Antinuclear autoantibodies (ANA) in Gulf War-related illness and chronic fatigue syndrome (CFS) patients

A. SKOWERA*, E. STEWART*, E. T. DAVIS*, A. J. CLEARE†, C. UNWIN†, L. HULL†, K. ISMAIL†, G. HOSSAIN†, S. C. WESSELY† & M. PEAKMAN* *Department of Immunology and †Department of Psychological Medicine, Guy's, King's & St Thomas' School of Medicine, King's College London, London, UK

SUMMARY

It is established that veterans of the 1991 Gulf War have an increased frequency of experiencing multiple symptoms. The underlying mechanism of these ailments is unclear, although they do not correspond to any clearly defined syndrome. The most common symptoms overlap with those of chronic fatigue syndrome (CFS). CFS was recently associated with a novel subtype of antinuclear autoantibody (ANA) that reacts with nuclear envelope (NE) antigens. NE autoantibodies are not known to be linked with any distinct clinical condition, but have been observed in patients with unusual mixed chronic autoimmune disorders and connective tissue diseases. In this study we examined whether NE ANAs are a feature of patients with CFS and symptomatic Gulf War veterans (sGWV). We studied the prevalence of ANA in 130 sGWV, 90 well Gulf War veterans (wGWV), 128 symptomatic Bosnia and Era veterans (sBEV), 100 CFS patients, and 111 healthy control subjects matching for age and sex. We found no significant difference in the prevalence of ANAs between any of the groups. None of the patients/or veterans we studied had ANA of the NE type. Our results show that multisymptom illness due to CFS or related to Gulf War service is not associated with antinuclear autoimmunity.

Keywords   antinuclear autoantibodies   Gulf War related illness   chronic fatigue syndrome   nuclear envelope autoantibodies

INTRODUCTION

Ill health in Gulf War veterans has attracted a great deal of attention, and has been termed 'Gulf War Syndrome' (GWS). There is no sound evidence for a new syndrome. Similar to veterans of previous conflicts, particularly the Vietnam War, where a detailed study of veterans was undertaken, Gulf veterans do report multiple symptoms more frequently than non-Gulf veterans [1]. The aetiology of ill health in Gulf veterans remains controversial. There are a number of proposed explanations for ill health in Gulf veterans: chemical weapons [2–4], post-traumatic stress disorder [5], or that the vaccination schedule designed to protect against exposure to biological weapons could have caused dysregulation in the immune system [6].

Some of the symptoms reported in veterans are similar to those found in various autoimmune conditions. These include joint and muscle aches, chronic fatigue, rashes and sleep disorders. Serological abnormalities have also been reported including hypergammaglobulinemia and abnormal serum proteins [7].

These symptoms overlap with those of chronic fatigue syndrome (CFS), and hence it is not surprising that some Gulf War veterans reporting illness fulfil criteria for symptom-based definition of chronic fatigue syndrome [8,9].

CFS is characterized by severe, disabling fatigue and other symptoms, including musculoskeletal pain, sleep disturbance, impaired concentration, and headaches [10–12]. The cause of CFS is poorly understood. Currently no diagnostic test exists for CFS, which can only be diagnosed after other medical and psychiatric causes have been excluded.

A recent report [13] has suggested that CFS is associated with a substantial increase in the prevalence of a subtype of antinuclear antibody (ANA), namely autoantibodies to nuclear envelope (NE) antigens. This is an uncommon form of ANA, which to date has only been associated, albeit rarely, with unusual mixed, chronic autoimmune disorders including primary biliary cirrhosis, chronic hepatitis, primary Sjögren's syndrome, systemic rheumatic diseases, systemic lupus erythematosus (SLE) and variably with vasculitis, thrombocytopenia, and Raynaud's phenomen [14–19], in each of which it is a rare finding. It has been suggested that such autoantibodies might be one consequence of prolonged immune activation, claimed to be a feature of both CFS and Gulf War-related illness [20,21]. Equally, it has been proposed that environmental toxins may elicit NE ANAs.

Correspondence: M. Peakman, Department of Immunology, Guy's, King's & St Thomas' School of Medicine, Rayne Institute, 123 Coldharbour Lane, London SE5 9NU, UK

E-mail: address: mark.peakman@kcl.ac.uk

In the present report, we study large cohorts comprising epidemiologically derived symptomatic and asymptomatic Gulf War veterans, appropriate military controls, symptomatic civilians fulfilling criteria for CFS, and healthy civilians, in order to test the hypothesis that autoantibodies to NE antigens are a feature of CFS and illness associated with Gulf War service.

MATERIALS AND METHODS

Study groups

The serum samples for this study were drawn from a subset of patients in a previously reported large Phase I epidemiological study of Gulf War veterans [1,22,23]. We now report a nested case control study randomly selected from the larger cohort, in which cases were symptomatic Gulf War veterans, compared to both well Gulf war veterans, and symptomatic military personnel who had served in either Bosnia or were nondeployed military personnel (Era) during the same time frame. The case definition for the symptomatic Gulf War veterans included reporting two or more symptoms and a score of less than 72·2 on the Short Form (SF)-36 Physical Functioning (PF) subscale (i.e. lower than the 10th centile of the SF-36 PF in the baseline control, group).

A total of 348 serum samples from servicemen and service-women (342 males, 6 females; age range 25–59 years) were tested blind to status. Amongst these were 130 symptomatic Gulf War Veterans (sGWV), 90 well Gulf War Veterans (wGWV) and 128 symptomatic military personnel, either veterans of the Bosnia conflict or nondeployed (Era) controls (sBEV). In addition, we studied 100 civilian-CFS patients (47 male, 53 female; age range 18–57 years) that were referred to our specialist clinic and satisfied the standard Center for Disease Control criteria for a diagnosis of CFS [10].

Finally we tested 111 serum samples from control subjects (blood donors of the South-east Thames Blood Transfusion Service); 49 were male and 62 female and the age range was 18–57 years. These control subjects were matched with the CFS sample for age and sex demographics. However, since veterans are predominantly male, the control group for this comparison comprised all male control subjects and one female selected at random.

Serum from a patient with SLE known to be positive for NE ANA was aliquoted and stored at –80°C and used as a positive control for indirect immunofluorescence screening.

In summary therefore, we report a unique study that includes epidemiologically derived symptomatic and asymptomatic Gulf War veterans, appropriate military controls, symptomatic civilians fulfilling criteria for CFS, and normal civilians. Ethical permission for this study was obtained from the King's NHS Trust Ethical Committee.

Indirect immunofluorescence

Anti-nuclear autoantibodies were detected by indirect immuno-fluorescent (IIF) microscopy using commercially available Hep-2 slides (Binding Site, Birmingham, UK). IIF was performed according to the protocol suggested by the manufacturer. In brief, the slides were incubated with the human sera at 1:40 dilution in PBS, pH 7·2 for 30 min in a humid chamber at room temperature. After washing (3 times) with PBS, the autoantibodies were revealed using rabbit antihuman IgG conjugated with FITC (Dako Ltd, Ely, UK) in 1:50 dilution and incubation was carried for another 30 min at room temperature in the dark. The slides were then washed 3 times and examined under a fluorescence microscope (Olympus BX40, Olympus, Southall, UK). In each assay negative control sera as well as positive control sera were always included. Slides were examined independently by three of the authors (AS, ES and ETD, of which ETD has in excess of 20 years experience of diagnostic IIF assays) who were blinded to the source of the samples. Samples testing positive for ANA were titrated in doubling dilutions to end-point extinction.

Statistical analysis

The distribution of ANA amongst the different study groups was compared by Fisher exact probability test using 2×2 tables.

Results

Antinuclear antibodies detected using indirect immunofluorescence (IIF) microscopy

We detected ANA in all of the study groups, with several different patterns of staining being observed (Table 1). The sGWV group had 8% positives (10/130), amongst which the ANA

Table 1. Antibody staining patterns in study groups

Patterns	Homogeneous	Speckled	Nucleolar	Nucleolar dots	Nucleolar membrane	Speckled/ nucleolar	Nucleolar/ homogeous	Total [sum of positives (%)]
Study groups								
sGWV (*n* = 130)	4	3	2	1				10 (8%)
wGWV (*n* = 90)		1	2					3 (3%)
sBEV (*n* = 128)	7	2	1	1				11 (9%)
HC group A (for GW group) (*n* = 51)	2	2	2					6 (12%)
CFS (*n* = 100)		9	7			1	1	18 (18%)
HC group B (for CFS) (*n* = 111)	7	6	4		1			18 (16%)

sGWV, sick Gulf War veterans; wGWV, well Gulf War veterans; sBEV, sick Bosnia and Era veterans; CFS, Chronic Fatigue Syndrome patients; HC, Healthy controls.

patterns were mainly homogeneous and speckled (7/10), or nuclear/nuclear dots (3/10). wGWV had 3% positives (3/90) of whom two out of three had a nuclear staining pattern and one speckled. The sBEV group had 9% positives (11/128) of which nine were homogeneous and speckled, and two had nuclear/nuclear dots. The results were compared with 51 age and sex matched healthy controls. This group had 12% (6/51) positive results (various patterns).

We found no statistical difference in the frequency and prevalence of ANA amongst the veterans study groups when comparing with each other or with matching healthy control subjects (Table 2).

CFS patients had a similar prevalence of ANA (18%, 18/100) to the matched healthy control subjects (16%, 18/111). In the CFS group the positive samples were mainly of speckled pattern (9/18); some had the nuclear pattern (7/18) and the rest had other patterns (2/18). 59% of the positive results were found in female CFS patients.

In the matching healthy control subjects, amongst various patterns, we found one individual with a pattern characteristic of NE antigens (Fig. 1) and 67% of the positive results were female subjects.

To examine whether the strength of positivity of ANA differed between study groups, positive samples were titrated to extinction. None of the ANAs tested were positive beyond a dilution of 1/160. In each of the study groups, approximately half of the positive samples had titres of 1/80 and half of 1/160. There was

Table 2. Results for statistical comparison (Fisher exact probability test) all study groups and appropriate control groups (all $P > 0.05$)

	P-value	Unadjasted odds ratio	95% CI
sGWV versus wGWV	0·25	2·41	0·6, 9·0
sGWV versus sBEV	0·82	0·89	0·4, 2·2
sGWV versus HC	0·39	0·62	0·2, 1·8
CFS versus HC	0·86	1·01	0·5, 2·3
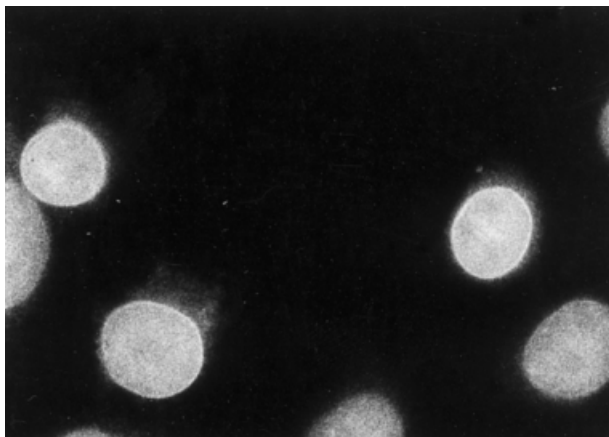
Fig. 1. Photomicrograph of indirect immunofluorescence staining pattern of autoantibodies to nuclear envelope (NE) antigens, demonstrated by characteristic peri-nuclear ring pattern. Substrate, Hep2 cells. Original magnification × 40.

no significant difference in the distribution of high and low ANA titres between any of the study groups.

DISCUSSION

Since the Persian Gulf conflict ended in 1991 there has been considerable interest in the increased number of Persian Gulf War veterans from coalition forces reporting unexplained illness. Many hypotheses have been proposed to explain this phenomenon [2–7,24] but no single explanation accounts satisfactorily for the symptoms. In the present study, we sought evidence for the presence of ANAs in patients with Gulf War-related illness. Our study was prompted by three considerations. First, many of the symptoms experienced by Gulf War veterans are similar to those experienced by patients with CFS [24]. The aetiology of CFS is also unknown, but a recent report has suggested that CFS patients have a high prevalence of a rare ANA, reactive with nuclear envelope antigens and giving a characteristic ring pattern on immunofluorescence with Hep-2 cells as substrate [13]. For this reason we sought evidence of a serological link between CFS and Gulf-War related illness.

Our second consideration was that many of the symptoms reported by Gulf-War veterans are reminiscent of those seen in the nonorgan-specific autoimmune diseases. These disorders are characterized by a high prevalence and wide variety of different autoantibodies to nuclear antigens, giving rise to numerous patterns of immunofluorescence on Hep-2 cells and numerous molecularly defined nuclear antigens [14,16–18,25]. In the present study we sought evidence of a similar, widespread manifestation of antinuclear autoimmunity.

Our third reason for carrying out the study was to examine whether there was serological evidence, in the form of multiple autoantibodies, to support the proposal that both CFS and Gulf-War related illness exhibit features of a T helper-2 (Th2) type of polarized immune response. It has been suggested that Gulf War veterans were exposed to stimuli that would strongly favour a T helper 2-biased (Th2) cellular immune response [6], such as:

- multiple vaccinations received in a short space of time (large antigen load can deviate the immune response towards Th2 [26,27];
- vaccines, including those against biological warfare agents, given in stressful active service conditions (high levels of stress have been shown to deviate the immune response towards Th2 [28–31]);
- pertussis toxin used as an adjuvant by United Kingdom forces in the Gulf (which causes Th2 deviation [32,33]).

Antibody production by B lymphocytes is mainly promoted and supported by the Th2 cytokines IL-4, IL-6 and IL-10 [34], and in the context of an autoimmune response, production of autoantibodies may be considered to represent evidence of a shift towards Th2 type immunity [35–37].

Our results demonstrate that there is no increase in prevalence of ANA in either patients with CFS or those with Gulf War-related illness compared with control subjects. None of the patterns of ANA seen were especially characteristic of either condition. In addition, we did not detect any NE patterns of ANA.

These results differ substantially from those reported by Konstantinov *et al.* who found the sera of 52% of the CFS patients' reacted with nuclear envelope antigens by IIF [13]. As far as could be ascertained the protocols were identical other than

that Konstantinov *et al.* [13] used a different source of Hep-2 slides to those employed in our study. It is conceivable that the results could have arisen as a consequence of differences in sensitivity, although we were able to detect other ANAs and one NE pattern in a control subject. Another difference is the higher percentage of female CFS subjects used in that study (85%), which is greater than in our study (53%). It is known that the general prevalence of autoantibodies is greater in female population, as shown in the healthy control subjects in the present study (67%), and this could account for the discrepancy, but not for the lack of association with either CFS or Gulf War related illness.

Nishikai *et al.* [38] also failed to detect autoantibodies reactive with nuclear membrane components by IIF amongst Japanese CFS patients, seeing instead a predominant 'diffuse/speckled' pattern on Hep-2 cells.

Another study [39] detected 15% ANA positive cases amongst CFS patients. In that paper they combined the results from two different chronic fatigue clinics (Boston and Seattle) in which there were differences in means of recruitment of CFS patients (different method of prescreening) as well as significantly varying results for ANA (Boston −8%; Seattle −20%).

Two additional studies found some evidence of ANA prevalence (50%), in Japanese children with nonspecific chronic symptoms similar to CFS (diagnosed as 'autoimmune fatigue syndrome' in these studies) [40,41]. Although no studies have been carried out previously on autoimmune phenomena in war veterans, extensive analysis of white blood cell number and phenotype, and serum immunoglobulin levels, revealed no abnormality in veterans of the Vietnam War [42].

We conclude that patients' multisymptom illness due to CFS (seen in our specialist clinic) and illness related to Gulf War service are not associated with increased frequency of antinuclear autoimmunity.

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