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Delayed Lymphocyte Apoptosis in Rheumatic Heart Disease

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The present study was conducted on the implication of apoptosis and some factors that may control it, in the pathogenesis of rheumatic heart disease. 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, 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 investigated; (c) fifty subjects of matched age and sex as control. All the subjects were subjected to (a) routine clinical and laboratory investigations and acute phase reactants (ASO, ESR, CRP) and throat culture; (b) measurement of CD95 (sFas); (c) detection of apoptotic cells by PI, geimsa, AO stains and assessment of apoptotic index; (d) DNA fragmentation detection of mRNA and its grade and serum levels in pg/ml for IL-1α, IL-1β, TNF-α and IFN-γ. These investigations were repeated after 6 months from completion of treatment. The results in the acute stage (ARHD) revealed significant decrease in the level of CD95 and percentage of apoptotic cells by the three stains, significant increase of mRNA expression and serum levels of IL-1α, TNF-α ($P<0.001$) and IL-1β, IFN-γ ($P<0.05$) with significant negative correlation between cytokines, CD95 and percentage of apoptotic cells. All parameters showed insignificant difference between mild to moderate and severe cases and returned to normal values in the inactive status (1ARHD) except IL-1α. It was concluded that anticytokine therapy for TNF-α and IL-1α should be added to the treatment strategy at the very beginning to prevent or attenuate the inhibition of apoptosis and reactive process in the valves.

Key words: Lymphocyte, rheumatic, apoptosis

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Introduction

Recent evidence indicates that alterations in cell survival contribute to the pathogenesis of a variety of diseases including autoimmune diseases (Thompson, 1995). A direct linkage between the control of programed cell death (apoptosis) and autoimmune diseases is yet to be demonstrated and will have obvious implications for the understanding of the pathogenesis of these diseases. One of the central signaling pathways of apoptosis in the immune system is the soluble Fas (CD95). It is expressed by mature T-cells, but expression is enhanced upon activation by antigen, thus rendering T-cells more sensitive to Fas L-mediated apoptosis (Lee and Shacter, 2001).

The cytokines which are known to be secreted by T-helper 1 and 2 and macrophage cells in response to infection and inflammation have been found to play an important role in the pathogenesis of autoimmune diseases (KraKauer ***et al.***, 1999). Some reported defined pro-inflammatory cytokines as the candidate mediators of B-cell destruction in the pancreas by apoptosis in autoimmune diabetes (Delaney ***et al.***, 1997). However, TNF is one of the most important physiological control of apoptosis. The critical role of TNF mediated apoptosis has been recognized in a wide variety of situations including immunomodulation and autoimmunity (Rath and Aggarwal, 1999). Sidoti-de Fraisse ***et al.*** (1998) and Natoli ***et al.*** (1998) reported that TNF regulates cell growth by apoptotic, nonapoptotic and antiapoptotic mechanisms. Andersen ***et al.*** (2000) postulated that there is synergistic effect of TNF-α, IL-1β and IFN-γ in the functional inhibition and induction of cell death in pancreatic B-cell in autoimmune diabetes through a synergistic activation of mitogen-activated protein kinase activity. IL-1α, like TNF-α, is a central mediator in the cytokine network, controlling important functions in the immune system (Loppnow ***et al.***, 2001) and implicated in the pathogenesis of rheumatic heart disease (Morris ***et al.***, 1993; Narin ***et al.***, 1995; Hafez ***et al.***, 2001).

Since there is no report in the literature concerns with the implication of apoptosis in the pathogenesis of rheumatic heart disease, we planned to study this process and some of the factors that may control it, based on both the documented and suggested knowledges, CD95, IL-1α, IL-1β, TNF-α and IFN-γ assessments were included in the investigation.

Materials and Methods

The rheumatic subjects were diagnosed according to modified Jones criteria update (Anonymous, 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:

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 (G1a)

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 plain chest radiography, together with echocardiographic diagnosis of mitral and/or aortic valvulitis with mild to moderate regurgitation.

Severe carditis (G1b)

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: especial systolic and/or basal diastolic murmur of grade III/VI and cardiomegaly with or without congestive heart failure. Plain chest radiography confirmed cardiac enlargement and echocardiography revealed severe mitral and/or aortic regurgitation. All 35 patients on admission received prednisone 2 mg 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 and 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 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.

All the subjects were subjected to the following

- Clinical diagnosis and assessment of activity and severity.
- X-ray chest, Electrocardiography (ECG), Echocardiography (ECHO).
- Complete blood picture, Antistreptolysin O (ASO), Erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and throat culture.

- Measurement of CD95 (sFas) (Cifon ***et al.***, 1994).
- Detection of apoptotic cells by propidium iodide (PI) stain geimsa stain, acridine orange (AO) stain and DNA fragmentation.
- Detection of mRNA and its grade and serum levels in pg/ml for IL-1α, IL-1β, TNFα and IFN-γ. (Chomczynski and Sacchi, 1987; Chretien ***et al.***, 1989; Xie and Rothblum, 1991; Yamamura ***et al.***, 1993; Becker ***et al.***, 1996 and Kawakami ***et al.***, 1997).

These investigations were repeated after 6 months from completion of treatment.

Measurement of CD95

The levels of CD95 (sFas) were determined by the following enzyme linked immunosorbent assay (ELISA) using two anti-human Fas monoclonal antibodies, DX-2 (IgG1) and DX-3 (IgG2a), that were generated as described previously by Cifone ***et al.*** (1994). These antibodies react with distinct epitopes in the extracellular domain. Anti-Fas antibody (DX-3; 10 μg ml^{-1} in 0.01 μm phosphate buffered saline, pH 7.2 (PBS) was coated onto 96 well micro titer plates (Sumililon multiplate MS-8496 F, Sumitomo Co, Ltd., Japan) and incubated at 37°C for 3 h. Blocking performed with 1:2 dilution with PBS of Block Ace (Dai Nihon Seiyaku Co, Ltd., Japan) at 37°C for 2 h. Then sera were added and incubated at room temperature for 1 h. After washing five times with 0.05% Tween/PBS, 10 μg ml^{-1} biotin conjugated anti-Fas antibody (DX-2) in 0.05% Tween/PBS containing 5% mouse serum was added and incubated at room temperature for 1 h. After washing five times, 1:150 diluted ABC solution (Vectastain ABC kit Elite pK-6100, Vector Laboratories Inc., Burlingame, CA) was added and incubated at room temperature for 1 h. After washing five more times, o-phenylendiamine (OPD) and 0.03% H_2O_2 in citrate buffer (pH 5.0) were added and incubated at room temperature for 30 min. OD490 was measured on an automated plate reader (Model 3550-UV Microplate Reader, Bio-Red, Hercules, CA) and the levels of sFas were determined by comparison to the standard curve obtained using recombinant sFas (19, 9.5, 4.75, 2.37, 1.18. 0.59. 0.29 and 0.14 ng ml^{-1}). The lower limit of detection was 0.59 ng ml^{-1}. (Tokano ***et al.***, 1996).

Detection of apoptotic cells by Giemsa stain, Acridine Orange stain (AO), propiduim iodide stain (PI) and DNA fragmentation

Lymphocyte separation

Cells were obtained from whole EDTA-blood using lymphocyte-separating solution (Ficoll-Paque; Pharmacia, Uppsala, Sweden) by the method of Boyum (1968). Lymphocytes were washed twice with sterile PBS pH 7-6.

Culture of lymphocyte

Cells were suspended at 107 ml^{-1} in RPMI 1640 (GIBCO) containing 25 mM glucose 20 mML-glutamine, 92 um gentamycin 20 μg/ml (Walkersvlle MD) and 25 mM HEPES buffer (pH 7.3: GIBCO).

Cells were suspended in sterile PBS (0.05M PO_4 0.15M NaCl, pH 7.2). The cell suspension were divided into 4 aliquots to measure the apoptosis at zero time (base lime), then 24, 48 and 72 h, cells were incubated (at 37°C in 5% Co_2). After incubation, cells were centrifuged (200xg 5 min), resuspended in ice-cold PBS and analyzed on a Coulter counter.

Assessment of cell viability

At time zero and then at subsequent times, cells were removed from culture and counted on a hemocytometer. Cell viability was determined by trypan blue dye exclusion test, one volume of trypan blue (0.4% GIBCO) was added to 5 volumes of cells at room temperature for 5 min.

Detection of apoptotic cells

Propidium Iodide stain

Cells were stained with Hoechst 33342 and Propidium iodide (PI) and visualized using fluorescence microscopy as described by Lee and Shacter (1998). A minimum of 200 cells were counted per sample and were classified as follows:

- Live cells (normal nuclei, blue chromatin with organized structure).
- Membrane intact apoptotic cells (bright blue chromatin that is highly condensed, marginated, or fragmented).
- Membrane permeable apoptotic cells (bright red chromatin, highly condensed or fragmented).

The extent of apoptosis was calculated as the percentage of total apoptosis divided by the total number of cells counted.

Giemsa stain

At time zero and at subsequent times, cells were removed from each culture , fixed in methanol, harvested on slides and slides were stained with May Grunwald Giemsa and examined by oil immersion light microscope (Wyllie and Morris, 1985). For assessment of the percentage of cells showing morphology of apoptosis 500 cells/slide were examined for each case at different times (0, 24, 48, 72 h). Lymphocyte were considered apoptotic if they exhibited the highly characteristic morphological features of chromatin aggregation, nuclear pyknosis and cytoplasmic vaculation. The apoptotic Lymphocyte percentage at different times was calculated.

Acridine orange stain (AO)

One drop of cell suspension was added to one AO solution (10 μg ml^{-1} in PBS), mixed gently on a slide and immediately examined with an Olympus HB-2 microscope with fluorescence attachment. Green fluorescence was detected between 500 and 535 nm cells exhibit bright fluorescent condensed nuclei (intact or fragmented) were interpreted as apoptotic cells and

expressed as a percentage of the total number viable cells, which exhibited a green and diffusely stained intact nucleus (Kumagai and Coustansmith, 1995).

Assessment of apoptotic index

For this analysis in each case the mean number of lymphocyte cells per field was registered in 15 randomly chosen fields. The apoptotic lymphocytes were then counted and expressed as apoptotic index (number of apoptotic cells total number of cells^{-1}). (Del Vecchio ***et al.***, 1991).

DNA Fragmentation

Total DNA was extracted with 0.5 ml saturated phenol followed by 0.5 ml chloroform:isoamyl alcohol (24:1) before centrifugation at 7000 g, 4°C for 10 min. DNA in the supernatant was precipitated by adding 0.02 ml of 5 min NaCl and 1 ml of absolute ethanol. After centrifugation at 9500 g for 15 min, the pellets were air-dried prior to suspending in 0.1 ml TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). RNA was eliminated by Rnase digestion (0.1 mg ml^{-1} Rnase, incubated at room temperature for 1 h). The DNA was electrophoresed using 1.8% agarose gel and visualized by ethidium bromide staining (Maruyama ***et al.***, 2000).

Measurement of serum levels of IL-1α, IL-1β, TNFα, IFNγ

Commercially available ELISA Kits (Research and Diagnostics Systems U.K) Specific for IL-1α, 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, 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 (10 U ml^{-1}). PBMC were separated by standard density gradient centrifugation (Ficol Hi Paque method).

RT-PCR

Total RNA was extracted from freshly isolated un-stimulated 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) oligonucleotids 5', 3' and primer sequences was used:

IL-1α	Sense	CACCCTCTGTCACCTGCTCAACA
	Antisense	GGTTCCTCGCTGCCTCCAAGACTC
IL-1β	Sense	GAATTCTGCAGTCCCAGCTCTGTGCAA.
	Antisense	GAATTCCACAGTCATATCCACAATAG.
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, 45 s) 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 expression of mRNA student's t-test for serum levels and pearson correlation test for correlation between the different parameters (Armitage, 1997).

Results

Analysis of the results revealed the following findings: Significant decrease in CD95 (sFas) in the serum (ng ml^{-1}) of patients with ARHD compared to after being in the inactive status (1ARHD) ($P<0.01$) and control ($P<0.01$). No significant difference between either patients with 1ARHD and control or between G1a and G1b (Table 1 and Fig. 1).

The apoptotic lymphocytes index detected by PI, Geimsa and AO are parallel and the apoptosis was found to be decreased only in G1 compared to G2 and G3 at $P<0.01$ and < 0.01, respectively. No significant difference between either G2 and G3 or G1a and G1b at (Table 2, Fig. 2-7). Agarose gel electrophoresis for DNA of apoptotic lymphocytes showed fragmentation of DNA (Fig. 8).

Table 1: Mean±SD of the level of CD95 (sFas) ng ml^{-1} in the studied groups at O time and after 72 h

	CD95 (ng ml^{-1})				
Time of examination	G1	1a	1b	G2	G3
At 0 time	1.28±0.36	1.29±0.31	1.260.42	5.11±1.26	5.63±1.24
At 72 h	7.92±1.43	7.98±1.62	7.84±1.61	29.42±3.82	31.34±4.21

G1 vs G2 and G1 vs G3: highly significant at P<0.01 G2 vs G3 and G1a vs G1b: non-significant.

Table 2: Mean+SD of the percentage of apoptotic PBLCs by the different types of stains at 0 time and at 72 h

	Apoptotic Index					
	PI stain		Geimsa		AO	
Groups	0	72 h	0	72 h	0	72 h
G1	3.21	9.16	5.12	14.27	4.86	11.36
	+0.54	+0.86	+0.43	0.82	+0.42	+1.46
1a	3.16	9.11	4.93	14.61	5.24	11.12
	0.52	+0.74	+0.62	+0.79	+0.61	+1.42
1b	3.32	9.21	5.22	13.93	4.63	11.54
	+0.47	+0.72	+0.41	+0.81	+0.53	+1.63
G2	16.43	51.83	25.72	62.14	30.82	61.38
	+4.61	+5.12	+2.84	+5.44	+3.54	+6.71
G3	16.17	52.10	26.31	62.68	31.11	61.81
	+3.96	+5.24	+2.61	+6.23	+3.92	+5.87

PBLCs: Peripheral blood lymphocyte cells, PI: Propidium iodide stain, AO: Acridine orange stain,
G1 vs G2 and G1 vs G3 highly significant at P<0.01, G2 vs G3, G1a vs G1b non-significant

Table 3: The grades of mRNA expression of interlukins in the studied groups

		Percentage of mRNA expression			
Groups	Grade of expression	IL-1α	IL-1β	TNFα	IFNγ
G1	-	0.0	57.2	17.2	65.6
	+	0.0	42.8	34.3	34.3
	++	22.8	0.0	48.5	0.0
	+++	77.2	0.0	0.0	0.0
G2	-	5.1	97.2	91.7	97.2
	+	57.1	2.8	8.3	2.8
	++	37.2	0.0	0.0	0.0
	+++	0.0	0.0	0.0	0.0
G3	-	96.0	96.0	98.0	96.0
	+	4.0	4.0	2.0	4.0
	++	0.0	0.0	0.0	0.0
	+++	0.0	0.0	0.0	0.0

G1 vs G2 and G1 vs G3 highly significant for IL-1α and TNFα at P<0.001 and significant at P<0.05 for IL-1β and IFNγ G2 vs G3 highly significant at P<0.01 for IL-1α and non-significant for IL-1β and TNFα and IFNγ

Table 4: Mean±SD of the serum levels (pg/ml) of the IL-1α, IL-1β, TNFα and IFNγ in patients with RHD and control

	Serum levels of the cytokines (pg ml^{-1})			
Groups	IL-1α	IL-1β	TNFα	IFNγ
G1	176.7±8.6	22.4±3.6	136.3±10.8	39.8±4.1
G2	61.2±2.51	14.2±3.1	41.4±6.1	12.1±2.4
G3	7.4±2.9	16.1±2.4	41.6±3.2	12.2±3.9

G1 vs G2 and G1 vs G3 highly significant for IL-1α and TNFα at P<0.001 and significant at P < 0.05 for IL-1β and IFNγ G2 vs G3 highly significant at P<0.01 for IL-1α and non-significant for IL-1β, TNFα and IFNγ

Table 5: Serum levels (pg ml^{-1}) and m RNA expression in the mild to moderate (G1a) and severe (G1b) ARHD at diagnosis before treatment

	Serum levels		M RNA expression		
Cytokines	G1a	G1b	Grade	G1a	G1b
IL-1α	172.1	204.5	-	0.0	0.0
	+12.4	+7.5	+	0.0	0.0
			++	22.7	0.0
			+++	77.3	100.0
IL-1β	21.6	26.4	-	72.8	42.9
	+4.1	+3.4	+	27.2	52.8
			++	0.0	0.0
			+++	0.0	0.0
TNFα	131.6	139.6	-	18.1	0.0
	+4.6	+6.1	+	36.4	28.6
			++	45.5	57.1
			+++	0.0	14.5
IFNγ	38.2	41.4	-	72.8	44.9
	+4.1	+4.8	+	27.2	57.1
			++	0.0	0.0
			+++	0.0	0.0

Serum levels in G1a vs G1b significantly at P<0.05 for IL-1α and non-significant for IL-1β, TNFα and IFNγ.
m RNA expression in G1a vs G1b significant at P<0.05 for IL-1α and TNFα and non-significant for IL-1β and IFNγ

Table 6: Correlation (r) between the level of CD95 (sFas) (ng ml^{-1}) and the percentage of lymphocyte apoptotic cells detected by PI, Geimsa , AO stains and between the results of the different stains with each other at 0 time and at 72 h in G1

	At 0 time			At 72 h		
	CD95	AO	Geimsa	CD95	AO	Geimsa
P1	0.73**	0.83**	0.79*	0.78*	0.77**	0.78**
Geimsa	0.81**	0.78**		0.86**	0.82**	
AO	0.76*				0.81**	

** P<0.001

Table 7 : Correlation (r) between serum levels of cytokines (pg/ml) and CD95 and the percentage of apoptotic lymphocytes detected by the three stains in G1 at 72 h

	Serum levels of cytokines (pg/ml)			
% of apoptotic cells at 72 h	IL-1α	IL-1β	TNFα	IFNγ
CD95	-0.71**	-0.48*	-0.66**	-0.41*
PI	-0.63**	-0.42*	-0.69**	-0.43*
Geimsa	-0.61**	-0.44*	-0.63**	-0.44*
AO	-0.58**	-0.46*	-0.59**	-0.42*

* : P<0.05, ** : P<0.001

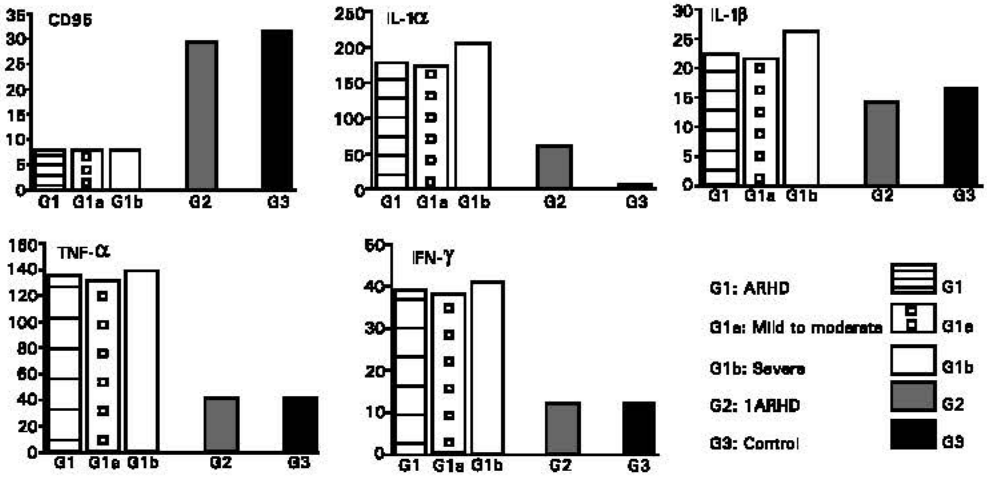

Fig. 1: Histogram of the serum level of CD95 at 72 h (ng ml^{-1}), IL-1α, IL-1β, TNF-α and IFN-γ (pg ml^{-1}) in the three groups

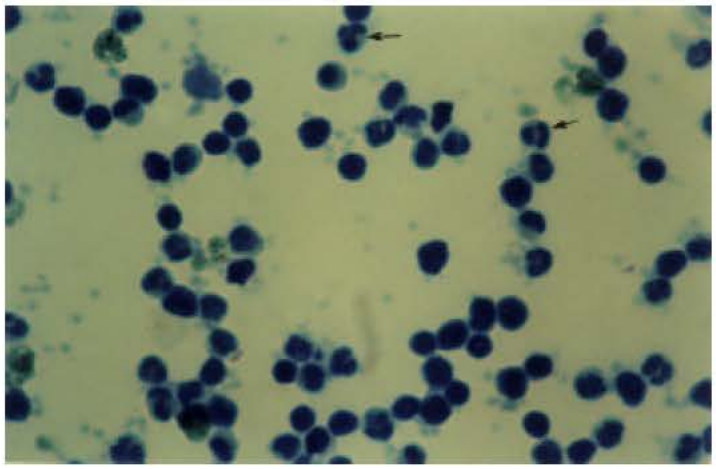

Fig. 2: A photomicrograph of blood lymphocytes (PBLCS) of ARHD *in vitro* culture showing small number of apoptotic cells stained by Giemsa Stain. Apoptotic cells have condensed fragmented nuclei, vacuolated cytoplasm and decrease cell volume (Original magnification X 100 May Giemsa)

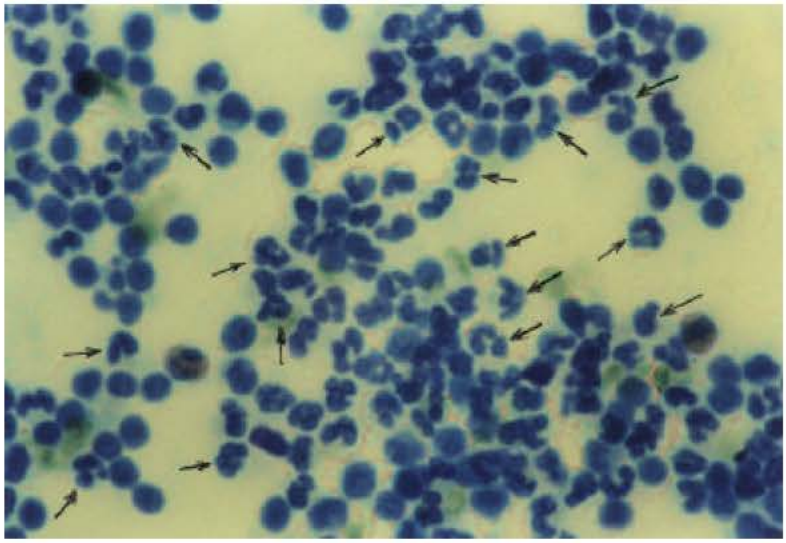

Fig. 3: A photomicrograph of blood lymphocytes (PBLCS) of ARHD *in vitro* culture showing big number of apoptotic cells stained by Giemsa Stain. Apoptotic cells have condensed fragmented nuclei, vacuolated cytoplasm and decrease cell volume (Original magnification X 100 May Giemsa)

Fig. 4: A photomicrograph of blood lymphocytes (PBLCS) of ARHD *in vitro* culture showing small number of apoptotic cells as seen fluorescent microscope stained by Acridine Orange Stain. Apoptotic cells have bright, fluorescent, condensed nuclei reduction of cells have viable cells show green diffusely stained intact nuclei (Original magnification X 100 Acridine Orange)

Fig. 5: A photomicrograph of blood lymphocytes (PBLCS) of ARHD *in vitro* culture showing big number of apoptotic cells as seen fluorescent microscope stained by Acridine Orange Stain. Apoptotic cells have bright, fluorescent, condensed nuclei reduction of cells have viable cells(v) show green diffusely stained intact nuclei (Original magnification X 100 Acridine Orange)

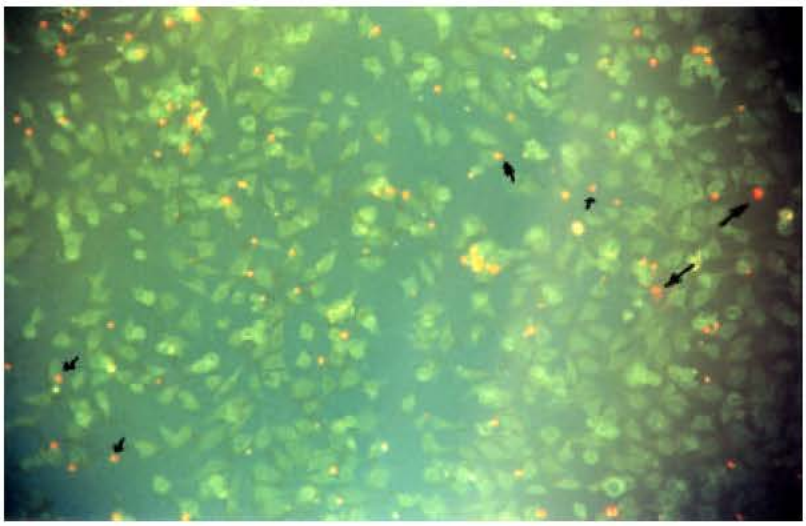

Fig. 6: A photomicrograph of human blood lymphocytes (PBLCS) of ARHD in vitro culture showing small number of apoptotic cells as seen by fluorescent microscope stained by Preputium Iodide Stain. Membrane intact Apoptotic cells (bright blues chromatin that is highly condensed, marginated of fragmented) and membrane permeable apoptotic cells (bright red chromatin highly condensed or fragmented). (Original magnification X 100 Preputium Iodide)

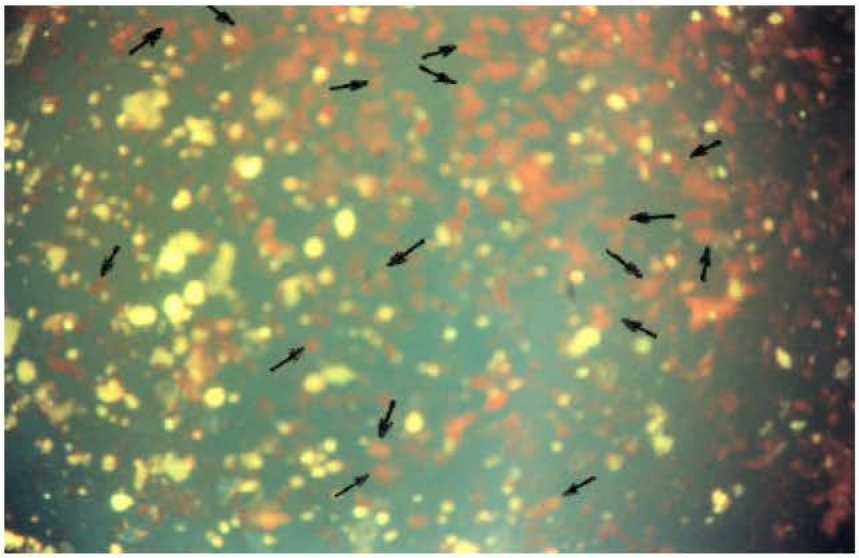

Fig. 7: A photomicrograph of blood lymphocytes (PBLCS) of IARHD in vitro culture showing bigl number of apoptotic cells as seen by fluorescent microscope stained by Preputium Iodide Stain. Membrane intact Apoptotic cells (bright blue chromation that is highly condensed, marginated or fragmented) and membrane permeable apoptotic cells (bright red chromatin highly condensed or fragmented). (Original magnification X 100 Preputium iodide)

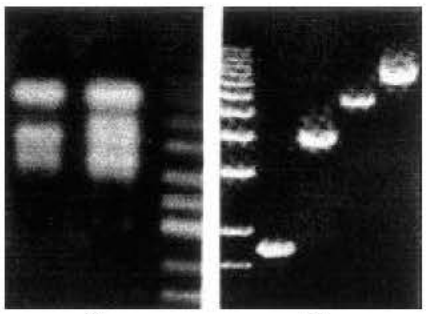

Fig. 8: DNA fragmentation in apoptotic cells after culture for 72 h; (A) decreased DNA fragmentation in patients with ARHD. (B) marked fragmentation of DNA with step ladder appearance in patients with IARHD

Detection of mRNA of IL-1α, IL-1β, TNF-α and IFN-γ (Table 3 and Fig. 9) revealed that mRNA expression in G1 is significantly more than G2 and G3. The level of significance is at $P<0.01$ for IL-1α and TNF-α and $P<0.05$ for IL-1β and IFN-γ. No significant difference between G2 and G3. Serum levels of cytokines are mirror images of mRNA (Table 4 and Fig. 1). The levels in G1a compared to G1b is significantly higher in G1b at $P<0.05$ for IL-1α and non-significant for IL-1β, TNF-α and IFN-γ. mRNA expression in G1a compared to G1b is significantly higher in G1b at $P<0.05$ for IL-1α and TNF-α and non-significant for IL-1β and IFN-γ (Table 5).

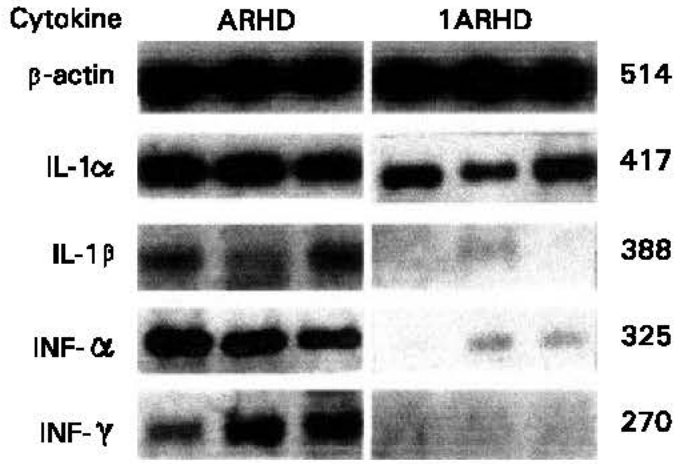

Fig. 9: The expression of the indicated cytokines mRNA were detected by semiquantitative RT-PCR. The result of RT-PCR for β-actin demonstrate the loading of equal amounts of DNA on the gel

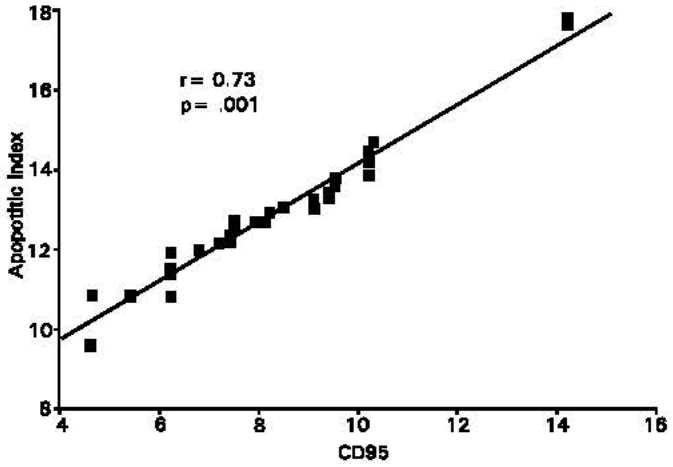

Fig. 10: Correlation between apopototic index and CD95

For patients with ARHD, Pearson correlation test between CD95 and apoptotic lymphocyte cells index detected by the different types of stains and between results of the different stains with each others revealed highly significant correlation for every test ($P<0.001$) (Table 6). On the other hand Pearson correlation test between serum levels of cytokines and CD95 and the apoptotic lymphocytes index detected by the three stains in G1 at 72 h, revealed significant negative correlation at $P<0.01$ for IL-1α and TNF-α and at $P<0.05$ for IL-1β and IFN-γ (Table 7).

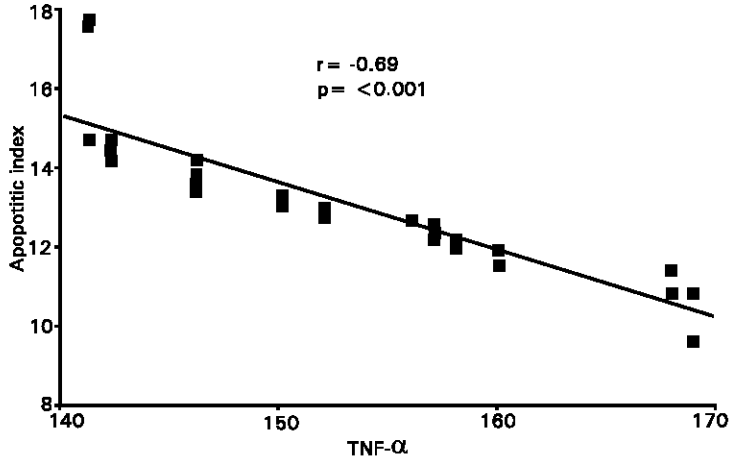

Fig. 11: Correlation between apoptotic index and TNF α

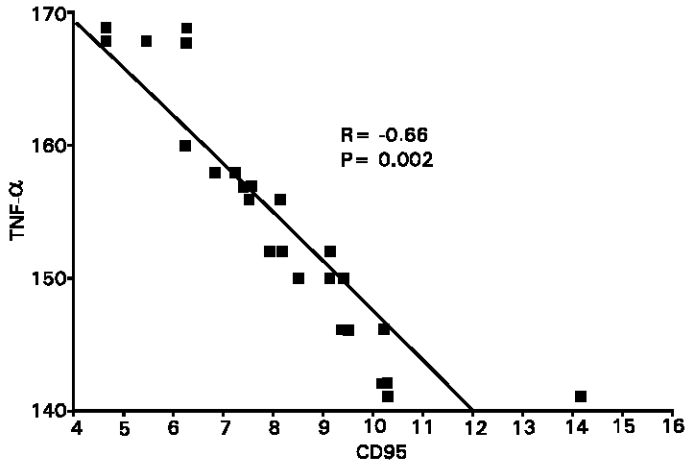

Fig. 12: Correlation between TNF α and CD95

Fig. 10-12 illustrate the curves of correlation between apoptotic index for lymphocytes stained by PI and CD95 serum level in ARHD showing significant positive correlation (r = 0.73, P< 0.001) and between apoptotic index and TNF-α and between apoptotic index and TNF-α and CD95 and TNF-α which show significant negative correlation (r=-0.69, P<0.001 and r = -0.66, P<0.002, respectively).

Discussion

An autoimmune disease can result from failure to remove autoimmune cells that arise during immune response to an antigen (Thompson, 1995). One of the important factors regulating cell

death in lymphocytes is the cell surface receptor sFas (CD95), a member of TNF receptor family (Scaffidi *et al.*, 1998). Stimulation of CD95 on activated lymphocytes can induce apoptosis. The death-inducing signal complex (DISC) is formed by interaction of FADD/MORT-1 with the death domain of CD95. This interaction activates caspase-8 and caspase-3 which proceed the process of apoptosis (Lee and Shacter, 2001).

This is the first study on the role of CD95 and programmed cell death in the pathogenesis of rheumatic heart disease (RHD). In this study we found a decreased CD95 expression in active rheumatic heart disease (ARHD) with no significant difference between mild to moderate and severe clinical presentation. The decreased levels turned to be in-significantly different from the healthy subjects. It is known that stimulation of Fas receptor on activated lymphocytes can induce spoptosis. The death-inducing signal complex (DISC) is formed by interaction of FADD/MORT-1 within the death domain of Fas. This interaction activates caspase-8 and caspase-3 which proceed the process of apoptosis (Lee and Shacter, 2001).

The present finding of increased production of mRNA and consequently high levels of circulating cytokines in ARHD is important. IL-1α and TNFα levels were highly significant increased at $P<0.001$ which indicate their possible important role in the initiation of apoptosis. Zhang *et al.* (2000) reported that stimulation of the cells with TNF-α has been shown to generate 2 signals, one that initiates programmed cell death and another that leads to activation of the transcription factors $_k$B (NF-$_k$B), which induces inhibitors of apoptosis and promotes the production of pro-inflammatory factors. TNF-α strongly activates NF-$_k$B through a second class of adapter protein TNFR-associated factors and this transcriptional factors regulates the expression of antiapoptotic gene products (Beg and Baltimore, 1996; Van Antwerp *et al.*, 1996) such as antiapoptotic members of the Bcl-2 family and the inhibitor of apoptosis c-1AP1 and c-1AP2. TNF-α also activates sphingosine-1-phosphate which is a lipid derived mediator shown to prevent the cytotic action of TNF-α (Osawa *et al.*, 2001). Nicholson and Thomberry (1997) suggested that TNF-α may be cytotoxic to B cells by activating cysteine proteases (caspases) that initiate the proteolytic cascade.

Interleukin-1 (IL-1) is a key mediator in the cytokine network controlling important functions in the immune system. Activation of IL-1 beta is performed enzymatically by caspase-1. This molecule is the funding member of the caspase family of enzymes, which are involved in maturation of cytokines, in initiation and execution of apoptotic processes (Loppnow *et al.*, 2000). However, the still significantly increased IL-1 level inspite of the decline in the patients in the inactive state while TNF-α, IL-1β IFN-γ, mRNA and serum levels, CD95 and percentage of apoptotic cells returned to normal values in patients with 1ARHD, points to two possibilities, I) the effect of IL-1α on programmed cell death through stimulation of the macrophages to secrete more TNF-α and II), direct effect of IL-1α on CD95 receptors leading to inhibition of CD95 production and hence inhibition of the process of apoptosis. The remittent increase of IL-1α even in 1RHD is against the II possibility.

Andersen ***et al.*** (2000) postulated that there is synergistic effect of TNF-α , IL-1β and IFN-γ in the functional inhibition and induction of cell death. The last two cytokines showed only increased mRNA and serum values at P<0.05, which raise the possibility of their more local effect in the valves.

Lane ***et al.*** (1993) found infiltration of the heart by inflammatory cells that secrete IL-1α and TNF-α and that local secretion of these cytokines contributes to increased levels of IL-1α and TNF-α in the serum. Fraser ***et al.*** (1997) studied rheumatic Aschoff nodules and the level of cytokines in relation to them and found that IL-1α and TNF-α are expressed within macrophages in all stages of Aschoff nodules. They suggested that this is required for T and B lymphocytes activation and aggregation. However, based on this study, further investigations are needed for the detection or not of IL-1β and/or IFNγ in the rheumatic valves.

In the process of apoptosis, cell nuclei and cytokeleton changes first, then membrane blebbing, caspase-3 activation occur and fragmentation of chromosomal DNA is last (Maruyama ***et al.***, 2000). In this research work we studied the percentage of apoptotic cells in the peripheral blood by different methods of stains to endorse the assessment. Mc Gahon ***et al.*** (1995) and Maruyama ***et al.*** (2000) reported that the order of apoptotic features such as morphological changes or nuclear condensation is not the same in the different types of cells and several methods are needed. The results are the same with the different stains and revealed low count of apoptotic lymphocytes in patients with ARHD compared to control and being within normal count in the inactive status. This is confirmed by the DNA fragmentation found in the lymphocytes of such patients. Correlation analysis revealed high significant correlation between CD95 and the percentage of apoptotic lymphocytes by the different stains and between the results of the different stains. The accumulating reactive mononuclear cells 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). Roberts ***et al.*** (2001) studied the valve tissues from rheumatic patients with valvular heart disease who required valve replacement and found expression of the adhesion molecule VCAM-1. The present correlation analysis highligtened these findings by the significant negative correlation between IL-1α, TNF-α, IL-Iβ, IFN-γ and CD95 and percentage of apoptotic lymphocytes by the different types of stains. The significance of the results is very high (P<0.001) with IL-1α and TNF-α and only at P<0.05 with IL-1β and IFN-γ.

Taken together, the present study is the first one to investigate the implication of CD95 and programmed cell death of lymphocytes in the pathogenesis of RHD. The inhibition of CD95 and consequently of apoptosis is mainly due to the effect of TNF-α and possibly IL-1β and IFN-γ . The still increased level of IL-1α in the inactive state together with the normal level of CD95 and percentage of apoptotic lymphocytes, indicates no major role of IL-1α in the initiation of apoptosis, but it is very important in the process of inflammation in the valves (Lane ***et al.***, 1993). The inhibition of apoptosis lead to accumulation of auto-reactive lymphocytes which migrate to

the valves, infiltrate and secrete cytokines. Hence, to stop early the process of rheumatic activity, we have to add to the therapeutic strategy anti TNF-α and anti IL-1α. The first to stop the effect of TNF-α of sFas and both the first and second to attenuate the process of reactivity in the valves.

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