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Susceptibility to Over Production of Cytokines in Acute Rheumatic Carditis and Their Role in the Pathogenesis

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The objective of this research work is to study the susceptibility to hyper-responsiveness to A-B hemolytic streptococcal antigen by overproduction of cytokines and their role in the pathogenesis of rheumatic carditis. The study included: (a) 35 unrelated subjects diagnosed as acute rheumatic carditis (10 severe and 25 mild to moderate grade)admitted to the hospital, treated and after improvement as evaluated by clinical examination, ECG , Echo, ESR and CRP, were discharged to be followed up in the outpatient cardiology clinic; (b) the thirty five patients during their follow up and after 6 months from completion of treatment were reinvestigated; (c Thirteen unrelated non rheumatic subjects complaining only from recurrent streptococcal pharyngitis were taken up during the attack of pharyngitis; (d) Fifty subjects of matched age and sex as control; (e) Eleven multiplex rheumatic families including 22 parents, (4 mothers had history of recurrent streptococcal infection), 40 Sibs (6 with acute rheumatic carditis, 15 with inactive rheumatic carditis, 7 with recurrent streptococcal pharyngitis and 11 normal). All the subjects were subjected to: (a) Routine clinical and laboratory investigations and acute phase reactants (ASO, ESR, CRP) and throat culture; (b) detection and grading of mRNA and estimation of the serum level for IL-1α, IL-1B, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ, TNFα. For the unrelated rheumatic subjects before and after treatment and 6 months from completion of treatment; c) for the multiplex families from the pedigrees: HLA-haplotype concordance, N measure for inheritance, and lod score of linkage. The results showed that increased mRNA production and serum levels of IL-1α, IL-1B, TNFα before and still after treatment. The first 2 continue to be high in the inactive status after 6 months from completion of treatment IL-1α is the most significant one. Subjects with recurrent streptococcal pharyngitis showed normal values. Family studies revealed an inherited tendency for over production of IL-1α through a dominant genetic control, which may be the case with IL-2 and possibly TNFα. In conclusion, treatment should not be stopped except after normalization of IL-1α.

Key words: Susceptibility, cytokines, rheumatic carditis

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Introduction

Immunoregulatory dysfunction in individuals susceptible to develop rheumatic fever, after infection with group (A) B-hemolytic streptococci have been reported (Hafez *et al.*, 1987, 1988, 1990; Gerbase, 1994; Bhatnagar, 1999). The cytokines which are known to be secreted by T-helper$_1$, T-helper$_2$ (Th_1, Th_2) and macrophage cells in response to infection and inflammation have been found to play an important role in the pathogenesis of auto immune diseases (Liblau *et al.*, 1995 and Krakauer *et al.*, 1999). Distinct and mutually exclusive profiles of cytokine expression have been described. Cells differentiation is highly sensitive to the detailed conditions prevailing within the micro environment where they recognize antigen (Paul and Seder, 1994 and Abbas *et al.*, 1996). Th_1 cells preferentially produce IL-2 and IFNγ which promote cellular immune responses against intracellular pathogens, whereas Th_2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 which promote humoral immunity by aiding in B-cell growth and differentiation. Macro phages secrete the proinflammatory cytokines IL-1, IL-6, IL-8, IL-10, IL-12 and TNFα which play vital roles in host defense but when present in excess in the course of inappropriate autoimmune responses, can have self destructive effects (Krakaeur *et al.*, 1999). Several studies have investigated serum levels of some interleukins in rheumatic fever (Yein *et al.*, 1997; Bhatnagar *et al.*, 1999; Hafez *et al.*, 2001). However, the literature is still poor in reports clarifying the susceptibility for over production of cytokines by family studies and their role in the pathogenesis and severity of rheumatic carditis.

The objective of this work is to study the role of cytokines in the pathogenesis of rheumatic fever carditis and the susceptibility to exaggerated production of cytokines through studies on multiplex rheumatic families.

Materials and Methods

The study included the parents and all Sibs of 11 multiplex rheumatic families (with more than one rheumatic sib), and unrelated subjects with rheumatic carditis. The rheumatic subjects were diagnosed according to modified Jones criteria update (Heart American Guidelines for diagnosis of rheumatic fever, 1992) and admitted to the Pediatric Cardiology Unit, University Children Hospital at Mansoura, Egypt and then after improvement followed up regularly in cardiology outpatient clinic. The subjects included in the study were enrolled into the following groups:

Unrelated subjects:

Group 1 (G1): Included 35 patients with active rheumatic heart disease (ARHD) 12 females, 14 males with age ranging from 5 to 16 years (mean 8.1 ± 2.8). They were subdivided into:

mild to moderate carditis: 25 patients , 15 females and 10 males with age range 5 to 16 years (mean 8.3 ± 1.8). They were diagnosed according to the following: apical systolic and/or basal diastolic murmur of grade II/VI. No cardiac enlargement in plains chest radiography, together with echocardiographic diagnosis of mitral and/or aortic valvulitis with mild to moderate regurgitation.

Severe carditis: 10 patients, 6 females and 4 males with age range 8 to 15 years (mean 10.7 ± 2.8). They were diagnosed according to the following: apical systolic and/or basal diastolic murmur of grade III/VI , and cardiomegaly with or without congestive heart failure. Plains chest radiography confirmed cardiac enlargement and echocardiography revealed severe mitral and/or aortic regurgitation. All 35 patients on admission received prednisone 2mg kg^{-1} day^{-1} till erythrocyte sedimentation rate (ESR) and c-reactive protein (CRP) became normal , then tapered for 2 weeks with the addition of 75 mg kg^{-1} day^{-1} acetyl salicylic acid for 8 weeks with tapering in the final 2 weeks. For patients with congestive heart failure, classic therapy were added (Fyler, 1992).

Group 2 (G2): The 35 patients with ARHD after improvement and being clinically and laboratory (ESR, CRP) in an inactive status (1ARHD), were followed up regularly in the outpatient clinic. Investigations have been done 6 months after completion of therapy with no recurrent attack of acute rheumatic fever. They were on regular penicillin prophylaxis.

Group 3 (G3): Included 13 normal children suffering only from recurrent streptococcal pharyngitis (RSP) proved bacteriologically and serologically to be due to group A-B hemolytic *streptococci*. They were 8 females and 6 males with age range 5 to 15 years (mean 9.1 ± 1.6). Sampling was done during the last attack of pharyngitis before taking any antibiotics .

Group 4 (G4): Included 50 healthy children, 29 females and 21 males with age range 5 to 14 years (mean 9.2 ± 1.4) who had not suffered any sore throat or other infections in the past 6 months. They were taken as control group.

Multiplex rheumatic families: Eleven rheumatic multiplex families were included in the study. They comprise: (a) 22 clinically normal parents, 4 mothers had history of recurrent streptococcal infection (they were free at the time of sampling and 2 out of them were tonsilectomized during their childhood period). The parents were consanguineous in 2 families; (b) 40 Sibs, out of them: 6 were diagnosed as acute rheumatic fever carditis (first attack and of mild to moderate grade) and included in the group of unrelated subjects with ARHD; 15 with 1ARHD (last attack of recurrent acute rheumatic activity was since less than one year in 5 families, number 2,4,7,8,10, and the other 10 was since more than one year); 7 with RSP and 11 were normal.

All the subjects were subjected to the following:

a) Clinical diagnosis and assessment of activity and severity.
b) X-ray chest, Electrocardiography (ECG), Echocardiography (ECHO).
c) Complete blood picture, Antistreptolysin O (ASO), Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and throat culture.
d) Detection of mRNA and its grade and serum levels in pg/ml for IL-1α IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ and TNFα

(Chomczynski and Sacchi, 1987; Chretien *et al.*, 1989; Xie and Rothblum, 1991; Yamamura *et al.*, 1992, 1993, Becker *et al.*, 1996; Kawakami *et al.*, 1997). This was repeated after treatment and after 6 months from completion of treatment.

Measurement of serum levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ and TNFα: Commercially available ELISA Kits (Research and Diagnostics Systems UK) Specific for IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNFα and IFNγ were used. Briefly, standards or samples containing the tested cytokine reacted with a monoclonal capture antibody and a polyclonal detection antibody. After incubation and washing to remove any unbound antibody enzyme reagent, the detection reagent (Substrate) was added and incubated. The reaction was stopped with sulphuric acid and the plate was read of the appropriate wave length. A standard curve was plotted and tested cytokines concentrations were determined by interpolation from this curve. All samples were tested in duplicate and all plasma samples were studied simultaneously.

Detection of cytokine-mRNA: Detection of IL-1α IL-β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ and TNFα messenger RNA (mRNA) was carried as follows: Isolation of peripheral blood mononuclear cells (PBMC) 10-20 ml of peripheral venous blood was collected into sterile pyrogen-free disposable syringes anticoagulated with endotoxin-free heparin (10μ ml^{-1}). PBMC were separated by standard density gradient centrifugation (Ficol Hi Paque method).

RT-PCR: Total RNA was extracted from freshly isolated unstimulated PBMC using an acid phenol-chloroform extraction method. Quantification of total RNA was performed spectrophotometrically. Cytokine cDNA of PBMC was obtained by

reverse transcriptase (RT) then amplified by polymerase chain reaction (PCR) using specific primers. The following commercially available (Clontech Laboratories, Inc, palo Alto, USA) oligonucleotide 5′ and 3′ and primer sequences was used:

IL-1α :		
	Sense	CACCCTCTGTCACCTGCTCAACA
	Antisense	GGTTCCTCGCTGCCTCCAAGACTC
IL-1β :		
	Sense	GAATTCTGCAGTCCCAGCTCTGTGCAA.
	Antisense	GAATTCCACAGTCATATCCACAATAG.
IL-2 :		
	Sense	ACTCACCAGGATGCTCACAT
	Antisense	AGGTAATCCATCTGTTCAGA
IL-4 :		
	Sense	CTTCCCCCTCTGTTCTTCCT
	Antisense	TTCCTGTCGACCGTTTCAG
IL-6 :		
	Sense	ATGTAGCCGCCCCACACAGA
	Antisense	CATTCATCTTTTTCAGCCAT
IL-8 :		
	Sense	ACAAGCTTCTAGGACAAGCC
	Antisense	ACTTCTCCACAACCCTCTGC
IL-10 :		
	Sense	ATGCCCCAAGCTGAGAACCAAGACCCA
	Antisense	CTCAAGGGGCTGGGTCAGCTATCCCA
IL-12 :		
	Sense	CCAAGAACTTGCAGCTGAAG
	Antisense	TGGGTCTATTCCGTTGTGTC
IFNγ :		
	Sense	AGTTATATCTTGGCTTTTCA
	Antisense	ACCGAATAATTAGTCAGCTT
TNFα :		
	Sense	GAGTGACAAGCCTGTAGCCCATGTTGTAGCA
	Antisense	GCAATGATCCCAAAGTAGACCTGCCCAGATC.

PCR was carried out in a Thermal Cycler (Perkin Elmer Cetus) for 30 cycles of denaturation (96°C, 1 min) , annealing (60°C, 45s) and extension (72°C, 1 min). The amplified gene products were separated by electrophoresis on 2% agarose gel and visualized by UV light illumination using ethidium bromide staining. The amount of cytokine PCR products was graded as follows: - , no band detected, in the undiluted cDNA sample: + , band detected in the undiluted cDNA sample but not in the 1:5 diluted cDNA: ++, band detected in the 1:5 diluted cDNA but not in the 1:25 diluted cDNA: +++, band detected in the 1:25 - diluted cDNA.

Statistical analysis: The collected data were exposed to statistical analysis using chi-square with linear trend for comparing the grades of transcribed of mRNA, Student's t-test for serum levels, and Kandel correlation test for correlation between interleukin levels and Erythrocyte Sedimentation Rate (ESR) and (CRP) (Armitage, 1983). To test for HLA concordance in sibling pairs, the chi-square test (X^2) was used .

To test whether the response with increased secretion of IL-1α is inherited (we took the IL-1α in the family studies since it is the most important interleukin based on our present results and it is the only cytokine found to be increased than normal range: ±2SD of normal in the Sibs with inactive rheumatic heart disease) we used the N measure given by Green and Low (1984) to test the null hypothesis of no inheritance.

To test for the link between any possible immunogenetic control on the response of the gene responsible for the expression of IL-1α mRNA and HLA, we used the lod score estimations for testing the null hypothesis of no linkage.

$$Z(m) = Log\ 10\ \frac{p(m)}{P(0)}$$

Where P(m) is the likelihood of the data for an assumed value (m). For a dysfunction to be associated with HLA, a value of Z greater than 2 is significant at the 0.01% level (Morton, 1983).

Results

The results of mRNA (Fig. 1) and serum levels of circulating cytokines were parallel (Tables 1 and 2). The effect of therapy on the cytokines levels and the relationship between cytokine and Erythrocyte Sedimentation rate (ESR) and C-Reactive protein (CRP) were analyzed.

Unrelated subjects

Active rheumatic heart disease (ARHD): Significant high mRNA and serum levels of IL-1α, IL-2 and TNFα was found in ARHD. IL-1α mRNA expression was significantly higher than IL-2 and TNFα mRNA expression (Table 5). The mRNA and serum level of IL-1α, IL-2, TNFα were higher in severe compared to mild to moderate ARHD before and after treatment (Tables 3 and 4). Levels of other cytokines were significantly higher in patients than control. The mRNA and circulating IL-1α were reduced significantly after treatment but still significantly higher than controls, IL-2, TNFα and control (Tables 3 and 4 and Fig. 2). On the other hand, significant difference was found between IL-2 and TNFα (Table 5). Levels of other cytokines were not significantly different from control (Tables 3 and 4 and Fig. 2).

The subjects after being in an 1ARHD status after 6 months after completion of therapy the mRNA and circulating IL-1α, and IL-2 were significantly lower than at the time of activity but higher than control (Tables 1, 2 and 3). IL-1α mRNA expression was higher than IL-2 ($P = 0.002$).

Subjects with recurrent *Streptococcal pharyngitis* (RSP): mRNA and serum levels of all cytokines were significantly low in subjects with RSP than the rheumatic patients but insignificantly different from control (Tables 1 and 2).

Relationship between cytokines and disease activity: Before treatment, all cytokines correlated with ESR and CRP, while after treatment IL-1α IL-2 and TNFα showed no correlation (Table 6).

Fig. 1: Products of the polymerase chain reaction (PCR) on a 2% agarose gel. Lane 1: 100 bp ladder marker, in the following lanes between brackets are the bp; Lane 2: IL-1α (417); Lane 3: IL-1β (388); Lane 4: IL-2 (344); Lane 5: IL-4 (344); Lane 6: IL-6 (225); Lane 7: IL-8 (348); Lane 8: IL-10 (328); Lane 9: IL-12 (355); Lane 10: IFNγ, (270); Lane 11: TNFα (325).

Table 1: Grades of cytosine m-RNA expression in patients with rheumatic heart disease in 2 groups (ARHD & IARHD), group of children with RSP and controls

Groups	Grade	IL-1α	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12	IFN-γ	TNF-α
Group 1	-	0.0	57.2	14.2	34.2	28.5	57.2	51.8	34.4	65.6	17.2
	+	0.0	42.8	42.1	65.8	71.5	42.8	42.8	65.6	34.4	34.3
	++	22.8	0.0	37.1	0.0	0.0	0.0	0.0	0.0	0.0	48.5
	+++	77.2	0.0	6.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Group 2											
	-	5.1	97.2	47.2	94.4	91.7	97.2	97.2	94.4	97.2	91.7
	+	57.1	2.8	25.0	5.6	8.3	2.8	2.8	5.6	2.8	8.3
	++	37.2	0.0	27.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	+++	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Group 3											
	-	92.4	85.7	92.4	92.4	92.4	92.4	92.4	92.4	85.7	92.4
	+	7.6	14.3	7.6	7.6	7.6	7.6	7.6	7.6	14.3	7.6
	++	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	+++	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Group 4											
	-	96.0	96.0	94.0	96.0	96.4	96.4	98.0	98.0	96.4	98.0
	+	4.0	4.0	6.0	4.0	4.0	4.0	2.0	2.0	4.0	2.0
	++	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	+++	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

G1: active rheumatic heart disease (ARHD), G2: inactive rheumatic heart disease (IARHD), G3: recurrent *Streptococcal pharyngitis* (RSP), G4: Controls.

Table 2: Serum levels (mean ± SD) of cytokines (pg ml^{-1}) in the four studied groups.

Groups	IL-1α	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-12	IFN-γ	TNF-α
Group 1	176.7±8.6	22.4±3.6	133.2±8.1	81.0±4.6	37.6±12.1	21.6±2.8	51.6±6.4	60.3±6.7	39.8±4.1	146.3±10.8
Group 2	61.22.51	14.2±3.1	30.5±4.3	12.4±2.1	7.4±2.4	9.6±2.8	12.2±2.4	45.2±5.1	12.1±2.4	41.4±6.1
Group 3	6.4±3.1	16.4±4.5	13.1±5.4	13.6±4.1	7.2±5.7	9.6±2.8	12.3±2.8	47.1±6.2	12.9±3.1	47.2±6.2
Group 4	7.4±2.9	16.1±2.4	12.2±2.9	13.1±2.6	7.5±1.9	9.3±3.1	11.9±3.4	46.7±3.7	12.2±3.9	41.6±3.2

G1: active rheumatic heart disease (ARHD), G2: inactive rheumatic heart disease (IARHD), G3: recurrent *Streptococcal pharyngitis* (RSP), G 4: control.

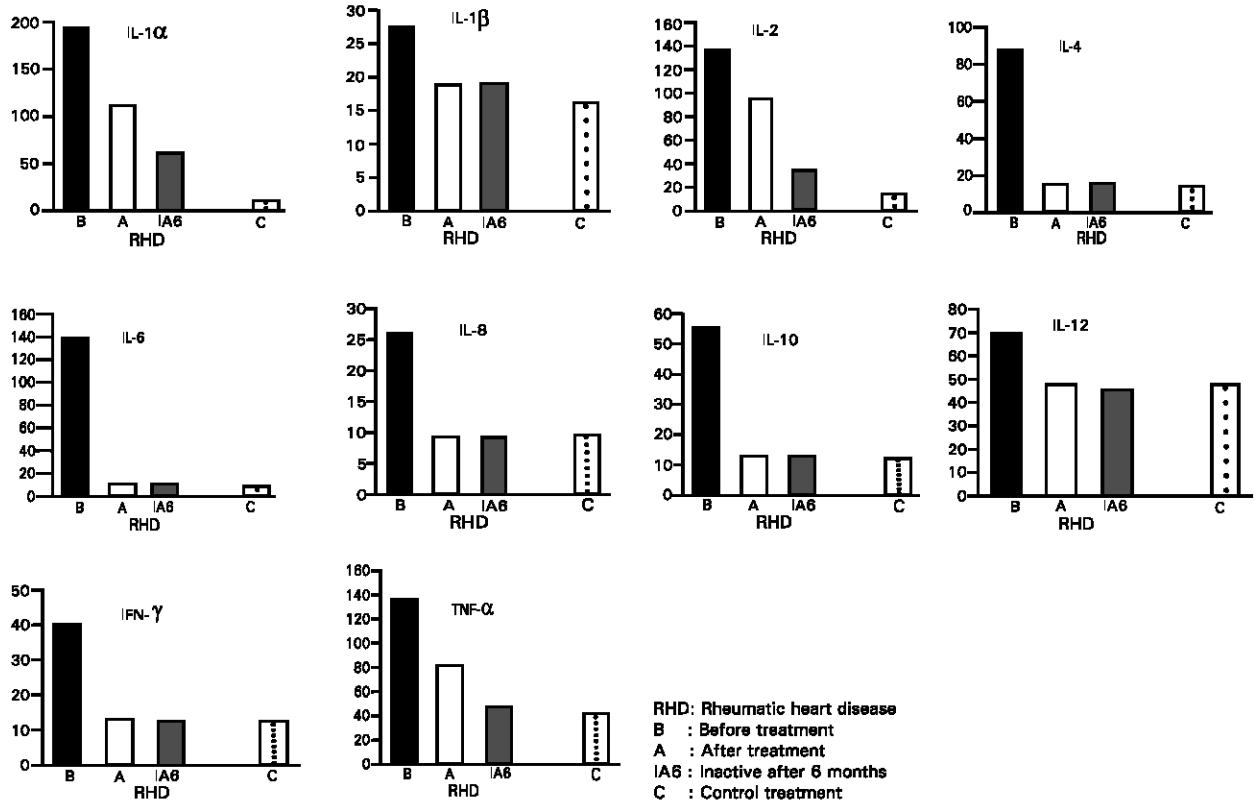

Fig. 2: Histograms of the serum levels of cytokines before and after treatment for patients in the active stage and in the inactive stage of RHD and control. Serum levels of the cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IFNγ, TNFα), expressed as the mean picograms per milliliter.

Table 3: The grades of production of m RNA of cytokines in patients with active rheumatic heart disease (ARHD) before treatment (B)and after treatment (A) treatment.

		ARHD								P-value				
		Mild to moderate				Severe								
Cytokines		-	+	++	+++	-	+	++	+++	P1	P2	P3	P4	P5
IL-1α	B	0.0	0.0	22.7	77.3	0.0	0.0	0.0	100.0	0.001	0.001	0.047	0.001	0.001
	A	0.0	22.8	45.4	31.8	0.0	0.0	42.9	57.1			0.031		
IL-1β	B	72.8	27.2	0.0	0.0	42.9	52.8	0.0	0.0	0.02	0.02	0.096	0.09	0.56
	A	95.4	4.6	0.0	0.0	95.4	4.6	0.0	0.0			0.52		
IL-2	B	9.1	50.0	36.3	4.5	28.6	28.6	28.6	14.3	0.02	0.02	0.032	0.001	0.002
	A	18.2	50.0	31.8	0.0	28.6	57.1	14.3	0.0			0.046		
IL-4	B	13.2	68.2	0.0	0.0	42.9	57.1	0.0	0.0	0.03	0.03	0.36	0.33	0.32
	A	100.0	0.0	0.0	0.0	100.0	0.0	0.0	0.0			0.51		
IL-6	B	22.7	77.3	0.0	0.0	42.9	57.1	0.0	0.0	0.001	0.001	0.073	0.18	0.42
	A	96.4	4.6	0.0	0.0	85.7	14.3	0.0	0.0			0.16		
IL-8	B	72.8	27.2	0.0	0.0	42.9	57.1	0.0	0.0	0.01	0.01	0.18	0.31	0.28
	A	95.4	4.6	0.0	0.0	100.0	0.0	0.0	0.0			0.72		
IL-10	B	54.5	45.5	0.0	0.0	42.9	57.1	0.0	0.0	0.01	0.01	0.084	0.08	0.32
	A	95.5	4.5	0.0	0.0	85.7	14.3	0.0	0.0			0.072		
IL-12	B	31.8	68.2	0.0	0.0	44.9	57.1	0.0	0.0	0.04	0.03	0.21	0.26	0.32
	A	100.0	0.0	0.0	0.0	85.7	0.0	0.0	0.0			0.62		
IFN-γ	B	72.8	27.2	0.0	0.0	44.9	57.1	0.0	0.0	0.04	0.04	0.06	0.41	0.42
	A	95.4	4.6	0.0	0.0	100.0	0.0	0.0	0.0			0.07		
TNF-α	B	18.1	36.4	45.5	0.0	0.0	28.6	57.1	14.5	0.02	0.02	0.03	0.02	0.02
	A	36.4	40.9	22.7	0.0	42.8	28.6	28.6	0.0			0.092		

P1 = (B) vs(A) for mild to moderate, P2 = (B) vs (A)for severe, P3 = (B)and (A) between mild to moderate and severe, P4 = (A) for mild to moderate vs control, P5 = (A)for mild to moderate vs IARHD (Table 1).

Table 4: The serum levels (pg ml^{-1}) of the cytokines (mean ±SD) in patients with active rheumatic heart disease patients before(B) and after(A) treatment.

		ARHD				P-value				
		Mild to moderate		Severe						
Cytokines		Mean	+SD	Mean	+SD	P1	P2	P3	P4	P5
IL-1a	B	172.1	12.4	204.5	7.6	0.001	0.001	0.045	0.0001	0.001
	A	111.4	11.4	118.6	6.8			0.039		
IL-1B	B	21.6	4.1	26.4	3.4	0.02	0.02	3.8	0.07	0.52
	A	18.4	2.6	19.2	2.2			6.3		
IL-2	B	162.6	8.1	144.4	2.2	0.02	0.02	0.020	0.000	0.003
	A	94.4	6.1	100.2	7.8			0.046		
IL-4	B	40.8	4.1	43.1	6.1	0.03	0.03	0.81	0.46	0.41
	A	13.1	3.7	14.9	4.3			0.63		
IL-6	B	126.2	6.1	143.4	5.1	0.000	0.000	0.073	0.186	0.51
	A	8.6	4.1	9.4	4.3			0.13		
IL-8	B	27.6	2.6	27.6	3.6	0.01	0.01	0.12	0.42	0.46
	A	9.6	2.3	9.0	2.4			0.74		
IL-10	B	54.2	5.2	59.4	4.3	0.01	0.01	0.092	0.093	0.41
	A	11.3	3.1	10.6	3.7			0.81		
IL-12	B	67.6	4.7	69.6	3.3	0.03	0.03	0.13	0.24	0.12
	A	47.1	3.6	47.4	4.1			0.24		
TFNγ	B	38.2	4.1	41.4	4.8	0.04	0.04	0.16	0.38	0.36
	A	12.1	4.6	12.8	3.3			0.72		
TNFα	B	131.6	4.6	139.6	6.1	0.002	0.002	0.086	0.03	0.03
	A	73.1	5.1	81.8	4.7			0.091		

(B) Multiplex rheumatic families: Based on our results (Fig. 3) analyzed our pedigrees of the multiplex rheumatic families taking only IL-1α which was found being the most important one in the pathogenesis of rheumatic carditis to be at high level after treatment and after 6 months and also the only one increased in Sibs with 1ARHD. Table 7 illustrates the parental haplotypes and Table 8 the HLA-haplotype concordance. The values according to the scores of concordance showed that: (a) the identical haplotypes score in rheumatic sibpairs is very high and the values are significantly different from the expected according to Mendelian segregation ($P < 0.001$); (b) the haploidentical haplotypes is very high in the sibpairs of subjects with RHD and RSP. The values are significantly different from the expected ($P < 0.001$); C) significant difference ($P < 0.001$) between the Haplotype assortment scores in the two groups of sibpairs. To test whether this hyper responsiveness is inherited, we used the N measure given by Green and Low (1984). Accordingly our test statistic 3.14 which is highly significant to reject the null hypothesis of no inheritance ($P < 0.001$).

Lod scores (z) for linkage (Table 9) show linkage between an immunogenetic control on the gene responsible for IL-1α and HLA system since z is more than 3 in dominant model ($P < 0.001$). Residual X^2_1 indicated that the values cannot be explained by the recessive model ($X^2 = 8.84$, $P < 0.05$) but fits the dominant model ($X^2 = 4.28$, $P > 0.05$).

Table 5: Statistical comparison between the grades of m RNA production of the Different cytokines in active rheumatic heart disease.

Time of sampling	IL-1α vs IL-2	IL-1α Vs IL-6	IL-1α vs IL-10	IL-1α vs TNFα	IL-2 vs IL-6	IL-2 vs IL-10	IL-2 vs TNFα	IL-6 vs TNFα	IL-6 vs IL-10	TNFα vs IL-10
B. T.(X^2)	37.28	58.0	58.0	43.26	15.18	10.52	3.17	19.21	6.05	19.67
(P)	0.0001	0.0001	0.0001	0.0001	0.00016	0.014	0.36	0.0001	1.013	0.0001
A.T.(X^2)	23.19	49.11	52.29	26.05	26.01	31.0	2.15	15.71	0.74	19.87
(P)	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.34	0.0014	0.38	0.0001

vs= versus B.T. = before treatment A.T. =after treatment

Table 6: Person correlation coefficient (r) between serum levels of cytokines and ESR and CRP in active rheumatic heart disease (ARHD)

	ARHD			
	B		A	
Cytokines	ESR	CRP	ESR	CRB
IL-1α	0.667***	0.562***	0.211	0.223
IL-1β	0.414*	0.426*	0.391	0.394*
IL-2	0.542**	0.551**	0.156	0.216
IL-4	0.416*	0.512**	0.414*	0.421*
IL-6	0.414*	0.420*	0.408*	0.461*
IL-8	0.436*	0.513**	0.426*	0.432*
IL-10	0.462*	0.481*	0.432*	0.463*
IL-12	0.462*	0.482*	0.398*	0.412*
IFNγ	0.448*	0.409*	0.442*	0.398*
TNFα	0.574**	0.548**	0.224	0.212

B: before treatment A : after treatment p: * = <0.05, ** = <0.01, *** = <0.001

Table 7: Parental HLA heplotypes in the 11 multiplex families

	Mother		Father	
Pedigree No	a	b	c	d
1	A2B17DR4	Aw19B12DR5	A1B15DR4	A11B-DR4
2	A2B15DR4	A-B14DR4	A28B12DR4	A1B12DR4
3	A2B15DR4	A29B27DR1	A9B12DR4	A2B14DR1
4	A3B5DR1	A29B15DR4	A1B17DR4	A2B23DR1
5	A10B21DR4	A-B8DR5	A2B4DR1	A29B-DR3
6	A9B5DR4	A3B5DR1	A1B12DR4	A28B22DR1
7	A1B12DR4	A28B14DR1	A28B37DR4	Aw19B14DR1
8	A1B12DR1	A28B14DR1	A29B5DR5	A10B15DR4
9	A3B5DR4	A28B5DR4	A2B-DR4	A1B5DR4
10	A10B21DR5	A-B8DR1	A-B40DR7	A29B-DR3
11	A28B37DR4	Aw19B14DR1	A9B18DR5	A2B14DR2
12	A1B8DR1	A10B14DR4	A10B8DR4	A10B14DR1

Table 8: HLA-haplotype concordance for subjects with rheumatic heart disease and those with *Streptococcal pharyngitis*.

	Pairs of rheumatic siblings			Pairs of subjects with RHD &RSP		
	Observed			Observed		
Concordant haplotypes	n	%	Expected %	n	%	Expected %
2	18	81.8	25	0	0.0	25
1	4	18.2	50	20	83.3	50
0	0	0.0	25	4	16.7	25

P1: Observed of pairs of rheumatic siblings vs expected = <0.001 P2: Observed of pairs of subjects with RHD and RSP vs expected = <0.001
P3: Observed of pairs of subjects with RHD vs observed of pairs of subjects with RHD and RSP = <0.001

Table 9: Lod scores (z) for linkage between genetic control on the gene responsible for the expression of IL-1α mRNA and HLA.

	Proportion linked to HLACm					Estimates		
Genetic model	0.9	0.7	0.5	0.3	0.1	m	z	Residual X^2_1
Recessive	1.87	4.13	3.76	1.78	0.37	0.16	1.2	8.84
Dominant	4.79	3.61	2.42	1.24	0.14	3.15	3.9	4.73

Discussion

In response to antigen stimulation, lymphocytes and macrophages differentiate into cytokine producing cells. The agents that stimulate lymphocytes can stimulate macrophages either by direct cell contact which is genetically controlled by MHC class II or by producing certain cytokines that can stimulate the cells to produce specific cytokines. The nature of the stimulant determines whether the cytokine accumulates intra cellularly or is secreted (Krakauer *et al.*, 1999). Recently, it has become apparent that after antigen stimulation, the cytokine production not only involves the acute transcription of cytokine genes but also includes a prior phase of chromatin remodeling that occurs during the initial differentiation of native cells (Viola and Rao, 1999). However, there are several attempts that various components of

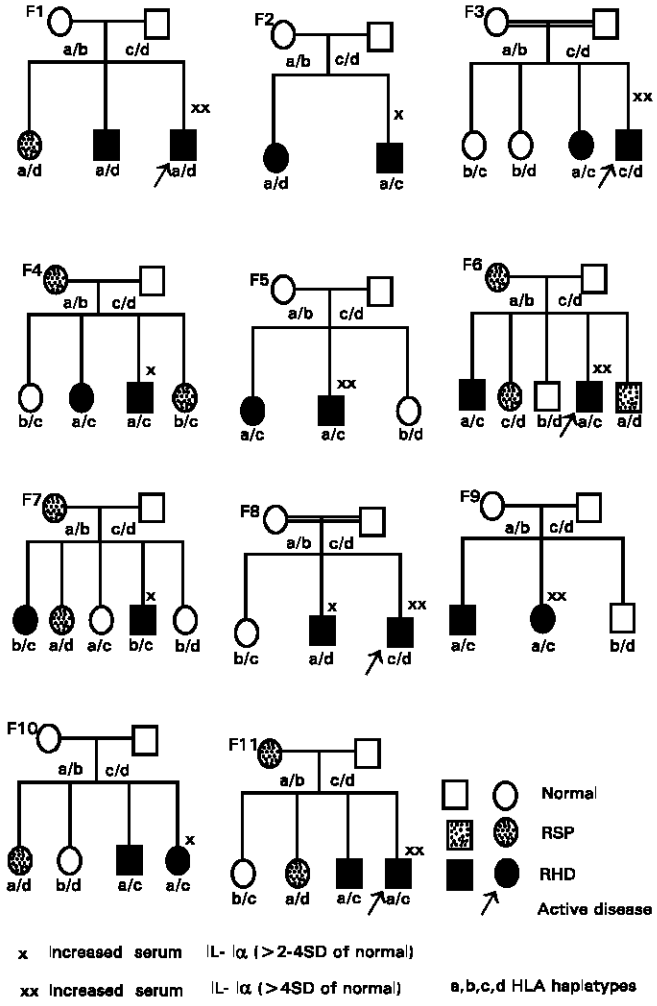

Fig. 3: The pedigrees of the 11 multiplex rheumatic families

Streptococcus pyogens are potent inducers of inflammatory cytokines from various cells (Tomai *et al.*, 1990 and Kotb *et al.*, 1993). Key factors that influence different-tiation are the dose and route of antigen administration, the engagement of co-stimulating receptors, and the local concentrations of cytokines (Paul and Seder, 1994 and Constant and Bottomly, 1997). Inherited immunoregulatory dysfunction in individuals susceptible to develop rheumatic fever has been reported (Hafez *et al.*, 1987, 1988, 1990; Gerbase *et al.*, 1994; Bhatnagar *et al.*, 1999). This may highlight and explain the hyper responsiveness of some cytokine genes to certain antigens.

Based on the present study, IL-1B expression is different and independent from that of IL-1α. Yegin *et al.* (1997) found that serum IL-1β insignificantly increase in ARF and 1ARHD and returned to undetectable levels. Also Straub *et al.* (1997) detected IL-1β in three cases out of 20 patients. However, Krakauer *et al.* (1999) reported three members of IL-1 family α , β and Ra expressed by three genes of different structures located close to one another on the long arm of human chromosome 2. Acres *et al.* (1997) suggested that the pattern of expression of the genes for IL-1α and IL-1β are regulated independently, which may explain our finding. Miller *et al.* (1989) reported that blood mononuclear cells cultures from rheumatic children produced more TNFα and IL-2 than control. Morris *et al.* (1993) reported significantly elevated serum levels of IL-1α and IL-2 in ARHD at all intervals up to 48 weeks, which is in agreement with our findings. Narin *et al.* (1995) found that patients with acute rheumatic fever on admission revealed significantly higher IL-1α and IL-2 production than that after three months where the serum levels were not significantly different from neither that of control nor individuals with streptococcal pharyngitis. They suggested that this indicate that monocytes/macrophages and T cells in IARHD are not functioning in a highly active state, which is not the same we found with IL-1α and IL-2, in spite that we used prednisone in the treatment as they did. Lane *et al.* (1993) found infiltration of the heart by inflammatory cells that secrete IL-1 and TNFα and that local secretion of these cytokines contributes to increased levels of IL-1 and TNFα in the serum. They concluded that local production of these cytokines promoted the induction of post-infection autoimmune myocarditis. This confirms that our present results are mirror image to the local secretion of the infiltrating cells. Furthermore, Fraser *et al.* (1997) studied rheumatic Aschoff nodules and the level of cytokines in relation to them and found that IL-1α and TNFα are expressed within macrophages (Aschoff and Antischkow cells) in all stages with Aschoff nodules, while IL-2 was found only in the third stage or lymphocyte rich lesions. This goes with the finding in this study which indicate that IL-1α plays the major role from the initiation of autoimmune process and together with IL-2 and TNFα continue the inflammatory process with high concentration even after normalization of the ESR and CRP.

Samsonov *et al.* (1995) reported rise in CD4 helper subsets associated with a rise in IL-1α and IL-2 during the acute phase. Narin *et al.* (1995) reported correlation between the production of IL-2 in ARF and IARHD and the percentage of CD4 + and CD25 + cells and no correlation with percentage of CD8 + cells. The observed expansion of CD4 + cells population may be associated with an increased capacity of T-helper cells to generate IL-2 receptors. Bhatanagar *et al.* (1999) found that activation of CD4 + cells in ARF by streptococcal pyogenic erythrogenic toxin A caused an increase in IL-2 and IFNγ production, while IL-4 and IL-10 production was markedly decreased. In IARHD they found lowered IL-2 and IFNγ and augmented IL-4 and IL-10 production, and suggested that CD4 cells in ARF endowed with TH1-type function, whereas CD4 cells in 1ARHD exhibit a TH2 like cytokine pattern. However, other than the increased production especially of IL-2 in the period of activity , these findings are not consistent with ours.

Kutukculer and Narin (1995) found significantly high serum IL-8 in ARF patients which decrease within 3 months. They also found the concentration of IL-8 to be normal in IARHD and subjects with only SP. Our present results are in agreement with the later but not with the earlier finding, since we found the concentration of IL-8 to normalize after treatment and significantly correlate with erythrocyte sedimentation rate (ESR) and C-Reactive protein (CRP). Yegin *et al.* (1997) reported significant changes in serum values of IL-6, Il-8 and TNFα in the acute phase, on day 7 of treatment and after treatment.

In the subjects having only *Streptococcal pharyngitis* (SP), the expression of all the studied cytokines insignificantly different from control. The same finding has been reported by Morris *et al.* (1993). However, it is known that the majority of persons who experience streptococcal pharyngitis do not develop rheumatic fever (Wannamaker *et al.*, 1951 ; Siegel *et al.*, 1961 Stollerman, 1975). In rheumatic fever patients there is inherited increased avidity for adherence of RF-associated strains to pharyngeal cells through a dominant susceptibility gene (s) and in addition some may have also another dominant gene (s) responsible for secretory immunoglobulin A deficiency linked to HLA (Williams and Gibbons, 1972; Selinger *et al.*, 1978, Reed *et al.*, 1980, Hafez *et al.*, 1989, 1995).

To infer if there is susceptibility to over production of proinflammatory and inflammatory cytokines in rheumatic subjects, when being exposed to the stimulation by streptococcal antigens either directly or through a dysfunction in an immunogenetic control influencing the gene expression of the

cytokine, it was a must to study families having more than one sib affected. We selected only IL-1α for the pedigrees analysis since our results revealed that it is the most important cytokine in the pathogenesis and did not decline to normal level after treatment or even in the inactive status after 6 months. In addition it was the only increased on in the Sibs with 1ARHD. However, some important findings have been emerged. First, in spite of the recurrent streptococcal pharyngitis in some mothers and Sibs, they had no rheumatic affection.

Also an important finding which parallel our usual clinical observation is that susceptibility of recurrent throat infection is through maternal transmission which points to the possibility of mitochondrial inheritance or x-linked autosomal dominant genetic defect. Second, genetic analysis using HLA-haplotype concordance and N measure revealed that the susceptibility of hyper responsiveness to the antigen stimulation is inherited. Third, linkage studies suggest that a dominant susceptibility immunogenetic control possibly on chromosome number 6 to the IL-1α gene located on chromosome number 2 is linked to the HLA system. However this indicate the possibility of similar mechanism with IL-2 and may be TNFα which needs another planned family study.

Taken to together, the present study investigated the subject from different aspects and indicate the possibility of genetic susceptibility to respond to certain streptococcal antigen(s) by secretion of certain cytokines in different grades in association with hyper responsiveness to produce autoantibodies that cross-react with a specific tissue. Also , the possibility of stimulation of certain cytokine gene (s) by damaged tissue resulting from the autoimmune reaction. In rheumatic arthritis Hafez *et al.* (2001) found increased production of IL-6 and IL-10 which is not the case in carditis, and fortunately the process ends with no sequelae. On the other hand in carditis the increased cytokine concentrations together with T lymphocytes and macrophages infiltration may help and result in collagen synthesis and damage to heart valves. However, the relation we found between the degree of concentration of IL-1α , IL-2 and TNFα and the severity of carditis indicate variability in the genetic susceptibility and hence the degree of response. Furthermore, the increased levels of IL-1α and to less extent IL-2 after 6 months from completion of treatment means that the rheumatic process is still present in spite of the clinical presentation and normalization of ESR and CRP , and hence the liability to more damage of the valves and/or recurrence of activity.

However, the changes in the concentration of cytokines in the peripheral blood is a mirror image to their concentration in the affected tissues, which is the valves and cardiac tissues in rheumatic carditis. It was reported that apoptosis is inhibited in autoimmune diseases (Mountz *et al.*, 1994). Failure to remove autoimmune cells that arise during development or that develop as a result of somatic mutation during an immune response can result in autoimmune disease (Thompson, 1995). Hafez *et al.* (not yet published) found decreased CD95 and inhibited apoptosis in children with active rheumatic fever. The accumulating reactive mononuclear which secrete the cytokines in the blood, migrate from blood into tissues by a highly complex process involving a cascade of adhesion molecules that are responsible for selective tissue migration known as homing receptors (Picker *et al.*, 1990, Hu *et al.*, 1992). Roberts *et al.* (2001) studied the valve tissues from rheumatic patients with valvular heart disease who required valve replacement, and found expression of VCAM-1. Furthermore, Hafez *et al.* (not yet published) found increased VCAM-1 and 1CAM-1 in the blood of children with acute rheumatic fever.

For patients with active carditis whatever the degree of severity, we used prednisone in the regimen as we mentioned before in the subjects and methods (Fyler *et al.*, 1992). Although many physicians use steroids for all patients with cardiac involvement, several studies do not show an advantage of steroids over salicylates in the management of patients with mild or moderate carditis, and recommended to reserve steroids for patients with severe carditis and/or cardiac failure (UK and US Joint Report, 1955, 1960, 1965 and Ayoub, 1995). Other studies reported that steroids result in more prompt resolution of the inflammatory process than aspirin and so can decrease the load on the heart (Dorfman *et al.*, 1961, Feinstein, 1961, Bywaters and Thomas, 1966 ; EL-Said *et al.*, 1998). Fyler (1992) reported the importance of using steroid therapy initially for all children with active carditis and unequivocal valve involvement, and later switch to salicylates in order to reduce valve damage. Knudsen *et al.* (1987) reported that glucocorticords inhibit transcriptional and post-transcriptional expression of interleukin-1 in U 937 cells, this inhibition is likely to occur in vivo. Thus the anti-inflammatory and immunosuppressive effects of glucocarticoids may, in part, stem from their suppression of IL-1 expression (Krakaur *et al.*, 1999).

So far, we recommend: (a) to consider the serum level of Il-1α in the laboratory criteria for follow up of subjects with rheumatic carditis and evaluation of Sibs in mulicase families; (b) treatment should not be stopped except after normalization of serum level of IL-1α; C) researches are important for future therapeutic approaches to block mediated responses to the stimulating streptococcal antigens since this, may help to prevent or reduce valvular damage.

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