Non-correlation of in vivo and in vitro parameters of Epstein–Barr virus persistence suggests heterogeneity of B cell infection

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Following primary infection Epstein–Barr virus (EBV) establishes a persistent infection which is maintained for the life-time of the host. EBV can be found in a small number of circulating B cells, but the nature of the virus–cell interaction has not been fully established. Several assay systems are used to quantify persistent EBV infection, including PCR amplification of EBV DNA and spontaneous outgrowth of lymphoblastoid cell lines in culture. More recently, outgrowth of EBV-positive B cell tumours in severe combined immunodeficient (SCID) mice inoculated with peripheral blood mononuclear cells (PBMC) from normal EBV-seropositive donors has also been used to study B cell infection in vivo. In the present study we have compared the results of these two biological assay systems with PCR detection of EBV DNA and a regression assay as a measure of host T cell immunity to EBV. PBMC from ten normal EBV-seropositive donors were studied and although each test gave consistent results on repeat assays, no correlation was found between any of the assays tested. This result suggests that each assay measures a separate aspect of EBV persistence in B cells, and indicates a previously unrecognized degree of heterogeneity in the B cell population in which EBV resides.

Introduction

Epstein–Barr virus (EBV) is a lymphotropic herpesvirus which, on infection in vitro, immortalizes human B cells to yield continuously proliferating lymphoblastoid cell lines (LCL) (Pope et al., 1968). The majority of the human population undergo subclinical infection with EBV during childhood (Niederman et al., 1970), and the virus then establishes a lifelong persistent infection in two cellular sites – squamous epithelial cells and B lymphocytes (Sixbey, 1989). Virus replication in oropharyngeal epithelium accounts for spread of the virus between individuals, and latent infection of B lymphocytes establishes persistence. This persistent infection is controlled by immune mechanisms – particularly EBV-specific cytotoxic T cells (CTL). These can be detected in the circulation in all normal seropositive individuals and are directed against all the viral genes expressed in B cells immortalized by EBV in vitro [EB viral nuclear antigens (EBNA) 1, 2, 3a, 3b, 3c, leader protein (LP), latent membrane protein (LMP)-1, 2a, 2b] with the exception of EBNA1 (Murray et al., 1992). However, the actual site of virus latency and the interrelationship between B cell and epithelial cell infection remains controversial. Most available evidence supports a model in which EBV establishes a non-productive infection in a pool of self-renewing B lymphocytes during primary infection which persists thereafter, despite CTL activity, by virtue of their restricted viral gene expression (Klein, 1994).

One of the difficulties in studying in vivo EBV persistence is the very low level of circulating virus-infected B cells. Classically, a limiting dilution culture system has been used to quantify these cells (Lam et al., 1991; Yao et al., 1985a): this measures the number of virus-infected cells in T cell-depleted peripheral blood mononuclear cells (PBMC) which give rise to spontaneous LCL. This technique estimates the level of EBV-carrying B cells in the circulation at $1 \times 10^5$ to $1 \times 10^6$ (Lam et al., 1991). In this in vitro assay system, outgrowth of LCL can be suppressed by the viral DNA polymerase inhibitor acyclovir.
(Collins, 1983), or by serum containing neutralizing antibody, indicating that they arise indirectly by release of virus from the in vivo EBV-carrying B cells and infection of bystanding normal B cells (Rickinson et al., 1977; Lewin et al., 1987). Thus, only cells with the capacity to enter a productive cycle in culture are measured in this assay.

More recently, PCR analysis has been used to detect and quantify EBV DNA in PBMC when a value similar to that obtained in the culture assay has been calculated (Miyashita et al., 1995). However, PCR detection of EBV DNA does not necessarily equate with biologically active virus.

Using the assays described above to monitor viral load, immunosuppressed patients such as transplant recipients can be shown to have raised numbers of virus-carrying cells in peripheral blood (Yao et al., 1985b). This is a consequence of low or absent EBV-specific CTL activity, which is suppressed by drugs (Crawford et al., 1981). Between 1 and 10% of these patients develop EBV-associated B lymphoproliferative disease (BLPD) (Thomas et al., 1995), but the relationship between the increased viral load and BLPD development remains obscure.

Recently, the severe combined immunodeficient (SCID) mouse, which lacks B and T cell immunity (Bosma et al., 1983), has been used as a model for BLPD because it develops EBV-associated tumors after inoculation of PBMC from normal donors (Visvanathan et al., 1989). However, PCR detection of EBV DNA does not necessarily equate with biologically active virus.

Methods

- **Blood donors and cell preparation.** Peripheral blood was collected from ten healthy EBV-seropositive donors with their informed consent. PBMC were separated from heparinized blood by Ficoll-Hypaque (Pharmacia) gradient centrifugation and washed in wash medium (RPMI 1640: Gibco) containing 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.7% (v/v) 7.5% NaHCO₃, 2% (v/v) 1 M HEPES buffer and 2% (v/v) foetal calf serum. The cells were either processed further for in vitro culture or resuspended in culture medium (RPMI 1640 containing 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 10%, v/v, foetal calf serum) for intraperitoneal (i.p.) injection into SCID mice.

- **Spontaneous outgrowth of LCL.** PBMC were depleted of T cells by E-rosetting using 5,2-aminophenylisothiouronium bromide hydrobromide (AET; Sigma)-treated sheep red blood cells. The E-negative cells were plated at 2 × 10⁵ per well in round-bottom wells (Falcon) in culture medium (see above). Cultures were incubated at 37 °C in 5% CO₂ and fed weekly by replacement of half the medium. Plates were examined regularly and scored for wells containing actively growing, immortalized cells after 6 weeks.

- **Regression assay.** EBV-specific CTL activity was assessed by in vitro regression assays following the standard method (Moss et al., 1978). Briefly, PBMC obtained from peripheral blood were infected with concentrated B95-8 EBV, suspended in culture medium (see above) and seeded (200 µl per well) into five replicate wells of a 96-well flat-bottomed microtitre plate (Sterilin) in serial doubling dilutions from 20 × 10³ to 0·6 × 10³ cells/ml. The cultures were fed weekly by replacement of half the medium, and after 4 weeks the wells were examined under an inverted microscope for features of regression. The strength of CTL activity was expressed as the regression index (RI), which was determined as the reciprocal of the lowest concentration of cells (× 10³ cells/ml) showing regression in three or more of the five wells multiplied by 100 (Whittle et al., 1984).

- **Tumour outgrowth in SCID mice.** CB-17 SCID mice were obtained from a breeding colony at the London School of Hygiene and Tropical Medicine under the supervision of G. J. Bancroft. Mice were maintained in microinoculator cages and fed autoclaved feed and water without antibiotics. Groups of three to five animals were injected i.p. with 4 × 10¹⁰ to 10 × 10¹¹ unfractionated PBMC each mouse (Moss et al., 1988). Animals were celled either on showing signs of illness or after a previously determined time-limit of 100 days, and examined for macroscopic evidence of tumours which were harvested together with lung, liver and spleen. Tissue samples were snap-frozen in liquid nitrogen and fixed in neutral buffered formalin for histopathological and immunohistochemical studies.

- **PCR for EBV DNA detection.** DNA was extracted from PBMC by routine methods (Sambrook et al., 1989). To detect the presence of EBV DNA in samples, PCR was carried out using primer sets which amplify sequence of the EBV genome within the EBNA2 gene (Boyle et al., 1991). The sensitivity of the primers was determined using Namalwa, an EBV-positive Burkitt’s lymphoma (BL) cell line which is known to carry one or two integrated EBV genomes per cell (Henderson et al., 1983). Tenfold dilutions of Namalwa cells (10⁶, 10⁵, 10⁴, 10³, 10², 10¹) containing the corresponding number of EBV genomes were mixed with 10⁶ cells of the EBV-negative BL cell line Ramos. Total DNA was extracted and subjected to PCR along with 1 µg of DNA from test samples. Sterile distilled water was always included in every PCR run as template-free negative control. PCR products (10 µl) were run on 2% (w/v) agarose gel (Sigma) and Southern blotted onto nylon membranes (Amersham). The membranes were pre-hybridized for at least 2 h at 68 °C in hybridization solution (6 × SSC, 5 × Denhardt’s solution, 0·5%, w/v, SDS and 100 µg denatured salmon sperm DNA). EBNA2-specific plasmid probes (50 ng; Addinger et al., 1985) were labelled with [32P]dCTP (Amersham) by random priming, denatured by boiling and added to membranes. After overnight hybridization at 68 °C, the membranes were washed once in 2 × SSC–0·1% (w/v) SDS at 68 °C for 30 min. The membranes were exposed to Hyper-MP films (Amersham) at −70 °C for 2 days. The autoradiographs were scanned through a gel scanner and the intensity of each positive PCR band measured. A standard curve was generated (see Fig. 1a). The test samples were compared with the control cell dilutions to obtain a semi-quantitative value of EBV genome copy number per sample.
Histopathology and immunohistochemistry. Tissue sections of formalin-fixed material were routinely stained with haematoxylin and eosin (H&E) and tested by in situ hybridization for expression of EBV-encoded RNAs (EBERs) (Howe & Steitz, 1986). Frozen sections were routinely stained for EBNA expression by indirect anti-complement immunofluorescence using known positive and negative human sera (Reedman & Klein, 1973).

Results

Peripheral blood obtained from each of ten healthy, EBV-seropositive volunteers on at least two occasions was tested for each of the four parameters of persistent EBV infection outlined above. The results are shown in Table 1.

(1) Tumour outgrowth in SCID mice. Between $4 \times 10^7$ and $10 \times 10^7$ PBMC from each of the ten donors were injected i.p. into each of at least six SCID mice. PBMC from nine out of the ten (90%) donors consistently gave rise to tumour outgrowth within a 100 day period, whereas PBMC from one donor (no. 4) consistently failed to grow. The incidence of tumour outgrowth in the mice was constant for each of the nine donors on at least two occasions but varied between donors from 13 to 90%.

A representative selection of tumours was examined by H&E, EBNA and/or EBERs staining and all tumours were found to be EBV-positive B cell lymphomas.

(2) Spontaneous outgrowth. PBMC were depleted of E-rosette-positive T cells and plated at $2 \times 10^4$ cells per well on at least two separate occasions. E-negative cells from nine of the ten (90%) donors gave spontaneous outgrowth of LCL, whereas cells from one donor (no. 2) failed to yield LCL. For the nine donors giving a positive result, the incidence of outgrowth ranged from 2 to 49% of wells, and was similar for the same donors on different occasions.

(3) PCR detection of EBV DNA. PCR amplification of the EBNA2 gene sequence was carried out using 1 µg of test DNA from donor PBMC. The primers were sensitive enough to detect one copy of the EBV genome (from one Namalwa cell) in a mixture of $10^6$ EBV-negative cells (Fig. 1a, b). EBV DNA was detected in PBMC from five out of ten (50%) of the donors tested, and this was consistent on repeat testing of PBMC obtained on at least two different occasions (Fig. 1b). The autoradiographs were scanned in order to quantify the signal intensity of the bands. Comparison with the intensity of the signals given by the Namalwa dilutions gave an estimation of the viral load. The EBV DNA copy number in PBMC from those donors giving a positive signal varied from $2 \times 10^4$ to $100 \times 10^4$ per $10^6$ PBMC. All donors were found to be carrying the type 1 strain of EBV. PCR analysis using EBV type 2-specific primers did not yield any positive results in any test samples (data not shown). To confirm the estimated EBV load in test samples obtained by densitometric comparison with Namalwa cells, experiments were carried out where DNA from a positive donor (no. 10) was serially 10-fold diluted in EBV-negative cells and PCR was carried out. The genome copy number was estimated to be $10^6$ in $10^6$ PBMC, which was in agreement with our findings (Table 1). In spite of spontaneous in vitro outgrowth of an LCL from PBMC from donor no. 8, EBV DNA was not detected in the donor’s peripheral blood.

Table 1. Comparison of results of EBV copy number, tumour outgrowth in SCID mice, spontaneous outgrowth in vitro, and regression assay from ten healthy EBV-seropositive donors

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>EBV copy number ($\times 10^3$) per $10^6$ PBMC</th>
<th>No. of PBMC injected per SCID mouse ($\times 10^3$)</th>
<th>No. of tumours/no. of SCID mice (%)</th>
<th>No. of wells showing spontaneous outgrowth/no. of wells (%)†</th>
<th>Regression index (RI)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>$&lt;1$</td>
<td>5–10</td>
<td>6/11 (55)</td>
<td>4/93 (4)</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>5–10</td>
<td>1/8 (13)</td>
<td>0/80 (0)</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>$&lt;1$</td>
<td>5–10</td>
<td>3/6 (50)</td>
<td>1/45 (2)</td>
<td>40</td>
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<tr>
<td>4</td>
<td>$&lt;1$</td>
<td>4–5</td>
<td>0/6 (0)</td>
<td>46/181 (25)</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>800</td>
<td>5</td>
<td>10/12 (83)</td>
<td>9/90 (10)</td>
<td>27</td>
</tr>
<tr>
<td>6</td>
<td>$&lt;1$</td>
<td>4–5</td>
<td>3/8 (38)</td>
<td>6/100 (6)</td>
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<td>7</td>
<td>20</td>
<td>4–6</td>
<td>3/8 (38)</td>
<td>3/100 (3)</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>$&lt;1$</td>
<td>4–5</td>
<td>7/9 (78)</td>
<td>39/80 (49)</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>4–5</td>
<td>8/11 (73)</td>
<td>11/27 (41)</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>1000</td>
<td>5</td>
<td>9/10 (90)</td>
<td>7/110 (6)</td>
<td>27</td>
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</table>

* Each animal within a particular test group received the same number of PBMC from a particular donor.
† Accumulated results from at least two separate experiments.

In vivo and in vitro parameters of EBV do not correlate
Fig. 1. (a) Standard curve obtained using the EBV-positive cell line Namalwa. Serial 10-fold dilutions of Namalwa cells were mixed with a 10^6 EBV-negative Ramos cells, DNA extracted and PCR carried out with EBNA2 primers. The PCR products were run on 2% agarose gel, transferred to nylon membrane and hybridized with ^32P-labelled EBNA2-specific plasmid probe. The autoradiograph was scanned through a densitometer, the volume of each band measured (x 10^3; y-axis) and a standard curve was generated. The scale on the x-axis from 1 to 7 denotes 10^6, 10^5, 10^4, 10^3, 10^2, 10 and 1 Namalwa cells, respectively, in a background of 10^6 EBV-negative Ramos cells. (b) PCR analysis of EBV DNA in PBMC from ten healthy seropositive donors. DNA was amplified using EBNA2 primers. PCR products (10 µl) were separated on 2% (w/v) agarose gel, Southern transferred to nylon membrane and hybridized with a ^32P-labelled EBNA2-specific probe. M, HaeIII-digested φX174 DNA size marker; +, DNA from one Namalwa cell in a background of 10^6 EBV-negative Ramos cells; −, 10^6 Ramos cells; H2O, sterile distilled water. Lanes 1–10, DNA from PBMC from donors 2 (lane 1), 3 (lane 2), 1 (lane 3), 5 (lane 4), 6 (lane 5), 10 (lane 6), 7 (lane 7), 8 (lane 8), 9 (lane 9) and 4 (lane 10).

Table 2. Ten donors ranked for each parameter measured (EBV copy number, tumour outgrowth in SCID mice, spontaneous outgrowth in vitro and regression assay)*

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>EBV copy number</th>
<th>Tumour outgrowth in SCID mice</th>
<th>Spontaneous outgrowth in vitro</th>
<th>Regression assay†</th>
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<td>10</td>
<td>10</td>
<td>10</td>
<td>6</td>
<td>5.5</td>
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</table>

* Spearman’s rank correlation: lowest value given lowest rank, highest value given highest rank.
† Regression assay ranked according to the cell concentration giving rise to a regression index (RI). Analysis of the data when using the ranked values of RI gave similar results.

To determine whether the endogenous EBV isolate of this particular donor could be amplified by PCR, DNA was extracted from the LCL cells and EBNA2 PCR was carried out. Our PCR system was capable of detecting the endogenous virus thus indicating that the negative EBNA2 PCR results for PBMC from donor no. 8 were due to the presence of less than one genome copy per 10^6 cells and not due to the inability to detect the particular isolate.

(4) Regression analysis. Donor PBMC were infected with EBV in vitro and cultured in doubling dilutions from 20 x 10^6 to 0.6 x 10^6 cells/ml in five replicate wells. Cultures were scored for regression/outgrowth after 4 weeks of culture. Cells from all ten donors showed regression, giving a regression index of either 10 (donor 8), 20 (donors 4, 6, 7), 27 (donors 5, 10) or 40 (donors 1, 2, 3, 9).

(5) Statistical analysis. Spearman’s rank correlation was used to compare the four sets of results with each other. The ranking is shown in Table 2. Values for each of the variables were ranked separately in ascending order of magnitude and Spearman’s rank correlation (denoted by r_s) was calculated. Overall, the value of r_s was between −0.13 and 0.49. Comparing r_s on each occasion with critical values showed little or no relationship between any of the ranks (all P-values > 0.1).

Discussion

This study used statistical analysis to compare four different assays which are regularly used as research tools to study EBV persistence and immunity in the normal and immunocompromised hosts. All ten donors gave positive results in at least one assay indicating that each was persistently infected with EBV. However, although each assay gave consistent
results when repeated on PBMC from the same donor taken at
different time-points, when the results of the different assays
were compared no correlation was found. This is a surprising
result which suggests that each assay measures a different
parameter of virus persistence in vivo.

The PCR assay detected EBV DNA in 50% of donors
tested, and this included the one donor whose PBMC did not
give spontaneous outgrowth in culture. This value is in line
with PCR results from other laboratories (Gopal et al., 1990).
PCR is not a biological assay and it is therefore expected to
detect all the EBV DNA present in the samples whether
infected or non-infected. In addition, the primers used in
this study were designed to amplify a region of the EBV
genome in the EBNA2 gene, which is present and amplifiable
in both type 1 and type 2 isolates (although all donors were
subsequently found only to carry type 1 virus). Since all donors
demonstrated evidence of EBV carriage in B cells in at least one
of the other assays, the negative PCR results in 50% of the
donors tested must be due to the level of EBV DNA being
below the sensitivity of the assay. The PCR method used was
shown to be sensitive enough to detect the one to two genome
copies present in one Namalwa cell in a background of 10^6
EBV-negative cells, and therefore EBV DNA in the 50% of
donors who gave negative results must have been at a level
below one genome per 10^6 PBMC. The results could not have
arisen due to an inability of the PCR to detect endogenous
virus since we successfully amplified the virus in a spontaneous
LCL from one donor tested (no. 8) whose PBMC gave negative
PCR results.

The two biological assays, that is spontaneous LCL
outgrowth in vitro and tumour outgrowth in vivo in SCID mice,
both gave positive results with PBMC from 90% of donors;
these levels of detection are consistent with other published
results (Picchio et al., 1992). However, in each case PBMC from
one donor consistently gave negative results, but these were
different donors in each assay. Since both these donors were
found to have type 1 EBV isolates by PCR, the lack of
outgrowth could not be attributed to the less vigorous in vitro
growth promotion properties of type 2 isolates (Rickinson et
al., 1987). The lack of correlation between the two assays
probably reflects the quite distinct mechanisms of outgrowth
of B cells in the in vivo and in vitro systems. Early studies using
spontaneous in vitro outgrowth from T cell-depleted PBMC
populations from normal donors show that this is pre-
dominantly a two-step process where virus-carrying cells
undergo lytic infection early in the culture period and the virus
released infects and immortalizes co-cultured normal B cells
(Rickinson et al., 1977). This process is inhibited by the drug
acyclovir, which blocks lytic reactivation (Collins, 1983), and
by neutralizing antibody, which prevents secondary infection;
only very rarely have B cells capable of direct outgrowth in
culture been detected (Lewin et al., 1987). Outgrowth of LCL
is also prevented by co-cultured, autologous T cells which
contain an EBV-specific cytotoxic population (Moss et al.,
1978) and these cells are therefore removed or inactivated
prior to culture. Both LCL and tumours arising in SCID mice
inoculated with seropositive PBMC exhibit a similar pheno-
type (Rowe et al., 1991). However, in contrast to LCL, tumour
outgrowth in SCID mice is not blocked by acyclovir (Boyle et
al., 1992). The presence of CD4^+ or CD8^+ T cells in the
inoculum is an absolute requirement for their growth (Veronesne
et al., 1992; I. Johannessen, unpublished results), suggesting
that SCID tumours arise from a subpopulation of EBV-carrying
B cells which have the capacity for direct outgrowth in vivo if
supplied with T cell-derived soluble factors and which are quite
distinct from the EBV-carrying cells which give rise to
spontaneous LCL. The present study lends support to this
suggestion and further suggests that tumour production in
SCID mice is a valid in vivo model for BLPD. BLPD tumours
often contain a large infiltrate of non-malignant CD4^+ T cells
which may act as a ‘feeder’ population, supplying essential
soluble factors to initiate and maintain tumour growth (Perera
et al., 1998).

Since the two biological assays tested here show no
correlation and are clearly testing very different aspects of EBV
persistence, the lack of their correlation with levels of EBV
DNA measured by semi-quantitative PCR, although con-
trasting with a published report (Miyashita et al., 1995), is not
unexpected. It confirms the suspicion that not all EBV DNA-
carrying cells have the ability to release virus in culture or
undergo direct proliferation in a SCID mouse. An alternative
explanation for these results would be that the biological
assays are inherently variable, giving inconsistent results
which make any comparison invalid. However, this is not the
case in this study since each assay was repeated on at least two
different occasions and found to give consistent results.

The final statistical analysis showed no correlation between
levels of EBV-specific CTL-activity as measured by a regression
assay, and any of the assays for B cell infection used. This
suggests that the T cells are not directly controlling the level
of B cell infection in the circulation, and supports the
suggestion that many of the EBV-carrying B cells are invisible
to T cell immunosurveillance because of their restricted viral
gene expression (Klein, 1994). The high level of EBV-specific
cytotoxic T cells which is maintained in the peripheral blood of
seropositive donors may therefore be targeting B cells in
lymph nodes, tissues or epithelial sites which are possibly at a
different stage of activation/differentiation and capable of
expressing all the latent viral genes known to be recognized by
these T cells (Murray et al., 1992).

The data presented here indicate a degree of heterogeneity
in the EBV-carrying B cell population in the peripheral blood of
normal individuals and clarify the role of individual assay
systems in the study of EBV persistence in the normal and
immunocompromised host.

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