of either chemokine (C-C motif) receptors (CCR) (11,12) or Toll-like receptors (TLR) by parasite-derived molecules (13). The results suggest that the observed reduction in mortality during the early acute phases of infection may be due to the down-regulatory effects of IL-4, but the long-term effects of this interleukin are detrimental, possibly because of the ability of this interleukin to inhibit pro-inflammatory antiparasitic products (14). Thus while a number of studies have shown that Th2 cytokines can have detrimental roles (15), therefore deficient in the Th2

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cytokines IL-4 or IL-10 have been shown to exhibit increased susceptibility to early T.gondii infection (16). IL-10 has inhibitory effects on macrophages, that IL-10 antagonized the ability of IFN-γ primed macrophages to kill intracellular T.gondii, and it was identified as a factor induced by T.gondii infection that contributed to the suppression of T cell function (17). These findings contributed to a model in which T.gondii induces IL-10 production to limit the host immune response and favor parasite replication. Thus, IL-10 was established as a vital player in the control of immunopathology during toxoplasmosis (18).

Patients and Methods:

Two hundred-seventy pregnant women (in the first trimester-12 gestational weeks) admitted to AL-Yarmouk teaching hospital suspected with toxoplasmosis, between (1-5-2010 and 1-12-2010), and 40 healthy pregnant women also in the first trimester as control groups. From each female included in this study 10 ml of venous blood were collected . For serum collection, the tube was centrifuged for 10 minutes at 4°C at 450 Xg. The serum was then aspirated using a Pasteur pipette and dispensed into sterile glass tubes (1 ml in each) and stored at -20°C until used. Enzyme Linked Immunosorbent Assay for the detection of IgM antibodies for Toxoplasma gondii in serum.

Reagents: Materials provided with the kit: (BioCheck, Inc. Foster City, CA, CA; 94404); Microtiter wells: purified Toxoplasma antigen coated wells (12x8 wells); Enzyme conjugate reagent; Sample diluents; Negative control; Cut-off calibrator; Positive control; Wash buffer concentrate (20X); TMB reagent; Stop solution. Reagent Preparation: All reagents should be allowed to reach room temperature (18-25°C) before use; one volume of wash buffer (20X) was diluted with 19 volumes of distilled water. Assay Procedure: The desired number of coated wells was placed into the holder; 1:40 dilutions of test samples, negative control, positive control, and calibrator were prepared by adding 5 µl of the sample to 200 µl of sample diluent. They were mixed well; 100 µl of diluted sera, calibrator, and controls were dispensed into the appropriate wells. For the reagent blank, 100 µl sample diluent were dispensed into 1A well position. The holder was tapped to remove air bubbles from the liquid and mixed well; the wells were incubated at 37°C for 30 minutes; at the end of incubation period, liquid from all wells was removed. The microtiter wells were rinsed and flicked 4 times with diluted wash buffer (1X) and then once with distilled water; 100 µl of enzyme conjugate were dispensed in each well. They were mixed gently for 10 seconds; the wells were incubated at 37°C for 30 minutes; enzyme conjugate was removed from all wells. The microtiter wells were rinsed and flicked 4 times with diluted wash buffer (1X) and then once with distilled water; 100 µl of TMB reagent were dispensed into each well, mixed gently for 10 seconds; the wells were incubated at 37°C for 15 minutes; 100 µl of stop solution (1N HCl) were added to stop reaction; mixing gently was performed for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely and before reading the result, it should be made sure that there are no air bubbles in each well; the optical density (O.D.) was read at 450nm within 15 minutes with a microwell ELISA reader (Titertek Multiskan, Finland). Calculation of the Results: the mean of duplicate cut-off calibrator values Xc was calculated; the mean of duplicate positive control (Xp), negative control (X n) and patient samples (Xs) was calculated; the Toxoplasma IgM index of each sample was calculated by dividing the mean values of each sample (X) by calibrator mean value, Xc. Interpretation of Results: Negative: Toxo M index less than 0.90 is negative for IgM antibody to T.gondii. Equivocal: Toxo M index between 0.91-0.99 is equivocal. Sample should be retested. Positive: Toxo M index of 1.00 or greater is positive for IgM antibody to T.gondii and indicates the probability of current or recent Toxoplasma infection. Calculation of Sensitivity and Specificity of the ELISA Test Sensitivity = a ÷ (a +c), Specificity = d ÷ (b +d) a = True positive , b = False positive , c = False negative , d = True negative

Enzyme Linked Immunosorbent Assay for the detection of IL-4, IL-10, IL-12p40, IFN-γ in serum: Kit contents; materials provided with the kits: (Beckman Coulter, France for IL-4 and Bio-Source, Europe S.A. for IL-10, IL-12p40, IFN-γ). Microtiter plate: 12x8 wells pre-coated with a monoclonal anti-human interleukin (IL-4 IL-10 and IL-12p40) or IFN-γ antibody (ready-to-use); recombinant human interleukin standard (IL-4, IL-10, IL-12p40) or IFN-γ; biotiny monoclonal antibody; streptavidin alkaline phosphatase or streptavidin-HRP conjugate; diluent; washing solution; substrate; stop solution. Assay Procedure: Before carrying out the assay procedure of an interleukin (IL-4, IL-10, IL-12p40) or IFN-γ determination, the kit was left at room temperature (18-25°C) for 30 minutes to equilibrate, as suggested by the manufacturer. After that, the assay was carried out following the instructions in the kits leaflet, which are summarized in the following steps: Serial concentrations (they were dependent on the parameter investigated as suggested by the kits manufacturer) of the standard were made using the diluents; aliquot (50 µl) of the standard or sample was added to the well, and the plate was incubated for two hours at room temperature with shaking; the well was washed with three cycles of washing using the washing solution, with the aid of a micotiter plate washer; an aliquot (50 µl) of biotinylated antibody was added to the well and the plate was incubated for 30 minutes at room temperature; the washing step was repeated (three cycles); an aliquot (100 µl) of streptavidin alkaline phosphatase or streptavidin-HRP (it was dependent on the parameter investigated as suggested by the kits manufacturer) conjugate was added to the well and the plate was incubated for 30 minutes at room temperature; the washing step was repeated (three cycles);

an aliquot (100 µl) of substrate was added to the well and the plate was incubated for 20 minutes at room temperature with shaking; An aliquot (50 µl) of stop solution was added to the well and the absorbance was read at a wave length of 450 nm using ELISA reader.

Calculation of the Results: The sample results were calculated by interplotation from a standard curve that was performed in the same assay as that for the sample (Figures 1,2,3,4 for IL-4, IL-10, IL-12p40 and IFN-γ, respectively), using a curve fit equation.

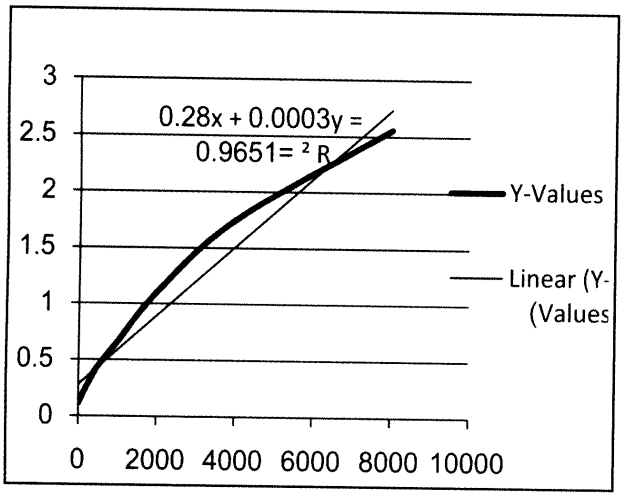

Figure 1: Standard curve of IL-4 serum level.

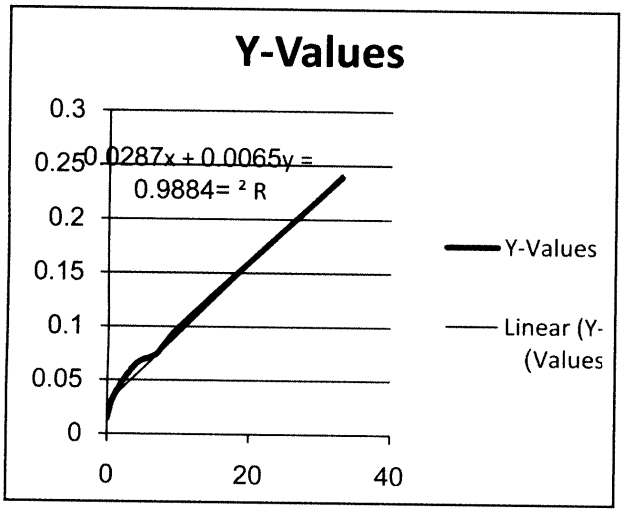

Figure 2: Standard curve of IL-10 serum level.

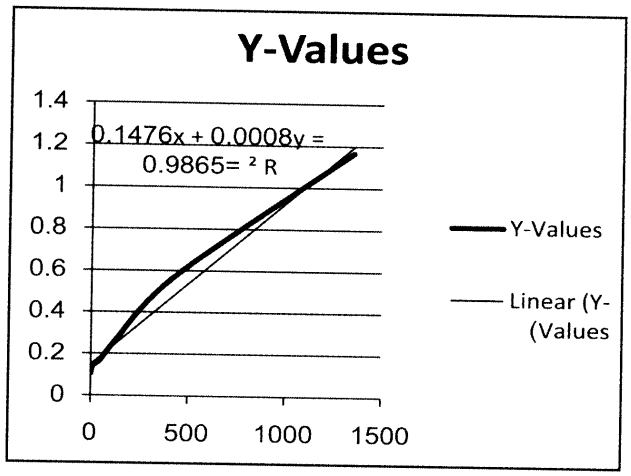

Figure 3: Standard curve of IL-12p40 serum level.

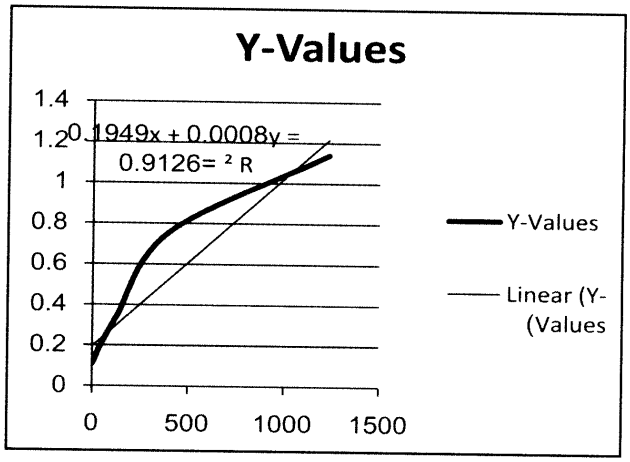

Figure 4: Standard curve of IFN-γ serum level.

Statistical Methods: The values of all parameters were presented as mean ± standard error (S.E.), and significant differences between means were assessed by ANOVA F test, the least significant difference (LSD) or Duncan>s test, using the computer programme Social Package for Statistical Analysis (SPSS) version 7.5, in which a probability (P) equals or less than 0.05 was considered significant.

Results: From the total of two hundred and seventy pregnant women suspected with toxoplasmosis, 60 patients were found to have acute toxoplasmosis disease (showed positive IgM antibodies against T.gondii), this number represent a high frequency of the infection (22.22%) by ELISA method from total patients. All pregnant women who undergo spontaneous abortion have significant increase of IgM antibodies level against T.gondii infections.The sensitivity and specificity of ELISA kit had been calculated and present as 88.46% sensitivity of ELISA methods and100% specificity of ELISA. Most infections of acute toxoplasmosis for pregnant women patients between age groups (20-24) years and (30-34) years (table1).

Table 1: Age distribution of acute toxoplasmosis patients.

Age groups	No.of patients	Percentage%
20-24	22	36.7
25-29	9	15
30-34	15	25
35-39	9	15
40-44	5	8.3
Total patients no.	60	100

Total patients with acute toxoplasmosis showed a significant decreased mean serum level of IL-4 as compared to the control (125 vs. 409 pg/ml) (table 2).

Table 2: Serum level of IL-4 in acute toxoplasmosis patients.

Groups	No.	Mean ± S.E. (pg/ml)	P≤
Controls	40	409±19.8	0.05
Acute toxoplasmosis patients	60	125±21.3	

Significant difference (P≤ 0.05) as compared to the corresponding controls. The same patients showed significantly decreased mean serum level of IL-10 as compared to controls (200 vs. 521 pg/ml) (table 3).

Table 3: Serum level of IL-10 in acute toxoplasmosis patients.

Groups	No.	Mean ± S.E. (pg/ml)	P≤
Controls	40	521±14.4	0.05
Acute toxoplasmosis patients	60	200±16.7	

Significant difference (P≤ 0.05) as compared to the corresponding controls. A significant increased mean serum level of IL-12p40 in total acute toxoplasmosis patients as compared to control subjects (590 vs. 250 pg/ml) (table 4).

Table 4: Serum level of IL-12p40 in acute toxoplasmosis patients.

Groups	No.	Mean ± S.E. (pg/ml)	P≤
Controls	40	250±20.2	0.05
Acute toxoplasmosis patients	60	590±17.9	

Significant difference (P≤ 0.05) as compared to the corresponding controls. These patients showed also significantly increased mean serum level of IFN-γ in total acute toxoplasmosis patients as compared to controls (56.61 vs. 9.72 IU/ml) (table 5).

Table 5: Serum level of IFN-γ in acute toxoplasmosis patients.

Groups	Number	Mean ± S.E. (IU/ml)	P≤
Controls	40	9.72±1.2	0.05
Acute toxoplasmosis patients	60	56.61±1.7	

Significant difference (P≤ 0.05) as compared to the corresponding controls.

Discussion:

ELISA method for the detection of IgM anti-toxoplasma antibodies was chosen in this study and not IgG antibodies because acute toxoplasmosis is usually diagnosed on the basis of IgM antibody detection, in acute infections, IgG and IgM antibodies level generally rise within one to two weeks of infection. The presence of elevated levels of T.gondii specific IgG antibodies indicates that infection has occurred but does not distinguish between recent infection and infection acquired in the distant past. Detection of T.gondii specific IgM has been used as an aid in determining the time of infection by the persistence of IgM antibodies up to 18 months after infection (19). Toxoplasmosis acquires its importance for two reasons. First, it can cause fetal infection if it is acquired during pregnancy, with-predictable manifestations in the fetus and neonate. Second, it is an important cause of morbidity and mortality among immunocompromised patients (20). For normal pregnancy to be established, a Th2 type immune response must be induced by the immune system at the maternal-fetal interface (21). Women who undergo spontaneous abortion may have a stronger Th1-response (22). So, infection of a pregnant mother by T.gondii may induce a Th1 CMI and hence lead to apoptosis then abortion. This can be explained by results about decreased levels of Th2 cytokine production such as IL-4 and IL-10 and increased Th1 cytokine like IFN-γ, in addition, immunity during the early stages of T.gondii infection is largely dependent on NK cell and macrophage interactions that result in the production of IFN-γ and IL-12, this explain increased serum level of IFN-γ and IL-12 during acute toxoplasmosis infection. Because T.gondii can be transmitted from a recently infected mother to her fetus, a rapid and accurate diagnosis of the infection is critical for establishing proper clinical care (23). When a pregnant woman is found to be infected with T. gondii, the next step is to determine whether the fetus is infected. Physicians most often use polymerase chain reaction (PCR) testing of amniotic fluid to diagnose congenital toxoplasmosis (24). In the current study, the entire cases positive for T.gondii, their pregnancy was ended with abortion.This issue was investigated where levels of many sex hormones, most notably estrogens and progesterone, are vastly increased during pregnancy, and consequently their effects on the immune system can be profound. The normal physiological role of these changes would appear

to be to protect the developing fetus from the mothers immune response. Although this hormonal manipulation of the immune system serves to prevent the fetus from being rejected, it also has consequences for parasitic infection. The ability of pregnancy to affect the immune system and indeed of the immune system to affect pregnancy has two important consequences for parasitic infection. First, pregnancy will favor the survival of many parasites that require a type 1 response to control them. Second, parasitic infections that induce a strong type 1 response will adversely affect pregnancy. Both of these scenarios have been demonstrated with the protozoan parasite T. gondii (25). Moreover, if infection occurs in the first trimester, when hormone levels are low and there is little Th2 bias, the chance of transmission to the fetus is low, although the chance of abortion is high. the Th1 response induced early during T. gondii infection will induce abortion early in pregnancy. In contrast, during the late stages of pregnancy, the strong Th2 bias and the diminished NK cell, macrophage, and CD8+ T-cell function may facilitate parasite survival and increase the likelihood of congenital transmission (26). However, studies indicate that induction of a strong type 1 cytokine response at the fetal-maternal interface may also result in rejection of the fetus. Thus, such a response could contribute to spontaneous abortion during acute toxoplasmosis in a pregnant female (27).

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