Comparison of Tissue Distribution, Persistence, and Molecular Epidemiology of Parvovirus B19 and Novel Human Parvoviruses PARV4 and Human Bocavirus

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Background. PARV4 and human bocavirus (HBoV) are newly discovered human parvoviruses with poorly understood epidemiologies and disease associations. We investigated the frequencies of persistence, tissue distribution, and influence of immunosuppression on replication of these viruses.

Methods. At autopsy, bone marrow, lymphoid tissue, and brain tissue from human immunodeficiency virus (HIV)–infected individuals with acquired immunodeficiency syndrome (AIDS) and those without AIDS and from HIV-uninfected individuals were screened for parvovirus B19, PARV4, and HBoV DNA by means of quantitative polymerase chain reaction analyses.

Results. B19 DNA was detected both in HIV-infected study subjects (13 of 24) and in HIV-uninfected study subjects (8 of 8), whereas PARV4 DNA was detected only in HIV-infected study subjects (17 of 24). HBoV DNA was not detected in any study subjects. The degree of immunosuppression with HIV infection did not influence B19 or PARV4 viral loads. B19 or PARV4 plasma viremia was not detected in any study subjects (viral load <25 DNA copies/mL). A significantly older age distribution was found for study subjects infected with B19 genotype 2, compared with those infected with B19 genotype 1. Two genotypes of PARV4 were detected; study subjects carrying prototype PARV4 (genotype 1) were younger (all born after 1958) than those infected with genotype 2 (PARV5; study subjects born between 1949 and 1956).

Conclusions. Tight immune control of replication of B19 and PARV4 was retained despite profound immunosuppression. Recent genotype replacement of PARV4, combined with absent sequence diversity among genotype 1 sequences, suggests a recent, epidemic spread in the United Kingdom, potentially through transmission routes shared by HIV.

The Parvoviridae are a genetically diverse family of small nonenveloped DNA viruses with linear single-stranded genomes. Until last year, the only parvoviruses known to infect humans were parvovirus B19, a member of the Erythrovirus genus, and adenoassociated viruses (AAVs) in the Dependovirus genus. Recently, 2 additional human parvoviruses have been discovered [1, 2]. PARV4 was detected in a large-scale screening of exogenous DNA and RNA sequences in a panel of blood samples from individuals with acute infections of undiagnosed etiology [1]. PARV4 does not resemble any other known mammalian parvoviruses and probably will be classified as the sole member of a new parvovirus genus. At the same time, a second novel human parvovirus was discovered in pooled respiratory samples [2]. The virus has been named “human bocavirus” (HBoV), since it is most closely related to 2 other parvoviruses, bovine parvovirus 1 and a minute canine virus in the Bocavirus genus (i.e., bovine/canine) of Parvoviridae.

HBoV infections have been found to be widely distributed among young children worldwide [3–12], with
SUBJECTS AND METHODS

Study subjects. The autopsy tissue samples used in this project were obtained from the Edinburgh Medical Research Council HIV Brain and Tissue Bank at Western General Hospital, Edinburgh. Consent for use of postmortem tissue was obtained from the Lothian Research Ethics Committee. For the studies of viral load in autopsy tissue, lymphoid (lymph node or spleen), brain (frontal or occipital lobe), and bone marrow tissue samples were obtained from 13 HIV-infected individuals with AIDS at time of death, 11 HIV-infected individuals without AIDS, and 8 HIV-uninfected individuals (table 1). Samples were stored at −80°C for use in subsequent polymerase chain reaction (PCR) analyses. All but 2 of the study subjects with AIDS were untreated with antiretroviral drugs before death. All pre-AIDS study subjects had a CD4 count of ≥200 cells/μL at the time of death, except for 1 study subject with a CD4 count of 167 cells/μL. All presymptomatic study subjects died from AIDS-unrelated causes [23]. Plasma samples were obtained from a total of 36 previously untreated HIV–1–infected individuals at varying stages of disease progression, which was reflected in their CD4 lymphocyte counts (table 1). Plasma samples were obtained from 40 low-risk HIV-negative individuals who were approximately age matched to the HIV-positive individuals; samples were collected over the same period of time (2003–2005).

DNA extraction. Tissue samples were extracted as described elsewhere [24]. Extracted DNA was quantified by spectrophotometry at 260/280 nm. A maximum of 0.5 μg DNA was assayed by PCR. DNA was extracted from 0.4-mL volumes of plasma by use of the QiaAmp DNA blood minikit (Qiagen). Extracted DNA was resuspended in 50 μL elution buffer, and 5 μL was assayed by PCR.

Amplification of parvovirus B19, HBoV, and PARV4 DNA sequences. Nested-PCR methods were used for amplification of B19, HBoV, and PARV4 DNA. B19 primers were based on...
a highly conserved region in the NS gene, with the following sequences (position of the 5' base in B19-Au sequence M13178 in parentheses): outer sense, GTGTATCAGAAATTGTTTTCTTGGTGGCT (2478); inner sense, TTYTGTGGACTTAGTTGCTCG (2867); inner antisense, ACTGGRGTAAAGCATGGG (2984); and outer antisense, TAACTTTAGCTAGATAAATAC (3038). For screening HBoV and PARV4 DNA, we used previously described primer sequences from the NS gene [12]. A shared amplification protocol for each target was used [12].

Measurement of viral load and sequencing. Nested PCRs for the 3 virus targets were sensitive for single copies of the target sequence [12], enabling use of limiting dilution [24] for quantitation of positive samples. For the autopsy samples, initial screening was performed by use of 0.5 μg DNA from each sample, and positive samples were titrated further in 5-fold dilution steps until limiting dilution was observed in 4 or 8 replicate reactions; viral loads then were calculated from the frequencies of positive reactions at limiting dilution [25] and were expressed as copies per million cells. Plasma samples were screened for B19 and PARV4 sequences by means of PCR using 5 μL of the extracted DNA (40 μL of the original sample). Based on single-copy detection [12], the assay had a theoretical sensitivity of 25 DNA copies/mL of plasma.

To rule out the presence of impurities in extracted DNA that could reduce PCR sensitivity, 1-μg quantities of extracted DNA from 15 different autopsy tissues and 3–5-μL volumes of 8 different plasma samples were amplified in reactions spiked with 10 copies of B19 or PARV4 DNA. No inhibition of amplification was detected in any sample, when compared with the 10-copy amplification controls.

Nucleotide sequencing. PARV4-positive samples were sequenced in the region amplified by the screening primers, by use of the Big Dye Terminator kit (Applied Biosystems). Because the B19 amplicon obtained from screening was short, sequencing used the following alternative set of primers in the NS gene: outer sense, GUGGUAARAAAAAYACMCUGUGG (1638); inner sense, SUGUUCAGURAUAGGCAGUGG (1716); inner antisense, AAGUAUGUCACCAUGUGGU (1943); and outer antisense, GUAASCCAGUCAGGGCGUGCAUC (2048). All samples positive with the screening primers were able to be amplified with the sequencing controls.

Accession numbers. The nucleotide sequences of B19 and PARV4 obtained in this study have been submitted to GenBank and have been assigned accession numbers EF371379–EF371417.

RESULTS

Detection of parvoviruses in autopsy tissue. DNA samples extracted from lymphoid tissue (lymph node or spleen, depending on availability), bone marrow, and samples of brain tissue (cerebral cortex) were screened for parvovirus DNA by means of nested PCR using sets of B19-, HBoV-, and PARV4-specific primers (table 2). B19 DNA was frequently detected in study subjects from all 3 categories (HIV negative, pre-AIDS, and AIDS), whereas PARV4 DNA was detected only in those study subjects with HIV coinfection (7 of 11 pre-AIDS study subjects and 10 of 13 study subjects with AIDS). In contrast to the frequent detection of B19 and PARV sequences, HBoV DNA was not detected in any study subjects. Because of the previously described association of HBoV with respiratory infections, we also assayed autopsy lung samples from 3 study subjects with AIDS, but PCR analysis showed that all samples were negative for HBoV DNA (data not shown).

Samples positive for B19 and PARV4 DNA were quantified by limiting-dilution PCR (figure 1). Viral loads varied widely within each tissue-sample type and subject category, ranging from undetectable (<6 DNA copies/10^6 cells) to several samples with >1000 copies of B19 or PARV4/10^6 cells. When data from all 3 subject categories were combined, the highest B19 viral loads were found in bone marrow samples, with a median level of 582 DNA copies/10^6 cells versus 92 DNA copies/10^6 cells in lymphoid tissue (P = .07, Mann-Whitney nonparametric U test) and 69 DNA copies/10^6 cells in brain tissue (P = .006) (figure 1A). Among the HIV-infected study subjects, a similar difference was found in PARV4 viral loads in bone marrow versus lymphoid tissue (median values of 220 and 46 DNA copies/10^6 cells, respectively; P = .0005) (figure 1B).

Neither B19 nor PARV4 showed an association between increased viral load and immunosuppression (figure 1). For B19, median viral loads for HIV-uninfected individuals had a range of values similar to that for the pre-AIDS and AIDS subject categories (e.g., median viral load in lymphoid tissue from HIV-
Figure 1. Viral loads for parvoviruses B19 (A) and PARV4 (B) in different tissues from the study subjects, plotted on a log scale (y-axis). Bars indicate median values for positive samples (filled circles). The significance of the differences in viral loads between subject categories was calculated by use of the Mann-Whitney U test.
uninfected individuals was 203 DNA copies/10⁶ cells, compared with 120 and 46 DNA copies/10⁶ cells for pre-AIDS study subjects and those with AIDS, respectively [P > .05]). Among HIV-infected study subjects, a correlation between CD4 lymphocyte count and B19 viral loads was not found in any of the tissues examined, as determined by means of the nonparametric Spearman's rank correlation test (R = −.01 to .04). Using previously determined HIV viral loads for the subjects in this study group [23, 26], we also found no association between the extent of B19 infection and the extent of HIV infection in any of the tissues examined (R = −.22 to .20).

Although PARV4 was found only in the lymphoid tissue and bone marrow of HIV-infected individuals, there was no correlation between immunosuppression and PARV4 viral load. Viral loads were similar in pre-AIDS study subjects and those with AIDS (figure 1B), and there was no correlation between PARV4 viral load and CD4 lymphocyte counts or HIV viral loads in the different tissues (R = −.47 to .03).

Detection of B19 and PARV4 viremia. To investigate whether persistent infection with B19 or PARV4 was associated with viremia, plasma samples from a second set of HIV-infected and -uninfected study groups (table 1) were tested, by PCR analysis, for B19 and PARV4 DNA. Despite a high assay sensitivity (25 copies DNA/μL [see Subjects and Methods]), all samples from both groups were negative for both B19 and PARV4 sequences (table 2). The individuals in these study groups were demographically similar to the HIV-positive study subjects from whom autopsy samples were obtained and also showed a range of CD4 lymphocyte counts that substantially overlapped with that of the latter group (table 1). Given the likelihood that a substantial proportion of the tested individuals were persistently infected with B19 and PARV4, negative findings from analysis of their plasma samples indicate that persistent infection with either virus is not associated with frequent viremia.

B19 and PARV4 genotypes. Amplified DNA from samples positive for both B19 and PARV4 was sequenced to identify which genotypes infected the study subjects (figure 2). Among HIV-infected and -uninfected study subjects, sequences of B19 genotypes 1 and 2 were detected (12 [60%] and 8 [40%] study subjects, respectively), with a significantly different age distribution between genotypes (figure 2A). The median year of birth for genotype 1–infected study subjects was significantly later than that for genotype 2–infected study subjects (1963–1964 vs. 1955, respectively; P = .004). Genotype 2 sequences differed from genotype 1 sequences by 9.5% in the region sequenced (positions 1737–1922 in the NS region of the B19-AU prototype sequence) and showed a highly constrained pattern of variability; all substitutions between and within genotypes were confined to synonymous sites (i.e., non–amino acid changing).

The pattern of sequence variability among PARV4 sequences was remarkably similar to that of the B19 sequences. Two variants of PARV4 were detected and differed from each other by 13% in the region sequenced (positions 1455–1569 in the complete PARV genome sequence NC_007018); all substitutions again were confined to synonymous sites. Fourteen of the sequences were identical to NC_007018 (referred to as “genotype 1”), and the remaining 4 ( provisionally termed “genotype 2”) showed greater intragroup variability (mean pairwise distance of 3.1%). Genotype 2 infections were found only in those born before 1956 (median year of birth was 1953, compared with 1964 for those with genotype 1 infections [P = .003; figure 2B]).

DISCUSSION

Persistence of human parvovirus infections. As indicated by their frequent detection in autopsy tissue, both B19 and PARV4 showed evidence for the lifelong persistence of infection, which contrasted markedly with the findings for HBoV. The high frequency of detection of B19 in this study is consistent with previous PCR-based evidence for frequent, potentially lifelong persistence of infection [19, 27]—that is, strong anti-B19 cytotoxic T cell reactivity among seropositive individuals [28] and the appearance of the IgG4 subclass of antibody to B19, which usually is associated with chronic viral infection [29]. Indeed, ongoing immunoreactivity to B19 at sites of replication has a potential etiological role in several chronic inflammatory diseases, such as arthritis [22, 30–35], although high frequencies of B19 detection also have been reported in a variety of tissues ( synovium, liver, bone marrow, myocardium, and lymphoid tissue) in study subjects without inflammatory disease [14–19].
As indicated by the results of the current study, persistence may be the usual outcome of B19 infection [14], and the detection of B19 may be incidental to inflammatory diseases [15, 36]. Since a high frequency of B19 infection was detected in lymphoid cells in this and a previous study (figure 1A) [19], the higher viral loads in inflamed tissues, such as synovial tissue in subjects with arthritis, may have arisen through the migration of B19-infected inflammatory cells into affected tissues.

Although the highest B19 and PARV4 viral loads were detected in bone marrow (which is consistent with the propensity of parvoviruses to target dividing cells) and both were found in lymphoid tissue, only B19 was detected in the brain autopsy samples. The spread of B19 into the central nervous system (CNS) occurred in all study subjects and was not related to immunosuppression (figure 1A); these findings are consistent with the frequent detection of B19 DNA sequences in prefrontal cortex samples in a recent study [37]. The detection of B19 DNA sequences in autopsy brain samples is unlikely to be simply a spillover effect associated with the entry of lymphoid or stem cells into the CNS; PARV4 and B19 viral loads were similar in lymphoid and bone marrow–derived cells, but PARV4 was consistently absent from the brain samples (table 2).

Association of B19 and PARV4 infections with HIV infection. A surprising finding in the current study was the similarity in B19 viral loads between immunocompetent and immunosuppressed individuals (figure 1A). Despite the destruction of the immune system in patients with terminal AIDS, B19 replication remained under tight control, with no evidence of increased viral loads in autopsy tissue from the study subjects with AIDS, compared with pre-AIDS and HIV-uninfected individuals; B19 or PARV4 viremia was undetectable in a larger study population of 76 individuals that included several with low CD4 lymphocyte counts. The behavior of B19 and PARV4 contrasted markedly with other persistent DNA viruses, such as human cytomegalovirus, for which reactivation leading to clinically significant disease frequently develops during AIDS. As another example, the ubiquitously distributed and persistent small DNA virus torquetenovirus (TTV) also shows evidence of increased viral replication in immunosuppressed individuals [38–40]. Using the same autopsy samples that were analyzed in the current study, an ∼1000-fold increase in TTV viral load was found in the bone marrow, lymphoid tissue, and brain tissue samples from the study subjects with AIDS, compared with the samples from pre-AIDS and HIV-uninfected study subjects (S. J. Willey, G. J. Hughes, S. Lucas, J. E. Bell, and P. Simmonds, unpublished data).

While B19 DNA sequences were detected in study subjects in all 3 categories, detection of PARV4 DNA was confined to HIV-infected individuals (table 2). Three hypotheses might explain this restricted distribution. It is possible that, in immunocompetent individuals, the immune system controls PARV4 replication to such an extent that PARV4 DNA becomes undetectable in autopsy samples, whereas reactivation of PARV4 occurs in immunosuppressed individuals through HIV infection. Alternatively, PARV4 infection may be nonpersistent in immunocompetent individuals, but the pre-AIDS study subjects and those with AIDS in the current study may have developed a persistent infection after reexposure to PARV4 while infected with HIV. Finally, it is possible that exposure and infection with PARV4 is restricted to the HIV-infected group because of shared routes of transmission, such as sexual contact or parenteral exposure. The latter possibility is supported by the finding of a history of injection-drug use in each of the PARV4-infected study subjects.

Without an antibody assay for PARV4, it is currently difficult to distinguish between these hypotheses. However, if the replication of PARV4 is controlled by the immune system in a way that is similar to B19 replication, then several observations in the current study point toward the third hypothesis. First, evidence from the measurement of PARV4 viral loads in pre-AIDS study subjects or those with AIDS does not indicate that the degree of immunosuppression had any effect on viral replication (figure 1B). If the lack of PARV4 detection in HIV-uninfected individuals was the result of immune control, then viral loads would be expected to be much lower in the pre-AIDS group, since they retain a modestly functioning immune system, compared with the study subjects with AIDS. Second, there was clear differentiation in PARV4 infection status, with concordant results obtained from lymphoid and bone marrow samples from all but 1 individual. There was similarly a distinct distribution of PARV4 viral loads among infected individuals from negative samples (figure 1B). If PARV4 infection was under varying degrees of immune control at different stages of HIV disease progression, such a clear differentiation between HIV-infected and -uninfected individuals would be unlikely. Future investigations of autopsy samples from HIV-infected individuals, non–injection-drug users (IDUs), and HIV-uninfected IDUs will help resolve the underlying reasons for the association of PARV4 infection with HIV infection in the current study.

Molecular epidemiology of B19 and PARV4 infections. Understanding of the epidemiology and transmission patterns of B19 has advanced greatly, owing to the discovery of a temporal succession of infections with genotypes 1 and 2 over the past 60–70 years [19], as well as recent evidence of much higher rates of sequence change in parvoviruses than had been thought previously [41, 42]. Our finding of a significantly older age distribution among study subjects infected with B19 genotype 2 closely reproduced findings from a Finnish study that demonstrated that B19 variants detected in biopsy samples corresponded to the original infecting strains [19]. The observed disappearance of genotype 2 infections in all but 1 individual...
born after 1965 closely matches data in the current study, in which the last genotype 2 infection was detected in an individual born in 1962 (figure 2A); this similarity provides some evidence for long-range B19 transmission networks between quite distantly separated countries in northern Europe.

Evidence was obtained for an equivalent temporal succession of infection with 2 different PARV4 genotypes in the study group. Infection with divergent variants of PARV4, provisionally referred to as genotype 2 in the current study and corresponding to the previously described PARV5 variants [13], was restricted to study subjects born in 1956 or earlier; this age range was even more sharply differentiated from that for genotype 1, compared with the difference observed between the B19 genotypes. If PARV4 infection is associated with needle sharing or HIV infection (as discussed in the previous subsection), then the time scale for this succession would be substantially more recent than that for B19. Thus, PARV4 infection might have occurred only after 1982, when the first cases of HIV infection occurred in Edinburgh.

This hypothesis of a very recent source of PARV4 infection in this risk group is supported by the nucleotide-sequence diversity of PARV4. No sequence variation was observed in the 13 genotype 1 sequences of 114 bp. The recently described high rates of sequence change in B19 and canine parvovirus [41, 42] can be used to infer maximum time since divergence for these variants, based on the assumption that substitution rates at synonymous sites in the viral nonstructural regions are similar between different genera of Parvoviridae. By application of the observed mean substitution rate of \(1.8 \times 10^{-4}\) substitutions per site per year (95th percentile range, \(7.5 \times 10^{-5}\) to \(3.0 \times 10^{-4}\) substitutions) [41], the PARV4 variants that infected the study subjects, as well as the individual in whom PARV4 was originally discovered, would have diverged within the last 14 years (range, 8–34 years) before death. This prediction indicates a very recent introduction and rapid dissemination of PARV4 infection in the HIV/IDU risk groups in Edinburgh in the 1980s–1990s. In contrast, the same calculation applied to B19 genotype 1 variants from the study subjects (mean pairwise distance, 0.47%) predicted an earlier average divergence time of 52 years, which is remarkably consistent with the observed replacement of B19 genotype 2 by genotype 1 in the 1960s (figure 2) [25].

Rapid turnover and changes in transmission dynamics of genetic variants of B19 and PARV4 revealed by these new models of parvovirus evolution may apply more broadly to other members of Parvoviridae. The combined set of 125 published sequences from the NP1 gene of HBoV sequences, including those from geographically disparate sources such as Sweden [2], Canada [8], Japan [5], and Australia [3], showed a mean pairwise distance of 0.003, which corresponds to a predicted time of origin of 34 years (range, 21–83 years). As an intriguing alternative to the hypothesis that HBoV was not detected in autopsy samples because infections were nonpersistent (see above), the genetic data support the possibility that HBoV also might have spread to Scotland very recently and was not circulating during the childhood years of the study subjects (1930s–1970s), when HBoV infection usually seems to be acquired. Further studies, including the development of serological assays for antibody to HBoV, are clearly required in order to understand more fully the transmission dynamics and persistence of HBoV, PARV4, and B19 infections.

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