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Cell-mediated immunity in anorexia nervosa

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SUMMARY

Twelve patients with anorexia nervosa were studied for cell-mediated immunity in terms of delayed hypersensitivity reactions to recall antigens, lymphocyte transformation responses to T-cell mitogens, and numbers of circulating leucocytes and T-cell subpopulations. Compared to controls, all patients had reduced cutaneous reactions and four were anergic. There was a mild leucopenia in patients and both $T4^+$ and $T3^+$ numbers were slightly reduced. Mean peak transformation responses for patients were slightly lower than controls for phytohaemagglutinin, but not for concanavalin A; however, patients required greater doses of mitogens to elicit peak transformation responses. Plasmas from patients did not contain inhibitors of transformation responses. We conclude that there are functional cellular abnormalities associated with the undernutrition of anorexia nervosa.

Keywords anorexia nervosa lymphocyte transformation delayed hypersensitivity

INTRODUCTION

Protein-energy malnutrition (PEM) is the most common cause of immunodeficiency in man. PEM is not restricted to the developing world and is often seen in hospital patients in developed countries (Weinseir *et al.*, 1979). Depressed cell mediated immunity (CMI) is the most consistent immune deficit in PEM with reduction of delayed hypersensitivity reactions *in vivo* (McMurray, Watson & Reyes, 1981) and depressed lymphocyte transformation responses to mitogens *in vitro* (Neumann *et al.*, 1975). These abnormalities are rapidly corrected by refeeding (McMurray, Watson & Reyes, 1981). T-lymphocyte numbers are reduced in the circulation and this may cause the functional abnormalities (Chandra. 1974, 1977).

PEM, however, rarely occurs in isolation, and is often complicated by infection that may both impair nutrition and alter immune function. In addition, it is difficult to identify which factors of PEM are responsible for altered CMI.

In contrast, patients with anorexia nervosa form a relatively homogeneous population with few of these problems. They are primarily energy deficient (Crisp, 1970; Crisp & Stonehill, 1971) and associated infection is unusual (Crisp, 1967, 1970; Dally, 1969; Bowers & Eckert, 1978); indeed infection may be less common than in normally nourished controls. Immune responses in anorexia nervosa have received little attention, but some reports suggest that aspects of CMI are normal (Armstrong-Esther *et al.*, 1978; Golla *et al.*, 1981; Dowde *et al.*, 1984).

To investigate more fully the relationship of PEM to CMI, we have carried out a detailed study of patients with anorexia nervosa.

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MATERIALS AND METHODS

Subjects. Twelve female patients with anorexia nervosa aged 19–25 (mean 22·8) years were studied on the day after admission to hospital. All had markedly low weights for height assessed by percentage of desirable body weight (median 73·2% range 84–62%, Metropolitan Life tables). One patient was receiving chlorpromazine. Control subjects were 13 healthy females aged 23–55 (mean 35) years, with a median percentage of desirable body weight of 99% (range 78–130%), only one healthy subject had a percentage desirable body weight below 90%. All were fasted overnight, and between 8 and 11 a.m. a blood sample was taken and skin tests with recall antigens performed.

Delayed hypersensitivity skin testing. Antigen solution (100 μl) was injected intradermally into the volar aspect of each forearm. Four recall antigens were used; 1% *Candida albicans* (Hollister Stier Ellehart, USA), 10 u purified protein derivative (PPD, Evans), 3% trichophytin (Hollister Stier Ellehart, USA) and varidase (10 u streptokinase, 2·5 u streptodornase; Lederle, Gosport, UK). Normal saline and 'Mantoux diluent' (Evans), were injected as control reagents. At 48 h, reactions were recorded as the average of two 90° diameters (mm) both of erythema and of induration. Results are expressed as the mean diameters of reactions to individual antigens and as the mean sum of mean diameters of reaction to all antigens with each subject group.

Cell preparation. Plasma was separated from 40 ml venous blood containing heparin (5 u/ml, preservative free heparin; Leo Laboratories, Princes Risborough, UK) by centrifugation at 200 *g* for 10 min at room temperature. The cellular fraction was reconstituted to its original volume in EMEM/PBS-EDTA medium. This was prepared by mixing one volume of Eagle's minimum essential medium, containing Earle's salts (EMEM, GIBCO, Paisley, UK) supplemented with 2 mmol/l l-glutamine (GIBCO, Paisley, UK), 2×10^5 u/l penicillin (Glaxo, Greenford, Middlesex, UK), 0·1 g/l streptomycin (Glaxo, Greenford, Middlesex, UK) and 24·1 mmol/l sodium bicarbonate, with an equal volume of Dulbecco's phosphate buffered saline (PBS, Flow, Irvine, UK) containing 0·01 mol/l ethylenediamine tetra-acetic acid disodium salt (EDTA, Sigma, Poole, Dorset, UK) and adjusted to pH 7·2. Mononuclear cells were isolated on Ficoll-Hypaque density gradients (Ferrante & Thong, 1978) and washed three times in EMEM-PBS/EDTA medium.

Viability of isolated mononuclear cells was determined by trypan blue exclusion (Boyse, Old & Chouroulmlkou, 1964), and the concentration and purity by staining with 0·1% (wt/vol) methylene blue in 173 mmol/l aqueous acetic acid. The number of monocytes was estimated by staining for non-specific esterases (Yam, Lin & Crosby, 1971; Tucker, Pierre & Jordan, 1977) using commercial reagents (Technicon Instruments, Tarrytown, NY, USA). Total and differential leucocyte counts were performed on a 1·0 ml sample of the initial heparinized blood.

T-cell sub-populations. Mononuclear cells were depleted of monocytes by adherence to Petri dishes (Sterilin) at 37°C for 30 min. T-cell populations in the non-adherent population were estimated by indirect immunofluorescence using the monoclonal antibodies OKT3, OKT4 and OKT8 (Ortho Diagnostic Systems) and results expressed as concentrations in peripheral blood.

Lymphocyte transformation responses to mitogens. Mononuclear cells were resuspended in 10% pooled AB or autologous plasma in EMEM, to $0{\cdot}75 \times 10^9$/l viable, non-specific esterase negative cells and 100 μl were distributed into 96-well plates (Nunc, Paisley, UK) containing 50 μl of EMEM or dilutions of purified phytohaemagglutinin (PHA, Wellcome Diagnostics, Beckenham, UK) or concanavalin A (Con A; Miles Yeda) in EMEM. Cultures were incubated at 37°C in a humidified atmosphere of 7% CO_2 in air. At 48 h, 50 μl ^{3}H-thymidine (37 MBq/l EMEM—specific activity 185 GBq/mM—Amersham International) was added and cells re-incubated for 24 h. Cultures were harvested onto glass fibre filters with wash solutions of PBS, 0·61 mol/l trichloroacetic acid and methanol. Radioactivity was measured by liquid scintillation counting and results expressed as dpm/culture.

The effect of patients' plasmas on transformation responses of lymphocytes from a healthy subject. The influence of patients' plasmas on transformation responses was investigated by supplementing cultures of cells from a healthy subject with test plasmas either from patients or from control subjects. Cells were prepared as described above and stimulated by addition of 50 μl PHA (1·15 mg/

l). Test plasmas were evaluated at concentrations (vol/vol) of 10%, 20%, and at 10% test mixed with 10% pooled AB plasma, in three separate experiments.

Statistical methods. Comparison of data from patients and controls were made by Students' *t*-test for the leucocyte populations and lymphocyte transformation peak heights; Wilcoxon's rank sum test for skin testing; and chi-square analysis for the frequency distribution of peak heights of transformation responses at different mitogen doses.

The study was approved by the ethical committee of St Thomas' Hospital. All patients gave informed consent.

RESULTS

Delayed hypersensitivity reactions

Both erythema and induration were reduced in patients compared to control subjects (Table 1). Four patients were unresponsive (anergic) to all antigens and the remainder exhibited reduced sums of diameters of reactions. None of the patients responded to either *Candida albicans* or trichophytin. Only five patients responded to PPD, but the magnitude of reaction in these patients was greater than that for controls, thus the means were little different. The reaction to varidase was markedly impaired in all patients.

Table 1. Delayed hypersensitivity skin reactions of nine patients with anorexia nervosa and seven controls

	Induration		Erythema	
Antigen	Controls	Anorexia	Controls	Anorexia
Candida	4·8±1·2	0	8·4±0·9	0
PPD	6·1±4·1	6·6±3·0	8·5±3·9	8·1±3·4
Trichophytin	1·1±1·1	0	1·4±1·4	0
Varidase	18·4±7·6	4·0±2·4	22·0±7·6	2·4±1·6
Sum of diameters	30·6±9·2*	10·3±3·9*	40·4±8·6**	9·4±4·1**

Controls versus patients, * = not significant; ** = $P<0·01$, by Wilcoxons' rank sum test. Values as mean ±s.e.m. As diameters of reaction (mm) and sum of diameters.

Table 2. Circulating leucocyte populations in controls and anorexia nervosa.

	Controls	Anorexia
Total WBC	5·7±0·4 (12)	4·4±0·5 (11)
PMN	2·9±0·3 (9)	2·2±0·4 (8)
MON	2·5±0·4 (9)	1·9±0·2 (8)
Esterase$^+$	0·16±0·13 (11)	0·19±0·13 (9)
T3$^+$	1·72±0·62 (8)	1·64±0·76 (6)
T4$^+$	1·18±0·48 (7)	1·05±0·71 (6)
T8$^+$	0·59±0·24 (7)	0·61±0·30 (6)
T4$^+$/T8$^+$	2·06±0·1 (7)	1·82±0·2 (6)

Leucocyte counts as $\times 10^9$ cells/l of peripheral blood WBC: white blood cells; PMN: polymorphonuclear leucocytes; MON: mononuclear leucocytes. Number of subjects in parenthesis. Values are mean ±s.e.m.

Cell preparations

Mononuclear cell yields, purity and viability were similar for controls and patients (data not shown). Functional assays and studies of surface markers on T-lymphocytes were therefore assumed to be on comparable cell preparations.

Circulating leucocyte populations

Total leucocyte, polymorphonuclear, mononuclear, $T3^+$ and $T4^+$ numbers were decreased in anorexia nervosa, but monocyte and $T8^+$ numbers were increased compared to controls. These differences were small and did not attain statistical significance.

Lymphocyte transformation responses

Responses of cells from patients or healthy subjects were dependent on the mitogen used and the source of plasma used to supplement the media, but within any individual set of culture conditions there were no significant differences between subject groups in terms of magnitude of either the peak height of mean dose-response curves (Fig. 1) or the mean of individual peak responses (data not shown). However, dose-response curves of cells from patients were displaced to the right so that greater mitogen concentrations were required to elicit reactions of similar magnitude to controls. Analysis of the frequency that peak responses occurred at each dose of mitogen demonstrated significant differences for patients cells compared to healthy subjects stimulated with Con A (AB plasma, $P < 0{\cdot}005$; autologous plasma $P < 0{\cdot}0005$) but not with PHA. Unstimulated cells of patients and healthy subjects incorporated similar amounts of thymidine (data not shown).

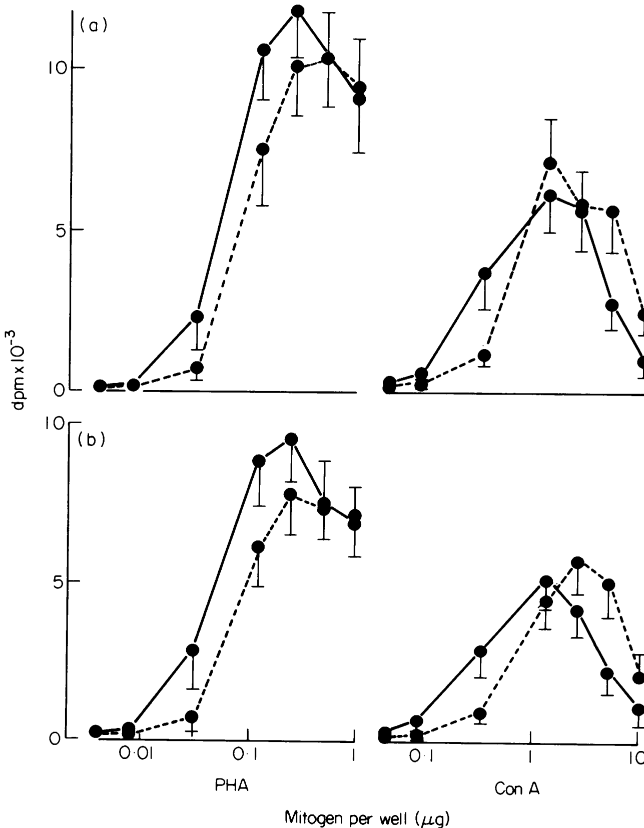

Fig. 1. Mean dose-response curves (±s.e.m.) for controls ($n = 13$), solid line, and anorexic patients ($n = 11$), dashed line, to stimulation by Con A and PHA in cultures supplemented with (a) pooled AB or (b) autologous plasma.

Effect of patients' plasmas on the transformation responses of lymphocytes from a healthy subject
When control cells were cultured with plasma from patients or healthy subjects at 10%, 20% or 10% + 10% AB plasma there were no statistically significant differences in responses (mean dpm ± s.e.m., patients versus controls: 10%: 9857 ± 1419 versus 10597 ± 1441; 20%: 10338 ± 999 versus 9341 ± 1258; 10% + 10% AB: 9431 ± 591 versus 7927 ± 987).

DISCUSSION

These are many reports of depressed CMI in PEM. However, the picture is unclear as PEM is not a discrete entity and represents a spectrum of malnourished states in which concurrent infection may also be present. In contrast, patients with anorexia nervosa form a relatively homogeneous group whose diet is carbohydrate deficient, yet adequate in protein and fat (Crisp, 1970; Russell, 1983; Crisp & Stonehill, 1971); and despite marked malnutrition patients are resistant to infection (Crisp, 1967, 1970; Dally, 1969; Bowers & Eckert, 1978).

Delayed hypersensitivity skin reactions were reduced in anorexia nervosa patients and this is common with PEM in starving children (McMurray *et al.*, 1981) and in malnourished hospital patients (Weinser *et al.*, 1979), but differs from other reports of normal reactivity in anorexia nervosa (Armstrong-Esther *et al.*, 1978; Golla *et al.*, 1981; Dowde *et al.*, 1984).

Reduced numbers of circulating T cells, T-helper and to a lesser extent T-suppressor subsets are a feature of PEM (Chandra, 1974, 1977; Ferguson *et al.*, 1974; Chandra, Gupta & Singh, 1982). Similar, but less marked changes in anorexia nervosa were observed in the present study. Golla *et al.* (1981) did not find changes in T or B lymphocytes numbers in anorexia nervosa; only four of those studied were at their nadir weight.

Lymphocyte function *in vitro* was abnormal in the present study, with displacement of transformation dose-response curves to the right, so that patients' cells required greater doses of Con A and PHA to elicit responses of comparable magnitude to controls. Thus, although an abnormality was present, a detailed dose-response curve was needed to reveal it, and would have been missed if only peak responses had been examined.

The higher peak lymphocyte transformation responses observed by Golla *et al.* (1981) in patients with anorexia nervosa at the nadir of their weight, compared to those being refed, may be explained by changes of the position of peak responses that result from refeeding. Thus, if the refed group were assessed using mitogen concentrations that were supra-optimal—but produce less than maximum stimulation—and compared to the nadir group who were tested at their optimal mitogen concentration, responses of the latter would appear to be increased.

Sera from children with PEM impair transformation responses of normal lymphocytes (Beatty & Dowdle, 1979). However, in the present study there was no evidence for a similar effect in anorexia nervosa, for although peak responses were lower in autologous plasma compared with AB plasma, this occurred for both patients and controls. Furthermore, plasma from patients alone or in combination with AB plasmas did not impair transformation responses of cells from a healthy subject, which suggests that patients' plasmas do not contain inhibitors of, and also contain sufficient nutrients to sustain, lymphocyte transformation responses.

The abnormalities reported in the present study are probably due to multiple factors, as in addition to a primary carbohydrate deficiency (Crisp, 1970; Crisp *et al.*, 1976; Crisp & Stonehill, 1971), moderate copper, zinc and iron binding protein deficiencies occur in anorexia nervosa (Casper *et al.*, 1980; Bryce-Smith & Simpson, 1984; Dowde *et al.*, 1984).

Although the control subjects studied were older than the patient group, there was no correlation between age and differences in the parameters studied for either group. As only small differences in lymphocyte populations were observed in the present study, it is unlikely that these are responsible for the functional abnormalities observed *in vivo* and *in vitro*.

There is no reason to assume that this study suggests predisposition to infection in patients, and the results are not compatible with the observation that infection is unusual in anorexia nervosa (Crisp, 1967; Dally, 1969; Bowers & Ekert, 1978). Indeed, reduced skin reactions in ths study may reflect increased lymphatic clearance of antigen rather than true anergy (Pepys, 1984) as reduced

skin reactivity in patients was not associated with shifts of individual lymphocyte transformation dose-response curves.

In conclusion, CMI is abnormal in anorexia nervosa and is due to a qualitative defect in T cell function which would be undetected in investigations not incorporating full dose-response curve evaluation of lymphocytes transformation responses.

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