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# Tumour necrosis factor production and cell-mediated immunity in anorexia nervosa

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## SUMMARY

Fourteen patients with anorexia nervosa (AN) were studied for the production of tumour necrosis factor (TNF), the activation of the interferon (IFN) system and cell-mediated cytotoxicity (CMC) and the results were compared with 16 age-matched healthy women. AN patients had significantly increased spontaneous TNF production by peripheral blood mononuclear cells (PBMC) *in vitro* ($16 \pm 5$ U/ml *versus* $4 \pm 3$ U/ml in the control group; $P < 0{\cdot}05$), although no TNF was detectable in the plasma from either group. TNF production *in vitro*, following stimulation of PBMC by phytohaemagglutinin (PHA) or tumour cells, was similar in AN patients and controls; however, lipopolysaccharide (LPS) induced TNF production was found to be lower in AN ($P < 0{\cdot}1$). CMC was significantly lower in AN patients ($4 \pm 2$ *versus* $10 \pm 3$ in controls, expressed as lytic units/$10^6$ cells; $P < 0{\cdot}05$), but no difference could be found between AN and controls in IFN activity as reflected by the level of the IFN-induced enzyme 2′-5′ oligoadenylate synthetase (2-5A) in PBMC. Beta-endorphins in the plasma were higher in the AN group ($P < 0{\cdot}05$) but these levels could not be correlated to those of IFN, CMC or TNF. Defective CMC and increased TNF production by PBMC in patients with anorexia nervosa may possibly result from the nutritional deficiencies and neuroendocrine abnormalities associated with the disease, and may contribute to the pathophysiology of AN.

**Keywords** anorexia nervosa tumour necrosis factor interferon β-endorphins natural killer

## INTRODUCTION

The immunological aspects of anorexia nervosa (AN) have received little attention (Cason *et al.*, 1986), yet several important processes which occur in AN may have a profound effect on immune functions. These include the presence of multiple nutritional deficiencies which are a well-recognized cause of immunodeficiency in humans, leading most consistently to a depressed cell-mediated immunity (Cason *et al.*, 1986). Many studies have shown that the neuro-endocrine system can control immune functions (Blalock, Harbour-McMenamin & Smith, 1985). Thus, the diverse endocrine changes reported in AN, affecting not only sex hormones but also cortisol, opioid activity and other neurotransmitters (Halmi, 1987) may lead to inevitable changes in the immune system. Stress and depression, which are often associated with AN, may also affect immune responses by as yet undefined mechanisms (Denman, 1986).

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Tumour necrosis factor (TNF), also known as cachectin, is a cytokine with numerous metabolic and immunologic activities (Beutler & Cerami, 1986). Of particular interest is its ability to mediate weight loss by several mechanisms including central suppression of food intake, suppression of lipoprotein lipase and catabolic effects on energy storage tissues (Tracey, Lowry & Cerami, 1987; Socher, Friedman & Martinez, 1988; Plata-Salaman, Oomura & Kai, 1988). These effects of TNF have been well documented both *in vitro* (Torti *et al.*, 1985; Lee *et al.*, 1987) and in experimental animals (Olif *et al.*, 1987; Tracey *et al.*, 1988b). The effect of TNF in mediating cachexia in cancer and in chronic infections in humans has not yet been unequivocally demonstrated (Beutler, 1988). We have recently found that induced TNF production by human peripheral blood mononuclear cells (PBMC) *in vitro* is significantly increased following acute starvation (Vaisman, Schattner & Hahn, 1989). In the present study we have examined PBMC from patients with AN and prolonged caloric deprivation for TNF production, interferon (IFN) system activity, cell-mediated cytotoxicity (CMC) and the relation of these parameters to plasma β-endorphins.

## MATERIALS AND METHODS

*Patients*

Fourteen female patients with AN, aged 13–20 (mean 16·2) years, were studied after admission to hospital. All had markedly low weights for height (median 59%, range 50–80%) with a self-imposed weight loss of 24–45% from prior weight (median 30%). All patients met the revised criteria for diagnosis of AN (Halmi, 1987) and none had any associated or intercurrent illnesses, nor were they receiving any medication when studied. Control subjects were 16 age-matched healthy women whose weight was in the range of 88–116% of desirable body weight (median 100%). After obtaining informed consent, all subjects were fasted overnight and a blood sample was taken between 8 and 10 A.M. for routine haematological and biochemical testing and for the immunological studies detailed below.

*Preparation of effector cells*

Twenty millilitres of peripheral blood were drawn into heparinized syringes and subsequently separated by density centrifugation on Ficoll–Hypaque gradients (Pharmacia Fine Chemicals, Uppsala, Sweden). Separated PBMC were washed with phosphate-buffered saline (PBS), pH 7·2, and suspended in RPMI 1640 medium, supplemented with antibiotics and 10% heat-inactivated fetal calf serum (RPMI–FCS).

*Target cells*

The target cells used were from the human carcinoma cell line, HeLa (ATCC CCL 2·1). They were maintained in RPMI–FCS. This cell line is sensitive to the cytotoxic activity of both the non-adherent and the adherent fractions of PBMC, as measured by 4-h and 12-h $^{51}$Cr release assays (unpublished).

*Assay of natural CMC*

Cell (contact) mediated cytotoxicity was assayed as previously described (Hahn *et al.*, 1989). Approximately 24 h prior to the assay, target cells were seeded into 9-mm microwells of 96-well microtitre test plates at $5 \times 10^4$ cells/100 $\mu$l per well. Eight double dilutions, in duplicate, of effector PBMC were then added, giving eight effector : target ratios, the highest of which was 10 : 1. The target cells were sensitized by addition of cycloheximide (CHI, Sigma) at 40 $\mu$g/ml which enhances the vulnerability of target cells to cytotoxicity but has no effect on the cytotoxic activity of effector cells. After 16 h at 37°C, 5% $CO_2$, target viability was evaluated using neutral red vital dye. The mean OD of control CHI-treated targets without effectors, was derived from eight replicate wells. Percentage of cytotoxicity was calculated according to the formula:

$$\% \text{ cytotoxicity} = \frac{\text{Dye uptake by target cells incubated with effector cells} + \text{CHI}}{\text{Dye uptake by target cells with CHI alone}}$$

A lytic unit (LU50) is defined as the number of effector cells required to cause lysis of 50% of the target cell monolayer. This is derived from the titration curve obtained from the various effector cell concentrations. LU50/$10^6$ cells is the number of lytic units derived for $10^6$ effector cells.

Treatment of target cells with 40 $\mu$g/ml CHI for 16 h causes reduced uptake of vital dye. This, however, results from inhibition of cell proliferation and not from cell death as measured by trypan blue dye exclusion. No difference was observed in the proportion of dye-excluding cells between CHI-treated and untreated target cells.

*Spontaneous and induced production of TNF*

PBMC were suspended at a concentration of $5 \times 10^6$ cells/ml in RPMI-FCS and 100 $\mu$l aliquots were incubated at 37°C for 16 h in the absence of inducer or in the presence of the following inducers: phytohemagglutinin (PHA, Wellcome), 20 $\mu$g/ml; bacterial lipopolysaccharide extracted from *Escherichia coli* (LPS; Makor Chemicals, Jerusalem), 10 $\mu$g/ml; or HeLa cells ($5 \times 10^4$ cells seeded 24 h prior to application of PBMC).

*Quantification of TNF activity*

Cytotoxicity was quantified as described (Hahn *et al.*, 1989). Twenty-four hours after seeding HeLa cells in 9-mm microwells at $5 \times 10^4$ cells/well, cell-free PBMC supernatants or plasma samples were applied to the cells in serial dilutions in the presence of 40 $\mu$g/ml CHI. Sixteen hours later, cell death was quantified by measuring the uptake of neutral red. One unit was defined as the concentration at which 50% of the target cells were killed.

An internal standard was included in each TNF bioassay. All TNF and CMC assays on patient and control material were performed in balanced groups.

*Identification of cytotoxicity as TNF*

To confirm that cytotoxicity was due to the production of TNF, neutralization of cytotoxic samples was performed using a monoclonal anti-TNF-alpha antibody (gift of BASF/Knoll, Ludwigshafen, FRG); 200 ng/ml of the monoclonal antibody (1 mg protein/ml) neutralize 1 ng/ml TNF-$\alpha$. Each sample was incubated for 24 h at 4°C with and without the anti-TNF antibody, then retested for cytotoxicity by bioassay as described. An excess of antibody (20 $\mu$g/ml) was used in all cases. The antibody preparation alone was not cytotoxic for the target cells.

*Assay for the IFN-induced enzyme 2′-5′ oligo-isoadenylate synthetase (2-5A) in PBMC*

This enzyme is present in various cells including PBMC and its level increases following exposure to all types of IFN both *in vitro* and, as we have shown, *in vivo* (Schattner *et al.*, 1981; Schattner & Revel, 1988). Measurement of the 2-5A in these cells can be conveniently and accurately used to determine the state of activation of the IFN system and its response to various pathological conditions. Our assay procedure has been previously reported in detail (Schattner & Revel, 1988). Briefly, $10^6$ PBMC were lysed by buffer containing 0·5% 'Nonidet'-P40, and the cytosol frozen at −70°C. For the enzyme assay, duplicates of 0·01 ml extract were mixed with poly (ri) : (rC)-agarose beads, and a reaction mixture containing $^{32}$P-alpha-ATP was added. Following incubation, 1 unit of bacterial alkaline phosphatase was added for 1 h at 37°C, the beads were then removed and the supernatant put on an alumina column, washed by glycine–HCl buffer, pH 2, and counted in the $^3$H-channel of a Tricarb scintillation counter. This procedure measures the $(A2'P)_nA$ nucleotides formed by the 2′-5′-oligoadenylate-synthetase, which activate ribonucleases, inhibit mRNA translation, and thus mediate the anti-viral and other important effects of IFN on cells (Revel, 1979).

**Table 1.** Cell-mediated cytotoxicity (CMC), tumour necrosis factor (TNF) and β-endorphin levels in the plasma and stimulated peripheral blood mononuclear cells (PBMC) supernatants of 12 patients with anorexia nervosa (AN) and 12 healthy subjects

| | AN Patients | Control subjects | P |
|---|---|---|---|
| CMC (LU50/$10^6$ cells)* | 4±2 (0·5–5) | 10±3 (4–14) | <0·05 |
| Spontaneous PBMC† TNF (U/ml) | 16±5 (10–29) | 4±3 (0–8) | <0·05 |
| PHA-induced PBMC TNF (U/ml) | 87±54 (34–243) | 84±50 (29–224) | NS |
| LPS-induced PBMC TNF (U/ml) | 60±32 (26–134) | 120±135 (17–441) | <0·1 |
| HeLa-induced PBMC TNF (U/ml) | 71±68 (11–230) | 76±77 (18–294) | NS |
| Plasma TNF | Undetectable | Undetectable | — |
| Plasma β-endorphins (pmol/*l*) | 5·38±3·2 (<3–11·2) | 3·45±1·2 (<3–6) | <0·05 |

Results are mean±s.d. with range in parentheses.
* LU50/$10^6$ cells, lytic units per $10^6$ effector cells (see Materials and Methods).
† Non-stimulated PBMC. LPS, lipopolysaccharide; NS, not significant.

*Beta-endorphins in plasma*
These were extracted by adsorption to anti-β-endorphin antibody sepharose columns, followed by elution with 0·025 M HCl and quantification by radioimmunoassay (Immuno nuclear, Stillwater, MN) with a minimal detectable amount of <3 pmol/*l*.

*Statistical analysis*
The significance of the difference between the groups was evaluated by Student's *t*-test.

## RESULTS

Despite their marked weight loss, the haematological and biochemical profiles of the patients with AN were not significcantly different from those of the healthy controls (not shown). Thus haemoglobin, white blood cells, platelets and differential counts, serum proteins, electrolytes, glucose and liver and kidney function tests were essentially normal in patients and control subjects alike.

Twelve patients with AN and the same number of healthy subjects were studied for CMC and for spontaneous and stimulated TNF production. Low levels of CMC were found in all AN patients; mean CMC was 4±2 LU50/$10^6$ cells (range 0·5–7, median 4 LU50/$10^6$ cells) as compared with a mean CMC of 10±3 LU50/$10^6$ cells (range 4–14, median 9 LU50/$10^6$ cells) in the control group (Table 1) ($P<0·05$), and similar results for normal adults (13±6 LU50/$10^6$ cells), as previously reported by us (Hahn *et al.*, 1989). Spontaneous TNF release by PBMC (in the absence of any inducer) was significantly increased in all AN patients with a mean±s.d. of 16±5 U/ml as compared with only 4±3 U/ml in healthy subjects (Table 1) ($P<0·05$). Induced TNF production, however, was not significantly different between the two groups regardless of whether the inducer was PHA, LPS or tumour cells. However, LPS-induced TNF production was found to be considerably lower in AN than in controls, almost attaining statistical significance ($P<0·1$). To confirm that bioactivity was due to TNF-α, prior incubation of the cytotoxic PBMC supernatants with an excess of anti-TNF-α monoclonal antibody was performed (Tracey, Lowry & Cerami, 1988a), and completely neutralized cytotoxicity in all cases (not shown). No TNF activity was detectable in any of the plasma samples tested.

Mean levels of the IFN-induced enzyme 2-5A in PBMC of 14 AN patients (expressed as ct/min per μg protein) were 68470 ct/min as compared with a mean of 70330 ct/min in the 16 healthy subjects studied (not significant). The levels of 2-5A showed considerable variability, however, the distribution was similar in both groups and individual results showed no correlation to the other variables examined.

Beta-endorphin levels in the plasma of healthy controls ($n=16$) was 3 pmol/*l* or less in all cases but four (3·7, 4, 4·1 and 6 pmol/*l*). Of the 14 patients with AN, seven had β-endorphin levels in plasma which were over 3 pmol/*l* and in three of them (21%), levels were increased over the normal values of 7±2 pmol/*l* (10·8, 11·1 and 11·2 pmol/*l*). The mean±s.d. level of β-endorphins in AN (5·38±3·2) was found to be higher than in the healthy control group (3·45±1·2) ($P<0·05$). However, patients with elevated plasma β-endorphins were not unique in any of the other variables tested.

## DISCUSSION

Our study group of 14 patients with AN met all accepted diagnostic criteria for the disease and had a median weight loss of 30% with no associated illnesses or medication at the time of the study. Nevertheless, their haematological and biochemical profiles were similar to the healthy, matched control group of 16 subjects. Patients with AN have primarily a carbohydrate deficiency with possible associated deficiencies of copper, zinc and iron binding protein, while protein and fat intake are generally considered to be adequate (Cason *et al.*, 1986). Malnutrition is a well-established cause of immunodeficiency in humans (Cameron, 1985) but this is mainly related to protein-energy deficiencies, where recurrent and chronic infection often complicates the picture. Few studies have focused on immune responses in AN, and results are controversial (Armstrong-Esther *et al.*, 1978; Golla *et al.*, 1981; Cason *et al.*, 1986).

We have examined three closely interlinked defence-mediating cytokine systems in AN: TNF, IFN and their relation to CMC. The finding of increased spontaneous production of TNF-α by PBMC of patients with AN was specific (neutralized by monoclonal anti-TNF-α antibody), significant (four-fold increase compared with normal controls; $P<0·05$), and intriguing. Our inability to detect biologically active TNF in plasma despite its constitutive production by PBMC may be due to its

short half-life (6 min) in plasma (Beutler & Cerami, 1986). Therefore, quantification of the production of TNF by PBMC *in vitro* can be used to overcome these problems, and has been suggested to reflect TNF production *in vivo* (Aderka *et al.*, 1985; Zembala *et al.*, 1988). This particularly applies to the yield of TNF by PBMC incubated in medium in the absence of any stimulation (spontaneous production). Stimulated TNF production in AN patients was not significantly different from that of controls, regardless of the inducer used, except for the lower response to LPS (Table 1), suggesting that cells which are already activated to produce a cytokine, may respond poorly to further stimulation (Preble *et al.*, 1983; Bowen, Lane & Fauci, 1985; Magilavy & Rothstein, 1988). The absence of a concomitant activation of the IFN system in AN lends further significance and specificity to the finding of increased spontaneous TNF synthesis. If this is indeed indicative of the situation *in vivo*, then the question of the possible inducer of TNF in AN must be addressed. Increased TNF production in humans has so far been encountered only in diseases due to infectious agents, and possibly in cancer and in autoimmune diseases (Beutler, 1988; Schattner, 1988), none of which are present in AN. TNF, however, is regarded as a mediator of acute phase-reactants (Tracey *et al.*, 1987) which are known to be elevated during stress and in patients with AN (Donohoe, 1984). It can be speculated that the neuroendocrine changes or the nutritional deficiencies which are associated with AN may mediate the induction of TNF, but this remains to be confirmed experimentally. Our recent observation of increased TNF production by inducer-treated PBMC *in vitro* during acute starvation (Vaisman *et al.*, 1989) may be related to the present findings. Others have also found that a dietary change, i.e. supplementation with n-3 unsaturated fatty acids, may alter stimulated TNF production in healthy volunteers (Endress *et al.*, 1989). However, the postulated inducer of TNF in AN has yet to be determined.

CMC was markedly lower in AN patients than in control subjects ($P<0{\cdot}05$). We have made similar observations in acute starvation and others have also noted a depressed natural killer (NK) cell activity in protein-caloric malnutrition which was corrected by proper dietary intake (Salimonu *et al.*, 1983). This apparently does not lead to a greater susceptibility of AN patients to infections or neoplasia (Cason *et al.*, 1986) and does not appear to be due to a defective IFN system, since we found a normal distribution of 2-5A levels. These results are somewhat contradictory to reports of NK cytotoxicity enhancement by TNF as well as by $\beta$-endorphins (Mathews *et al.*, 1983; Ostensen, Thiele & Lipsky, 1987), which were also elevated in some of our AN patients. Finally, we examined the relation between plasma $\beta$-endorphins and the IFN system as assessed by a sensitive indicator, the IFN-induced enzyme 2-5A in PBMC. In one previous study endogenous opiates were found to be increased in AN, returning to normal levels with weight recovery (Kaye, Ebert & Gwirtsman, 1984), and in another study $\beta$-endorphin levels were normal in patients with AN (Gerner & Sharp, 1982). If endogenous opioid peptides are indeed increased in AN as a result of stress or other causes, they may mediate immunosuppression (McCain *et al.*, 1982; Shavit *et al.*, 1984). In particular, the IFN system may be depressed as a result of a structural similarity of IFN-$\alpha$ and endorphins, which may also adversely affect NK cell activity (Greening, 1984; Shavit *et al.*, 1984, 1985). Although we found increased $\beta$-endorphin plasma levels in individual AN patients, most had normal reactivity and the cases of increased $\beta$-endorphins were not associated with lower 2-5A or CMC more than the other patients or the controls.

The group of 14 AN patients included in the present study showed no substantial difference in plasma $\beta$-endorphins or IFN-system activation when compared with healthy controls on a regular diet, but they did have a significantly lower CMC and a consistent increase in spontaneous TNF production *in vitro*. The mechanism(s) mediating these changes remain(s) unclear, as does their significance *in vivo*. However, enhanced TNF production may have deleterious effects in AN (Tracey *et al.*, 1987; Plata-Salaman *et al.*, 1988) such as a central effect resulting in a further suppression of food intake or increased tissue catabolism.

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