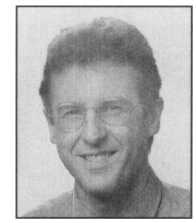
On the Trail of a New Virus

THE CALVERT LECTURE 1997



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Human parvovirus B19 was discovered in 1975[1]. The first clinically significant illness associated with B19 infection was hypoplastic crisis in children with sickie-cell anaemia[2]. Development of specific serological assays showed that patients with fifth disease (erythema infectiosum) also had acute B19 infection[3]; an aetiology that is now widely accepted.

Human parvovirus B19 is a member of the genus Erythrovirus of the family Parvoviridae. B19 is a small single-stranded DNA virus, whose genome is a single linear molecule of only 5596 nucleotides; composed of an internal coding sequence of 4830 nt, flanked by the terminal repeat sequences of 383 nt each. The major nonstructural protein, NS1, is encoded by the left side of the genome, and is essential for viral replication. Structural proteins, VP1 and VP2, are encoded by the right side of the genome and form the viral capsid. Parvovirus B19 has not been shown to infect other animals, and the other animal parvoviruses have not been shown to infect humans.

B19 infection is common in humans, with a seroprevalence in developed countries of 5% in children, 60% in blood donors and 85% or more in the elderly. In temperate climates, there is an increased prevalence from late winter to early summer and increased infection rates every four to five years. B19 is transmitted in the community by the respiratory route causing infection both sporadically and in outbreaks, which are apparent in schools. Transmission has also occurred among medical laboratory staff working with native virus. B19 virus can also be found in donated blood, the incidence of which is estimated between 1:3300 and 1 :50,000, and infection has been transmitted by clotting factor concentrates. Although nosocomial transmission and resultant outbreaks in both paediatric and adult wards have been documented, this is infrequent [4].

B19 infection may be asymptomatic or may result in a wide range of clinical manifestations depending on host factors such as age, presence of anti-B19 antibody, immunocompetence and red cell fragility. B19 is the aetiological agent of erythema infectiosum, transient aplastic crisis in patients with shortened red cell survival, acute and chronic arthritis and chronic anaemia in immunocompromised patients. B19 infection has also been associated with fetal hydrops, congenital red cell aplasia, vasculitis syndromes, glomerulonephritis with nephrotic syndrome, meningitis, encephalopathy, peripheral neuropathy, myocarditis, hepatitis, systemic lupus erythematosus, necrotising histiocytic lymphadenitis (Kikuchi's disease), pulmonary disease following paediatric heart transplantation, conjunctivitis, Kawasaki disease, congenital abnormalities and chronic fatigue syndrome[4].

An outbreak of B19 infection in Northern Ireland

My work began with the description of an outbreak of B19 infection in Northern Ireland. Testing patient serum using an in-house enzymeimmunoassay assay (EIA) for anti-B19 IgM, 133 cases of acute B19 infection were diagnosed by the Regional Virus Laboratory from 1984 to July 1991. An increased prevalence (103 of 133 cases) occurred during 1989-1990. Of the total 133 cases, the ratio of female to male was 3.4:1. The age range was 4-63 years with a mean of 28 years. Clinical manifestations of infection from 1984 - 1991 included rash (n=22), arthralgia (n=35), rash and arthralgia (n=70), aplastic crisis in patients with shortened red cell survival (n=3), fetal death following maternal infection (n=1), Henoch-Schonlein purpura (n=1), and lymphadenopathy (n=1). This outbreak coincided with an outbreak in England and Wales[5].

Study of the incidence of fetal death in B19-infected pregnant women.

Although B19 was known to cause fetal death, the incidence of this following maternal B19 infection was unknown. During 1989, the outbreak year, we collected and later tested the serum from 2400 pregnant women at 12 weeks gestation for the presence of anti-B19 IgM by enzymeimmunoassay. Eight of these were positive. We followed these pregnancies and found that one resulted in spontaneous abortion at 26 weeks. The incidence of fetal death following maternal infection was therefore 12.5%, in general agreement with the major UK study of 186 pregnancies which showed a 9.2% incidence of fetal death. No congenital abnormalities were noted in the 7 surviving infants, either at birth or at 3 years of age[5].

TABLE

Clinical details and results of serum PCR for B19 DNA from seven patients with persistent B19 infection at acute infection and at follow-up assessment. Genotyping of these B19 isolates is shown in Figure 2. (see reference numbers 16 & 17).

Patient number	Age onset	Sex	Symptoms at onset	B19 DNA at onset	Follow-up interval (months)	Duration of symptoms (months)	Symptoms at follow-up	B19 DNA at follow-up
24	10	F	Rash	NT	55	<1	-	+
39	49	F	Arthralgia in knees	+	61	<1	-	+
40	29	F	Arthralgia in knees	+	61	61	Arthralgia in knees	+
41	54	F	Rash	+	60	<1	-	+
46	22	F	Rash	NT	50	<1	-	+
50	29	F	Aplastic crisis	+	26	26	Chronic haemolytic anaenia	+
51	17	F	Arthralgia in knees and shoulders	NT	65	65	Arthralgia in knees and shoulders, Chronic fatigue syndrome	+

NT not tested.

Production of a monoclonal antibody to B19 capsid proteins

A highly conserved B19 epitope is known to be encoded by amino acids 328 - 344 of B19 viral Protein 2. Antibody attachment to this epitope neutralises the virus in erythroid culture.

Using avidin-biotin immunohistochemistry, 3H8 labelled erythroid cells with and without viral inclusions in lung sections from 4 fetuses with histology suggestive of intrauterine B19 infection. No staining was observed in other cell types or fetal lung sections from negative controls. The isotype of 3H8 was IgG1[6].

Study of the cellular distribution of blood group P and B19 antigens in B19 infected bone marrow

In 1993, blood group P antigen was implicated as the cellular receptor for parvovirus B19[7]. In response to this, we examined the bone marrow of 61 AIDS patients and selected 3 bone marrow biopsies which were positive for B19 DNA by nested PCR and B19 capsid proteins by avidin-biotin immunohistochemistry using monoclonal antibody, 3H8. These sections were then stained by a double-fluorescent labelling technique using 3H8 linked to Texas Red followed by a monoclonal antibody to P antigen linked to fluorescein. In all 3 cases an identical pattern of cellular distribution of the two antigens was seen, providing further evidence for P as the virus receptor (Figure 1)[8].

Study of the molecular epidemiology of parvovirus B19

The dominant influences on the clinical manifestations of B19 infection are those of the host. Namely, age, presence of anti-B19 antibody, immunocompetence, red cell fragility, and blood group P antigen status. However, it is possible that the make-up of a particular B19 strain may also contribute[9]. To study the molecular epidemiology of B19 infection, we developed a viral typing method using PCR - single-stranded conformational polymorphism (SSCP) assay. A nested PCR method was developed using oligonucleotide primers specific for a region within the B19 non-structural gene. The first reaction amplified a 369 bp fragment, and the second amplified an internal 284 bp fragment (B19 nucleotides 1399-1682). This 284 bp fragment, after visualisation on agarose gel electrophoresis was then typed using SSCP. The principle of SSCP is that electrophoretic mobility of a DNA molecule in a gel is sensitive to both its size and shape. Therefore, a mutated sequence causes altered folding which is detected as a change in mobility. And in general, the method has a higher sensitivity for short fragments of 100 - 200 bases[10]. The method was optimised to 100% sensitivity, and was able to detect a single mutation in the 284 bp fragment. The method was then applied to 50 virus isolates from patients with different symptoms and geographical locations.

Five types were demonstrated, each of which had a unique nucleotide sequence. In all, 6 mutations were detected, all of which were silent, consistent with the

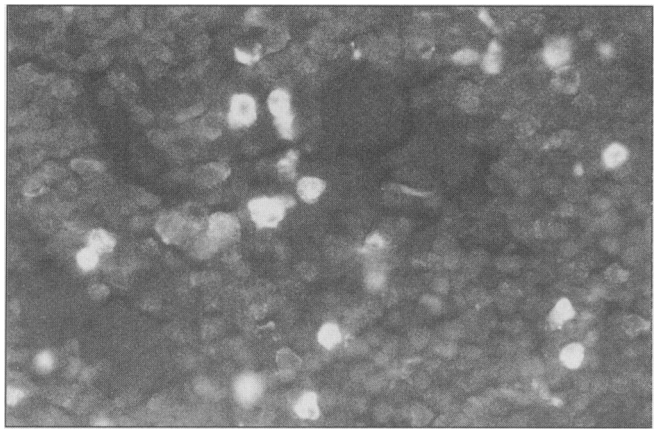

Figure 1a

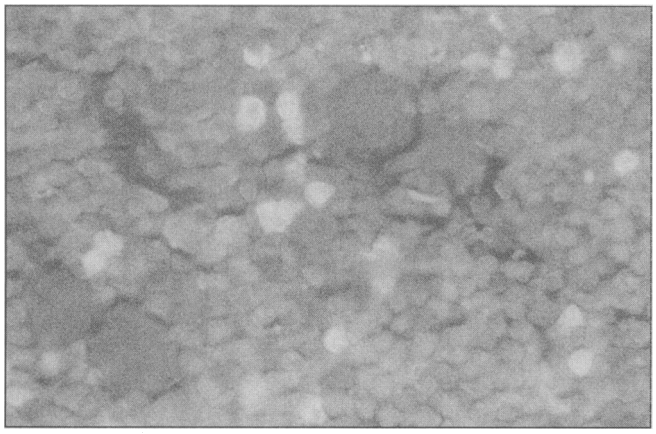

Figure 1b

Figure 1a. B19 infected bone marrow. stained sequentially with monoclonal antibody to B19 antigen (3H8) linked to fluorescein and monoclonal antibody to blood group P antigen (a-P) linked to texas red. Figures 1a and 1b were viewed with filters of emission wavebands at 515nm (to detect bound Streptavidin - FITC) and 615nm (to detect bound Streptavidin - Texas Red), respectively. Both monoclonal antibodies show the same pattern of cellular distribution (see reference number 8).

conserved nature of this region and its crucial role in B19 replication. Among the 50 isolates, types 3 and 4 accounted for 92%. A correlation was seen between SSCP type and country of origin. Type 3 predominated in Japan and the UK, whereas type 4 predominated in the USA. Also, type 3 strains predominated among females, whereas there were approximately equal numbers of strain types 3 and 4 among males, a finding which remains to be explained. Within the Japanese group, although type 3 strains predominated overall, strains isolated from 1981 to 87 consisted of types 1,2, 3, and 4, whereas strains isolated from 1990 to 94 consisted almost entirely of type 3. There was no correlation between SSCP type and clinical illness [11].

Study of the role of B19 in the pathogenesis of rheumatoid arthritis

B19 arthralgia occurs in up to 80% of infected adults, affecting the hands, wrists, knees, and cervical spine. Most patients are women, and rheumatoid factor may be present or may rise following B19 infection. B19 DNA has been detected in the synovial fluid, cells, and tissue of patients with serologically proven B19 infection. On this basis, B19 was implicated in the pathogenesis of RA.

First, we hypothesised that if B19 plays a role in RA, then rheumatoid synovium would contain B19 DNA significantly more frequently than controls. We examined 52 patients undergoing elective orthopaedic surgery; 26 test patients with RA and 26 controls with osteoarthritis (OA)

All synovia were negative for both antigens. Using nested PCR to detect B19 DNA, all sera were negative. However, in synovium,10 of 26 RA patients and 9 of 26 OA patients were positive (P = 0.77). All patients with B19 DNA in synovium had serum anti-B19 IgG[12].

Second, we hypothesised that if B19 plays a role in RA, then RA patients at the time of acute joint swelling may exhibit B19 DNA in the serum, synovial fluid and synovial fluid cells significantly more frequently than controls. A total of 29 patients with acute joint swelling requiring knee joint aspiration were assessed; 18 test patients had RA, and 11 control patients had non-rheumatoid (non-RA) disease.

Serum and synovial fluid from au patients from both groups were anti-B19 IgM negative. Serum, synovial fluid and synovial fluid cells from all patients tested negative for serum B19 DNA[13]. From, these 2 studies, we concluded that a role for B19 in the pathogenesis of RA was not supported.

Presence of viral DNA in synovium is not indicative of viral replication unless intracellular B19 protein is also identified. P antigen was looked for in synovial sections as it might act as the viral receptor in synovial cells, as it does in erythroid cells. However, since P was not detected in any case, the mechanism of viral entry into these cells is unclear. In addition, in vitro cultures of human synovial cells are resistant to B19 infection. There are therefore two theories which may account for a persistent arthropathy following B19 infection and possibly also the presence of B19 DNA in synovium. First, as the joint symptoms coincide with appearance of specific IgG, viral persistence may occur in an extraarticular site such as the bone marrow, with prolonged symptoms being generated by immunopathological mechanisms. Intermittent viraemia may contaminate synovium and explain positive PCR results. However against this, in the present study all 52 sera were PCR-negative. Second, local viral replication may occur in another cell type, for example the macrophage, with excretion of a factor causing synovial inflammation. The B19 non-structural protein, NS1, would be a candidate as it is cytotoxic in vitro[12].

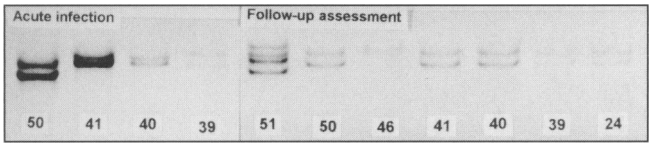

Figure 2. SSCP analysis of PCR products from the serum of 7 patients with persistent B19 infection (Table). Each specimen showed double—stranded DNA of 284bp (nt 1399 to nt 1682) on conventional agarose gel electrophoresis. PCR products were denatured at 90°C in formamide and subjected to electrophoresis in a Mutation Detection Enhancement acrylamide gel (AT Biochem) in 90mM Trisborate, pH 8.3 / 4mM EDTA at a constant power of 20W for 5 hours at 23°C in a vertical gel electrophoresis apparatus (Bio-Rad). The gel was stained by a silver staining method (Promega), and viewed and photographed on a light box (see reference numbers 16 and 17). Reproduced with permission of Scandinavian University Press.

Follow-up study of acute B19-infected persons

In immunocompetent persons, prompt viral clearance and disease resolution is the rule. However, reports have described persistent B19 infections in apparently normal persons and an association with autoantibody production and autoimmune disease. These reports prompted us to perform a follow-up study in 53 persons with confirmed B19 infection and without a known immune defect, to determine the natural history, immune response, and incidence and significance of autoantibody production and virus persistence. Fifty-three patients testing positive for serum anti-B19 IgM were followed for approximately 4 years. At follow-up, clinical symptoms were recorded and blood taken. A control group was included, matched for age and sex.

Seven patients presenting with rash had resolved within a month. Of 42 cases of arthralgia, 19 had resolved within a month, and 23 persisted for 1-7 years. In two cases with persistent arthralgia, chronic fatigue syndrome (CFS) was also present. Of 3 patients presenting with aplastic crisis, 2 were asymptomatic at follow-up and one required occasional blood transfusion for a chronic anaemia. There was one case of fetal death following maternal infection. Apart from one patient with persistent arthralgia who had been diagnosed as having rheumatoid arthritis, there were no cases with other symptoms suggestive of autoimmune disease. At follow-up, all test and control patients were serum anti-B19 IgM negative. All 53 test patients and 45 controls were IgG positive (2-tailed P value = 0.008)[14].

These sera were then tested for antibodies to the unique region of B19 VP1. B19 structural proteins, VP1 and VP2, form the viral capsid in a ratio of 1:25, respectively. They are identical, except for an additional 227 amino acids at the amino-terminal of VP1. This so-called unique VP1 region projects from the virus surface and its recognition by the human immune system is crucial in disease resolution. Serum was tested for antibodies to this region by immunoblot, using 11 synthetic overlapping peptides incorporated onto nitrocellulose strips. At follow-up all test and control patients were negative for antibodies to this region. Serum from the time of acute infection was available for 33 test patients, 16 of which were positive. However, presence or absence of these antibodies did not predict particular symtoms of their duration. Of the 11 peptides, 3 were variably recognised; numbers 2, 8 and 9. These peptides correspond to 2 regions, which encode neutralising viral epitopes. Although, immune recognition of this region is crucial in disease resolution surprisingly all patients tested negative for these antibodies at convalescence. The most likely explanation may be that while antibody binding of these linear epitopes may neutralise the virus, the most important neutralising epitopes are conformational and therefore undetectable in an immunoblot[14].

Seven test and no control patients had serum autoantibody at a titre greater than or equal to 160. These antibodies consisted of antinuclear, gastric parietal cell, antireticulin, antmitochondrial and rheumatoid factor. Only one of these patients had clinical illness at follow-up; a 48 year old woman with serum rheumatoid factor of 1920 iu/ml and a 4 year history of polyarthritis, who had been diagnosed as having RA. In conclusion, in most cases it would seem that these autoantibodies did not have clinical relevance[15].

Serum was then tested for B19 DNA by nested PCR. Seven Test and no control patients were positive (2-tailed P value = 0.016). All seven with persistent infection were women, only 3 of which were symptomatic with arthralgia, arthralgia and CFS and chronic anaemia, respectively (Table). There was no obvious relationship between B19 persistence, autoantibody production and immune recognition of the unique region of VP1. For the 7 persistently infected patients, serum from the time of acute infection was available for four, all of which contained B19 DNA. SSCP assay (Figure 2) showed identical types in 5 of 7 follow-up isolates, suggesting an advantage to the virus of this particular type. In 2 of the 4 cases for which both acute and follow-up PCR product was available patient numbers 40 and 41, the SSCP type at follow-up was different from that at presentation, demonstrating nucleotide substitution occurring during persistent infection. In addition, 2 virus types were shown to co-exist in patient 51, with chronic fatigue syndrome (Figure 2). This is a phenomenon not previously demonstrated for B19 but known to occur with Aleutian mink disease parvovirus, which is also prone to persistent infections. We speculated that as B19 non-structural protein is required for replication, DNA sequence

changes in this region may modify viral replication, possibly promoting persistence[16,17].

Study of the role of B19 in chronic fatigue syndrome

Results of the follow-up study indicated that 2 of 53 patients with acute B19 infection developed CFS, which was still present after 4 years. One of these patients was shown to be persistently infected, and SSCP assay showed the co-existence of 2 virus types. In view of this we examined serum from 22 cases of CFS, according to the diagnostic criteria of the Centers for Disease Control, and 12 normal controls. Regarding serum ant-B19 IgM, 3 of 22 test and 2 of 12 control cases were positive; a surprising result, although not statistically significant. Regarding serum anti-B19 IgG, 15 of 22 test and 8 of 12 control cases were positive; again not significant. All test and control cases were PCR-negative.

We also tested muscle biopsies from 6 patients with CFS and 6 control persons for B19 DNA by nested PCR; one of each group was positive, a non-significant difference[18]. From these 2 studies, we concluded that a role for B19 in the pathogenesis of CFS was not supported.

Study of B19 infection in HIV-1 infected patients

Acute B19 infection in AIDS patients may lead to persistent infection and bone marrow suppression. Persistent B19 infection is typically manifest by pure red cell aplasia, and is associated with a lack of humoral responsiveness to the unique region of VP1. Human immunoglobulin, containing these antibodies, is the only specific treatment. To determine the incidence and significance of B19 infection in AIDS, we tested the bone marrow and assessed the clinical status of 61 HIV infected patients. Reasons for bone marrow biopsy were investigation of anaemia, various cytopenias and suspected lymphoma. The bone marrow of 23 control patients assumed to be HIV negative was also examined. Bone marrow biopsy had been performed in these patients for investigation of lymphoma, leukaemia, anaemia, platelet abnormalities, multiple myeloma, and raised serum IgM.

13 test and no control patients had B19 DNA detected in bone marrow by nested PCR (2-tailed P value = 0.016). Of the 13 infected patients, 11 were CDC group 4, reflecting the known correlation between B19 persistence and level of immunosuppression. However surprisingly, only 2 of these 13 had a haemoglobin below 9g/dl.

One explanation may be that the virus is present at very low concentration in bone marrow, as in all cases it was detected only by nested and not by one—step PCR. The sensitivity of the nested PCR was of the order of 10 genome copies per ml. While the sensitivity of the one—step PCR was of the order of 100,000 genome copies per ml. Unfortunately, serum from the time of bone marrow biopsy was unavailable. Our conclusion, which is consistent with studies using serum, is that low titre B19 persistence in the bone marrow may be common and frequently subclinical in AIDS patients [19].

ACKNOWLEDGEMENTS

I would like to acknowledge the support of Dr Peter Coyle, Dr Percy Ferguson, Professor Tadasu Nunoue and Professor Peter Behan. For financial support, I would like to thank the Royal Victoria Hospital for a one year research fellowship, the Department of Health, the British Council in Japan, The Royal Society, and the Persistent Virus Disease Research Foundation.

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