

Immune responsiveness in chronic fatigue syndrome

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Summary: We have endeavoured to find immunological indications of chronic virus infection in patients with chronic fatigue syndrome (myalgic encephalomyelitis) and to investigate immune responsiveness to viruses in such patients in comparison with normal subjects and patients with muscular dystrophy. Levels of circulating IgM immune complexes were elevated (above the 95% normal control range) in 10 (17%) of 58 patients with chronic fatigue syndrome, which was not significantly different from the normal controls or from dystrophy controls (by Mann Whitney U test). Levels of IgG complexes were only increased in 10% of patients.

Lymphocyte proliferation in response to concanavalin A (Con A), assessed by increase in ³H-thymidine incorporation, did not differ between 14 patients and 18 normal subjects. The proliferative response to Coxsackie B virus antigen did not differ between chronic fatigue patients and normal subjects when expressed either as an increase in counts or as a stimulation index. Adjustment of the counts in relation to the proliferation response to Con A, as an indication of the overall proliferative response of the cell preparation, did not reveal any hidden difference. IgM antibodies to Coxsackie B viruses were not found in any of 20 patients and in 1 of 20 dystrophy controls. Significant levels of neutralizing antibodies to Coxsackie B viruses 1–5 were found in 6 out of 19 (32%) patients compared with 4 out of 17 (24%) dystrophy controls, which does not differ from currently expected normal incidence. Antibody titres to other respiratory viruses were also not notably different between the patient and control groups.

In conclusion we can find no evidence for a definable viral aetiology for the chronic fatigue syndrome, neither in terms of a persistent infection nor an altered ability to respond to virus.

Introduction

Chronic fatigue syndrome (CFS),^{1,2} myalgic encephalomyelitis (ME)³ and post-viral fatigue syndrome^{4,5} are terms used to describe a state of tiredness and physical and mental fatiguability together with a variety of other variable symptoms for which there is no obvious cause. It has been suggested that the symptoms may be the result of a chronic virus infection that has not been adequately dealt with by the immune system,^{4–6} thus one of the synonyms (post-viral fatigue syndrome) for the condition. The evidence for this stems from the indication that the start of symptoms often, though by no means always, dates from an acute illness attributed to a virus infection.⁶

Investigations of the immune system have shown numerous abnormalities^{4,7} but the most notable feature of these investigations is their inconsistency.⁷ Many investigators, particularly in North America, have considered the chronic fatigue syndrome to be a consequence of infection with Epstein Barr virus (EBV), but enteroviruses, particularly group B Coxsackie viruses (CBV), have

also been suggested to be initiators of the disease process;^{5,6} other infectious agents may also be involved. Evidence for persistence of enterovirus in CFS derives from the detection of CBV IgM antibodies in serum,^{4,6,8} the isolation of enterovirus from the stools,⁵ the detection of circulating enteroviral antigen⁵ and the presence of enteroviral RNA in muscle biopsies from patients.⁹ Among more general indications of disturbed immune function are one report of reduced lymphocyte response to a mitogenic lectin,⁴ the presence of IgM containing immune complexes in the circulation⁵ and variations in T helper and T suppressor cell levels in the blood.⁴ In general, electrophysiological studies of muscle have revealed little if anything abnormal.

The aim of this study was to investigate patients with CFS, firstly to see whether they have circulating immune complexes, either IgG- or IgM-containing, which would suggest a continuing immune response and a continuing source of antigen, and, secondly, to look for IgM antibodies to CBV and other viruses which would imply a recent infection or possibly a continuing one with incomplete switching to IgG production. Also, we

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could see how our patients compare with those of other investigators, who have found some 30% of patients have anti-CBV IgM⁹ which would be useful for purposes of definition in a disease for which the diagnostic criteria are not precise or universally accepted. Indeed it is highly arguable whether CFS represents a single disease or a syndrome consisting of several symptoms of different origin.

Cell-mediated immune responses are also important in controlling virus infections and an indication of such immunity can be obtained from investigation of the proliferative response to viral antigen of blood lymphocytes *in vitro*. Therefore we endeavoured to assess the reactivity of patients to CBV by investigation of their lymphoproliferative response to CBV antigen. Finally, we have looked for antibodies to a variety of common pathogens to see whether there is any indication of a recent or continuing viral infection.

Materials and methods

Patients

Patients were selected from those referred to the muscle clinic on account of unexplained chronic fatigue, either by their family doctors or hospital physicians. The patients fitted the recent Oxford consensus definition,¹⁰ namely, that fatigue is the principle symptom, that it is severely disabling with an effect on physical and mental functioning and that such fatigue should have been present for 50% of the time or more for at least 6 months, with or without an acute viral infection at onset. In those patients in whom the history was clear enough to make an assessment, approximately half (15/31) had a history indicating a viral infection immediately preceding their symptoms. The range of duration of symptoms in the patients investigated was 1 to 20 years, and with those of long standing disease an accurate history around the time of onset was difficult. The age range was 14–61 years and 32 were male and 26 female. The common causes of fatigue had been excluded both on clinical grounds and by extensive investigation either prior to referral or in the course of the clinical assessment, which excluded hypothyroidism, inflammatory myopathy, myasthenia gravis, and anaemia or any other haematological or malignant condition. Patients had a percutaneous muscle biopsy¹¹ for histological and histochemical examination to exclude myopathy. Patients agreed to a detailed psychological/psychiatric assessment as part of the comprehensive evaluation carried out at the clinic, but these results were not involved in the diagnosis of chronic fatigue.

Normal controls were mostly laboratory person-

nel of an approximately similar age range, 21–48 years, and sex distribution, 20 male and 17 female. A group of control patients was also studied; these patients was diagnosed as having Duchenne, Becker, facio-scapular-humeral or myotonic dystrophy.

Immune complex assays

Immune complexes in serum were determined by the polyethylene glycol (PEG) precipitation method⁵ on sera that had been stored at –80°C until use. To obtain a final PEG concentration of 2%, 400 µl serum was mixed with 1200 µl 2.67% PEG; this was then left at 4°C for 3 days. It was then spun at 2000 rev/min for 25 minutes, the supernatant poured off and the precipitate washed with 2% PEG and spun again. The precipitate was then dissolved in 100 µl barbitone saline buffer and the IgM and IgG contents determined by radial immuno-diffusion using antibodies to human IgM and IgG (Sigma) and human serum of known IgM and IgG content suitably diluted as a standard. Results are expressed as µg of precipitated immunoglobulin per ml serum.

Lymphocyte proliferation in response to Concanavalin A and Coxsackie B4

Mononuclear cells were separated from blood using Lymphoprep (Nycomed) and cultured in RPMI 1640 with 20% heat-inactivated human AB serum. Culture mixtures contained 1.25×10^6 cells/ml and consisted of 0.1 ml for the Concanavalin A (Con A) cultures and 0.2 ml for the virus-activated cultures which were carried out in 96-well round bottomed plates. Con A (Sigma) was used at a final concentration of 25 µg/ml. The virus preparation was Coxsackie B4 complement fixing antigen (Virion) diluted 1/4 in 1640 and then 10 µl added to the 200 µl cultures. This had been shown previously to be an optimally stimulating dose. Control antigen (Virion) was used similarly. For the final 6 hours of culture 0.5 µCi ³H-thymidine was added. Cultures were done in quadruplicate and harvested at 3 days for the Con A and 6 days for the virus cultures.

ELISA test for Coxsackie B IgM

This was carried out as previously described^{12,13} using a µ-antibody capture technique. The cut off was taken as the negative serum control mean + 3 s.d.

Coxsackie B neutralization tests

These were carried out as previously described to Coxsackie B 1–5.¹⁴

Complement fixing antibodies

Standard techniques were used¹⁴ to detect complement fixing antibody to herpes simplex, cytomegalovirus, mumps, measles, influenza A, influenza B, adenovirus, *Mycoplasma pneumoniae*, psittacosis, Q fever and respiratory syncytial virus.

IgM antibody to EBV

This was carried out using the Gull commercial kit by immunofluorescence.

Results

Immune complexes

There was no significant difference between the immune complex content of normal control and patient sera as assessed by Mann Whitney U test both for IgM and IgG complexes (Figure 1). If values outside (above) the 95% normal range are considered, because the use of standard deviation is not an appropriate derivation with very skewed distributions, then there are 1 out of 37 normals, by definition, and 10 out of 58 patients with elevated levels of IgM complexes, though three of these are only marginally above the cut off. Of these 10 patients 6 showed elevated levels of IgG complexes (Figure 2); there are no samples with only IgG complexes. Results are expressed as μg of precipitated protein per ml serum; the median values of $8 \mu\text{g/ml}$ for IgM and $3 \mu\text{g/ml}$ for IgG were the same for both normal control and disease groups. A group of 15 muscular dystrophy patients showed a similar range of values to the normal controls (Figures 1 and 2).

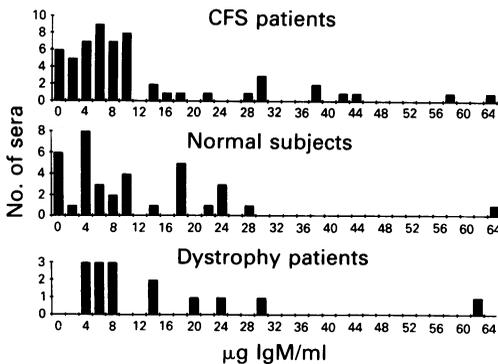


Figure 1 IgM immune complexes in serum from 58 patients with CFS, 15 patients with various muscular dystrophies and 37 normal controls, expressed as μg PEG precipitable IgM per ml serum.

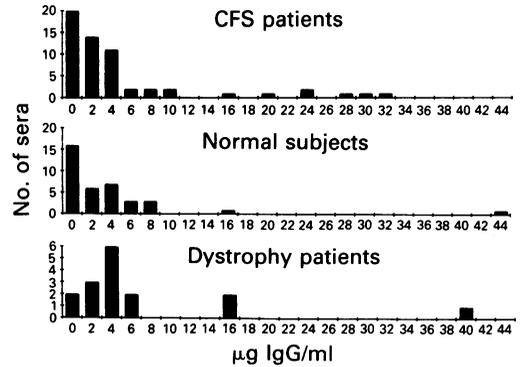


Figure 2 IgG immune complexes in serum from 58 patients with CFS, 15 patients with various muscular dystrophies and 37 normal controls, expressed as μg PEG precipitable IgG per ml serum.

Lymphocyte proliferation

When the response to Con A was expressed as the increase in counts compared with unstimulated cultures there was no difference between patient ($n = 14$) and control ($n = 18$) cell cultures (Figure 3), and indeed the scatter of values looks very similar; if the results were expressed as a stimulation index, a less useful way of expressing Con A stimulation, again there was no difference (data not shown).

The way to express the proliferation data with the virus stimulated cultures was more problematical. The proliferative response can be expressed as a simple increase of the counts in the antigen stimulated cultures above the 'control antigen' cultures or, more usually, as a stimulation index, the ratio of counts in the antigen cultures to the 'control antigen' cultures.¹⁵ The 'control antigen' cultures always showed thymidine incorporation above that of completely untreated cultures, and variably so. The increases in counts are shown in Figure 4a, where there is no difference between the patient and control groups, though there is a suggestion of a bimodal nature of the response in the patient group. However, it was thought that, as the Con A response might give an indication of the overall proliferative capacity of the particular cell population under investigation, then we should also express the CBV response in relation to the Con A incremental response (Figure 5a). The possible bimodal nature of the responsiveness of the patients' cell cultures that is apparent in the uncorrected increase in counts data (Figure 4a) disappears when this is done. Then we calculated the stimulation indices (Figure 4b), and no difference between controls and patients was found, nor was any revealed when the data were

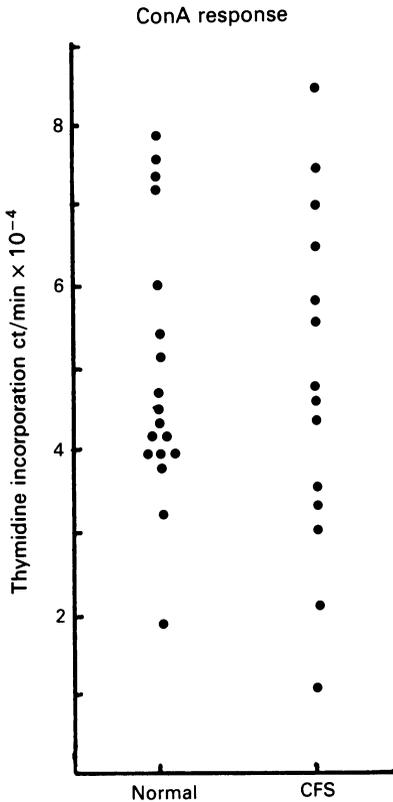


Figure 3 Concanavalin A stimulation of proliferation of blood lymphocytes from 14 patients with CFS and 18 normal controls, expressed as increase in ct/min of stimulated cultures above unstimulated cultures.

corrected in relation to the Con A proliferation (Figure 5b).

Cultures from a small number of muscular dystrophy patients showed values within the range of the normal and disease cultures described.

IgM anti-Coxsackie antibody

A significant level of IgM antibody to Coxsackie B4 was not found in any of 20 patients with CFS and in only 1 out of 20 patients with muscular dystrophies.

Neutralizing antibody to Coxsackieviruses

Levels of neutralizing antibody to Coxsackieviruses B1–B5 in 19 chronic fatigue patients and 17 patients with muscular dystrophies showed no difference in distribution (Table 1).

Other antibody studies

Of 17 muscular dystrophy and 19 CFS patients investigated for antibodies to various respiratory viruses only 1 patient was found to have a significant titre, a dystrophy patient with antibody to *Mycoplasma pneumoniae*. Four CFS patients had a moderately raised titre to influenza B, 2 patients, 1 CFS and 1 dystrophy had moderately raised titres to influenza A and 1 CFS patient had a moderately raised titre to adenovirus. This frequency and distribution of raised titres which are suggestive of recent infection are as expected of any group of subjects. All these patients were negative for anti-EBV IgM.

Discussion

The viral theory of the chronic fatigue syndrome proposes that there is a persistent infection that leads to the symptoms either by a direct effect of virus on muscle, as can be inferred from the demonstration of CBV RNA in the muscle,⁹ or indirectly via a systemic effect of the response to the virus, which can be exemplified by a similarity between some of the symptoms of CFS and those caused by administration of alpha interferon.¹⁶ Another approach to the symptoms proposes that they are mainly of a psychogenic nature,¹⁷ though quite possibly initiated by a viral infection and the accompanying debility, and that symptoms may then be compounded by 'unfitness' accompanying reduced habitual physical activity.¹⁸ The work of Behan and colleagues⁴ which revealed different patterns of T lymphocyte subset abnormality in patients of short or long duration of symptoms is compatible with the concept that patients of different duration of disease may differ in nature. It could be suggested that symptoms may initially relate to a virus infection, but in some individuals behaviour patterns become established and are maintained after the virus infection has resolved.

The suggestion of a persistent viral infection comes largely from investigations of EBV serology, mostly in the USA, which, however, has given a very unclear picture,^{1,7} and also from investigations of CBV in Britain. A higher incidence of neutralizing antibody to CBV, 22% compared with 9% in controls, was found in patients who had recently presented with chronic fatigue syndrome⁶ and more recently a higher incidence of IgM anti-CBV was found in patients, 37% compared with 9% in controls, indicating a recent or persistent infection.⁹ More direct evidence of CBV infection has been provided by obtaining positive cultures of CBV from stools of 22% of patients compared with 7% of controls.⁵ Further, it was possible to detect an enteroviral antigen, VP1, common to most

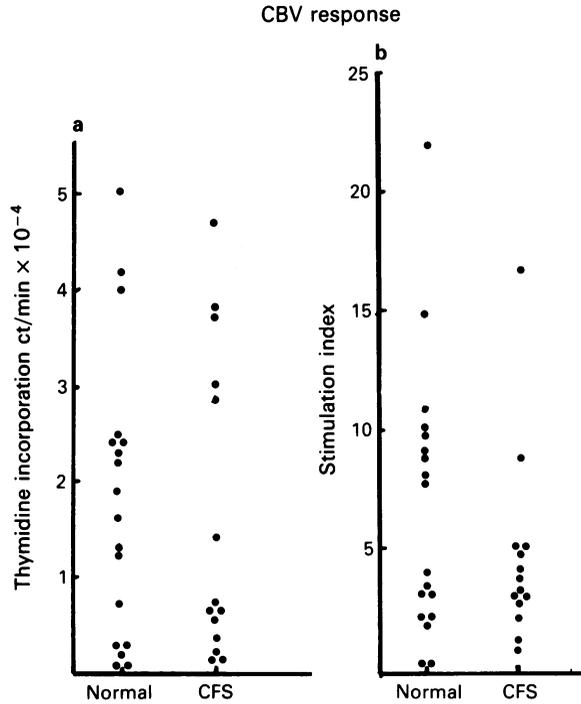


Figure 4 Coxsackie B4 virus stimulation of proliferation of blood lymphocytes from 14 patients with CFS and 14 normal controls expressed as: (a) increase in ct/min of virus stimulated cultures above control antigen stimulated cultures. (b) stimulation index, the ct/min of virus stimulated cultures divided by the ct/min of control antigen stimulated cultures.

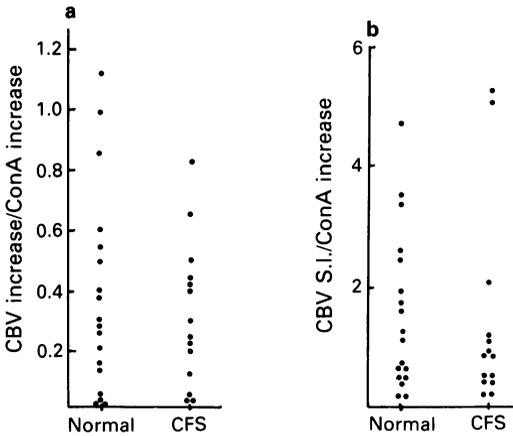


Figure 5 Coxsackie B4 response in relation to Con A response. (a) Increase in ct/min of Coxsackie B4 response divided by Con A increase. (b) Stimulation index of Coxsackie B4 response divided by Con A increase ($\times 100$).

Table I Neutralising antibody to Coxsackie B virus

Patients	Antibody titre		
	≤ 128	256	≥ 512
Muscular dystrophy (17)	7	6	4
Chronic fatigue (19)	6	7	6

enteroviruses, in the serum of 50% of patients, though no control subjects, in this study, showed such antigen.⁵ This study was done on patients with a wide range of duration of symptoms and it was suggested that there is a strong correlation between clinical improvement and disappearance of VP1 in those patients investigated for a period of time. The incidence of VP1 was particularly high in those patients with IgM complexes; 73% of patients had circulating IgM immune complexes.

Our finding is of a very much lower incidence of immune complexes, 17%, and even though sizeable differences between different laboratories using a similar technique can occur, it has to be suggested

that we are looking at different populations of patients, though both we and the London group did not include an infectious onset in our criteria for diagnosis. However, of those with high immune complexes who were assessable for an infectious onset, 5 out of 6 had such an onset. Our finding of an absence of IgM antibody to CBV, in a group of patients at least half of whom had indications of a preceding infection, done in the same laboratory as previous investigations, also suggests a different population of patients, though there are no obvious differences in diagnostic criteria between our patients and those investigated in Glasgow,⁶ though these patients were probably of short duration of disease.

Duration of disease is very likely to be another factor contributing to variability of results between different groups, as already discussed,⁴ some groups mainly having patients of short duration of symptoms,⁶ others having patients with a broad range of duration.⁵ The incidence of 25% for significant levels of neutralizing antibody to Coxsackieviruses in patients with CFS is similar to previous results,⁶ where control incidence was 9%, but more recent control studies have found a higher incidence, 25%, in the normal population,¹⁹ which shows the variations that can occur with time even in a normal population.

The finding that there was no difference in the lymphocyte proliferative responsiveness to CBV, again, does not indicate any particular aberration in response to this virus, though the wide variation in response of the control cells to the viral antigen and of all cells to the 'control antigen' makes this a less precise determination than that of antibody or immune complex levels.

In conclusion we have found no evidence of an abnormal cell-mediated immune response to Coxsackie B virus, nor did we find any evidence for a general defect in T cell activity, as indicated by responsiveness to the T cell mitogen Concanavalin A, in patients with the chronic fatigue syndrome. There was no serological evidence for increased incidence of infection with Coxsackievirus or various respiratory viruses, nor was there any overall difference in the incidence of circulating immune complexes in the patients. However, there is probably a considerable heterogeneity among patients with CFS, particularly with regard to duration of disease and the presence or absence of an acute infectious onset. Therefore the finding that there was a small percentage of patients with elevated levels of immune complexes may be important in defining a sub-group of patients.

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