(Reprinted from Nature, Vol. 274, No. 5673, pp. 756–761, August 24, 1978)

Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study

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Results from a prospective sero-epidemiological study initiated in Uganda in 1971 indicate that children with high antibody titres to Epstein-Barr virus structural antigens are at high risk of developing Burkitt's lymphoma. These findings strongly support a causal relationship between the Epstein-Barr virus and Burkitt's lymphoma but suggest that the oncogenic potential of the virus is realised only in exceptional circumstances.

ALTHOUGH there has never been any direct epidemiological evidence that Burkitt's lymphoma (BL) is caused by the Epstein-Barr virus (EBV), there is considerable circumstantial evidence. The virus, first isolated from a biopsy specimen taken from a child with BL[1], is well known for its ability to transform human B lymphocytes[2,3] and for its *in vivo* oncogenic potential in non-human primates[4,5]. BL patients have been found to have higher levels of antibodies against a wide range of EBV-determined antigens (EBV capsid antigen(s) (VCA), early antigens (EA) and membrane antigens (MA)) than unaffected children of the same age from the same area[6,7]. In addition, multiple copies of viral genomes (EBV/DNA) and EBV-determined nuclear antigen (EBNA) have often been detected in BL cells[8,9]. On the other hand, BL has a highly restricted geographical distribution[10–12] which led to the suggestion that an arthropod-borne agent might be the cause[11], whereas EBV is widespread and not known to be arthropod-borne. Nevertheless, by the end of the 1960s the evidence suggesting that EBV was a necessary factor for the development of BL was sufficiently strong to justify a large prospective sero-epidemiological investigation.

Rationale and design

As infection with EBV is nearly ubiquitous among African children, it was necessary to show that children developing BL were infected with, or reacted to, EBV in a different way from the general population. The difference was to be, for example, in the age at infection, the degree of infection, or the response to infection. It was possible that the EBV serological profile peculiar to BL patients might have arisen as a result of the disease because the proliferating tumour cells could harbour the virus, though elevated EBV antibody titres are not regularly seen in patients with other B-cell lymphomas[13]. To exclude that possibility, serum samples had to be obtained from patients before the onset of disease and this necessitated a large prospective study. A model was provided by the demonstration of EBV as the cause of infectious mononucleosis (IM)[14,15]. Serological tests showed that only students who had avoided EBV infection before entering Yale University were at risk of developing IM. Any association of BL with EBV was likely to be more complex and we formulated four hypotheses[16,17].

Our first (the null hypothesis) was that there is no causal relationship between EBV and BL. The EBV serological profile of children who will later develop BL should not differ from that of controls matched for age, sex and locality. The high reactivities observed in BL patients after diagnosis would reflect a secondary reactivation of a passenger virus. Our second hypothesis (the IM model) was that BL develops shortly (within 2 yr) after a primary infection by EBV. Sera collected from patients before the incubation period would lack antibodies to EBV. According to our third hypothesis, BL

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develops in children who have had a long and heavy exposure to EBV. The sera of 'future' BL patients would exhibit high antibody titres long before diagnosis of BL. Our fourth hypothesis was that EBV has a causal role in BL, but that the latent periods between infection and clinical onset were long and variable and timely intervention of cofactors was necessary. Before diagnosis the reactivity of the sera from BL patients might differ from that of controls but in an unpredictable manner. This hypothesis could not be tested in a study of reasonable size.

We selected the West Nile District of Uganda (Fig. 1) as the study area as the incidence of BL was high and the epidemiology well documented[18,19]. If either our second or third hypothesis was correct, the study would also yield information about the length of the period between primary infection and onset of disease.

Feasibility studies in 1968 and 1969 showed that within individuals, antibody titres to EBV were quite stable over 18 months[20]. About 10% of children had titres greater than 1/160 and 10% had titres less than 1/10. Assuming that either group (according to the second and third hypotheses) might have a fivefold greater risk than normal, then 30 cases of BL would be needed to ensure a high probability of a positive finding. We estimated that in 5 yr approximately 35 cases would arise in 37,000 4–8-yr-olds living in the study area, which was selected from the most densely populated parts of four adjacent counties (Aringa, Maracha, Terego and Madi (Fig. 1)).

Organisation of the survey

We explained our purpose at public meetings, and a few days before the collection of blood in each locality we made personal visits and registered all members of each household. Parents brought their children, 2 or 3 d later, to a central point, usually within 2–3 km of their home. More than 85% of eligible children were included in the study in most parishes. However, in Aringa county adequate cooperation was not obtained and so part of Ayivu county was incorporated into the study (Fig. 1).

Venous blood was taken whenever possible, but from very young children a finger or heel prick was used. Thick and thin blood films were also obtained for malarial parasite investigation. Serum samples were transferred in insulated boxes containing cooling packs and within 6 h placed in a domestic refrigerator (temperature approximately 4 °C). On the following day, sera were separated and stored in a freezer (at −70 °C). Sera were shipped periodically in liquid nitrogen from Arua to the International Agency for Research on Cancer (IARC), Lyon, for long term storage in liquid nitrogen.

From February 1972 to September 1974, serum samples were obtained from about 42,000 children up to 8 yr old. The search for new cases of BL among those children was intensified from January 1973 by a team which visited regularly all health centres, dispensaries and hospitals in the survey area. All suspected cases were traced and examined clinically. If BL was still suspected, the following specimens were obtained before treatment: (1) a needle biopsy and touch smears for cytological evaluation; (2) a surgical biopsy fixed in formalin sent to the Department of Pathology of Makerere University, Uganda; (3) another biopsy sent to the IARC for coded evaluation by pathologists familiar with BL (G. O'Conor, National Cancer Institute and D. Wright, University of Southampton, with the help of N. Muñoz, IARC); (4) a piece of tumour, to be frozen, sent to H. zur Hausen's laboratory in Erlangen for detection of EBV/DNA[21]; (5) touch smears with fresh tumour tissue, air- and acetone-fixed, sent to IARC for detection of

Table 1 Patients diagnosed with BL in the West Nile District of Uganda in the period 1968–77 by year of diagnosis and county of residence

	Aringa, Maracha Terego, Madi		Other counties		Total	
1968	7		2		9	
1969	12		4		16	
1970	7	42	8	33	15	75
1971	10		11		21	
1972	6		8		14	
1973	12		11		23	
1974	4		2		6	
1975	9	31	9	32	18	63
1976	4		5		9	
1977	2		5		7	

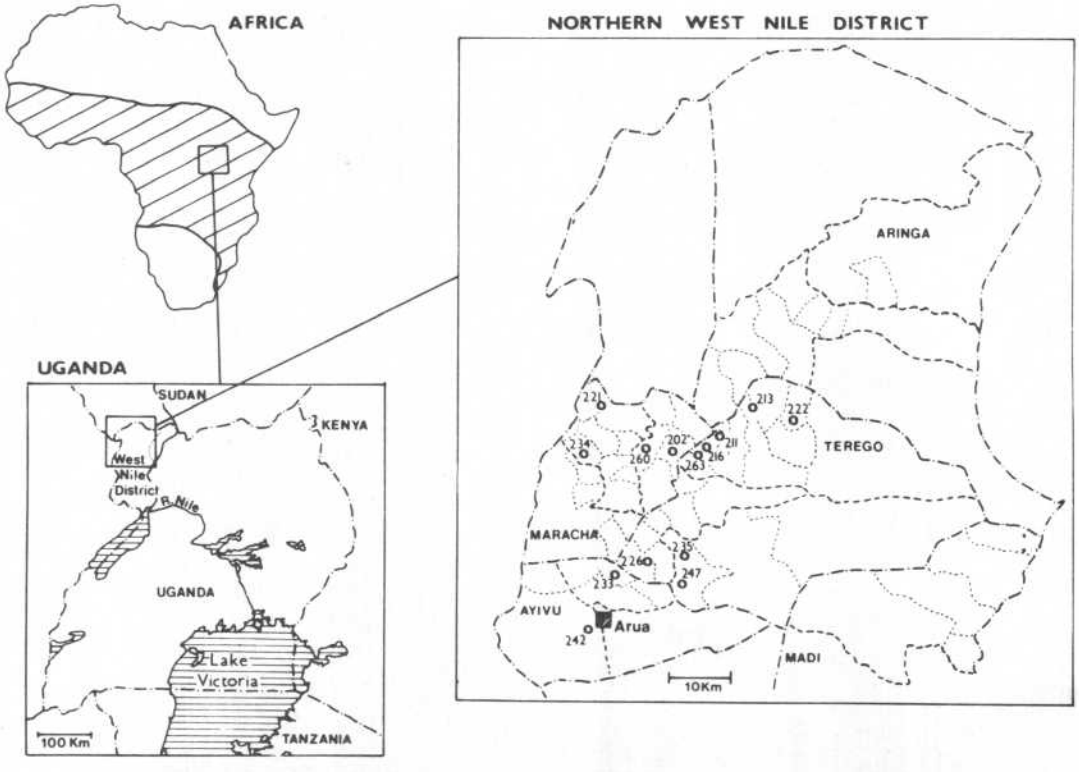

Fig. 1 Map of Africa showing the study area –·–·–, County boundaries; – – –, sub-county boundaries; · · ·, parish boundaries; ○, BL cases.

Table 2 Demographic, clinical and pathological data of the 14 BL patients who were bled before diagnosis

Case no.	Sex	Age (yr)	Diagnosis Date	Site of tumour	Outcome	Pathology	EBV/DNA in tumour
202	F	5	May 1973	R. mand./ovary	Died Oct. 1973	Confirmed	ND
211	M	5	Sept. 1973	Cervical/lymph nodes	Alive Dec. 1977	Consistent	ND
213	F	3	Sept. 1973	R. mand./ovary	Died Dec. 1973	Consistent	ND
216	F	6	Dec. 1973	Both max.	Died April 1974	Consistent	ND
221	F	6	Sept. 1974	R. orbit	Alive Dec. 1977	Consistent	54 g.e.
222	M	4	Sept. 1974	Both max./both mand.	Alive Dec. 1977	Consistent	116 g.e.
226	F	10	Feb. 1975	R. orbit/liver	Died Feb. 1975	Confirmed	49 g.e.
233	M	5	July 1975	Orbits/liver	Died Nov. 1975	Consistent	ND
234	M	8	July 1975	R. max./BM	Died Dec. 1975	Consistent	30 g.e.
235	M	10	July 1975	L. mand.	Alive Dec. 1977	Confirmed	38 g.e.
242	M	12	Sept. 1975	L. mand.	Alive Dec. 1977	Consistent	ND
247	M	5	Nov. 1975	Orbits/liver	Died Mar. 1976	Consistent	Negative
260	M	5	Nov. 1976	R. orbit/R palate	Died Nov. 1977	Unclass.* lymphoma	Negative
263	M	8	Nov. 1976	L. max./L clavicle	Alive Dec. 1977	Consistent	40 g.e.

Mand., mandible; Max., maxilla; BM, bone marrow; R, right; L, left; ND, not done because of insufficient tumour tissue; g.e., genome equivalents per tumour cell (determined by nucleic acid reassociation kinetics[21]).
*The three pathologists described this tumour as retinoblastoma, metastatic neuroblastoma and unclassified lymphoma, respectively.

EBV nuclear antigen[22]; (6) frozen serum samples from the child and other members of the family, and (7) thick and thin blood films for malarial burden comparison with films taken from the patient before diagnosis.

Three types of control were used for comparison with the serum of each new BL patient: (1) serum from a neighbour of the same age and sex selected at random from the main survey, who was revisited and bled again together with his whole family; (2) four control sera selected from the serum bank at IARC taken from children of the same age, sex and locality as the BL patient and bled in the main survey at about the same date, and (3) sera from a random sample of the surveyed population[23].

Antibodies to VCA

Between 1973 and November 1977, 31 children had been clinically diagnosed with BL in the study area (Table 1), but

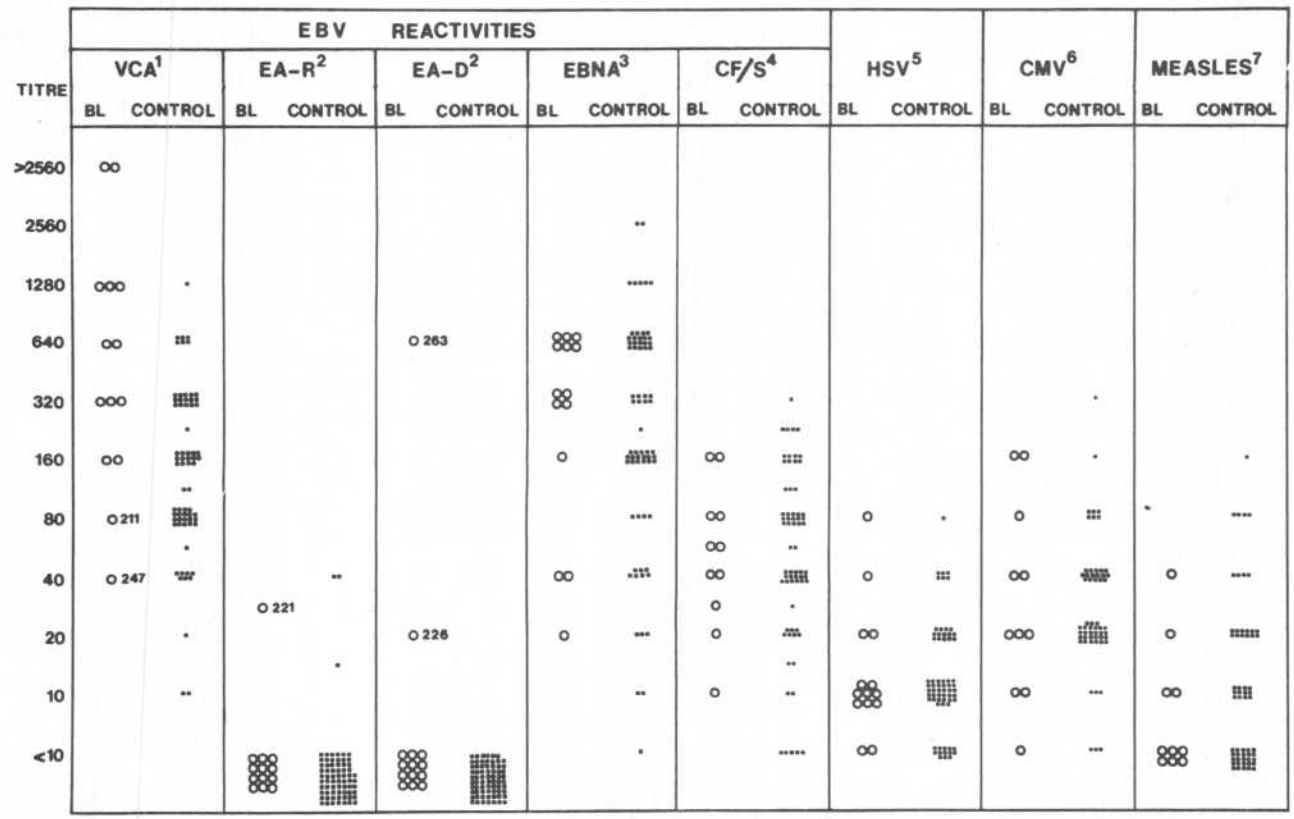

Fig. 2 Serological reactivities of 14 pre-BL and 69 matched controls. (1) Antibody titres to EBV structural antigens (VCA). Indirect immunofluorescence test[24], IARC results. (2) Antibody titres to EBV early antigens, EA-R, restricted component; EA-D, diffuse component. W. H.'s results. (3) Antibodies to EBV nuclear antigen (EBNA), anti-complement immunofluorescence test[22], IARC results. (4) Complement-fixing antibody titres to soluble EBV antigen[27], IARC results. (5) Antibody titres to HSV structural antigens. Indirect immunofluorescence test[28]. P. F.'s results. (6) Antibody titres to CMV structural antigens. Indirect immunofluorescence test. P. F.'s results. AD169 strain of CMV was grown on human embryonic lung fibroblasts and sera were tested by immunofluorescence as in ref. 25. (7) Complement-fixing antibodies to measles virus[29].

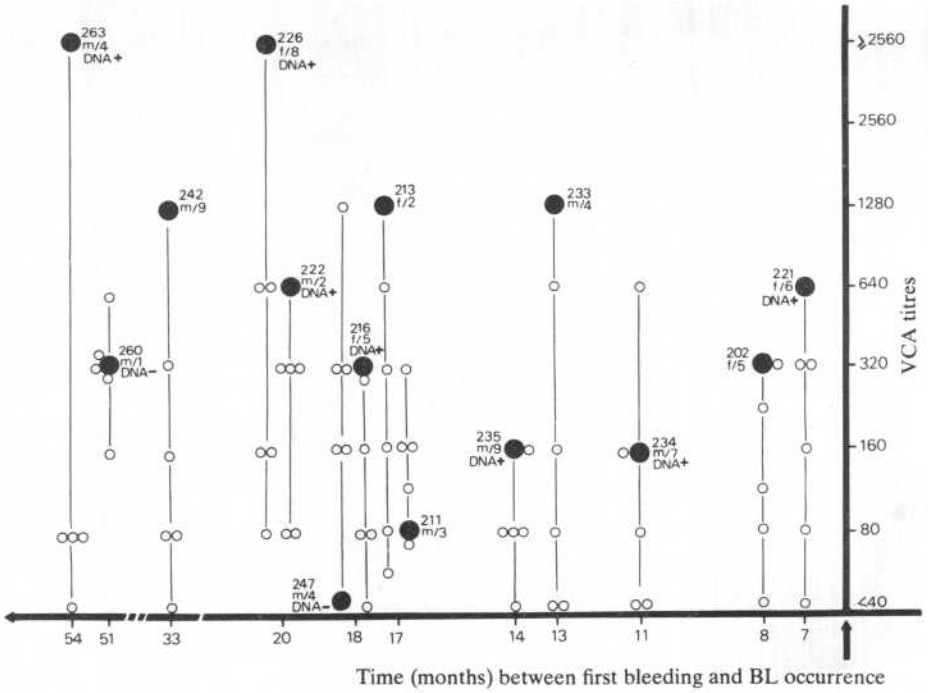

Fig. 3 VCA antibody titres in sera collected from BL cases before tumour manifestation and from controls. ●, BL cases (sex/age (yr)), EBV-DNA in tumour; ○, control.

only 14 originated from the bled population. Figure 1 shows the geographical location of these 14 cases. Fourteen BL cases is less than expected during a nearly 5-yr follow-up of the studied cohort. As Table 1 shows, there was a decline in incidence of BL during the study period. Of the 31 cases, only three were missed at the original bleeding, the rest were either in a region not yet covered by the field team, or were too old for inclusion.

Demographic, clinical and pathological data for these 14 patients are given in Table 2. Most patients were diagnosed soon (2–4 weeks) after the family had observed the first signs. The interval between initial serum collection and the onset of BL varied from 7 to 54 months (Fig. 3).

EBV serological testing[22,24–27] by immunofluorescence and complement fixation was carried out blind in the IARC laboratory (VCA, EA, EBNA and CF/s) in Lyon and in W.H.'s laboratory in Philadelphia (VCA, EA-R and EA-D, EBNA) for the 14 patients ('pre-BL' and 'post-BL' diagnosis), for the 13 randomly selected matched controls (pre- and post-; the serum for one control patient could not be found in the serum bank), for the 56 neighbour controls, and for family members. Detailed results are available on request to us. Figure 2 shows the antibody titres of pre-BL sera and matched controls.

Whereas no significant differences were observed for EA and EBNA reactivities between pre-BL sera and controls, higher titres of antibodies to VCA were seen in the pre-BL sera than in matched controls. The geometric mean titre (GMT) for both BL patients (425.5) and controls (125.8) was higher in IARC than in Philadelphia (BL patients, 176.7; controls, 52.7) but the ratio of the GMTs of patients and controls was about 3.4 to 1 in both places. Titres obtained at IARC have regularly been higher than those from Philadelphia because different cell lines were used as the source of antigen (Jijoye at IARC and EB3 in Philadelphia) and because of differences in the cut-off points taken in the immunofluorescent microscopy.

The differences in anti-VCA titres in pre-BL sera and in the sera of the 69 controls were statistically significant (IARC, $t = 3.00$, $P = 0.01$; Philadelphia, $t = 2.65$, $P = 0.02$—two-sided tests). In the IARC results (Fig. 3), seven of the 14 pre-BL sera had a VCA titre higher than any of their controls, a further three had titres at least as high as any control, and the remaining four had VCA titres lower than their highest ranking controls. The probability (two-sided) of observing such a distribution by chance is 0.008 (non-parametric test), a value close to that obtained by the t test. The Philadelphia results gave almost identical values, as seven of the 14 cases had a higher VCA titre than corresponding controls.

In Fig. 4, the VCA titres found in children who were later to develop BL are compared with the GMTs of a random sample of the population in the survey area[23]. The titres of all but two of the patients are higher than the mean of those in the corresponding age group in the normal population. The risk of developing BL was estimated to be approximately 30 times higher for those with a VCA titre two dilutions or more above the normal population GMT standardised for age, sex and locality.

Detection of EBV genomes by nucleic acid hybridisation reassociation kinetics[21] was attempted in nine cases and was positive in seven (Table 2). In two cases, no EBV/DNA could be detected. Detection of the EBV-specific nuclear antigen (EBNA)[22] was attempted in eight biopsies and gave concordant results.

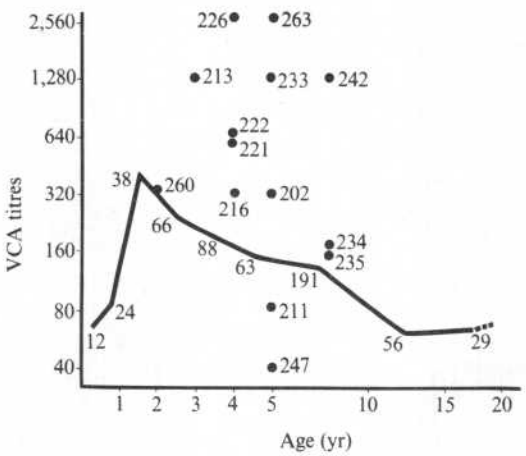

Fig. 4 VCA antibody titres in sera collected from BL cases (●) before tumour manifestation compared with antibody titres in a random sample of the population of the study area (——). Numbers against solid line indicate number of sera tested at these ages in the random sample.

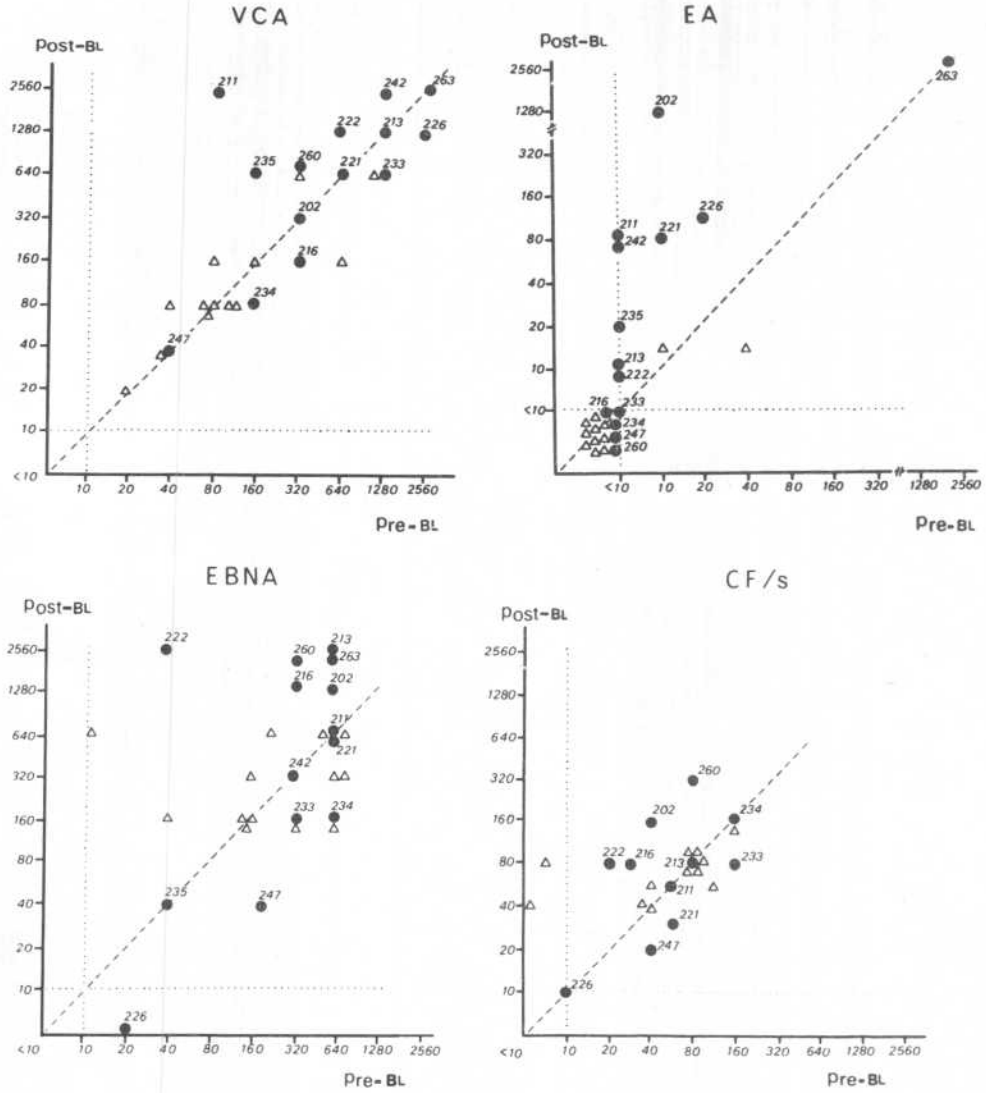

Fig. 5 EBV serological reactivities in cases (●) and controls (△) before and after clinical onset of BL.

Serological activity and development of BL

Figure 5 compares EBV reactivities before and after the onset of disease. Antibody titres to VCA showed a remarkable stability, indicating that the high antibody levels previously associated with BL were present long before clinical onset. Of the other EBV activities, only EA antibodies developed, in eight of the 14 BL patients, subsequent to the initial bleeding. In seven of these, antibodies were directed against the EA-R component. One further case had very high EA-D antibody titres long before tumour development, which remained high after diagnosis (case 263). The EBV antibody titres of the randomly selected controls changed very little between initial bleeding and the clinical onset of BL in the matched patient. No differences were observed in sera from family members of cases compared to control families, taken at the main bleeding and after the disease onset of the case.

The pre-BL and post-BL sera as well as control sera were tested blind for immunofluorescent antibodies to herpes simplex virus (HSV) and cytomegalovirus (CMV) and complement-fixing antibodies to measles virus at the Center for Disease Control, Atlanta. Sera from BL patients before and after diagnosis showed no marked differences in antibody levels relative to those of controls (Fig. 2). These results are in contrast to those of Hilgers *et al.*[30], who reported greater antibody titres to herpes viruses in sera from BL patients after clinical onset of disease than in sera from matched controls.

There was no marked difference between the number of malarial parasites in BL cases before diagnosis and in controls, but at diagnosis, patients had significantly fewer parasites than controls, possibly because they had taken anti-malarial drugs.

Epidemiological evidence

Ten of the 14 patients had antibody titres to VCA as high as, or higher than, any corresponding controls long before tumour development. These results strongly support our third hypothesis that children with long and heavy exposure to EBV are at increased risk of developing BL. Elevated EBV antibody titres might indicate either that the virus is causally related to BL or merely that BL arises in persons with a longstanding defect in their cell-mediated immunity. If the latter were the case, elevated antibody titres to other viruses would have been expected, especially to other herpes viruses, and to CMV in particular as it seems likely that this virus, like EBV, remains latent in B lymphocytes[31,32]. We therefore interpret our results as offering strong support for a causal relationship between EBV and BL.

Of the four BL patients who did not have elevated EBV antibody titres before disease onset, one (case 211) had a long history of tumorous cervical lymph nodes and exhibited antibodies to EA-D, characteristics atypical of BL. No tumour tissue was available from this patient to test for EBV markers. Case 260 was diagnosed as retinoblastoma, metastatic neuroblastoma or unclassified lymphoma by the pathologists and had no detectable EBV/DNA in the tumour tissue. The remaining two cases (234 and 247) had typical features of BL. Case 234

had detectable EBV/DNA in tumour tissue, while case 247 did not. Of the seven patients whose tumour tissue exhibited EBV markers, six had antibody titres to VCA before diagnosis as high or higher than their matched controls (Fig. 3).

The data suggest that the association of BL with predisease VCA titres is strongest in, and may be confined to, cases characterised by EBV/DNA in the tumour cells, and that among these cases high VCA titres are necessary for the disease. Thus it is possible that, in tropical regions, there are two types of BL with different aetiologies—one associated with EBV and the other not. The EBV-associated tumour would have high VCA titres long before clinical onset of disease and have detectable EBV genomes and EBNA in the tumour cells. The other lymphomas would not have abnormal VCA titres before onset of disease and their tumours would lack EBV markers (viral genome or EBNA). The latter would seem to constitute most childhood lymphomas in temperate climates[13], while they are the exception in tropical areas, and their aetiology is unknown.

The lack of increase in anti-EBV activities other than VCA (EA, EBNA and CF/s) in BL patients before diagnosis might hinder interpretation of the higher VCA titres as evidence that EBV has a causal role in BL. But the various EBV antibodies do not develop simultaneously nor do they persist for equal periods after primary infection[33]. Antibodies to VCA appear soon after primary infection, first as IgM, then as IgG and the VCA titres tend to remain stable with only a moderate tendency to decline. EA antibodies also develop soon after primary infection, but decline rapidly and disappear within months. They would reappear in cases of reactivation of the latent infection, or possibly on reinfection (if this occurs). Antibodies to EBNA usually develop long after primary infection (a month to a year). Thus high VCA antibody titres may reflect the severity of the original primary infection, relating either to the infective dose or to the lack of proper host control due, for example, to an early age at primary infection or to a simultaneous severe attack of malaria. VCA antibody titres might then represent a marker for the remaining pool of EBV-carrying cells in a latent stage long after primary infection by the virus. The effect of oncogenic viruses in animal systems is enhanced when the virus is administered to newborn animals[34] and very early infection with the EBV has been suggested to initiate the induction of BL[35]. Our results are compatible with such a possibility. That pre-BL sera (with one exception) did not exhibit EA antibodies indicates that the long standing EBV infection was not chronically active.

Need for other factors

It has been suggested[36–38] that BL arises as a result of immunological disorders in children exposed since early infancy to heavy malarial infection. The elevation of EBV/VCA antibody titres might accordingly be considered a consequence of failure to control the viral infection. High VCA titres in patients with Hodgkin's disease are believed to reflect impaired cell-mediated immunity[39]. But if this were the case for BL candidates, high titres of antibodies to CMV and HSV could have been expected, and these were not found either before or after diagnosis. The high antibody levels to VCA seems to be a specific phenomenon.

The time-space clustering and the seasonal variation (refs 18 and 40 and B. Lachet, N. E. D. and G. de T. in preparation) observed for BL imply a short interval (less than 18 months) between a final triggering event and the clinical onset of BL. Infection with EBV occurs too early to be the triggering factor and cannot be responsible for the geographical distribution of BL, but may be responsible for initiating the malignant process while holoendemic malaria or some other environmental factor(s) would promote the clinical manifestation of BL. A causal relationship between malaria and BL might be demonstrated by means of anti-malarial treatment and IARC has begun such a project in the North Mara district of Tanzania, where chloroquine tablets are distributed twice a month to children aged 0–10 yr (ref. 42).

In conclusion, our results, taken in conjunction with the established *in vitro* transforming activity[2,3] of the virus and the demonstration of its oncogenicity in new world primates[4,5], offer strong support for a causal association between EBV infection and the development of BL. However, as even in the West Nile district of Uganda less than one child in a thousand develops BL, other cofactors are required. It thus seems that the potential oncogenicity of EBV is realised only in exceptional circumstances. Final proof of causality would be the prevention of BL by intervention against EBV. The development and testing of a suitable vaccine[43] could be one means of achieving this, but considerable logistic and ethical problems would have to be surmounted[44,45].

The Government of Uganda kindly permitted this study. For practical help and advice we thank R. Depue (NCI), G. Henle (Philadelphia), J. Higginson (IARC), G. Kafuko (East African Virus Research Institute), G. Klein (Karolinska Institute), A. Linsell (IARC), R. Morrow (Harvard School of Public Health), C. Olweny (Uganda Cancer Institute), R. Owor (Makerere University), M. Pike (University of Southern California) and M. Schneidermann (NCI). We thank the medical officers, other government officials, chiefs, elders and people of the West Nile district of Uganda for help and cooperation. We also thank the field teams for their contribution, and Colette Bonnardel, Bernadette Charnay, Marie-Claude Favre-Beaut and Marie-France Lavoue for data processing and serology testing. This study was supported by contract no. NO1 CP 43296 between the NCI and the IARC, and by grant (no. Ha 449/12).

Received 29 March; accepted 30 June 1978.

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Printed in Great Britain by Henry Ling Ltd., at the Dorset Press, Dorchester, Dorset