Human pegivirus RNA is found in multiple blood mononuclear cells in vivo and serum-derived viral RNA-containing particles are infectious in vitro

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Human pegivirus (HPgV; previously called GB virus C/hepatitis G virus) has limited pathogenicity, despite causing persistent infection, and is associated with prolonged survival in human immunodeficiency virus-infected individuals. Although HPgV RNA is found in and produced by T- and B-lymphocytes, the primary permissive cell type(s) are unknown. We quantified HPgV RNA in highly purified CD4+ and CD8+ T-cells, including naïve, central memory and effector memory populations, and in B-cells (CD19+), NK cells (CD56+) and monocytes (CD14+) using real-time reverse transcription-PCR. Single-genome sequencing was performed on viruses within individual cell types to estimate genetic diversity among cell populations. HPgV RNA was present in CD4+ and CD8+ T-lymphocytes (nine of nine subjects), B-lymphocytes (seven of ten subjects), NK cells and monocytes (both four of five). HPgV RNA levels were higher in naïve (CD45RA+) CD4+ cells than in central memory and effector memory cells (P<0.01). HPgV sequences were highly conserved among subjects (0.117 ± 0.02 substitutions per site; range 0.58–0.14) and within subjects (0.006 ± 0.003 substitutions per site; range 0.006–0.010). The non-synonymous/synonymous substitution ratio was 0.07, suggesting a low selective pressure. Carboxyfluorescein succinimidyl ester (CFSE)-labelled HPgV RNA-containing particles precipitated by a commercial exosome isolation reagent delivered CSFE to uninfected monocytes, NK cells and T- and B-lymphocytes, and HPgV RNA was transferred to PBMCs with evidence of subsequent virus replication. Thus, HPgV RNA-containing serum particles including microvesicles may contribute to delivery of HPgV to PBMCs in vivo, explaining the apparent broad tropism of this persistent human RNA virus.

INTRODUCTION

Human pegivirus (HPgV; originally called GB virus C/hepatitis G virus and GB virus type C) is an RNA virus classified as a member of the newly assigned genus Pegivirus within the family Flaviviridae (Adams et al., 2013; Stapleton et al., 2012). Although HPgV infection is common, with 1–4 % of healthy US blood donors being viraemic at the time of donation, it is not clearly associated with any human disease (Bhattarai & Stapleton, 2012; Mohr & Stapleton, 2009). Due to shared modes of transmission, HPgV prevalence is high among human immunodeficiency virus (HIV)- and hepatitis C virus (HCV)-infected individuals (up to 42 %) (Mohr & Stapleton, 2009; Rey et al., 2000; Stapleton et al., 2011). Several studies, although not all, found an association between persistent HPgV infection and prolonged survival in HIV-infected individuals (Herlglake et al., 1998; Nunnari et al., 2003; Tillmann et al., 2001; Toyoda et al., 1998; Vahidnia et al., 2012; Williams et al., 2004; Xiang et al., 2001; Zhang et al., 2006). At least three viral proteins inhibit HIV replication in vitro, including the non-structural NS3 and NS5A proteins and the envelope (E2) protein (George et al., 2012; Jung et al., 2007; Xiang et al., 2006, 2012). Furthermore, HPgV infection is associated with reduced immune activation in HIV-infected individuals, presumably contributing to the observed improvement in HIV clinical outcomes (Bhattarai et al., 2012b, 2013; Maidana-Giret et al., 2009; Stapleton et al., 2011).
et al., 2009, 2012b, 2013). Recent studies found that the HPgV E2 protein inhibits T-cell receptor (TCR)- and IL-2 receptor (IL2R)-mediated signalling, which probably contributes to the reduction in T-cell activation (Bhattarai et al., 2012b, 2013; Maidana-Giret et al., 2009; Stapleton et al., 2009, 2012b, 2013).

HPgV was initially thought to be hepatotropic, and early studies found HPgV RNA in liver biopsies; however, virus was also detected in spleen, bone marrow, cerebrospinal fluid, PBMCs and T- and B-lymphocytes (George et al., 2006; Mellor et al., 1998; Pessoa et al., 1998; Radkowski et al., 2000; Tucker et al., 2000). As HPgV was thought to cause non-A, non-B, non-C hepatitis, evidence of replication in liver tissue was aggressively sought (Madejón et al., 1997; Saito et al., 1997). Although HPgV RNA was found in liver tissue, evidence supporting virus replication in hepatocytes was inconclusive or lacking (Berg et al., 1999; Fan et al., 1999; Laskus et al., 1997). Specifically, HCV RNA levels were consistently higher in the liver of HCV/HPgV co-infected individuals, despite a higher concentration of HPgV RNA in serum, and the median liver:serum ratio of HPgV RNA was <1.0, consistent with serum contamination of liver tissue (Pessoa et al., 1998). Several additional studies did not find evidence of HPgV replication in the liver (Berg et al., 1999; Fan et al., 1999; Pessoa et al., 1998), and, following liver transplantation, HPgV serum RNA levels did not decrease significantly, unlike HCV RNA levels (Berg et al., 1999). Similarly, HPgV negative-strand RNA, indicative of active virus replication, was not detected in liver samples but was detected in bone marrow and splenic tissues in some subjects (Kisiel et al., 2013; Laskus et al., 1997; Radkowski et al., 2000).

Consistent with lymphotropism, HPgV RNA was detected in PBMCs obtained from HPgV-infected subjects, and virus replication was observed following infection of healthy donor PBMCs and by maintaining PBMCs from HPgV-infected humans in culture ex vivo (Fogeda et al., 1999, 2000; George et al., 2006; Mellor et al., 1998; Rydze et al., 2012; Shimizu et al., 1999; Xiang et al., 2000, 2004). Although HPgV replicates efficiently in humans with the mean serum viral load (VL) typically greater than 1 \times 10^7 genome equivalents (GEs) ml^{-1}, replication is poor in vitro and is reduced in PBMCs following T-cell activation (George et al., 2003; Rydze et al., 2012; Tillmann et al., 2001). Recent studies observed a reduction in T-cell activation and proliferation markers in subjects with HPgV/HIV co-infection compared with HIV-monoinfected individuals (Bhattarai et al., 2013; Maidana-Giret et al., 2009; Rydze et al., 2012; Stapleton et al., 2012b), suggesting that HPgV may replicate preferentially in naive T-cells. In addition, activation markers on NK cells and B-cells are reduced in HPgV/HIV co-infected individuals, raising the possibility that HPgV may alter the activation of additional immune cell types in vivo (Bhattarai et al., 2012b, 2013; Maidana-Giret et al., 2009; Stapleton et al., 2009, 2012b, 2013). To understand better the distribution of HPgV in PBMCs and to study potential mechanisms of HPgV transmission, we examined highly purified blood mononuclear cells for HPgV RNA and characterized sequence diversity within and between serum and cells obtained from infected subjects. In addition, serum-derived HPgV RNA-containing particles were assessed for their ability to deliver viral RNA to PBMCs.

**RESULTS**

PBMCs were obtained from 14 HIV/HPgV co-infected subjects receiving combination antiretroviral therapy, and with documented HIV RNA concentrations of less than 48 copies ml^{-1} for 11–163 months prior to donation for use in these studies. There were two female and 12 male subjects and the mean HPgV plasma VL was 9.5 \times 10^7 GEs ml^{-1}. The age, race and gender of the subjects and the mode of HIV transmission are shown in Table 1.

**HPgV RNA is present in multiple PBMCs**

Highly purified T-lymphocytes (CD3^+ /CD4^+ and CD3^+ /CD8^+) and their respective naive (CD45RA^+) , central memory (CM; CCR7^+/CD45RA^-) and effector memory (EM; CCR7^-/CD45RA^-) subsets, B-lymphocytes (CD3^- /CD19^+), NK cells (CD3^- /CD56^+) and monocytes (CD3^- /CD14^+) were isolated from PBMCs obtained from HPgV-infected subjects by a three-step process of Ficoll-Hypaque purification and immunoaffinity column enrichment followed by cell sorting (Fig. 1). The cells were washed five times in large volumes during these three purifications to remove adherent virus, and the final purity of isolated cells was greater than 97 % for all preparations (examples shown in Figs 2 and 3). HPgV RNA was present in all PBMCs studied (n=13), and in the B-cells of seven of nine subjects, the NK cells of four of five subjects and the monocytes of four of five subjects (Fig. 3d). The mean HPgV RNA concentration in PBMCs was 667 GEs per 10^4 cells, whilst the mean HPgV RNA concentration was 734 GEs per 10^4 cells in B-cells, 42 GEs per 10^4 cells in NK cells and 38 GEs per 10^4 cells in monocytes. Typically, 1 \times 10^5–1 \times 10^6 purified cells were used for RNA extraction; however, the minimum number of cells obtained for RNA extraction was 4.5 \times 10^4 purified B-cells in one subject, and HPgV RNA was detected in this sample.

To assess the possibility that the HPgV RNA detected in purified cells simply reflected virus adherent to cells, an equal number of PBMCs (1 \times 10^6–5 \times 10^6) from five HPgV-viraemic subjects were incubated in PBS or 0.25 % trypsin-EDTA (GIBCO) for 1 min, neutralized with 800 μl complete RPMI 1640 with FBS and washed three times with PBS and counted. Total cellular RNA was extracted and the HPgV RNA was quantified by reverse transcription (RT)-PCR. The mean reduction in HPgV RNA was 42 %, and thus the majority of viral RNA was not removed by trypsin treatment (Fig. 3e). The reduction in viral RNA in each subject is also shown (Fig. 3f). The lower limit of detection for HPgV RNA for the quantitative real-time
PCR assay was 21 GEs per test sample (Souza et al., 2006; Rydze et al., 2012). Given the numbers of blood cells used for RNA extraction, the values reported for all subjects were more than 100-fold higher than the limit of detection. Among the nine subjects with purified CD4⁺ and CD8⁺ T-cells, HPgV RNA concentration was greatest in CD4⁺ cells with a mean of 2010 GEs per 10⁴ cells, followed by CD8⁺ cells at 672 GEs per 10⁴ cells (Fig. 4a, b). Within CD4⁺ T-cells, HPgV RNA concentration was highest in naïve cells with a mean value of 1330 GEs per 10⁴ cells compared with 391 and 88 GEs per 10⁴ CM and EM cells, respectively. Because of the broad inter-subject variation in HPgV viral RNA concentration, the proportion of total RNA in the different CD4 and CD8 subpopulations (naïve,

<table>
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<th>Subject no.</th>
<th>Age (years)</th>
<th>Sex</th>
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<th>CD4 cells (%)‡</th>
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<td>42</td>
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<td>C</td>
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<tr>
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<td>12</td>
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<tr>
<td>13</td>
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<tr>
<td>14</td>
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<td>M</td>
<td>C</td>
<td>MSM</td>
<td>277 (19)</td>
<td>7.95</td>
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*C, Caucasian; AA, African–American; His, Hispanic; NA, Native American.
†MSM, men who have sex with men.
‡CD4 value=cells mm⁻³.
§Determined as log₁₀ GEs ml⁻¹.

**Table 1. Demographics and clinical characteristics of the subjects studied**

**Fig. 1.** Cell purification approach. CD3⁺ or CD3⁻ cells were enriched from PBMCs using anti-CD3 conjugated magnetic beads (CD3⁺ column). Following enrichment, CD3⁺ T-cells were sorted into CD4⁺ and CD8⁺ populations using FACS, and these cells were further sorted into naïve (CD45RA⁺), CM (CCR7⁺ CD45RA⁻) and EM (CCR7⁻ CD45RA⁻) subpopulations. CD3⁻ cells were further sorted by FACS into CD19⁺ B-cells, CD56⁺ NK cells and CD14⁺ monocyte populations.
CM and EM) was analysed for each subject. Similar to the absolute number, the proportion of HPgV RNA present in naïve CD4+ T-cells (82%) was highest, and this was significantly higher than that found in CM (39%) or EM (15%) cells, respectively ($P<0.01$; Fig. 4c). Similarly, within CD8+ T-cells, naïve cells averaged 1565 GEs per $10^4$ cells whilst CM and EM averaged 1466 and 204 GEs per $10^4$ cells, respectively (Fig. 4b). Unlike CD4+ cells, the proportion of HPgV RNA was not significantly different among the different CD8+ T-cell subpopulations (Fig. 4d). Nevertheless, in both CD4+ and CD8+ T-cells, more subjects had HPgV RNA detected in the naïve population than in CM or EM cells, consistent with HPgV reducing activation and proliferation of naïve T-cells and as observed in HPgV-infected subjects (Stapleton et al., 2012b).

**HPgV RNA-positive serum particles deliver viral RNA and carboxyfluorescein succinimidyl ester (CFSE) to multiple blood mononuclear cell types**

Previous studies found that serum HPgV particles separate into two distinct particle types during saline flotation gradient centrifugation (Xiang et al., 1998), including a very-low-density particle fraction (buoyant density <1.07 g ml$^{-1}$) and a more heterogeneous denser fraction (>1.11 g ml$^{-1}$) (Bhattarai et al., 2013; Xiang et al., 1998). The heavier particles had a density similar to that described for vesicles of endocytic origin (exosomes: 1.10–1.19 g ml$^{-1}$; Meckes & Raab-Traub, 2011). Incubation of both particle types with a commercial exosome isolation method only precipitated HPgV RNA from the heavier particle type, suggesting that these particles include serum microvesicles (SMs) with properties of exosomes (Bhattarai et al., 2013). To determine whether the particles isolated using this commercial exosome isolation are taken up by PBMCs, HPgV RNA-containing particles were purified from serum obtained from randomly selected HPgV-infected subjects and labelled with CFSE (Fig. 5a), and CFSE-positive or -negative particles were incubated with PBMCs obtained from three healthy subjects. Following 3 h incubation, 27.5% of PBMCs were CFSE positive compared with PBMCs incubated with CFSE-negative SMs (Fig. 5b). As CFSE is cell permeable, trace amounts of CFSE in the wash buffer could transfer CFSE to PBMCs. To ensure that CFSE in the buffer was not responsible for cell labelling, PBMCs were incubated with the final buffer obtained after washing the CFSE-positive SMs. CFSE was not detected in these PBMCs (Fig. 5b). All PBMC cell types studied (CD3+ T-cells, CD19+ B-cells, CD56+ NK cells and CD14+ monocytes) demonstrated uptake of CFSE following incubation with CFSE-labelled SMs (Fig. 5c).

To determine whether the serum HPgV RNA-containing particles isolated by the same method delivered viral RNA...
to PBMCs, healthy donor cells were incubated with HPgV RNA-containing particles in triplicate for 16 h at 37 °C. Cells were washed and the HPgV RNA was quantified in the cell pellets with and without trypsin treatment to remove cell-surface attached particles, as described elsewhere (Smith et al., 2014). HPgV RNA was detected in the PBMCs incubated with SMs, and trypsin reduced the amount of cell-associated HPgV RNA by approximately fivefold (Fig. 5d; \( P < 0.01 \)). In contrast, serum obtained from HPgV RNA-viraemic subjects was dialysed to remove divalent cations, and viral RNA was quantified with or without trypsin treatment (1 min, 25 °C). Serum HPgV RNA was reduced by 99.96 % following trypsin treatment (Fig. 5e). PBMCs were then incubated with either 500 μl serum or with SMs prepared from 500 μl serum for 16 h at 37 °C. Cells were washed and subsequently maintained in cell culture for 5 days. Following trypsin treatment, PBMCs incubated with SMs had significantly more HPgV RNA than those incubated in serum (Fig. 5f; \( P < 0.01 \)), suggesting that SMs and other particles precipitated by the exosome isolation kit may function as an efficient method of HPgV viral RNA transmission to uninfected cells. Cell-associated HPgV RNA levels remained relatively constant for 5 days in culture, whilst HPgV RNA increased in cell-culture supernatants (Fig. 5g). As HPgV RNA-containing particles purified by the exosome purification method were able to deliver CFSE to healthy PBMCs (Fig. 5b), and HPgV RNA increased over time in cells following trypsin treatment (Fig. 5g), these data are consistent with delivery of HPgV RNA from serum particles, including SMs and possibly virions.

### Limited HPgV sequence diversity among different cell types

Single-genome sequencing (SGS) was performed using primers to amplify sequences within the NS2/3 coding region. In total, 225 individual sequences were generated...
from four subjects’ serum, PBMCs and CD4+, CD8+, CD19+, CD14+ and CD56+ cells (Fig. 6a, b). Phylogenetic analysis of all 225 single-genome nucleotide sequences showed identity and clustering of each sequence within each subject, indicating no cross-contamination (data not shown). The mean genetic distance between consensus sequences of each subject was 0.11 ± 0.02 substitutions per site (range 0.58–0.14). Within the sequences of each subject, however, few nucleotide changes were found. The mean genetic distance between sequences within subjects was 0.006 ± 0.003 substitutions per site (range 0.006–0.010). A total of 250 nt changes among 84 294 nucleotide positions were found. These base changes were distributed among all cell types and serum. There were very few non-synonymous changes observed, and from the 250 nucleotide changes found in the 225 sequences, only 19 resulted in amino acid changes, with the overall non-synonymous/synonymous substitution ratio being 0.07, suggesting low selective pressure. A phylogenetic tree of the nucleotide sequences from each subject is shown in Fig. 6(c), illustrating the relative genetic homogeneity between sequences obtained from each compartment within each subject. There was no significant difference in genetic diversity between compartments in any patient (P > 0.05).

The deduced amino acid sequences were aligned for the virus isolates amplified from each cellular compartment and serum (example shown in Fig. 7). Only 19 amino acid changes among the 28 098 amino acid positions were observed. All variant amino acid residues occurred only once in each subject with the exception of I41M, which was seen in more than one subject. I41M was found in one sequence each of subject no. 3 CD4+ cells, no. 13 PBMCs and no. 10 serum. Thus, little amino acid variability was identified in these subjects.
DISCUSSION

Nearly 20 years following its discovery, the primary site(s) of replication and cellular receptor(s) for HPgV entry remain unidentified. Although early studies proposed that the virus was hepatotropic, several lines of evidence suggested that the virus is lymphotropic (Fogeda et al., 1999, 2000; Xiang et al., 2000, 2001). Consistent with this hypothesis, PBMCs from HPgV-infected people maintained in culture ex vivo released virus into culture medium for at least 35 days (Rydze et al., 2012), and serum-derived virus from infected individuals is capable of infecting PBMCs in vitro, although replication is variable and dependent upon both the source of the virus and the source of the recipient PBMCs (George et al., 2003).

Fig. 5. HPgV RNA-containing particles from HPgV-infected serum deliver CFSE and viral RNA to PBMCs. (a) HPgV RNA-containing particles were isolated from infected subject serum samples using an exosome isolation reagent. HPgV RNA-positive particles were labelled with CFSE (CFSE Pos) or not (CFSE Neg), conjugated to microbeads and analysed by flow cytometry. (b) PBMCs were incubated with CFSE-negative SMs, CFSE-positive SMs or the final wash buffer of CFSE-positive SMs for 3 h, and CFSE delivery was measured by flow cytometry. (c) Percentage of T-cells (CD3⁺), B-cells (CD19⁺), monocytes (CD14⁺) and NK cells (CD56⁺) within PBMCs that demonstrated CFSE following incubation. (d) HPgV RNA-positive particles were added to PBMCs for 16 h and, following washing, the cells were treated with trypsin to remove surface-associated HPgV RNA. The amount of intracellular HPgV RNA (trypsin treated) or all cell-associated HPgV RNA (PBS treated) was quantified. (e) In contrast, the amount of serum HPgV RNA detected was reduced by 99.96% by trypsin treatment. (f) Healthy donor PBMCs were incubated with HPgV RNA-containing SMs or serum for 16 h, washed and maintained in culture. Following trypsin treatment, HPgV RNA was significantly greater in PBMCs incubated with particles isolated using the exosome isolation reagent compared with serum. (g) Following incubation with HPgV RNA-containing particles, RNA was quantified in cell-culture supernatants and in PBMCs (trypsin treated). Cell-associated HPgV GE was quantified per 0.1 μg total RNA, whilst culture supernatant HPgV GE was quantified per 1 μg total RNA.
We examined the localization of HPgV RNA in multiple peripheral blood cell types. Among 14 subjects, HPgV RNA was present in PBMCs and in highly purified populations of T- and B-lymphocytes, consistent with previous studies (Fogeda et al., 2000; George et al., 2006; Mellor et al., 1998). Because HPgV is associated with decreased T-cell activation and proliferation (Bhattarai et al., 2012a; Maidana-Giret et al., 2009; Stapleton et al., 2012b) and with an increased proportion of double-negative (CD4−/CD8−/CD3+) T-cells (Bhattarai et al., 2012a; Maidana-Giret et al., 2009; Stapleton et al., 2012b), we examined HPgV RNA in naïve, CM and EM subpopulations of both CD4+ and CD8+ T-cells. HPgV RNA concentration was highest in the naïve (CD45RA+) T-cells compared with EM or CM T-cells,

**Fig. 6.** Limited HPgV sequence diversity among different cell types and serum. (a) Phylogenetic trees illustrate the relationships between the single-genome sequences obtained from each of the four subjects. The mean genetic distance between sequences within each subject was 0.006 ± 0.003 substitutions per site. The GenBank accession number for the outgroup is given (AF121950). (b) Number of HPgV single-genome sequences obtained from the various cell types per subject. (c) Phylogenetic tree of consensus sequences from the four subjects studied. The mean genetic distance between consensus sequences of subjects was 0.117 ± 0.024 substitutions per site.
HPgV sequences are highly conserved in serum and PBMC cell types. Alignment of predicted HPgV NS2/3 amino acids from single-genome amplification of serum, PBMCs, NK cells (CD56⁺) and monocytes (CD14⁺) viruses for one subject. The consensus amino acid sequence is shown on top, and amino acids that differed are indicated below.

![HPgV NS2/3 amino acid alignment comparing cell populations and serum for subject 12.](http://vir.sgmjournals.org)

<table>
<thead>
<tr>
<th>CD14</th>
<th>CD56</th>
<th>PBMC</th>
<th>Serum</th>
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<td>ARGAYLFOSGSMSPSWKKGKLLEMDAALVEFLFHTOGCHS11HEARTLCQGCWHELPSVARREDYTVLVVPQDVHELHPGVPPAPV13BCHGFLGVKXAI1TGEP01LEPGMVYWLO7ATR</td>
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<td>ASQAYLFOSGSMSPSWKKGKLLEMDAALVEFLFHTOGCHS11HEARTLCQGCWHELPSVARREDYTVLVVPQDVHELHPGVPPAPV13BCHGFLGVKXAI1TGEP01LEPGMVYWLO7ATR</td>
<td>ASQAYLFOSGSMSPSWKKGKLLEMDAALVEFLFHTOGCHS11HEARTLCQGCWHELPSVARREDYTVLVVPQDVHELHPGVPPAPV13BCHGFLGVKXAI1TGEP01LEPGMVYWLO7ATR</td>
</tr>
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Consistent either with infection of naïve T-cells or with infection of haematopoietic lymphoid precursors that maintain viral infection during differentiation. Alternatively, HPgV infection might reduce differentiation from naïve to effector cells via decreasing activation and proliferation.

HPgV RNA was also detected in highly purified monocytes and NK cells in four of five subjects, and thus new peripheral blood cell targets for HPgV were identified. Consequently, if the primary target of HPgV is a haematopoietic stem cell, it must be a progenitor of both lymphoid and myeloid cells. Preliminary experiments do not suggest an increased permissiveness of peripheral blood CD34⁺ cells (E. T. Chivero, N. Bhattarai, E. L. Mohr and J. T. Stapleton, unpublished observations); however, these cells represent a small proportion of haematopoietic stem cells. As NK cells obtained from HPgV-infected individuals have reduced activation markers compared with HPgV-uninfected individuals (Stapleton et al., 2013), the finding of HPgV RNA in NK cells raises the possibility that HPgV alters NK cell function, which might contribute to persistent infection and potentially to subclinical immune suppression. Studies to assess the effect of HPgV infection of NK cells are underway.

Although some of the HPgV RNA detected in the purified cell populations may represent adherent virus, the fact that cells went through three purification steps with high volume washes in between and after each step, and that 58% of cellular HPgV RNA was not removed by trypsin treatment whilst 99.96% of serum virus was removed by trypsin, argue that the majority of the RNA was intracellular. In addition, several groups have shown that HPgV is produced by healthy, uninfected PBMCs following addition of HPgV-positive serum, and virus is produced by PBMCs obtained from HPgV-viraemic donors ex vivo (Fogeda et al., 1999, 2000; George et al., 2006; Mellor et al., 1998; Rydze et al., 2012). Finally, incubation of HPgV RNA-containing particles prepared by a commercial exosome purification method transferred CFSE and viral RNA to PBMCs and, following trypsin treatment of these cells, viral RNA increased 10-fold in culture supernatants whilst remaining...
stable in the cells during 5 days in culture (Fig. 5b–d). Taken together, these data support a broad tropism for HPgV in PBMCs including B- and T-lymphocytes, NK cells and monocytes.

An alternative reason why HPgV is found in diverse PBMC cell types is that virions and/or viral RNA in SMs are produced by a different permissive cell type capable of being taken up by multiple cell types in peripheral blood, either by specific cellular receptors or by non-traditional interactions between the cells and SMs. HPgV RNA in plasma is highly associated with lipids (Xiang et al., 1998), including lipid-associated microvesicles that exhibit properties of exosomes (Bhattarai et al., 2013). Although little is known about HPgV entry, the low-density-lipoprotein receptor appears to be involved in virus entry (Agnello et al., 1999). Given the universal distribution of low-density-lipoprotein receptor on different human cells, lipid-associated virus and/or serum microvesicles may be taken up by this mechanism. Studies are under way to assess this possibility. Consistent with this, the finding of minimal HPgV genetic diversity within the four types of highly purified blood mononuclear cell types compared with serum, with a mean non-synonymous-to-synonymous substitution ratio of 0.07 per site, supports a single source of virus production with subsequent delivery of virus to different cell types that support low levels of replication. Of note, HPgV and other members of the genus Pegivirus do not have a predicted nucleocapsid protein at the N terminus of the polyprotein (Stapleton et al., 2011); thus, it is tempting to speculate that cellular-derived microvesicles are involved in virus release and subsequent cellular infection.

The mean serum HPgV VI was 9.5 × 10^7 GEs ml^-1 in these subjects, similar to previous reports (George et al., 2003; Rydze et al., 2012; Tillmann et al., 2001). The mean number of cell-associated HPgV genome copies in this cohort was 677 GEs per 10 000 PMBCs and within specific cell types ranged from 38 to 2010 GEs per 10 000 cells. There are approximately 2 × 10^12 lymphocytes present in adult humans (Alberts et al., 2002). If infected cells produce only one progeny per cell, the maximum number of infected cells would be 1–6%, varying among cell types and among individuals. It is likely that infected cells produce far more than this; thus, the rate of HPgV-infected PBMCs is likely to be similar to HIV infection in CD4^+ T-cells, where approximately one in every 100–400 CD4^+ T-cells has replicating virus (Haase, 1999).

In summary, we detected HPgV RNA in T- and B-lymphocytes, NK cells and monocytes obtained from infected humans. HPgV RNA concentrations were low in all cell types studied but were enriched in naive CD4^+ T-cells compared with effector or memory CD4^+ T-cells. HPgV RNA-containing particles precipitated by a commercial exosome isolation kit delivered viral RNA to uninfected PBMCs, and viral RNA replicated within these cells ex vivo. Together, these data provide insight into the diverse immune cell tropism of HPgV.

**METHODS**

**Subjects.** HPgV/HIV-1 co-infected, HPgV monoinfected and healthy HPgV/HIV-1-negative subjects were invited to participate in the study. Following written informed consent, blood was obtained to test for HPgV RNA and to prepare PBMCs and T-cell subpopulations. For HIV-1-infected subjects, HIV VI was tested on the day of blood sampling using the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test (Roche) by the University of Iowa Hospitals and Clinics clinical laboratory. All HPgV/HIV-1 subjects were selected based on prior studies showing that they had previously had HPgV viremia and were on combination antiretroviral therapy, and that they had non-detectable HIV-1 VLs (<48 copies ml^-1; Roche) for a minimum of 6 months prior to blood sampling. This study was approved by the University of Iowa Institutional Review Board.

**Cell purification.** PBMCs were purified from heparinized blood using gradient density centrifugation (Lymphocyte Separation Media; Mediatech). Following two washing steps, CD3^+ cells (T-cells) were enriched by positive selection using the AutoMax magnetic system according to manufacturer’s instructions (Miltenyi Biotec). Washed CD3^+ -enriched cells were incubated with anti-CD4 and -CD8 antibodies and washed CD3^- cells were incubated with anti-CD19, -CD56 and -CD14 antibodies. After further washing, the cells were purified by FACS on an Aria flow cytometer (Becton Dickinson Biosciences). CD3^+ -enriched cells were also incubated with CD45RA and CCR7 antibodies, and both CD4^+ and CD8^+ T-cell naive (CD45RA^+), CM (CCR7^+ CD45RA^−), and EM (CCR7^− CD45RA^-) subsets were purified by FACS. Purified cells were counted using the Countess automated cell counter (Invitrogen). The following antibodies (and their labels) from BD Biosciences were used: anti-CD3 (V450), anti-CD4 (FITC), anti-CD8 (Alexa Fluor 700), anti-CD19 (PerCP–Cy5.5), anti-CD45RA (allophycocyanin), anti-CCR7 (phycoerythrin), anti-CD14 (allophycocyanin–Cy7) and anti-CD56 (Alexa Fluor 700). CD3 microbeads were obtained from Miltenyi Biotec.

**HPgV RNA quantification.** RNA was extracted immediately following cell sorting using the manufacturer’s recommended protocol (RNAeasy; Qiagen). Real-time RT-PCR was used to quantify HPgV RNA in a 30 µl PCR mix containing 15 µl Taqman Mastermix, 0.75 µl Superscript 3, 0.3 µl Rox reference dye, 11 µl template RNA and a primer/probe set designed to amplify a conserved HPgV 5’ non-translated region, as described previously (George et al., 2003; Rydze et al., 2012). The probe and primer sequences were: probe, 5’-FAM-TGACCGGATTATTGGACCTCAACCC-TAMRA-3’; sense primer, 5’-GGCAGGGCGGAAA-3’ and antisense primer, 5’-CTTAAGACCCACCTATAGTGCGTACC-3’. Amplifications were performed using an Applied Biosystems 7500 real-time PCR system. Thermocycling conditions were 50°C for 20 min, 95°C for 2 min and 50 cycles at 95°C for 15 s and 58°C for 1 min. HPgV RNA concentration was determined using a standard curve generated from serial dilutions of an in vitro-transcribed HPgV RNA, as described previously (George et al., 2003; Rydze et al., 2012). HPgV RNA GEs were normalized per 10^6 cells.

**SGS.** RNA from serum, PBMCs and CD4^+, CD8^+, CD14^+, CD19^+ and CD56^+ cells from four subjects was reverse transcribed with random hexamers and the resulting cDNA diluted prior to amplification of the NS2/3 protein coding region using nested PCR with Expand High Fidelity PCR polymerase. Primers 5’-ATTGAGTGCTGTGGTATGGTGCCG-3’ (outer sense) and 5’-GATGAAGCCCCATGGGAAAA-3’ (outer antisense) were used in the first round, followed by amplification with primers 5’-TGCTGTCCTAGATGAGTGG-3’ (inner sense) and 5’-CGTCAAGCATGTGTCAGCCG-3’ (inner antisense). PCR conditions were one cycle of 94°C for 2 min, 40 cycles of 94°C for 15 s, 55°C for 15 s and 72°C for 30 s, and one cycle of 72°C for 10 min. Following agarose gel analysis, PCR products generated at
Identification and staining of serum particles including SMs. Preparations enriched for SMs were purified from clarified HIV-uninfected, HPgV-infected human serum obtained using Total Exosome Isolation Reagent (Invitrogen) according to the manufacturer’s instructions. Isolated SMs (200 μl) were incubated with 5 μM CFSE dye (Invitrogen) for 30 min at 37 °C. Stained SMs were washed three times in 15 ml PBS by centrifugation for 45 min at 3000 g using Ultra-15 centrifugal filter units (100 kDa; Amicon). After the overnight incubation, the SMs were resuspended in PBS and fixed with 50 μl aldehyde/sulfate latex beads (Invitrogen) at room temperature. After 1 h, 100 μl glycine (100 mM) and 100 μl BSA (10%, v/v) were added to the reaction and incubated for an additional 30 min at room temperature. Beads were washed in PBS (three times) and pelleted by centrifugation for 2 min at 10 000 g prior to flow cytometry analysis.

Uptake of serum particles enriched by exosome isolation reagent by blood mononuclear cells. Unstained or CFSE-stained SMs (50 μg) were incubated with PBMCs (1 × 10^6 cells ml^-1) obtained from three independent healthy donors for 3 h at 37 °C. Cells were washed and resuspended in PBS prior to labelling with the following antibodies: anti-CD3 (V450), anti-CD19 (PerCP-Cy5.5), anti-CD14 (allophycocyanin–Cy7) and anti-CD56 (Alexa Fluor 700) (BD Biosciences). Cells were washed in PBS and fixed with 4% paraformaldehyde for 15 min and analysed by flow cytometry using a Becton Dickinson LSR II.

Infection of PBMCs with HPgV RNA-positive particles. Freshly isolated PBMCs from an HPgV-negative subject were infected with HPgV-positive serum or particles isolated by the exosome isolation reagent from an equivalent amount of serum overnight. Cells were washed three times with PBS and cultured in fresh medium containing RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 IU penicillin ml^-1 and 100 μg streptomycin ml^-1. Cells were harvested, washed three times with either PBS, or first trypsinized (1 min) and washed with citrate buffer (pH 6). HPgV RNA concentration was quantified by real-time RT-PCR on days 1, 3, 5 and 7, as described above.

Statistics. Statistics were performed using GraphPad software v.4.0 (GraphPad Prism Software). Mean HPgV RNA quantities within compartments were calculated. Wilcoxon signed-rank tests were used to compare differences between the genetic distances of compartments with P<0.05 considered statistically significant.

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