Original Article

CD-Markers as an immunological aspect in patients with visceral leishmaniasis

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Summary:

Back ground: Visceral leishmaniasis is an endemic protozoan disease in Iraq. Recovery from this disease confers a solid and permanent immunity. Immunological assessment of our patients was carried out and the results showed a significant reduction in the percentage of CD3, CD56 and a significant increase in the percentage of CD19 in the peripheral blood lymphocyte of VL in comparison with control group.
Patients and methods: Indirect immunofluorescence technique analysis was performed to detect the percentage of CD3, CD19and CD56 positive lymphocytes.
Results: Our results in the patients groups showed decrease in the percentage of CD3, CD56 and increase in the percentage of CD19**.** Follow up of patients after treatment showed a significant increase in the percentage of CD3, CD56, but they were still lower than their normal range .
Conclusion:- CD3,CD56, were decreased in VL infection then increased after treatment but it was less than its normal range, while CD19 was increased in VL when compared with control group.
Key words: CD-Markers profile, leishmania donovani, amastigote form

Introduction

Leishmaniasis is a spectrum of diseases caused by *Leishmania* species, protozoan of the order kinetoplastidia. They present a wide clinical spectrum ranging from cutaneous lesions to fatal visceral disease; they are distributed through 88 countries (1).There are about 30 species of sand flies, which can transmit different species of Leishmania (2). Leishmania is a genous of the family Trypanosomatidae, with two forms, an amastigote which occurs in the mammalian host and a promastigote which occurs in the insect host. Infection of man by this protozoan result in a disease called leishmaniasis (3). In Iraq, according to the reports of the Communicable Disease Control Center in Ministry of Health (MOH), infectious diseases including leishmaniasis remain on the list of major causes of morbidity and mortality(4) The parasite has a digenetic life cycle, alternating between a flagellated extra cellular promastigote from that multiplies within the gut of the sand fly (*phlebotomus spp*. and *Lutozomyia spp.*) and non flagellated amastigote from replicative within the host macrophage and other cells of the reticuloendothelial system (5). CD-markers are glycoprotein's characterized in two populations of lymphocytes (T and B-lymphocyte) and natural killer cells. Most T-helper cells express CD4,where as most T-cytotoxic cells express CD8, NK cells express CD16 and CD56, and B cells express CD19, CD21,CD32 and CD35.The CD4 cells are involved in the regulation function (helper/inducer) of the immune response and the T-CD8 cells have suppressive and cytotoxic activity. T-cell function releasing involves the respective recognition of CD4 and CD8 by class II and class I MHC molecules which represent their natural ligands(6)

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Materials and Methods:

Patient's study group

Patient's groups included in this study were divided as follows**:**
A-Confirmed VL group.
Blood samples were collected from 50 children less than six years of age who had positive bone marrow smears for VL; those were diagnosed in Al-Mansoor Children Hospital, Al-Kadhimiya Children Hospital, Central Children Hospital and Al-Ilwia Children Hospital.
B-Control groups
B1-Endemicity control
Fifty blood samples were collected from children less than six years of age living in an endemic area (Al-Suwaira district) and who were apparently healthy.
B2-Healty control
Forty blood samples were collected from children less than six years of age from different primary health centers in Baghdad with no history of living in endemic areas with VL and who were apparently healthy by physical examination.
A sufficient amount of blood was collected in an anticoagulant container and plain tube for cell mediated immunity from fifty children with disease (before administrating of sodium stibogluconate injection) and twenty five children who were

followed–up until the end of therapy (28 days of sodium stibogluconate therapy). Each blood sample obtained in anticoagulant-tube centrifuged as soon as possible and serum was separated, in addition to lymphocyte separation. The Isopaque-Fecol technique originally described by Boyum (7) was used for isolation of mononuclear cells.

2-Methods

Indirect Fluorescent Antibody Test (IFAT) For Counting Lymphocyte Subsets

A-Principle:- Indirect immunofluorescence for the detection of CD-antigen depends on two steps; the first step leads to the binding of primary antibody to specific cell antigen. The second step allows the detection of specific CD-antigen when anti-mouse immunoglobulin IgG fluorescinated conjugate is added to be examined by immunofluorescent microscope. The positive samples show an apple green fluorescence corresponding to areas of cell surface where primarily antibody bound.

Procedure, according to the method of (8).

B- Assay procedure

1-Lymphocyte suspension was adjusted to contain 2×10^6cell/ml, 45µl of lymphocyte suspension was transferred in tube and 5 µl of monoclonal antibody (CD3, CD19, and CD56) was added, mixed well and incubated at (2-8C^o) for 30 minutes.

2-Lymphocyte suspension was centrifuged two times at 1500g for 5 minutes and the supernatants was aspirated and discarded and cell pellet was resuspended in PBS/BSA.

3-Fifty µl of fluorescent conjugate (diluted 1:80 in PBS/BSA) was added and incubated for 30 minutes at (2-8C^o) in the dark.

4-Washing was repeated as in the step 2.

5-The cell pellets were resuspended in 200 µl of PBS/BSA, a drop was delivered by Pasture pipette and placed in the center of clean slide with cover slip.

C-Calculation of the results

Slides were examined under 40X-magnification of a fluorescent microscope. Their dark green staining identified positively labeled cells. Two hundred cells were counted to determine percentage of reactivity of the tested monoclonal antibodies.

Statistical analysis:Data were analyzed statistically using SPSS program version 10. Results were expressed using simple statistical parameters. Analysis of quantization data was done using t-test and ANOVA (analysis of variance). Acceptable level of significance was considered to be less than 0.05

Results:

Results of bone marrow smears fifty bone marrow smears showed moderate to sever megaloblastosis, an increased number of plasma cells and megakaryostic hyperplasia with abnormal morphology. Amastigotes appeared as round or oval bodies found intracellularly in monocytes and macrophages, extracellular leishman bodies are also seen in the stained smears (figure1)

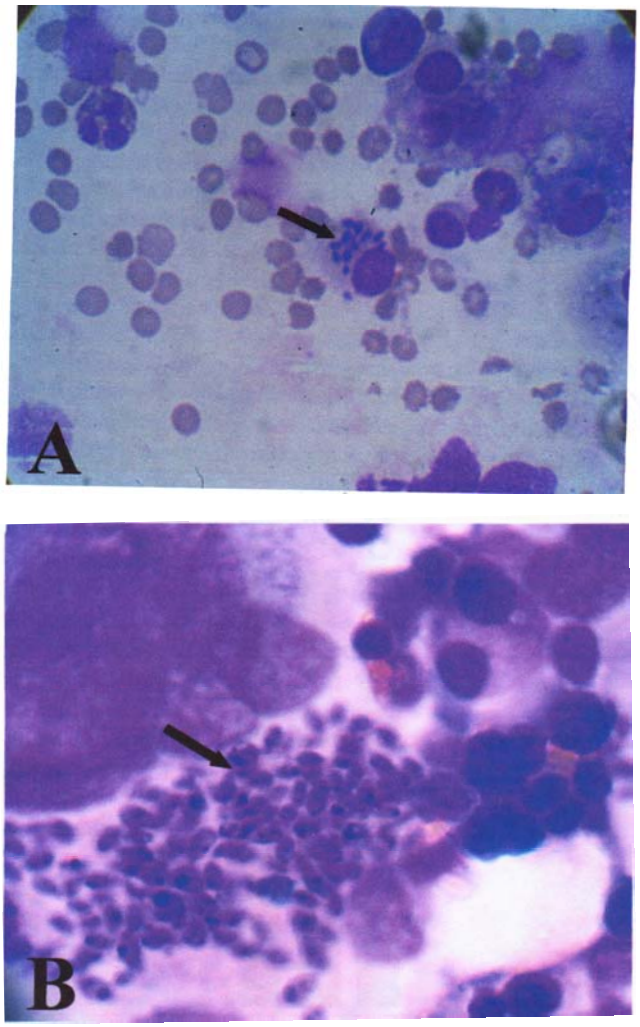

Figure (1): **Bone marrow smears showing intratracellular and extracellular Leishmania donovani bodies in patiens with VL.**
A-Viability of Leishmania amastigote in infected macrophage (×100X.
B-Amastigotes release from died macrophage to infect other macrophages magnification power of a and b × 100X.

Lymphocyte subsets enumeration

One method for enumeration of lymphocyte subsets immunofluorescence was compared in healthy children using CD19, CD3 &CD56

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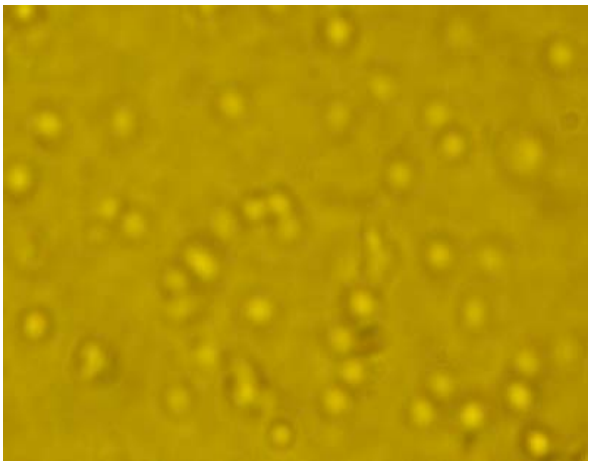

Figure (2): Slides of indirect immunofluorescence microscope at 490 nm without exposure to UV-light to see lymphocyte subsets.(400 x).

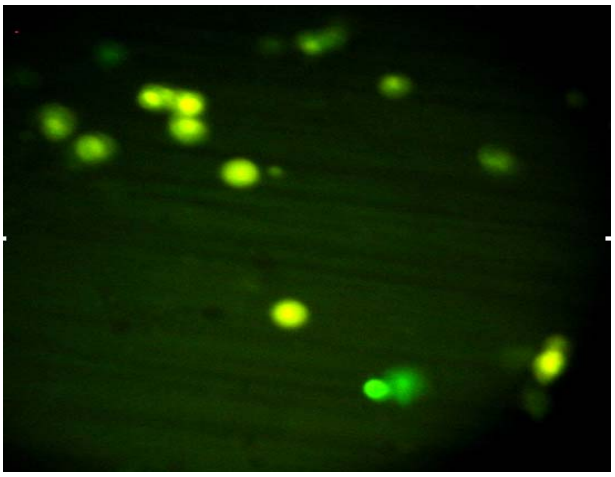

Figure (3): Slides of indirect immunofluorescence microscope at 490 nm. Positive cells give green-apple when stained with FITC-labeled antibodies after exposure to UV-light to see lymphocytes. (400 x) Lymphocytes subsets counting by Indirect Fluorescent Antibody Test (IFAT) .

B-cells(CD19): CD19 was higher in pretreated VL group (13.5 ±0.34)% than in the control group (9.5±0.34) % and the difference was significantly important (p<0.01). CD19 in post treated group was (11.2±0.38)% which was significantly lower than pretreated group but still higher than control group (p<0.01).(Table1).Fig(4).

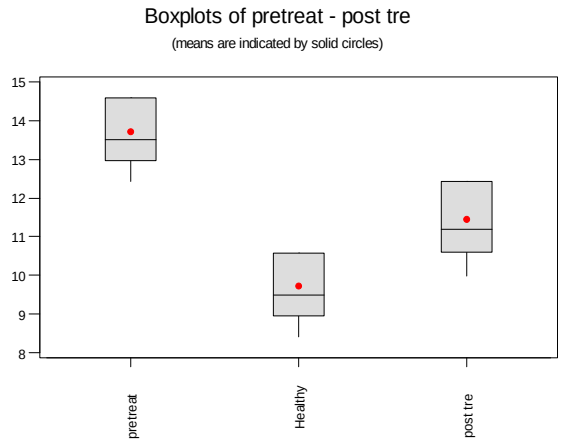

Figure (4): Percentage of CD19 among pretreated VL, healthy control and post treated VL by immunofluorescence test.

T-Cells (CD3): CD3 was lower in the pretreated VL group (54.6±1.26)% than control group (73.2±0,71)% with significantly difference (p<0.01).In the post CD3 was (68.2± 1.2) % significantly higher than that in the pretreated group
(p<0,01).There was a significant difference between post treated and control

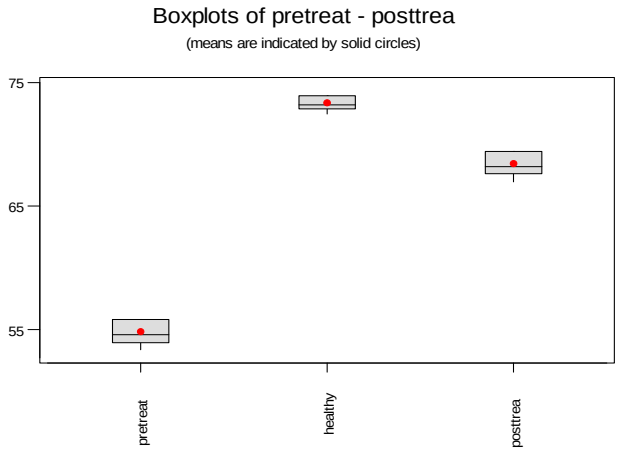

Figure (5) Percentage of CD3 among pretreated VL, healthy control and post treated VL by immunofluorescence test.

Natural killer cells (CD56): CD56 in the pretreated VL group was lower (9.1 ±0.37))%than in the control group (12.2 ±0.32))%with significant difference between both groups(p<0.01)**.**CD56 in the post treated group was (11±0.25) %significantly higher than pretreated group. There was a significant
difference between post treated and control groups (p<0.01) (Table1).(Fig.6)

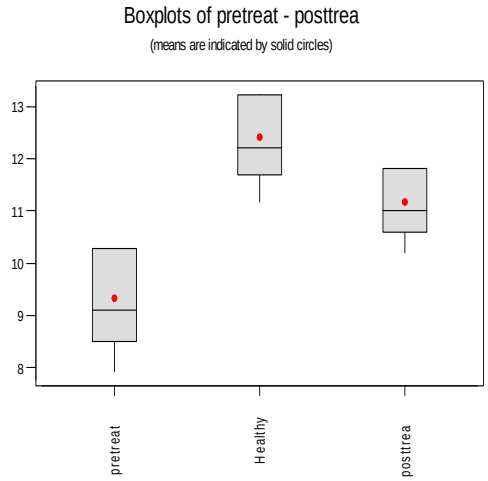

Figure (6): **Percentage of (CD56) among pretreated VL, healthy control and post treated VL by immunofluorescence**

Table (1): Percent of total T-lymphocyte subsets in patients with VL before and after sodium stibogluconate therapy

CD-Marker	Pretreated VL Group (Mean ± SE)	Healthy control group (Mean ± SE)	Post treated VL group (Mean ± SE)
CD19	13.5 ±1.08	9.5 ±1.08	11.2±1.22
CD3	54.6±1.26	73.2±0.71	68.2 ±1.2
CD56	9.1±1.19	12.2±1.03	11±0.81

Discussion:

Lymphocyte subset enumeration by IFAT : Total-T-lymphocyte CD3+reduction in patients with VL was observed in comparison with control. This could be associated with reduction of T-Lymphocyte proliferation. The same results reported by Ho et al.,(9) who noted the proliferative response of ,Kenyan patients with VL were severely depressed to heterogeneous as well as Leishmania antigen. Suppression may be mediated by macrophage, either by defective antigen processing and presentation, or by elaboration of suppressive mediators like IL-10, TGF-ß and prostaglandin E2 (10).Lower percentage of CD56 (natural killer marker) was shown in patients with VL in comparison with control. Lowering of NK cell numbers in peripheral blood may result from their localization in tissue, like liver and spleen. It is known that NK cells, acting as innate immune effector cells and may play an important role in the early stages of Leishmania infection, they respond rapidly and serve as source of activating cytokine like IFN-γ and TNF-ą, which not only inhibit the growth of parasite during initial stages of infection but also influence the differentiation ofCD4-T-cells towards Th1phenotype (11,12).The results of this study have revealed that there was a significant increase of CD8T-cells in patient with VL in comparison with control. These results in agreement with results of Colmenares *et al*.,(13) who confirmed the increase of CD8 T-cells in VL cases ,however, Rohtagi *et al*., (14) stated that peripheral blood CD8 cell count was normal in acute and uniforrmaly low in the cronic cases. This may be explained by the presence of IL-2from macrophage and Th1 which increase CD8+ division. Results have been indicated that CD19 was significantly higher in patients with VL in comparison with control. The increased number of B cells may result from polyclonal activation of B-cells by *Leishmania* antigens (*15).* The antigen could be presented by the B-cells to the T-cells this leads to the development of CD4+Th2 (16). Th2-cells through its cytokines (IL-10 and IL-4) inhibit macrophages activation and is involved in antibody production. The antibody role of B-cells against *Leishmania donovani* infection was noted by Smelt et al., (17) who reported that B-cells deficient mice cleared parasite more rapidly from liver and infection failed to established in the spleen. Treatment resulted in a significant increase in the percentage of CD56, CD3 compared with pretreated group but still lower in comparison with control In contrast the percentage of CD19 showed a significant decrease in comparison with pretreated group and still higher than control. These results confirmed the results of previous studies Ghosh *et al.(18)* who showed that CD4+/CD8+ratio returned to normal value three months after recovery .Neogy *et al*.,(19) stated that follow -up B-lymphocyte population during and after chemotherapy for the period of eight months showed the clinical improvement in kala-azaqr patients resulting from treatment.

Conclusion:

CD3,CD56, were decreased in VL infection then increased after treatment but it was less than its normal range, while CD19 was increased in VL when compared with control group.

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