ASSESSMENT OF SOME CYTOKINES PRODUCTION (Interleukin- 4, IL-10, IFN – γ) IN MALNOURISHED CHILDREN IN BAGHDAD

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Abstract

This study included the assessment of some humoral and cell mediated immunity factors particularly some cytokines level in relation to nutritional status in both twenty four malnourished Iraqi children and fourteen healthy children as control group with age group between (1-36)months . The serum levels of interleukin -4, IL-10 and IFN γ were measured in both groups by using ELISA method, the results showed that significant decrease in serum level of IFN γ in malnourished children than control (P. value < 0.05) .while significant elevation of IL-4 and IL-10 in all malnourished children as compared to healthy control subjects (P.value < 0.01) .also, the results showed that significant differences in weight and length between patients and control groups by using BMI.

Introduction

Malnutrition in children is a major public health problem in many developing countries about 280 million children younger than five years are reported to be malnourished (1, 2). It is a global problem, having adverse effect on the survival, health performance and progress of population in the developing countries. It may be due to improper or inadequate food intake of essential vitamins and minerals or may results from inadequate absorption of food (3). The constitutional manifestations of nutritional reflect are growth failure, lack of weight gain, edema, abnormal skin pigmentation, chronic diarrhea and recurrent infections (2, 4).

Malnutrition can lead to lower the average of intelligence, stunted, growth and increased susceptibility to disease extreme weight loss, sever cases of malnutrition can lead to death (5 , 6).The main nutrition problems include inadequate intakes of energy ,vitamin and protein, iodine deficiency disorders, iron deficiency anemia (7).

Multiple abnormalities in the immune response, including cell mediated immune responses, secretary IgA antibody production, phagocyte function and complement, NK cell activity, T –cell number, ratio of T –cell subsets and cytokine production have been described in connection with protein energy malnutrition (2).

The characterization of T-cell response are either Th-1 response (dominated by the production of interferon IFN- γ, associated with cell mediated immunity or Th2 –type response(characterized by production of interleukin- 4 and IL-5) participate in humoral immunity is important because it provides a basis of understanding how T–cell contribute to resistance or susceptibility to different infections (15) .Th-1 cells produce IL-2 ,IFN- γ and tumor necrosis factor mediated immunity to viral and bacterial pathogens , whereas Th2 cell production , IL-4 ,IL-5 ,IL-6 ,IL-10 and IL-13 are involved in allergic diseases and in defense against parasite infections (2 ,8).

Moreover, Th1 and Th2 cells amplify and shape of the immune responses that mediated protection of the host during infectious disease (9) .The purpose of this study was to assess the effects of malnutrition on the production of some cytokines IL-4, IL-10 and IFN γ .

Subject, Material AND Methods

***Subject**

Twenty four (24) hospitalized malnourished Iraqi children with malnutrition were the subject of this study. The cases were admitted to the central teaching hospital of pediatrics in Baghdad. They were (8) female and (16) male, their ages ranged between 1-36 months. The diagnosis of malnourished children was based on clinical and physical examination including weight and length .Fourteen healthy children (14) who met the same age and sex were considered as a control group ,this group included (9) male and (5) females ,their ages ranged between 1-36 months . The anthropometric measurements (weight, height/ length) were applied with reference to their weight for ages as percentage of the expected weight according to Gomez classification (10). The weight measurement by the mother were ask to remove all heavy objects from the child and the weight is taken to nearest 100gm in addition, length of children were measured by using tape.

BMI (Body mass index)

The body mass index is used to asses' dietary and environmental factors influencing stunting and other signs of poor group status of children by using anthropometric measurement , weight and height (11), by the
Using the CDC growth chart (United States of BMI to monitor and track patients according to WHO). BMI was calculated by following equation (12).

BMI = weight (kg) / height 2 (meter) 2

Blood sample

Three to five ml (3-5) blood from each child was withdrawn by using disposable syringe. The blood was placed in plastic disposable tube, it was left to clot at room temperature, and then the serum was separated by centrifugation at 3000 rpm for (5-10) min, divided then stored at -20 °C . until used.

Estimation of serum IL-4 and IL-10

ELISA test is intended to be used for quantitative determination of native and recombinant human IL-4 or IL-10 in solution such as cell culture, serum or plasma. Interleukin kit is a solid sandwich enzymes linked immunosorbent assay (ELISA). A monoclonal antibody specific for IL is coated on to wells of micro titer plate. Sample or standard are pipetted in to these wells , followed by addition of biotinylated second antibody (conjugate) during the first incubation , the human IL considered as antigen binds simultaneously capture to the antibody on one site and to the solution phase biotinylated on second site . After removal of excess second antibody, Streptovidin-peroxidase is added; this binds to the biotinylated antibody, after a second incubation and washing to remove the entire unbound enzyme. Substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of IL- present in the original specimens (13).
The procedure of IL-4 and IL-10 determination according to the manufactures as (MABTECHAB AB, COMPANY) and was done briefly as follow:-

Day 1:-

1- One hundred microliter of mAb IL-10was added /well for coated ELISA plate and then plate was incubated overnight at (4-8) °C .

Day 2:-

2- Washed twice with PBS.
3- The plate was blocked by addition of 200 µL / well of incubation buffer (PBS – tween 20 containing 0.1% BSA) , incubated for 1 hour at room temperature, then washed five time with PBS containing 0.05% tween 20.
4- The IL-4 or IL-10 standard was prepared by reconstituting contents of recombination human IL-4 standard (2 mg), this gives a stock solution of 10 mg /ml to the prepare standard concentration were 1000, 100, 10, 1 and 0.1pg/ml.
5- 100 ml of sample or standard was added per each well, incubated for 2 hours at room temperature and then washed the plate five times with PBS containing 0.05% tween 20.
6- 100 ml of mab IL-4 or IL-10 biotin conjugate was added at all wells .incubated for 1 hour at room temperature, and then washed five times with PBS containing 0.05% tween 20.
7- Substrate solution (P-nitrophel-phosphate PNPP 100 ml) was added for each well, and then measured the optical density (O.D.) at 450nm for substrate in an ELISA reader after suitable developing time. The calculation was done depending on standard curve.

Estimation of serum interferon γ level

Principle

ELISA IFN γ method was used for quantitative determination of human interferon in solution such as cell culture supernatant, plasma or serum. The immunoenzymatic assay of IFN is a sandwich type assay with two immunological steps as mention in principle of IL-4 (14) Test procedure for human γ IFN kit according to MABTECH AB, company)As in IL-4 and IL-10.

Stastical analysis

The following statistical data analysis approaches were used in order to analyze and assess the results of the study:

I. Descriptive data analysis including (mean value,standard deviation , 95%confidence interval for population mean value ,graphic presentation and using gluster – bar chart and tables) .

И. Inferential data analysis

There were used to accept or reject the statistical hypotheses, which included the following:

1. Kolmogorov-smirov (Z) for testing the normal distribution function.
2. Binomial test for testing a difference between two category normal scales of dichotomous random variables.
3. Leven test for testing the homogeneity of variance for equality.
4. Variance and ANOVA technique of means (testing of coincidence) with using (L.S.D.)
5. Fisher exact probability –test a difference between two categories nominal scale of dichotomous random variable in the (2X2) association table.
6 .Chi-square-tests for testing a difference between several category nominal scales of dichotomous random variables.

Results:-

Age, sex and body mass index distribution.

In order to study the relationship between the nutritional status and immunity certain immunological parameters were examined in (24) children with malnutrition and (14) control children. Ages of the 14 normal healthy children were ranged between 1-36 months, the results revealed that no significant differences in age distribution between normal and malnourished groups (P>0.05). Table (1, 2). In addition, by using multiple comparisons significant by (L.S.D.) method between the different age groups in the control and study samples showed no statistical differences in different cytokines level in both control and malnourished children by using exact fissure test (f). Table (2).

Of the 14 normal healthy controls, 9 children were male and 5 were female .whereas, the 24 malnourished children included 16 males and 8 females .there were no significant differences in sex distribution between control and malnourished group. Table (3). Weight and length of 24 malnourished children and 14 control healthy children were taken from 1 to 36 months, According to CDC growth charts, United States (of BMI for age percentiles for boys and girls). The results showed that high significant differences at P=0.000 was obtained between the observed frequencies of the BMI in two study groups .see table – 4, Figure (1)

Table (1): Testing the coincidence among different age groups in control and study samples for the studied parameters

Groups	Parameters	S.O.V.	Sum of Squares	F	Sig.
Control	IFN γ	Between Groups	14437.5	0.236	0.869
		Within Groups	203942.9		
		Total	218380.4		
	IL-4	Between Groups	428.548	0.514	0.681
		Within Groups	2776.667		
		Total	3205.214		
	IL-10	Between Groups	2722.619	13.650	0.001
		Within Groups	664.881		
		Total	3387.5		
Patients	IFN γ	Between Groups	1235.778	0.471	0.706
		Within Groups	17508.06		
		Total	18743.83		
	IL-4	Between Groups	2210.069	0.910	0.454
		Within Groups	16188.89		
		Total	18398.96		
	IL-10	Between Groups	324.444	0.954	0.433
		Within Groups	2266.889		
		Total	2591.333		

Table (2): Multiple comparison significant by (L.S.D.) method between the different age groups in the control and study samples

Groups	Dependent Variable	(I) Age (month)	(J) age (month)	Sig.	C.S.
Control	IFN γ	up to 6 m.	up to 12 m.	0.940	NS
			up to 18 m.	0.565	NS
			> 18 m.	0.567	NS
		up to 12 m.	up to 18 m.	0.578	NS
			> 18 m.	0.570	NS
		up to 18 m.	> 18 m.	0.884	NS
	IL-4	up to 6 m.	up to 12 m.	0.281	NS
			up to 18 m.	0.389	NS
			> 18 m.	0.288	NS
		up to 12 m.	up to 18 m.	0.881	NS
			> 18 m.	0.843	NS
		up to 18 m.	> 18 m.	1.000	NS
	IL-10	up to 6 m.	up to 12 m.	0.001	HS
			up to 18 m.	0.000	HS
			> 18 m.	0.009	HS
		up to 12 m.	up to 18 m.	0.148	NS
			> 18 m.	0.025	S
		up to 18 m.	> 18 m.	0.002	HS
Patients	IFN γ	up to 6 m.	up to 12 m.	0.591	NS
			up to 18 m.	0.718	NS
			> 18 m.	0.270	NS
		up to 12 m.	up to 18 m.	0.863	NS
			> 18 m.	0.448	NS
		up to 18 m.	> 18 m.	0.397	NS
	IL-4	up to 6 m.	up to 12 m.	0.523	NS
			up to 18 m.	1.000	NS
			> 18 m.	0.441	NS
		up to 12 m.	up to 18 m.	0.467	NS
			> 18 m.	0.116	NS
		up to 18 m.	> 18 m.	0.394	NS
	IL-10	up to 6 m.	up to 12 m.	0.108	NS
			up to 18 m.	0.344	NS
			> 18 m.	0.339	NS
		up to 12 m.	up to 18 m.	0.472	NS
			> 18 m.	0.532	NS
		up to 18 m.	> 18 m.	0.959	NS

Table (3): Descriptive Statistics of gender groups in control and study samples for the studied parameters

Groups		Gender	N	Mean	Std. Deviation	Std. Error Mean
Control	IFN γ	Male	9	271.67	146.01	48.67
		Female	5	248	107.27	47.97
	IL-4	Male	9	54.22	18.05	6.02
		Female	5	47	10.37	4.64
	IL-10	Male	9	77.78	19.22	6.41
		Female	5	77	10.37	4.64
Patients	IFN γ	Male	16	75.94	33.08	8.27
		Female	8	80.38	17.84	6.31
	IL-4	Male	16	120	27.87	6.97
		Female	8	138.13	26.72	9.45
	IL-10	Male	16	97.81	12.51	3.13
		Female	8	97.88	5.89	2.08

Table (4): Association of BMI indicator among control and study groups

Groups	Count	IFN γ		Total	C.S. P-value
		Normal value	Abnormal value		
Control	Count	14	0	14	F.E.P.T. P=0.000 HS
	% within Groups	100.0%	0.0%	100.0%	
	% within BMI	100.0%	0.0%	36.8%	
	% of Total	36.80%	0.00%	36.80%	
Patients	Count	0	24	24	
	% within Groups	0.0%	100.0%	100.0%	
	% within BMI	0.0%	100.0%	63.2%	
	% of Total	0.0%	63.2%	63.2%	
Total	Count	14	24	38	
	% within Groups	36.8%	63.2%	100.0%	
	% within BMI	100.0%	100.0%	100.0%	
	% of Total	36.8%	63.2%	100.0%	

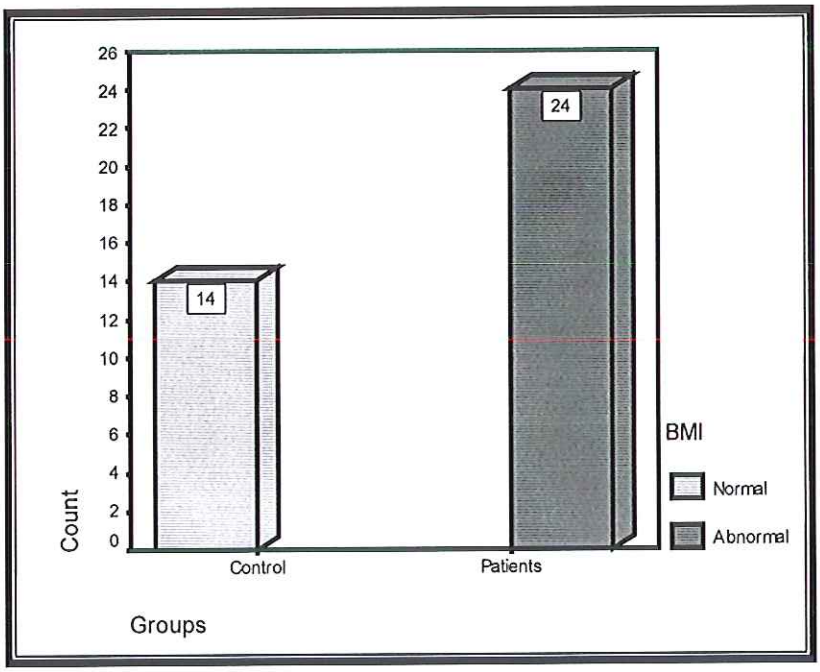

Figure (1): Cluster Bar chart for BMI indicator among control and study groups

Testing statistic for normal dis tribution function assumption showed that all the studied indicators (IL-4.IL-10 and INF- γ) in both groups independently having normal distribution function with p. value> 0.05.(table 5 ,6,7)

Table (5): Testing the Normal distribution function in control and study samples for the studied parameters outcomes

One-Sample Kolmogorov-Smirnov Test					
Groups			IFN γ	IL-4	L-10
Control	N		14	14	14
	Normal Parameters	Mean	263.21	51.64	77.5
		Std. Deviation	129.61	15.7	16.14
	Kolmogorov-Smirnov Z		0.613	0.578	0.554
	Asymp. Sig. (2-tailed)		0.847	0.892	0.919
Patients	N		24	24	24
	Normal Parameters	Mean	77.42	126.04	97.83
		Std. Deviation	28.55	28.28	10.61
	Kolmogorov-Smirnov Z		0.594	0.377	0.913
	Asymp. Sig. (2-tailed)		0.871	0.999	0.375

A Test distribution is Normal.
B Calculated from data.

Table (6): Descriptive statistics of different parameters in control and study samples

		N	Mean	Std. D.	Std. Error	95% Confidence Interval for Mean		Min.	Max.
						Lower Bound	Upper Bound		
IFN γ	Control	14	263.21	129.61	34.64	188.38	338.05	35	410
	Patients	24	77.42	28.55	5.83	65.36	89.47	15	130
IL-4	Control	14	51.64	15.7	4.2	42.58	60.71	35	95
	Patients	24	126.04	28.28	5.77	114.1	137.98	550	170
IL-10	Control	14	77.5	16.14	4.31	68.18	86.82	50	115
	Patients	24	97.83	10.61	2.17	93.35	102.32	70	110

Table (7): Coincidence's testing among the two independent groups (control and study) groups for the studied different parameters

Parameters	Levene's Test for Equality of Variances		t-test for Equality of Means			C.S. P-value
	F	Sig.	T	df	Sig. (2-tailed)	
IFN γ	30.025	0.000	5.289	13.74	0.000	HS P<0.01
IL-4	4.933	0.033	-10.424	35.963	0.000	HS P<0.01
IL-10	1.817	0.186	-4.692	36	0.000	HS P<0.01

Estimation of IL-4 serum level:-

The results appeared that a significant increase in mean serum level of IL-4(mean = 126.04) (Std. D =28.28) in malnourished children group in compares to control normal group (mean= 51.64) (Std. D = 15.7) and with high significant differences HS P.value <0.01) table(6) figure(2) .also , coincidence's testing among the two independent groups (control and study) revealed a high differences in serum level of IL-4 between patient and control group by using fissure test (F=4.933) and the comparative was high significant (Table 6,7) .

Table (8): Association of IL-4 indicator among control and study groups

Groups	Count	IL-4		Total	C.S. P-value
		Normal value	Abnormal value		
Control	Count	13	1	14	F.E.P.T. P=0.000 HS
	% within Groups	92.9%	7.1%	100.0%	
	% within IL-4	81.3%	4.5%	36.8%	
	% of Total	34.2%	2.6%	36.8%	
Patients	Count	3	21	24	
	% within Groups	12.5%	87.5%	100.0%	
	% within IL-4	18.8%	95.5%	63.2%	
	% of Total	7.9%	55.3%	63.2%	
Total	Count	16	22	38	
	% within Groups	42.1%	57.9%	100.0%	
	% within IL-4	100.0%	100.0%	100.0%	
	% of Total	42.1%	57.9%	100.0%	

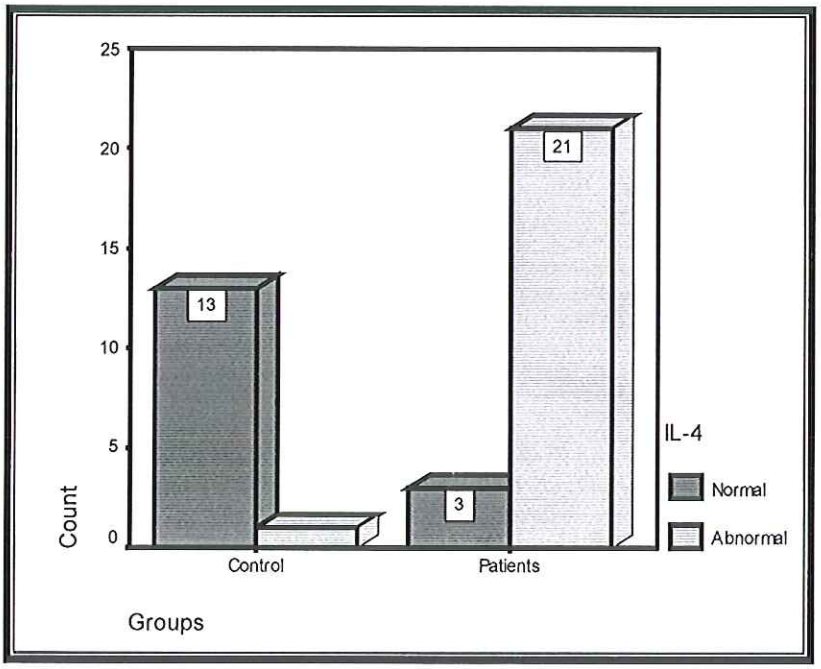

Figure (2): Cluster Bar chart for IL-4 indicator among control and study groups

***Estimation of serum level of IL-10**

The estimated level of IL-10 in sera of all study groups included both patient and normal control subjects were carried out.

The level of IL-10 was found to be significant increase in all malnourished children group (mean = 97.83) compared to all control group (mean = 77.5) with (P. value <0.01). On the other hand, the statistical analysis revealed that high significant differences in level of IL-10 in different age group among control group by using multiple comparison significant by (L.S.D.) method between the different age groups in the control and study samples (table 2 ,9) and fig.-3

Table (9): Association of IL-10 indicator among control and study groups

Groups	Count	IL-10		Total	C.S. P-value
		Normal value	Abnormal value		
Control	Count	13	1	14	F.E.P.T. P=0.000 HS
	% within Groups	92.9%	7.1%	100.0%	
	% within IL-10	68.4%	5.3%	36.8%	
	% of Total	34.2%	2.6%	36.8%	
Patients	Count	6	18	24	
	% within Groups	25.0%	75.0%	100.0%	
	% within IL-10	31.6%	94.7%	63.2%	
	% of Total	15.8%	47.4%	63.2%	
Total	Count	19	19	38	
	% within Groups	50.0%	50.0%	100.0%	
	% within IL-10	100.0%	100.0%	100.0%	
	% of Total	50.0%	50.0%	100.0%	

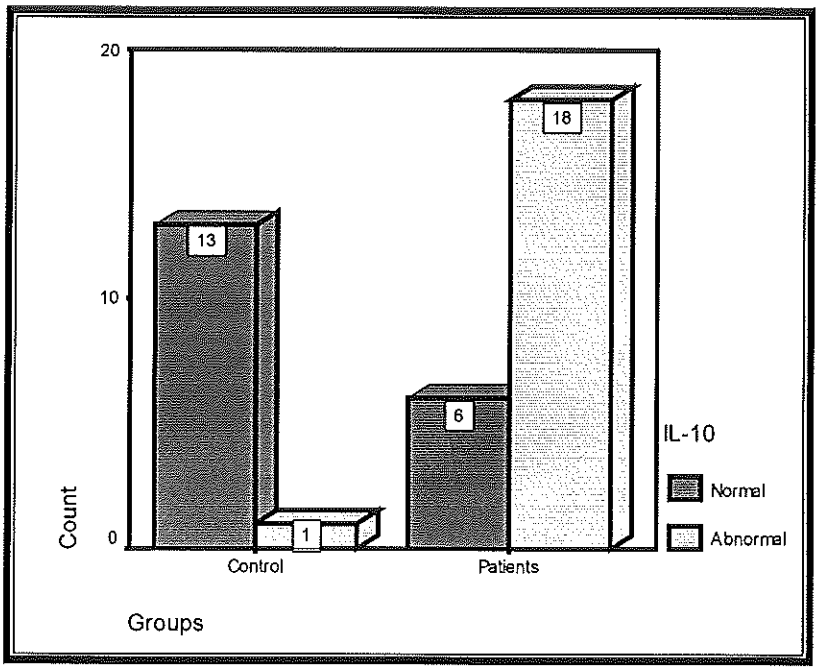

Figure (3): Cluster Bar chart for IL-10 indicator among control and study groups

*Estimation of serum level of γ IFN:-

IFN was found at high level in all control children group (mean=263.21 pg/ml) in compare with malnourished children group (mean=77.42pg/ml). While there is no significant differences in mean of INF γ serum level in different age groups in same group table 2, 10 and fig.4.

Table (10): Association of IFN γ indicator among control and study groups

Groups	Count	IFN γ		Total	C.S. P-value
		Normal	Abnormal		
Control	Count	14	0	14	F.E.P.T. P=0.393 NS
	% within Groups	100.0%	0.0%	100.0%	
	% within IFN γ	38.9%	0.0%	36.8%	
	% of Total	36.8%	0.0%	36.8%	
Patients	Count	22	2	24	
	% within Groups	91.7%	8.3%	100.0%	
	% within IFN γ	61.1%	100.0%	63.2%	
	% of Total	57.9%	5.3%	63.2%	
Total	Count	36	2	38	
	% within Groups	94.7%	5.3%	100.0%	
	% within IFN γ	100.0%	100.0%	100.0%	
	% of Total	94.7%	5.3%	100.0%	

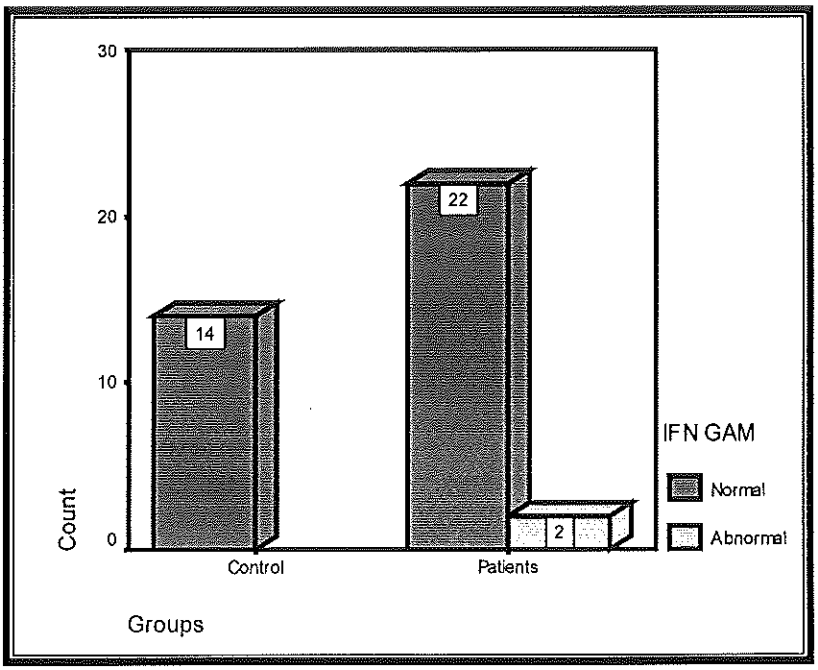

Figure (4): Cluster Bar chart for IFN γ indicator among control and study groups in diagnostic by the effects of malnutrition .

Discussion

Protein energy malnutrition PEM is a real health problem in developing countries that effect the growth and development of young children (2, 15) .The present study was undertaken to investigate the importance of malnutrition influencing the functions of the immune system in selected groups of Iraqi children giving the important of this subject as revealed by the fact that defective nutrition affects all types of the immune response (16).

PEM protein energy malnutrition is important cause of immunocompetence impairment and consequently PEM can result in illness and death due to infectious disease (17) .The impairment are seen in several immune responses such as ,secretary IgA , phagocyte function , the complement system and antibody affinity and their production (18) . This study revealed that serum level of IFN γ was significant differences between control and malnourished children indicated low level of IFN – γ in malnourished children in compared with control group but the serum level of IL-4 and IL-10 were higher in malnourished group .Our results are similar to that reported by (Najera et al., 2001 and Rodriguez et al., 2003) (2, 8). These reduction in serum level of INF is probably due to over production of other cytokines cause inhibit IFN production and reduce cell mediated immunity . Generally the malnourished children characterized by recurrent infection and combination of increase consumption in antigen-antibody reaction (20) or impaired hepatic synthesis (21) or to excess loss of complement through inflammation or injured tissues such as the intestine (22). Previously, the alteration in the capacity to produce some cytokines in malnourished have been reported (Rikimaured et al., 1998) observed that lymphocytes were unable to secrete normal quantities of cytokines or to achieve adequate immunological function and proposed altered physiological function of immune system may lead to impairment of immune response. The serum level of some cytokines were identified in peripheral blood of malnourished

children showed reduce production of IFN- γ and increase the serum level of interleukin-4 and interleukin -10 in compared with control group these observation are in agreement with results of previous studies (19 ,20) .
indicated that CD8 T-cell diminished IL-2 production induce energy (failure to proliferate to antigen) that inhibits autocrine of IL-2 production (23).therefore , the reduction in IL-2 expressing cells in malnourished children may be due to the reduced number of CD45R0 cells under / and reduced the capacity of CD45R0 cells to produce cytokines .

IFN- γ is a key cytokines in the development of type 1 immune response, which are required for the elimination of pathogen (24) .as well as; IFN- γ induces differentiation and activation of monocytes /macrophage and enhance their activation and their microbial effecter function (25). Moreover, IL-2 stimulates IFN- γ production (2, 20, and 26) and therefore reduced expression further inhibits IFN- γ production may be also related to the impaired CMI shown in children with malnutrition.
The data obtained in the present study showed a significant increase in IL-4, production by CD4+ and CD8+ cells from malnourished children , also the results obtained in the present study showed a significant decrease in IFN produced γ ed by CD4 and CD8 cells.

IL-10 is a suppressive cytokine that may contribute to the decrease production of IL-2 and γ IFN.therefore, interleukin -10 may be an important immunosuppressive factor related to the impaired immune response observed in malnourished children ., In addition, it has been reported that IL-10 can decrease the cytotoxic function of CD8+ Tcell when it is added before or at the time of activation (30); similar results were obtained for CD4+T-cell (31).
An important increase in the serum level of IL-10 in malnourished children (Th -2 cytokines) is widely regarded as suppressor factor for type -1 immune response (2 ,27) .induction of higher level IL-10 has been associated with inhibition of IL-2 production by innate cells has been reported in connection with a large number of pathogens (28).moreover.IL-4 has a direct effect on CD4 + cell cause suppression of IL-2 and IFN- γ secretion (29). The result of this study showed that serum level of IL-4 and IL-10 were significantly increased their production in malnourished children when compared to control group. This finding may be attributed for alter the balance of type -1 and type -2 T cell (humoral and cell mediated immunity) responses leading to increase the capacity, tendency and sensitivity to infection associated with malnutrition.
The results of this study revealed there was a significant differences in weight and height (body mass index) between healthy control and malnourished groups .Dattani and preece (1998) (4) stated that malnutrition can cause short stature ,this point is compatible with our results (table -4) as significant differences in lengths and weight between control and malnourished groups were obtained .

Generally, nutritional status of the host could alter the function and the activities of the immune system and consequently influence the host response to infection. The commonest sites affected are the lungs, urinary and gastroenteritis tract in addition to the blood stream (33) Although, the early identification of patient with suboptimal nutritional status can allow the implementation of nutritional intervention to enhance the ability of the body to fight infection and disease (2).therefore, this study was conducted to identify possible functional indicators and acute nutritional deprivation .

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