New Type B Isolates of Epstein–Barr Virus from Burkitt’s Lymphoma and from Normal Individuals in Endemic Areas

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SUMMARY

All Epstein–Barr virus (EBV) isolates can be classified as type A or type B depending upon the identity of their EBV nuclear antigen (EBNA) 2 protein. The great majority of isolates examined to date encode an EBNA 2A protein like that of the reference type A strain B95-8. Type B virus strains, encoding an antigenically distinct EBNA 2B protein, have as yet only been rescued from rare Burkitt’s lymphoma (BL) cell lines of African origin (Jijoye, AG876). Our recent finding that type B isolates are less efficient than type A in in vitro transformation assays prompted us to determine (i) the relative contribution the two types of virus make to the incidence of BL in endemic areas of Africa (Kenya) and New Guinea and (ii) the relative incidence of infection with these two types in the normal population in these same areas. On the first point, EBNA 2 gene typing using specific DNA probes showed that four of ten recently established Kenyan BL cell lines and two of four BL cell lines from New Guinea carried type B virus isolates. To address the second point, spontaneous lymphoblastoid cell lines were established from the blood of normal virus carriers and typed for EBNA 2 at the protein level; a significant proportion (>20%) of the normal population in both the above BL-endemic areas were infected with type B isolates. This is the first indication of the widespread nature of type B virus infection in any community and the first isolation of such viruses from a non-BL source. The reproducible size of the EBNA 2B protein encoded by all type B isolates irrespective of their geographical origin, and of the EBNA 1 protein encoded by all type B isolates from one area, contrasted markedly with the extreme variability in the size both of EBNA 2A and of EBNA 1 seen generally among type A isolates. This suggests that the number of type B virus strains in existence worldwide could be quite limited. Most importantly, the data suggest that type B viruses, despite their relatively poor performance in in vitro transformation assays, can contribute at least as efficiently as can type A viruses to the pathogenesis of BL.

INTRODUCTION

The Epstein–Barr virus (EBV) shows a striking association with two human tumours, Burkitt’s lymphoma (BL) and nasopharyngeal carcinoma (NPC) both of which exhibit an unusual geographical distribution. This is particularly interesting because the virus itself is ubiquitous, being found as a widespread and largely asymptomatic infection in all human communities (see Epstein & Achong, 1986). To date, there is little evidence to support the view that these different consequences of EBV infection reflect the existence of different viral strains. Indeed virus isolates from different regions of the world, or more importantly from patients with different virus-associated diseases, have proved to be remarkably similar when their genomes
have been compared by restriction enzyme analysis (Bornkamm et al., 1980, 1984). Accordingly much more attention has been given to the identification of non-viral cofactors, which are clearly involved in the pathogenesis of both BL and NPC, as a means of explaining the unusual distribution of these tumours.

More recently, however, the question of EBV strain variation has received new impetus with the observation that two long-established BL cell lines of West African origin, Jijoye (Pulvertaft, 1964) and AG876 (Pizzo et al., 1978), carry virus strains which are clearly different from the prototype B95-8 strain in the BamHI WYH region of the genome (Dambaugh et al., 1984; Adldinger et al., 1985). Subsequently the cloning of DNA fragments from the non-homologous regions of the M-ABA (a B95-8-like isolate) (Polack et al., 1984) and the Jijoye (Adldinger et al., 1985) virus genomes has provided probes which can distinguish all EBV isolates as either 'type A' (B95-8-like) or 'type B' (Jijoye-like) in the BamHI WYH region. These differences in fact stem from sequence divergence within the open reading frame encoding the nuclear antigen EBNA 2 (Hennessy & Kieff, 1985; Dillner et al., 1985; Rymo et al., 1985; Muller-Lantzsch et al., 1985). Thus the EBNA 2 type B protein found in Jijoye and in AG876 cells is significantly smaller (75K mol. wt.) and is antigenically distinct from the corresponding (85K) EBNA 2 type A protein present in B95-8 and in many other EBV-carrying cell lines (Dambaugh et al., 1984; Rowe et al., 1985).

Such variation in EBNA 2 structure is most surprising in view of the central role which this protein is thought to play in the process of EBV-induced B cell transformation (Miller et al., 1974; Fresen et al., 1978, 1980; Bornkamm et al., 1982; Skare et al., 1985). Indeed we have recently shown significant biological differences between type A and type B virus isolates when tested in the in vitro transformation assay, where type B transformants grew out unusually slowly and were difficult to expand into cell lines (Rickinson et al., 1987). Given this background, it became important (i) to establish the relative contributions that type A and type B virus isolates make to the high incidence 'endemic' form of BL as found in Africa and New Guinea and (ii) to determine whether the relative frequencies of type A- and type B-associated tumours reflect the prevalence of these two types of agent in the community at risk or whether one or other type carries a higher lymphomagenic risk. The present work addresses these two questions by examining (i) EBV-positive BL cell lines established from cases of the disease arising in Kenya and New Guinea and (ii) spontaneous EBV-positive lymphoblastoid cell lines (LCLs) established from healthy virus carriers resident in these same BL-endemic areas.

METHODS

BL cell lines. The BL cell lines of African origin described here were recently established from cases of EBV genome-positive BL arising in high incidence areas of Kenya (Lake Victoria, Machakos, Meru). The origin and characteristics of these BL cell lines are more fully described elsewhere (Rowe et al., 1985b; Rooney et al., 1986). Of the BL cell lines of New Guinea origin, two were recently established from biopsy material as above (QIMR-WW1-BL, QIMR-WW2-BL) and two others were established several years earlier (QIMR-AMB-BL, QIMR-GOR-BL). The lines were routinely maintained by twice-weekly subculture in RPMI 1640 medium supplemented with 2 mM-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 15% (v/v) foetal calf serum from selected batches (Flow Laboratories).

Spontaneous LCLs. Blood samples (20 ml) were taken from healthy residents of the above BL endemic areas of Kenya and New Guinea, placed in an equal volume of RPMI 1640 containing 10 units/ml heparin, and air-freighted at ambient temperature to the host laboratory (Kenyan samples to Birmingham, U.K.; New Guinea samples to Brisbane, Australia). As controls, blood samples were also obtained from Caucasian donors both in U.K. and in Australia; these included healthy donors, acute infectious mononucleosis patients and rheumatoid arthritis patients. Mononuclear cells were prepared from such blood samples as described elsewhere (Moss et al., 1978) and seeded at doubling dilutions (2 × 10^6 to 0.12 × 10^6 cells per well) in 0.2 ml flat-bottomed microtest plate wells, both alone and on a feeder layer of human embryo fibroblasts. The cells were maintained in RPMI 1640 medium containing 2 mM-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% (v/v) foetal calf serum, and supplemented with 0.1 μg/ml cyclosporin A (CSA) to avert any non-specific T cell activation (Rickinson et al., 1984). These cultures were observed over an 8 to 16 week period for evidence of spontaneous transformation. Positive cultures were thereafter maintained by regular refeding until sufficient cell numbers were available for protein analysis; in some cases this took more than 6 months from the initial seeding of the cultures.
EBNA 2 typing of BL and normal cell lines

Control cell lines. Control cell lines of known EBNA 2 type were as follows: type A, BL42 (Lenoir et al., 1985), M-ABA, CC34-5 (Zimber et al., 1986) and X50-7 (Rowe et al., 1985a); type B, Jijoye (Pulvertaft, 1964).

EBNA 2 gene typing. Five μg of DNA isolated from the various cell lines was digested by BamHI, the fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose filter (Southern, 1975). Two parallel filters were hybridized with inserts of pM-BamH2 (Polack et al., 1984) and pJ-HKA7 (Adldinger et al., 1985), respectively, as described by Zimber et al. (1986).

EBNA 2 protein expression. Protein samples from spontaneous LCLs were separated by discontinuous polyacrylamide gel electrophoresis and blotted onto nitrocellulose as previously described (Rickinson et al., 1987). Excess protein-binding sites on the blotted filters were blocked by incubation for 2 h in 20 mM-Tris-buffered saline pH 7.5 containing 5% dried skimmed milk (TBS-milk). Three parallel filters were then incubated at 4°C overnight with selected human sera of known EBNA reactivity diluted 1:100 in TBS-milk. Mo serum was anti-EBNA 2A/B−, anti-EBNA 1+; PK serum was anti-EBNA 2A+, anti-EBNA 1+; Am serum was anti-EBNA 2B+, anti-EBNA 1−. After incubation with the primary antibody the filters were washed in phosphate-buffered saline (PBS)-0.1% Tween 20 and incubated for 2 h with 125I-labelled Protein A (Amersham) diluted to 0.1 gCi/ml in TBS-milk. Following extensive washing in PBS-Tween the filters were dried and subjected to autoradiography for 1 to 5 days with an intensifying screen. Mol. wt. determinations were made from protein standards (Sigma) which had been stained with remazole dye (Griffith, 1972) and run on the same gel.

RESULTS

EBNA 2 typing of endemic BL cell lines

The 10 recently established BL cell lines of Kenyan origin and the four BL cell lines from New Guinea were all EBV genome-positive and had chromosomal translocations characteristic of BL (Rooney et al., 1986; D. J. Moss, unpublished observations). Fig. 1 shows representative results obtained when BamHI-digested DNA from each of the above cell lines was separated electrophoretically, transferred to nitrocellulose filters and the filters were hybridized with 32p-labelled DNA from the clones pM-BamH2 (M-ABA-derived type A probe, Fig. 1a) and pJ-HKA7 (Jijoye-derived type B probe, Fig. 1b). The intensity of the signal varied considerably from cell line to cell line despite the fact that each lane received the same amount of DNA; this may well reflect differences in virus-producer status between the various lines since even a small number of productively infected cells within any culture will greatly increase the ratio of virus:cell genomes in DNA preparations. In each case, the BL cell lines clearly showed preferential hybridization with one or other of the two probes. Thus, as shown in Fig. 1, the QIMR-WW1-BL, KYU-BL, MAK-BL, OBA-BL and MWI-BL lines gave a clear signal with the type A-specific probe whereas QIMR-WW2-BL, ELI-BL, CHEP-BL, WAN-BL and MUK-BL reacted strongly with the type B probe.

Two additional features of the results in Fig. 1 require comment. First, the unusual doublet of bands seen with WAN-BL (Fig. 1b) suggests some heterogeneity of the resident virus in this line, a result similar to that recently reported for another BL line by Zimber et al. (1986). Secondly, the virus strains associated with QIMR-WW1-BL and with QIMR-WW2-BL cells gave unusually large BamHI fragments encompassing that region of the viral DNA detected by the present probes; in each case this reflects the loss of a BamHI site, leading to fusion of the BamHI F fragment either to BamHI H (QIMR-WW1-BL) or to BamHI YH (QIMR-WW2-BL), as has been observed with certain other virus strains (Bornkamm et al., 1984; Zimber et al., 1986). Note that the presence of an additional BamHI site in the viral DNA of the M-ABA control line results in hybridization of the type A probe to a 1 kb fragment in this case (Zimber et al., 1986).

On the basis of their preferential hybridization to one of the two DNA probes, each of the 14 BL cell lines could be identified as carrying either a type A or a type B virus isolate. The results obtained from the complete panel of BL lines are summarized in Table 1 and indicate that type B isolates, hitherto thought to be quite rare, are associated with a substantial percentage of BL cases both in Kenya as well as in New Guinea.

EBNA 2 typing of spontaneous LCLs from BL-endemic areas

Blood samples were taken from healthy donors from the same BL-endemic areas of Kenya and New Guinea and the lymphocytes were cultured in medium containing CSA in an attempt to generate spontaneous EBV-carrying LCLs. Lines were successfully established from 25 of 40
Fig. 1. Hybridization of $^{32}$P-labelled type A-specific (a) and of type B-specific (b) probes to filters containing separated $Bam$HI DNA fragments from the cell lines indicated. Each lane received 5 μg DNA.

Individual blood samples received from Kenya, and from 14 of 25 samples received from New Guinea. The EBNA 2 type of these lines was then determined from protein preparations separated by SDS-PAGE and analysed by immunoblotting with type-specific sera. Representative results from 10 spontaneous LCLs of Kenyan origin are shown in Fig. 2, the X50-7 and Jijoye lines being included throughout as EBNA 2A-positive and EBNA 2B-positive controls respectively. Immunoblotting with serum Mo confirmed that all the LCLs expressed an EBNA 1 protein whose mol. wt. varied to some extent between isolates (Fig. 2a). When extracts of the same lines were probed using serum PK with reactivity against EBNA 1 and EBNA 2A,
EBNA 2 typing of BL and normal cell lines

Table 1. EBNA 2 type of endemic BL cell lines

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<thead>
<tr>
<th>Cell line</th>
<th>Geographical origin</th>
<th>EBNA 2 gene type</th>
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<tbody>
<tr>
<td>KIE-BL</td>
<td>Kenya</td>
<td>A</td>
</tr>
<tr>
<td>KYU-BL</td>
<td>Kenya</td>
<td>A</td>
</tr>
<tr>
<td>LIV-BL</td>
<td>Kenya</td>
<td>A</td>
</tr>
<tr>
<td>MAK-BL</td>
<td>Kenya</td>
<td>A</td>
</tr>
<tr>
<td>MWI-BL</td>
<td>Kenya</td>
<td>A</td>
</tr>
<tr>
<td>OBA-BL</td>
<td>Kenya</td>
<td>A</td>
</tr>
<tr>
<td>CHEP-BL</td>
<td>Kenya</td>
<td>B</td>
</tr>
<tr>
<td>ELI-BL</td>
<td>Kenya</td>
<td>B</td>
</tr>
<tr>
<td>MUK-BL</td>
<td>Kenya</td>
<td>B</td>
</tr>
<tr>
<td>WAN-BL</td>
<td>Kenya</td>
<td>B</td>
</tr>
<tr>
<td>QIMR-WW1-BL</td>
<td>New Guinea</td>
<td>A</td>
</tr>
<tr>
<td>QIMR-AMB-BL</td>
<td>New Guinea</td>
<td>A</td>
</tr>
<tr>
<td>QIMR-WW2-BL</td>
<td>New Guinea</td>
<td>B</td>
</tr>
<tr>
<td>QIMR-GOR-BL</td>
<td>New Guinea</td>
<td>B</td>
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five of 10 LCLs (ODH, SER, IGU, MUG, WANJ) and the X50-7 control line revealed an EBNA 2A band in the 85K to 90K size range (Fig. 2b). Conversely, as shown in Fig. 2(c), serum Am with strong anti-EBNA 2B but no anti-EBNA 1 reactivity detected the EBNA 2B protein as a 75K/79K doublet in the other five LCLs (ALU, AND, NZI, OJA, MUN) as well as in the Jijoye control line. Note the very weak cross-reactivity of the Am serum with the EBNA 2A band in the appropriate type A lines, emphasizing that the predominant antibody response in this donor is type-specific.

The overall results, summarized in Table 2, show that six of 25 Kenyan isolates and three of 14 New Guinea isolates were of type B. One particularly interesting feature of the immunoblotting data on the Kenyan LCLs was the apparently constant size both of the EBNA 1 protein (79K) and of the EBNA 2B doublet (75K/79K) in all six type B isolates (Fig. 2 and data not shown). By contrast there was marked variability between type A isolates in the sizes both of their EBNA 1 and of their EBNA 2A proteins. Only two of the three New Guinea LCLs of type B could be grown to sufficient cell numbers to allow direct comparison with those of Kenyan origin. Nevertheless the results shown in Fig. 3(a) once again illustrate the uniformity in size of the EBNA 2B protein, for the two New Guinea lines showed the same 75K/79K doublet as already described above for all six Kenyan lines as well as for the control BL cell line, Jijoye, originally derived in West Africa. In a parallel immunoblot, presented in Fig. 3(b), both New Guinea lines produced an EBNA 1 band that ran slightly below the EBNA 1 of the Kenyan cell lines.

Finally, it is worth noting that Table 2 also includes EBNA 2 typing data on 100 spontaneous LCLs derived from Caucasian donors (50 in U.K., 50 in Australia) during the course of this work. Of these, three lines carried type B virus isolates and expressed an EBNA 2B doublet which was again similar to that described above (data not shown).

Discussion

The availability of EBNA 2 type-specific probes, capable of distinguishing type A and type B virus isolates at the DNA or at the protein level, makes possible comparisons between these two families of viruses in terms of their disease association and of their geographical distribution. The present work and the recent reports of Zimber et al. (1986) and Ernberg et al. (1986) together show that type B isolates are associated with a significant proportion (around 40%) of BL tumours arising in the high incidence areas of Africa and New Guinea.

The present study has gone on to examine whether the relative frequencies of type A- and type B-associated tumours in endemic areas merely reflect the prevalence of the two types of agent in these communities or, conversely, whether one or other type of virus confers a higher lymphoma risk. The prevalence of type A and of type B virus infection in normal populations has been estimated by EBNA 2 typing of a panel of spontaneous EB virus-positive LCLs newly


Fig. 3. Comparison of EBNA 2B and of EBNA 1 proteins in spontaneous type B virus-carrying lines established from normal Kenyan and from normal New Guinea donors. (a). EBNA 2B detected with Am serum as a 75/79K doublet in each of the above lines. (b) EBNA 1 detected with Mo serum as a 79K protein in the Kenyan lines and as a 75K protein in the New Guinea lines.

Table 2. EBNA 2 types of spontaneous LCLs from normal donors

<table>
<thead>
<tr>
<th>Location</th>
<th>Type A</th>
<th>Type B</th>
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<tr>
<td>Kenya (BL-endemic areas)</td>
<td>19/25 (76%)</td>
<td>6/25 (24%)</td>
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<tr>
<td>New Guinea (BL-endemic areas)</td>
<td>11/14 (79%)</td>
<td>3/14 (21%)</td>
</tr>
<tr>
<td>Caucasian controls</td>
<td>97/100 (97%)</td>
<td>3/100 (3%)</td>
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established from the blood samples of randomly selected donors. Although this approach runs the risk of underestimating the incidence of type B virus infection because type B transformants are more difficult to establish as lines, every effort was made to adopt a culture protocol which would eliminate such a bias (Rickinson et al., 1987). This approach avoids many of the disadvantages inherent in serological screening of donor sera for EBNA 2A-specific or EBNA 2B-specific antibodies. In this regard, many asymptomatic virus carriers do not have detectable anti-EBNA 2 antibody titres (Sculley et al., 1984) and so screening is uninformative, whereas in

Fig. 2. Expression of EBNA 1, EBNA 2A and EBNA 2B proteins in spontaneous lymphoblastoid cell lines established from normal individuals from BL-endemic areas of Kenya. EBNA proteins were detected by SDS–PAGE and immunoblotting. (a) Expression of EBNA 1 (70K to 85K) in all the cell lines detected with a human serum (Mo) having high anti-EBNA 1 reactivity, no reactivity against other EBNA species and unusually low reactivity against antigens of the virus productive cycle. (b) Expression of EBNA 2 (85K to 90K) in type A virus-carrying lines detected with a human serum (PK) having a high anti-EBNA 2A reactivity. PK serum also recognizes EBNA 1 in all the cell lines. (c) Expression of EBNA 2 (75K/79K doublet) in type B virus-carrying lines detected with a human serum (Am) having high anti-EBNA 2B and no anti-EBNA 1 reactivity. Am serum displays a weak cross-reactivity with the EBNA 2A protein in type A virus-carrying lines. The additional bands seen in some lanes with Am serum (above the 116K and below the 66K markers) represent antigens of the virus productive cycle and reflect the high virus producer status of these particular lines; the very weak signal shown by the IGU cell line in the 75K region of the gel was not reproducibly observed on repeated testing.
at least some seropositive individuals the anti-EBNA 2 response includes antibodies which are cross-reactive between EBNA 2A and 2B (T. B. Sculley et al., unpublished results), thus making it difficult to identify the type of infecting virus. Dual infection with type A and type B viruses is of course a formal possibility, but there was no evidence for this in the present study i.e. no spontaneous LCLs were established containing type A virus-positive and type B virus-positive subpopulations.

The analysis of spontaneous LCLs suggests that a substantial proportion (>20%) of the normal population in the BL-endemic areas of Kenya and New Guinea are infected with type B virus strains. To our knowledge this is the first demonstration of the widespread nature of type B virus infection; indeed the type B isolates described here are the first to be obtained from a source other than BL tissue. Note that in Western communities, type A viruses are overwhelmingly predominant and, not surprisingly, the small number of EBV-positive BLs arising in such areas usually carry type A isolates (Zimber et al., 1986; Ernberg et al., 1986). The relative contributions that type A and type B viruses make to the incidence of endemic BL appears to reflect the prevalence of infection with these agents within the general population in these areas. There is no obvious difference between the two in terms of lymphomagenic risk.

Such findings are interesting in light of our recent observation that type B viruses, though capable of initiating proliferation in resting human B cells just as well as type A viruses, nevertheless produce transformants that grow poorly and are much more difficult to establish as permanent cell lines (Rickinson et al., 1987). This re-emphasizes the differences between the in vitro transformation system, which requires continued expression of the full spectrum of latent viral proteins to maintain cell growth transformation (Dambaugh et al., 1986), and the much more complex situation in vivo where virus infection is part of a multi-stage oncogenic sequence culminating in the malignant transformation of a cell at a particular stage in the B cell differentiation pathway (Gregory et al., 1987). If EBNA 2 expression is required in the sequence leading to BL, then the EBNA 2A and 2B proteins would appear to be functionally equivalent in this particular respect. It is worth noting here that expression of EBNA 2 is often down-regulated in newly established BL cell lines (Rowe et al., 1987a), a finding which suggests that the malignant clone emerging in vivo no longer requires EBNA 2 expression for its continued growth.

The evolutionary relationship between type A and type B virus isolates is obscure. If they have indeed evolved from a common progenitor, then divergence of the EBNA 2 coding sequences seems to have occurred without equivalent divergence of other known viral genes. An alternative possibility is that the type B isolate could have been generated by recombination in the EBNA 2 region between a type A virus and one of the related B lymphotropic herpesviruses of Old World monkeys (Dambaugh et al., 1984; Zimber et al., 1986). One interesting aspect of the present results concerns the degree of inter-isolate variability within each of the two virus types. Although individual type A isolates even from the same geographical area differ markedly in the sizes of their EBNA 2A and EBNA 1 proteins, all type B isolates worldwide encode a very similar 75K/79K EBNA 2B doublet and isolates from the same geographical area tend to encode the same size EBNA 1 protein (present report and Rickinson et al., 1987). Reference to previously published data on the mol. wt. of the latent membrane protein (Rowe et al., 1987b) also suggests a greater uniformity among type B isolates. This raises the possibility that there are only a limited number of type B virus strains in existence worldwide, perhaps reflecting strict constraints which the EBNA 2B protein places on the structure of other latent viral gene products with complementary functions in the virus life cycle. Given the pronounced (but not exclusive) segregation of type B isolates within equatorial regions, it could be that some special feature of this environment is required for effective competition between type B isolates and the ubiquitous type A family of viruses.

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