Transactions of the Royal Society of Tropical Medicine and Hygiene 105 (2011) 86–94

Contents lists available at ScienceDirect

Transactions of the Royal Society of Tropical Medicine and Hygiene

journal homepage: http://www.elsevier.com/locate/trstmh

Burkitt's lymphoma: maximising the use of fine needle aspirates by long-term preservation for diagnosis and research

Susan Van Noorden[a,*], Irvin A. Lampert[a], Shao-An Xue[b], Dimitrios Lykidis[a,1], John A. Phillips[c,2], Elizabeth Molyneux[d], Beverly E. Griffin[e]

[a] Department of Histopathology, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK
[b] Department of Immunology and Molecular Pathology, University College London Medical School, Royal Free Hospital, London NW3 2PF, UK
[c] Burkitt's Lymphoma Unit, Kamuzu Central Hospital, Lilongwe, Malawi
[d] Department of Paediatrics, Queen Elizabeth Hospital, Blantyre, Malawi
[e] Division of Medicine, Imperial College London, St Mary's Hospital Medical School, Praed Street, London W2 1PG, UK

ARTICLE INFO

Article history:
Received 28 October 2009
Received in revised form 5 November 2010
Accepted 5 November 2010
Available online 18 December 2010

Keywords:
African childhood tumours. Fine needle aspirate. Cytological fixative. Transport medium. Immunocytochemistry. EBV gene expression

ABSTRACT

Fine needle aspirates from Burkitt's lymphoma and other tumours transferred directly into ThinPrep® PreservCyt® (Cytyc UK Ltd, Crawley, UK) buffered alcohol fixative retain their cellular and viral antigens and nucleic acids for many months at ambient temperatures. Despite the presence of blood and debris, cells dried onto slides from droplets and post-fixed in formalin, or sections of paraffin-embedded cell blocks from formalin post-fixed pellets, prove adequate for morphology, immunocytochemistry, in-situ hybridization and molecular biological analyses. Where there is lack of expertise in making thin smears or hospitals lack pathology laboratories and services, PreservCyt® provides an excellent medium for transport elsewhere for diagnosis and research.

1. Introduction

Endemic Burkitt's lymphoma (eBL), even in the HIV/AIDS era, is still the most common childhood cancer in sub-Saharan Africa[1,2] and, as in Denis Burkitt's era, in this geographical setting it remains a cancer of the rural poor living in the 'Great Lake Regions' of Africa.[3] In two Malawian hospitals alone, Queen Elizabeth Hospital for Children in Blantyre and Kamuzu Central Hospital in Lilongwe, about 250 new, clinically-evaluated Burkitt's lymphoma (BL) cases are admitted annually, and numerous other children return with relapses within months for re-examination and further treatment. The standard treatment is with cyclophosphamide which in some patients results in recovery after dramatic shrinkage of the tumour. The drug acts on proliferating cells which may be stimulated by the presence of Epstein-Barr Virus (EBV) in the lytic phase. A follow-up study[4] of 73 evaluable patients from Lilongwe, treated with multiple high doses of cyclophosphamide only, gave survival data as 71% for patients (52) with BL confined to the head and 29% for those (21) with primary abdominal tumours. Forty children were alive at a mean follow-up time of 59 months; the rest of the patients may have been resistant or only partially responsive to this therapy. Multi-drug therapy for resistant tumours may prove successful in another 10% or so of cases,[5] but for many African children, there remains no affordable cure.

BL, a non-Hodgkin B-cell lymphoma, is the most rapidly proliferating of all documented tumours. This is presumably largely as a consequence of the translocation of the

* Corresponding author. Fax: +44 208 740 7417.
E-mail address: s.vannoorden@imperial.ac.uk (S. Van Noorden).

[1] Present address: Department of Molecular Biology & Genetics, Democritus University of Thrace, Building 10, Panepistimioupoli, Dragana, Alexandroupoli 68100, Greece.

[2] Present address. 60 Old Rossorry Road, Enniskillen, Fermanagh BT74 7LF, UK.

0035-9203/$ – see front matter © 2010 Royal Society of Tropical Medicine and Hygiene. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.trstmh.2010.11.001

c-myc oncogene from its normal position on chromosome 8 to regions on chromosomes 2, 14 (the major translocation site) or 22, where it then comes under the control of immunoglobulin gene promoters. The presence and expression of the oncogenic Epstein-Barr virus (EBV), in most eBLs, may also influence tumour growth. In vitro, EBV has the capacity to 'immortalise' B-lymphocytes, causing them to proliferate indefinitely; its role in vivo remains unclear.[6] Over time, the complexity of non-Hodgkin's lymphomas has come to be better recognised,[7] but as yet there is no agreed consensus on what characterises BL, although some of its 'markers' are agreed.[8] The World Health Organization[9] has broadly defined three categories of BL, the endemic (e) form, associated with malaria and largely confined to children below the age of 18, where EBV is present in nearly 100% of the tumours; the sporadic (s) form, with no geographic, climatic or age association and less than 50% association with EBV and a third form, HIV-(or immunodeficiency) associated, where EBV positivity varies widely. Morphologically, it is almost impossible to differentiate among these species. Histological, immunophenotypic and genetic investigations combined with molecular analyses are needed for definitive characterisation.[7] Even these may prove unreliable, and are currently outside the expertise generally available in equatorial Africa. The fact that neoplastic lymphocytes are apparently genetically much less stable than normal lymphocytes[10] proves a confounding problem in a tumour with doubling times measurable in hours and highly prone to chromosomal alterations.

Spina et al.[11] noted that most of our recent knowledge of differences between BLs comes from the use of tumour-derived cell lines, and emphasised that cells in culture are not truly representative of the situation in vivo. We concur with these statements[12] but note, from more than twenty years' experience on studies of BL in Malawi, the difficulties encountered in sampling, processing and storing tumour material for further work locally or for transporting to other centres. The magnitude of the problem is reflected in the work of Tumwine et al.[13] in Italy who carried out a tissue microarray analysis of Ugandan BLs using archived formalin-fixed, paraffin-embedded biopsies. Of 600 such biopsies, only 129 (21.5%) were deemed adequate for microarray; most cases were excluded from their study due to the 'inadequacy of the material examined'. Thus, although material for study on eBLs should be available in quantities that could support further definition and evaluation, the problems of retrieval and storage have not been solved. Until they are, most research will be carried out on surgical biopsies from patients in developed countries, or on cell line-derived materials.

In Malawi and by inference some other sub-Saharan countries, tissue biopsy samples are not routinely taken from children, as in the few centres that have surgical facilities, the surgeons' time is at a premium for other problems. In other centres, pathology laboratories to process the material are not generally available. However, fine needle aspirates (FNAs) can give a rapid result with little trauma for the patient. Carefully taken, since the cells are fragile, these can be prepared as smears on microscope slides. For many years, in the absence of diagnostic facilities in the Malawian centres, alcohol-fixed smears have been sent to London for diagnosis. They are then stained for morphology by the May-Grunwald-Giemsa, (MGG) method and examined microscopically. Such materials can also have limited use for immunocytochemistry and molecular methods. However, tests have often proved to be constrained in scope by the poor quality of the smears and paucity of material, even when handled by experienced individuals.

Here, we have explored whether tumour aspirates ejected directly into ThinPrep® PreservCyt® non-gynae fixative (Cytyc UK Ltd, Crawley, UK) might overcome some of the problems inherent in smear preparations. This buffered methanol solution, in general use in cytology for preparing cytospin and ThinPrep®' monolayers for morphological and other diagnostic methods, protects cell structure, antigenic sites and nucleic acid integrity. Although, for practical reasons, the prescribed pre-fixation wash to lyse and remove red blood cells and other proteinaceous materials was not used, the procedure was successful for the eBL aspirates. We have now examined over 100 individual tumour samples stored (even over long periods) and transported at ambient temperatures and find them suitable for cytological, immunocytochemical, in situ hybridisation and molecular biological (PCR/RT-PCR) assessment. We show here data obtained from clinically diagnosed eBLs, using this fixation.

Long-term aims that require good clinical material involve identifying the (presently elusive) role(s) for EBV in eBLs[6] and markers that will differentiate patients likely to respond to cyclophosphamide from non- or partial-responders, both goals that hopefully will result in alternative, better treatments for this tumour. Samples allowing accurate diagnosis are of paramount importance for such studies. This communication is intended to show the potential of a simple preparation and storage method that can be used in developing countries to aid diagnosis and further the achievement of such aims.

2. Materials and Methods

2.1. Smear preparations

Fine needle aspirates from the tumour were smeared on slides, fixed in alcohol and stored for transport to London. There they were stained by the MGG method (blue cytoplasm, pink nuclei). Sometimes additional smears were air-dried for 1 hr, fixed in 10% formal saline for 10 min then covered with the methanolic polyethylene glycol cytological preservative, Cytofixx (CellPath Ltd, Newtown, UK) which seems to protect cellular antigens indefinitely.[14]

2.2. Cell suspensions in fixative

Tumour aspirates were ejected directly into PreservCyt® non-gynae fixative. Several aspirates were taken from each patient to increase the amount of material, and suspensions were stored at ambient temperature (often quite high in Malawi) to await transport and analysis. Stored this way, materials survive for several years.

Where the original smears proved inadequate for diagnosis, cells were deposited on charged slides in droplets (1.5 μl) from the settled, liquid-fixed material. This simple method was chosen as conserving more material than making cytospins. Several separate deposits could be made on a single slide for different immunocytochemical tests. After drying for 1 hr and post-fixation in formal saline for 10 min the preparations were stained or, for storage, covered with Cytofixx.

2.3. Cell blocks

In addition, cell blocks were made from centrifuged portions of the liquid-fixed material, post-fixed in 10% formal saline, infiltrated with 1.5-2% agar and processed to paraffin.[15] Such blocks provide numerous sections, allowing for many tests on a small amount of cellular material. Two-micron sections, collected on charged slides, were treated as routine paraffin sections for immunocytochemistry and in situ hybridization (ISH).

2.4. Immunocytochemistry (immunoperoxidase)

Immunostaining was initially done for the germinal center B-lymphocyte phenotype characteristic of eBL (CD10, CD20, Bcl6, Ki 67 [>90%], all characteristically positive, and CD3, Bcl2, characteristically negative). The protein product of the EBV BZLF-1 gene, Zebra/zta, was also investigated in some samples. There are other well-known tumours of African children that may present in a similar way to eBL but require different treatment. Where the clinical, morphological and immunocytochemical data suggested a need, additional diagnostic immunocytochemical tests were performed; such tumours included Hodgkin's lymphoma (CD30), Wilms' tumour (WT1), neuroblastoma and retinoblastoma (neurofilaments, neuron-specific enolase, protein gene product 9.5, synaptophysin, S-100), glioma (glial fibrillary acidic protein) and muscle tumours (desmin, muscle-specific actin).

Paraffin sections from cell blocks were de-paraffinised in xylene (x 3) and rehydrated in methanol using graded dilutions from 100% to 70%. Cytofixx was removed from cell preparations in 95% methanol (x 2) and preparations placed in 70% alcohol. Endogenous peroxidase was irreversibly blocked on both types of preparation by immersion for 30 min in 0.3% hydrogen peroxide in methanolic (70% methanol), 0.01 M phosphate-buffered 0.85% sodium chloride, pH 7.2 (phosphate-buffered saline, PBS). Preparations were rinsed in water, and antigen retrieval was carried out by heating preparations for 20 min in a domestic microwave oven on full power in 0.01 M citrate buffer, pH 6.0 (for CD20, Ki67, CD3) or 0.01 M TRIS/0.0003 M EDTA ((Tris) (hydroxymethyl)methylamine/ethylene diamine tetra-acetic acid) buffer, pH9 (for CD10, Bcl6, Bcl2).

Non-specific background staining was blocked with non-immune serum from the same species as that providing the second antibody. Preparations were incubated overnight at 4 °C with the primary antibodies at predetermined optimal dilutions in PBS containing 0.1% bovine serum albumin and 0.1% sodium azide (Table 1). Negative controls consisted of antibody diluent alone. Formalin-fixed paraffin sections of tonsil were used as positive controls for the lymphoid markers and cells from the BL-associated cell line, P3HR1, for EBV Zebra/zta. The secondary antibody, labeled with a horseradish peroxidase polymer ([ImmPRESS™, Vector Laboratories, Peterborough, UK] or Novolink [Leica Microsystems, Milton Keynes, UK]) was applied to cells for 30 min. After rinsing in PBS the enzyme was developed for 10 min in 0.01% hydrogen peroxide with 0.05% diaminobenzidine tetrahydrochloride (DAB) (Sigma Aldrich, Poole, UK) in PBS for the ImmPRESS™ preparations, or as directed for the DAB reagents in the Novolink kit. The reaction produces an insoluble dark brown end-product at the antigenic site. Nuclei were counterstained blue with haematoxylin, if desired; after dehydration through 70-100% methanol and clearing with pure xylene, synthetic resin mountant and a coverslip were applied.

2.5. In situ hybridization

ISH for small EBV-encoded nuclear non-coding RNAs (EBERs) 1 and 2 was performed as a diagnostic test for EBV using cell deposits or paraffin sections, with P3HR1 cells similarly treated as a positive control. A kit (NCL-EBV-K, Leica Microsystems) was used according to the manufacturer's instructions. The dark blue end-product is partially soluble in alcohol, so preparations were mounted in Ultramount, a permanent aqueous mountant (Dako Ltd, Ely, UK).

2.6. PCR and RT-PCR

These protocols were carried out on a few PreservCyt®-fixed FNAs as a feasibility study, aimed at showing whether such tumour samples could be used for identifying the presence of EBV repetitive *(BamH1* W) and single copy (BZLF1) EBV genes and multicopy RNAs (EBERs 1 and 2). Cultured P3HR1 cells fixed in PreservCyt® for 4 months were used as positive control.

The DNA was subjected to PCR for detection of the EBV *BamH1* W major (IR1) repeat and products were identified after electrophoresis on agarose gels using primers and protocols given elsewhere.[16]

An Agilent 2100 Bioanalyzer (Agilent Technologies, Cheadle, UK) was used, as described,[17] to determine the state of purity of the RNA extracted from six samples that had been stored for 5-14 months and contained cells mixed with varying amounts of blood. Cultured P3HR1 cells fixed in PreservCyt® for 4 months were used as positive controls.

For the RT-PCR work, RNA was reversibly transcribed using an AMV first strand cDNA synthesis kit (Invitrogen Ltd, Paisley, UK). RT-PCR to detect separately the EBV non-coding transcripts, EBER1 and EBER2, using primer pairs 5'-AGGACCTACGCTGCCCTAGA and 5'-AAAACATGCGGACCACCAGC (for EBER1) and 5'-AGCCGTTGCCCTAGTGGTTTC and 5'-CGGACAAGCCGAATACCCTTC (for EBER2), respectively. Primers for the virus-encoded replication activator intermediate early transcript from BZLF1 are those used in earlier studies on eBL.[18]

Table 1
Reagents for immunocytochemistry and in situ hybridization (ISH)

Mouse monoclonal (M) or rabbit polyclonal (R) antibody to:	Dilution	Commercial source	Catalogue no.
Lymphocyte markers			
CD3 M (T-lymphocytes)	1/50	Leica Microsystems[a]	NCL-L-CD3-565
CD10 M (B-lymphocytes, germinal centre)	1/20	Leica Microsystems	NCL-L-CD10-270
CD20 M (B-lymphocytes)	1/250	Dako[b]	M0755
CD30 M (Hodgkin's lymphoma, diagnostic of Reed-Sternberg cells)	1/50	Dako	M0751
Bcl2 M (B-lymphocytes, follicular area)	1/200	Dako	M0887
Bcl6 M (anti-apoptotic transcription factor, germinal centre B-lymphocytes)	1/25	Dako	M7211
Neural markers			
Neurofilaments M	1/50	Dako	M0762
Neuron-specific enolase M	1/600	Dako	M0873
Protein gene product 9.5 M	1/10	Leica Microsystems	NCL-L-PGP9.5
Synaptophysin R	1/50	Dako	A0251
S-100R	1/1000	Dako	Z0311
Glial fibrillary acidic protein R	1/100	Dako	Z0334
Muscle markers			
Desmin M	1/40	Dako	M0760
Muscle-specific actin M	1/600	Dako	M0635
Other markers			
Zta/Zebra (for Epstein-Barr virus)	1/10	E.Manet (gift)	Z125
Ki67 M (nuclear proliferation marker)	1/50	Leica Microsystems	NCL-L-Ki67-MM1
WT1 M (diagnostic of Wilms' tumour)	1/100	Leica Microsystems	NCL-L-WT1-562
Other reagents			
ThinPrep® PreservCyt® solution (non-gynae)		Cytyc UK[c]	70097-002
Cytofixx dropper		CellPath Ltd[d]	NHB-0200-00A
3,3'-Diaminobenzidine tetrahydrochloride dihydrate		Sigma Aldrich Chemical Co.[e]	D5637
ImmPRESS reagent kit, peroxidase universal anti-Mouse/Rabbit Ig		Vector Laboratories[f]	MP-750
Novolink polymer detection system		Leica Microsystems	RE7 140-CE
Epstein-Barr virus probe ISH kit		Leica Microsystems	NCL-EBV-K
Ultramount permanent aqueous mountant		Dako	S1964
cDNA synthesis kit		Invitrogen[g]	12328-040

[a] Leica Microsystems UK Ltd, Milton Keynes, UK; [b] Dako UK Ltd, Ely, UK.[c] Cytyc (UK) Ltd., Crawley, UK.[d] CellPath Ltd, Newtown, UK.[e] Sigma-Aldrich Co. Ltd., Poole, UK.[f] Vector Laboratories Ltd., Peterborough, UK. [g] Invitrogen Ltd, Paisley, UK.

3. Results

3.1. Cytological staining

Morphological diagnosis was initially made on the MGG stained smears with reference to the clinical information. Typical BL cells are round and about the size of a macrophage nucleus, whereas cells of diffuse large B-cell lymphoma (DLBCL), by comparison, are considerably larger. There can be overlap between some characteristics of BL and DLBCL,[8] however, DLBCL appears to be rare in children from sub-Saharan Africa. The BL cell nucleus occupies most of the cell, the chromatin is dispersed with accentuation on the nuclear membrane and two to six prominent nucleoli are usually seen. The intensely basophilic rim of cytoplasm may contain vacuoles. Mitotic cells are often present, as are macrophages, which account for the classical 'starry sky' description of BL preparations.

When smears were of poor quality, but apparently contained cellular material, liquid-fixed cell preparations provided a useful diagnostic alternative. After formalin post-fixation, both paraffin sections and cell preparations from the liquid-fixed FNAs could be stained satisfactorily with haematoxylin and eosin for diagnosis, but not with Giemsa.

3.2. Immunocytochemistry

For further confirmation of diagnosis, as well as to assess tumour markers associated with BL, standard immunoperoxidase staining was carried out. This proved to work well on both the cell deposits and paraffin sections from the suspensions (Figures 1–3). Diagnostic immunocytochemical tests distinguished eBL (CD10+, CD20+, Ki67+ with >90% positivity, Bcl6+, Bcl2-, CD3-) from most other childhood tumours and confirmed the clinically and morphologically suggested diagnoses. These markers are consistent with a germinal centre B-cell derivation of eBL. The protein product of the EBV BZLF1 gene, Zebra/zta, identified earlier in some Malawian BLs,[18] and essential for EBV replication was identified in sub-populations of cells in some samples (Figure 4). Positive and negative control staining was satisfactory throughout.

Despite occasional non-specific staining of the blood and debris surrounding the dispersed cells in some preparations, the lymphoma or other tumour cells could be distinguished easily. Where a haematoxylin nuclear counterstain was performed together with an immunostain, unreactive cells showed a blue-stained nucleus, but no brown immunoperoxidase reaction. In the absence of this nuclear counterstain (for greater contrast of the

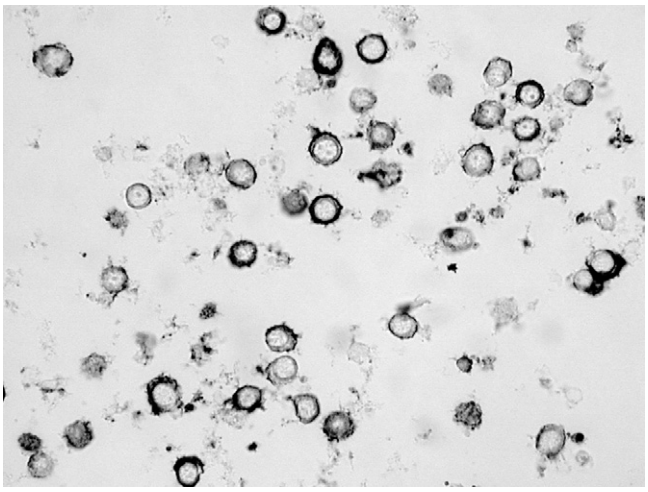

Figure 1. Fine needle aspirate from Malawian child fixed in PreservCyt buffered alcohol. Immunoperoxidase stain for CD20 on paraffin section of formalin-post-fixed sample. Most cells are positive. Cell membrane localisation.

immunostain) unreactive cells appeared as refractile negative images.

Table 1 gives the source and dilution of the antibodies and other reagents used in this study.

Table 2 shows the age distribution of samples from 100 patients where smears and liquid-fixed preparations were available. For 32 of these patients, cytological diagnosis of BL was possible on the smear alone. In 22 this was confirmed on liquid-fixed cells, 10 of the liquid-fixed samples were not further tested. In a further 28 cases, the smear was cellular but inadequate for diagnosis. Immunocytochemistry on liquid-fixed cells in these cases was helpful in 24, not helpful because of inadequate sample in 4. In the remaining 40 cases, neither the smear nor the liquid-fixed material was diagnosable initially, being either blood only or an inadequate sample. Of the total diagnosable cases, 47 were BL, 3 were uncertain, 6 were non-BL (3 undetermined, 1 epithelial tumour, 1 lymphoma, 1 probable glioma); 44 were inadequate (blood or scanty material). Table 3 shows detailed results on 12 selected cases, comprising both eBL and other tumours.

3.3. ISH for EBERs (1 and 2)

ISH for the combined EBER (1 and 2) RNAs was effective on the PreservCyt®-fixed cell preparations (Figure 5) and formalin post-fixation was not essential. In general, whether as whole cells on slides or as paraffin sections, better results were obtained on PreservCyt®-fixed material than on long-stored, alcohol-fixed smears that had not been Cytofixx-protected. Protected formalin-fixed smears were sometimes useful, but the end-result depended strongly on the quality of the smear. This variable was removed by using the liquid-fixed cells.

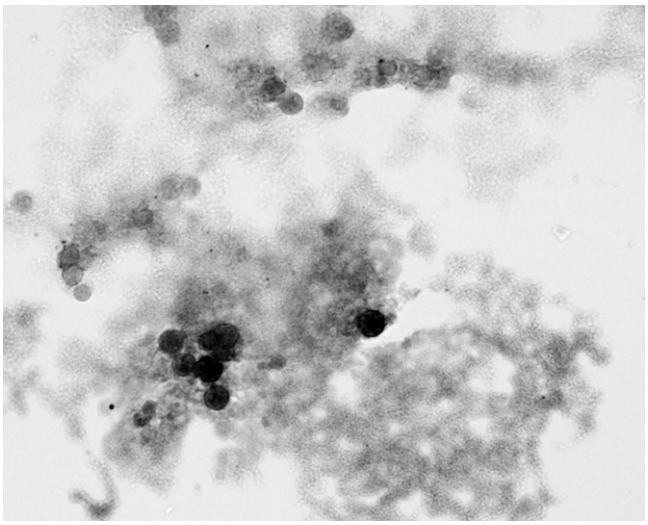

Figure 2. Fine needle aspirate from Malawian child fixed in PreservCyt buffered alcohol. Immunoperoxidase stain for Bcl6 on cells deposited on slide from PreservCyt fluid, post-fixed in formalin. Nuclear localisation.

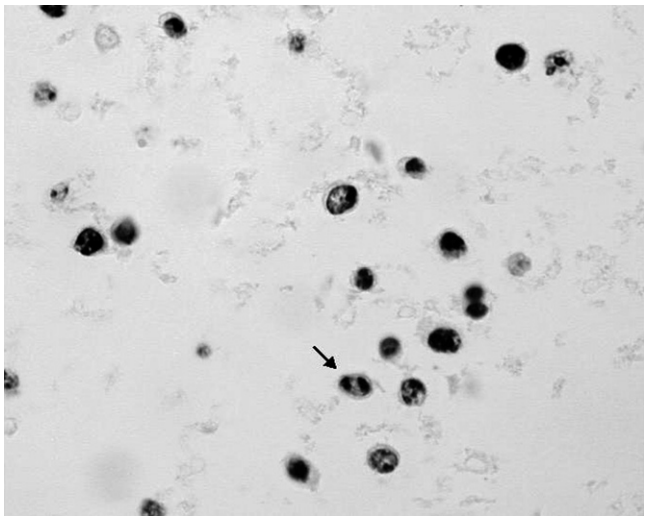

Figure 3. Fine needle aspirate from Malawian child fixed in PreservCyt buffered alcohol. Immunoperoxidase stain for Ki67 on paraffin section of formalin-post-fixed sample. Most cells are positive. Nuclear localisation, some cells in mitosis (arrow).

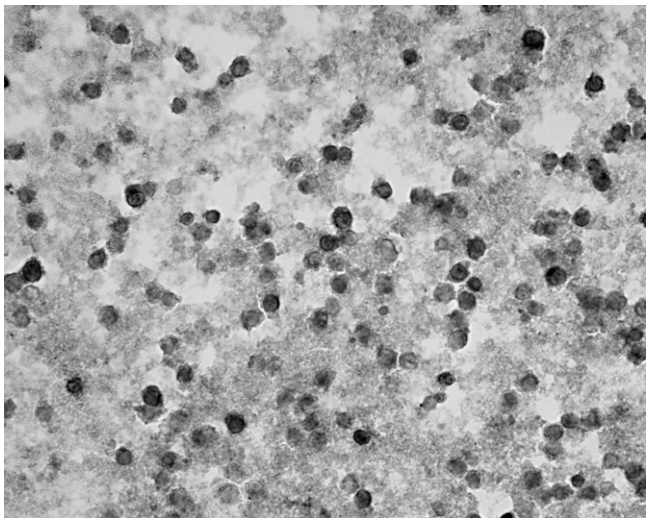

Figure 4. Fine needle aspirate from Malawian child fixed in PreservCyt buffered alcohol. Immunoperoxidase stain for Zebra/zta protein on cells deposited on slide from PreservCyt fluid, post-fixed in formalin. Many cells are positive. Nuclear and cytoplasmic localisation.

3.4. PCR and RT-PCR

Successful extraction and PCR of DNA (Figure 6) and RT-PCR of RNA (Figure 7) from the PreservCyt®-fixed eBL samples depended on the concentration of cells among the debris, purer preparations giving better yields. Bioanalysis of RNA samples for a 'housekeeping' gene, beta-actin, showed that control pure cell cultures (BL-associated P3HR1 cells) fixed in PreservCyt® for 4 months gave undegraded RNA. Patient samples, although often partially degraded, were still adequate for RNA analysis and showed positivity for EBER1 (Figure 7), EBER2 and BZLF1 (in some tumours).

4. Discussion

eBL continues to affect the lives of children in sub-Saharan Africa and remains little understood. Ideally, research and diagnosis would be carried out on formalin-fixed, paraffin-embedded biopsies of the tumours.

Table 2
Age distribution of 100 cases provided from January 2007 to January 2009 as both smears and liquid-fixed material

Age in years	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Male (n = 60)	1	1	5	4	7	3	8	9	3	6	2	5	2		1	1			1
Female (n = 40)		2	4	3	6	9	3	5	2	1	1	2	2						

Table 3
Detailed results on typical cases

Cytology diagnosis	CD10	CD20 IC	Ki67 IC	Bcl6 IC	Bcl2 IC	Zebra/zta IC	EBER1&2 ISH
BL Case 1	++	+++	+++	+	neg	nd	++++
BL Case 2	nd	++	++	+	neg	nd	+++
BL Case 3	++	+++	++	+	neg	+	+
Probably BL							
Case 4	+++	+++	+++	+	neg	+	+++
BL Case 5	+++	+++	+++	+	+/-	nd	+++
BL Case 6	+++	+/-	+++	+	neg	+/-	++
BL Case 7	+++	+++	+++	+	neg	+/-	+++
BL Case 8	++	++	++	+	neg	nd	+++
Large B cell lymphoma							
Case 9	+	+	+	+/-	+/-	+/-	+
Neuroblastoma							
Case 10	neg	neg	neg	neg	neg	+/-	neg
NotBL							
Case 11	nd	neg	+++	+/-	neg	+/-	neg
Reactive polymorphic lymphoid							
Case 12	neg	+	neg	neg	+/-	nd	neg

IC = immunocytochemistry; ISH = in situ hybridisation; BL = Burkitt's lymphoma. +++ = most cells strongly positive; ++ = many cells definitely positive; + = many cells positive but some with weak reaction; +/- = some cells positive, neg = negative; nd = not done

However, given Malawi's prevalent shortage of surgical and pathological facilities, biopsy of the tumours is not an option and diagnosis is heavily dependent on clinical features. Some BL tumours may be difficult to distinguish from others that require different treatment. Pathological diagnosis is helped by cellular morphology on MGG-stained smears from FNAs but pathologists are scarce; hence smears may be sent elsewhere for diagnostic confirmation, and often some prove inadequate.

In this pilot feasibility study we have shown that a simple procedure can provide ample material for diagnosis and research. In the 100 cases shown in Table 2, there were 32 good preparations and a further 24 that could not be diagnosed on the poor initial smear but could be assessed on the liquid-fixed samples. The immunocytochemical diagnostic tests served well to distinguish BL from non-BL cases and thus prevent inappropriate treatment of many of the patients. We did not aim at complete pathological diagnosis, but rather to exclude non-BL cases from the drug regime used to treat this disease. A non-BL lymphoma profile (e.g. case 9 in Table 3) could indicate to the clinician that the patient might need referral for a surgical biopsy and further investigation. Until vast progress is made in enlarging surgical, pathological and technical facilities in developing countries, some wastage of material will have to be accepted. Even if 50% of the samples proved inadequate, abundant material should be available for a future research project on this disease.

With the PreservCyt® fixation method described in this paper, smear-making expertise is not essential. Samples are fixed immediately and cell and viral properties survive even high ambient temperatures for at least a year. We have had results identical to the original ones on reassessing samples stored for up to four years. Many samples

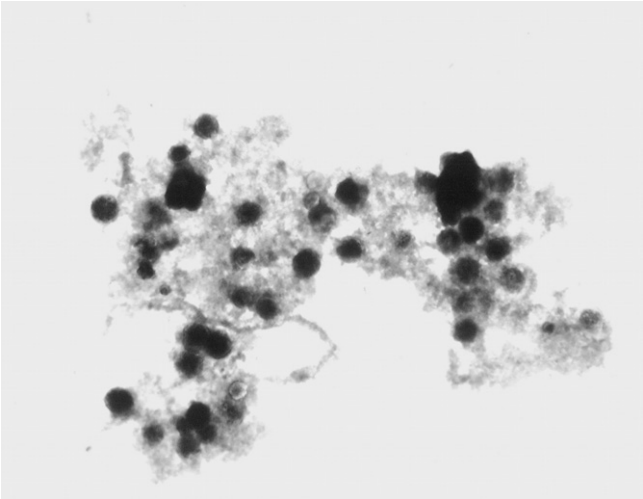

Figure 5. Fine needle aspirate from Malawian child fixed in PreservCyt buffered alcohol. In situ hybridisation for EBERs 1 and 2 RNAs on paraffin section of formalin-post-fixed sample. Alkaline phosphatase marker. Most cells are positive. Nuclear localisation.

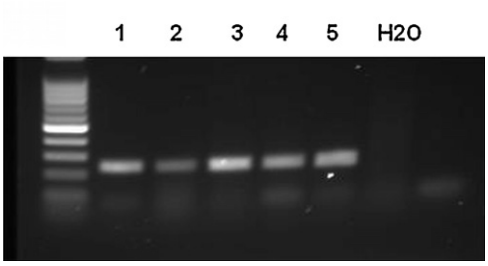

Figure 6. Detection of EBV *BamH1* W DNA (IR1) by PCR. Total DNA was extracted from five samples of fine needle aspirates kept in PreservCyt for varying lengths of time. After amplification by PCR (39 cycles), all five samples were positive. 100 bp DNA marker ladders, at left.

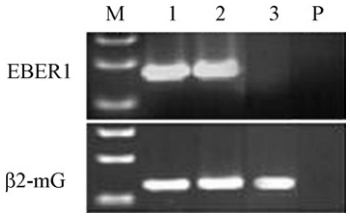

Figure 7. Detection of EBER1 expression by RT-PCR. Total RNA was extracted from two samples of fine needle aspirates kept in PreservCyt fixative for over two years (lanes 1 and 2) or from EBV negative human PBMCs (lane 3), and reversibly transcribed using random hexanucleotide primers as described[12]. EBER1 expression (top panel) was detected by RT-PCR (39 cycles) using a pair of EBER 1 specific primers (given, Materials and Methods). In similar experiments, EBER2 expression alone (unlike in earlier studies[18]) was examined in seven tumours; all were positive (data not shown). Beta-2-microglobulin (bottom panel) was used as a housekeeping gene control. Track M contains a 100 bp DNA ladder, and P (control), primers only.

were adequate for immunocytochemistry (Figures 1–4) or molecular biological protocols (Figures 5–7), as indicated in Table 3. Despite the presence of debris and red blood cells in FNAs, scattered immuno- or ISH-positive tumour cells were easily identified in cell preparations whether taken directly from the fixative or processed as paraffin sections from pelleted material. Formalin post-fixation gave better immunostaining than PreservCyt® alone, particularly for the lymphoid and nuclear markers, and provided a useful substitute for primary fixatior in formalin (not possible for these Malawian samples). Notably, however, when a reasonably competent smear contained blood only, the liquid-fixed material proved not to be a useful alternative as it, too, contained only blood. Thus expertise in taking FNAs and appreciation of the fragility of these tumour cells up to the point where they are fixed is important.

We did not investigate the *c-myc* translocation by fluorescence in situ hybridisation (FISH), but since our ISH and PCR methods show that nucleic acids are well preserved, such tests could undoubtedly be incorporated into future studies. Notably, however, in studies aimed at distinguishing among the B-cell malignancies, Chuang et al[19] were able to dispense with the *c-myc* ISH tests in distinguishing BL (mainly in children) from diffuse large B-cell lymphoma (DLBCL) (adults), relying only on morphology and an immunocytochemical panel of antibodies. In Malawi, DLBCL is rare among children, as is eBL among adults. Out of nearly 1700 cases of eBL recorded in Kamuzu Central Hospital in Lilongwe in the past 15 or so years, many of which were seen by one of us (JAP), only 12 of the patients were over 18 years old. From reported data, many or most of these probably harbour the major t(8:14) *c-myc* translocation, but chromosomal alterations at other cell sites are common.[20] One of the potential advantages of the protocol described in this paper is that samples could in future be available to sort out the cytogenetic events that influence the behaviour of eBLs.

In this feasibility study, we have confirmed consistently the expression of EBV lytically-related genes in some cells in some eBL patients (Figure 4). However, unlike in earlier studies,[18] patient response to therapy was often unknown. Thus, we could not confirm whether BZLF1 gene expression was accompanied by better tumour response to therapy. Future work will address this question.

This work was initiated in order to obtain material that could be diagnosed rapidly and accurately by morphology and immunocytochemistry, in order to prevent children with tumours other than eBL from being inappropriately medicated. It was further aimed at acquiring accurately diagnosed tumour material for future work. We intend to explore further the role of EBV, which is found, before therapy, in nearly all cases of this unique children's tumour. We also hope to confirm markers that distinguish between tumours that respond to chemotherapy and those that do not. We have accomplished the first of our aims and the others will hopefully follow.

In conclusion, PreservCyt® (and possibly other liquid cytology fixatives) is an inexpensive and convenient medium for transporting FNAs at ambient temperatures from centres lacking complete pathology services, as recently confirmed.[21] In that it allows for long-term retention of cellular properties and diagnostically relevant assays, this procedure should encourage a greater use of primary materials in future research.

Authors' contributions: The project was devised by BEG, IAL and SVN with input from EM and JAP; SVN made the cell preparations, did the immunostaining and in-situ hybridisation and analysed the results with IAL; IAL diagnosed the samples, analysed the results with SVN and photographed the preparations; SAX did the RT-PCR for EBV RNA and analysed and interpreted the data; DL did the PCR for DNA and analysed and interpreted the data; EM and JAP supervised the provision and transport of FNAs and supplied patient histories from Malawi; BEG directed the project and wrote the paper with SVN. All authors critically read and contributed to the draft versions of the manuscript and read and approved the final version. BEG, SVN and IAL are guarantors of the paper.

Acknowledgements: The authors thank Professor Gordon Stamp for his great support of this project both scientifically and with laboratory facilities and gratefully acknowledge the assistance of the technical staff of the Histopathology Department, Hammersmith Hospital. We also acknowledge the help of the staff of the Burkitt's lymphoma wards in Blantyre and Lilongwe. We thank Dr Evelyne Manet

(Lyon, France) for the gift of antibody to the zta/Zebra protein.

Funding: We thank the Leukaemia and Lymphoma Research Fund, 43 Great Ormond Street, London WC1N 3JJ, UK for grant no. 07031 in partial support of this work. DL was supported by grant no. 2002/6416 from Cambridge Antibody Technology, Milstein Building, Granta Park, Great Abington, Cambridge CB21 6GH, UK.

Conflicts of interest: None declared.

Ethical approval: Not required.

References

1. Parkin DM, Ferlay J, Hamdl-Chérif M, Sitas F, Thomas JO, Wabinga H et al. *Cancer in Africa epidemiology and prevention*. IARC Scientific Publications No.153. Lyon: IARC Press; 2003. Chapter 5, Childhood Cancer; p 381-96.
2. Sinfield RL, Molyneux E, Banda L, Borgstein E, Broadhead R, Hesseling P, et al. Spectrum and presentation of pediatric malignancies in the HIV era: experience from Blantyre, Malawi, 1998–2003. *Pediatr Blood Cancer* 2006;**48**:515–20.
3. Burkitt DP. A sarcoma involving the jaws in African children. *Brit J Surg* 1958;**46**:218–23.
4. Kazembe P, Hesseling PG, Griffin BE, Lampert I, Wessels G. Long term survival of children with Burkitt lymphoma in Malawi after cyclophosphamide monotherapy. *Med Paediatr Oncol* 2003;**40**:23–5.
5. Hesseling P, Molyneux E, Kamiza S, Israels T, Broadhead R. Endemic Burkitt lymphoma: a 28-day treatment schedule with cyclophosphamide and intrathecal methotrexate. *Ann Trop Paediatr* 2009;**29**:29–34.
6. Bornkamm GW. Epstein-Barr virus and its role in the pathogenesis of Burkitt's lymphoma: an unresolved issue. *Semin Cancer Biol* 2009;**19**:352–65.
7. Ferry JA. Burkitt's lymphoma: clinicopathologic features and differential diagnosis. *The Oncologist* 2006;**11**:375–83.
8. Rosenwald A, Ott G. Burkitt lymphoma versus diffuse large B-cell lymphoma. *Ann Oncol* 2008;**19**:67–9.
9. Jaffe E, Harris NL, Stein H, Vardiman JW, editors. *World Health Organization classification of tumours, Vol 3: Pathology and genetics of tumours of the haematopoietic and lymphoid tissues*. Lyon: IARC Press; 2001.
10. Seshadri R, Kutiaca RF, Trainor K, Matthews C, Morley AA. Mutation rate of normal and malignant human lymphocytes. *Cancer Res* 1987;**47**:407–9.
11. Spina D, Leoncini L, Megha R, Gallorini M, Disanto A, Tosi P, et al. Cellular kinetic and phenotypic heterogeneity in and among Burkitt's and Burkitt-like lymphomas. *J Pathol* 1997;**182**:145–50.
12. Xue SA, Labrecque LG, Lu QL, Ong SK, Lampert IA, Kazembe P, et al. Promiscuous expression of Epstein-Barr virus genes in Burkitt's lymphoma from the central African country Malawi. *Int J Cancer* 2002;**99**:635–43.
13. Tumwine LK, Campidelli C, Righi S, Neda S, Byarugabe W, Pileri SA. B-cell non-Hodgkin lymphomas in Uganda: an immunohistochemical appraisal on tissue microarray. *Hum Pathol* 2008;**39**:817–23.
14. Maxwell P, Patterson AH, Jamison J, Miller K, Anderson N. Use of alcohol- fixed cytospin protection by 10% polyethylene glycol in immunocytology external quality assurance. *J Clin Pathol* 1999;**52**:141–4.
15. Yeoh GPS, Chan KW. Cell block preparation on residual ThinPrep®sample. *Diagn Cytopathol* 1999;**21**:427–43.
16. Labrecque LG, Barnes DM, Fentiman IS, Griffin BE. Epstein-Barr virus in epithelial cell tumours: a breast cancer study. *Cancer Res* 1995;**55**:39–45.
17. Lykidis D, Van Noorden S, Armstrong A, Spencer-Denei B, Li J, Zhuang Z, et al. Novel zinc-based fixative for high quality DNA, RNA and protein analysis. *Nuc Acids Res* 2007;**35**:e85.
18. Labrecque LG, Xue SA, Kazembe P, Phillips J, Lampert I, Wedderburn N, et al. Expression of Epstein-Barr virus lytically related genes in African Burkitt's lymphoma: correlation with patient response to therapy. *Int J Cancer* 1999;**31**:6–11.
19. Chuang S-S, Ye H, Du M-Q, Lu C-L, Dogan A, Hsieh P-P, et al. Histopathology and immunohistochemistry in distinguishing Burkitt lymphoma from diffuse large B- cell lymphoma with very high proliferation index and with or without a starry-sky pattern. *Am J Clin Pathol* 2007;**128**:558–64.
20. Boerma EG, Siebert R, Kluin PM, Baudis M. Translocations involving 8q24 in Burkitt lymphoma and other malignant lymphomas: a historical review of cytogenetics in the light of today's knowledge. *Leukemia* 2009;**23**:225–34.
21. Tang W, Harmon P, Gulley ML, Mwansambo C, Kazembe P, Martinson P, et al. Viral response to chemotherapy in endemic Burkitt lymphoma. *Clin Cancer Res* 2010;**16**:2055–64.