Journal of General Virology (2001), **82**, 3011–3019. Printed in Great Britain

Circulating tumour necrosis factor-α and interferon-γ are detectable during acute and convalescent parvovirus B19 infection and are associated with prolonged and chronic fatigue

Jonathan R. Kerr,[1] Faraj Barah,[2] Derek L. Mattey,[3] Ian Laing,[4] Stephen J. Hopkins,[5] Ian V. Hutchinson[6] and David A. J. Tyrrell[7]

[1] Department of Microbiology, Royal Brompton Hospital, National Heart and Lung Institute, Imperial College School of Medicine, Sydney Street, London SW3 6NP, UK

[2,5,6] Department of Virology[2], North Western Injury Research Centre[5] and School of Biological Sciences[6], University of Manchester, Manchester, UK

[3] Staffordshire Rheumatology Centre, Stoke-on-Trent, UK

[4] Department of Biochemistry, Manchester Royal Infirmary, Manchester, UK

[7] Formerly of the MRC Common Cold Unit, Salisbury, Wiltshire, UK (now closed)

To investigate whether cytokine responses may have a bearing on the symptoms and outcome of parvovirus B19 infection, circulating cytokines were measured during acute infection ($n = 51$), follow-up of acute infection ($n = 39$) and in normal healthy controls ($n = 50$). At acute B19 virus infection (serum anti-B19 IgM-positive), patients ranged in age from 4 to 54 years, with a mean age of 28·2 years. The male:female ratio was 1:4·1 and symptoms were rash ($n = 15$), arthralgia ($n = 31$), fatigue ($n = 8$), lymphadenopathy ($n = 4$), foetal hydrops ($n = 3$), transient aplastic crisis ($n = 2$), neutropenia ($n = 2$), myelodysplasia ($n = 1$), thrombocytopenia ($n = 1$) and pancytopenia ($n = 1$). Of these patients, 39 were contacted after a follow-up period of 2–37 months (mean of 22·5 months). In comparison with normal controls, detectable IL-6 was associated with acute B19 virus infection (26%; $P = 0{\cdot}0003$), but not with follow-up (6%; $P = 0{\cdot}16$). Detection of interferon (IFN)-γ was associated with acute B19 virus infection (67%; $P < 0{\cdot}0001$) and follow-up (67%; $P < 0{\cdot}0001$). Detection of tumour necrosis factor (TNF)-α was associated with acute B19 virus infection (49%; $P < 0{\cdot}0001$) and follow-up (56%; $P < 0{\cdot}0001$). IL-1β was detected in acute infection (20%), but not at follow-up. At acute B19 virus infection, detection of serum/plasma IL-6 was associated with rheumatoid factor ($P = 0{\cdot}038$) and IFN-γ ($\geqslant$ 7 pg/ml) was associated with fatigue in those patients of $\geqslant$ 15 years of age ($P = 0{\cdot}022$). At follow-up, fatigue was associated with IFN-γ ($\geqslant$ 7 pg/ml) and/or TNF-α ($\geqslant$ 40 pg/ml) ($P = 0{\cdot}0275$). Prolonged upregulation of serum IFN-γ and TNF-α appears to represent a consistent host response to symptomatic B19 virus infection.

Introduction

Human parvovirus B19, discovered in 1975 (Cossart *et al.*, 1976) and first linked with human disease in 1981 (Pattison *et al.*, 1981), is a small single-stranded DNA virus classified as a member of the family *Parvoviridae*, genus *Erythrovirus*, whose tropism is primarily for erythroid precursors. Parvovirus B19 is the only parvovirus that has been clearly linked with disease in humans. B19 virus replicates only in human cells and is autonomous, i.e. not requiring the presence of a helper virus.

Specific antiviral antibody production is thought to represent the major defence against B19 virus, as human normal immunoglobulin (Ig) frequently clears the virus from peripheral blood and results in clinical improvement in immunosuppressed persons (Kurtzman *et al.*, 1989; Schwarz *et al.*, 1990) and also as specific antibody protects against infection both *in vivo* and *in vitro*. Following acute B19 virus infection, there is

Author for correspondence: Jonathan Kerr.
Fax +44 207 351 8443. e-mail j.kerr@ic.ac.uk

a progressive change in the anti-B19 antibody subclass and specificity, with increasing recognition of conformational VP1/2 epitopes with a decreasing recognition of linear VP1/2 epitopes, which may be accompanied by a switch from IgG3 to IgG4. In addition, cytotoxic lymphoproliferative responses have been demonstrated recently against VP1/2 antigens in persons with past B19 virus infection (von Poblotzki *et al.*, 1996). The particular progression of these events in an individual may be mediated by the type of $CD4^+$ T-cell response (Franssila *et al.*, 1996; Wagner *et al.*, 1995), as has been shown for other viruses (Goodbourn *et al.*, 2000; Hunter & Rainer, 2000).

B19 virus infection has been associated with an extremely wide variety of clinical manifestations. Acute B19 virus infection may be asymptomatic in 50% of infected children and in symptomatic persons is associated classically with childhood rash illnesses, erythema infectiosum, arthralgia, foetal death, transient aplastic crisis (TAC) in those with shortened red cell survival and pure red cell aplasia in immunocompromised persons (Kerr, 2000). Less common clinical associations of B19 virus infection include various skin eruptions, haematological disorders, such as neutropenia, hepatobiliary disease, neurological disease and rheumatic disease, including chronic fatigue syndrome (CFS) (Kerr, 2000). However, except for predispositions such as shortened red cell survival and immunosuppression, factors that determine symptomatology following B19 virus infection are not understood. Therefore, we hypothesized that variation in the cytokine response to B19 virus infection may play an important role in resultant clinical manifestations.

Although there are few reports on cytokine responses to B19 virus infection, cytokine dysregulation has been linked with B19 virus-associated haemophagocytosis and pancytopenia (Watanabe *et al.*, 1994), arthritis (Wagner *et al.*, 1995) and myocarditis (Nigro *et al.*, 2000). Parvovirus B19 NS1 protein upregulates IL-6 transcription by binding the NF-κB site in the IL-6 promoter, suggesting that IL-6 upregulation may be important in the pathogenesis of B19 virus infection (Moffatt *et al.*, 1996). Corcoran and colleagues demonstrated that lymphocytes from convalescent adults produced high levels of IL-2 and interferon (IFN)-γ in response to both VP1 and VP2 proteins (Corcoran *et al.*, 2000).

To investigate the possibility that cytokine responses to acute parvovirus B19 infection have a bearing on the clinical manifestations and outcome of infection, we examined B19 virus-infected patients both at the time of acute infection and again at follow-up for B19 virus markers, autoantibodies and serum cytokines [IL-1β, IL-2, IL-6, IL-10, tumour necrosis factor (TNF)-α and IFN-γ].

Methods

■ **Patient enrolment, assessment and serum collection.** Patients with acute B19 virus infection ($n = 51$) were identified by the Department of Virology, Manchester Royal Infirmary, UK, from 1998 to 2000 by detection of serum anti-B19 IgM. In all cases, these patients were bled at the time of, or shortly after, the onset of new symptoms, which had not been present previously, and all patients were well and healthy (by their own assessment) prior to the onset of these symptoms. In all cases, the serum anti-B19 IgM test was positive, while tests for markers of acute infection by other agents were negative. Following this, patients were contacted and, with their consent, visited at home in order to obtain a detailed history and to draw a blood sample. A total of 39 patients was successfully followed up. Clinical details on these patients will be described elsewhere. A total of 50 normal healthy control persons was also enrolled in the study. These persons were employed by the Manchester Royal Infirmary, UK, and were enrolled with their consent.

Blood samples were collected in pyrogen-free blood collection tubes using the Vacutainer system (Becton Dickinson), separated by centrifugation and serum stored at −20 °C until the time of analysis. DNA was extracted from EDTA–anticoagulated blood by phenol–chloroform extraction. All patient and control serum samples were tested for anti-B19 VP2 IgM, anti-B19 VP1/2 IgG, anti-B19 NS1 IgG, B19 virus DNA, rheumatoid factor (RF) and antinuclear antibodies (ANA). Serum and EDTA-treated plasma were tested for the cytokines IL-1β, IL-2, IL-6, IL-10, TNF-α and IFN-γ. All DNA samples were tested for B19 virus DNA.

■ **Qualitative parvovirus B19 antibody testing.** Serum anti-B19 VP2 IgM was detected by ELISA (Biotrin), according the manufacturer's instructions. Serum anti-B19 VP1/2 IgG and NS1 IgG were detected by Western blot (Mikrogen), according to the manufacturer's instructions. Serum was tested also for anti-B19 VP1 IgG by indirect immunofluorescence staining using recombinant baculovirus-infected Sf9 insect cells expressing B19 virus VP1 (Kerr *et al.*, 1995).

■ **Nested PCR for B19 virus DNA.** DNA was extracted from samples of 100 µl of serum by phenol–chloroform extraction. A 5 µl sample of DNA extract was added to 45 µl of a PCR mixture containing 10 mM Tris–HCl (pH 8·3), 50 mM KCl, 1·5 mM $MgCl_2$, 0·01% (w/v) gelatin, 200 µM dATP, 200 µM dCTP, 200 µM dGTP, 200 µM dTTP, 0·5 µM primer 1 (5′ AATACACTGTGGTTTTATGGGCCG 3′), 0·5 µM primer 6 (5′ CCATTGCTGGTTATAACCACAGGT 3′) and 1·25 U Ampli*Taq* DNA polymerase (Perkin Elmer). The second reaction utilized the same mixture (above), but with 0·5 µl primer 2 (5′ GAAAACTTTCCATTTAATGATGTAG 3′) and 0·5 µl primer 5 (5′ CTAAAATGGCTTTTGCAGCTTCTAC 3′), instead of primers 1 and 6 (Durigon *et al.*, 1993). Primers 1, 2, 5 and 6 correspond to nucleotides 1399–1422, 1498–1525, 1576–1600 and 1659–1682, respectively, of the NS1 gene of B19 virus genomic DNA (Shade *et al.*, 1986). For both first and second steps, dsDNA was initially denatured for 6 min at 95 °C, followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. PCR using primers 1 and 6 yielded a product of 284 bp and PCR using primers 2 and 5 yielded a product of 103 bp (Durigon *et al.*, 1993). Positive controls contained viraemic serum at dilutions of $1:10^3$ and $1:10^6$; the negative control contained distilled water. PCR products were subjected to gel electrophoresis using a 1 kb ladder as the molecular mass marker (Life Technologies) and the separated DNA molecules were stained with 1% ethidium bromide and visualized by ultraviolet transillumination. The sensitivity of this PCR assay has been shown to be in the order of 1–10 genome copies (Durigon *et al.*, 1993).

■ **Autoantibody measurement.** Sera were tested for the presence and titre of RF using the Serodia-RA Latex Particle Agglutination kit (Fujirebio), according to the instructions of the manufacturer. ANA were detected using human epithelioma type 2 cells by standard indirect immunofluorescence staining.

■ **Cytokine ELISA.** Quantification of plasma cytokine levels (IL-1β, IL-2, IL-6, IL-10, TNF-α and IFN-γ) was performed in duplicate using

Table 1. Acute B19 virus infection

Results of χ^2 analysis of the relationships between clinical manifestations, B19 virus markers, autoantibodies and cytokines. OR with 95% CI (in parentheses) and *P* values are shown in relation to IL-1β, IL-6, TNF-α (> 250 pg/ml) and IFN-γ ($\geqslant$ 7 pg/ml).

Patient	Detectable IL-1β ($n = 10$)	Detectable IL-6 ($n = 13$)	TNF-α > 250 pg/ml ($n = 10$)	IFN-γ $\geqslant$ 7 pg/ml ($n = 30$)
Age $\leqslant$ 20 years at onset ($n = 12$)	OR 5·67 (1·25–25·73) $P = 0·05$	–	OR 5·5 (1·21–25·01) $P = 0·05$	–
Males ($n = 10$)	OR 6·45 (1·18–45·01) $P = 0·027$	–	–	–
Fatigue ($n = 8$)	–	–	–	OR 6·36 (1·46–27·67) $P = 0·022$*
RF ($n = 16$)	–	OR 6·98 (1·09–230·01) $P = 0·038$	–	–
IL-1β ($n = 10$)	–	–	OR 152 (9·6–6040·12) $P < 0·0001$	–

*In those $\geqslant$ 15 years of age at onset.

specific ELISA kits (Diaclone), according to the manufacturer's instructions. For IL-1β, the six standards ranged from 15·6–500 pg/ml and the minimum detectable dose was less than 5 pg/ml. For IL-2, the six standards ranged from 31·25–1000 pg/ml and the minimum detectable dose was less than 10 pg/ml. For IL-6, the six standards ranged from 6·25–200 pg/ml and the minimum detectable dose was less than 2 pg/ml. For IL-10, the six standards ranged from 12·5–400 pg/ml and the minimum detectable dose was less than 5 pg/ml. For TNF-α, the six standards ranged from 25–800 pg/ml and the minimum detectable dose was less than 10 pg/ml. For IFN-γ, the six standards ranged from 12·5–400 pg/ml and the minimum detectable dose was less than 5 pg/ml. In each case, the optical density of known standards was used to construct a calibration curve that enabled the calculation of the cytokine level in each test sample using the optical density of that sample plotted against the calibration curve; the mean cytokine values $\pm$SD were then calculated for each sample.

■ **IL-6 bioassay.** As B19 virus NS1 protein has been shown to upregulate IL-6 transcription (Moffatt *et al.*, 1996) and as we found detectable IL-6 in only 26% cases of acute B19 virus infection and 6% of follow-up cases, we tested these samples by IL-6 bioassay to determine any additional bioactivity. Thawed plasma and serum samples were heated at 56 °C for 30 min before bioassays using the B9 hybridoma. Briefly, samples were serially diluted in microplates with RPMI medium (containing 5% foetal calf serum, gentamicin at 50 U/ml and 2-mercaptoethanol at 50 µmol/l) from an initial dilution of 1:81 for serum and 1:3 for plasma samples. The B9 cells were diluted in the same medium and added to the microplate wells in an equal volume to give a final concentration of $0·5–1·0 \times 10^{-4}$ cells/ml. After 3 days, the plates were centrifuged at 300 ***g*** for 10 min and the supernatants 'flicked' sharply from the microplates. After the addition of 100 µl of 3-(4,5-dimethyIthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) at 1 mg/ml in RPMI, the cultures were agitated for 20 s and incubated at 37 °C and 5% CO_2 for 4 h. A 100 µl vol. of 10% SDS in 0·01 mol/l HCl was added to dissolve the formazan precipitate, prior to determining the absorbance values at 570 nm on a microplate reader (Holt *et al.*, 1991). IL-6 concentrations were calculated by reference to the 1st International Standard for IL-6 (NIBSC).

■ **Statistical analysis.** The strength of association between clinical variables/B19 virus markers with cytokine levels was estimated using odds ratios (OR) and relative risks with 95% confidence intervals (CI). Levels of significance were determined using 2 × 2 contingency tables by either χ^2 or Fisher's exact analysis. Where appropriate, Yates' (χ^2) or Haldane (OR) corrections were applied.

Results

Correlation of IL-6 ELISA and bioassay results

Of the 51 patients with acute B19 virus infection, 13 patients had detectable levels of IL-6 by ELISA; five of these were also detected by IL-6 bioassay. Three of 39 patients at follow-up had detectable IL-6 levels by ELISA, of which none was detected by IL-6 bioassay. In all cases, plasma IL-6 levels of less than 40 pg/ml by ELISA were not detected by the bioassay, but ELISA levels of greater than 40 pg/ml were detected by the bioassay, but were reduced by a factor of 10. Therefore, ELISA was approximately 10-fold more sensitive than the bioassay. Statistical tests were applied to the IL-6 ELISA results only.

Normal healthy controls

The ages of these persons ranged from 22 to 63 years, with a mean age of 33·4 years. The male:female ratio was 1:3. All persons had normal blood haematology, biochemistry and erythrocyte sedimentation rates (ESR). Of the 39 control persons, 37 were positive for serum anti-B19 IgG and eight had NS1 antibodies. All control persons were negative for serum B19 virus DNA and for the cytokines IL-1β, IL-2, IL-6 and IFN-γ. Six controls had detectable plasma IL-10 (all less than 2 pg/ml). Seven control persons had detectable TNF-α, but in only three of these was this level higher than 30 pg/ml.

Table 2. Symptoms, B19 virus markers, autoantibodies and serum cytokines

Status of 13 patients with fatigue (not relieved by rest and resulting in significantly reduced activities), which persisted since the time of acute B19 virus infection. All patients had fatigue for the entire follow-up period, the onset of which coincided with the onset of acute B19 virus infection.

	Patient												
	1	2	6	7	8	9	11	20	22	29	31	32	37
Sex	M	F	F	F	F	F	F	F	F	F	F	M	F
Age at onset of B19 virus infection/fatigue (years)	26	42	44	25	34	35	40	27	46	46	36	46	34
Time since acute B19 virus infection (months)	4	19i	23	24	26	22	7	30	27	26	25	30	27
Diagnosis of fatigue*	PF	CFS	PF	PF	CFS	PF	CFS	CFS	PF	PF	PF	CFS	PF
Arthralgia (without swelling/redness)	–	+	+	–	+	+	+	+	+	+	–	+	–
Arthralgia duration (months)†	–	19	23i	–	26	2	7	2	1	26	3	30	1
Other‡	–	RDA	CH	–	Th	–	C	–	–	–	–	–	–
B19 virus markers/autoantibodies													
Serum B19 virus DNA	+	+	–	–	+	–	+	–	+	–	–	+	–
Leukocyte B19 virus DNA	+	–	–	–	–	–	+	–	–	–	–	–	–
Serum anti-B19 VP1/2 IgG	+	+	+	+	+	+	+	+	+	+	+	–	+
Serum anti-B19 NS1 IgG	+	+	–	–	–	+	–	+	–	–	+	–	+
RF	–	+	+	–	+	+	–	–	–	+	–	–	+
ANA	–	–	–	–	H300§	–	–	–	–	–	–	–	–
Serum cytokines (pg/ml)													
IL-1β	–	–	–	–	–	–	–	–	–	–	–	–	–
IL-2	–	–	38·02	–	–	–	–	–	–	–	–	–	–
IL-6	–	39·45	–	–	–	–	–	–	–	–	–	–	–
IL-10	–	–	–	–	4·11	–	–	–	–	–	–	–	–
TNF-α	115·24	85·69	–	–	55·89	390·18	40·67	105·22	104·87	–	–	41·08	38·03
IFN-γ	13·02	6·01	12·04	190·12	5·32	–	4·02	–	–	15·64	7·21	26·51	17·34
C-reactive protein (normal, < 5 μg/ml)	1·14	0·17	0·10	44·0	0·36	0·99	1·83	4·8	0·77	10·3	0·44	1·47	1·92
SAA (normal, < 6 μg/ml)	–	–	–	61·78	–	4·18	–	–	–	63·54	12·95	–	–

* PF, prolonged fatigue (> 1 month); CFS, chronic fatigue syndrome.
† i, intermittent; remitting and relapsing.
‡ R, Raynaud's syndrome; A, abdominal pain; D, diarrhoea; C, carpal tunnel syndrome; H, Heberden's nodes; Th, hyperthyroidism.
§ H, homogeneous; 300, titre of 300.

Table 3. Patients with acute B19 virus infection, follow-up of B19 virus infection and controls

Clinical details, B19 virus markers, autoantibodies and circulating cytokines. Percentages are given in parentheses. NT, not tested.

	Acute B19 virus infection	Follow-up of B19 virus infection	Normal controls
Number of patients	51	39	50
Mean age (range in years)	28·2 (4–54)	–	33·4 (22–63)
M:F ratio	1:4·1	1:4·1	1:3
Arthralgia	31 (61)	11 (28)	0
Fatigue	8 (16)	13 (33)	0
Serum anti-B19 VP1/2 IgM	51 (100)	0	0
Serum anti-B19 VP1/2 IgG	42 (82)	38 (97)	37 (74)
Serum anti-B19 NS1 IgG	7 (14)	16 (41)	8 (16)
Serum B19 virus DNA	42 (82)	10 (26)	0
Leucocyte B19 virus DNA	NT	4 (10)	0
Rheumatoid factor (RF)	16 (31)	14 (36)	0
Anti-nuclear antibody (ANA)	9 (18)	6 (15)	1 (2)
IL-1β	10 (20)	0	0
IL-2	3 (6)	1 (2)	0
IL-6	13 (26)	3 (6)	0
IL-10	2 (4)	5 (10)	6 (12)
TNF-α	25 (49)	22 (56)	7 (14)
IFN-γ	34 (67)	26 (67)	0

Acute B19 virus infection

These patients ($n = 51$) ranged in age from 4 to 54 years, with a mean age of 28·2 years. The male:female ratio was 1:4·1. Symptoms at the time of acute B19 virus infection were rash ($n = 15$), arthralgia ($n = 31$), fatigue ($n = 8$), lymphadenopathy ($n = 4$), foetal hydrops ($n = 3$), TAC ($n = 2$), neutropenia ($n = 2$), myelodysplasia ($n = 1$), thrombocytopenia ($n = 1$) and pancytopenia ($n = 1$). Serum anti-B19 VP1/2 IgM was detected in all of these persons. Serum anti-B19 VP1/2 IgG was detected in 42 patients. Serum anti-B19 NS1 IgG was detected in seven patients. Serum B19 virus DNA was detected in 42 patients. RF and ANA were detected in 16 and 9 persons, respectively.

B19 virus-infected persons during the acute phase of infection had detectable IL-1β (20%), IL-2 (6%), IL-6 (26%), IL-10 (4%), TNF-α (49%) and IFN-γ (67%). Numbers of persons with TNF-α levels less than 50 pg/ml, more than 50 pg/ml and more than 1000 pg/ml were 8, 24 and 18%, respectively. Numbers of persons with IFN-γ levels less than 10 pg/ml, more than 10 pg/ml and more than 50 pg/ml were 18, 33 and 16%, respectively. Detectable IL-1β was associated with the male sex ($P = 0{\cdot}027$), TNF-α ($P = 0{\cdot}016$) and TNF-α > 250 pg/ml ($P < 0{\cdot}0001$). Detectable IL-6 was associated with RF ($P = 0{\cdot}038$). Age of onset $\leqslant 20$ years was associated with detectable IL-1β ($P = 0{\cdot}05$) and TNF-α > 250 pg/ml ($P = 0{\cdot}05$). Fatigue was associated with IFN-γ $\geqslant 7$ pg/ml in those $\geqslant 15$ years of age ($P = 0{\cdot}022$), but not with detectable IFN-γ ($P = 0{\cdot}1$) (Table 1).

Follow-up of B19 virus infection

Of the 51 patients, 39 with acute B19 virus infection were contacted after a follow-up period of between 2 and 37 months (mean of 22·5 months) (the follow-up period for 37 of these persons was at least 7 months). At this time, 19 patients were found to have symptoms that began at the time of acute infection and which persisted throughout the follow-up period. These symptoms were arthralgia ($n = 5$), arthralgia and fatigue ($n = 6$), fatigue ($n = 7$), lymphadenopathy ($n = 1$) and purpura, which was known to be due to thrombocytopenia ($n = 2$). Five patients fulfilled the CDC criteria for CFS (Table 2) (Fukuda *et al.*, 1994). All patients had normal blood haematology, biochemistry and ESR. All B19 virus-infected persons at follow-up were negative for serum anti-B19 VP2 IgM and all except for patient #32 were positive for serum anti-B19 VP1/2 IgG: patient #32 tested negative for anti-B19 IgG by both Western blot and fluorescent antibody tests. Serum anti-B19 NS1 IgG was detected in 16 persons. Serum B19 virus DNA was detected in 10 persons and leukocyte B19 virus DNA was detected in four persons. RF and ANA were detected in 14 and 6 persons, respectively.

B19 virus-infected persons at follow-up had detectable IL-1β (0%), IL-2 (2%), IL-6 (6%), IL-10 (10%), TNF-α (56%) and IFN-γ (67%). Numbers of persons with TNF-α levels less than

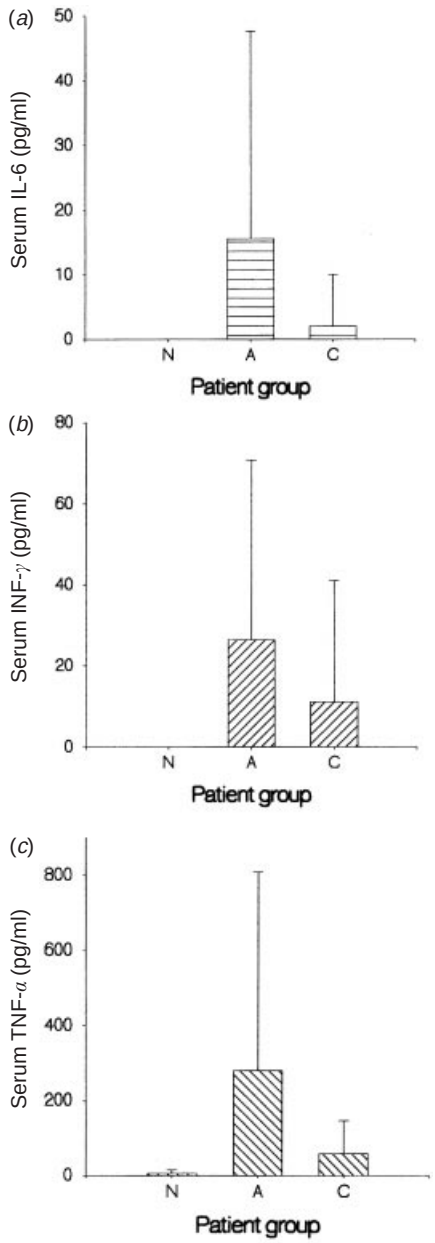

Fig. 1. Mean ± SE of the mean serum levels of (*a*) IL-6, (*b*) IFN-γ and (*c*) TNF-α (pg/ml) in normal healthy controls (N) ($n = 50$), patients with acute B19 virus infection (A) ($n = 51$) and patients followed for a mean of 22·5 months after acute B19 virus infection (C) ($n = 39$).

50 pg/ml, more than 50 pg/ml and more than 1000 pg/ml were 15, 41 and 0%, respectively. Numbers of persons with IFN-γ levels less than 10 pg/ml, more than 10 pg/ml and more than 50 pg/ml were 41, 23 and 3%, respectively. Fatigue was associated with IFN-$\gamma \geqslant 7$ pg/ml and/or TNF-$\alpha \geqslant 40$ pg/ml ($P = 0{\cdot}0275$).

Predictors of outcome of B19 virus infection

In order to assess the value of each symptom and marker present at onset in predicting the outcome of B19 virus infection and markers present at follow-up, relative risks were determined. The only cytokine predictor for the outcome of B19 virus infection was that of detectable serum IL-2 at acute infection. In the three patients in whom serum IL-2 was detected, all had cleared the virus from peripheral blood and were symptom-free at follow-up. There was a trend towards an association between detectable serum IL-2 at acute infection and undetectable TNF-α levels at follow-up ($P = 0{\cdot}14$).

Cytokine levels in control patients, acute B19 virus infection and follow-up of B19 virus infection

In terms of symptoms, B19 virus markers and cytokines, B19 virus-infected persons at onset and follow-up were significantly different from the normal controls (Table 3)

The detectable cytokines encountered most frequently in B19 virus infections and follow-up were IL-6, IFN-γ and TNF-α (detectable levels of other cytokines measured were found much less frequently). Fig. 1 shows mean levels ± SE for levels of IL-6, IFN-γ and TNF-α, respectively, in normal persons and in persons at the time of acute B19 virus infection and follow-up. In comparison with the normal controls, detectable IL-6 was associated with acute B19 virus infection (26%; $P = 0{\cdot}0003$), but not with follow-up of B19 virus infection (6%; $P = 0{\cdot}16$) (Fig. 1*a*). Detection of IFN-γ was associated with acute B19 virus infection (67%; $P < 0{\cdot}0001$) and follow-up (67%; $P < 0{\cdot}0001$) (Fig. 1*b*). Detection of TNF-α was associated with acute B19 virus infection (49%; $P < 0{\cdot}0001$) and follow-up (56%; $P < 0{\cdot}0001$) (Fig. 1*c*).

Discussion

The present study documents the cytokine dysregulation that occurs in patients both at the time of acute B19 virus infection and after a mean follow-up period of 22·5 months. Prolonged/chronic fatigue occurred in 13 of 39 patients followed-up and, in these patients, there was a significant association between fatigue at follow-up with IFN-$\gamma \geqslant 7$ pg/ml and/or TNF-$\alpha \geqslant 40$ pg/ml ($P = 0{\cdot}0275$).

IL-6

As parvovirus B19 NS1 protein upregulates IL-6 transcription in haemopoeitic cells, it was suggested that IL-6 may be important in the pathogenesis of certain B19 virus-associated phenomena, such as autoimmunity and arthralgia (Moffatt *et al.*, 1996), and elevated IL-6 levels have been demonstrated in three infants with lymphocytic myocarditis (Nigro *et al.*, 2000). It appears that IL-6 may be important during the acute phase of B19 virus infection as it was detected in 25% of patients and was associated with RF and increased serum amyloid A (SAA) levels; however, it is probably not important during the ensuing months as IL-6 was detected in only 6% of patients at follow-up.

IL-2

In the present study, we found that a detectable IL-2

response at acute infection appeared to protect against chronic symptoms, chronic viraemia and TNF-α upregulation. A placental IL-2 response has been shown also to correlate with prevention of B19 virus transfer to the developing foetus (Jordan *et al*., 2001). These findings suggest the importance of cytotoxic T lymphocytes in an efficient host response to B19 virus infection.

TNF-α and IFN-γ

Several reports document that TNF-α and IFN-γ may be important in the host response to parvovirus B19 infections with diverse clinical symptoms, including haemophagocytosis and pancytopenia (Watanabe *et al*., 1994), arthritis (Wagner *et al*., 1995) and myocarditis (Nigro *et al*., 2000). Corcoran and colleagues demonstrated that lymphocytes from convalescent adults produced high levels of IL-2 and IFN-γ in response to both VP1 and VP2 proteins; however, there was a significant deficit of IFN-γ production in response to VP1 and VP2 by lymphocytes from recently infected children (Corcoran *et al*., 2000) and this may explain, in part, why childhood parvovirus infections are more frequently asymptomatic as compared to B19 virus infections that occur later in life. Among the animal parvoviruses, raised levels of serum/plasma TNF-α have been associated with enteritis due to canine parvovirus (CPV) (Otto *et al*., 1997) and both TNF and IFN play a role in the pathogenesis of Kilham rat virus-induced autoimmune diabetes mellitus in rats (Chung *et al*., 1997).

These findings are consistent with previous reports of long-term B19 virus DNA persistence following infection (Kerr, 2000) and with a state of immune activation in many individuals that may persist for years following acute B19 virus infection, possibly with the prime purpose of controlling B19 virus replication. Apoptosis is a feature of infection with various human and animal parvoviruses, including parvovirus B19 (Moffatt *et al*., 1998), feline panleukopenia virus (Ikeda *et al*., 1998), parvovirus H-1 (Ohshima *et al*., 1998), CPV (Bauder *et al*., 2000), minute virus of mice (MVMi) (Segovia *et al*., 1999) and adeno-associated virus (AAV) (Zhou & Trempe, 1999). The TNF-α signalling pathway has been shown to be important in apoptosis due to parvoviruses H-1 (Rayet *et al*., 1998) and B19 virus (Sol *et al*., 1999). In the case of parvovirus H-1, virus-infected and NS1-expressing U937 promonocytic cells showed activation of CPP32 ICE-like cysteine protease with resultant apoptotic changes, in a manner similar to that resulting from exposure of these cells to TNF-α (Rayet *et al*., 1998). In the case of parvovirus B19, virus-infected and NS1-expressing erythroid cells were sensitised to TNF-α-induced apoptosis (Sol *et al*., 1999).

B19 virus-associated fatigue

A prolonged state of immune activation may result in a chronic deterioration in health and clinical symptoms such as fatigue and the CFS. Although this is the first documentation, to our knowledge, of an association between these cytokines and fatigue in the setting of B19 virus infection, these findings are consistent with previous reports on fatigue of unknown aetiology. Patients with CFS have been shown to have elevated levels of both serum IFN-γ (Rasmussen *et al*., 1994) and TNF-α (Patarca *et al*., 1994; Moss *et al*., 1999) as compared to control cases. IFN-γ appears to be a key mediator in cytokine dysregulation in the post-Q-fever fatigue syndrome (Penttila *et al*., 1998). In addition, recombinant IFN-γ administration has been shown to result in fatigue symptoms (Mani & Poo, 1996). Recently, new therapies which target TNF-α or its receptor have been shown to improve mood and ameliorate fatigue (Choy & Panayi, 2001).

B19 virus-associated arthralgia/arthritis

The pathogenesis of B19 virus-induced rash and arthralgia is not clearly understood. It has been assumed that, because the appearance of these symptoms coincides with the appearance of specific IgG, they are mediated by immune complex deposition (Anderson *et al*., 1985). TNF-α is known to be important in rheumatoid arthritis (RA) as it stimulates fibroblasts, chondrocytes and osteoclasts to produce matrix metalloproteinases which destroy joint tissue (Shingu *et al*., 1993) and as administration of inhibitors of TNF-α and its action protect the majority of patients from joint damage (Feldmann & Maini, 2001). Although the present study found that within the B19 virus-infected group, detectable serum TNF-α was not associated with arthralgia at either acute infection or follow-up: at acute infection, 61% of patients within this group had arthralgia and 49% had raised serum TNF-α, whereas at follow-up, 28% had arthralgia and 56% had raised serum TNF-α. In addition, these patients contrasted markedly with normal controls at both acute infection and follow-up (Table 3). As this was a small study group and the relationship between TNF-α and arthritis is complex, it is possible that long-term TNF-α upregulation following B19 virus infection may play a role in the pathogenesis of B19 virus arthralgia.

Although there is circumstantial evidence linking B19 virus with RA, studies on the role of B19 virus in the pathogenesis of RA are conflicting and have failed to consistently demonstrate a plausible role for the virus. However, Takahashi *et al*. (1998) and Ishii *et al*. (1999) have demonstrated both a high prevalence of disease-specific persistence of infectious B19 virus DNA in rheumatoid synovium, along with increased production of TNF-α by immune cells infected with B19 virus. Therefore, B19 virus-mediated upregulation of TNF-α may be a crucial mechanism in the possible link between B19 virus and RA.

Parvovirus oncosuppression

Particular members of the family *Parvoviridae*, including H-1 virus, the prototype strain of MVM and AAV types 1 and 2, prevent tumour formation in laboratory animals (Rommelaere & Cornelis, 1991). This may also be relevant in humans and

studies have shown an inverse correlation between the risk of cervical cancer and AAV seroprevalence (Sprecher-Goldberger *et al.*, 1971; Mayor *et al.*, 1976; Georg-Fries *et al.*, 1984). In addition, the presence of AAV DNA in the uterine cervix appears to protect against the development of cervical cancer associated with human papillomavirus (Coker *et al.*, 2001). The mechanism of this phenomenon is unknown and, as it was shown by *in vitro* experiments that parvoviruses are inefficient in inducing TNF-α in mammalian cells (Schlehofer *et al.*, 1992), a primary role for TNFα was thought to be unlikely. However, findings of the present study may warrant a re-evaluation of this assumption, as both TNF-α and IFN-γ are known to suppress tumour formation and are efficacious in the treatment of certain cancers (Lejeune *et al.*, 1998).

Conclusion

In conclusion, we report that circulating TNF-α and IFN-γ levels are raised during acute and convalescent parvovirus B19 infection and are associated with prolonged and chronic fatigue. These findings shed new light on our knowledge of the host response to this infection, its chronicity in certain individuals and the pathogenesis of B19 virus-associated fatigue. As B19 virus infects most people worldwide, these findings suggest that cytokines produced during the years following acute infection in certain individuals may predispose to the development of other diseases and provide clues as to the mechanism of other observed phenomena, such as parvovirus-mediated oncosuppression.

This study was supported by the Chronic Fatigue Syndrome Research Foundation (CFSRF), UK, and had the approval of the Central Manchester Local Research Ethics Committee (LREC).

References

Anderson, M. J., Higgins, P. G., Davis, L. R., Willman, J. S., Jones, S. E., Kidd, I. M., Pattison, J. R. & Tyrrell, D. A. J. (1985). Experimental parvoviral infection in humans. *Journal of Infectious Diseases* **152**, 257–265.

Bauder, B., Suchy, A., Gabler, C. & Weissenbock, H. (2000). Apoptosis in feline panleukopenia and canine parvovirus enteritis. *Journal of Veterinary Medicine Series B* **47**, 775–784.

Choy, E. H. S. & Panayi, G. S. (2001). Cytokine pathways and joint inflammation in rheumatoid arthritis. *New England Journal of Medicine* **344**, 907–916.

Chung, Y. H., Jun, H. S., Kang, Y., Hirasawa, K., Lee, B. R., Van Rooijen, H. & Yoon, J. W. (1997). Role of macrophages and macrophage-derived cytokines in the pathogenesis of Kilham rat virus-induced autoimmune diabetes: diabetes-resistant biobreeding rats. *Journal of Immunology* **159**, 466–471.

Coker, A. L., Russell, R. B., Bond, S. M., Pirisi, L., Liu, Y., Mane, M., Kokorina, N., Gerasimova, T. & Hermonat, P. L. (2001). Adeno-associated virus is associated with a lower risk of high-grade cervical neoplasia. *Experimental and Molecular Pathology* **70**, 83–89.

Corcoran, A., Doyle, S., Waldron, D., Nicholson, A. & Mahon, B. P. (2000). Impaired gamma interferon responses against parvovirus B19 by recently infected children. *Journal of Virology* **74**, 9903–9910.

Cossart, Y. E., Field, A. M., Cant, B. & Widdow, D. (1976). Parvovirus-like particles in human sera. *Lancet* **1**, 72–73.

Durigon, E. L., Erdman, D. D., Gary, G. W., Pallansch, M. A., Torok, T. J. & Anderson, L. J. (1993). Multiple primer pairs for polymerase chain reaction (PCR) amplification of human parvovirus B19 DNA. *Journal of Virological Methods* **44**, 155–165.

Feldmann, M. & Maini, R. N. (2001). Anti-TNF alpha therapy of rheumatoid arthritis: what have we learned? *Annual Review of Immunology* **19**, 163–196.

Franssila, R., Soderlund, M., Brown, S. B., Spaan, W. J. M., Seppala, I. & Hedman, K. (1996). IgG response to human parvovirus B19 infection. *Clinical & Diagnostic Virology* **6**, 41–49.

Fukuda, K., Straus, S. E., Hickie, I., Sharpe, M. C., Dobbins, J. G., Komaroff, A. L. & The International Chronic Fatigue Syndrome Study Group (1994). The chronic fatigue syndrome: a comprehensive approach to its definition and study. *Annals of Internal Medicine* **121**, 953–959.

Georg-Fries, B., Biederlack, S., Wolf, J. & zur Hausen, H. (1984). Analysis of proteins, helper dependence, and seroepidemiology of a new human parvovirus. *Virology* **134**, 64–71.

Goodbourn, S., Didcock, L. & Randall, R. E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus counter-measures. *Journal of General Virology* **81**, 2341–2364.

Holt, I., Cooper, R. G. & Hopkins, S. J. (1991). Relationships between local inflammation, interleukin-6 concentration and the acute phase protein response in arthritis patients. *European Journal of Clinical Investigation* **21**, 479–484.

Hunter, C. A. & Reiner, S. L. (2000). Cytokines and T cells in host defense. *Current Opinion in Immunology* **12**, 413–418.

Ikeda, Y., Shinozuka, J., Miyazawa, T., Kurosawa, K., Izumiya, Y., Nishimura, Y., Nakamura, K., Cai, J., Fujita, K., Doi, K. & Mikami, T. (1998). Apoptosis in feline panleukopenia virus-infected lymphocytes. *Journal of Virology* **72**, 6932–6936.

Ishii, K. K., Takahashi, Y., Kaku, M. & Sasaki, T. (1999). Role of human parvovirus B19 in the pathogenesis of rheumatoid arthritis. *Japanese Journal of Infectious Diseases* **52**, 201–207.

Jordan, J. A., Huff, D. & DeLoia, J. A. (2001). Placental cellular immune response in women infected with human parvovirus B19 during pregnancy. *Clinical and Diagnostic Laboratory Immunology* **8**, 288–292.

Kerr, J. R. (2000). Pathogenesis of human parvovirus B19 in rheumatic disease. *Annals of the Rheumatic Diseases* **59**, 672–683.

Kerr, J. R., O'Neill, H. J., Deleys, R. J., Wright, C. & Coyle, P. V. (1995). Design and production of a target-specific monoclonal antibody to parvovirus B19 capsid proteins. *Journal of Immunological Methods* **180**, 101–106.

Kurtzman, G. J., Frickhofen, N. K., Kimball, J., Jenkins, D. W., Nienhuis, A. W. & Young, N. S. (1989). Pure red-cell aplasia of 10 years' duration due to persistent parvovirus B19 infection and its cure with immunoglobulin therapy. *New England Journal of Medicine* **321**, 519–523.

Lejeune, F. J., Ruegg, C. & Lienard, D. (1998). Clinical applications of TNF-α in cancer. *Current Opinion in Immunology* **10**, 573–580.

Mani, S. & Poo, W. J. (1996). Single institution experience with recombinant gamma-interferon in the treatment of patients with metastatic renal cell carcinoma. *American Journal of Clinical Oncology* **19**, 149–153.

Mayor, H. D., Drake, S., Stahmann, J. & Mumford, D. M. (1976). Antibodies to adeno-associated satellite virus and herpes simplex virus in sera from cancer patients and normal adults. *American Journal of Obstetrics and Gynecology* **126**, 100–104.

Moffatt, S., Tanaka, N., Tada, K., Nose, M., Nakamura, M., Muraoka, O., Hirano, T. & Sugamura, K. (1996). A cytotoxic nonstructural protein, NS1, of human parvovirus B19 induces activation of interleukin-6 gene expression. *Journal of Virology* **70**, 8485–8491.

Moffatt, S., Yaegashi, N., Tada, K., Tanaka, N. & Sugamura, K. (1998). Human parvovirus B19 nonstructural (NS1) protein induces apoptosis in erythroid lineage cells. *Journal of Virology* **72**, 3018–3028.

Moss, R. B., Mercandetti, A. & Vojdani, A. (1999). TNF-α and chronic fatigue syndrome. *Journal of Clinical Immunology* **19**, 314–316.

Nigro, G., Bastianon, V., Colloridi, V., Ventriglia, F., Gallo, P., D'Amati, G., Koch, W. C. & Adler, S. P. (2000). Human parvovirus B19 infection in infancy associated with acute and chronic lymphocytic myocarditis and high cytokine levels: report of 3 cases and review. *Clinical Infectious Diseases* **31**, 65–69.

Ohshima, T., Iwana, M., Ueno, Y., Sugiyama, F., Nakajima, T., Fukamizu, A. & Yagami, K.-i. (1998). Induction of apoptosis *in vitro* and *in vivo* by H-1 parvovirus infection. *Journal of General Virology* **79**, 3067–3071.

Otto, C. M., Drobatz, K. J. & Soter, C. (1997). Endotoxemia and tumor necrosis factor activity in dogs with naturally occurring parvoviral enteritis. *Journal of Veterinary Internal Medicine* **11**, 65–70.

Patarca, R., Klimas, N. G., Lugtendorf, S., Antoni, M. & Fletcher, M. A. (1994). Dysregulated expression of tumor necrosis factor in chronic fatigue syndrome: interrelations with cellular sources and patterns of soluble immune mediator expression. *Clinical Infectious Diseases* **18** (Suppl. 1), S147–S153.

Pattison, J. R., Jones, S. E., Hodgson, J., Davis, L. R., White, J. M., Stroud, C. E. & Murtaza, L. (1981). Parvovirus infections and hypoplastic crisis in sickle-cell anaemia. *Lancet* **1**, 664–665.

Penttila, I. A., Harris, R. J., Storm, P., Haynes, D., Worswick, D. A. & Marmion, B. P. (1998). Cytokine dysregulation in the post-Q-fever fatigue syndrome. *Quarterly Journal of Medicine* **91**, 549–560.

Rasmussen, A. K., Nielsen, H., Andersen, V., Barington, T., Bendtzen, K., Hansen, M. B., Neilsen, L., Pedersen, B. K. & Wiik, A. (1994). Chronic fatigue syndrome – a controlled cross-sectional study. *Journal of Rheumatology* **21**, 1527–1531.

Rayet, B., Lopez-Guerrero, J. A., Rommelaere, J. & Dinsart, C. (1998). Induction of programmed cell death by parvovirus H-1 in U937 cells: connection with the tumor necrosis factor alpha signalling pathway. *Journal of Virology* **72**, 8893–8903.

Rommelaere, J. & Cornelis, J. J. (1991). Antineoplastic activity of parvoviruses. *Journal of Virological Methods* **33**, 233–251.

Schlehofer, J. R., Rentrop, M. & Mannel, D. N. (1992). Parvoviruses are inefficient in inducing interferon-β, tumor necrosis factor-α, or interleukin-6 in mammalian cells. *Medical Microbiology and Immunology* **181**, 153–164.

Schwarz, T. F., Roggendorf, B., Hottentrager, B., Modrow, S., Deinhardt, F. & Middeldorp, J. (1990). Immunoglobulins in the prophylaxis of parvovirus B19 infection. *Journal of Infectious Diseases* **162**, 1214.

Segovia, J. C., Gallego, J. M., Bueren, J. A. & Almendral, J. M. (1999). Severe leukopenia and dysregulated erythropoiesis in SCID mice persistently infected with the parvovirus minute virus of mice. *Journal of Virology* **73**, 1774–1784.

Shade, R. O., Blundell, M. C., Cotmore, S. F., Tattersall, P. & Astell, C. R. (1986). Nucleotide sequence and genome organization of human parvovirus B19 isolated from the serum of a child during aplastic crisis. *Journal of Virology* **58**, 921–936.

Shingu, M., Nagai, Y., Isayama, T., Naono, T., Nobunaga, M. & Nagai, Y. (1993). The effects of cytokines on metalloproteinase inhibitors (TIMP) and collagenase production by human chondrocytes and TIMP production by synovial cells and endothelial cells. *Clinical and Experimental Immunology* **94**, 145–149.

Sol, N., Le Junter, H., Vassias, I., Freyssinier, J. M., Thomas, A., Prigent, A. F., Rudkin, B. B., Fichelson, S. & Morinet, F. (1999). Possible interactions between the NS-1 protein and tumor necrosis factor alpha pathways in erythroid cell apoptosis induced by parvovirus B19. *Journal of Virology* **73**, 8762–8770.

Sprecher-Goldberger, S., Thiry, L., Lefèbvre, N., Dekegel, D. & de Halleux, F. (1971). Complement-fixation antibodies to adeno-associated viruses, cytomegaloviruses and herpes simplex viruses in patients with tumours and in control individuals. *American Journal of Epidemiology* **94**, 351–358.

Takahashi, Y., Murai, C., Shibata, S., Munakata, Y., Ishii, T., Ishii, K., Saitoh, T., Sawai, T., Sugamura, K. & Sasaki, T. (1998). Human parvovirus B19 as a causative agent for rheumatoid arthritis. *Proceedings of the National Academy of Sciences, USA* **95**, 8227–8232.

von Poblotzki, A., Gerdes, C., Reischl, U., Wolf, H. & Modrow, S. (1996). Lymphoproliferative responses after infection with human parvovirus B19. *Journal of Virology* **70**, 7327–7330.

Wagner, A. D., Goronzy, J. J., Matteson, E. L. & Weyand, C. M. (1995). Systemic monocyte and T-cell activation in a patient with human parvovirus B19 infection. *Mayo Clinic Proceedings* **70**, 261–265.

Watanabe, M., Shimamoto, Y., Yamaguchi, M., Inada, S., Miyazaki, S. & Sato, H. (1994). Viral-associated haemophagocytosis and elevated serum TNF-α with parvovirus B19-related pancytopenia in patients with hereditary spherocytosis. *Clinical and Laboratory Hematology* **16**, 179–182.

Zhou, C. & Trempe, J. P. (1999). Induction of apoptosis by cadmium and the adeno-associated virus Rep proteins. *Virology* **261**, 280–287.

Received 6 June 2001; Accepted 17 August 2001