

Ministry of Higher
Education and
Scientific Research



University of Babylon
College of Science
Department of Biology

Characterization of Cell Mediated Immune Responses induced by Visceral Leishmania Major Infection

A Thesis

Submitted to The College of Science of the University of
Babylon in Partial Fulfillment of The Requirements
for The Degree of Doctor of Philosophy in
Biology/ Zoology

By

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Sha'aban 1429

Certification

This is to certify that the preparation and organization of this thesis have been done by the graduate student *Faheim M. D. Alshebly* under our supervision at the college of Science/ Babylon University in partial fulfillment of the requirements for the degree of doctor of philosophy in Biology-Zoology.



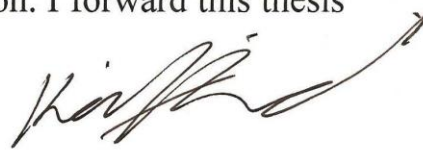
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Dedication

To the soul of my dear father...

Faheim

Acknowledgment

First of all, I would like to thank our God the Merciful and the Almighty for helping me in completing this work,

I would like to express my sincere thanks, gratitude and deep appreciation to my supervisor Prof. Dr. Nidhal Abdul Mohymen, who suggested and set the research plan, for her encouragement and firm direction. I give her my deepest gratitude and thanks.

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I also dedicate my best wishes and gratitude for all people I forgot to mention .

Faheim

Summary

Leishmaniasis are a certain diseases caused by some protozoan parasitic species of the genus *Leishmania*. However, the visceral leishmaniasis (V.L.) is a form of the disease where the pathogens attack some internal organ's of human such as liver, spleen and intestine.

Knowledges concerning the responses of the human immune system to the parasites are not adequately studied especially in Iraq.

The main aim of the present study is to investigate the cell mediated immune (CMI) response of the naturally infected patients attended Al-Dewanyia Children hospital, during Oct.2006 to Dec.2007 and were diagnosed as visceral Leishmaniasis .

The present study included eighty children of different ages (forty patients, twenty in post healing stage of the same disease and twenty apparently healthy children as control .

The expression of certain activation markers such as CD3, CD4, CD8 ,CD19,CD45RA, CD45RO, CD1a, CD14, CD22, CD44, CD71 and HLA-DR on the peripheral blood lymphocytes (PBL) of the studied children were detected and certain cytokines of the studied groups were estimated.

Cellular expression of CD3 ,CD4,CD8 and CD19 were detected by using direct dual immunofluorescence staining technique while the, CD45RA, CD45RO,CD1a ,CD22,CD44,CD71,and HLA-DR were detected by using immunocytochemistry staining technique (DAB).The concentrations of each of the interferon- γ (IFN- γ), tumor necrosis factor (TNF- α), interleukin four (IL-4) and interleukin ten (IL-10) in the serum of the studied groups were determined by sandwich enzyme linked immunosorbent assay (ELISA).

The distribution of different lymphocyte subsets were compared in different study groups and the results showed that there was a insignificant difference in CD8, CD19 and slightly increase significant difference in CD4 while there was no significant difference in CD3 in comparison with control group.

The results of immunocytochemistry staining showed high significant increase in CD14, CD22, CD44, CD45RA, CD71 and HLA-DR while CD1a showed significant decrease than control group.

The result of cytokines determination showed that there was significant increase in IL-4, IL-10, and TNF-alpha but slightly increase in acute group in comparison to control group and same thing with post healing group.

Significant increase in total serum IgG and IgM levels were observed in patients with V.L. compared to control, but we show decrease in total serum IgA level in patient, and the result revealed an increase in complement C3 and C4 serum level in V.L. patient group, were is no significant differences between post healing and control .

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List of abbreviations

Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency Syndrome
BF	Beta complement factor
C	Complement
CD	Cluster of differentiation
CL	Cutaneous leishmaniasis
CMI	Cell mediated immunity
CSA	Crude soluble antigen
CSF	Colony stimulating factor
D.W.	Distilled water
DAB	3,3 di aminobenzidin
DAT	Direct agglutination test
DTH	Delayed type hypersensitivity
ELISA	Enzyme linked immunosorbent assay
FITC	Fluorescein iso thiocyanate
G-CSF	Granulocyte colony stimulating factor
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen-D related
HRP	Horse raddish peroxidase
HSP	Heat shock protein
ICAM	Intra cellular adhesion Molecule
IFAT	Indirect immunofluorescent antibody assay
IFN-gamma	Interferon gamma
IL	Interleukin
KD	Killo Dalton
L.	Leishmaniasis
LSA	Leishmanial soluble antigen
McAb	Monoclonal antibody

M-CSF	Macrophage colony stimulating factor
NO	Nitrous oxide
PBC	Peripheral blood cell
PBS	Phosphate buffer saline
RPE	Rhodamin phycorhien
Sb	Sodium stibogluconate(pentostam)
Th	Thymocyte helper cells
TMB	Tetramethylbenzidine
TNF-alpha	Tumor necrosis factor-alpha
VCAM	Vascular cell adhesion Molecule
V.L.	Visceral leishmaniasis
WHO	World health organization

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

﴿ فَتَعَالَى اللَّهُ الْمَلِكُ الْحَقُّ وَلَا تَعْجَلْ بِالْقُرْآنِ
مِنْ قَبْلِ أَنْ يُقْضَىٰ إِلَيْكَ وَحْيُهُ وَقُلْ رَبِّ زِدْنِي
عِلْمًا ﴾

صدق الله العلي العظيم
سورة طه الآية (114)

Table1: Age, Sex & Laboratory parameters in different study groups .

No.	age	gender	Hp %	WBC	hepatosplenomegaly	group type
1	1.50	Male	6.90	6,000	Moderate enlargement	Acute
2	0.35	Female	5.40	4,500	Sever enlargement	Acute
3	0.80	Male	7.60	8,800	Moderate enlargement	Acute
4	0.50	Male	10.00	7,500	Moderate enlargement	Acute
5	2.00	Female	9.00	6,400	Moderate enlargement	Acute
6	2.00	Female	11.00	4,500	Slight enlargement	Acute
7	2.00	Male	7.00	8,000	Sever enlargement	Acute
8	3.00	Male	9.90	8,400	Moderate enlargement	Acute
9	2.11	Male	8.70	4,800	Slight enlargement	Acute
10	1.00	Male	7.50	7,600	Moderate enlargement	Acute
11	1.60	Male	7.50	4,200	Slight enlargement	Acute
12	0.60	Female	9.10	5,300	Slight enlargement	Acute
13	1.00	Male	6.80	5,600	Slight enlargement	Acute
14	1.00	Male	7.90	3,800	Slight enlargement	Acute
15	2.00	Male	5.80	5,000	Moderate enlargement	Acute
16	0.60	Female	8.00	3,000	Sever enlargement	Acute
17	1.70	Female	8.80	4,500	Slight enlargement	Acute
18	0.10	Male	9.90	6,000	Sever enlargement	Acute
19	3.00	Male	10.00	5,300	Sever enlargement	Acute
20	1.40	Female	8.00	4,200	Sever enlargement	Acute
21	2.00	Female	9.00	3,400	Sever enlargement	Acute
22	5.00	Male	6.60	4,400	Sever enlargement	Acute
23	6.00	Male	10.80	0	No enlargement	Acute
24	3.00	Male	11.00	9,800	No enlargement	Acute
25	1.00	Male	8.00	3,000	Sever enlargement	Acute
26	1.10	Female	8.00	3,000	Sever enlargement	Acute
27	0.80	Male	8.00	8,000	Moderate enlargement	Acute
28	1.30	Female	9.50	6,000	Slight enlargement	Acute
29	2.70	Male	10.70	4,800	Slight enlargement	Acute
30	1.00	Male	8.60	4,000	Slight enlargement	Acute
31	1.80	Female	9.50	5,600	Slight enlargement	Acute
32	1.30	Female	9.80	6,000	Moderate enlargement	Acute
33	1.50	Female	5.40	3,800	Moderate enlargement	Acute
34	1.50	Male	10.00	4,200	Slight enlargement	Acute
35	0.60	Female	12.00	0	Moderate enlargement	Acute
36	0.80	Female	11.00	6,000	Slight enlargement	Acute
37	1.60	Female	8.00	3,000	Sever enlargement	Acute
38	1.70	Female	9.00	6,000	Moderate enlargement	Acute
39	1.00	Female	7.80	8,200	Slight enlargement	Acute
40	1.60	Female	8.00	5,000	Moderate enlargement	Acute

41	1.20	Female	9.40	4,800	No enlargement	Post healing group
42	1.90	Male	9.00	3,400	No enlargement	Post healing group
43	0.80	Male	8.90	4,100	No enlargement	Post healing group
44	0.30	Female	8.40	3,600	No enlargement	Post healing group
45	0.70	Male	10.00	8,000	No enlargement	Post healing group
46	1.30	Female	6.90	2,800	No enlargement	Post healing group
47	1.00	Female	7.80	8,200	No enlargement	Post healing group
48	5.00	Male	10.00	6,000	No enlargement	Post healing group
49	3.00	Female	9.70	3,200	No enlargement	Post healing group
50	2.60	Female	10.50	5,000	No enlargement	Post healing group
51	1.30	Female	10.00	4,500	No enlargement	Post healing group
52	1.60	Male	9.00	4,800	No enlargement	Post healing group
53	1.70	Male	10.00	6,000	No enlargement	Post healing group
54	0.80	Female	8.00	4,500	No enlargement	Post healing group
55	2.60	Female	12.00	11,000	No enlargement	Post healing group
56	2.00	Female	10.00	9,000	No enlargement	Post healing group
57	2.10	Female	12.00	7,000	No enlargement	Post healing group
1	1.90	Female	11.00	8,000	No enlargement	control
2	2.00	Male	11.00	6,000	No enlargement	control
3	4.00	Male	12.00	7,500	No enlargement	control
4	1.20	Female	10.80	8,000	No enlargement	control
5	0.11	Male	9.50	4,800	No enlargement	control
6	1.80	Male	9.00	3,900	No enlargement	control
7	2.00	Male	8.80	8,000	No enlargement	control
8	0.80	Male	10.00	6,000	No enlargement	control
9	2.00	Male	10.80	6,200	No enlargement	control
10	1.30	Male	12.00	6,000	No enlargement	control
11	2.80	Male	12.40	7,000	No enlargement	control
12	2.00	Female	11.00	5,900	No enlargement	control
13	0.90	Male	13.00	7,600	No enlargement	control
14	2.30	Male	12.60	8,000	No enlargement	control
15	1.20	Female	12.00	4,400	No enlargement	control
16	1.50	Male	12.00	6,000	No enlargement	control
17	2.90	Male	13.00	7,000	No enlargement	control
18	2.00	Female	12.00	12,000	No enlargement	control

Table 2: PBL CD markers, Cellular expression stained by FITC, RPE & DAB.

No .	age	gender	CD 8	CD 4	CD 3	CD 19	CD 1a	CD 14	CD 22	CD 44	CD45 RO	CD45 RA	CD 71	HLA DR	Group type
1	1.50	Male	45.8	37.0	35.0	18.7	41.9	58.8	37.8	20.6	36.00	28.00	33.0	71.00	Acute
2	0.35	Female	40.0	35.0	21.5	28.0	43.0	60.6	38.0	25.9	45.00	28.00	34.0	63.00	Acute
3	0.80	Male	40.8	38.0	18.6	28.0	42.5	78.0	41.0	17.0	39.00	36.00	56.0	69.00	Acute
4	0.50	Male	39.0	38.0	20.9	24.8	40.6	79.0	51.0	26.0	43.00	33.00	51.0	75.00	Acute
5	2.00	Female	47.0	31.7	18.0	28.0	39.8	43.0	28.0	37.0	72.00	29.00	47.0	49.70	Acute
6	2.00	Female	42.0	37.0	19.6	27.0	43.0	65.0	38.0	27.0	51.00	45.00	59.0	78.00	Acute
7	2.00	Male	40.8	36.0	20.8	27.0	40.0	70.0	30.0	23.7	49.00	40.00	47.0	79.00	Acute
8	3.00	Male	47.0	30.7	22.0	25.0	38.8	59.0	45.0	29.0	46.00	39.00	51.0	81.00	Acute
9	2.11	Male	41.7	35.0	21.0	27.0	43.0	51.0	23.0	24.0	52.00	38.00	37.0	70.70	Acute
10	1.00	Male	49.7	35.6	18.8	30.9	42.0	62.0	46.0	22.0	45.00	47.00	67.0	78.00	Acute
11	1.60	Male	40.0	37.5	25.0	22.0	31.9	48.0	31.0	37.0	39.00	46.00	66.0	69.00	Acute
12	0.60	Female	43.6	44.0	17.5	30.9	44.0	58.0	39.0	19.0	41.00	41.00	72.0	78.00	Acute
13	1.00	Male	41.0	37.0	24.0	24.0	40.7	66.0	49.0	13.0	46.00	46.00	56.0	76.60	Acute
14	1.00	Male	39.0	38.0	18.0	30.0	42.5	52.0	35.0	16.0	39.00	39.00	70.0	82.00	Acute
15	2.00	Male	43.0	36.0	25.0	23.6	41.8	49.0	42.0	23.0	42.00	42.00	58.0	72.00	Acute
16	0.60	Female	45.0	37.0	23.0	25.0	38.6	62.0	42.6	18.0	38.00	38.00	72.0	81.00	Acute
17	1.70	Female	43.0	34.0	21.0	27.0	44.8	44.5	42.6	20.0	41.00	41.00	30.0	86.50	Acute
18	0.10	Male	40.7	38.5	22.0	27.8	41.0	63.0	51.0	30.7	49.00	51.00	60.0	66.00	Acute
19	3.00	Male	42.0	36.0	22.6	27.0	38.0	74.0	66.0	29.7	45.00	56.00	54.0	62.00	Acute
20	1.40	Female	43.0	40.8	17.5	32.0	40.6	71.0	52.0	23.0	58.00	53.00	56.0	68.00	Acute
21	2.00	Female	42.7	36.8	22.7	26.5	49.0	62.0	61.0	27.0	62.70	64.00	63.0	73.00	Acute
22	5.00	Male	47.6	37.8	25.0	21.8	42.0	69.0	49.0	21.0	63.00	47.00	61.0	77.00	Acute
23	6.00	Male	42.0	36.5	20.0	27.0	39.0	95.0	86.0	36.0	41.00	55.00	64.7	86.00	Acute
24	3.00	Male	45.6	31.7	28.7	17.0	40.0	57.0	53.0	30.5	40.50	49.00	62.0	73.00	Acute
25	1.00	Male	42.0	36.0	21.0	28.5	46.0	57.0	50.0	21.0	63.00	42.70	57.0	66.00	Acute
26	1.10	Female	41.5	36.0	21.5	26.9	41.0	73.0	61.0	35.0	41.00	61.00	61.0	69.00	Acute
27	0.80	Male	41.0	37.0	26.5	23.0	26.0	47.0	55.0	29.0	59.00	32.00	36.0	68.00	Acute
28	1.30	Female	45.7	31.0	20.5	29.0	47.0	69.0	49.0	22.0	38.00	48.00	63.0	58.00	Acute
29	2.70	Male	42.0	35.0	27.0	19.0	45.0	47.0	46.0	19.7	55.00	87.00	55.0	71.00	Acute
30	1.00	Male	43.0	37.9	26.0	20.0	40.0	76.0	48.0	16.0	47.00	61.00	62.0	81.00	Acute
31	1.80	Female	41.0	37.7	25.7	21.0	42.0	88.0	54.0	18.0	43.00	62.50	70.0	70.00	Acute
32	1.30	Female	42.0	36.5	29.0	19.0	36.7	62.0	56.0	20.7	49.00	43.00	44.0	67.00	Acute
33	1.50	Female	43.0	37.0	24.6	24.0	61.0	42.0	48.0	11.0	36.00	40.00	34.0	62.00	Acute
34	1.50	Male	44.0	30.7	24.6	24.0	40.7	56.0	54.5	23.0	63.00	39.00	49.0	59.00	Acute
35	0.60	Female	56.0	37.0	33.0	12.6	36.0	75.0	47.0	19.0	61.00	44.00	35.6	66.00	Acute
36	0.80	Female	47.5	36.8	31.5	18.5	46.0	74.0	56.0	14.0	52.00	56.00	59.0	72.00	Acute
37	1.60	Female	22.0	37.0	20.5	28.9	95.0	4.70	49.0	45.7	52.00	58.00	95.0	80.00	Acute
38	1.70	Female	53.6	33.0	31.0	16.8	4.70	90.0	95.0	53.0	95.00	95.00	75.0	78.00	Acute
39	1.00	Female	43.6	46.0	22.7	27.0	55.0	44.0	33.0	60.0	56.00	48.00	66.0	69.00	Acute
40	1.60	Female	44.0	33.6	34.0	14.0	46.0	32.0	38.0	63.0	58.00	45.00	63.0	57.00	Acute

41	1.20	Female	27.0	11.0	28.0	11.0	38.0	43.0	41.0	27.0	30.00	51.00	41.0	83.00	Post healing group
42	1.90	Male	28.0	14.0	27.0	11.0	42.0	39.0	36.0	48.0	48.00	49.00	38.0	40.00	Post healing group
43	0.80	Male	25.5	10.0	26.8	5.00	58.0	62.0	69.0	72.0	64.00	20.00	30.0	61.00	Post healing group
44	0.30	Female	19.0	19.0	28.0	13.6	86.0	81.0	73.0	91.0	37.00	44.00	76.0	71.00	Post healing group
45	0.70	Male	31.6	8.80	27.0	9.00	56.0	42.0	35.0	63.0	49.00	52.00	31.0	39.00	Post healing group
46	1.30	Female	21.0	12.8	30.0	10.0	49.0	32.0	28.0	83.0	56.00	61.00	24.0	29.00	Post healing group
47	1.00	Female	30.6	17.0	29.6	12.6	29.0	45.0	34.0	36.0	38.00	47.00	52.7	31.00	Post healing group
48	5.00	Male	25.6	14.0	28.0	12.0	17.0	36.0	13.0	32.0	80.00	53.00	34.0	51.70	Post healing group
49	3.00	Female	27.8	15.0	33.0	14.0	38.0	42.0	26.0	33.0	56.00	86.00	31.8	38.00	Post healing group
50	2.60	Female	21.8	6.00	26.0	7.70	28.0	37.0	47.0	39.0	33.00	90.00	26.0	34.00	Post healing group
51	1.30	Female	20.9	10.0	25.0	10.8	48.0	62.0	29.0	63.0	53.00	29.00	42.0	35.00	Post healing group
52	1.60	Male	22.6	6.00	22.0	6.00	58.0	38.0	56.0	61.0	66.00	90.00	51.0	66.00	Post healing group
53	1.70	Male	21.0	8.00	26.7	12.0	47.0	33.0	28.0	29.0	31.00	28.00	30.9	90.00	Post healing group
54	0.80	Female	15.0	10.0	23.0	6.00	51.0	36.0	31.0	28.0	46.00	53.00	28.0	36.70	Post healing group
55	2.60	Female	23.0	30.5	28.0	8.00	23.0	34.0	31.0	27.0	40.00	56.00	23.0	39.00	Post healing group
56	2.00	Female	22.0	9.50	20.0	8.00	44.0	40.0	28.0	86.0	52.00	95.00	3.80	30.00	Post healing group
57	2.10	Female	20.0	8.00	23.0	10.0	56.0	39.0	31.0	90.0	53.00	55.00	31.0	37.00	Post healing group
1	1.90	Female	24.0	15.0	24.0	12.0	61.0	50.0	19.5	8.60	4.50	23.00	14.0	36.00	control
2	2.00	Male	24.0	16.0	23.0	17.0	40.7	22.0	16.0	14.0	8.00	19.00	16.0	33.00	control
3	4.00	Male	35.0	14.6	37.0	7.50	52.0	35.0	21.0	12.0	5.00	15.00	17.0	24.00	control
4	1.20	Female	28.0	17.0	30.0	11.0	55.0	33.0	12.0	11.0	11.00	21.00	12.0	28.00	control
5	0.11	Male	34.0	15.0	27.0	13.0	33.0	12.0	3.00	13.0	10.70	16.00	13.0	10.70	control
6	1.80	Male	24.0	12.0	25.0	14.7	51.0	25.0	9.00	20.0	12.00	18.00	17.0	25.00	control
7	2.00	Male	20.0	13.0	23.0	9.00	46.0	38.0	19.0	18.0	3.70	14.00	20.0	19.00	control
8	0.80	Male	23.0	13.6	27.0	12.7	20.0	13.0	20.0	15.0	3.50	9.00	29.0	11.00	control
9	2.00	Male	24.0	13.0	27.0	13.0	37.0	15.0	22.0	13.0	5.70	22.00	15.0	32.00	control
10	1.30	Male	25.0	12.5	31.0	15.0	23.0	4.70	16.0	19.0	13.00	23.00	13.7	20.00	control
11	2.80	Male	26.0	12.6	20.0	10.0	39.0	17.0	23.0	55.0	13.00	17.00	16.0	23.00	control
12	2.00	Female	24.0	15.0	31.0	12.0	28.0	10.7	21.0	21.0	10.00	21.00	17.0	20.00	control
13	0.90	Male	23.0	15.0	31.0	12.0	27.0	13.9	18.0	11.0	9.00	19.00	16.0	16.00	control
14	2.30	Male	29.0	7.00	34.0	7.00	31.0	30.7	15.0	9.00	11.00	13.00	13.0	21.00	control
15	1.20	Female	24.0	10.0	25.0	9.00	40.0	35.0	13.0	16.0	7.70	19.00	15.7	24.00	control
16	1.50	Male	34.0	5.50	28.0	8.00	34.0	26.0	17.0	7.00	5.60	21.00	18.0	29.00	control
17	2.90	Male	17.0	5.30	28.0	12.0	38.0	35.0	23.0	9.50	9.50	20.00	14.0	20.00	control
18	2.00	Female	26.0	9.00	32.0	8.00	46.0	40.0	25.0	8.60	4.70	23.00	16.7	35.00	control

Table3: Serum Cytokines levels in study groups.

No.	age	gender	IL-4	IL-10	IFN-gamma	TNF-alpha	Group type
1	1.50	Male	469.30	296.32	2.53	289.85	Acute
2	0.35	Female	371.38	320.33	6.94	215.38	Acute
3	0.80	Male	521.62	593.98	2.76	497.79	Acute
4	0.50	Male	600.11	51.26	6.26	498.02	Acute
5	2.00	Female	573.95	593.01	3.74	179.03	Acute
6	2.00	Female	495.46	528.21	8.32	232.06	Acute
7	2.00	Male	547.79	432.53	7.74	242.19	Acute
8	3.00	Male	783.24	315.00	6.67	420.33	Acute
9	2.11	Male	560.87	493.64	4.67	434.04	Acute
10	1.00	Male	723.35	160.94	8.00	360.16	Acute
11	1.60	Male	675.67	371.90	2.03	215.07	Acute
12	0.60	Female	641.08	105.73	3.19	297.00	Acute
13	1.00	Male	652.43	533.49	5.15	566.31	Acute
14	1.00	Male	547.79	429.65	3.34	281.53	Acute
15	2.00	Male	534.70	253.40	3.19	214.59	Acute
16	0.60	Female	534.00	246.93	2.24	128.41	Acute
17	1.70	Female	495.46	147.96	8.78	350.62	Acute
18	0.10	Male	482.38	726.96	8.97	479.92	Acute
19	3.00	Male	521.62	214.92	8.67	668.79	Acute
20	1.40	Female	547.79	121.09	5.00	381.01	Acute
21	2.00	Female	403.89	142.22	9.10	356.58	Acute
22	5.00	Male	979.46	366.42	5.02	457.27	Acute
23	6.00	Male	521.62	193.59	1.35	211.21	Acute
24	3.00	Male	508.54	352.01	2.19	244.97	Acute
25	1.00	Male	521.62	284.80	6.31	692.02	Acute
26	1.10	Female	861.73	270.40	3.46	476.94	Acute
27	0.80	Male	454.16	256.00	6.82	367.04	Acute
28	1.30	Female	871.89	236.79	9.37	325.60	Acute
29	2.70	Male	600.11	241.60	8.47	249.34	Acute
30	1.00	Male	443.14	337.61	2.69	457.27	Acute
31	1.80	Female	534.70	361.62	3.34	139.11	Acute
32	1.30	Female	508.54	161.90	3.94	552.01	Acute
33	1.50	Female	521.62	180.53	7.56	413.78	Acute
34	1.50	Male	547.79	136.85	9.45	182.17	Acute
35	0.60	Female	560.87	181.25	2.54	174.86	Acute
36	0.80	Female	844.86	197.43	4.88	144.38	Acute
37	1.60	Female	678.60	182.21	5.24	370.88	Acute
38	1.70	Female	403.89	130.61	9.08	310.71	Acute
39	1.00	Female	416.98	157.98	6.67	305.94	Acute
40	1.60	Female	573.95	149.34	7.08	153.41	Acute

41	1.20	Female	76.18	74.30	1.42	83.22	Post healing group
42	1.90	Male	81.41	66.14	1.60	91.47	Post healing group
43	0.80	Male	76.18	82.28	1.83	21.85	Post healing group
44	0.30	Female	163.10	81.90	9.74	99.21	Post healing group
45	0.70	Male	87.95	71.90	4.29	25.31	Post healing group
46	1.30	Female	195.22	95.48	3.24	77.86	Post healing group
47	1.00	Female	152.74	66.14	6.10	63.45	Post healing group
48	5.00	Male	180.83	67.58	2.09	29.50	Post healing group
49	3.00	Female	208.30	89.73	3.19	96.81	Post healing group
50	2.60	Female	161.79	41.65	1.86	68.63	Post healing group
51	1.30	Female	91.88	78.05	1.45	22.35	Post healing group
52	1.60	Male	205.68	64.22	1.75	51.03	Post healing group
53	1.70	Male	147.40	88.68	2.05	30.69	Post healing group
54	0.80	Female	90.57	69.02	2.48	74.78	Post healing group
55	2.60	Female	76.18	66.14	3.10	75.07	Post healing group
56	2.00	Female	204.38	59.90	1.04	43.21	Post healing group
57	2.10	Female	169.64	81.26	1.17	24.03	Post healing group
1	1.90	Female	76.18	91.17	2.61	61.77	control
2	2.00	Male	205.68	114.63	1.02	80.34	control
3	4.00	Male	174.79	106.94	1.16	94.04	control
4	1.20	Female	208.30	70.94	1.41	26.00	control
5	0.11	Male	203.07	46.93	1.01	83.82	control
6	1.80	Male	212.22	59.42	1.45	87.39	control
7	2.00	Male	206.99	64.10	1.67	77.26	control
8	0.80	Male	214.84	51.26	1.37	85.01	control
9	2.00	Male	201.84	73.82	2.93	61.77	control
10	1.30	Male	210.92	48.85	1.17	27.81	control
11	2.80	Male	286.16	67.10	1.41	13.98	control
12	2.00	Female	213.53	51.74	2.37	75.48	control
13	0.90	Male	212.22	50.30	3.55	97.52	control
14	2.30	Male	192.60	19.09	1.75	68.92	control
15	1.20	Female	206.99	26.77	1.20	58.20	control
16	1.50	Male	200.45	44.53	2.63	56.41	control
17	2.90	Male	199.14	40.09	1.98	58.20	control
18	2.00	Female	199.84	47.41	2.37	74.88	control

Table4:Immunoglobulin isotopes and complement (C3& C4) in study groups .

No .	age	gender	C3	C4	IgG	IgM	IgA	Group type
1	1.50	Male	1,320	743	5,700	1,490	172	Acute
2	0.35	Female	172	64	7,100	1,700	687	Acute
3	0.80	Male	375	171	7,980	977	945	Acute
4	0.50	Male	925	346	10,300	1,770	859	Acute
5	2.00	Female	172	374	5,820	859	605	Acute
6	2.00	Female	1,790	306	6,800	1,920	103	Acute
7	2.00	Male	580	374	13,400	1,290	773	Acute
8	3.00	Male	1,120	513	8,000	2,230	859	Acute
9	2.11	Male	1,590	632	12,000	2,740	193	Acute
10	1.00	Male	1,640	404	10,300	916	773	Acute
11	1.60	Male	1,000	140	6,240	1,630	605	Acute
12	0.60	Female	888	360	5,030	859	687	Acute
13	1.00	Male	244	131	3,190	390	600	Acute
14	1.00	Male	1,330	360	5,030	488	183	Acute
15	2.00	Male	745	562	8,000	1,630	112	Acute
16	0.60	Female	1,040	546	4,000	1,100	204	Acute
17	1.70	Female	1,080	687	5,900	2,400	773	Acute
18	0.10	Male	1,370	822	1,900	390	560	Acute
19	3.00	Male	2,200	687	8,600	1,040	238	Acute
20	1.40	Female	963	73	1,200	2,080	103	Acute
21	2.00	Female	1,280	465	2,900	537	605	Acute
22	5.00	Male	709	404	10,800	1,560	560	Acute
23	6.00	Male	1,200	743	5,700	1,490	172	Acute
24	3.00	Male	172	64	7,100	1,700	687	Acute
25	1.00	Male	375	171	7,980	977	945	Acute
26	1.10	Female	925	346	10,300	1,770	859	Acute
27	0.80	Male	172	374	5,820	859	605	Acute
28	1.30	Female	1,790	306	6,800	1,920	103	Acute
29	2.70	Male	580	374	3,400	1,290	773	Acute
30	1.00	Male	1,120	513	8,000	2,230	859	Acute
31	1.80	Female	1,590	632	1,200	2,740	193	Acute
32	1.30	Female	1,640	404	10,300	916	773	Acute
33	1.50	Female	1,000	140	6,240	1,630	605	Acute
34	1.50	Male	888	360	5,030	859	687	Acute
35	0.60	Female	244	131	3,190	390	600	Acute
36	0.80	Female	1,330	360	5,030	488	183	Acute
37	1.60	Female	745	562	8,000	1,630	112	Acute
38	1.70	Female	1,040	546	4,000	1,100	204	Acute
39	1.00	Female	1,080	687	5,900	2,400	773	Acute
40	1.60	Female	1,370	822	1,900	390	560	Acute

41	1.20	Female	320	150	4,640	344	595	Post healing group
42	1.90	Male	244	60	3,900	330	773	Post healing group
43	0.80	Male	269	73	3,540	300	605	Post healing group
44	0.30	Female	375	50	5,030	390	112	Post healing group
45	0.70	Male	152	82	4,640	320	605	Post healing group
46	1.30	Female	150	73	5,040	344	560	Post healing group
47	1.00	Female	153	64	4,250	488	530	Post healing group
48	5.00	Male	430	60	4,050	916	687	Post healing group
49	3.00	Female	172	55	4,030	586	605	Post healing group
50	2.60	Female	160	50	3,900	682	690	Post healing group
51	1.30	Female	220	82	9,850	749	590	Post healing group
52	1.60	Male	150	73	5,040	344	570	Post healing group
53	1.70	Male	153	64	4,250	488	560	Post healing group
54	0.80	Female	430	60	4,050	916	530	Post healing group
55	2.60	Female	172	55	4,030	586	103	Post healing group
56	2.00	Female	160	50	3,900	682	690	Post healing group
57	2.10	Female	220	82	9,850	749	590	Post healing group
1	1.90	Female	430	254	6,240	639	570	control
2	2.00	Male	150	37	2,840	390	560	control
3	4.00	Male	320	120	4,260	802	530	control
4	1.20	Female	172	64	3,190	344	103	control
5	0.11	Male	195	64	2,500	310	112	control
6	1.80	Male	195	50	2,400	300	687	control
7	2.00	Male	293	110	6,650	692	605	control
8	0.80	Male	160	73	2,840	440	595	control
9	2.00	Male	269	101	2,600	537	773	control
10	1.30	Male	150	55	2,850	344	605	control
11	2.80	Male	140	64	2,500	311	112	control
12	2.00	Female	430	254	6,240	639	687	control
13	0.90	Male	150	37	2,840	390	859	control
14	2.30	Male	320	120	4,260	802	580	control
15	1.20	Female	172	64	3,190	344	770	control
16	1.50	Male	195	64	2,500	310	112	control
17	2.90	Male	195	50	2,400	300	687	control
18	2.00	Female	293	110	6,650	692	605	control

1.Introduction

The fact that the number of patients suffering from Leishmania infections is increasing and their serious consequences are evidently overlapping more thorough our knowledge than the host factors that are responsible for resistance and susceptibility for the needed disease .

Leishmaniasis presents a wide spectrum of manifestations with either integumentary or visceral involvement. Visceral leishmaniasis or the kala-azar, the most sever of leishmaniasis disease caused by *L. donovani* and *L. infantum* in the old world and *L. chagazi* in the new world, is chronic disease that is characterized by fever, enlargement of spleen and liver, weight loss, anemia and leukocytopenia. It's a progressive infection with fatal outcome in the absence of more result in divers clinical and immunopathological situations. The capacity of parasite to cause this wide of diseases manifestations depends upon their ability to evade the immune defence mechanism by performing a well-tuned orchestra of host-parasite interaction. The survival strategies of the parasites lead to the suppression of cell mediated immunity (Saha *et al.*, 2006).

During disease evolution there is extensive parasite multiplication attaining a high parasite burden in the spleen and liver. Where as in the most common forms of integumentary leishmaniasis parasite growth is controlled, anti-leishmania cell-mediated immune responses (CMI) is mounted (Nylen *et al.*, 2007). A lake of anti-leishmania CMI has been considered as a hall mark of visceral leishmaniasis (Chemli, 2006) there has been described a transient incapacity of mounting an effective CMI in V.L. based on the absence of lymphocyte proliferative response as well as IFN-gamma

production upon Peripheral blood cell pbmc *in vitro* cell stimulation by leishmanial antigen (Galdads *et al.*, 2005).

T-cell mediated immunity plays a central role in host responses to intracellular parasite (Pompeu *et al.*, 2001).

Cytokines are central elements in the development of an immune response and have received a great deal of attention in both human and experimental leishmaniasis. Cure for leishmaniasis are related to the predominance of Th1 response, since this leads to the production of IFN-gamma and activation of parasite infected macrophage. In contrast, a Th2 response with interleukin-4 and interleukin-10 production often results in disease progression (Barral *et al.*, 1995).

Infection of leishmania in human is characterised by the appearance of anti-leishmanial antibodies in the sera of the patients (Stark *et al.*, 2006). In some studies the presence of antibodies against *L. brazilienses* infection in the sera of infected patient has been critically monitored and utilized for the diagnosis and prognosis of the disease (Romero *et al.*, 2005).

Aims of this study are:

1. To assess the effect of *L. donovani* on the immune system of patient diagnosed to have kala azar fever by checking the following items:
2. To evaluate the peripheral blood lymphocytes surface antigen (CD marker) like: CD3, CD4, CD8, CD19, CD45RA, CD45RO, CD1a, CD14, CD22, CD44, CD71 and HLA-DR antigen.
3. To study Cytokines profiles using IL-4, IL-10, IFN-gamma and TNF-alpha.
4. To assess of humoral immune response by checking of total immunoglobulins (IgG, IgM and IgA) and complement (C3 and C4).

2. Literature Review

2.1. Parasite and its life cycle:

There are three subspecies of *Leishmania donovani* complex that have clinical significance: *L. donovani chagasi*, *L. donovani donovani* and *L. donovani infantum*. Although the epidemiologic information on each of these sub species varies, the methods of laboratory diagnosis, life cycle, clinical symptoms, treatment and prevention and the control of each one basically identical (Zeibeg, 1997).

In the vertebrate host, *Leishmania* parasite survives and multiply intracellularly in mononuclear phagocytes as non motile amastigotes, about 2-4 μm in diameter (Fig. 1). Recent studies indicate that cells are other than mononuclear phagocytes, for example fibroblasts, may also harbor the parasite (Solbach and Laskay, 2000).

Transmission to the vertebrate host is from *Phlebotomous* sandfly, in which parasite develops and replicates as 20 μm flagellated promastigotes, in the fly the parasites undergo developmental events starting with the amastigote in the blood meal, containing through several stages of promastigote maturation and culminating with the infectious metacyclic form. The environmental cues which trigger this event are not well understood, but temperature and pH appear to play a role (Handman, 2001).

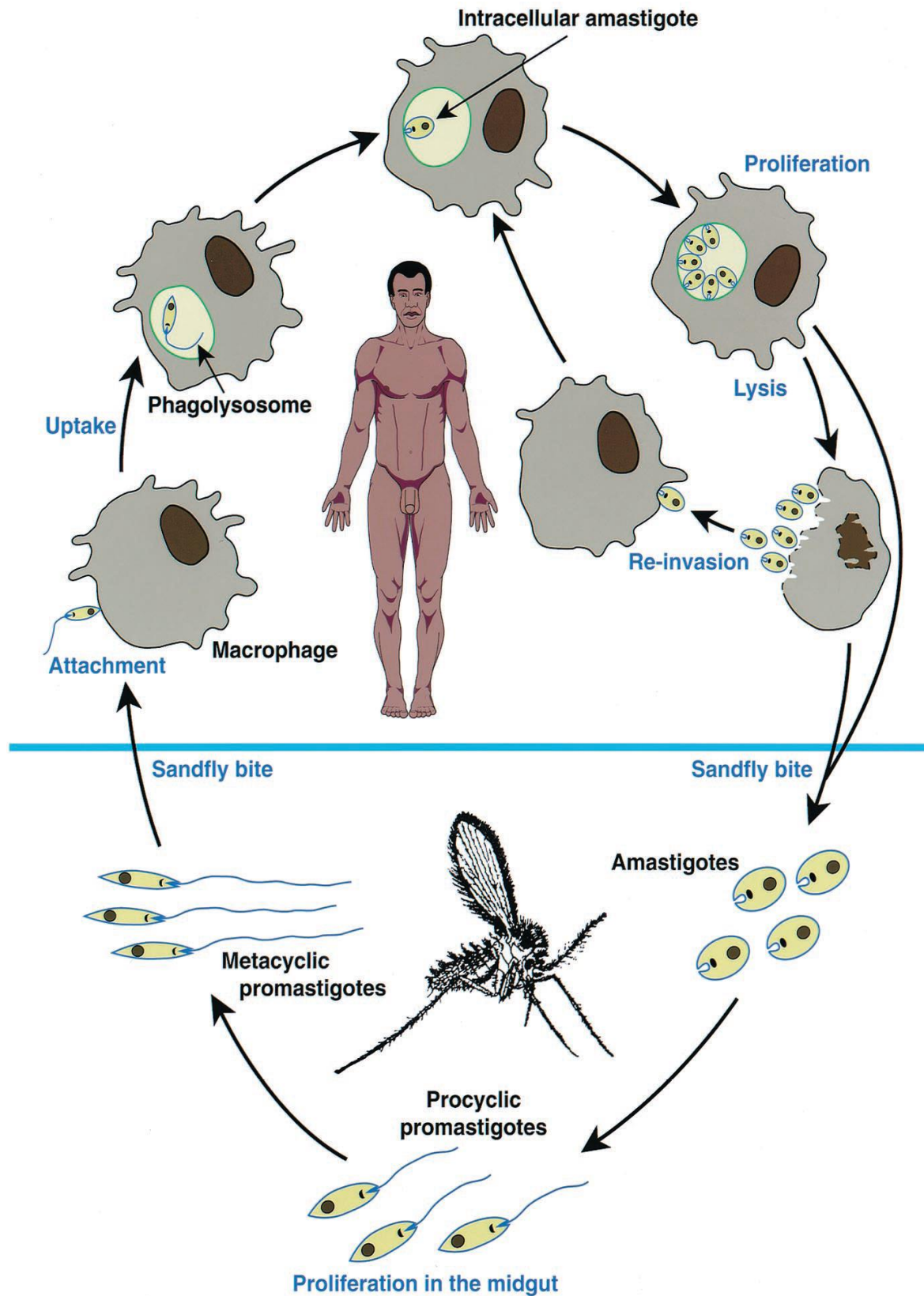


Figure 2.1: Schematic diagram of the *Leishmania* digenetic life cycle. (Adapted from Handman, 2001).

2.2. Clinical symptoms:

The name of Kala azar has been originated from India to mean black fever which refers to the hyperpigmentation of skin during the disease. Alternatively, the term might be derived from the word “Kal” meaning death which signifies the fatality of the disease (Brahmachari,1928 cited by Saha 2006).

Visceral leishmaniasis is also called dum dum fever, often present with a non descript abdominal illness, hepatomegally, and splenomegally. Early stages of disease may resemble malaria with development of fever and chills.

The onset of such symptoms are gradual and it follow an inoculation period ranging from 2 weeks to 18 months (Zeibig, 1997),with diarrhea as well as anemia, pancytopenia, weight lost and hypergammaglobulinemia (Stark , 2006).

2.3. Epidemiology:

Leishmania donovani complex is known to be an endemic in areas of the Middle East, India, Africa, the mediterranean Basin, Central and South America, Asia exception of Canada, Chile and Uruguay (Stark *et al.*, 2006). According to WHO report 1998, there are 1-1.5 million cases of Cutaneous leishmaniasis (CL) and 500000 cases of visceral leishmaniasis (V.L.) each year and population of 350 million is at the risk of the lose due to leishmaniasis and they are close to 2.4 million all over the world (Desjeux, 2004).

2.4. Cell Mediated immune responses in human V L:

It is well documented that V.L. is characterized by suppression of CMI, which is proved from the unresponsiveness of the patients to the leishmania skin Test (LST) or Montenegro test. This test measures a DTH reaction to an intradermal injection of leishmanial antigen (Manson – Bahrpe, 1961).

Containment of the disease following a successful treatment is associated with a strong cell mediated DTH response (Reiner, 1995). The cell mediated Immune suppression is also evident from blastogenesis assay of the peripheral blood mononuclear cells (PBMCs) (lymphoproliferation) from untreated V.L. patients (Neogy *et al.*, 1988). Reductions in proportion of the helper T-cells and the Immunosuppression (Koech, 1987), is rapidly reversible with effective chemotherapy.

The control and protection of V.L. In general, is dependent on the IFN- γ induced Innate and adoptive cellular Immune responses, which induce the intracellular Killing by activated macrophage (Ribeiro-de-Jesus *et al.*, 1998).

From Murine models studies of *L.major* infection, a clear Th1/Th2 polarization with distinct patterns of cytokines productions by CD+4 T cells i.e , IFN γ /IL-2 associated with resistance and IL-4/IL-10 associated with susceptibility was determined (Heinzel *et al.*, 1989) .

The existence of such distinct immunologic spectrum in human V.L. remains unclear. Different reports suggest that mixed Th1 and Th2 kind of responses are elicited upon leishmania infection in humans (Bahrencheer *et al.*, 1995).

Under this mixed Th1/Th2 milieu, a suppressed Th1 response along with an elevated Th2 is the hallmark of the active disease while protective immunity is achieved by upregulation of Th1 response after a successful chemotherapy (Kubar and Fragaki, 2005). Inability of the PBMCs of the V.L. patients to produce IL-2 and IFN - γ and their restoration after chemotherapy in response to the leishmanial antigens is also documented elsewhere (Holaday *et al.*, 1993).

It is well known that IFN - γ induced microbicidal activity against both the promastigote and a mastigot of *L.donovani* in monocyte derived human

macrophage, in H₂O₂ – dependent (Murray *et al.*, 1983), as well as in No mediated pathway (Vouldoukis *et al.*, 1997) Therefore, high parasite burden which is favored in active V.L. patient can be explained by the deficiency of IFN – γ during disease.

Upon infection with *L. donovani* the monocyte from healthy individuals. Pretreated with IFN – γ , were shown to produce increased TNF- α and IL – 1, two important elements in the host response to microbial infection. This suggested potential mechanism by which IFN – γ mediates its effects (Reiner *et al.*, 1990).

There are reports that show PBMCs isolated from all active V.L. patients failed to produce IL – 1 and TNF-alpha equally, upon stimulation with *L. chagasi* antigen lysate (Ho *et al.*, 1992).

Serum level of IL – 4 was found to be high in the V.L. patients compared to no or low level of IFN- γ (Zwingeberger *et al.*, 1990). The considerable level of IFN – γ is secreted at the very initial stages of the exposure to the parasites as observed in the seroconverted or sub clinically area (Bacellar *et al.*, 1991).

Although the reasons behind high levels of INF – γ secretion at the initial phase of infections and the decline in its production at the active stage of the disease is not clear, this INF – γ is found to come from the NK cells and might be important as component of innate Immune mechanism for the macrophage activation (Nylen *et al.*, 2003). Importance of IL – 10: IL – 10 was initially characterized as at cytokine, but later on, it was proved to be a pleiotropic cytokine, secreted from different cells types including the macrophages, (Saha *et al.*, 2006). Experimental evidences indicate that IL – 10 plays an important regulatory role in the progression of V.L.. The suppressive ability of IL – 10 on the TNF – alpha mediated microbicidal

activity of macrophages is well established for other diseases (Oswald *et al.*, 1992). Investigation on Brazilian patients showed that IL – 10 productions from *L. chagasi* antigen stimulated PBMC cultures of acute V.L.. Which was significantly higher than in cured individuals, where as asymptomatic leishmania skin test (LST) Positive individuals had no release of IL – 10 (Holaday *et al.*, 1993). In active V.L. patients production of high levels of IL – 10 mRNA is reported from bone marrow aspirate, lymph nodes, PBMCs, and splenic aspirate and the levels decrease after successful chemotherapy (Kenney, 1998). These studies also showed detectable levels of TNF – alpha mRNA in the bone Marrow , lymph node , PBMC or the splenic aspirate samples from the infected individuals though their specific cellular source were not determined . This is interesting because the production of TNF – γ at mRNA level is in contrast with the studies where evidences suggest that there is suppression of the Th1 kind of response, observed from the inability to produce TNF – γ by PBCs in active V.L..

The suppression of the leishmania specific Th1 kind of response in the patients is the main reason for the disease susceptibility and this suppression is regulated by IL – 10 (Bacellar *et al.*, 1991; Carvalho *et al.*, 1992) .

Involvement of other cytokines in Immune regulation of Haman V.L.:

IL – 4 is considered to be the signature cytokines of Th2 response, the role of IL – 4 ordinary considered suppression type cytokine , since Th2 response with IL – 4 and IL – 10 production which they results in disease progression (Saha *et al.*, 2006) .

- Involvement of different cells in immune regulation of human V.L. :

Studies on the involvement of the different immune cells in the maintenance of Th1/Th2 environment through the secretion of different cytokines help to understand the nature of Immunomodulation in V.L. . Most of the T cell clones derived from the PBCs of active V.L. patients and asymptomatic subjects (Seropositive and DTH positive) were CD8⁺ with negligible CD4⁺ , while the situation is reversed in the cured individuals with more numbers of CD4⁺ then CD8⁺ cells (Saha *et al.*, 2006).

CD8⁺ T cells isolated from the a symptomatic subjects produced high amounts of IFN – γ , which strongly suggests a role of CD8⁺ in human resistance to leishmania infection However , a unique population of CD4⁺ cells producing both IFN – γ and IL – 5 is found to contribute in the control of infection in asymptomatic subjects (Mary *et al.*, 1999) . An earlier study demonstrated higher number of CD4⁺ clones than CD8⁺ clones in the asymptomatic DTH positive individuals , although clones isolated from active V.L. were mostly CD8⁺ (Saha *et al.*, 2006) . Addition of CD8⁺ clones isolated from acute Kala – azar patient augmented IL – 10 production from the PBMCs cultures of same patients after recovery, suggesting that CD8⁺ T cells may have a role in mediating IL – 10 production during active disease (Gasim *et al.*, 1998).Further observation suggested that CD8⁺ cells are likely to mediate the endogenous IL – 10 secretion rather than antigen – specific IL – 10 , and thus the immunocompetence of CD8⁺ T cells could probably play an important role in progressive V.L. (Holaday, 2000) . Other study by the same author postulated that CD8⁺ suppressor T cells (Ts) population and Th1 CD4⁺ population have counter – regulatory effects for susceptibility and resistance , and predicated that the basic immunoregulatory mechanism during infection is The prevention of endogenous IL-10 secretion mediated

by CD⁺8 cells by the activity of leishmanial antigen – specific Th1 CD⁺4 cells .

2.5. Immunologic Markers:-

Lymphocyte and other leukocytes express a large number of different molecules on their surfaces.

Some of these molecules appear at particular stages of cell differentiation or activation for short periods , while others are characteristics of different cell lineages , such molecules , which can be used to distinguish cell populations which are called markers and many of them that can be identified by specific monoclonal antibodies (McAb) (Van Dongen & Adriaansen , 1996) and called clustered of differentiation (CD) among them:

2.5.1. CD1a: called thymocyte antigen T cells self – reactive to the CD1 family of Ag – presenting molecules also display Ag and CD1 Isoform (CD1a , b and c) specificity , many TcR interactions with self – CD1 are strong enough to be demonstrable in standard cellular assay or in combination with cytokines (Brigl, and Brenner, 2004), CD1a, CD1b, and CD1c restricted T cells recognizing lipid Ag have also been clearly delineated (Moody *et al.*, 2004) . CD1d – relative cells have been implicated in tumor surveillance (Terabe *et al.*, 2000), and early host defense where several studies have found microbial foreign Ags that stimulate these cells CD1, including α – sphingolipids , lipophosphoglcans and phosphatidyl inositol - mannosides (Mattner *et al.*, 2005), Foreign lipid Ag – reactive CD1a , CD1b and CD1c , restricted T cells seam much like foreign peptide specific MHC class I – and class II restricted T cells , except for the recognition of lipid – containing Ags rather than peptide . It has been proposed that this subset participates in the adaptive immune response (Beckman *et al.*, 1994). Both the CD1a – reactive and the CD1a- , CD1b- , CD1c- reactive T cells that are self – reactive may participate early in the immune response in the absence of

foreign Ag recognition, producing cytokines (Brigl *et al.*, 2004), instructing dendritic cell (DC), maturation and activating other cells such as NK and macrophages cell (Leslie *et al.*, 2002) , that participate in host defense .

2.5.2. CD3

CD3 is also called OKT3, Member of immunoglobulin superfamily on 11q23 Complex of delta, epsilon, gamma, zeta and eta chains of integral membrane glycoproteins that associates with T cell antigen receptor (TCR), and is required for TCR cell surface expression and signal transduction TCR-CD3 complex consists of either TCR alpha/beta or TCR gamma/delta heterodimers coexpressed at the cell surface with CD3 delta and epsilon defects cause autosomal recessive severe combined immunodeficiency (no T cells, normal B cells, normal NK cells (Roifman, 2004).

2.5.3. CD4

CD4 is also called OKT4, Nonpolymorphous glycoproteins belonging to immunoglobulin superfamily expressed on surface of T helper cells; serve as coreceptor in MHC class II-restricted antigen induced T cell activation CD4⁺ CD25⁺ T cells maintain peripheral tolerance and prevent autoimmunity (Taams and Akbar, 2005).

2.5.4. CD8

CD8 is also called T cell suppressor/cytotoxic,OKT8, cell surface glycoprotein, member of immunoglobulin superfamily; at 2p12 Heterodimer of an alpha and a beta chain linked by two disulfide bonds; heterodimer on thymocytes and homodimer on peripheral blood T cells MHC class I restricted receptor; binds to nonpolymorphic region of class I molecules; may increase avidity of interactions between cytotoxic T cell and target cell during antigen-specific activation Can kill target cells by recognizing peptide-MHC complexes on them or by secreting cytokines capable of signaling through death receptors on target cell surface CD8 alpha cells promote survival and

differentiation of activated T cells into memory CD8⁺ T cells, which may become clonal (but not malignant) in the elderly (Clambey *et al.*, 2005).

2.5.5. CD14:

CD14 is also called lipopolysaccharide (LPS) receptor, monocyte differentiation antigen Pattern recognition receptor that detects antigenic molecules on the surface of bacteria (lipoteichoic acid on gram positive, LPS on gram negative), myobacteria (glycolipids), fungi (mannans), as a part of the innate immune system. Adaptive immune system, by contrast, refers to lymphocytes recognizing microorganism proteins via T cell receptors and antibodies

Soluble form of CD14 is secreted by liver and monocytes; in low concentrations it confers LPS responsiveness to cells which are otherwise CD14 negative

Macrophages with a multiprotein complex of CD14, MD-2 and TLR4 bind to LPS, causing macrophage activation and release of cytokines; overstimulation may cause toxic shock syndrome

Detection of lipopolysaccharide induces IL-12 production (mediated by CD14), producing interferon gamma, which steers immune system away from allergy driven Thelper2 phenotype, associated with IgE production

Mutations can prevent adequate inflammatory response to infection, leading to systemic infections (Le Van *et al.*, 2001).

2.5.6. CD19

CD19 Corecepted with CD21, earliest B cell antigen in fetal tissue. Regulates B cell development, activation and differentiation. It may be defined that intrinsic and antigen receptor-induced signaling thresholds critical for clonal expansion of the B cell pool and humoral immunity (Tedder and Isaacs, 1989).

2.5.7. CD22 :

CD 22 is a B – lymphocyte – specific, 140 KD all surface glycoprotein, and a member of the singlecs (Sialis acid – binding) Immunoglobulin Ig like lectins family of proteins (Nitschke *et al.*, 2001). Functional studies in CD22 – deficient mice have demonstrated that CD22 plays an important role in downregulating B – cell receptor (BCR) – mediated signaling. Cross – linking of CD22 with antibodies in vitro has been shown to augment cell proliferation, to promote class switching and to increase the Ig secretion (Tuscano *et al.*, 1996).

CD22 was originally identified as B – cells associated a dhesion protein that appeared to function in the regulation of B – cells activation (Walker and Kenneth, 2007). CD22 is an inhibitory coreceptors, is required to modulate the antigen receprorsigna (in response to cause from local microemironment. This is essential to ensure that an appropriate humoral response is mounted against pathogens, but that reactivity to self antigens and autoImmunity is a voided (Grimaldi *et al.*, 2005).

2.5.8. CD44

CD44 is also called CD44s (standard isoform) or CD44H, lacks all alternative exons; all references below are to standard isoform unless stated otherwise

Family of cell surface glycoproteins with numerous isoforms and posttranslational modifications involving glycosylation (CD44v6 is common variant) generated by alternate splicing of mRNA

Important in epithelial cell adhesion (cell-cell and cell-matrix) to hyaluronate in basement membranes, and maintaining polar orientation of cells; this process may contribute to tissue remodeling and may stimulate cell proliferation and migration (Aruffo *et al.*, 1990).

2.5.9. CD45 The leukocyte common antigen.

Almost all hemopoietic lineage cells express CD45, the leukocyte common antigen. CD45 is a cell surface glycoprotein with tyrosine phosphatase activity and a molecular weight of 180 – 220 kDa. Only mature thrombocytes, mature erythrocytes and some of their progenitors lack CD45 expression (Woodford - Thomas and Thomas, 1993 ; Thomas, 1994; Trowbridge and Thomas, 1994; Poppema *et al.*, 1996). Although CD45 expression may comprise up to 10% of the cell surface, in general there is an increase with maturation of white blood cells and decrease with red cell maturation (Trowbridge, 1991; Hermiston *et al.*, 2003).

CD45 Isoforms

CD45 is known to exist as several isoforms in many species. These isoforms are generated by alternative splicing of exons (A, B and C respectively) (Justement, 1997). On this basis, eight possible isoforms have been Predicted , although not all of them have been observed to date . Possible splicing of additional exons has been reported (Virts *et al.*, 1998). Nomenclature for the isoforms generated by alternative splicing of the classic 3 exons is according to transcripts included in the resulting molecule: CD45RO for absent exons 4, 5 and 6), CD45RABC (for vexons 4, 5 and 6 transcribed). CD45 isoform expression varies between leukocyte subpopulations and changes according to activation and de – activation processes (Beverley, 1992 ; Clement, 1992; Bleesing, and Fleisher, 2003). The most commonly known isoform switch was reported to be that which occurs after activation of resting T cells in humans , with an isoform switch from CD45RA to CD45RO (Suarez *et al.*, 2002).

Equilibrium is between CD45 monomers and dimmers exists on the cell surface for all isoforms. The total CD45 phosphatase activity in a cell is determined by this equilibrium, which is in turn controlled by the isoforms

expressed. For example, in naïve T – cells, the expression of mixed larger RABC isoforms would shift the equilibrium toward monomers because the negative charges and bulky O – linked glycoconjugates in their extracellular domain act as a repulsive barrier to homodimerization. In support of this, early fluorescence resonance energy transfer analysis suggests that CD45 mainly exists as monomers in resting cells (Mittler *et al.*, 1991). Consequently, total CD45 phosphatase activity would be higher, maintaining Src family protein tyrosine kinases in a "primed" state capable of full activation upon TCR stimulation. During the initial phase of T cell activation, CD45 activity remains high, facilitating sustained TCR signaling during a primary immune response (Desai *et al.*, 1993). TCR signaling induces the expression of splicing factors that mediate the exclusion of exons A, B and C in CD45 (Lynch and Weiss, 2000). Due to the slow turnover of surface CD45, it takes several days for RAC isoforms on the cell surface to be replaced by RO (Birkeland *et al.*, 1989; Deans *et al.*, 1989). The expression of RO in activated T cells shifts the equilibrium toward dimers due to its more efficient homodimerization, which renders it subject to negative regulation by the inhibitory wedge. Thus, this isoform switch may act as part of a negative feedback loop, contributing to the cessation of the immune response to avoid undesirable tissue injury (Hermiston *et al.*, 2003).

Functions of CD45

The complex functions of CD45 are not yet entirely understood, although significant progress has recently been made (Hermiston *et al.*, 2003). CD45 is an important factor in regulating T and B cell activation and maturation (Poppema *et al.*, 1996; Altin and Sloan, 1997; Yamada *et al.*, 2002; Irles *et al.*, 2003) as well as thymic selection (Justement, 1997; Alexander, 2000). It positively and negatively regulates Src family protein

tyrosine kinases, which adjust the signaling cascade in B and T cells (Ashwell and D'Oro, 1999; Penninger *et al.*, 2001).

CD45 regulates the threshold level of activation of lymphocytes above a specific set point, therefore the involvement of CD45 in other immune functions including cytokine receptor mediated signaling was recently proven (Penninger *et al.*, 2001; Sasaki *et al.*, 2001; Fernandis *et al.*, 2003; Zhou *et al.*, 2003). CD45 also plays an important role in the function of myeloid cells. CD45RO is increasingly expressed as myeloid cells mature, however CD45RA is present in only small amounts on mature myeloid cells (Caldwell *et al.*, 1991).

2.5.10. Transferrin receptor (tfr, CD71)

The membrane glycoprotein transferring receptor is a house-keeping receptor that constitutively cycles between the plasma membrane and the endosomal compartment (Cheng *et al.*, 2004). The tfr binds iron – loaded transferrin at the cell surface and is internalized and transported to early sorting endosomes, where the complexed iron is rapidly separated from transferrin. Subsequently, the tfr- transferrin complex accumulates within recycling endosomes and then recycles to the plasma membrane (Alarcon and Fresno, 1998). In addition to being an iron transporter, the transferring receptor (tfr) has been shown to play a role in T cell activation. Stimulation of the tfr with specific abs results in T cell proliferation, IL-2 secretion, and protein kinase c activation., it appears to play as a costimulatory role in T-cell activation (Cano *et al.*, 1990; Salmeron *et al.*, 1995). There is also an important role for the tfr in early events in the formation of the immunological synapse (Batista *et al.*, 2004). There has been a known correlation between the number of cell surface transferring receptors and the rate of cell proliferation (Larrick and Cresswell, 1979; Kuhn, 1989; Richardson, 2002).

2.5.11. Major histocompatibility complex (MHC)

The MHC spans a 3-5 mega base region on the short arm of chromosome 6 that consist of over 200 genes (Goldsby *et al.*, 2000) arranged into three subregions encoding three classes of molecules.

Class I: is expressed on the surface of nearly all nucleated cells and platelets(Goldsby *et al.*,2000). The HLA class 1 molecule bind to the peptide fragments derived from endogenous antigens and present them for recognition by the T-cell receptor (TCR) of CD8 T-lymphocytes(Roitt *et al.*, 1998).

Class II: encodes glycoproteins expressed on a few cell types includes B lymphocytes, activated T cells, macrophages, dendritic cells, inflamed vascular endothelium and some epithelial cells. The MHC class II molecules present processed antigenic peptides to CD4 T-lymphocytes (Chapel *et al.*, 1999). Class I and class II genes are both member of the immunoglobulin gene family (Goldsby *et al.*, 2000).

HLA-DR (peptide-MHC complex) can activate CD4 T-cell and induced the proliferation and secretion of various cytokine, TNF-alpha released by the antigen presenting cells plays an important role in this process. It leads to the formation of oxygen radical that is able to kill intracellular parasite. Activated CD4 T-cells can stimulate antibody formation in B-cells (Burmester *et al.*, 2003).

Class III: these proteins are not relevant to antigen presentation and generally encode various secreted proteins that have immune functions including TNF-alpha and Beta, components of the complement factor (C4A, C4B, C2 and BF) and heat shock protein (HSP70) (Kelly *et al.*, 2003).

2.6. Role of T lymphocyte populations

The Immunity in human and experimental leishmaniasis is predominantly mediated by T lymphocytes (Sacks *et al.*, 2002). Initial studies

on mice using T cell-depleted mice (Skov and Twohy, 1974) and nude BALB/c mice (Murray *et al.*, 1987) have shown the importance of T lymphocytes for protection against *L. donovani* infection. Adoptive transfer of T cells, immune to Leishmania antigen, conferred resistance against *L. donovani* infection (Rezai *et al.*, 1980). Reconstitution experiments using nude BALB/c mice, and cell depletion experiments in euthymic mice using monoclonal anti-CD4 or anti-CD8 antibodies showed the necessity of both CD4⁺ and CD8⁺ T cells in the protection against *L. donovani* infection (Stern *et al.*, 1988). In *L. donovani*-infected BALB/c mice, there was a time course-related participation of different cell populations: L3T4⁺ (CD4⁺) cells are important in the initial two weeks of infection, when the parasite replication is still occurring, mainly in the formation of hepatic granulomas, but later this cell population decreases and is replaced by Lyt 2⁺ (CD8⁺) cells when the progress of the infection is controlled (McELrath *et al.*, 1988).

2.7. Mediators of inflammation

Cytokines are small proteins /glycoproteins 5 – 50 kDa produced by different cells including macrophages, fibroblasts and lymphocytes. They function as chemical messengers of the immune system, signaling from the innate to the adaptive immune system in infections, inflammation and trauma. More than a hundred different cytokines have been described. Similar functions can be mediated by more than one cytokine, sometimes in a synergistic way (Dinarello and Moldawer, 2002).

Cytokines signal through specific receptors on target cells of the immune system and exert their effect mainly locally, in a paracrine (effect on a neighboring cell) or autocrine (effect on the cell that produced the cytokine in a feedback loop) way, and modulate inflammatory responses. Nevertheless increased levels of cytokines may also be detected in the circulation in V.L.

as well as other inflammatory and infectious diseases (Robak and Gladalska, 1998).

2.7.1. Pro – inflammatory cytokines

Interferon – gamma (IFN – γ)

Interferon – gamma, mainly produced by activated CD4 and CD8 T – cells and NK cells, is a homodimeric glycoprotein having two subunits each of 21 to 24 k Da is a potent activator of mononuclear phagocytes inducing the respiratory burst, mechanism. IFN – γ also up regulate the expression of MHC class II molecules , leading to amplification the process of CD4 T – cell activation and promotes their differentiation to Th1 phenotype , and inhibits the proliferation of the Th2 type cells and thus tends to suppress humoral immunity. It did not only decrease the production of IL-4 by Th2 cells but also potently inhibit the effects of IL-4 on B – cells, particularly inhibiting IgE production. GKO mice , in which IFN – γ or IFN – γ receptor genes had been disrupted, showing several immunological defects including impaired macrophage activation , reduced NO production, reduced MHC class II molecules expression, reduced IgG2a and IgG3 serum levels and defective NK cells function (Benjamini *et al.*, 2000). IFN – γ is the primary lymphokine responsible for stimulating macrophage oxidative burst and activating antileishmanial activity in vitro (Murray & Delph, 2000). IFN – γ also induces the secretion of TNF – α upon activation of macrophages for intracellular killing of the amastigote by enhancing NO production (Bogdan *et al.*, 1996).

Immunological defects, including impaired macrophage activation, reduced nitrous oxide (NO) production, reduced MHC class II molecules expression, and IgG2a and IgG3 serum levels and defective NK cells function (Benjamini *et al.*, 2000). IFN – γ is the primary lymphokine responsible for stimulating macrophage oxidative burst and activating antileishmanial

activity in vitro (Murray & Delph, 2000). IFN – γ also induces the secretion of TNF – α upon activation of macrophages for intracellular killin of the amastigote by enhancing NO production (Bogdan *et al.*, 1996).

The efficacy of IFN – γ was tested by gene transfer experiments in which injection of mammalian expression plasmid bearing IFN – γ gene in normal and in IFN – γ gene disrupted infected BALB/C mice lead to controlled visceral leishmaniasis infection and reduced parasite burden (Taylor & Murray, 1998).

Tumor necrosis factor- alpha.(TNF – α)

TNF – α is a 17 KD protein composed of three identical subunits. It is produced mainly by monocytes and macrophages, but also by activated helper T- , B- and NK – cells, mast cells polymorphonuclear leukocytes, astrocytes and smooth muscle cells. Its main function appears to promote inflammation by inducing fever, shock, tissue injury, energy substrate mobilization, cachexia , bone resorption , differentiation and proliferation of hematopoietic cells and production of acute – phase proteins .

TNF – α is the most rapidly released cytokin under stress and has the ability to stimulate the production of other pro – inflammatory cytokines, such as IL– 1, IL- 6 and chemokines such as IL- 8 (Tracey *et al.*, 1987; Butler *et al.*, 1995) . TNF – α causes the activation and differentiation of monocytes and macrophages , acts as a chemotactic agent for monocytes and induces the expression of adhesion molecules on endothelial cells , which further induces the migration of leukocytes to the site of TNF – α release. TNF – α also promotes inflammation by upregulating the production of matrix metalloprotein – 1 (MMP – 1) and PGE2 (Dayer *et al.*, 1985). TNF – α reduces the expression of MHC class II proteins on macrophages and acts as a growth factor for monocytes by preventing programmed cell death (Mangan *et al.*, 1991; Watanabe and Jacob , 1991).

Resting T – cells does not respond to TNF – α and does not express the receptor, TNF – R. Instead activated T – cells increase the expression of the IL-2 receptor (IL-2R) and thus the proliferative response to IL-2 as well as the production of IL-2 in response to TNF – α . IL-2R expression is also increased on NK- cells upon exposure to TNF- α (Tracey *et al.*, 1987).

Endothelial cells , which are also major targets of TNF – α , increase their expression of adhesion molecules , such as intracellular adhesion molecule (ICAM) – 1 and vascular cell adhesion molecule (VCAM) – 1 , when responding to TNF – α (Osborn , 1990) . In addition to this, TNF – α recruits leukocytes to the site of inflammation by inducing angiogenesis and the release of chemokines produced by a variety of cells .

TNF – α appears to inhibit collagen by fibroblasts and to stimulate the resorption of proteoglycans in cartilage and inhibit their synthesis (Osborn, 1990). On the other hand, TNF – α has been shown to have an anti-inflammatory role by stimulating the release of corticotrophin, a hormone that stimulates the release of cortisol from the adrenal cortex, which indirectly downregulates inflammation (Tilders *et al.*, 1994).

Practically all cells have TNF – R and therefore respond to TNF – α . Two different forms of TNF – R, p55 and p75 have been described; TNF – Rp55 is ubiquitously expressed on all cells, whereas TNF – Rp75 is mainly found on hemopoietic and endothelial cells. Signaling through TNF – Rp55 has an effect on antiviral activity, fibroblast proliferation and induction of superoxide dismutase, whereas signaling through TNF – Rp75 affects the proliferation of thymocytes and cytotoxic T – cells (Tartaglia *et al.*,1992). The receptors are also produced naturally as soluble molecules, which inhibit the action of TNF – α by competition of binding with cell surface receptors (Aderka *et al.*, 1992).

2.7.2. Regulatory cytokines

IL – 10

Interleukin – 10 (IL-10) is a fascinating cytokine first identified by its ability to stop immune response by inhibiting production of a number of cytokines due to its suppressor and inhibitor ability , IL-10 was first called a cytokine synthesis inhibitory factor (Fiorentino *et al.*, 1989; Moore *et al.*, 2001). Biologically functional human IL-10 (hIL-10) is a 36 kDa dimer (Tan *et al.*, 1993; Windsor *et al.*, 1993) consisting of two 160 amino acid residue – long polypeptide chains (Vieira *et al.*, 1991).

Studies since then have indicated that it inhibits many aspects of immune function and that its synthesis is not restricted to Th2 cells as it is also produced by CD4⁺ Th0 and Th1 cells, CD8 – T cells, B cells, keratinocytes, various tumour cell lines and in particular monocytes/macrophages (Moore *et al.*, 2001) .

Also IL-10 functions as an effective immunoregulatory molecule based on two important functions: inhibition of cytokine synthesis and downregulation of antigen – presenting cell function (Fiorentino *et al.*, 1989; Ding *et al.*, 1993). This latter activity is largely due to the ability of IL-10 to downregulate both constitutive and induced expression of HLA-DR, HLA-DP and HLA-DQ (De-Wall Malefyt *et al.*, 1991), and co – stimulatory molecules such as B7-1 and 2 (Buelens *et al.*, 1995). It is generally thought that the inhibitory effect of IL-10 upon T cells is indirect via its ability to inhibit cytokine synthesis from monocytes (Ding *et al.*, 1993). Includes IL-1 , IL-6, IL-12 , IFN- α , TNF – α , granulocyte – macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF) , but also chemokines such as IL-8 and macrophage inflammatory protein (MIP) – 1 α (Fiorentino *et al.*, 1991) at both protein and mRNA levels . The mechanism by which IL-10 inhibits cytokine synthesis is somewhat contradictory. Some

studies have indicated that IL-10 inhibits TNF synthesis through blockade of gene transcription (Wang *et al.*, 1994), mediated possibly via effects on NF- κ B (Wang *et al.*, 1995). Other reports suggest the effect of IL-10 to be mainly posttranscriptional (Bogdan *et al.*, 1992). This is reflected in the observation that IL-10 will inhibit TNF production in either resting or pre – activated cells (Aste – Amezaga *et al.*, 1998). IL-10 can also induce the production of cytokine inhibitors include the IL-1 receptor antagonist (IL-1ra) (Chomarat *et al.*, 1995) and the release of both soluble TNF receptors: p55 and p75 culture (Joyce *et al.*, 1994).

Intereukin -4

Intereukin -4 (IL-4) is a 15-19 kDA glycoprotein produced by the Th2 sub – type of CD4⁺ T-lymphocytes and by mast cell precursors. IL-4 down regulates the production of IFN- γ by Th1 CD4⁺ T-lymphocytes, induces the proliferation of thymocytes and mature T-lymphocytes but blocks the IL-2 induced proliferation T-cells as well as the production of IL-2 dependent LAK cells. On the B- cells, IL-4 has a growth factor activity mediated via the production of soluble CD-23, and a differentiation activity leading to the production of IgE, IgM and IgG1. On monocytes, IL-4 induces an increased number of histocompatibility class I antigens and CD-23 receptors but inhibits the expression of IgG receptors. IL-4 blocks the production of IL-1 , IL-6 , TNF – α , PGE2 . G-CSF and stimulates the production of M-CSF and G-CSF by monocytes. IL-4 has also an action on eosinophiles by increasing the expression of CD-23 and inhibiting the expression of IgG receptors. By its pleiotropic activity, IL-4 is a key cytokine in the cytokine network that shows anti – inflammatory properties and is probably involved in mechanisms of allergy.(Banchereau, 1990).

2.8. Role of cytokines

T lymphocytes participate in the immune response to *L. donovani* infection by producing different cytokines. While euthymic *L. donovani*-infected BALB/c mice are able to control infection with granuloma formation and IFN- γ and IL-2 production, nude BALB/c mice neither form granulomas nor produce IFN- γ (Murray *et al.*, 1987). Human recombinant IFN- γ restores the ability of nude BALB/c mice to control *L. donovani* infection. Furthermore, anti-IFN- γ antibody abolishes granuloma formation (Squiers *et al.*, 1989), confirming the importance of this cytokine in protection. Moreover, depletion experiments using anti-IL-2 monoclonal antibodies and reconstitution using recombinant IL-2 showed a role for IL-2 in leishmanicidal activity apparently through the induction of IFN- γ , and in granuloma formation with involvement of L3T4⁺ and Lyt 2⁺ T cells (Murray *et al.*, 1992). Results in immune mice differ from those described for naive animals. Neutralization of IL-2 but not IFN- γ abolishes resistance, and granuloma formation is not affected by neutralization of IL-2 or IFN- γ (Murray *et al.*, 1993).

The role of IL-4 as a cytokine related to susceptibility has been recently questioned in experimental cutaneous leishmaniasis (Sacks *et al.*, 2002), but most studies on experimental visceral leishmaniasis had raised this question about the role of IL-4 in susceptibility from the beginning. In one study, besides predominant IFN- γ production in the initial and late phase of infection, IL-4 production was detected in the intermediary phase coinciding with peak of parasite burden in the susceptible strain, and no IL-4 production in the resistant mouse strain (Saha *et al.*, 1993). A progressive increase in IL-10 mRNA level in tissues during infection suggested a role in susceptibility (Melby *et al.*, 1998). In addition, the control of parasite growth in inner organs in BALB/c IL-10^{-/-} mice and in normal mice with IL-10 receptor

blockade by antibodies confirmed the role of IL-10 in susceptibility (Murphy *et al.*, 2001) (. Since IL-10 receptor blockade increased serum IFN- γ levels, a protective effect was initially attributed to the non-suppressed leishmanicidal effect of IFN- γ .

In contrast, IL-12 was shown to be linked to protection against the infection. IL-12 treatment of *L. donovani*-infected BALB/c mice significantly reduced the parasite burden with the participation of CD4+ and CD8+ T cells, NK cells and IFN- γ , IL-2 and tumor necrosis factor (TNF)- γ (Murray *et al.*, 1995) . But a distinct antimicrobial effect of IL-12, independent of IFN- γ .

2.9. Humoral responses in leishmaniasis:

Although it is evident that (CMI) play a very important role in the susceptibility or resistance and prophylaxis in response to chemo therapy, the role of humoral immune response cannot be ruled out as V.L. is marked by high levels of leishmania- specific antibodies (Neogy *et al.*, 1987), which appear soon after infection and before the development of cellular immunologic abnormalities. The role of these elevated antibodies dies resolution of the disease and protective immunity is mostly unknown. The occurrence of sub clinical or asymptomatic infection among a large population of individuals residen in the endemic areas with development of specific antibodies and or T- cell response to leishmanial antigens suggested naturally acquired immunity (Sacks *et al.*, 1987).

Infection of leishmania is human is characterized by the appearance of anti-leishmanial antibodies in the sera of the patients. In CL usually they are present at low levels during the active phase of the disease (Behin *et al.*, 1989).

In some studies the presence of antibodies against *L. braziliensis* infection in the sera of infected patients has been critically monitored and

utilized for diagnosis and prognosis of the leishmanial antibodies titers are well documented in V.L (Neogy *et al.*, 1987).

It is known that the Th1 cytokine IFN- γ probably up regulates isotypes IgG1 and IgG3 ,and Th2 cytokines IL-4 and IL-5 stimulates the production of IgG4 in human (Abbas *et al.*, 1996), the role of the elevated anti-leishmanial antibodies in V.L patients towards protection or pathogenesis is still unclear worldwide studies has revealed the elevated levels of IgG,IgM,IgE during disease (Atta *et al.*, 2004), and other studies postulated that IgG not only fails to provide protection against this intra cellular pathogen ,but it actually contributes to disease progression and passive administration of antibodies-leishmanial IgG resulted in later lesions in BALB/C mice with greater amount of IL-10 production (Miles *et al.*, 2005) .

Role of B cells and immunoglobulins

Polyclonal B cell activation is present both in human and experimental visceral leishmaniasis (Bunn-Moreno *et al.*,1985; Galvao-castro *et al.*, 1984), but the actual role of B cells or immunoglobulins in the immunity in visceral leishmaniasis have been poorly evaluated. Most of the data in this area have been obtained with cutaneous leishmaniasis models, where resistance was observed with depletion of B cells using anti-IgM antibody (Sacks *et al.*, 1984) or in BALB xid mice, lacking B-1 B cells (Hoerauf *et al.*, 1994), and susceptibility was increased by transfer of B cells (Hoerauf *et al.*, 1996) or administration of B-cell hematopoietic factor, IL-7 (34). In visceral leishmaniasis, enhanced resistance was recently shown in mutant mice that lack mature B cells (Smelt *et al.*, 2000).

To distinguish the effect of B cells from that of immunoglobulins on susceptibility, experiments were performed using mice genetically altered to contain no circulating antibody, with or without functional B cells, and mice

defective in Fc receptor. These studies showed that the circulating antibody is crucial for susceptibility to the development of cutaneous leishmaniasis (Kima *et al.*, 2000). Furthermore, amastigotes from the lesion of cutaneous leishmaniasis were shown to be coated by IgG, and internalization of immunoglobulin-coated amastigotes by macrophages was shown to lead to IL-10 production and consequent enhancement of intracellular parasite growth in vitro (Kane and Mooser, 2001). Similar mechanisms might be acting in visceral leishmaniasis.

2.10 Laboratory diagnosis:

The clinical diagnosis of V.L. is complex because its clinical features are shared with other commonly occurring diseases, such as malaria, typhoid fever and tuberculosis, many of the diseases can coexist with V.L. (in case of co-infection) sequestration of the parasite in the spleen, bone marrow or lymph nodes further complicate this issue (Sundar *et al.*, 2002). So the definitive diagnosis depends on laboratory investigations, Giemza stained slide of blood, bone marrow, lymph nodes aspirates and biopsies of the infected areas serve as the specimens of choice for demonstrating the diagnosing amastigote forms. Culture of blood bone marrow and other tissues may also be performed and these samples often show the promastigote forms (Zeibig, 1997).

2.10.1. Immunological diagnosis:

Antigen detection:

Antigen detection is more specific than antibody-based immunodiagnostic tests (Vinayak *et al.*, 1994; De-colmanares *et al.*, 1995). This method is also useful in the diagnosis of disease in cases where there is deficient antibody production (as in AIDS patients). De-colmanares *et al.*, (1995) have reported that two polypeptide fractions of 72-75 kD and 123 kD present in the urine of visceral leishmaniasis patient by using western blot technique. The sensitivity

of the 72-75 kD. fraction was 96% and specificity was 100%. Besides these antigens were not detectable within 3 weeks of anti-V.L. treatment, suggesting that the test has a very good prognostic value, and could be a power full non invasive method for V.L. diagnosis (De-colmenares *et al.*, 1995).

A latex agglutination test for detection Leishmanial antigen in urine of patients with V.L. has showed sensitivity rates between (68-100%) and specificity of 100% in preliminary trails. The antigens detected quite early during the infection and the results of animal experiments suggest that the amount of detectable antigens tends to decline rapidly following chemotherapy treatment (Attar *et al.*, 2001).

Antibody detection:

For several decades non specific methods, depends upon raised globulin levels, have been used in the diagnosis of V.L. some of the tests used for detecting these non specific immunoglobulins are Napier's formal gel or aldehyde test and the chopra antimony test. Since these tests depend upon raised globulins levels results can be false positive in other diseases that cause hyper-gammaglobulinemia like malaria, HIV, ricketsial infection and visceral larva migrance (Bary, 1976 ; Ichhpujani *et al.*, 1994). So lack of specificity as well as varying sensitivities of these tests render them highly unreliable.

Several immunodiagnostic methods which are more sensitive and specific have been developed. They are useful in identifying specific cases and can be used for community surveillance; the consistent presence of high levels of antibodies against parasite antigens can simplify diagnosis of V.L.. Several serological techniques are based on detection of these antibodies (Saunders *et al.*, 2002).

Direct agglutination test:

In 1988, a modified direct agglutination test (DAT) was reported to be useful in the diagnosis of V.L. and is being used in several endemic countries (Harith *et al.*, 1988). DAT in various studies has been shown to be (91-100%) sensitive and (72-100%) specific (Zijlstra *et al.*, 1991; Mengistu *et al.*, 1990). The test is based on direct agglutination of *Leishmania donovani* promastigote antigens that act specifically with anti-leishmanial antibodies in the serum specimens.

The antibodies cause agglutination of the promastigote this agglutination is visible with the naked eye as a mat or an enlarged button on the button of the wells of the microwell plates (Harith *et al.*, 1987; Harith *et al.*, 1988). The test was applied to sera of 280 inpatient of Baringo district a known focus of V.L. in Kenya when treated cases included the test showed a sensitivity of 100% and specificity of 99.3% (Harith *et al.*, 1986).

A new DAT for use with urine samples for the diagnosis of V.L. has been developed and compared with the conventional DAT with serum samples. The sensitivity of new DAT (90.7%) was almost the same as that of the conventional DAT (91%) the specificity of new DAT is (96.4%) (Islam *et al.*, 2004).

Like most antibody-based tests, DAT may yield or positively for a long time after complete cure and thus has not proved to be of much prognostic value (Harith *et al.*, 1988).

Enzyme Linked Immunosorbant Assay (ELISA):

ELISA has been used as a potential serodiagnostic tool for almost all infectious diseases, including leishmaniasis. The technique is highly sensitive but its specificity depends upon the antigen used. Several antigens have been

tried, the commonly used antigen is a crude soluble antigen (CSA) (Saundar *et al.* 2002). The sensitivity of ELISA using different concentration of CSA is reported to range from (80-100%) but cross reaction with sera from patients with trypanosomiasis, tuberculosis, and toxoplasmosis have been recorded (Bray, 1970; Smrkovski, 1977; Mengistu *et al.*, 1990; Choudhry *et al.*, 1990; Baqir and Al-Jeboori, 2001). The sensitivity and specificity of ELISA in diagnosis of V.L. could also be increased by the use of soluble antigens derived from promastigotes cultivated in a protein-free medium. One study done on 129 cases of V.L. patients showed a sensitivity of (95%) (Ryan, 2002). A comparison study was done among urine DAT and conventional DAT with serum samples and urine ELISA, the sensitivity of urine DAT (90.7%) was almost the same of urine ELISA (93.3%). The specificity of the urine DAT (96.4%) was nearly identical with that of the urine ELISA (97.3%) (Islam *et al.*, 2004).

Indirect fluorescent antibody technique (IFAT):

In the IFAT, the whole parasite antigens are put on microscope slides to which the test serum is applied. Reactive antibodies adhere to the parasite and are not removed by washing. Antihuman immunoglobulins prepared in goats or pigs and labeled with a fluorescent dye are applied. Fluorescence can be visualized by fluorescent if the test is positive, (Jassim *et al.*, 1998). The test is based on detecting antibodies which appeared in the early stage of infection and undetectable 6-9 months after cure (Manson-Bahar, 1971).

IFAT is a specific group, so it can not differentiate between species of leishmania (Niazi, 1980). Suggesting group reaction within the family Trypanosomatidae, ie; (cross reaction), therefore, positive reaction and false positive may occur with any leishmanial or trypanosomal disease. This will

limit it in regions where both trypanosomiasis and leishmaniasis are endemic (Bray, 1976).

Lower sensitivity than DAT, this sensitivity was 97% for IFAT is opposed to 100% for DAT in proved V.L. cases, and 80% as opposed to 86% in suspected cases (Jassim, 1998).

Immunochromatographic strip test:

Because of the condition prevailing in areas of endemicity, any sophisticated method cannot be employed on wider scale. There is a need for a simple, rapid and accurate test with good sensitivity and specificity, which can be used without any specific expertise (Sundar *et al.*, 2002).

Promising ready to use immunochromatographic strip test is based on a recombinant antigen rk39, which has been developed as a rapid test for use in difficult field condition (Sundar *et al.*, 2002). A recombinant antigen rk39 has been shown to be specific for antibodies in patients with V.L. caused by members of the *Leishmania donovani* complex (Burns *et al.*, 1993; Badaro *et al.*, 1996). This antigen, which is conserved in the kinesin region, is highly sensitive and predictable of the onset of acute disease. The antigen is derived from *Leishmania chagazi*. High antibody titers in immunocompetent patients with V.L. have been demonstrated.

The test is qualitative test, membrane based immunoassay for the detection of antibodies to V.L. in human serum. The nitrocellulose membrane is precoated with a novel recombinant V.L. antigen (rk39) on the test line region and chicken anti-protein A on the control line region. During testing the serum sample reacts with the dye conjugate (protein A – colloidal gold conjugate) which has been pre-coated in the test device. The mixture then migrates upward on the membrane chromatographically by capillary action to react with recombinant V.L. antigens on the membrane and generates a red

line. Presence of this red line indicates a positive result, while its absence indicates a negative result (Sundar *et al.*, 2002; Carvalho *et al.*, 2003). In the first extensive trial on 323 patients, Sundar and his team found that the strip test is 100% sensitive (confidence interval 98-100%), (Sundar *et al.*, 1998). Several studies from the Indian subcontinent reported the test to be 100% sensitive (Sundar *et al.*, 1998; Bern *et al.*, 2000; Sundar *et al.*, 2002).

Skin test:

Delayed type hypersensitivity (DTH) or T-cell mediated immunity is a group-specific immune response. The Montenegro skin test (Leishmania skin test) is a test for DTH specific to leishmaniasis, but its role is limited (Marsden, 1985; WHO 1995). The test is negative in acute cases of V.L. due to the absence of DTH and positive only in cases which have been cured (Marsden, 1986).

2.11. Differential diagnosis:

V.L. should be considered in the differential diagnosis of both short-term and long-term fever a combined by hepatosplenomegaly and anemia especially when there is a history of residence in an endemic area (Smith, 1998). The differential diagnosis includes: parasitic diseases (malaria with tropical splenomegaly syndrome, Schistosomiasis), as well as bacterial diseases (Miliary tuberculosis, typhoid fever, brucellosis), fungal diseases (Histoplasmosis) and other diseases (Leukemia, Lymphoma) in the immunocompromized patients especially as fulminant infection without characteristic splenomegaly (Herwaldt, 1996; Smith, 1998).

2.12. Treatment:

The pentavalent antimonial compound, sodium stibogluconate (pentostum) and megalumine antimonite (Glucontime), have been the mainstay of anti leishmanial chemotherapy for more than 40 years (Melby, 2004). These drugs have similar efficacies, toxicities and treatment regimens. Anaphylactic shock

is rare following administration (Pearson *et al.*, 1990; Smith, 1998; Melby, 2004). Currently for pentostam the recommended regimens are 20mg/kg body weight/24hrs intravenously or intramuscularly for 28 days. Repeated courses of therapy may be necessary in patient with severe infection (Smith, 1998; Melby, 2004).

An initial clinical response to therapy usually occurs in the first weeks (i.e. fever usually subsided and the patients felt well) (Smith, 1998). Hematological improvement with rising Hb and total with cell count occurs in 2-4 weeks (Smith, 1998) but complete clinical healing (i.e. regression of splenomegally and normalization of cytopenias) is usually not evident for weeks to a few months after completion of therapy, cure rate with this regimen is (80-100%), patient should be followed up for least 1 year to detect relapse (Smith, 1998; Melby, 2004).

Amphotricin B deoxycolate and newer lipid formulation are very useful in the treatment of antimonyl unresponsive V.L. (Melby, 2004) Amphotricin B deoxycolate at doses of 0.5-1.0 mg/kg each day or every other day for 14-20 doses, achieves a cure rate for V.L. of close to 100% but renal toxicity commonly associated with Amphotricin B was evident (Beaver *et al.*, 1984; Beaver *et al.*, 1995; Melby, 2004).

The lipid formulation of Amphotricin B is especially attractive for treatment of leishmaniasis because the drugs are concentrated in the reticuloendothelial system and are less nephrotoxic. The dose of liposomal amphotricin B is 3mg/kg on day 1-5, and again on day 10, has been shown to be highly effective (90-100%) cure rate for treatment of V.L. in immunocompetent children, some of whom were refractory to antimony therapy (Melby, 2004).

Also, pentomidine isothionate (iomidine), one of diamidine compounds is given intramuscularly in a dosage of 4 mg of base/kg of body weight daily for 10 days. The course may be repeated after a 10 day interval. An immediate hypotensive reaction and local pain at the site of injection are the major side effects (Beaver *et al.*, 1984).

2.13. Immunotherapy (Future treatment):

The treatment of V.L. remains unsatisfactory, and there is an urgent need to develop new thereapies to reduce drug and long – term hospitalization, and to prevent drug resistance. As successful treatment of Kala – azar is thought to depend, at least in part, on alterations in the host immune response to the parasite, direct manipulation of the immune response, either alone or in combination with drugs, may be a useful strategy for improving treatment regimens for V.L. (Nylen *et al.*, 2007).

In experimental *Leishmania donovani* infection in susceptible host, acquired resistance in the liver is initially regulated by multiple Th1 – and Th2 cell associated cytokines (Alexander *et al.*, 2000).

The medionism is primarily driven to completion by Th1 – type products, including IL-12 which induced IFN-4, acting in concert with TNF (Stager *et al.*, 2003). If unimpeded, the net result at infected liver Foci is the assembly of epitheloid granulomas within which intracellular parasites are killed by IFN-gamma and TNF-alpha activated macrophages (Murray, 2001).

This same inflammatory mechanism also supports the efficacy of conventional antileishmanial chemotherapy. T-cells and endogenous IL-12 and IFN – σ are required for expression of the V.L. action of pentavalent antimony (Sb) in experimental infection (Escobar *et al.*, 2001). TNF and IL – 4 also optimize the host response to (Sb) (Alexander *et al.*, 2000). The role of IL-4, ordinary considered suppression – Type cytolsines, appears to reflect its

less – well – appreciated capacity to foster Th1- cells development and help regulate initial IFN – γ secretion (Stager *et al.*, 2003).

Effects to take advantage of the preceding immunopharmacology have focused on IL-12 and IFN – γ and on raising the level of T – cells reactivity at the time of (Sb) treatment. Approaches in V.L. infection have included coadministration of (Sb) (i) with exogenous IL-12 or IFN- γ (Murray *et al.*, 2000) or (ii) with induction of endogenous IL- 12 and / or IFN – γ achieved by T – cell costimulation (Zubairi *et al.* 2004) , transfer of sensitized dendritic cells (Ghosh *et al.*, 2003), or injection of IL – 12 to induce endogenous IFN – γ (Murray *et al.*, 2001). These Experimental approaches enhance Sb's initial efficacy and / or durability of its effect.

Reports on administration of IFN – γ in The V.L. patients for therapy imply the functional importance of this cytokine to establish the immune environment for clearance of the parasite (Sundar *et al.*, 1997a).

Subjects, materials and methods:**3.1. Subjects selection:**

The study has been included sixty V.L. patients, they are divided into two groups: group 1: includes 40 patients whom were in the acute phase of the disease, and the second group includes 20 patients whom were in post healing stage (after chemotherapy). Their ages below 15 years. They were attendants of Al-Dewanyia children hospital from October 2006 to December 2007.

The diagnosis in each case was established by clinical examination done by a specialist doctor in the hospital and confirmed by laboratory investigations (DAT) rk39 kala-azar detect rapid test.

3.1.1. Controls:

Twenty apparently healthy children were enrolled as a control and checked by the same kit for detection of kala-azar.

3.1.2. Baseline data:

Baseline data about the patients were obtained from routine history and clinical examination this information arranged in a questionnaire including:

name, age, sex, address, disease duration, hepatosplenomegaly, fever, Hb% and white blood cell count.

3.2. Laboratory diagnosis:**3.2.1. Blood collection:**

From each patient and control, five ml venous blood was aspirated from a suitable vein after efficient disinfecting over the injection site. Blood samples were divided into two parts, Three ml immediately transferred to sterile heparinised vacutainer tubes for lymphocyte separation, and two ml of blood immediately transferred to sterile plain tubes for serum separation to be used in serological tests.

The unheparinized blood in plain tube was let to clot and then centrifuged at 1000 rpm for 5 minutes at 25°C to separate the serum and dispensed into sterile tightly closed Eppendorf tubes in 0.1 mls aliquots and stored at -20°C until assayed (Boyum, 1968).

3.2.2. Lymphocyte separation

The Isopaque-ficol technique originally described by Boyum in 1968 was used for lymphocyte separation.

Materials used in lymphocyte separation were:

- Phosphate Buffered Saline (PBS) (Flow laboratories, U.K).
- Lymphocyte separation medium (Ficol) (Flow laboratories, U.K).
- Centrifuge tube.
- Centrifuge (LKB, Macrospin R).
- Pasteur pipette.
- Improved Neubauer.
- Trypan blue.

Lymphocyte separation was done as following: three mls of blood which were collected in heparin containing (10 units/ml blood) tube was diluted with PBS 1:1 v/v. Then after dispense 4ml of lymphocyte separation medium into a 10ml centrifuge tube, a 3ml of the diluted blood sample were carefully layered on the top of the separation medium, then centrifuge for 30 minutes at 2500 rpm at 20°C, after centrifugation, the lymphocytes formed a gray-colored layer at the interface of the blood plasma and the separation medium, lymphocytes aspirated with a sterile Pasteur pipette, and transferred to a centrifuge tube containing at least three times the volume of PBS and suspend evenly. Then lymphocyte suspension was centrifuged at 1000 rpm for 10 minutes and the supernatant was aspirated and discarded and the cell pellet was resuspended in PBS by gentle drawing the cells in and out of a

pasteur pipette, then repeat centrifugation and resuspension in PBS another two times, then resuspend in the PBS for subsequent experimentation. The cells were calculated by counting in haemocytometer (improved Neubauer) and trypan blue. Then the cells were adjusted to 1×10^6 cells/ml in PBS. (Marian & Catherine, 1998).

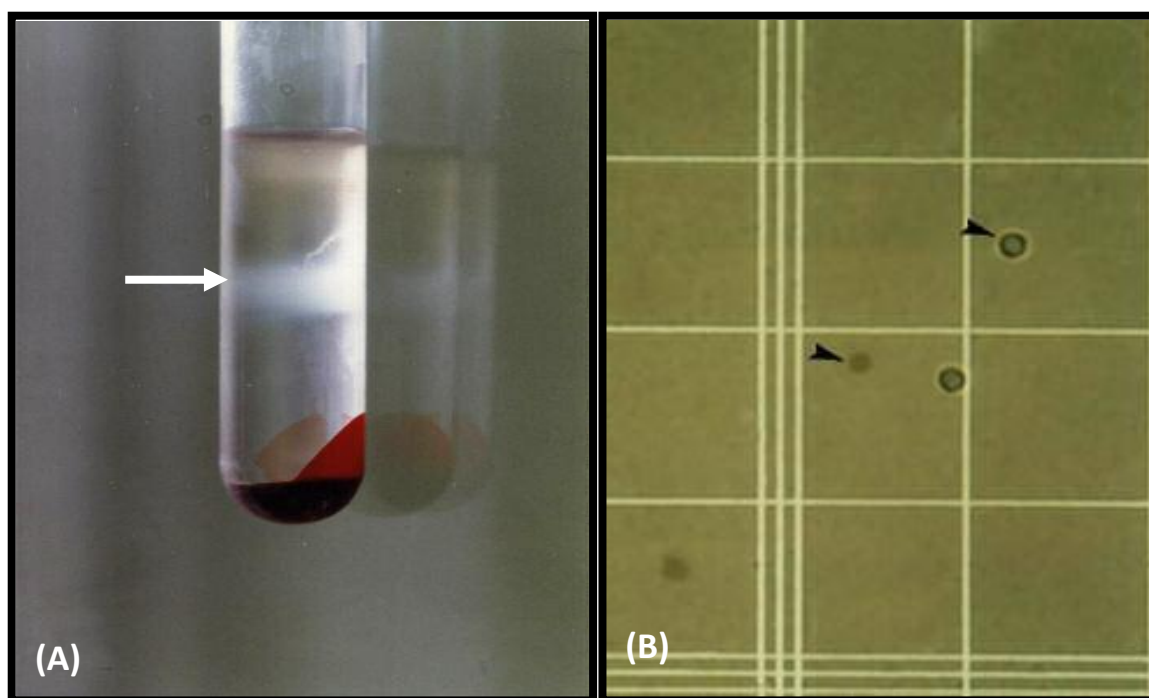


Figure 3.1: (A) Stratification of blood constituents after centrifugation lymphocyte layer is evident as a white buffy layer at the interface of blood plasma and separation medium (B) Assessment of lymphocyte viability by trypan blue exclusion test. As shown, the viable cells exclude the stain while the non-viable cell stained with trypan blue.

3.2.3. Slides preparation

Slides were prepared according to Marian & Catherine, 1998, with a simple modification in the type of fixative and as following:

Materials used in slides preparation:

- Cell suspension adjusted to 1×10^6 cells/ml.
- Immunofluorescent slides.
- PBS.
- Formalin.
- Acetone.
- Automatic adjustable pipette.

Preparation of fixative: Buffered formal acetone fixative, was prepared by mixing: 8ml of PBS, 38ml of D.W., 33.2 mls of formalin, and 60ml acetone.

Preparation of slide smears: Slides were prepared by dropping ten μ l of cell suspension on fluorescent slides, then smears were allow to dry slightly at the periphery of slide prior to adding 10 μ l of the fixative solution. After drying the fixative solution the slides were foiled with parafilm & kept at -20°C until used in immunostaining.

3.3. Direct immunoflourescence dual staining technique for the detection of CD4-CD8 and CD3-CD19 on PBL:

The principle

Fluorescen isothiocyanate (FITC) and Rhodamin-phycorethine (RPE) labeled monoclonal antibodies directly react with antigens on the surface of PBL. FITC and RPE are molecules that have the ability to absorb invisible, ultraviolet radiation, and then have the capability of emitting an apple green and orange red fluorescence colored light respectively after exposure to UV light.

Materials:

1. Blocking Reagent: normal goat serum in phosphate buffered saline (PBS) containing carrier protein.
2. Phosphate buffer saline (PBS).
3. Dual color reagents: DC011 Anti (CD4-RPE-CD8-FITC) labeled proteins respectively and DC005 Anti (CD3-FITC- CD19-RPE) labeled proteins respectively (SEROTEC, USA): with specificity to human CD4-CD8 and CD3-CD19 proteins. The powder reconstitute with 0.5 ml PBS (pH 7.4)+0.09% Sodium azide+1% Bovine serum albumin (BSA).
4. Glycerol carbonate buffer mounting medium formed from: 1.59g Na₂ Co₃ (BDH, UK) and 2.93 gm NaHCo₃ (BDH, UK) were dissolved in 1 liter of distilled water.
5. Precision pipette 50-200µl and tips (Finn pipette, Finland).
6. Eppendorf tubes.
7. Slide holders (10-slides each).
8. Timer with alarms (Junghans, Germany).
9. Tissue paper.
10. Incubator (Mettler, Germany).
11. Cover slips.
12. Immunofluorescence microscope. (Olympus, Japan)

Direct dual immunofluorescence procedure

The method of IF-labeling of fixed cells was done as described by (Batty,1986) as following:

Slides were prepared as described then, the pre-coated slides with lymphocytes were removed from freezer, allowed to reach room temperature, unwrapped and washed with PBS by dipping the slides into PBS- containing jar for about 5 minutes at room temperature. Slides were laid flat, smear-side

up, in humidity chamber then 10 μ l of 1/75 diluted fluorochrom (FITC) conjugated monoclonal antibodies were added to each smear, cover the chamber and slide were left undisturbed in incubator at 37°C for 50 minutes. Slides then transferred to staining jar filled with PBS at room temperature and PBS replaced twice at 5 minutes intervals, then drained and blotted gently.

Two drops of mounting media [(nine parts glycerol to one part of 0.2M carbonate buffer, pH=9 (Batty, 1986) to enhance fluorescence and retard fading on exposure to UV-light (Marian & Catherine, 1998)] placed on each smear of slides. Then cover slips were lowered into place slowly to avoid bubbles; cover slips may be sealed around edges with clear nail polish. Slides were examined then with fluorescence microscope at 490 nm; positive cells give green-apple when stained with FITC-labeled antibodies and exposed to UV light.

In each smear, the countable field was located and the total number of 100 cells was counted, the percentage of positive cells was calculated as following:

Percentage of positive cells= (No. of positive cells/ total No. of cells) $\times$ 100%

3.4. Immunocytochemistry staining techniques for the detection of CD22, CD14, CD45RA, CD45RO, CD1a, CD44 and HLA-DR protein on PBL:

The principle

A primary antibody reacts with an antigen on the surface of PBL. A biotinylated secondary antibody then reacts with the primary antibody. This is followed by the attachment of an enzyme-conjugated streptavidin to the biotins on the secondary antibody. The enzyme converts a substrate to a colored reaction product. High levels of signal amplification are achieved due to the binding of multiple units of secondary antibody to each primary

antibody, the binding of multiple enzyme-conjugated streptavidin molecules to each secondary antibody, and the enzymatic conversion of the substrate was recorded (Boyum,1968).

Materials :-

1- Immunoperoxidase Secondary Detection system. (Dako Cytomation, USA, Ref K0673) which contain the following components:

- Peroxidase block.
- Secondary Antibody: biotinylated goat anti-mouse IgG in PBS, containing carrier protein.
- Streptavidin–HRP.
- DAB chromogen.
- DAB substrate buffer.

2- Monoclonal Antibodies:

- Anti CD22, CD14, CD45RA, CD45RO, CD1a, CD44 and HLA-DR (Serotec).

3- DPX mounting medium (England).

4- Precision pipette 50-200µl and tips (Finn pipette, Finland).

5- Plain tubes (10 mls).

6- Eppendorf tubes.

7- Graduated cylinders.

8- Pasteur pipettes (10 ml).

9- Slide holders (10-slides each).

10- Timer with alarms (Junghans, Germany).

11- Tissue paper.

- 12- Incubator (Mettmert, Germany).
- 13- Gloves.
- 14- Cover slips.
- 15- PBS.
- 16- Humid chamber.
- 17- Washing bottle.
- 18- Counter stain (haematoxylin).
- 19- Light microscopy (ZEISS, West Germany).

Immunocytochemistry staining procedure

The method of immunoperoxidase staining done according to Johanston and Throp,(1982) with some modifications and as following; the lymphocytes precoated fluorescent slides were removed from freezer, allowed to reach room temperature, unwrapped and then dipping the slides into PBS-filled jar for about 5 minutes and slides were placed on a flat level surface (avoid attachment of slide to each other and should not allowed to dry out at any time), then enough drops of Peroxidase block were dropped to cover each well and incubated for 5 minutes at room temperature then rinse with PBS from a wash bottle, slides then placed in PBS wash bath for 2 minutes and excess buffer was taped and wiped around smears. Then, enough power block reagent (1/10 diluted in PBS) were applied for 5 minutes and excess blocking reagent was taped but not washed. Then, the coated lymphocytes were covered by 20 μ l of 1/30 diluted mouse monoclonal Abs (primary Ab) specific human CD-markers (CD22, CD14, CD45RA, CD45RO, CD1a, CD44 and HLA-DR). Slides then incubated at 37°C for 1hr, then unreacted monoclonal Ab were removed by three cycle of washing with PBS each two

minutes, after washing enough drops of Peroxidase block solution were dropped to cover each smear and incubated for 10 minutes in humid chamber at room temperature then rinse with PBS from a wash bottle, then slides were washed wiped around the smear. After that enough solution of biotinylated secondary antibody (anti-mouse Ab) were applied to cover each smear, distributed evenly over the precoated cells and slides then placed in humid chamber for 1 hour at 37°C and washed in buffer and bathed in PBS for 5 minutes then wiped around smear. Enough solution of streptavidin conjugated peroxidase were applied to cover the smear and slides were placed in humid chamber for 1 hour at 37°C then washed in buffer and bathed in PBS for 5 minutes then wiped around the wells. Slides then Developed in a substrate (DAB) working solution which prepared by addition of one drop of chromogen (DAB) to one ml of substrate buffer. Then enough drops of freshly prepared DAB working solution were applied to cover the section at room temperature for 10 minutes or until the color is observed then the reaction terminated by rinsing gently with distilled water from a washing bottle. Slides then placed in bath of hematoxyline for 30 seconds at room temperature. Slides were rinsed gently with distilled water from a wash bottle then rinsed under gently running tap water for 5 minutes. A drop of mounting medium (DPX) was placed onto the wet smear and the spot quickly covered with a cover slip. Slides were let to dry.

Slides were examined under 40X-magnification power of light microscope (ZEISS). The dark brown staining identified positive labeled cells. A total of 100 cells were counted to determine the percentage of reactivity of each of the tested monoclonal Abs. the percentage of positive cells calculated as following:

Percentage of positive cells= (No. of positive cells/ total No. of cells) ×100%

3.5. Quantitative determination of human serum cytokines:

Principles:

The assay is based on an oligoclonal system in which a blend of monoclonal antibodies (MAbs) directed against distinct epitopes of (IL-4, IL-10, IFN-gamma and TNF-alpha) were used. Where standard or samples containing this cytokines reacts with capture monoclonal antibodies (MAbs1°) coated on microtiter well and with (MAbs2 °) labelled with horse raddish peroxidase (HRP). After an incubation period allowing the formation of sandwich. Coated MAbs1°-cytokines MAbs2°-HRP. Bound enzyme-labelled antibodies are measured through a chromogenic reaction, after addition of stop solution the microtiter plate is read at appropriate wave length Beutler, (1987).

Materials for IL-4 determination:

1. ELISA kit for measurement of IL-4 (Biosource IL-4 EAISA kit, Belgiun), which contains:
 - a) Microtiter plate with 96 anti IL-4 coated wells.
 - b) Standard 0 in human plasma.
 - c) Standard 1 to 5 in human plasma.
 - d) Solution B (buffer with preservatives for

Assay procedure: it was done according to Biosource IL-4 EASIA kit. For run test:

- Pipette 100µl of solution B for all wells.
- Pipette 100 µl of each standard, control, sample into the appropriate wells.
- Pipette 50 µl of anti – IL-4 conjugate into all wells.
- Incubate for 2 hours at room temperature on a horizontal shaker at 700 rpm.
- Washing by using washer solution after dilution.

- Pipette 200 μ l of freshly prepared chromogenic solution into each well.
- Incubate the plate for 30 minutes at room temperature with shaking at 700 rpm, avoiding direct sun light.
- Pipette 50 μ l of stop solution into each well.
- At last we read absorbance at 450nm in ELISA reader.
- The O.D. values in the table

IFN-gamma assay procedure:

- Pipette 50 μ l of each standard, control, serum in the appropriate wells.
- Pipette 50 μ l of anti-IFN-gamma conjugate into the wells.
- Incubate for 2 hours at R.T. on horizontal shaker set at 700rpm.
- Washing by washing solution in washer 3 times.
- Pipette 200 μ l of freshly prepared chromogenic solution into each well after dilution with 1 vial of substrate buffer (0.2 mls of chromogen+1 vial buffer).
- Incubate the plate for 15 min. At R.T. with shaking at 700 rpm.
- Pipette 50 μ l of stop solution into each well.
- Read absorbance at 450nm by ELISA reader.

IL-10 assay procedure:

- Pipette 100 μ l of solution B for all wells.
- Pipette 100 μ l of each standard, control, sample into the appropriate wells.
- Pipette 50 μ l of anti – IL-10 conjugate into all wells.
- Incubate for 2 hours at room temperature on a horizontal shaker at 700 rpm.
- Washing using washer by using washer solution after dilution.
- Pipette 100 μ l from solution A into all wells, then 50 μ l of anti IL-10 conjugate into all wells.
- Incubate for 2 hrs at R.T. with shaking set at 700 rpm.
- Washing by washing buffer 3 times.
- Pipette 200 μ l of freshly prepared chromogenic solution into each well.
- Incubate the plate for 30 minutes at room temperature with shaking at 700 rpm, avoiding direct sun light.
- Pipette 50 μ l of stop solution into each well.

- At last we read absorbance at 450nm in ELISA reader.

TNF-alpha assay procedure:

- Pipette 50µl of incubation buffer for all wells.
- Pipette 200 µl of each standard, control, sample into the appropriate wells.
- Incubate for 2 hours at room temperature on a horizontal shaker at 700 rpm.
- Washing using washer () by using washer solution after dilution.
- Pipette 100 µl of standard 0 into all wells.
- Pipette 50 µl of anti – TNF-alpha conjugate into all wells.
- Incubate for 2 hours at room temperature on a horizontal shaker at 700 rpm.
- Washing using 3 times by using washer solution after dilution.
- Incubate for 2 hrs at R.T. with shaking set at 700rpm.
- Washing by washing buffer 3 times.
- Pipette 200 µl of freshly prepared TMB chromogenic solution into each well.
- Incubate the plate for 30 minutes at room temperature with shaking at 700 rpm, avoiding direct sun light.
- Pipette 50 µl of stop solution into each well.
- At last we read absorbance at 450nm in ELISA reader.

3.6. Quantitative measurements of serum immunoglobulines (IgA, IgG, IgM and complement components C3 and C4).

Principle of the test:

The concentration of immunoglobuline (IgA, IgG, IgM and complement components C3 and C4) were estimated in the sera of V.L. patients and healthy controls using a single radial immunodiffusion assay (SRIDA) by binding site kit (U.K.) according to the manufacturer instruction:

Equal volumes of sera and test samples were added to wells in an agarose gel containing monospecific antisera, the sample diffuse radially through this gel and this substance being assayed forms a precipitation ring with the specific antisera. Ring diameter was measured and reference values were correlated to reach actual concentrations (Mancini *et al.*, 1965).

This test was done as the follows:

1. After removing serum samples and immune-kit from the refrigerator, they were left at room temperature for 15 minutes.
2. The plate was removed from bag, and the lid was removed if the moisture is present, the un cover plate was allowed to remain at room temperature for approximately 15 minutes until moisture had evaporated.
3. Five 5µl from each serum sample were dispensed into wells.
4. The lid was replaced (closed firmly) and incubate at R.T. at inverted position for 48hours except in IgM 72 hours.
5. The immunoprecipitation ring diameter was measured with the (eye piece) viewer device which was capable of measuring the preceptating ring diameter in 0.1 mm in centemeter.

3.7. Statistical analysis

Statistical analysis was performed with the SPSS 15.01 statistical package for social sciences and also Excell 2003. Data analysis was done using student t-test for tables with means and standard deviations and analysis of variance (ANOVA) test if we have more than two groups. P-value of ≤ 0.05 was used as the level of significance.

Descriptive statistics for the clinical and laboratory results were formulated as mean and standard deviation (SD) (Alsahoeke *et al.*,1990) .

Results

4.1-Demographic and clinical results

Our study included 40 patients with V.L.. At baseline and 20 children after treatment with (Sb), and 20 children apparently healthy control

4.1.1 – Age and sex distribution:

It was found that the age group (one to two years) represented 50% of total patients number, while age group less than one year represented 35% and (2-3) years old age group represented 10% while 4-5 years old age group represented 5% as shown in figure 4-1 and table 4-1.

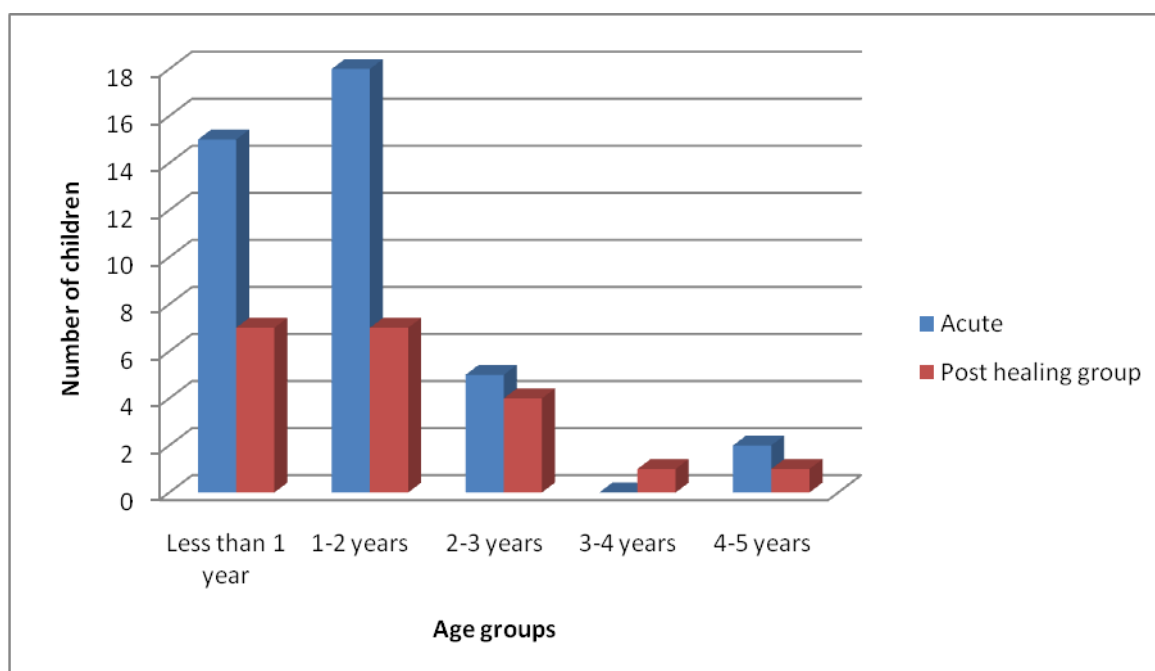


Figure 4-1: Frequency of V.L. patients according age groups.

Table one shows that the male was represented 52% and 47% were female of all patients, male to female ration was 1:1 figure 1.

Table 4-1: age and sex distribution in patient with V.L..

Age groups		Acute	Post healing	Total
Less than 1 year		15	7	22
1-2 years		18	7	25
2-3 years		5	4	9
3-4 years		0	1	1
4-5 years		2	1	3
Gender Type	Male	21	11	32
	Female	19	9	28
Total		40	20	60

4.1.2 – Clinical manifestation:

From table 4-2, and figure 4-2, we can read the following: Hb concentration showed a significant difference between acute and post healing and control group, in acute patient group the mean value was 8.64 ± 1.594 and in post healing 9.51 ± 1.34 represented versus 11.27 ± 1.291 in control.

There is no significant difference in WBC count between the acute and post healing group but a significant differences decrease in WBC count was found when compared with control and post healing and acute patient group figure 4-3.

Hepatosplenomegaly it was obvious that hepatosplenomegaly was diagnosed in all patients with acute and post healing table 4-3.

Table 4-2: Basic descriptive statistics of laboratory parameters in different study groups.

laboratory parameters	Clinical groups	Mean $\pm$ Std. Dev.	ANOVA Test
Hb concentration	Acute	8.637 $\pm$ 1.594	0.000**
	Post healing	9.505 $\pm$ 1.340	
	control	11.272 $\pm$ 1.291	
WBC count	Acute	5,190 $\pm$ 2,107	0.030*
	Post healing	5,641 $\pm$ 2,294	
	control	6,794 $\pm$ 1,813	

* Significant difference $p \leq 0.05$.

**Highly significant difference $p \leq 0.005$.

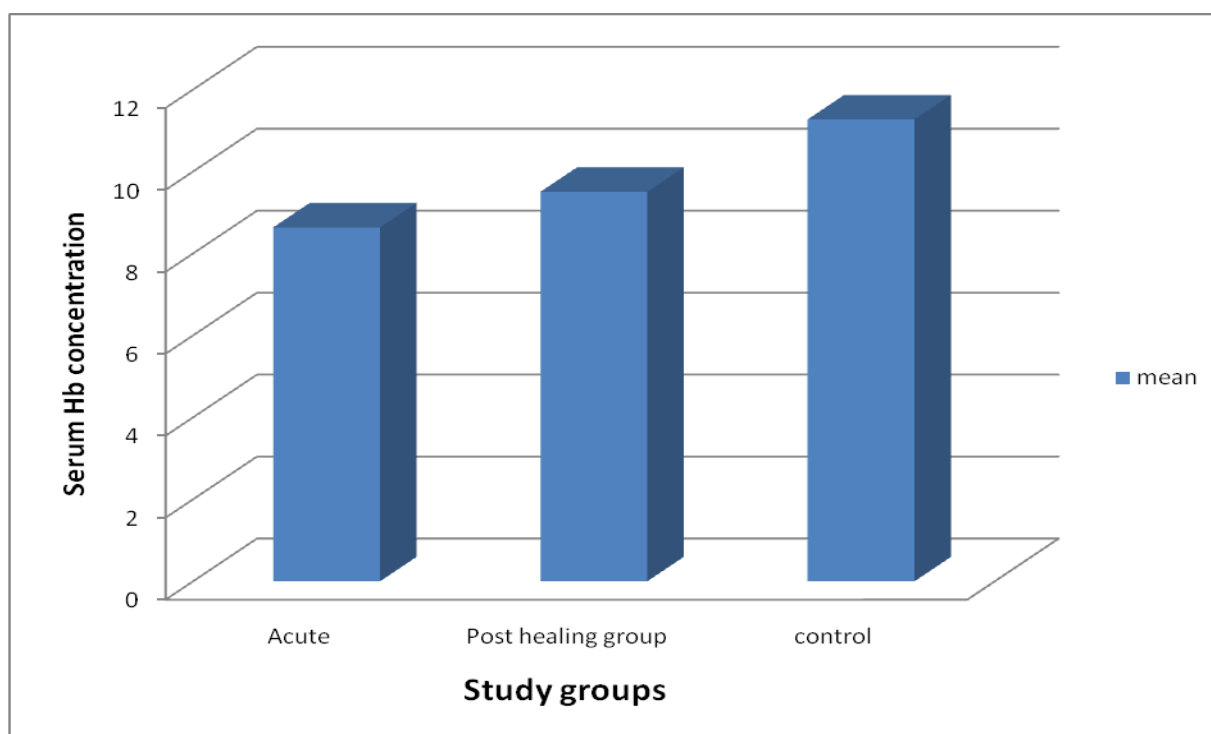


Figure 4-2: mean of serum hemoglobin in different study groups.

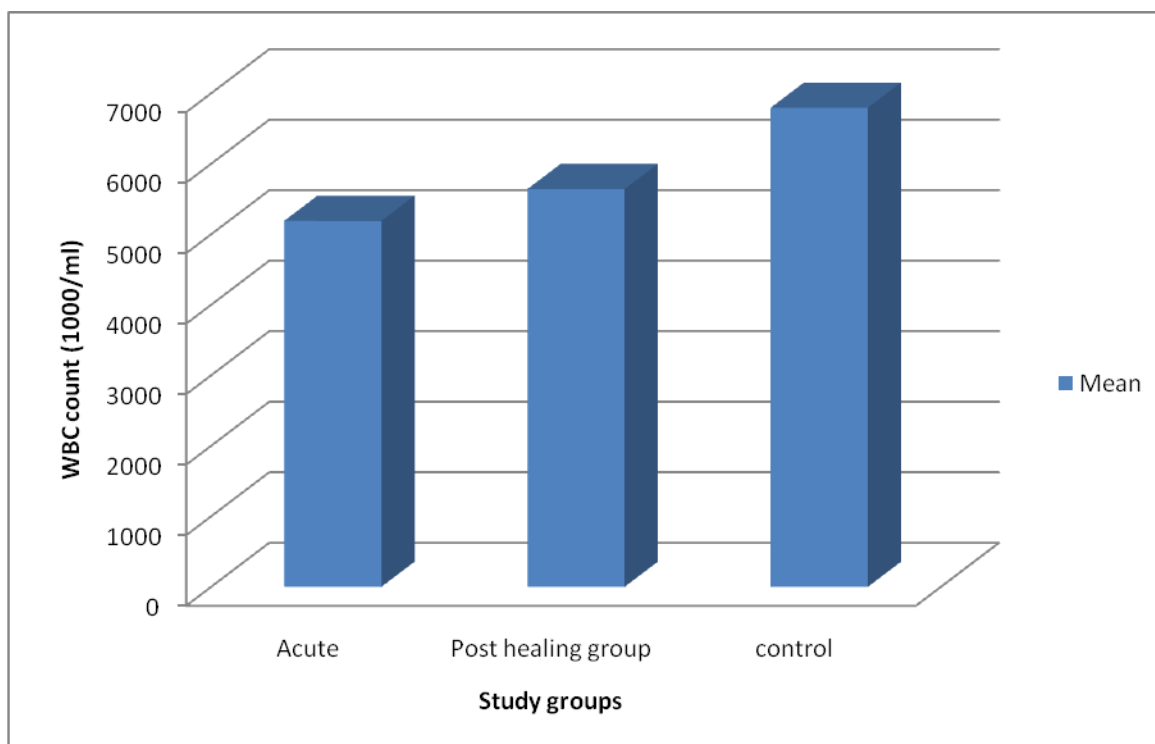


Figure 4-3: total mean of white blood cell count in different study groups.

Table 4-3: Frequency and percentage of hepatosplenomegaly in different study groups.

Hepatosplenomegaly	Clinical groups			Chi square Test
	Acute	Post healing	control	
	Count (%)	Count (%)	Count (%)	
No enlargement	2 (6%)	20 (100.0%)	20 (100.0%)	0.000**
Slight enlargement	14 (35.0%)	0 (0.0%)	0 (0.0%)	
Moderate enlargement	13 (32.5%)	0 (0.0%)	0 (0.0%)	
Sever enlargement	11 (27.5%)	0 (0.0%)	0 (0.0%)	
Total	40 (100%)	20 (100.0%)	20 (100.0%)	

**Highly significant difference $p \leq 0.005$.

4.2 lymphocyte subsets (CD-expression)

Phenotyping of peripheral blood lymphocyte (PBL) subsets (CD3, CD4, CD8 and CD19) of all groups had been done by direct immunofluorescent dual staining technique figure 4-10, beside using of immunocytochemistry for the detection of CD1a, CD14, CD22, CD45RO, CD45RA and HLA-DR protein were IgG1 as negative control figure 4-9.

4.2.1 Total T-lymphocytes (CD3):

Children with V.L. had shown that CD3+ cells percentage (23.682 ± 4.592) was slightly significant lower ANOVA 0.002 ($P < 0.005$). Than the control group ($27.944 \pm 4.304\%$) whereas no-significant differences between post healing ($26.535 \pm 3.193\%$) and control group, as shown in table 4-4.

4.2.2. T-helper/inducer lymphocytes (CD4+):

The percentage of CD4+ cells in V.L. children ($36.345 \pm 3.048\%$) was significantly differences ($p \leq 0.005$) with control group which was recorded ($12.283 \pm 3.536\%$) of total cells. And there was no statically significant difference between post healing ($12.329 \pm 5.945\%$) and control group as showed in table 4-4.

4.2.3 T-cytotoxic /suppressor lymphocytes (CD8+):

The results demonstrated in table 4-4 show that there were high statically significant differences in the mean of CD8+ expression on PBL between acute V.L. children ($43.097 \pm 4.950\%$) and health control group (25.777 ± 4.734) $p \leq 0.005$. The percentage of cells expressed (CD8+) in post healing group was (23.670 ± 4.367). There was no significant difference between acute group and post healing group results.

4.2.4 CD4+/CD8+ ratio:

The CD4+/CD8+ ratio has a special importance because it represents an idea that refers to the immunological balance between T-helper cell and T-

cytotoxic cells in the immune system, in the higher CD4+/CD8+ ratio the nearer balance point would be to T-helper cells, which mean lower cytotoxic activity and higher other form of CMI and humoral immunity. CD4+/CD8+ ratio was higher in V.L. patients (0.84) than that of the control group as we shown in table 4-4.

Table 4-4: Peripheral Blood CD markers cellular expression stained by dual immunofluorescence in different study groups.

Cell surface marker expression	Clinical group	mean±SD.	ANOVA test
CD 3	Acute	23.682±4.592	0.000**
	Post healing	26.535±3.193	
	Control	27.944±4.304	
CD 4	Acute	36.345±3.048	0.000**
	Post healing	12.329±5.945	
	Control	12.283±3.536	
CD 8	Acute	43.097±4.950	0.002**
	Post healing	23.670±4.367	
	Control	25.777±4.734	
CD4/CD8	Acute	0.843	0.000**
	Post healing	0.520	
	Control	0.476	

**Highly significant difference $p \leq 0.005$.

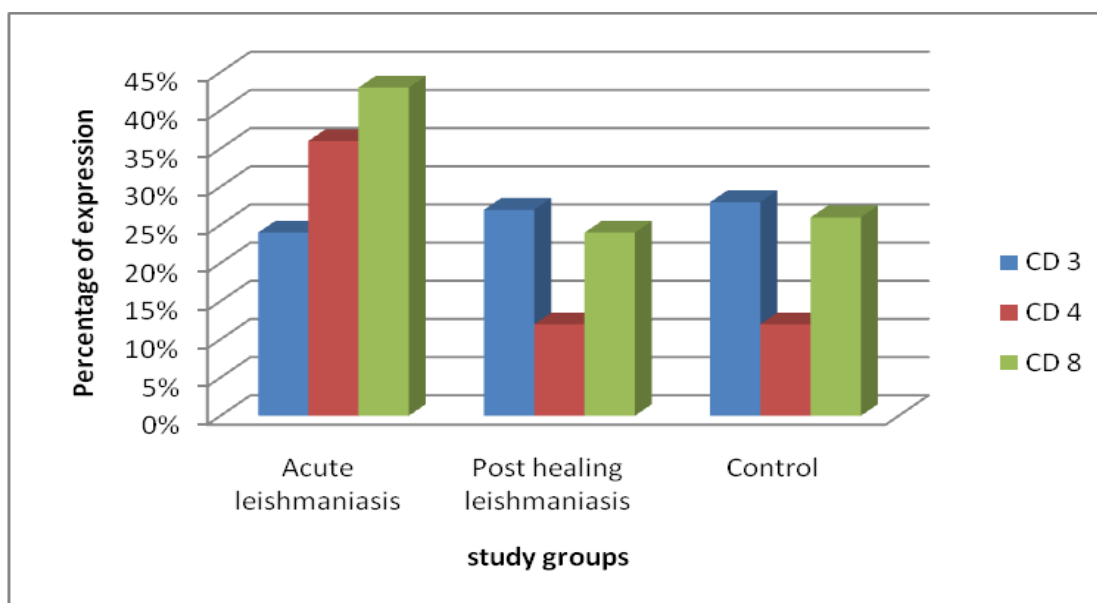


Figure 4-4: mean percentage of peripheral blood lymphocytes cellular expression of CD4 and CD8 in different study groups.

4.2.5 Total B-lymphocytes (CD19):

The percentage CD19+ cell in V.L. patients (27.944 ± 4.304) was significantly higher than that at control group (9.805 ± 2.704). CD19 in the post healing V.L. group was (9.805 ± 2.704) as shown in table 4-5.

4.2.6. B-lymphocytes (CD22+):

There was a high increase in CD22+ cells percentage in V.L. patients (47.912 ± 13.695) in comparison to CD22+ cells percentage of the control group (17.361 ± 5.519) which represented high significant differences ($p < 0.005$), and that between post healing (37.411 ± 15.652) and control group table 4-5.

4.2.10 HLA -DR:

Children in the group of acute V.L. had shown that HLA-DR cells percentage (71.437 ± 8.208) was significantly higher than that of control group (23.705 ± 7.449) see table and 4-5, figure 4-10. The percentage of this marker in the post healing group was (47.729 ± 19.263) which was high statistical differences with control group.

Table 4-5: Peripheral Blood B-lymphocyte CD markers cellular expression in study groups.

Cell surface marker expression	Clinical group	mean±SD	ANOVA test
CD 19	Acute	27.944±4.304	0.000**
	Post healing	24.455±4.731	
	Control	9.805±2.704	
CD 22	Acute	47.912±13.695	0.000**
	Post healing	37.411±15.652	
	Control	17.361±5.519	
HLA-DR	Acute	71.437±8.208	0.000**
	Post healing	47.729±19.263	
	Control	23.705±7.449	

**Highly significant difference $p \leq 0.005$.

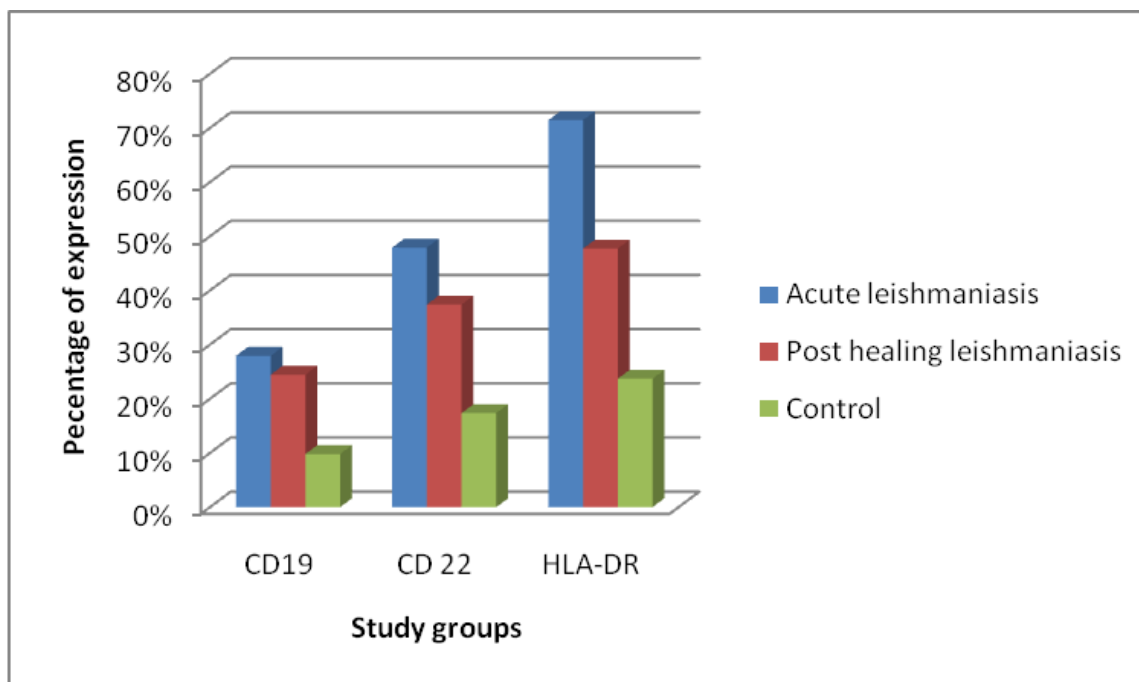


Figure 4-5: mean percentage of peripheral blood B-lymphocytes cellular expression of CD19, CD22 and HLA-DR in different study groups.

4.2.7 Monocyte differentiation CD14+:

The percentage of CD14+ cells of V.L. children (60.840 ± 16.654) was significantly higher ($p < 0.05$) than that of control (25.333 ± 12.462), Table 4-6. The percentage of CD14+ cells in the post healing V.L. group of, there was (43.588 ± 12.913), and represented significant differences in comparison with control group ($p < 0.05$), and there was a statistical significant difference between acute group and post healing group.

4.2.8 Antigen CD1a:

There was a relative increase in the percentage of CD1a+ cells percentage of V.L. children (42.415 ± 11.728) in comparison to CD1a cells percentage of the control group (38.983 ± 11.289) but this increase was not significantly ($p > 0.005$) table 4-6, in the post healing V.L. group CD1a+ cells were (45.176 ± 16.291) slightly more than acute group but not significant differences between it.

Table 4-6: Peripheral Blood monocyte CD markers cellular expression in study groups.

Cell surface marker expression	Clinical group	mean $\pm$ SD	ANOVA test
CD 14	Acute	60.840 ± 16.654	0.000**
	Post healing	43.588 ± 12.913	
	Control	25.333 ± 12.462	
CD 1a	Acute	42.415 ± 11.728	0.360NS
	Post healing	45.176 ± 16.291	
	Control	38.983 ± 11.289	

^{NS} No significant difference.

**Highly significant difference $p \leq 0.005$.

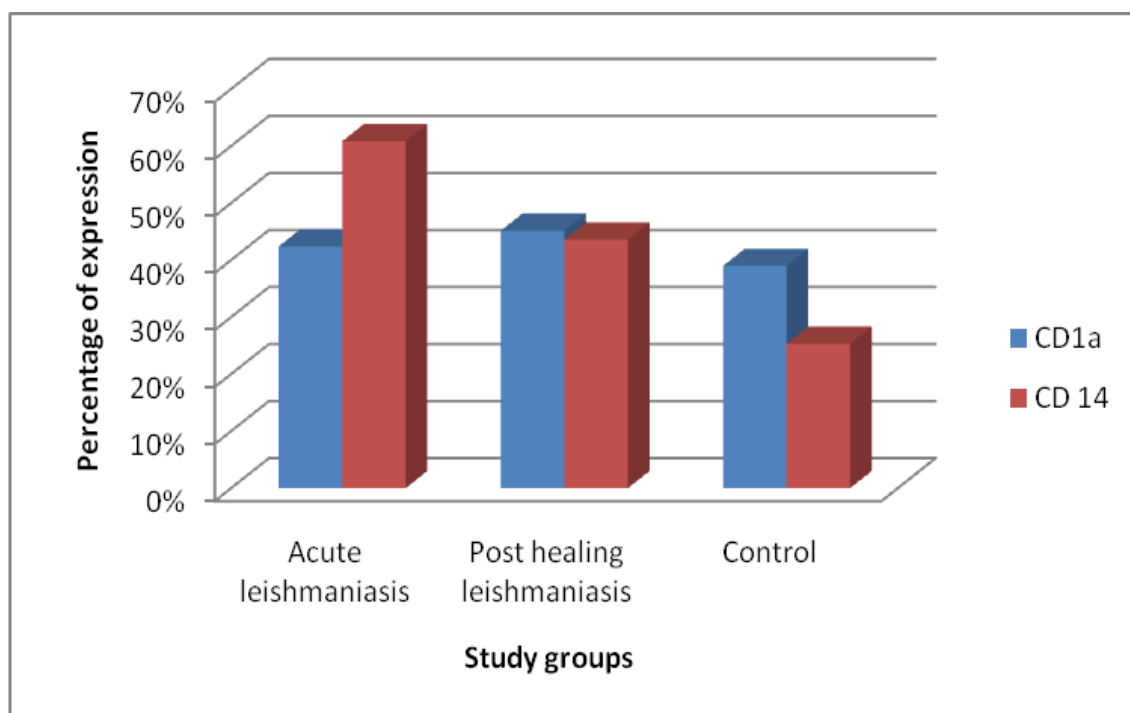


Figure 4-6: mean percentage of peripheral blood monocytes cellular expression of CD1a and CD14 in different study groups.

4.2.9 Hyaluronic acid CD 44+:

The percentage of CD44 cells in V.L. children (26.655 ± 11.788) was significantly higher from the control group (15.5944 ± 10.690) see table 4-7, CD44 in post healing group (53.411 ± 24.145) no statistical significant with control group but significantly differences with acute group.

4.2.10 Ttransferrin receptor, T9 antigen CD71:

Results of CD71+ in table 4-7 shows that there was highly significant difference in mean among V.L. children and healthy control group ($p < 0.005$). whereas higher percentage was reciprocal in V.L. acute group (56.282 ± 13.632) in comparison with control group percentage (16.283 ± 3.752). A statically significant difference between post healing V.L. group (34.952 ± 15.406) and control group.

Table 4-7: Peripheral Blood activation marker of lymphocyte CD markers cellular expression in study groups.

Cell surface marker expression	Clinical group	mean±SD	ANOVA test
CD 71	Acute	56.282±13.632	0.000**
	Post healing	34.952±15.406	
	Control	16.283±3.752	
CD 44	Acute	26.655±11.788	0.000**
	Post healing	53.411±24.145	
	Control	15.5944±10.690	

**Highly significant difference $p \leq 0.005$.

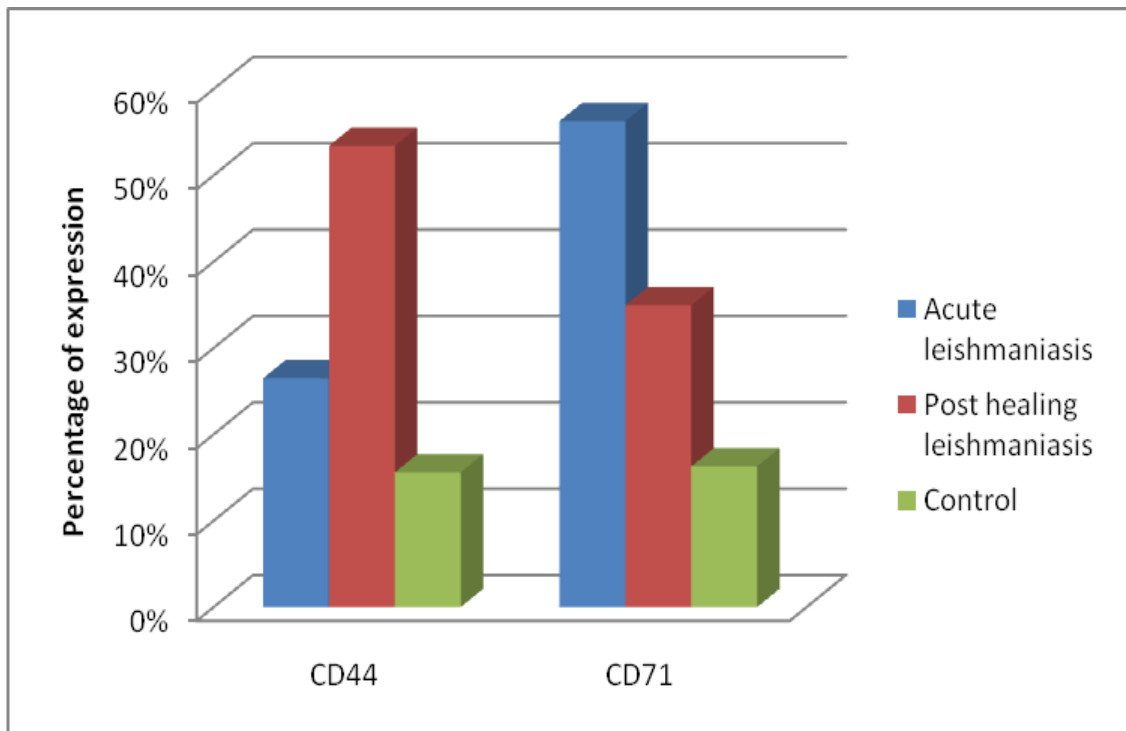


Figure 4-7: means of peripheral blood lymphocytes cellular expression of different activation markers in different study groups.

4.2.11 Leukocyte common antigen (LCA) CD45:

CD45 was detected in two isoforms CD45RA (Naïve lymphocyte) And CD45 RO (memory lymphocyte). According to ANOVA test, there was a highly statistical significant difference ($p \leq 0.005$) in mean of both CD45 RA and CD45 RO expression on PBL among V.L. children and other control group.

CD45RA percentage in the post healing V.L. group was (56.411 ± 22.217), CD45RO (48.941 ± 13.488) by which they significantly differ ($p < 0.005$) in comparison with control group CD45RA (18.500 ± 3.884) and CD45RO (8.200 ± 3.252), table 4-8.

Table 4-8: Peripheral Blood CD markers cellular expression of CD45 isoforms in study groups.

Cell surface marker expression	Clinical group	mean $\pm$ SD	ANOVA test
CD 45 RO Memory	Acute	49.780 $\pm$ 11.676	0.000**
	Post healing	48.941 $\pm$ 13.488	
	Control	8.200 $\pm$ 3.252	
CD 45 RA Naive	Acute	47.330 $\pm$ 13.789	0.000**
	Post healing	56.411 $\pm$ 22.217	
	Control	18.500 $\pm$ 3.884	

**Highly significant difference $p \leq 0.005$.

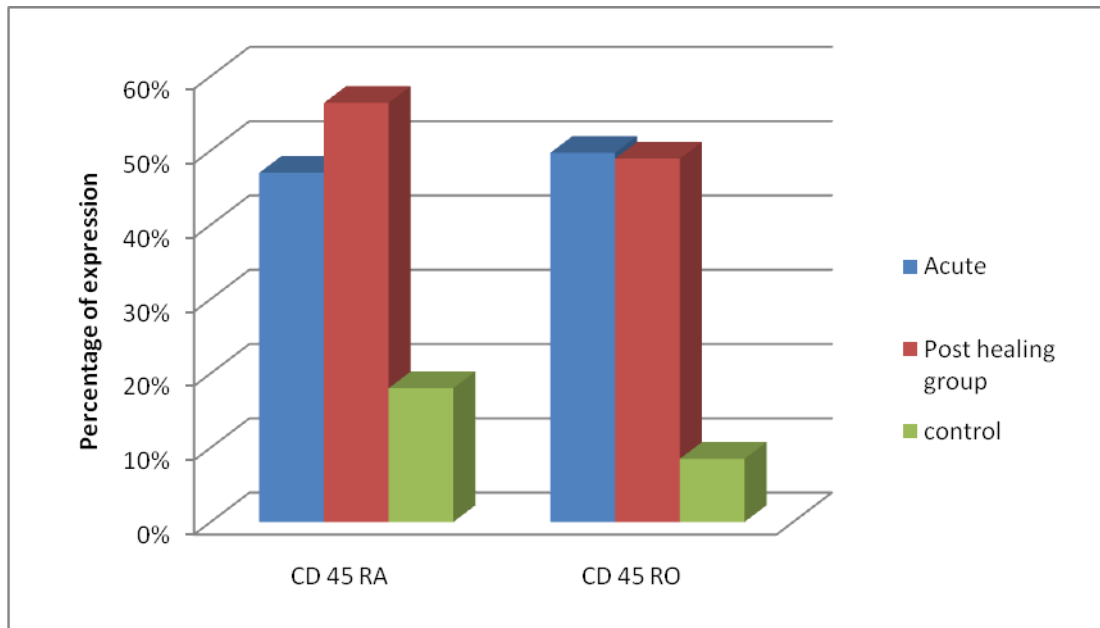


Figure 4-8: mean of peripheral blood cellular expression of naive (CD45 RA) and memory (CD45 RO) cells in different study groups.

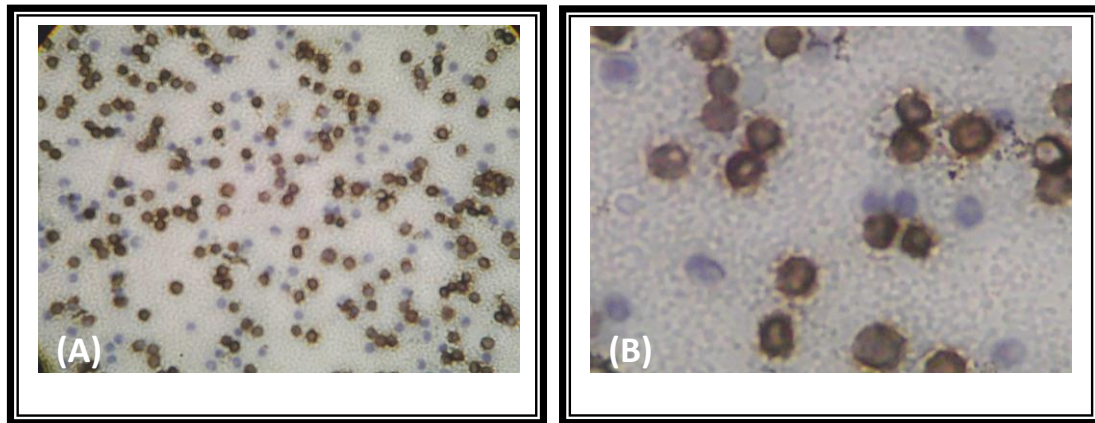


Figure4-9: Immunocytochemical staining of PBL from leishmania infected patients stained with specific mAb, visualized by peroxidase/DAB (brown) and counter stained with Meyer's hematoxylin.

A: Low power magnifications of 400X. B: High power magnifications of 1000X.

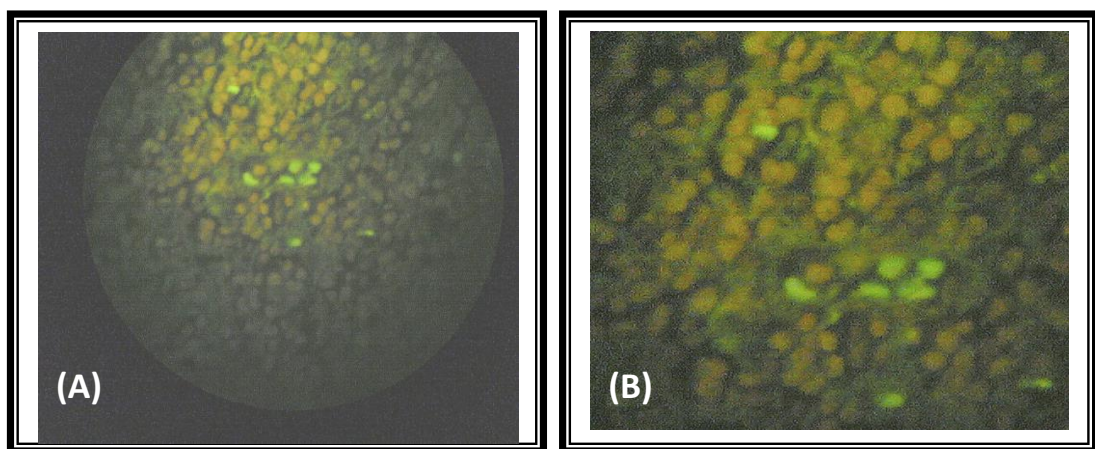


Figure 4-10: Direct Dual Immunofluorescence staining of PBL from leishmania infected patients stained with FITC- murine labeled anti-CD8 and RP- murine labelled anti-CD4.

A: Low power magnifications of 400X. B: High power magnifications of 1000X.

4.3 Serum cytokines level:

4.3.1 IFN-gamma level:

A significant increase ($p \leq 0.005$) of serum IFN- δ was recorded in acute group (5.167 ± 2.538 pg/ml) see (table 4-9), and significant differences ($p \leq 0.005$) between post healing (2.847 ± 2.189 pg/ml) and control group. Also we recorded a significant difference between acute and post healing, figure 4-11.

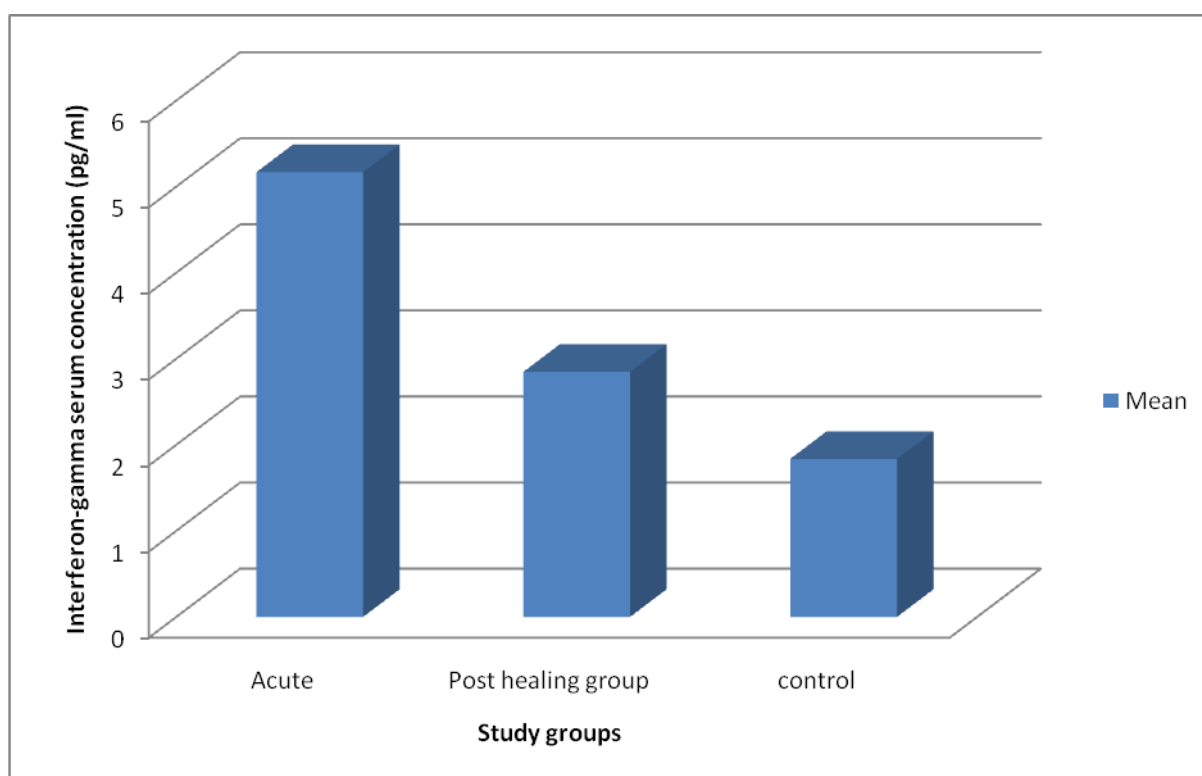


Figure 4-11: mean of serum interferon gamma in different study groups.

4.3.2 TNF- α

It is obvious that there was an increase in TNF- α level in the sera of patient with V.L. (328.939 ± 157.78 pg/ml) in comparison with control group (66.044 ± 23.564 pg/ml). There was no important differences between post healing and control. As shown in table 4-9 and figure 4-12.

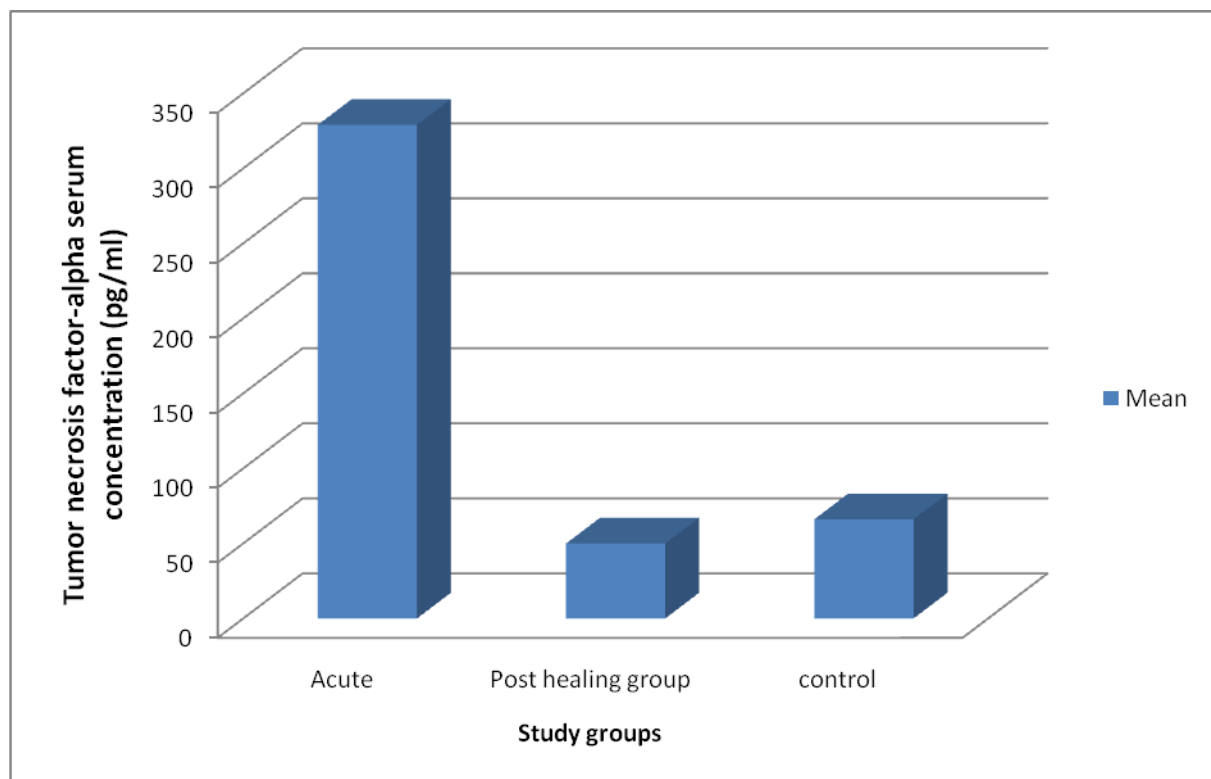


Figure 4-12: mean serum tumor necrosis factor alpha in different study groups.

4.3.3 IL-4

There was a significant increase in IL-4 level in the sera of children patient with V.L. (650.9 ± 263.35 pg/ml) table 4-9 and figure 4-13. The post healing group recorded a less significant ($p \leq 0.005$) value than acute patient group (133.50 ± 101.11 pg/ml) and that of the control (279.2 ± 249.12 pg/ml).

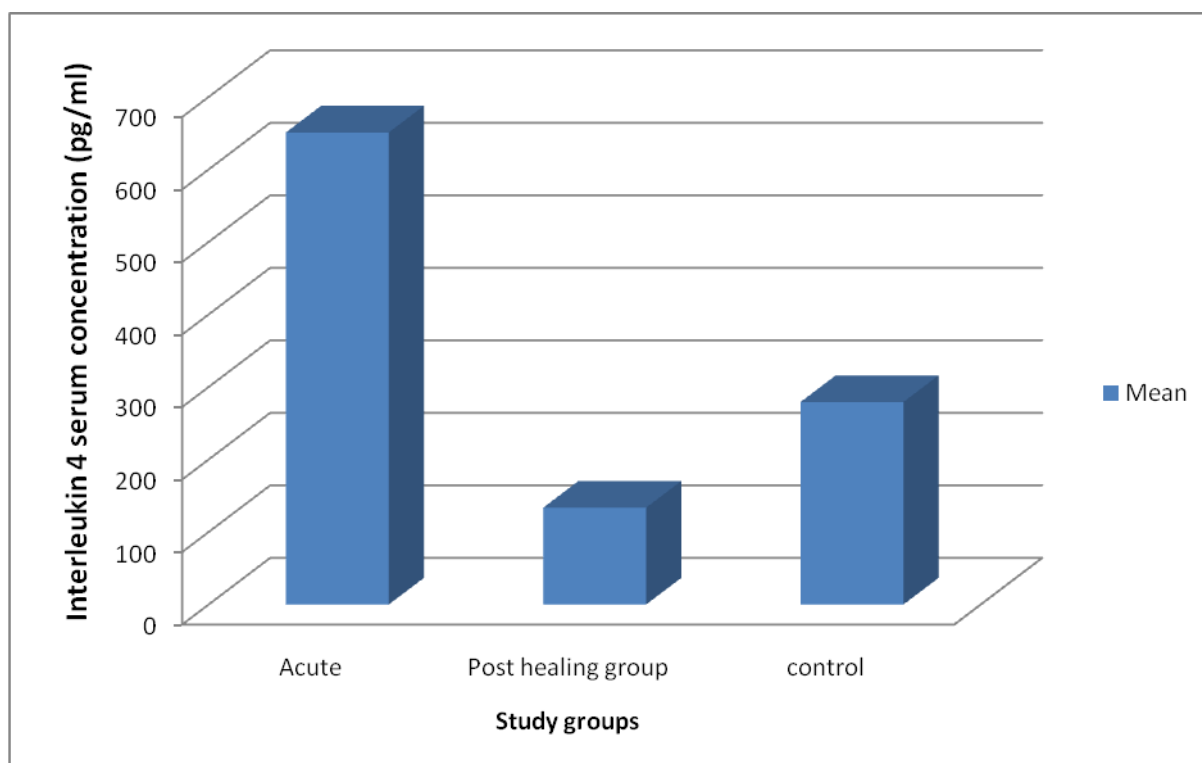


Figure 4-13: mean serum interleukin-4 in different study groups.

4.3.4 IL-10:

Table 4-9 and figure 4-14, shows that there was highly statistical differences between acute group (286.459 ± 155.81 pg/ml) and control group (56.949 ± 24.081 pg/ml) $p < 0.005$, and there was no statistical significant differences between post healing and control group.

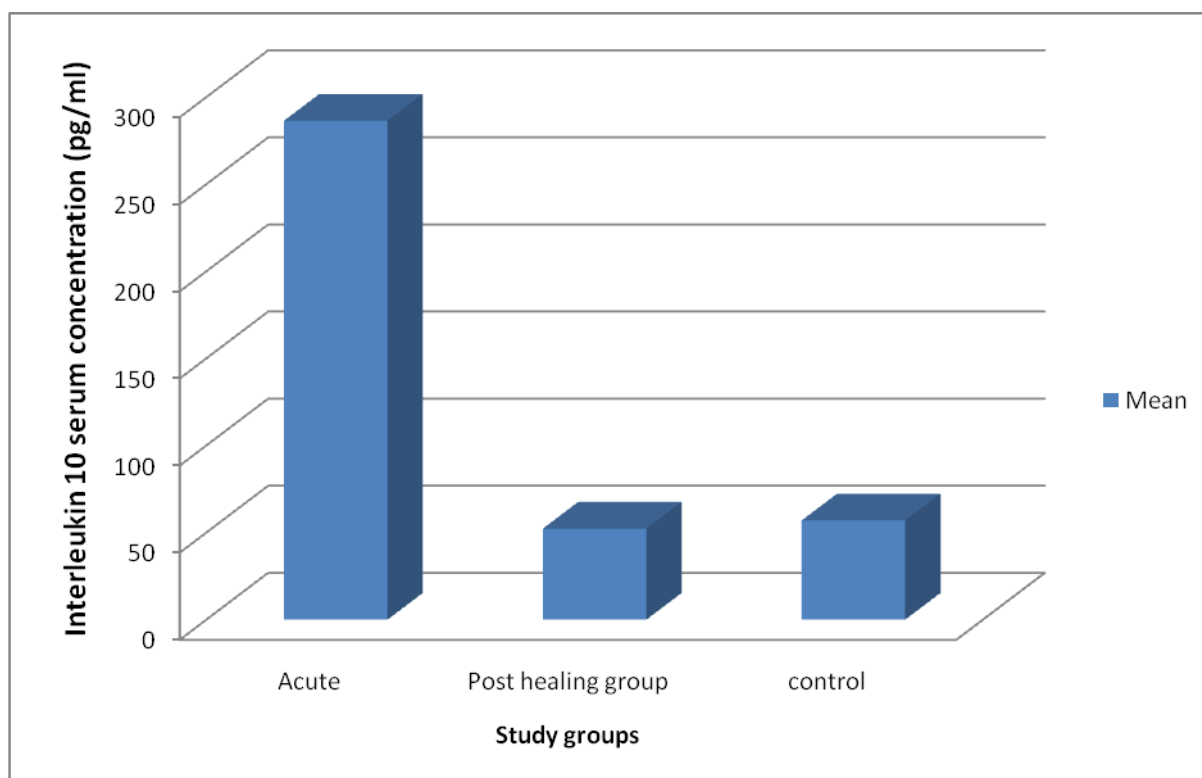


Figure 4-14: mean serum interleukin-10 in different study groups.

Table 4-9: Serum Cytokines levels in study groups.

Cytokines type	Clinical group	Mean $\pm$ SD (μ g/ml)	ANOVA
Interferon gamma	Acute	5.167 $\pm$ 2.538	0.000**
	Post healing	2.847 $\pm$ 2.189	
	Control	1.836 $\pm$ 0.741	
Tumor necrosis factor alpha	Acute	328.939 $\pm$ 157.78	0.000**
	Post healing	49.910 $\pm$ 32.032	
	Control	66.044 $\pm$ 23.564	
Interleukin 10	Acute	286.459 $\pm$ 155.81	0.000**
	Post healing	52.255 $\pm$ 21.511	
	Control	56.949 $\pm$ 24.081	
Interleukin 4	Acute	650.948 $\pm$ 263.35	0.000**
	Post healing	133.496 $\pm$ 101.11	
	Control	279.209 $\pm$ 249.12	

**Highly significant difference $p \leq 0.005$.

4.4 Immunoglobulin levels

4.4.1 Serum level of IgG:

The mean IgG level in patients with V.L. cases (6402 ± 2951 Mg/L) was significantly higher than mean value of healthy control (3719 ± 1596 Mg/L) ($p \leq 0.005$), while the mean level in post healing patient was (4941 ± 1901 Mg/L) and the value was higher than control as shown in table (4-10).

4.4.2-Serum level of IgM:

The mean level of IgM was (1369 ± 669 mg/l) was higher in patients with V.L. than control group (477 ± 184 Mg/l) ($p \leq 0.005$). Post healing mean value level of IgM (542 ± 210 mg/l) was significantly less than in V.L. patient and post healing group, table 4-10.

4.4.3 – Serum level of IgA:

The mean value of IgA (522 ± 286 Mg/l) in acute group shows that there is no significant differences between all groups, as shown in table 4-5 and figure 4-15.

Table 4-10: Mean of Immunoglobulin Isotypes (IgA , IgG and IgM)in study groups.

Immunoglobulin isotype	Clinical group	Mean $\pm$ SD (mg/l)	ANOVA
IgG	Acute	6402 ± 2951	0.001
	Post healing	4941 ± 1901	
	Control	3719 ± 1596	
IgM	Acute	1369 ± 669	0.000
	Post healing	542 ± 210	
	Control	477 ± 184	
IgA	Acute	522 ± 286	0.902NS*
	Post healing	553 ± 179	
	Control	531 ± 246	

**Highly significant difference $p \leq 0.005$.

*No significant difference $p \geq 0.05$.

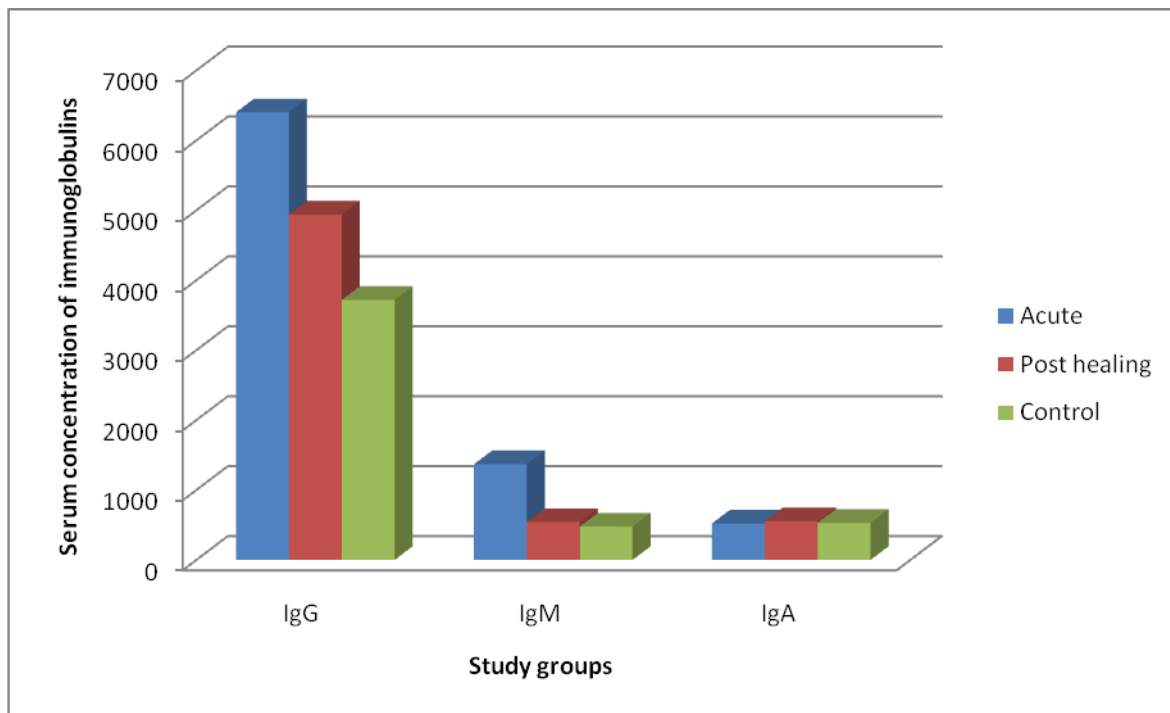


Figure 4-15: means serum concentration of different immunoglobulins in different study groups.

4.5 Complement component level:

4.5.1 Serum level of C3 complement:

The mean value of C3 complement was (995±512 mg/l) in patients with V.L. which was higher than control group (234.94±93.63 mg/l) ($p \leq 0.005$), and there was no significant differences between post healing and control group ($p \geq 0.005$) as shown in table 4-11.

4.5.2 Serum level of C4 complement:

The mean value of C4 was (417±49 mg/l) higher in children with V.L. than control group (94.05±64.07 mg/L) were ($p \leq 0.005$) and there was no significant differences between post healing and control group ($p > 0.005$) see table 4-11 and figure 4-16.

Table 4-11: Mean of Coplement (C3&C4)in study groups.

Complement type	Clinical group	Mean ±SD	ANOVA
C3	Acute	995±512	0.000
	Post healing	231.17±99.46	
	Control	234.94±93.63	
C4	Acute	417.49±215.33	0.000
	Post healing	69.7±23.57	
	Control	94.05±64.07	

****Highly significant difference $p \leq 0.005$.**

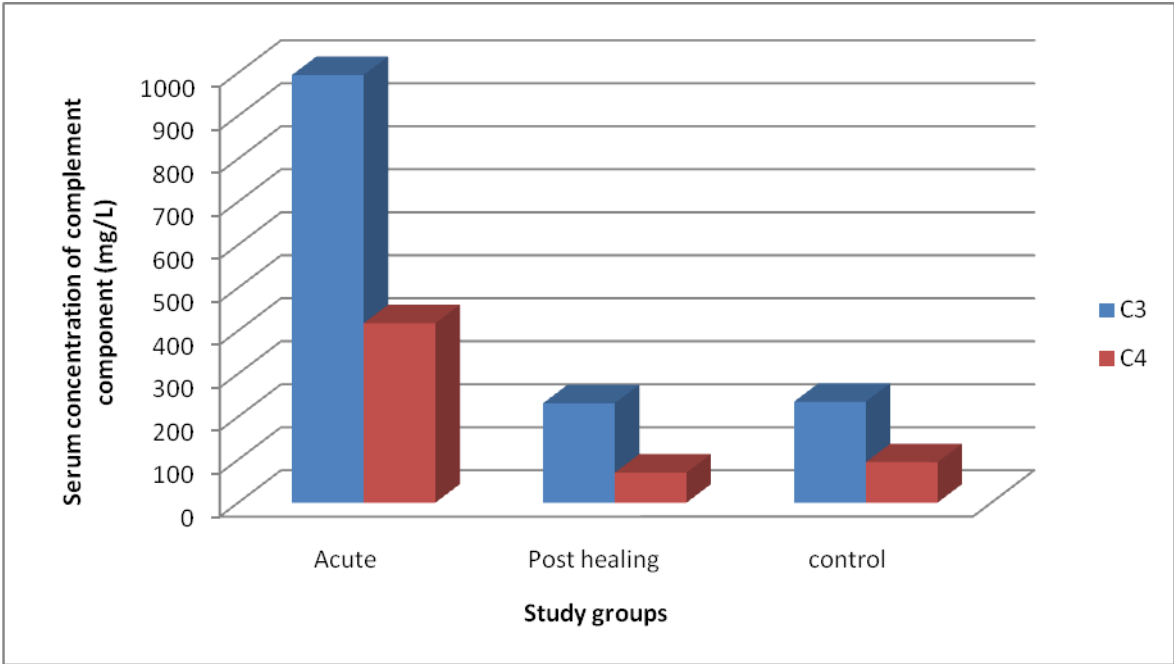


Figure 4-16: means serum concentration of C3 and C4 complement components.

Discussion

Our study characterized T cell and B cell responses in Kala-azar has been confined to peripheral blood from which an adequate number of lymphocytes can be routinely obtained. We studied serum cytokines levels and mononuclear cell subpopulation in the peripheral blood of patient with V.L. before and after antimony therapy were analysed. The percentage of activated monocyte, T cells and possibly B cells; and serum level of TNF- α , IFN- γ , IL-4 and IL-10 were studied.

5.1- Demographic and clinical discussion:

In the present study we studied 60 V.L. patients during active state (40 patients) and different time periods after treatment post healing group (20 patients) evaluating their cytokines and cells population proportion from their surface expression as in shown table (4-1).

5.1.1 Age

The age group under 1 year represents 35% of all patients enrolled and patients with age 1-2 years represent 50%, while 2-3 years patients represent 10% and group of patients up to 5 years represent 5%. So, the highest of V.L. was with age group 1-2 years old, age depending on infecting species and geographic area (Stark *et al.*, 2006).

5.1.2. Gender

In our study the male to female ratio is approximately 1:1(29:31), this result is in disagreement with (Stark *et al.*, 2006) who find that males have an increased incidence of infection as the proportion male to female have been reported around 2/1 this may be attributed to that majority of our studied samples ranged from 1-5 years and both sexes have the same exposure to the sand fly; while the Stark studied samples were older (16-25) years old and males were more exposed to sand fly bites as well as our specimen is small in comparison with Stark sample.

5.1.3 Clinical Features:

After an initial multiplication in the skin and causing a transient small lesion, the parasite can further multiply in bone marrow, liver, spleen and intestine (Stark *et al.*, 2006).

In our study, all the patients were presented with clinical features involving hepatosplenomegaly (liver size = 2-12cm) while spleen around (3-7cm) increasing in size, fever, and low blood cell count 5190 cell/ml with significant decrease in Hb% concentration of V.L. patient, while in post healing group, only the hepatosplenomegaly and gut enlargement due to ascetic in intestine lymphoid tissue. The infected organs began to get smaller after two weeks-12 months to return to normal size (Stark *et al.*, 2006).

5.2 Phenotypic characteristic of leukocytes in all groups

The functional activation and subpopulation of leukocytes were analysed using different surface expressed molecules:

5.2.1 Peripheral Blood Lymphocytes:

In our study, total T-lymphocyte CD3 reduction in patients with V.L. was observed in comparison with control. This could be associated with reduction of T-lymphocyte proliferation. The same results reported by (Ho *et al.*, 1983) who noted that the proliferative response of Kenyan patients with V.L. were more severely depressed to heterogenous as well as leishmania antigen was induced. It is well documented that V.L. is characterised by suppression of CMI, which is proved from the unresponsiveness of the patient to the leishmanian skin test (LST) or montingro test (Saha *et al.*, 2006) suppression may be mediated by microphage either by defective antigen processing and presentation or by elaboration of suppressive mediators like IL10 TGF γ and prostaglandin E2 (Saha *et al.*, 1995).

5.2.2 Percentage of T helper versus T cytotoxic cells

Resistance to infection with leishmania requires the stimulation of Th1-immune response characterized by the activation and proliferation of T lymphocytes capable of producing IL2, IFN γ , TNF- α and other cytokines which in turn induce host macrophages to produce reactive nitrogen intermediates that are critical for the killing of the intracellular parasites.

Using dual stain technique (FITC-CD8 and RPE-CD4) we found that elevated proportion of CD8⁺ cell and CD4⁺ in acute V.L., and production of high level of IFN- γ in those patients. This suggests a role of CD8⁺ cells in human resistance to V.L. and this result is in agreement with (Mary *et al.*, 1999), Yasmine *et al.*, (2002) gained a results in favor of role for CD8⁺ T cell in the control of primary infection.

In our results, the percentage of CD4⁺ cells has slightly increased (36.35%) in V.L. patients group than the control (12.28%), an earlier study demonstrated higher number of CD4⁺ clones than CD8⁺ clones in the a symptomatic individuals delayed hypersensitive positive individuals, although, clones isolated from active V.L. were mostly CD8⁺ (Saha *et al.*, 2006) another study showed that leishmaniasis patient and normal subjects have similar numbers of CD4⁺ and CD8⁺ T cell in PBMC (Maria Da –Cruz *et al.*, 2002) this can be explained because the patients with V.L. have low frequencies of *Leishmania* responder T-lymphocytes in the Blood (Coutinho *et al.*, 1984).

The current studies revealed that there was a significant increase of CD8⁺ T cell in V.L. acute patient in comparison with control, These results were in agreement with results of (Colmenares *et al.*, 2003) who confirmed the increase of CD8⁺ cell count was normal in acute and

uniformly low in chronic cases. This may be explained by the presence of IL-2 from macrophage and Th1 which increase CD8⁺ division. The role of CD8⁺ T cell against Leishmania infection was suggested by Stenger and Modlin (1998) who reported that CD8⁺ T cells were shown to be responsible for the conversion of susceptible BAIB/C mice into resistant phenotype after depletion of CD4⁺ T cells against L-major infection. CD8⁺ T-Cells isolated from the asymptomatic subjects produce high amounts of IFN- γ which strongly suggest a role of CD8 cells in human resistance to leishmania infection (Saha *et al.*, 2006).

5.3 Peripheral blood B lymphocyte:

5.3.1 CD19: In the current study CD19⁺ cells in V.L. (27.94%) were significantly increased than the control group (9.81) while, CD19⁺ in post healing group was (24.45%) were statistically decreased in comparison with control. We must refer to two important points first: it has been reported that intracellular amastigotes of Leishmania are able to actively destroy newly synthesized MHC-II molecules (Bogdan *et al.*, 1996), and second point: the leishmania have special surface glycoprotein called gp63, which converts (C3b into i C3b). This is favorable from the microorganism stand point is that because this conversion would favor phagocytosis clearance rather than lytic clearance (Matthews *et al.*, 2000). The conversion from Th1 into Th2 which involvement in major sick response an humoral immune response that mediated by B cells which will express both CD19⁺ and B220, indicating that the cells were exclusively B cells (Harris-David *et al.*, 2000). This study was confirmed by another study showing high percentage of B cells in acute group than control.

5.3.2. B cell expression CD22:

CD22 has been previously characterized as an activation marker of mature B-lymphocytes, is required to modulate the antigen receptor signal in response to cues from the local microenvironment (Walker *et al*., 2007) in the current study, CD22 (B-lymphocytes) showed a significant increase in differences in comparison to the control group. The increase in number of B-cells may result from polyclonal activation of these cells (Amit *et al*, 2004). The antibody role of B cell against *Leishmania donovani* infection was noted by (Smelt *et al*, 2000) who reported that B cells deficient mice cleared parasite more rapidly from livers and infection failed to be established in the spleen.

5.4 CD1a antigen:

In the present study, the percentage of these cells bearing CD1a antigen significantly decreases in V.L. patients (42.41 ± 11.72) in comparison with control group (38.98 ± 11.28), that's in line with post healing group (45.17 ± 16.29) ($p > 0.05$). The CD1a antigen is a MHC class I-like molecule which is used for the presentation of lipid antigens typically expressed on immature dendritic cells (DC) and langerhans cell in the blood but present in all organs (Burmester *et al.*, 2003), therefore, this basic information confirms our results. The function of the CD1a was involved in presentation of endogenous and bacterial lipid antigen to T-cells may also mediate thymic T-cell development where recognition of lipid containing Ags rather than peptides it has been proposed that this subset participate in host defence, therefore, these subset cell percentage is low (Leslie *et al.*, 2002).

5.5 Monocytes CD14:

The present finding reported a highly significant elevation in the percentage of CD14+ cells of V.L. patients group (60.84 ± 16.65) in comparison with control group (25.33 ± 12.46), we also found that

significant increase in post healing group in comparison with control as we show in table 4-6. Our study confirmed by Nylen, (2007) who reported the expression of CD14 was elevated in patients before treatment. CD14 expression by mature monocytes and macrophage were represented 60% PBMC (IeVan *et al.*, 2001). CD14 is receptor for complex of lipopolysacchride (LPS) which is a powerful mitogen, which leads to increased monocyte cell percentage and inflammatory mediators.

5.6 CD44 molecule expression

The result of this study revealed that there was relatively an increase in the percentage of CD44+ cells(Marginal significant differences) in comparsion to the control group, also in post healing group. This result is in agreement with that gained by (Martina *et al.*, 2004), cell expression of CD44 showed high increasing in V.L. patient than in asymptomatic patient.

When the skin (epiderm) exposed to bite by sand fly that is considered as a switch for CD44 activation were it is important in epithelial cell adhesion (Cell-Cell and Cell matrix) to hyaluronate in basement membrane and maintaining polar orientation of cells. This process may contribute to tissue remodeling and may stimulate cell proliferation and migration (Aruffo *et al.*, 1990).

5.7 Leukocyte common Antigen (LCA) CD45

CD45,the leukocyte common antigen is a haemopoitic cell specific tyrosine phosphate, many isoforms are gene related by alteration splicing of extracellular domains, importantly, human polymorephic variants that alters CD45 isoform expression are associated with autoimmune and infection diseases, establishing CD45 as important immune modulator with significant influence on disease burden (Tchilian and Bevera,2006).

The our results demonstrated significant perturbation of T-cell dynamics in V.L. patient who had fewer naïve T cell (CD45RA) than

control ($p < 0.005$) and higher memory T (CD45RO) cells than control $p < 0.005$. These data are readily explained by T cell proliferation and phenotypic differentiation, The increase in number of peripheral blood T cells with various phenotypic alteration including increase of CD45RO (memory phenotype) and reduced expression of CD45RA (Naïve phenotype). We observed remarkable differences in PML where the percentages of CD44⁺ CD45RO⁺ cells were shown in V.L. patient children also in post healing group, tended to central memory not to activation effector memory CD44^{hi} CD45^{hi} Ro were in control Naïve memory CD44^{low} CD45^{hi} Ro The increased differentiative step that govern conversion of effector cell to memory cells. However building on previous work done by (Kanegane and Tosato, 1996) and Ku *et al*, (2000) whom recorded that IL-15 is critical for memory cell differentiation and survival. Furthermore, IL-15 play a key role for supporting the basal homeostatic turn over of population (i.e the replacement proliferation of memory cells which maintain the total number of antigen specific memory cells (Lodoice *et al.*, 1998), or due to sustained exposure to antigen peptide (MHC complex order to persist (Carter, 1998).

5.8 Peripheral blood lymphocyte expression of early activation antigen CD71.

The transferrin receptor (CD71) is a member glycoprotein whose clearly defined function is to mediated cellular uptake of iron from plasma glycoprotein, transferring iron uptake from transferrin receptor, internalizing as endocytic recycle by receptor-mediated endocytosis and the release of iron from the protein due to decrease in endosomal pH (ponka and lok, 1999). In addition to be an Iron therefore, it has been receptor engagement, and that's why called early activation antigen (Salmeron *et al.*, 1995).

Our results showed a markedly increase in PBL expression CD 71 (mean 6.28%) in acute group with highly statistical differences compared with control group (16.28%) suggesting a state of early highly PBL activation state that could be occur specifically in V.L. patient. This result may give inclination that CD71 expression was up regulated in earlier stages of disease activity.

5.9 HLA-DR:

In our study we found statistically significant increase in the percentage of HLA-DR cells in the V.L. patients (71.44%) in comparison with control group (23.71%) also we found significant difference between post healing (47.73%) and control, on the other hand between acute V.L. group and post healing group. It has been suggested that one of the important immunoregulatory abnormalities in V.L. patients is related to HLA-DR+ cells and activation of lymphocytes by different stimuli increase their expression of surface markers (Goldsby *et al.*, 2000).

Our study confirmed by (Handman, 2001) who reported a supporting role for the MHC in resistance to a variety of infectious diseases including leprosy, schistosomiasis, malaria and leishmaniasis.

From all the above, we think that HLA-DR may take an important role in activation B-cell and CD4+ T-cell which leads to an activation of immune system response and induce the proliferation and secretion of various cytokines, TNF-alpha released by the antigen presenting cells plays an important role in this process. It leads to the production of oxygen radicals that are able to kill intracellular parasite. Activated T-cell can stimulate antibody production in B-cells. Both mechanisms are directed at the elimination of extracellular pathogens (Burmester *et al.*, 2003).

5.10 Cytokine profile:

The profile of T cell cytokine synthesis plays an important role in regulating the immune responses and determining the resistance or susceptibility to disease. In an experiment model of leishmaniasis the outcome of infection depend upon the activation of one of the two subsets of CD4⁺ cells Th1 and Th2. Resistance is associated with Th1-type response and susceptibility is associated with TH2 type response (Saha *et al.*, 2006).

In genetically predispose murine models of L. major infection, a clear Th1 / Th2 polarization with distinct pattern of cytokine production by CD4 T cell i.e. IFN- γ /IL-2 associated with resistance and IL-4/IL-10 associated with susceptibility was determined (Heinze *et al.*, 1989).

5.10.1 Interferon gamma

In this study, the result shows a highly significant increase serum level of IFN-gamma in patients (5.17pg/ml) in comparison with control. It is well known that IFN- γ induces activation at macrophage in (H₂O₂) depended (Murray *et al.*, 1983) as well as in (NO) mediated pathway (Vouldoukis *et al.*, 1997). Therefore a high parasite burden is favoured in active V.L. patients can be explained by the deficiency of IFN- γ during disease. These studies confirmed our results. Another study mentioned that the amount of IFN- γ raise to reach 200 pg/ml in V.L. patient during treatment (Nylen *et al.*, 2007). Whereas, another study referred to serum level of IL-4 it was found to be high in the Brazilian V.L. patient to no or very low level of IFN- γ (Saha *et al.*, 2006). Considerable level of IFN- γ is secreted at very initial stage of the exposure to the parasite as observed in the seroconverted or subclinically infected individuals in the endemic area (Bacellar *et al.*, 1991).

High levels of IFN- γ had been detected in serum are necessary in the maintenance balance between Th1 and Th2 responses. These results matched the results of previous researchers, (Gomes & Dos, 1998) who was found a mixed Th1/Th2 response of parasite specific T cells from both acute and chronic murine V.L..

5.10.2 Interleukin-10

Our results showed an increase IL-10 in acute group (286.64pg/ml) than the control group (56.65pg/ml) and there was no statistical difference between post healing and control group. These results confined to the results showed By (Margaret and David 2001) who stated that V.L. patients have an increased expression of IL – 10 mRNA an important strategy for down-regulating T-Cell response.

IL-10 was initially characterized as a Th2 cytokine but later on it was proved to be a pleiotropic cytokine, secreted from different cell types including the macrophage. Experimental evidence indicates that IL-10 may an important regulatory role in the progression of V.L.. The suppressive ability of IL-10 on the IFN- γ mediated microbicidal activity of macrophage is well established for other disease (Oswald *et al.*, 1992). These observations clearly indicates that the suppression of the leishmania specific Th1 kind of response in the patient is the main reason for the disease susceptibility and this suppression is regulated by IL-10 and IL-10 level is declined after successful chemotherapy (Galdas *et al.*, 2005).

In Fact in human severity of V.L. has been closely associated with increased levels of IL-10 and the use of anti-IL-10 antibody to block the IL-10 activity or IL-10 receptor blockade can be effective approach for the treatment of V.L. (Murray *et al.*, 2002).

5.10.3 Interleukin-4:

Concerning the results of serum level of 1L-4, it was found to be high in Acute group (V.L.) in comparison with control, Which leads that active V.L. is associated with elevated plasma levels of 1L-4 (Nylen *et al.*,2007). 1L-4 is considered to the signature cytokine of Th2 response. Althoughs it is known to correlate with disease in murine CL but not murine V.L., (Wilsonme *et al.*,2005), 1L-4 is reported to be present in the sera PBMC supernatant or as m RNA in Human V.L. (Sundar *et al.*, 1997b; Kenney *et al.*,1998), but there are reports stated that 1L-4 is not always produced in V.L. patients and that it has no Immunomodulatory effect in downregulating the Th1 response during disease (Bacellaro *et al.*,2000), these inconsistent detections of 1L-4 in V.L. patient might be due to the fact that there are disease specific soluble 1L-4 receptors in the serum of V.L.-patients ,Which can neutralize both the bioactivity and Immunologic detection of this cytokine (Sang *et al.* ,1999).

5.10.4 Tumor necrosis factor-alpha:

Th1-type product, including 1L-12 and it induced IFN- γ acting in concert with TNF- α (Henry *et al.*,2005) Circulating levels of TNF- α while it is mainly secreted by the activated macrophages were detected with active V.L. and during disease. It is interesting to consider that the highest levels of TNF- α were detected simultaneously with the highest levels of 1L-10, known in baluster of TNF- α secretion .we found that circulating levels of TNF- α decline as patients improved in experimental V.L., TNF- α had been shown to be a critical factor in disease control .In Human V.L. levels of TNF- α had been related to activity of disease (Salom *et al.*,1996). The discovery of elevated TNF levels in the sera of parasites disease suggest that this cylokine may play a part in host defence against parasitic infection (Scuderi *et al.*,2006).

5.11. Humoral Immune response:

5.11.1. Immunoglobulin levels:

Kala-azar- is also characterized by strong antibody titers. Cytokine elaborated by activated T cell induce the switching of B- lymphocyte to several IgG isotype and are thus obligatory for some humoral responses.

We found the mean of IgG level in patients with V.L. cases (1025.12mg/l) was producing plasma cells significantly higher than mean value of control (2500 mg/l) and post healing group (4300mg/l) than control.

The increased level of IgG can be explained to increase specific IgG resulted from infection together with the normal IgG in patient sera.

IFN- γ the th1 cytokine upregulates the Isotypes IgG2 and IgG3 in mice and probably IgG1 and IgG3 in humans. 1L-4 and 1L-5 Th2 cytokine stimulate the production of high levels of IgM, IgE and IgG iso types such as IgG1 in mice and IgG4 in humans (Abbas *et al.*, 1996). Analysis of leishmania antigens – specific Ig Isotype and IgG subclass in V.L. patient sera revealed elevated levels of IgG, IgM, IgA, IgE and IgG4. A marked elevation of IgG1 antibodies was observed in all these patients (Anam *et al.*,1999). All the above studies confirmed our finding. The high levels of antibodies in post heading could explain by the study of Hailu (1990) who reported that the increase levels of anti leishmanial antibodies may be present for a long time after treatment. IgA values not affected before and after therapy this agreed with Al-Orainey *et al.*, (1994).

5.11-2 Complements Level:

The mean level of C3 was significantly increased in patients (1160.1mg/l) comparison with that observed in control group (233.4mg/l). This result is in agreement with (Dominguez and Torano,

1999) who reported that infection with V.L. Stimulates production of C3 after the first weeks of infection. in contrast, Handman (2001) reported that the C3 level was reduced as a result to consumption by promastigote when enter the macrophage surface through the C3 receptor and binds to the surface molecules (LPG & GP63) of promastigote. Let us to explain how it occurs? The complement protein C3 b is one of the most potent immune opsonins. C3b binds to foreign material and promotes its uptake, via C3b receptors on phagocytic cell as expected, C3b binds to the leishmania protozoa. These results in uptakes by the macrophage. The leishmania have a special (Surface glycoprotein, called gp63 which converts C3b into IC3b. this favorable from the microorganism stand point because, this conversion would favor phagocytic clearance rather than lytic clearance. As stated earlier. leishmania are very resistant to deyradation once phagocytosed there for this conversion makes sense in terms of leishmanias survival. Another interesting point in is that IC3b may also promot phagocytosis by B cells. Because opsonin –mediated phagoytosis is non antigen specific phenomenon, it would result in humaral response against irrelevant epitopes as is explained later, is infective against leshmania (Saha *et al.*, 2006) our result disagreement with Ghosh *et al*, (2003) results who explained the decreased level of C3 than that observed in control. The difference between results may be due to gentic factors, some clinical parameters such as sex, age and parasites strains.

The mean serum level of the C4 was significantly increased in the infected group compared to that of the control group, this was related to activation classical or alternative pathway in V.L. patients (Paredes *et al.*,1997).In addition there may another cause of C4 elevation which could be due to circulating immune complex which were formed

as results of leishmania infection leading to stimulate the classical pathway depending on the parasite species (Ravanbod, 2000), another reason, the cytokines the 1L-4 and 1L-5 are recognised as helper for B-lympocyte and stimulate the production of high levels of IgE, IgM and non complement C4- Fixing IgG4 in human (Abbas *et al.*,1996) our results are not agreed with (Ague *et al.*,1981; Dominguez and Torano .1999) who reported that decreased level of C4 may be due to the activation of alternative.

Conclusions and recommendations:

6.1. Conclusions:

1. There is an increased expression of activation markers on the surface of PBLs of V.L. patients such as CD19, CD44, CD45RA, CD45RO, CD71 and HLA-DR antigens and phenotypic markers CD4, CD8 and CD22.
2. Cytokines are important to the outcome of V.L. which indicated by a significant elevation of serum levels of IFN-gamma, TNF-alpha, IL-4 and IL-10 in V.L. patients.
3. The PWBC count demonstrates no significant difference between patients and controls in response to V.L.. Furthermore, PWBC count in V.L. patients is slightly less than control and post healing group.
4. There is a significant increase in patient serum levels of total IgG, IgM has been reported in V.L. patients but there was no change in IgA level in infected children before and after therapy.
5. The third and fourth complement component (C3 and C4) levels were more elevated in V.L. patients sera and reduced after therapy.

6.2 Recommendations:

1. Re-evaluation of the activation markers and serum cytokines are in larger groups of patients and, if possible, with using other activation markers and cytokines.
2. Advanced molecular techniques like polymerase chain reaction can be used to identify the leishmania infection.
3. It is possible to use IFN- γ as therapeutic agents in V.L..
4. Studying the CMI characterisation with group who was cured from infection with Leishmania and began resistance to new infection. On the other hand, assessment of other parameters of CMI in those patients who have cured from infection, that may be involved in prevention of reinfection by the parasite.

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الخلاصة

تسبب امراض اللشمانيا انواعا من الطفيليات الوحيدة الخلية التابعة الى جنس اللشمانيا *Leishmania* . وان مرض اللشمانيا الاحشائية (V.L.) هو احد امراض هذا الجنس حيث يهاجم الطفيلي المسبب له الاعضاء الداخلية لجسم الانسان كالکبد والطحال والامعاء.

ان المعلومات التي تخص هذا النوع من الامراض وخاصة المتعلقة بالاستجابة المناعية في الانسان لا تزال قليلة وخاصة في العراق ،ولذلك فأن الهدف الرئيسي لهذه الدراسة هو التعرف على الاستجابة المناعية الخلوية (C.M.I) في المرضى المصابين طبيعياً بهذا المرض في مستشفى الديوانية للاطفال للفترة من تشرين الاول 2006 ولغاية كانون الاول 2007 .

شملت هذه الدراسة ستون مصابا بالمرض وبأعمار مختلفة اربعون منهم بالطور الحاد من الاصابة و عشرون في طور الشفاء كما تم اختيار عشرون طفلا بصورة عشوائية من الاصحاء لغرض المقارنة .

تم تحديد الاظهار الخلوي للمعلومات المناعية (CD marker) لكل من CD4، CD3 ، CD8 ، CD19 وذلك باستخدام طريقة التصبغ المناعي المتألق ، في حين حددت المعلومات المناعية التالية :- CD45RO، CD45RA ، CD1a، CD14، CD22، CD44، CD71 و HLA-DR وذلك باستخدام طريقة التصبغ المناعي الخلوي الانزيمي للخلايا للمفاوية في الدم المحيطي .

في حين تم تحديد الانتروفيرون كاما، العامل المنخر للخلايا السرطانية، الانترولوکين 4 و الانترولوکين 10 في مصل الدم المحيطي للعينات المدروسة باستخدام طريقة التفاعل المناعي المقترن بالانزيم (ELISA) .

ان توزيع اصناف الخلايا للمفاوية قد تم مقارنته بين مجاميع الدراسة المختلفة وقد اظهرت النتائج بان هنالك فرق معنوي في المعلم المناعي CD8 و CD19 وبصورة طفيفة للمعلم المناعي CD 4 بينما لا يوجد فرق معنوي للمعلم المناعي CD3 عند المقارنة مع مجموعة المقارنة.

اوضحت نتائج التصبغ المناعي زيادة وبفرق معنوي للمعلم المناعي CD14 ، CD 22 ، CD44 ، CD71 و HLA-DR بينما أظهر المعلم المناعي 1a انخفاض معنوي في نسبة الاظهار مقارنة بمجموعة السيطرة.

كما اظهرت نتائج قياس المدورات المناعية بان هنالك زيادة وبصورة معنوية لكل من العامل المنخر للخلايا السرطانية، الانترلوكين 4 و الانترلوكين 10 لكن الزيادة كانت طفيفة في الانترفيرون كما لمجموعة الطور الحاد مقارنة بمجموعة السيطرة ونفس الشيء مع مجموعة مابعد الشفاء.

كانت هناك زيادة معنوية في المصل لكل من IgG و IgM بالمرضى المصابين بالطور الحاد للشمانيا الاحشائية مقارنة بمجموعة السيطرة، لكن يوجد هنالك نقصان في IgA في المصل في المرضى المصابين بالطور الحاد للشمانيا الاحشائية. وبينت النتائج زيادة معنوية لكل من C3 و C4 في المصل في المرضى المصابين بالطور الحاد للشمانيا الاحشائية.



خواص الاستجابة المناعية الخلوية في المرضى المصابين باللشمانيا الاحشائية

أطروحة

مقدمة إلى كلية العلوم وهيئة الدراسات العليا في جامعة بابل كجزء من متطلبات
نيل درجة الدكتوراه فلسفة في
علوم الحياة / علم الحيوان

من قبل الطالب

فاهم محمد دغيم الشبلي

بكالوريوس/ماجستير علوم حياة - مناعة طفيليات