ORIGINAL ARTICLE

Impact of anorexia nervosa on activation characteristics of lymphocytes

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Abstract

OBJECTIVES: Anorexia nervosa (AN), a disease of chronic human starvation has a deep impact on the function of several organ systems. We hypothesized that disturbed cellular activation may contribute to complications in AN. We tested our assumption on short-term activation kinetics of lymphocytes.

PATIENTS AND METHODS: Blood was taken from 11 AN and 10 healthy adolescents. Peripheral blood mononuclear cells were isolated and CD4+ lymphocytes were then activated with phythohemagglutinin for the determination of calcium-influx and membrane potential. Moreover, cells were also activated by anti-CD3/anti-CD28 coated beads and three days after the prevalence of interleukin-2 positive CD4+ cells were determined.

RESULTS: After activation, more time was required to reach maximal calcium content in CD4+ cells of AN patients than in those of controls (control vs. AN (median, range): 86 [45–232] vs. 215 [59–235] second, $p<0.05$), but the rate of membrane potential alteration was similar. The number of interleukin 2 positive CD4+ cells was lower in AN (11.50 [7.60–15.30] vs. 13.50 [12.00–22.00] %, $p<0.05$). No association was detected between cell activation and any of clinical or anthropometric data of AN patients.

CONCLUSIONS: These results suggest that AN may have an impact on calcium handling of the cells and, hence, cell activation characteristics. We assume that altered calcium flux kinetics may contribute to complications present in AN.

Abbreviations

AN	- anorexia nervosa
APC	- allophycocyanine
CD	- cluster of differentiation
CDI	- Child Depression Inventory
$DiBAC_4(3)$	- *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol
DSM-IV	- Diagnostic and Statistical Manual of Mental Disorders, American Psychiatric Association
ECG	- electrocardiography
FITC	- fluorescein isothiocyanate
IL	- interleukin
PBMC	- peripheral blood mononuclear cells
IFN	- interferon
NK	- natural killer
PBS	- phosphate buffer saline
PHA	- phythohemagglutinin

INTRODUCTION

Anorexia nervosa (AN) is an extreme form of dietary calorie restriction. In association with starvation, the function of several major organ systems is affected. In advanced stage cardiovascular (Casiero & Frishman, 2006), haematological (Stricker, 1983), endocrine (Krassas, 2003) and musculo-skeletal disturbances (McLoughlin *et al*, 1998) may occur along with an increased susceptibility to infections. Many people with AN also suffer from depression, and it is believed that there may be a link between these two disorders (Corcos el al., 2000; Speranza *et al*., 2005). Energy restriction and nutritional deficiencies may exert a complex effect on cell function. Although effects of AN at cellular levels have not been elucidated yet, based on the signs and symptoms and co-morbid states we hypothesized that the excitability of cells in many tissues may be altered in AN. Theoretically, low excitability of neurons in some brain regions may contribute to depression (Neumeister *et al*., 2006); that of cardiomyocytes to bradycardia and arrhythmias (Sobie *et al*., 2006); that of muscle cells to muscular weakness (Green, 1998); that of endocrine glands to endocrine disturbances (Bonnefont & Mollard, 2003); and that of leukocytes to immune suppression (Freedman, 2006). A central element of cell activation process is a depolarization of cell membrane and a sudden increase of intracellular calcium content that is followed by other stimulatory steps. In this study we aimed to gather data about the cell activation in AN patients with no electrolyte disturbances. As a surrogate marker for the characterization of cellular activation we analyzed the calcium-flux patterns and membrane potential kinetics during ex vivo stimulation of CD4+ lymphocytes and demonstrated striking differences between AN patients and controls. We also measured activated leukocytes' interleukin-2 cytokine production as another measure of lymphocyte activation process.

METHODS

Patients

We investigated 11 Caucasian adolescents (10 girls and 1 boy) with AN. All the patients fulfilled DSM-IV criteria (Diagnostic and Statistical Manual of Mental Disorders, American Psychiatric Association, 1994) for restricting type AN (i.e., a body weight 15% lower than that expected for age and height or failure to make expected weight gain during period of growth, intense fear of becoming fat or gaining weight, disturbed body image, denial of the seriousness of the current low body weight and amenorrhea in postmenarcheal females). In addition to general physical status detailed data were obtained about the severity of depression measured by CDI (Child Depression Inventory). Severe depression was defined as CDI >16. According to previous evaluation, none of them suffered in hypo- or hyperthyreosis and hypercortisolemia. Each of them presented with serum electrolyte values within the normal range. Cardiac function was evaluated by ECG; QTc values were measured. 10 healthy girls of comparable maturity were enrolled as controls. Anthropometric data and clinical characteristics are summarized in Table 1.

At the time of sampling, each subject was free of infection. None of the subjects took nutrient supplements within 1 month and was vaccinated within 6 months before sampling. Informed consent appropriately has been obtained from all patients and caregivers and the Institutional Ethical Committee approved our study.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were separated by a standard density gradient centrifugation (Ficoll Paque, 25 minutes, 400 g, 22 °C) from 10 ml freshly drawn peripheral venous blood collected in Li-heparin treated tubes. PBMCs contained in the interphase were washed twice in phosphate buffer saline (PBS) and stored in 10% (vol/vol) dimethoxy sulfoxide in fetal calf serum at –80 °C until analysis. After thawing, the cells were washed twice in PBS and their viability was assessed by trypan blue exclusion.

Measurement of interleukin-2 secretion

PBMCs were plated at 2×10^5 cells/well into 96-well round-bottom plates (Sarstedt, Germany) in supplemented RPMI-1640 culture medium. Cells were stimulated with anti-CD3/anti-CD28 coated beads (Dynal Inc) for 24 hours at 0.3 bead/cell ratio. At the end of the culture period cells were stained with APC labeled CD4 antibodies. After washing, cells were permeabilised then incubated with FITC–labeled anti-IL-2 monoclonal antibodies (PharMingen, San Diego, CA, USA). The analysis was performed by a BD FACSAria flow cytometer. At least 10,000 cells were analyzed, and the data were processed using the FacsDiVa software. Mean fluorescence values of cytokines were analyzed.

Calcium flux measurements

Fluo-3-AM (Cat. No. F-1242) and Fura Red-AM (Cat. No. F-3021) were from Molecular Probes (Eugene, Oregon, USA). Cells were resuspended in Hank's balanced salt solution at 10^6–10^7 cells/ml; and loaded with dyes (4 μg/ml Fluo-3 AM and 10 μg/ml Fura Red AM supplemented with

Pluronic-F127) for 30 min at 30 °C. Cells were washed once, and stained with APC-labeled anti-CD4. After washing, cells were kept at room temperature (21 C) in the dark. A 500 µl aliquot was warmed to 37 °C prior to Ca flux assay. First a baseline (30 s) level was recorded. Than the tube was removed, 25 µg/ml phythohemagglutinin (PHA) added and the tube replaced. Recording was commenced as soon as cells traversed the laser line and continued for up to 10 minutes (600 s). Data were saved as FCS 3.0 files, and analyzed with Bioconductor's R-flowcyt package. The baseline-normalized ratio of mean fluorescence intensity of Fluo-3 to Fura Red was plotted against time, median fluorescence values per seconds were extracted. From each time units median values were calculated and plotted against time, with a lowess smoothing of f=0.2. Maximum fold-change of intracellular calcium and time to reach maximal calcium content have been measured.

Determination of membrane potential

To measure the changes in membrane potential, as a sign of lymphocyte activation, 300 nM *bis*-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$) dye (Molecular Probes, Eugene, OR, USA) was added to PBMCs stained with APC-labeled anti-CD4 and incubated for 15 minutes, then 25 µg/ml PHA was added before measurement and the fluorescence intensity of the T cell population was analyzed. $DiBAC_4(3)$ was prepared in dimethoxy sulfooxide according to the manufacturer's instructions.

Statistical analysis

Mann-Whitney rank tests was used for the comparison of lymphocyte IL-2 production, maximal fold-change of intracellular calcium and time to reach maximal calcium levels in control and AN subjects. The association between lymphocyte activation kinetics and length of QTc, or severity of depression was tested with Spearman correlation test.

Table 1. Clinical characteristics of healthy subjects and patients. Activation parameters of their stimulated lymphocytes.

Parameter	Healthy subjects	Patients with anorexia nervosa
Clinical characteristics		
number of subjects	10	11
age (years)	15.21 [14.47–17.10]	13.70 [12.05–17.36]
body mass index before disease (kg/m2)	–	22.04 [18.66–25.88]
actual body mass index (kg/m2)	23.41 [19.97–28.51]	14.88 [11.08–17.93]
duration of starvation (months)	–	8 [4–42]
number of subjects with severe depression (CDI > 16)	–	4
heart rate (1/min)	75 [68–83]	56 [39–80]
length of QTc interval (ms)	411 [363–438]	392 [359–442]
Lymphocyte activation markers		
prevalence of IL-2 positive cells (%)	13.50 * [12.00–22.00]	11.50 [7.60–15.30]
maximal fold-change in calcium content	1.98 [0.97–4.61]	1.44 [1.26–1.72]
time to maximal calcium levels	86 [45–232]	215 * [59–235]
slope of membrane-potential alteration (change in fold intensity/10 min)	1.30 [0.003–3.38]	1.20 [0.23–3.40]

* $p<0.05$

RESULTS

We found that the prevalence of IL-2 positive CD4+ lymphocytes after activation was lower in AN than in control subjects (median [range]: 11.50 [7.60–15.30] vs. 13.50 [12.00–22.00] %). The calcium-flux characteristics of CD4+ lymphocytes upon activation differed markedly in AN and healthy subjects as maximal calcium levels developed later in AN samples (215 [59–235] vs. 86 [45–232] second, $p<0.05$) (Figure 1). The dynamics of membrane potential alteration measured by $DiBAC_4(3)$ staining was similar in both groups. Our results are summarized in Table 1.

We also tested the association between lymphocyte activation parameters and patients' clinical status. However, no association was detected between patients' actual body mass index, duration of starvation, hormonal status, QTc intervals, or depression scores and the fold-change of calcium content, calculated time to maximal calcium level or IL-2 positive lymphocytes after stimulation.

DISCUSSION

Anorexia nervosa is a disorder with an extreme form of starvation. Chronic malnutrition is associated with different complications with an impact on several organ systems. As a result, endocrine disturbances, cardiovascular disorders and, in later stages, infections occur (Birmingham *et al.*, 2003; Brown *et al.*, 2005). In this study we tested our hypothesis that disturbed cell activation is present in AN and may contribute to these alterations.

In this study we demonstrated: lymphocytes behave differently after unspecific activation of cell membrane receptors in AN patients and healthy controls. This finding is supported by the results of two independent methods: IL-2 production was somewhat smaller, while time to reach maximal calcium content after activation was nearly double in patients with AN compared with controls.

In our recent study we analyzed cellular networks responsible for T-cell regulation along with cytokine

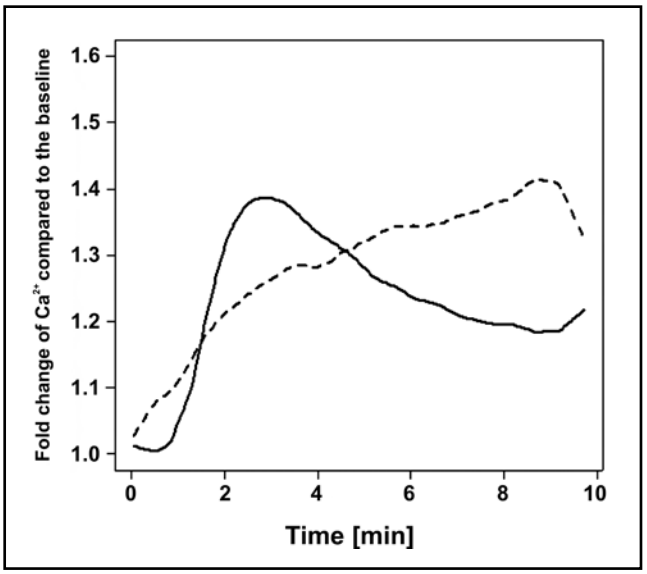

Figure 1. Intracellular calcium changes in lymphocytes after stimulation. Changes of intracellular calcium content after stimulation of lymphocytes of healthy subjects (continuous line) and of patients with anorexia nervosa (dotted line) with 25 µg/ml phytohemagglutinin are expressed as fold change to baseline value on vertical axis. Curves have been calculated using median values of individuals from both groups for each time point.

production capabilities. Similarly to this work, recently we measured lower than normal IL-2 production in another set of AN patients, but found similar prevalence in regulatory T cells, macrophages and dendritic cells and the production of IL-4, IL-10 and IFN-gamma (unpublished observation). This suggests that altered calcium flux kinetics and IL-2 production capacity is rather a marker of cellular impact of AN than a sign of general major immune disturbances in this phase of disease.

Our findings are the first data about calcium handling of lymphocytes in AN. However, some observations have been done in two other experimental models that share some similarity with AN.

Freitag and co-workers aimed to develop an immunological parameter that is sensible to the nutritional state (Freitag *et al*., 2000). They performed calcium-flux measurements and proliferation tests in lymphocyte of starving cats. However, their results were contradictory as they demonstrated higher calcium content, but impaired proliferation activity during starvation. The apparent explanation for this contradiction is the use of ionomycin for lymphocyte activation; this agent acts as a calcium ionophor via a receptor-independent manner and cannot be considered as a physiologic stimulus (Chatila *et al*., 1989). Therefore in spite of higher calcium levels even Freitag *et al.* concluded that acute starvation exerts an immunosuppressive effect. An immunosuppressive effect of starvation is a well documented phenomenon (Cunha *et al*., 2003). Interestingly, calcium-flux kinetics found in AN patients was similar to that reported in lymphocytes of patients with severe depression. Aldenhoff *et al*. enrolled 19 patients with depression and demonstrated that maximal intracellular calcium concentrations develop later and are stabilized at lower levels in activated lymphocytes (Aldenhoff *et al*., 1997). As the association of depression with AN is well documented we raised the question whether alteration of calcium-flux kinetics in AN is attributable to coexisting depression. Therefore we compared AN patients with and without depression. No association between calcium-flux parameters and depression score values was detected that may suggest an independent effect of AN (or starvation) on lymphocyte functions. It is worth to mention, however, that due to low patient number the statistical power is not enough to evaluate this question. Bradycardia generally occurs in AN patients: indeed, median heart rate in our investigated population was also below normal values. Bradycardia is likely a compensatory mechanism against energy restriction and is suggested to be the result of increased vagal tone and decreased thyroxin levels (Facchini *et al*., 2006). In a minority of patients, partly due to severe electrolyte disturbances QTc prolongation and severe arrhythmias may also develop and lead to sudden death. However, the mechanism has not been fully elucidated yet and other mechanisms contributing to disturbed cardiomyocyte activation are also plausible. As calcium influx is a crucial element in the activation of cardiac cells as well (Sobie *et al*., 2006), it was tempting to speculate, however, that altered calcium-flux characteristics measured on AN lymphocytes may be also a marker of a calcium-flux characteristics on cardiomyocytes. Therefore, we tested the association of calcium flux parameters and heart rate and QTc prolongation both in AN and controls, but no correlation was obtained suggesting that lymphocyte calcium flux is not convenient as a surrogate marker to predicting cardiomyocyte dysfunction.

What factors are responsible for altered lymphocyte activation in AN? As membrane potential is the primary determinant of transmembrane electrolyte fluxes, we tested the association of calcium flux with membrane potential. The lack of any link indicates that altered calcium flux characteristics in AN is not due to any unspecific alteration in membrane potential. Instead, it is more reasonable to postulate that the expression of calcium channels or the baseline intracellular calcium content is different in AN from the normal.

The physiologic factors possibly contributing to the alteration of cell activation characteristics are to be clarified. Electrolyte disturbances in advanced stage of AN may occur, but in this study each of our AN patients presented with normal electrolyte levels. While energy-restriction is definitely present in each patient, overt signs of vitamin or other nutrient deficiencies were not detected in any of our AN patients. Selective refeeding of patients with sequential monitoring of calcium-flux characteristics would address this question, but this study is unlikely to be performed due to ethical reasons.

Another mechanism of action is the alteration of endocrine milieu. The hormonal response to hypothalamic regulatory hormones such as thyroid releasing hormone, corticotropin releasing hormone, gonadotropin releasing hormone is disturbed leading altered thyroxin, cortisol and follicle-stimulating hormone/luteinizing hormone levels, respectively (Krassas, 2003). Data support that cortisol impairs calcium flux in NK-cells, another type of leukocytes (Masera *et al*., 1989). On the other hand, thyroxin increased calcium influx and contractility in cardiomyocytes (Kim *et al*, 2000) and increased the expression of calcium channels on muscle cells (Brodie & Sampson, 1990). However, the contribution of endocrine disturbances to the observed alterations in calcium flux is still hypothetical. Although none of our patients presented significant hypercortisolemia or hypothyreosis, in this study we did not focus on the characterization of endocrine milieu and our data do not provide an opportunity to evaluate the significance of endocrine milieu in the altered calcium-flux characteristics of AN lymphocytes.

In conclusion, we demonstrated a delayed response of lymphocytes to activation stimuli in starving patients. Although no association was detected between calcium flux kinetics, IL-2 production and clinical parameters, this finding may indicate a general disturbance in cell activation.

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