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CIRCULATING AND MITOGEN-INDUCED TUMOR NECROSIS FACTOR (TNF) IN MALNOURISHED CHILDREN

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Abstract Malnutrition in children is associated with an increased risk of infection and death. Multiple abnormalities in the inflammatory-immune response, including cytokine production, have been described in protein energy malnourished (PEM) children and could account for increased severity and frequency of infection. The aim of the present study was to determine whether there are abnormal basal tumor necrosis factor (TNF) serum concentrations in PEM children, to relate it with serum cortisol and plasma corticotrophin levels and to explore simultaneously the *in vitro* production of TNF by peripheral blood leukocytes (PBL). No differences were found in basal plasma corticotrophin and serum cortisol concentrations in malnourished as compared with normal, wellnourished control children. Basal TNF serum concentrations were significantly higher in malnourished children than in controls. Conversely, mitogen induced TNF production by PBL *in vitro* was significantly reduced in PEM children compared with controls. Abnormalities in circulating and mitogen-induced TNF production are present in malnourished children even in absence of elevated serum cortisol concentrations. These abnormalities potentially could modify inflammatory-immune responses to infectious stimuli in malnourished children.

Resumen ***Factor de necrosis tumoral (TNF) circulante e inducido por mitógenos en niños con desnutrición primaria.*** La desnutrición en los niños se asocia a un elevado riesgo de infección y muerte. En los niños desnutridos se han descripto múltiples anomalías de la respuesta inflamatoria e inmunitaria, e incluso en la producción de citokinas. Estas anomalías pueden ser responsables al menos en parte, de la mayor frecuencia y/o severidad de algunas infecciones. Nuestro trabajo consistió en determinar las concentraciones séricas basales del factor de necrosis tumoral (TNF) en niños desnutridos, correlacionarlas con las concentraciones séricas de cortisol y de corticotrofina plasmática y medir la producción *in vitro* de TNF por parte de los leucocitos de sangre periférica de los mismos niños, comparándolas con las correspondientes a niños controles eutróficos. No encontramos diferencias significativas entre ambos grupos en las concentraciones basales de cortisol o de corticotrofina. Los niveles séricos basales de TNF fueron significativamente mayores en el grupo de niños desnutridos. Inversamente, la producción de TNF *in vitro* por parte de los leucocitos de sangre periférica de los niños desnutridos estimulados con mitógenos fue significativamente menor. Los niños desnutridos parecen presentar anomalías en la producción de TNF, con valores basales elevados pero disminución de su producción frente a estímulos, aun en ausencia de concentraciones elevadas de cortisol. Estas anomalías pueden condicionar su respuesta frente a estímulos inflamatorios e infecciosos.

Key words: malnutrition, primary malnutrition, tumor necrosis factor

Malnutrition, particularly in children under 4 years old, has been associated with an increased risk of mortality. A strong and significant synergy was found between wasting and infections as predictors of children mortality in field studies[1, 2].

A myriad of abnormalities in the inflammatory and immune responses have been described (reviewed in [3]) in malnourished children. Particularly, when studied in absence of inflammatory stimuli, plasma concentrations of interleukin (IL) 6 and soluble receptors for TNF seem to be higher in PEM children than in controls[4]; i.e the inflammatory cascade appears to be activated *in vivo* in malnourished children, even in absence of obvious infection. However, the acute phase protein response seems to be impaired in malnourished children with clinical infections, or after diphteria-pertussis-tetanus (DPT) vaccination[5].

Diminished *in vitro* production of IL1, IL6, and TNF in PEM children have been reported[6, 7], but to our knowledge a potential correlation between circulating concentrations of TNF in basal conditions and the simultaneous ability of stimulated leukocytes to produce the cytokine *in vitro,* has not been explored in malnourished children.

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In the present paper, we studied basal serum TNF concentrations and mitogen-induced TNF production *in vitro* in a group of malnourished children without any clinical evidence of infection.

Since serum cortisol may be elevated in severely malnourished children[8], and hormones released by the pituitary-adrenal axis are known to modulate cytokine production[9] we also determined basal adrenocorticotrophin (ACTH) and cortisol concentrations in the same group of patients.

Subjects and Methods

Subjects: Nine children (4 female, 5 male; mean age 23.8 months, standard error of the mean (SEM) 4.9 months) with primary malnutrition attending as outpatients to the Nutrition Rehabilitation Unit at the Children's Hospital, La Plata, Argentina were studied. All of them were considered as having acute malnutrition according to national (see Table 1) and international standards[10]. Nine wellnourished (according to the same standards), healthy children (5 female, 4 male), mean age 17.5, (SEM 3.9 months), mean weigh for age Z score 0.005, (range -0.73 to 0.75), attending to the same unit for health control and dietary counselling, were recruited as a control group. Children with fever, obvious infection or other known pathologies were not included in the study. Informed parental consent and permision from the corresponding local authorities were obtained.

Hormone and cytokine assays: Sterile blood samples were drawn between 08:00 and 09:00 a.m. in fasting condition and divided in aliquots to be further processed. An aliquot was heparinized and used to obtain peripheral blood leukocytes (PBL) and for plasma ACTH determination. A second aliquot was allowed to coagulate, and serum samples were frozen (-20°C) for basal cortisol and TNF measurement.

PBL were obtained by centrifugation of heparinized blood samples at 100x g for 30 minutes; 200 000 cells in 0.1 ml of RPMI 1 640 were distributed in 96-well plates and incubated in 5% CO_2 atmosphere at 37°C, in the presence or absence of either concavalin A (Con A) Sigma, at concentrations of 0.1, 1,0 and 10.0 µg/ml, or phytohemaglutinin (PHA), from *Phaseolus vulgaris,* at 0.5, 1.0 and 10.0 µg/ml. After 72 hours, supernatants were collected and frozen at -20°C, until processed for TNF determination. To asess cellular viability, cell proliferation was measured after incubation of PBL for an aditional 4-hours period in the presence of tritiated thymidine; radioactivity incorporated by cells was determined in a Tracor Analytic β Counter.

Plasma determination of ACTH was performed in duplicate by an immunoradiometric assay[11]. Intra and interassay coefficients of variation were lower than 3 and 8% respectively. Serum cortisol concentrations were determined in duplicate with a commercial kit (Immunotech, Marseille, France); the intra and interassay coefficients of variation ranged between 6 and 9%.

TNF determinations were done by measuring the cytolytic effect of serum and supernatants on L929 cells[12]. Serum samples and supernatants were run at dilutions of 1/4 and 1/8, and 1/4, 1/8, and 1/16, respectively. Briefly, L929 cells (70 000 per well) were incubated in 5% CO_2 at 37°C, for 24 hours in 96-well microtiter plates in minimal essential medium containing 10% fetal calf serum. On the following day, samples were added to the cells monolayers in quadruplicate and in presence of actinomycin D (1 µg/ml). After a second 24-hours incubation period, 0.05% crystal violet in methanol-water was added for 30 minutes. Plates were then rinsed and 0.1 ml of 33% acetic acid per well was added. Plates were then read in a 7 530 Multiplate Reader (Cambridge Technology). The reader was calibrated with a plate having more than 95% cell destruction and absorbance at 595 nm was inversely proportional to TNF bioactivity. A TNF solution of known concentration, ranging between 15 and 9 500 units (U) of bioactivity per ml, from Genzyme (lot 82 437) was used as a standard in each assay[13]. The intra and interassay coefficients of variation were 9 and 11% respectively.

Statistical analysis: Results are expressed as means (SEM). Basal circulating hormone and TNF concentrations in malnourished and control children were compared by the Student's t test. Supernatants data were analyzed using a multifactorial analysis of variance, followed by Fischer's test for means comparison[14].

TABLE 1.– *Nutritional and laboratory parameters in malnourished children*

Pat.	Age (months)	Weight (kg)	Height (cm)	Z score (w/a I.S)[1]	Gomez mod. (w/a N.S)[2]	TNF (U/ml)	Cortisol (nM)	ACTH (pg/ml)
1	19	7 850	72.5	-3	II	3.6	427	12
2	15	8 020	72.5	-2	I	3.6	248	65
3	17	8 250	73	-2	I	2.7	323	152
4	14	6 950	70	-2	II	4	192	20
5	18	9 000	76	-2	I	4.9	53	56
6	60	13 500	98	-3	II	5.1	52	270
7	9	5 400	60.5	-3	II	3	282	25
8	32	9 550	79	-2	II	5.5	57	5
9	8.5	6 950	68	-2	I	0.6	79	18
Controls[3]	17.5	NA	NA	0.005	NA	1.8 (0.3)	249 (74)	51 (19)

NA: Normal for age as described in Subjects and Methods

[1] *Weight for age international standards (ref 10)*

[2] *Weight for age national standards (H. Lejarraga et al. Comité Nacional de crecimiento y desarrollo SAP 1994)*

[3] *Values are means (SEM)*

Results

Basal concentrations of ACTH, cortisol and TNF: As seen in Fig. 1, basal plasma ACTH (upper panel) and serum cortisol (middle panel) concentrations were within the normal range for controls. Malnourished children showed values somewhat above for ACTH, and below for cortisol, as compared with controls; but these differences were not statistically significant. Basal serum TNF concentrations (lower panel) in malnourished children were significantly higher than those found in controls ($P < 0.05$).

In vitro TNF production: Fig. 2 shows TNF concentrations in supernatans of PHA- and Con A-stimulated PBL (upper and lower panel respectively) from malnourished and control children. At the lower concentration tested, both mitogens failed to produce any measurable effect. With higher concentrations of both mitogens, a concentration-related response with increasing TNF production in the supernatants was apparent for both groups of children, but TNF concentrations were significantly lower in malnourished children compared with controls ($P < 0.05$).

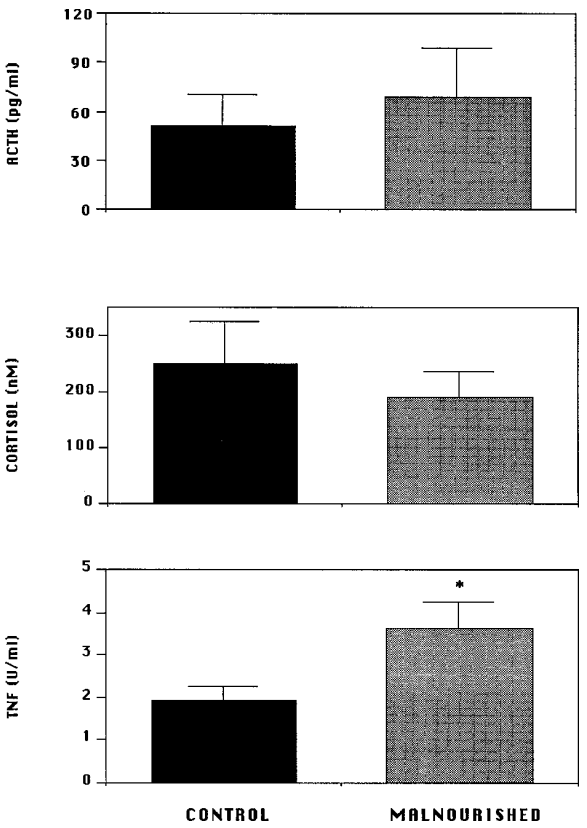

Fig. 1.– Plasma ACTH (upper panel), serum cortisol (middle panel) and serum TNF (lower panel) concentrations in control and malnourished children in basal condition. Values are mean ± Standard Error of the Mean. n = 9 children per group. * $P < 0.05$ vs control values.

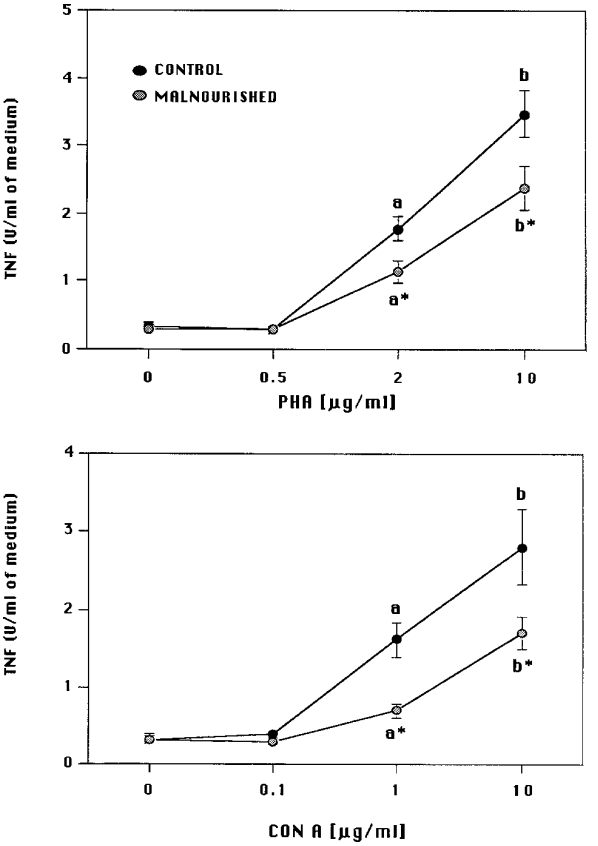

Fig. 2.– Tumor Necrosis Factor (TNF) production by Peripheral Blood Leukocytes (PBL) of normal control and malnourished children in the absence (concentration zero) or presence of several concentrations of phytohemaglutinin (PHA) (upper panel) and Concavalin A (Con A) (lower panel). Values are means ± SEM. n = 12 wells per point. a. $P < 0.05$ vs con-centration zero values. b. $P < 0.05$ vs values obtained with the middle concentration of either mitogen. * $P < 0.05$ vs control values.

A normal ability of cells from patients and controls to proliferate, (indicating satisfactory culture conditions) was observed in each experiment (data not shown).

Discussion

A strong association between malnutrition, infection and death has been reported in children[1, 2]. Bacterial infection is a well known potent stimulus for the acute inflammatory response; but only in the past few years, the description and characterization of IL1, TNF, IL6 and their soluble receptors has allowed a better understanding of the inflammatory response in man[15]. Acting in close cooperation, cytokines may be crucial in the host response to infection and, as demonstrated in some clinical situations[16, 17], their production is potentially related to the final outcome.

Several findings suggest that the inflammatory response may be abnormal in malnourished individuals[4-7]. It has been

observed that when studied in basal conditions, malnourished war prisoners have higher serum concentration of TNF than controls[18]. Malnourished HIV patients without concomitant diseases, have higher serum concentration of TNF and soluble TNF receptors than their wellnourished counterparts[19]. Children with primary PEM also have elevated serum concentration of IL6, C-reactive protein and soluble TNF receptors, irrespective of the presence of evident infection[4]. According to our data, basal serum TNF concentrations were significantly higher in malnourished than in wellnourished children, even in absence of obvious infection. Taken together, these findings suggest a basal activation of the inflammatory cascade in malnourished patients.

Conversely, in our *in vitro* design, PBL from malnourished patients produce lower concentrations of TNF in response to PHA and Con A, than PBL from controls. Similar findings have been described in PEM children for lipopolysaccharide (LPS)-induced IL1[6], IL6 and TNF[7] production. *In vivo* production of the acute phase reactants C-reactive protein and serum amyloid A in response to infection or after DPT vaccination, was also described as impaired in malnourished children and can be fully normalized after nutritional recovery[5].

Evidence of *in vivo* activation of the inflammatory cascade and simultaneously impaired *in vitro* cytokine production, has been described in the context of some clinical infections, such as typhoid fever[20] and meningococcal infection[21]. In human volunteers receiving a single intravenous endotoxin injection, the *in vitro* production of TNF by PBL in response to LPS was found significantly decreased three hours after the injection[22]. A similar phenomenon may be induced *in vivo* in mice by the administration of LPS, or a combination of IL1 and TNF[23]. The precise mechanism of low cytokine production capacity after *in vivo* exposure to stimuli remains unknown[20, 23].

It may be speculated that continuous exposition to multiple infectious agents produces a similar situation in malnourished children, even in absence of clinically evident infection. This altered status of chronic activation of the inflammatory cascade and simultaneously diminished cytokine production may account for an abnormal inflammatory response during malnutrition in children, and for the severity of some infections in the malnourished host.

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