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Detection of Epstein-Barr virus genomes in AIDS related lymphomas: Sensitivity and specificity of in situ hybridisation compared with Southern blotting

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Abstract

Eighteen cases of AIDS related, non-Hodgkin's lymphomas were examined for the presence of Epstein-Barr virus (EBV) genomes using in situ hybridisation with a ^{35}S-labelled probe. The results were compared with those obtained independently by Southern blot analysis with a ^{32}P-labelled probe of frozen tissue from the same tumours. Technically satisfactory results were obtained with both methods in 15 lymphomas. EBV DNA was detected in seven of 15 (47%) cases by in situ hybridisation and in eight of 15 (53%) cases by Southern blotting (including all the cases positive by in situ hybridisation). The results of EBV DNA detection by the two techniques were identical in 14 of 15 (93%) cases. In situ hybridisation gave no false positive results.

This study shows that the sensitivity and specificity of in situ hybridisation for the detection of EBV genomes in AIDS related lymphomas approaches that of Southern blotting, even when using routinely processed archival, paraffin wax embedded material.

Epstein-Barr virus (EBV) has been implicated in the pathogenesis of a range of benign and malignant lymphoproliferative and epithelial lesions.[1-11] Traditionally, the virus has been identified in tissues either by the use of immunocytochemistry to show the presence of EBV specific antigens in cytological preparations, or by the detection of EBV genomes in extracts of frozen tumour by filter-based nucleic acid hybridisation. Both these techniques require fresh or frozen tissue and this has limited the possibilities available for the study of EBV associated lesions in tissue sections.

Recently, methods have been described for showing the presence of EBV genomes in routinely processed paraffin wax embedded material by in situ hybridisation.[7 11-19] This technique is potentially of great value because it allows retrospective studies to be carried out on archival pathological material, and it provides information on the nature and distribution of cells infected with EBV which is not available with other methods. We have previously shown the value of this technique for studying both epithelial and lymphoproliferative EBV associated lesions.[11 12 16] In particular, we have shown that about a half of AIDS related lymphomas contain EBV genomes, as detected by paraffin wax section in situ hybridisation with radiolabelled probes.[17-19]

While previous investigations have shown the potential of paraffin wax section in situ hybridisation, there have been no systematic studies to assess the specificity and sensitivity of this technique for showing the presence of EBV genomes compared with established methods using filter hybridisation. Previous studies of EBV genome positive, AIDS related lymphomas have found a range of viral copy numbers comparable with that found in other types of EBV associated lymphoproliferation. AIDS related lymphomas provide, therefore, a good model for testing the sensitivity of paraffin wax section in situ hybridisation when applied to lymphoproliferative disorders induced by EBV.

Methods

The tumours were selected from a series of over 100 cases of non-Hodgkin's lymphomas from patients seropositive for human immunodeficiency virus (HIV), collected by the French-Danish Study Group on the pathology of AIDS related lymphomas. Criteria for inclusion were the availability of both routinely processed, paraffin wax embedded material and snap frozen tumour tissue. These criteria were met in 18 cases. All were surgical biopsy specimens of non-Hodgkin's lymphomas, selected from the archives of several different institutions participating in the study. Tissue was routinely fixed and processed before paraffin wax embedding, although the procedures were not standardised. The fixative used in each case is shown in the table, although precise details of the protocol used were not available. Paraffin wax blocks had been stored for up to three years. Cases were classified according to the updated Kiel classification,[20] with modifications as reported previously.[19]

The lymphomas were examined in separate laboratories for the presence of EBV DNA using both in situ hybridisation on paraffin wax sections and Southern blotting of snap frozen tissues. All studies were performed blind without knowledge of the results obtained in the other laboratory, and the findings were compared once all investigations had been completed.

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Histological type and results of EBV DNA detection by Southern blotting and paraffin wax section in situ hybridisation

Case No	Site of lymphoma	Lymphoma histology	Fiaxtive used	Epstein-Barr virus DNA	
				Southern blotting	In situ hybridisation
1	Lymph node	Burkitt	NBF	+	+
2	Lymph node	Burkitt	NBF	+	+
3	Lymph node	Immunoblastic (PC)	NBF	+	+
4	Lung	Immunoblastic (PB)	AAF	+	+
5	Palate	Immunoblastic (PB)	AAF	+	+
6	Lymph node	Immunoblastic (PB)	NBF	+	+
7	Gum	Immunoblastic (PB)	AAF	+	+
8	Lymph node	Burkitt	NBF	+	−
9	Lymph node	Burkitt	NBF	−	−
10	Bone marrow	Burkitt	NBF	−	−
11	Lymph node	Burkitt	NBF	−	−
12	Muscle	Burkitt	NBF	NS	−
13	Lymph node	Centroblastic	NBF	−	−
14	Lymph node	Centroblastic	AAF	−	−
15	Lymph node	Centroblastic	NBF	−	−
16	Lymph node	Immunoblastic (PB)	NBF	−	−
17	Gum	Immunoblastic (PB)	AAF	+	NS
18	Gum	Immunoblastic (PB)	AAF	+	NS

The results of EBV DNA detection using in situ hybridisation and Southern blotting were in agreement in 14 of the 15 lymphomas which gave technically satisfactory analyses.
AAF = acetic acid, alcohol, formaldehyde (Lillie's AAF).
NBF = neutral buffered formalin.
NS = not satisfactory.
PB = plasmablastic differentiation.
PC = plasmacytic differentiation.

PROBES

EBV DNA was detected using EBV *BamHI W* probes specific for the viral internal repeats. For in situ hybridisation total plasmid DNA was labelled with ^{35}S-dCTP by nick translation[21] to a specific activity of at least 1×10^9 dpm/μg. For Southern blot analysis, probes were labelled with ^{32}P-dCTP by the random primer method.[22]

For in situ hybridisation, probes for CMV (*EcoRI J* fragment of human cytomegalovirus and plasmid pBR322 (without the EBV insert) were used as negative controls.

IN-SITU HYBRIDISATION

ISH was performed on routinely fixed and processed, paraffin wax embedded tumour tissue as described previously.[12 19] Briefly, sections were cut onto slides coated with 3-aminopropyltriethoxysilane (Merck; Darmstadt, Germany) to prevent section loss,[23] baked overnight at 65°C, and dewaxed in xylene. Slides were incubated sequentially at room temperature in 0·2 N HCl (10 minutes) and 0·01% Triton X-100 (90 seconds) with intervening washes in 2 × SSC (0·3 M sodium chloride/0·03 M sodium citrate, pH 7·6). Sections were digested in pronase (1 mg/ml; Boehringer Mannheim, Germany) for 10 minutes at 37°C, washed in 2 × SSC, acetylated with 0·1 M triethanolamine/0·25% acetic anhydride for 10 minutes, and dehydrated. Hybridisation mixture (40 ng/ml ^{35}S-labelled probe, 0·25 mg/ml salmon sperm DNA, 50% deionised formamide, 2 × SSC, 10% dextran sulphate, and 50 mM dithiothreitol) was applied under siliconised coverslips, and probe and tissue DNA were denatured simultaneously on a 90°C heating block for six minutes. After overnight hybridisation at 37°C coverslips were removed in 50% formamide/0·1 × SSC at 37°C and then washed, first in 50% formamide/0·1 × SSC, for four hours at 37°C, and then in 2 × SSC and 0·1 × SSC, each for 30 minutes at room temperature. After dehydration in graded alcohols containing 0·3 M ammonium acetate slides were dipped in Ilford G5 emulsion (with 0·3 M ammonium acetate), exposed at 4°C for between two and eight days, developed in Kodak D19, fixed with Kodak rapid fixer, and counterstained with haematoxylin and eosin.

Cells were scored positive for viral DNA if they showed deposition of grains clearly in excess of the background level seen over random cells, and if hybridisation with the control probes was negative.

Controls: In all in situ hybridisation experiments the ^{35}S-labelled CMV and pBR322 probes were used on adjacent sections as negative probe controls. Sections of cells or tissues known to contain EBV—that is, oral hairy leucoplakia, acute infectious mononucleosis in tonsils, post transplantation lymphoma, or pellets of the EBV replicatively infected cell line P3HR1—were included in each batch of experiments as positive tissue controls. Non-hybridised sections and plain glass objective slides were included in each autoradiography batch as controls of background levels of signal. All slides were evaluated independently by two observers (SHD and GP). In cases of discrepancy, the case was re-examined by both observers and a consensus diagnosis reached.

SOUTHERN BLOT ANALYSIS

Total cellular and viral DNA was extracted from tumour samples (snap frozen after surgical removal) using standard procedures.[24] Briefly, cell lysis and proteinase K digestion was followed by phenol/chloroform extraction and ethanol precipitation. Ten μg of DNA was digested with the restriction endonuclease *Bam HI* (Bethesda Research Laboratories; Gothersburg, MD, USA) according to the supplier's recommendations. Digested DNA was size-fractionated by electrophoresis on 0·7% agarose gels in TRIS-borate buffer, hydrolysed for 15 minutes in 0·25 N HCl, denatured for 30

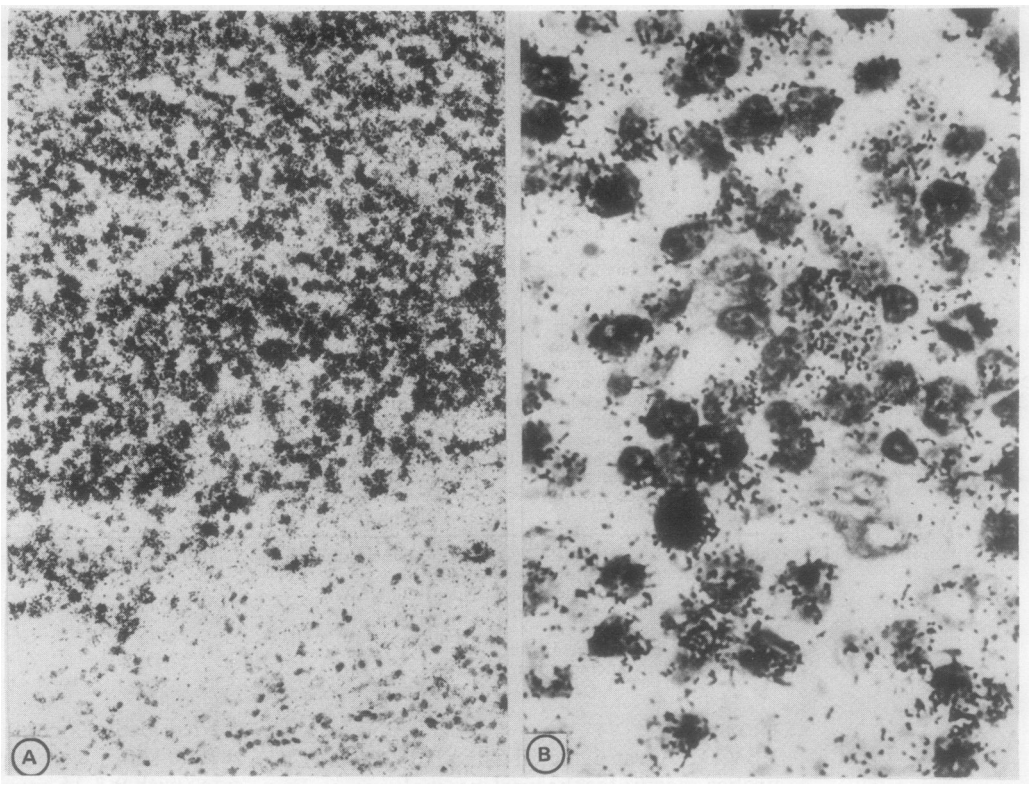

Figure 1 Partial involvement of a lymph node by Burkitt's type lymphoma in a patient with AIDS (case 1). Paraffin wax section in situ hybridisation with a ^{35}S-labelled EBV BamHI W probe. (A) Infiltrating lymphoma contains EBV DNA in every cell as shown by heavy deposition of silver grains. Residual stroma (lower part of field) shows only background signal. (B) At high power all identifiable tumour cells are heavily labelled (haematoxylin and eosin).

minutes before being transferred to nylon membrane (Hybond N, Amersham) according to the method of Southern.[25] Filters were hybridised with ^{32}P-labelled probe, as recommended by the manufacturer, and then washed at 60°C for 30 minutes each in 0·1% SDS/2 × SSC and 0·1% SDS/0·2 × SSC. Hybridised probe was detected by autoradiography for one to three days at −70°C using intensifying screens (Quanta III, Dupont; Wilmington, DE, USA).

Controls: Positive and negative controls for Southern blotting consisted of DNA extracted from Raji and BJAB cell lines, respectively.

Results

In two out of the 18 cases of AIDS related lymphomas in situ hybridisation was technically unsatisfactory because of high background signal in both test and control slides. In a third case Southern blot analysis could not be performed because of the poor yield of DNA extracted from the frozen block. Thus technically satisfactory results were obtained with both techniques in 15 cases (table).

In situ hybridisation identified EBV genomes in tumour cells of seven of 15 (47%) cases. As previously reported,[19] there was considerable variation in the number and distribution of EBV positive cells both among cases (figs 1 and 2), and from field to field within the same tumour. Positive control tissues showed a hybridisation pattern identical with that reported previously.[12 16] No signal over background was detected in any of the EBV positive cases when the CMV and pBR322 probes were sub-

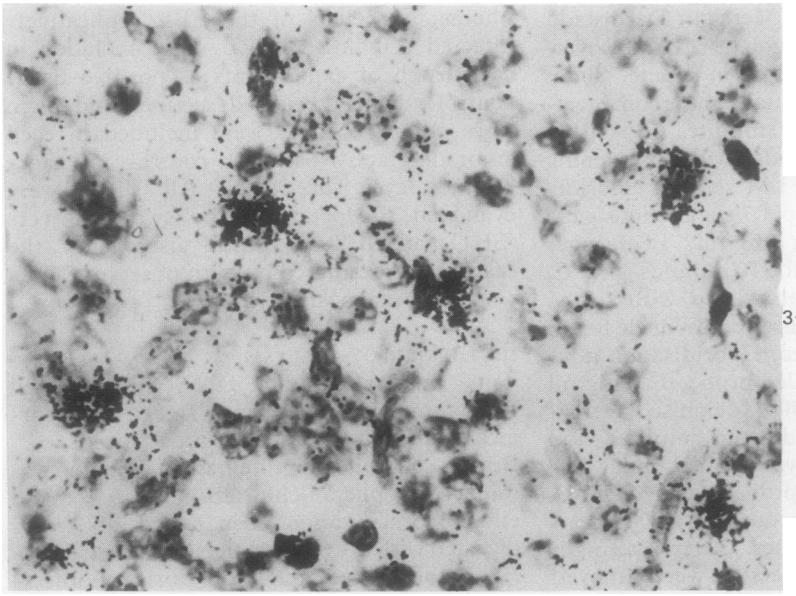

Figure 2 A polymorphic immunoblastic lymphoma with plasmablastic differentiation in an AIDS patient (case 4). Paraffin wax section in situ hybridisation with a ^{35}S-labelled EBV BanHI W probe shows the presence of EBV genome. The hybridisation pattern is much more heterogenous than that seen in fig 1, with scattered tumour blasts showing heavy autoradiographic labelling (haematoxylin and eosin).

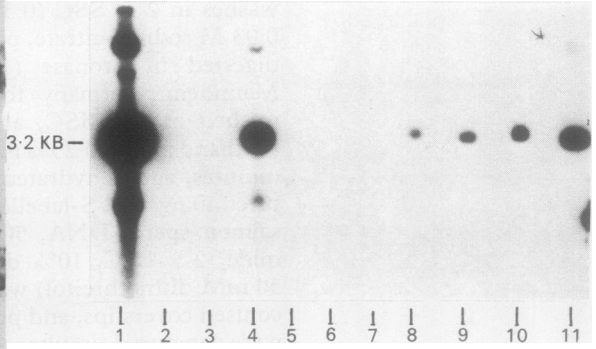

Figure 3 Southern blot analysis of nine cases of AIDS related lymphoma (lanes 3–11). The DNA in each lane was digested with BamHI and hybridised with the BamHI W probe. Five tumours (lanes 4, 8–11) show a positive 3·2 kilobase pair band corresponding to the BamHI W fragment of EBV. Lanes 1 (Raji) and 2 (BJAB) contain positive and negative control DNA, respectively.

stituted as negative controls for the EBV probe.

EBV DNA was detected in eight of 15 (53%) tumours by Southern blotting, including all cases positive by in situ hybridisation. In positive cases a 3·2 kilobase pair DNA fragment could be shown by hybridisation of the *BamHI W* probes to *BamHI* digests (fig 3). This band was also found in control DNA known to contain EBV genomes, and corresponds to the reported size of the *BamHI W* fragment of isolated EBV DNA. EBV genomes were identified in one lymphoma (case 8) by Southern blot analysis, but not by in situ hybridisation. Thus the two techniques gave identical results in 14 out of the 15 (93%) technically satisfactory cases.

Discussion

Traditionally, Southern blot analysis of DNA extracted from frozen tissue has been used as the "gold standard" when attempting to show the presence of viral genomes in tumours supposedly associated with EBV. The relative rarity of EBV associated lesions, however, combined with the need for frozen tissue for analysis, have made it difficult to collect large series of such cases. Paraffin wax section in situ hybridisation is, therefore, an attractive technique as it permits retrospective studies on archival material. Tissue structure is also maintained during in situ hybridisation and this, combined with the superior morphological detail of paraffin wax sections, allows viral sequences to be localised precisely within a tissue. Several recent studies have confirmed the value of paraffin wax section in situ hybridisation for EBV genome detection in both epithelial and lymphoproliferative lesions.[7 11-19]

Optimal conditions for performing EBV in situ hybridisation have been studied in various model experimental systems,[26-29] but there have been no previous systematic investigations to compare the sensitivity of paraffin wax section EBV in situ hybridisation with Southern blot analysis in the same tumours. In our study these two techniques have identical results for EBV detection in 14 of 15 (93%) cases of AIDS related lymphomas. The EBV carriage rates detected by in situ hybridisation and Southern blotting (47% and 53%, respectively), however, are similar to those found in a larger series of AIDS related lymphomas.[19] This suggests that the tumours in the present study can be taken to be representative of AIDS related lymphomas in general.

Discrepancies between the results of in situ hybridisation and Southern blot analysis might have been predicted in this study for several reasons. In general, the minimum average copy number per cell of a gene detectable by paraffin wax section in situ hybridisation will not be as low as that which can be shown by Southern blotting of fresh tissue. This is attributable to several factors, including the possible degradation of nucleic acids during fixation, the reduced access of probe to target DNA in fixed tissues, the restrictions placed on prehybridisation treatments by the need to retain good tissue morphology, and the introduction of increased background noise by the use of sensitive detection systems. This means that occasional lymphomas may exist in which tumour cells contain so few EBV copies that they fall below the limit of detection by in situ hybridisation, but they may still be identified by Southern blot analysis. Case 8 in our study may be an example of such a lymphoma.

On the other hand, the pattern of viral distribution in some tumours may mean that EBV can be detected by in situ hybridisation, but not by Southern blotting. This might occur if a tumour contains only very few EBV positive cells. The individual tumour cells may contain sufficient viral copies to be identified in sections by in situ hybridisation. When DNA is extracted for Southern blot analysis, however, these viral sequences may be so diluted by DNA from EBV negative cells as to be undetectable.

Another possible source of false negative results when using in situ hybridisation is related to fixation of the tissues. It has been shown that the sensitivity of in situ hybridisation is strongly affected by both initial delay in fixation and by the overall length of fixation.[30-32] Furthermore, certain fixatives such as Bouin's seem to be unsuitable if paraffin wax in situ hybridisation is subsequently used, possibly because they cause high levels of DNA degradation.[33 34] In our study fixation delay and overall fixation times for the different cases were not known. Standard prehybridisation procedures had, therefore, to be used, particularly with regard to the conditions of protease treatment used to expose target DNA. The close agreement of results obtained in this study by both in situ hybridisation and Southern blotting indicates that in most cases this did not affect the overall sensitivity of EBV detection. One lymphoma, which was negative by in situ hybridisation, was found to contain EBV by Southern blotting. Whether this false negative result was due to the tumour having a viral copy number below the detection limits by in situ hybridisation, or whether it reflected the use of an unusual fixation protocol is not clear.

We used ^{35}S-labelled probes in this study as in our experience these are more sensitive than non-isotopic probes for the detection of low abundance target sequences.[16] Other groups have described the use of non-isotopic probes for EBV genome detection in lymphomas by paraffin wax section in situ hybridisation.[14 15] Separate studies will be required to assess the sensitivity and specificity of these techniques.

We have only examined the sensitivity of paraffin wax section EBV in situ hybridisation when applied to AIDS related non-Hodgkin's lymphomas, but we believe these can be used as a model for lymphoproliferative disorders induced by EBV in general. Our results show that the sensitivity and specificity of paraffin wax section EBV in situ hybridisation using ^{35}S-labelled probes in these lesions approaches that of Southern blot analysis of frozen tissue extracts. This technique can, therefore, be used not only to provide information on the distribution and nature of cells infected with EBV in tissues, but also to identify EBV genome

positive tumours when fresh or frozen tissue is not available to perform more traditional analyses.

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Addendum

After this paper had been accepted, we were able to re-examine DNA from case 8 with Southern blotting to estimate the EBV copy number. The tumour contained an average of less than two copies of EBV per cell compared with control cell lines, confirming that the failure of in situ hybridisation to detect EBV DNA in this case could be attributed to the low viral copy number present.

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