Original article

Fas-induced apoptosis in malnourished infants

Background: *Malnutrition in children is frequently associated with an increased incidence of infection. Apoptosis of immune cells in undernourished organisms may cause an increase in the organism's susceptibility to diseases related to immune suppression. Lymphocyte apoptosis was described in malnutrition. The role of factor of apoptosis signal (fas,CD95) in apoptosis of lymphocyte populations in malnourished children is still unclear.*

Objective: *This study investigated apoptosis in T lymphocytes in different types of malnutrition and the role of Fas in lymphocyte apoptosis and its relation to clinical and laboratory parameters of malnutrition.*

Study design*: Sixty-three malnourished infants and children were compared to 27 healthy controls. Beside thorough history and clinical examination, laboratory investigations and flow cytometry assessment of T lymphocytes were done. The viability of T lymphocytes was determined by combination of fluorescence dye 7-amino actinomycin, CD95 and CD3.*

Results: *There was significant increase in apoptotic T-cells in the patients compared to the controls. There was up-regulation of Fas expression in $CD3^+$ cells. Furthermore $CD3^+/CD95^+$ cells were less viable than $CD3^+/CD95^-$ cells of the patients and than $CD3^+/CD95^+$ cells of the controls. All the clinical and laboratory parameters of the studied patients showed no significant correlations with any of the apoptotic indices.*

Conclusion: *Increased apoptosis in T lymphocytes in malnourished children may be the cause of the decrease in lymphocyte count in their peripheral blood. This in turn may be the cause of decreased cell mediated immunity and the more common occurrence of infection in such patients. Up-regulation of Fas may be the cause of apoptosis in T lymphocytes in these malnourished children.*

Keywords: Fas, apoptosis, malnutrition, flow cytometry, infection, T lymphocytes.

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INTRODUCTION

Malnutrition continues to be a major public health problem throughout the developing world[1]. In Egypt, the incidence of malnutrition was found to be 7.3%[2]. Previous authors have pointed out that malnutrition is the primary cause of secondary immunodeficiency[3,4]. This was related to changes in cellular immunity[4] and in peripheral lymphocyte subsets[5]. Malnutrition in children is frequently associated with an increased incidence of bacterial, fungal and viral infections. Around the world, each year 12 million children under 5 years of age die due to the malnutrition-infections cycle[5].

Apoptosis is a genetically controlled process in which the cell actively participated in its own destruction in response to various types of stress. Apoptosis plays a key role in the homeostatic regulation of the hematopoietic system[6]. Moreover, many studies have demonstrated that the apoptosis of immune cell in undernourished organisms may cause an increase in the organism's susceptibility to diseases related to immune suppression[7,8,9].

Lymphocyte apoptosis was described in peripheral blood and lymphatic organs during infection and malnutrition[10]. Lymphoid atrophy is a well recognized consequence of nutritional deprivation in animals, including man[11].

Factor of apoptosis signal (Fas) induces apoptosis in activated T cells when they are repeatedly stimulated by antigen and functions to maintain T cell tolerance by deleting auto reactive cells[12]. The functional role of Fas (CD95) in the immune system has been examined in a variety of experimental models[13]. The results of such studies support the idea that Fas/Fas ligand pathway is critically involved in the elimination of mature but self reactive lymphocytes. However, little has been

done to explore its function in apoptosis of lymphocyte populations in malnourished children.

The aim of this work is to study the apoptosis in T lymphocytes in different types of malnutrition, we also aim to assess the role of Fas in apoptosis of T lymphocytes and correlate these findings to the clinical and laboratory parameters of the studied patients.

METHODS

This study was an analytical case-control study carried out on 63 malnourished children admitted to Pediatrics Hospital, Assiut University in Egypt, 34 boys and 29 girls. Their ages ranged from 1.5 to 24 months with mean of 11.12 ± 6.36 months. Thirty five children presented with second-degree marasmus, 19 with third degree marasmus, and 9 with kwashiorkor. The clinical signs and symptoms of malnutrition, as well as weight deficits, were used to determine the type and severity of malnutrition according to the established values[14,15]. Forty four patients were with artificially fed, 15 were breast feeding and 4 were fed artificial and breast milk. They were admitted to hospital because of gastroenteritis, and ten of them had respiratory infections as well. The study also included 27 apparently healthy controls .They includes 15 females and 12 males. There ages ranged from 6 to 24 months with mean of 11.44 ± 4.77 months.

An informed written consent in accordance with Assiut University ethical committee guidelines was taken from cases and controls.

All patients and controls were subjected to thorough history taking and clinical examination. The following investigations were done:

Serum level of sodium (Na) and potassium (K) were determined by AVL 9180 analyzer, Roch diagnostic, Germany. Determination of serum calcium (Ca) level and liver and kidney functions were done by Beckman model CX-9 chemistry analyzer, Calif, USA. The EDTA blood was used for complete blood count Celltac E automated hematology analyzer, Tokyo, Japan and flow cytometric assessment were also performed.

Cell preparation and staining

T lymphocytes were determined by anti CD3 conjugated monoclonal antibody to phycoerythrin (PE) dye (clone MEM-57, EXBIO, Czech), combined with surface death receptor (CD95) conjugated with fluorescein isothiocyanate (FITC) dye (clone DX2, Pharmingen, BD) and fluorescence dye 7-amino actinomycin D (7AAD) (Pharmingen, BD) to study the viability of the cells. 7AAD is a fluorescent DNA-binding agent which intercalates between cytosine and guanine bases. Because of changes in cell membrane permeability, staining with 7AAD can be used to define dead (7AAD bright), apoptotic (7AAD dim) and live (7AAD negative) populations by flow cytometry (FCM) without the need of cell membrane permeabilisation[16,17].

Flow cytometry analysis

Analysis was performed with FACSCaliber FCM (Becton Dickinson, USA). A minimum of 10,000 total events were acquired to establish an analysis using CELL Quest software (Becton Dickinson, USA). The lymphocyte gate (R1) was determined manually on the basis of forward and right angle light scatter (FSC & SSC respectively). Cells were expressed on a scatter diagram combining CD95-FITC with CD3-PE fluorescence and a region (R2) was drawn around positive population for both. The marker for determining positive and negative cells was set according to the negative control. To quantify viability of positive cells within R2, regions (R3, R4 and R5) were drawn satisfying 7-AAD-negative (viable), dim (apoptotic) and bright (dead) respectively (Fig. 1). Other regions were drawn around the $CD3^+/CD95^-$ population and around all $CD3^+$ cells. To quantify viability of cells within these regions, the same regions (R3, R4 and R5) were drawn as above.

Statistical analysis

Analysis was done using SPSS (version 13), (SPSS, Inc., Chicago, IL). The numerical data were represented as mean ± SD for parametric quantitative data and median (range) for nonparametric quantitative data. Student's *t* test was used for parametric data. Mann-Whitney *U* test for nonparametric data in addition to the correlation studies. The difference was considered significant if probability (*p*) values were less than 0.05.

RESULTS

The present study revealed significant decrease in the weight, hemoglobin level, Na, K and Ca levels and significant increase in white blood cells (WBCs) count in the patients when compared to the controls (Table 1). As regards liver and kidney functions, they were within the normal range for age and sex according to Nicholson and Pesce18.

The current study revealed significant decrease in the percentage and absolute count of the CD3+ cells in the patients when compared to the controls (patients: 34.7%, 213; control: 67.1%, 1510 respectively; $p<0.001$). To investigate whether the decreased CD3+ cells in malnourished patients may

be caused by increased apoptosis or not, we studied their viability. CD3+ cells were less viable, more apoptotic in the patients when compared to the controls and the differences were highly significant ($p<0.001$). (Table 2).

To study the role of Fas antigen; we compared the viability of T lymphocytes that express CD95 and those which do not. The percentage of CD95 expression on the T lymphocytes as well as the percentage of apoptotic and dead cells expressing CD95 was significantly higher in the patients than the controls (Table 3).

There is no significant difference in the studied parameters second degree marasmus and third degree marasmus (Table 4 and Table 5).

CD3+CD95+ cells were significantly less viable (median 26.11%) than CD3+CD95- cells (median 85%) in the patients ($p<0.001$). Similarly the CD3+CD95+ cells were more apoptotic (median 49.3%) and dead (median 11.99%) than CD3+CD95- cells (median 11.5%, 0.82% respectively) with highly significant values ($p<0.001$). (Fig. 2).

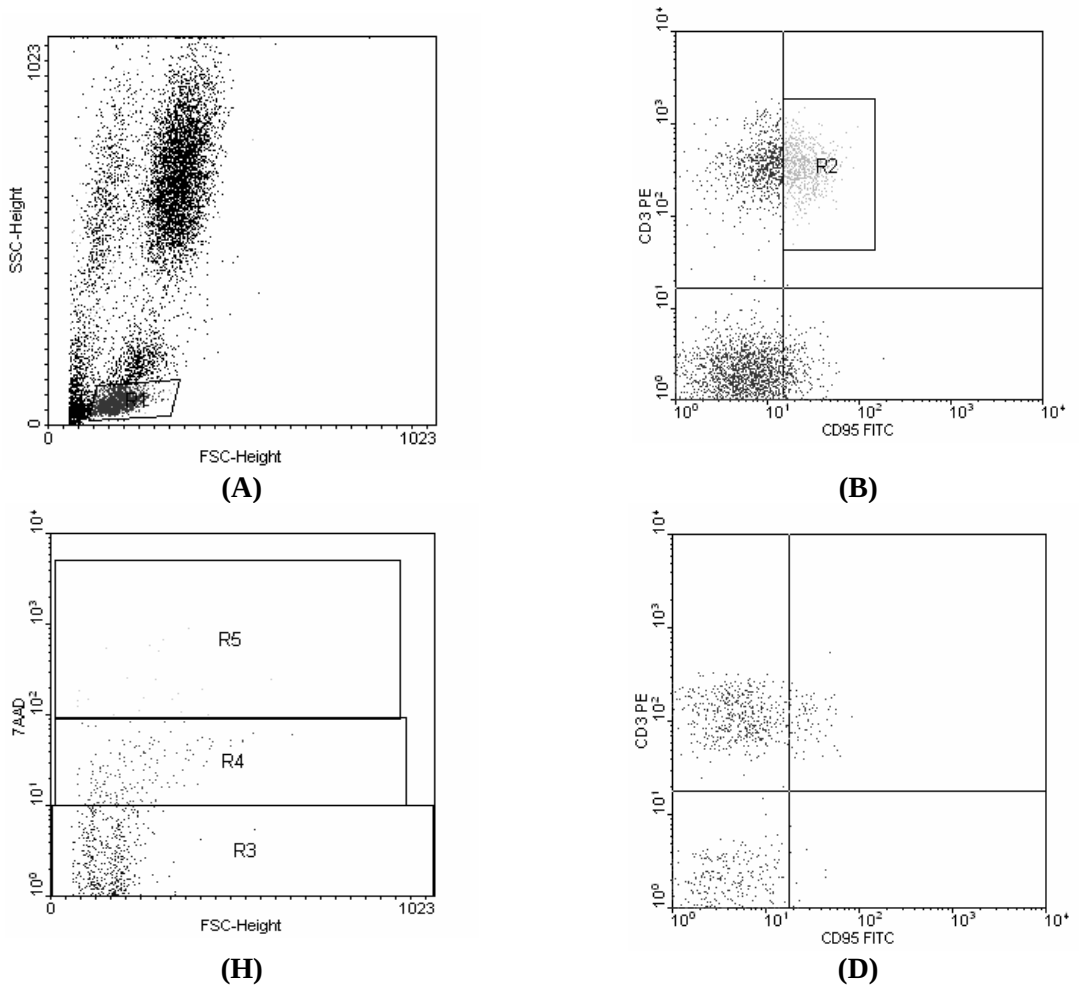

Figure 1. (A) Dot plot of FSC versus SSC showing a distinct population of lymphocyte cells (R1). (B) Dot plot of CD95-FITC versus CD3-PE gated on R1 showing lymphocytes population that co express CD3 and CD95 (R2) in patient. (C) Dot plot of FSC of $CD3^{+}/CD95^{+}$ve cells (gated on R2) versus 7AAD fluorescence, divided into viable cells negative for 7AAD (R3), apoptotic cells dim for 7AAD (R4) and dead cells bright for 7AAD (R5). (D) Dot plot of CD95-FITC versus CD3-PE gated on R1 showing lymphocytes population that express CD3 and CD95 in control.

Table 1. Comparison of weight, CBC and macro-mineral measurements between malnourished children and controls

	Patients (63)	Controls (27)	*p* value
Weight (kg)	5.1±1.61	9.7±1.07	<0.001
Weight percentile	below 5^{th}	between 10^{th}-50^{th}	-
Hb (g/dL)	9.2±1.71	11.6±0.67	<0.001
WBCs (10^9/L)	10.5±4.5	7.3±1.4	0.001
Platelets (10^9/L)	236±120	287±85.3	0.046
Na level (m.mol/L)	132.1±5.9	138.1±2.4	<0.001
K level (m.mol/L)	3.4±0.77	4.0±0.27	<0.001
Ca level (mg/dl)	7.1±1.25	9.5±.58	<0.001

Values are presented as mean± SD and test of significance is Student *t* test.
Hb: hemoglobin; WBCs: white blood cells; Na : sodium; K: potassium; Ca: calcium

Table 2. Comparison of $CD3^+$ cells percentage and absolute count and their viability between malnourished children and controls.

	Patients (63)	Controls (27)	*p* value
Percentage of $CD3^+$cells	34.7 (4.17-90.55)	67.1 (56.78-80.18)	<0.001
Absolute count of $CD3^+$cells	213 (10-1847)	1510 (507-4283)	<0.001
Viability of $CD3^+$ cells:			
Viable	43.75 (3.33-89.7)	81.54 (24.97-92.55)	<0.001
Apoptotic	37.92 (8.56-79.71)	14.24 (5.2-70.79)	<0.001
Dead	8.17 (0.67-48.1)	3.03 (0.57-7.7)	<0.001

Values by median (range) and test of significance is Mann-Whitney U test.

Table 3. Comparison of CD95 expression on $CD3^+$ cells and their viability between malnourished children and the controls.

	Patients (63)	Controls (27)	*p* value
Percentage of $CD3^+CD95^+$	10.23 (0.5-59.5)	5.58 (0.39-13.95)	0.010
Viability of $CD3^+CD95^+$:			
Viable	26.1 (1.69-79.17)	69.5 (22.47-88.8)	<0.001
Apoptotic	49.3 (17.25-85.9)	27.3 (10.43-65.17)	<0.001
Dead	11.9 (1.09-57.41)	7.36 (0.77-12.07)	<0.001

Values by median (range) and test of significance is Mann-Whitney U test.

Table 4. Comparison of $CD3^+$ cells percentage and absolute count and their viability between second degree and third degree marasmus.

	Second degree Marasmus (35)	third degree marasmus (19)	*p* value
Percentage of $CD3^+$cells	36.36±22.97	36.32±20.44	NS
Absolute count of $CD3^+$cells	420.17±519.65	364.68±376.73	NS
Viability of $CD3^+$ cells:			
Viable	45.63±23.91	43.83±26.74	NS
Apoptotic	38.84±18.63	42.95±20.48	NS
Dead	13.59±11.53	12.67±13.17	NS

Value presented as mean ±SD and test of significance is paired sample t test.

Table 5. Comparison of CD95 expression on $CD3^+$ cells and their viability between second degree and third degree marasmus.

	Second degree Marasmus (35)	third degree marasmus (19)	*p* value
Percentage of $CD3^+CD95^+$	13.42±12.95	17.45±17.97	NS
Viability of $CD3^+CD95^+$:			
Viable	29.99±20.04	32.76±24.62	NS
Apoptotic	50.51±17.1	48.92±18.31	NS
Dead	19±16.1	17.74±16.6	NS

Value presented as mean ±SD and test of significance is paired sample t test.

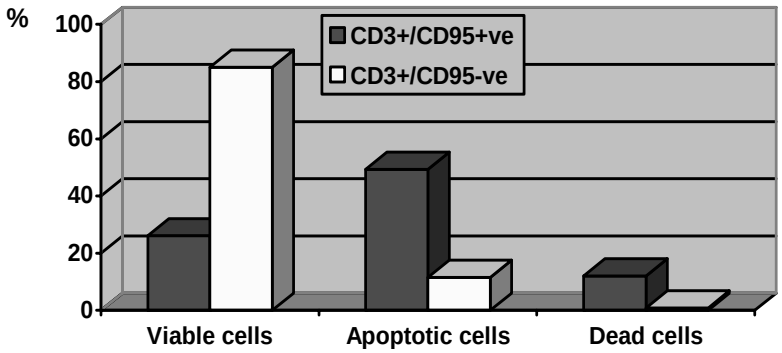

Figure 2. The percentage of viable, apoptotic and dead $CD3^+/CD95^+$ and $CD3^+/CD95^-$ lymphocytes in malnourished children (median), $p<0.001$.

When we compared artificially feed patients with breast feed patients there was no significant differences in the studied parameters. There were no significant differences in the studied parameters in cases of marasmus compared to cases of kwashiorkor.

All the clinical and laboratory parameters of the studied patients showed no significant correlations with any of the apoptotic indices.

DISCUSSION

Severe malnutrition occurs as a consequence of deficient food intake and/or low-protein diets. Malnourished children are more susceptible to infections than well-nourished children; in consequence, they are considered immune-deficient[5]. This suppression results from the increased morbidity and mortality of immune cells[19].

In this work, there was a decrease in the percentage and the absolute count of $CD3^+$ cells in malnourished children when compared to normal controls. This finding agrees with the results of previous studies[5,20,21]. El-Hodhod and his coworkers[10] concluded in their study that abnormality in peripheral blood lymphocytes in malnutrition is not limited to their abnormal count or disturbed subset ratio as previously reported but also can be due to enhanced apoptosis of these cells. They showed increase in early and late apoptosis in lymphocytes in protein energy malnutrition compared to the controls assessed by Annexin V using flow cytometry. These results fit the results of the present study which showed highly significant increase in apoptotic T lymphocytes in the patients compared to the controls as assed by 7AAD.

Another flow cytometry study done on rats with TUNEL assay by Ortiz et al[22] showed that severe malnutrition induced during lactation was associated with increased levels of spontaneous thymocyte apoptosis. They proposed that this apoptosis may be related to the marked thymic atrophy found. Moreover, thymus size correlated with the percentage of CD4 and CD8 T-lymphocytes in peripheral blood of malnourished infants in another study[23]. However, the molecular mechanisms are not yet well understood. On the other hand, many studies reported that deficiencies in micro minerals activate different pathways for signaling apoptosis[24-27]. A study for da Costa and his coworkers[28] showed that choline-deficient diet increased DNA damage in humans, without molecular explanation. A more recent study showed increased expression of Fas in the very early phases of choline-deficiency in rats[29].

The identification of pathways of apoptosis in T cells leads to predictions about the pathophysiologic consequences and practical implications of disrupting or augmenting these pathways[30]. For example, Cho et al[31]concluded that the inhibition of apoptosis in malnourished macrophages by allicin is a possible therapy for immune-suppressive diseases caused by malnutrition.

Therefore, we tried to investigate the role of Fas antigen in malnourished T lymphocyte apoptosis in humans. We found that malnourished patients' $CD3^+$ cells up-regulated Fas antigen and this up-regulation was highly statistically significant compared to the controls. In addition, we studied the relation between apoptosis and the expression of Fas antigen, and found highly significant relations between both. This might explain molecular basis for exacerbation of apoptosis in peripheral blood T lymphocytes in malnourished children.

In the current study, all the clinical and laboratory parameters of the studied patients showed no significant correlation with any of the apoptotic indices. In this respect, our results are in keeping with El-hodhod et al[10] who showed an increase in apoptosis in nonedematous and edematous types of malnutrition with no differences detected on comparing the two types. Indeed this may point to the fact that regardless of the severity of malnutrition and infection, once the process of under-nutrition starts increased apoptosis of lymphocytes occurs. This necessitates prevention of malnutrition -rather than treatment- by detecting it in the early and sub-clinical stage through regular weighing of all infants and children especially underprivileged ones.

In conclusion, the abnormality in peripheral blood lymphocytes in malnutrition can be explained by the enhanced apoptosis of these cells. The up-regulation of Fas may explain molecular basis for apoptosis in T lymphocytes in malnourished children. In addition there is no relation between any clinical or laboratory parameters with these apoptotic measures. It will be interesting to examine the effects of these apoptotic findings with the prognosis and outcome of the patients.

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