Phagocytic Index in Patients with Visceral Leishmaniasis in Iraq

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ABSTRACT:

BACKGROUND:

Visceral Leishmaniasis is an endemic protozoal 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 that a significant increase in phagocytic index in patients with VL in comparison with control group.

AIM OF THE STUDY: Determining phagocytic index.

METHODS:

Blood samples were collected from 50 patients suffering from VL confirmed parasitologically by bone marrow smears, 40 blood samples from apparently healthy children and 50 blood samples from children in an endemic area (Al-Suwaira district).Their age was less than six years in all groups, and who were apparently healthy.

RESULTS:

The immunological assessment of our patients was carried out and the result showed that a significant increase in phagocytic index in patients with Visceral Leishmaniasis in comparison with control group ($p<0.01$). While the result of endemic control group by ELISA, IFAT and IC were used to test 50 serum samples from healthy children who lived in an endemic area. A total of 48 cases of them were found to be negative at least by one of the above tests, IC showed the lowest cross reactivity rate (4%).

CONCLUSION:

Phagocytic index was higher in patients with Visceral Leishmaniasis in comparison with control group**.**

***KEY WORDS*:** staphylococcus aureus, leishmania donovani promastigotes

INTRODUCTION:

Leishmaniasis is anthropozoonoses due to infection with Leishmania parasite, for which there is, to date, no safe and effective vaccine [(1)]. It is aspectrum 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 [(2)].There are about 30 species of sand flies, which can transmit different species of Leishmania [(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)]. In spite of the long history of the disease in our country and the increasing numbers of reported cases, the true incidence, prevalence exposure rate, endemic foci, clinical aspects and reservoir animals are among many other facts about the disease that are incompletely studied. This is probably due to the lack of diagnostic tests that could be used not only in the detection of active cases of the disease, but also as an epidemiological screening test. Such test should be easy to perform not only in reference laboratories but also in district and in the study areas. In 1903 both Leishman and Donovani observed certain bodies in material from the spleen of patients with Kala-azar fever in India then Rogers in 1904 and Nicole in 1908 established the trypanosomatid nature of *Leishmania* parasite *in vitro* culture [(5)]. Adler and The odor in 1926 noted the relation of the disease to *phlebotomus sergenti* in Baghdad, visceral leishmaniasis was first mentioned in Iraq by Kulz,1916 [(6)].In immunological aspects, macrophages also act through reactive oxygen intermediate(ROI) that are generated following phagocytosis and stimulated the respiratory burst. When activated by cytokines, macrophages release more superoxide and hydrogen peroxide than normal resident macrophages and O2- independent killing mechanisms are similarly enhanced [(7)].

MATERIALS AND METHODS:

Patient's study group Patient's groups included in this study were divided as follows**:**

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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 a plain tube centrifuged as soon as possible and serum was separated , a liquated into portions to avoid repeated freezing and thawing and stored at -20Cº until used.

2-Methods

Phagocytic Index A-Principle:-

Phagocytes involves the ingestion of foreign materials. Ingestion can be determined by incubation of neutrophils with *Staphylococcus aureus* then intracellular *Staphylococcus aureus* can be seen microscopically [8].

Polymorph nuclear lymphocyte separation (PMNLS) PMNLS were isolated from the pellet as described by in [9] with slight modification as follows:- **1.**Five millimeters of heparinzed blood were mixed with 5% solution of Dextran and PBS in a 4:1 ratio and subsequent sedimentation at 37Cº. **2.** The supernatant was then harvested and holding with isotonic sterile calcium and magnesium free Hank's balanced salt solution (HBSS). **3.** Cell suspension was then centrifuge at 2000g for 10 minutes in a conical test tube and the supernatant discarded. After washing twice in HBSS, the pellet was gently resuspended in 200 µl of HBSS, and then adjusted to (5×10^6 cell/ml).

Bacterial culture *Staphylococcus aureus* isolate (supplied by Central Public Health Laboratory) was grown on nutrient agar over night at 37Cº. The bacterial growth was harvested and washed 3 times with PBS. The pellet resuspended in PBS and diluted to appropriate cell count (1×10^8 cell/ml).

B-Assay procedure:-

This was performed according to the procedure outlined (10) by adding 0.25ml of bacterial suspension (1×10^8 cell/ml) to suspension of PMNLS (1×10^6 cell/ml) in 0.25ml HBSS. The mixture was mixed and incubated in water bath at 37Cº for 30 minutes with continuous slow mixing then centrifuged at 1500 g for 5 minutes. The pellet was suspended in 200µl of HBSS. A drop was delivered to prepare thin smear and fixed with methanol. Smears were stained with diluted Giemsa stain for 10 minutes. The slides were then allowed to dry after washing with tap water and examined microscopically.

C-Calculation of the results Two hundred PMNS were counted and the percentage of phagocytic cells were determined. **Phagocytosis of Leishmania donovani promastigotes in vitro:** This was performed according to the procedure outlined [11] by adding 200µl of *Leishmania donovani* promastigotes suspension which was prepared at a concentration (1×10^9) to 100µl of heparinzed blood. Reagents were mixed and incubated in water bath at 37cº for 1 hour with slow continuous mixing. A drop was aspirated each 10 minutes to prepare thin smears, fixed with methanol and stained with diluted Giemsa stain for 10 min. The slides were then allowed to dry after washing with tap water and examined microscopically *Leishmania donovani* promastigotes.

Statistical analysis Data were analyzed statistically using SPSS program version 10. Results were expressed using simple statistical parameters. Analysis of quantitation 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)

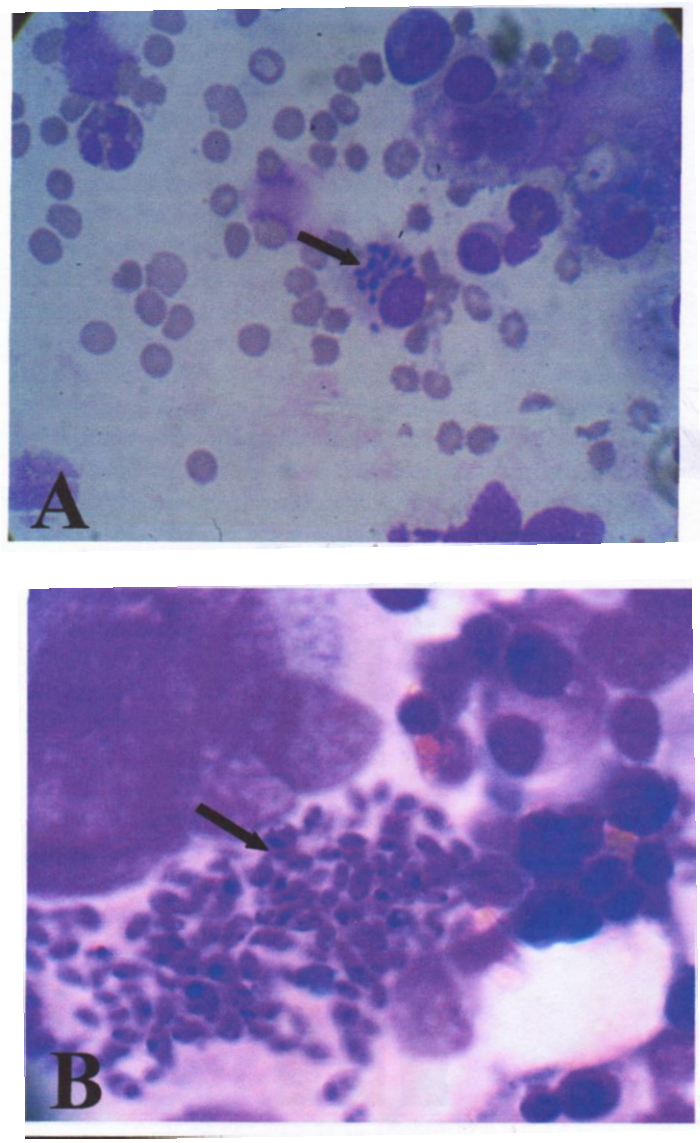

Figure (1): Bone marrow smears showing intratracellular and extracellular Leishmania donovani bodies in patiens with VL. A-viability of Leishmania amastigote in infected macrophage (×100X. B-amastigotes release from died macrophage to infect other macrophages magnification power of a and b × 100X.

Phagocytic Index: The phagocytic Index in patient with VL was (32.8 ± 1.87)% higher than that in the healthy control group (13.9 ± 1.15) % and the difference was high significant ($p<0.01$) (Figure 2). While the result of endemic control group by ELISA, IFAT and IC were used to test 50 serum samples from healthy children who lived in an endemic area. A total of 48 cases of them were found to be negative at least by one of the above tests, IC showed the lowest cross reactivity rate (4%).

Phagocytosis of Leishmania donovani promastigote in vitro. Follow up of phagocytosis *of Leishmania donovani* promastigote *in vitro* was done to demonstrate different stages of phagocytosis. Figure (3) showes that neutrophil attacks *L. donovani* promastigote then *L donovani* adheres to neutrophil which phagocytes *Leishmania* and possibly digested the ingested parasite and leave signs for this digestion.

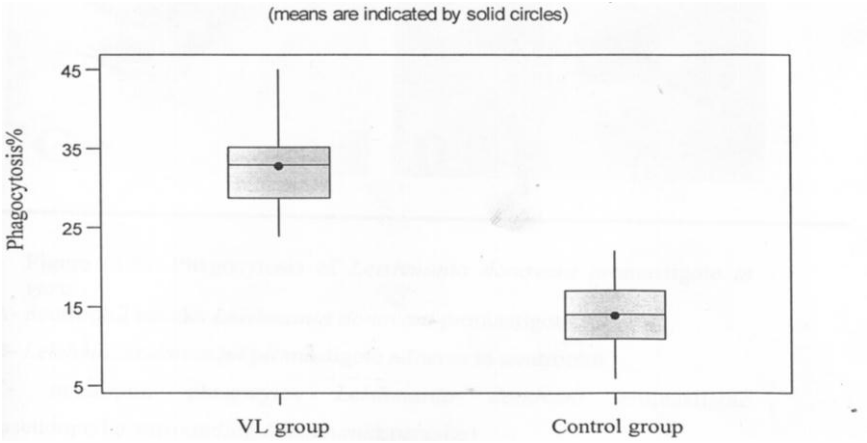

Figure (2): Phagocytic Index in patients with VL and healthy control group

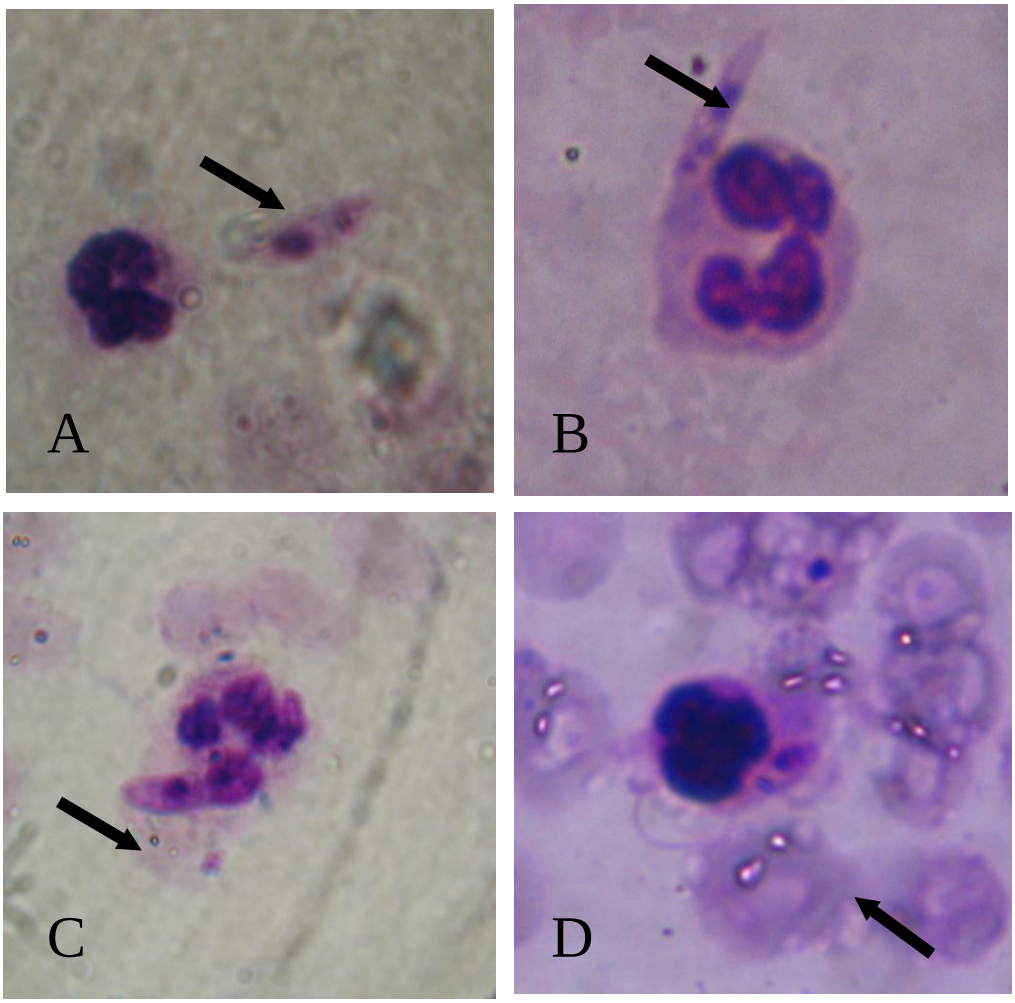

Figure (3): Phagocytosis of Leishmania donovani promastigote in vitro
A- neutrophil attacks Leishmania donovani promastigote.
B- Leishmania donovani promastigote adheres to neutrophil.
C- neutrophil phagocytes Leishmania dondvani promastigote (pseudopodia surrounding Leishmania parasite)
D- neutrophil possibly digested Leishmania donovani promastigote and leaves morphological sign for this digestion(magnification power of a, b, c, d x 100X).

DISCUSSION:

Visceral leishmaniasis is a fatal disease if left undiagnosed. A definitive diagnosis of visceral leishmaniasis currently requires demonstration of parasite by smears or culture from tissue, usually bone marrow or spleen, whereas an immunological diagnosis is based on indirect evidence (antibodies formed against parasite). For this reason, immunological methods should be used in practice only when the techniques are highly sensitive, very specific and carefully evaluated [12]. One of these methods is Phagocytic index.

Phagocytic Index. The ability of phagocytic cells to engulf *Staphylococcos aureus* increased in patients with VL in comparison with control. It is known that exposure to *Leishmania* parasites which avoid phagocytosis by various mechanism increases the ability of neutrophils to engulf them through complement and FC receptors as a mechanism of parasitism because neutrophils was found to be beneficial for parasite survival and acts as a vector for parasite entry into its main host macrophages [13]. Also results of this study showed that patients with VL have a greater ability to synthesize the chemokine interleukin-8 and granulocyte are not only producers but also the primary targets for IL-8. This chemokine plays an important role in chemotaxis and increase uptake of parasite by neutrophils [14]. Follow up of phagocytosis of *L .donovani* promastigote in *vitro* showed that neutrophils engulf L. *donovani* promastigote and possibly kill the ingested parasite. This could be explained by the fact that neutrophils are the first leukocyte that migrate to the site of infection and encounter the parasites [15]. Likewise it has been observed that 47percent of human blood neutrophils express CD28 and that CD28 signals through P13 kinas induces IFN-gamma [13]. IFN-gamma might result in augmentation of MHC class II expression in an autocrine manner and subsequent leishmanicidal function. That's mean neutrophils help both phagocytic killing and transfer to macrophages for better survival [16].

CONCLUSION:

Phagocytic index was higher in patients with Visceral Leishmaniasis in comparison with control group

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