The role of Epstein-Barr virus in Hodgkin's disease from different geographical areas

M Weinreb, P J R Day, F Niggli, J E Powell, F Raafat, P B Hesseling, J W Schneider, P S Hartley, F Tzortzatou-Stathopoulou, E R A Khalek, A Mangoud, U R El-Safy, F Madanat, M Al Sheyyab, C Mpofu, T Revesz, R Rafii, K Tiedemann, K D Waters, J C Barrantes, A Nyongo, M S Riyat, J R Mann

Children's Hospital, Ladywood Middleway, Birmingham, UK

Oncology Department
M Weinreb
P J R Day
J E Powell
J R Mann

Histopathology Department
F Raafat

Kinderspital, Zurich, Switzerland
F Niggli

Tygerberg Hospital, Cape Town, South Africa
P B Hesseling
J W Schneider

Red Cross Children's Hospital, Cape Town, South Africa
P S Hartley

Aghia Sophia Children's Hospital, Athens, Greece
F Tzortzatou-Stathopoulou

Zagazig University, Cairo, Egypt
E R A Khalek
A Mangoud
U R El-Safy

University of Jordan, Amman, Jordan
F Madanat

JUST, Jordan
M Al Sheyyab

Tawam Hospital, Abu Dhabi, UAE
C Mpofu
T Revesz

Markaz Pezeshki Shohadah, Teheran, Iran
R Rafii

Royal Children's Hospital, Melbourne, Australia
K Tiedemann
K D Waters

Hospital Nacional de Ninos, San Jose, Costa Rica
J C Barrantes

Nairobi Hospital, Nairobi, Kenya
A Nyongo
M S Riyat

Correspondence to:
Dr Michal Weinreb, Oncology Department, The Children's Hospital, Ladywood Middleway, Birmingham B16 8ET.

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Abstract

Recent studies have suggested that Epstein-Barr virus (EBV) may play a role in the aetiology of Hodgkin's disease. To determine the role of EBV in childhood Hodgkin's disease in different geographical areas, immunohistochemical staining and in situ hybridisation were used to analyse latent membrane protein 1 (LMP 1) and small nuclear non-transcribed RNAs (EBER-1) respectively. Testing for EBV within the Reed-Sternberg and Hodgkin's cells was carried out in childhood Hodgkin's disease from 10 different countries. The proportion of LMP 1 positive cases varied significantly, being 50% of cases from the United Kingdom (38/75), South Africa (9/18), Egypt (7/14), and Jordan (8/16), 60% from the United Arab Emirates (6/10), 70% from Australia (11/16), 81% from Costa Rica (34/42), 88% from Iran (7/8), 90% from Greece (20/22), and 100% of the 56 cases from Kenya. A sensitive polymerase chain reaction based EBV strain typing technique was established using archival tissues. EBV strain type 1 was shown to be predominant in childhood Hodgkin's disease from the United Kingdom, South Africa, Australia, and Greece. Type 2 was predominant in Egypt. EBV strain types 1 and 2 were both detected in some cases of childhood Hodgkin's disease in the United Kingdom, Costa Rica, and Kenya. The high incidence of EBV and the presence especially in developing countries of dual infection with both strain types 1 and 2 may reflect socioeconomic conditions leading to malnutrition induced immunological impairment. The possibility of HIV infection also needs to be explored.

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Keywords: Hodgkin's disease, Epstein-Barr virus, geographical area.

Epidemiologists have long suspected that a common virus may be a causative agent in Hodgkin's disease[1] and that the age of exposure to the virus may depend upon socioeconomic conditions.[2] Recent studies have suggested a link between Epstein-Barr virus (EBV) and Hodgkin's disease in adults and children.[3–5] In previous studies we analysed the EBV status of malignant tissues from 74 British[6] and 53 Kenyan[7] children diagnosed with Hodgkin's disease. We used monoclonal antibodies specific for EBV latent membrane protein 1 (LMP 1),[8] one of the key effectors of EBV induced cell transformation in vitro.[9] The results showed an almost complete concordance between EBV genome and LMP 1 presence in Reed-Sternberg and Hodgkin's cells. However the EBV positivity rates varied significantly between the British children[6] (50% of cases positive) and the Kenyan children[7] (100% positive). In the present study we have analysed EBV status in childhood Hodgkin's disease using immunohistochemical staining for LMP 1. Some cases were further investigated using a sensitive in situ hybridisation for the EBV encoded small nuclear non-polyadenylated RNAs (EBER-1), which are present at levels of 10^7 copies per cell in latently infected cells.[10]

At least two EBV types have been identified in most human populations.[11] The major differences that characterise the EBV 1 and EBV 2 genomes are in the latent infection cycle genes EBNA 2,[12] and EBNA 3A, 3B, and 3C.[13] These viral types differ not only in latent proteins, but also in their in vitro B lymphocyte transforming ability.[14]

In our present study, a sensitive polymerase chain reaction (PCR) method was used to analyse EBV strain types present in children with Hodgkin's disease from different parts of the world.

Methods

Subclassification according to the Rye convention[15] was carried out by two histopathologists on 277 formalin fixed paraffin embedded archival tissue samples from children (aged below 16 years) with Hodgkin's disease; 75 cases were from the United Kingdom, 48 from the Middle East (16 from Jordan, 10 from United Arabic Emirates (UAE), 8 from Iran, and 14 from Egypt), 22 were from Greece, 18 from South Africa, 16 from Australia, 42 from Costa Rica, and 56 from Kenya.

IMMUNOHISTOCHEMICAL STAINING

LMP 1 expression was investigated using a pool of four mouse monoclonal antibodies (CS1, CS2, CS3, CS4) specific for LMP 1.[16] Staining was developed by double layer alkaline phosphatase/antialkaline phosphatase[17] as previously described.[18] Archival lymph node biopsies from children with benign lymphoid hyperplasia were used as negative controls. As

Table 1 Basic descriptive statistics for 277 cases of childhood Hodgkin's disease

Country	Year of diagnosis (range)	No of cases	Male: female	Sex ratio	Median age	Range
UK	1957–92	75	55:20	2·75	10·0	2–14
Greece	1972–91	22	13:9	1·44	8·0	2–14
Jordan	1983–92	16	10:6	1·67	5·5	3–11
UAE	1983–92	10	9:1	9·0	6·5	4–12
Iran	NK	8	8:0	–	6·0	3–12
Egypt	NK	14	10:2	5·00	7·0	3–15
S Africa	1979–89	18	15:3	5·00	8·5	5–14
Australia	1982–87	16	11:5	2·20	12·0	4–14
Costa Rica	1986–92	42	35:7	5·00	7·0	2–13
Kenya	1981–91	56	38:13 (5 NK)	2·92	8·0	2–15

positive controls paraffin embedded EBV positive lymphoblastoid tumours from SCID mice and material from known EBV associated Hodgkin's disease cases were used.

IN SITU HYBRIDISATION

Formalin fixed paraffin embedded tissues from Hodgkin's disease lymph nodes were hybridised with a biotinylated oligonucleotide probe specific for the EBER-1 and EBER-2 mRNA.[19]

EBV STRAIN TYPING

For EBV detection in archival tissues we developed a sensitive PCR technique whereby DNA was extracted from formalin fixed, parafffin wax embedded tissues from LMP 1 positive Hodgkin's disease lymph nodes, using a method described previously.[7] The strain type of EBV was determined using differential PCR. Two pairs of oligonucleotide primers were used to amplify fragments from strains type 1 and 2 respectively. Common oligonucleotide primers directed against the polymorphic gene sequence of EBNA 3C detected both type 1 and type 2.[13] The sequence of the amplified fragments was verified using two unique cutting restriction endonucleases.[7] Type 2 strain specific primers were used to target the region of EBNA 2.[7]

DNA extracted from cell lines known to be EBV negative, EBV type 1 positive, and EBV type 2 positive were used as control material.

STATISTICS

Associations between biological findings (LMP and EBV positivity), clinical features of the disease (age, sex, clinical stage, histological subtype) and country of origin were investigated. For comparing proportions, the χ^2 test was used, while the Kruskal-Wallis test was used to compare age. Multiple logistic regression was used to determine which factors were independently associated with LMP 1 positivity.

Results

CLINICAL FEATURES OF THE CASES

Details of the children studied from each country are shown in table 1. The observed variation in the sex ratios was not statistically significant ($p=0·48$). There was, however, significant variation in the age at presentation between countries ($p<0·001$). Comparing each country in turn with the United Kingdom, the median age at diagnosis was significantly lower in Costa Rica ($p=0·0001$), Jordan ($p=0·0005$), Iran ($p=0·01$) and the UAE ($p=0·02$), and was of borderline significance for Egypt ($p=0·07$).

Table 2 shows the histological subtypes for each country. NS histological subtype (as a proportion of all subtypes) differed significantly between the countries ($p<0·002$).

The median age of children with the mixed cellularity subtype was significantly lower ($p=0·007$) than for the other histological subtypes.

LMP 1 STAINING

Archival material was available from 277 cases of childhood Hodgkin's disease. In each case the biopsy could be classified unequivocally by monoclonal antibody staining as LMP 1 positive in the malignant cell population, or as uniformly LMP 1 negative. The staining was restricted to the cytoplasm and cell membrane of Reed-Sternberg and Hodgkin's cells.

The results for children for each country are shown overall and in relation to histological subtype in table 3 . There is a significant difference in the proportion of LMP 1 cases between countries ($p<0·0001$). LMP 1 positivity was found in all histological subtypes, most frequently in the mixed cellularity disease ($p<0·001$).

Logistic regression analysis was carried out to determine which clinical factors were

Table 2 Histological subtype, country, and median age

	LP		NS		MC		LD	
Country (n)	n	(%)	n	(%)	n	(%)	n	(%)
UK (75)	14	(19)	37	(49)	19	(25)	5	(7)
Greece (21)	2	(10)	12	(57)	7	(33)	–	
Jordan (16)	1	(6)	7	(44)	8	(50)	–	
UAE (10)	5	(50)	–		4	(40)	1	(10)
Iran	–		–		–		–	
Egypt (13)	2	(15)	3	(23)	7	(54)	1	(8)
S Africa (17)	–		4	(24)	10	(59)	3	(18)
Australia (15)	1	(7)	11	(73)	3	(20)	–	
Costa Rica (42)	3	(7)	24	(57)	10	(24)	5	(12)
Kenya (55)	3	(5)	26	(47)	18	(33)	8	(15)
All cases (264)	31	(11·7)	124	(46·9)	86	(32·6)	23	(8·7)
Median age (years)	8·5		9·5		7·0		11	

LP=lymphocyte predominant; NS=nodular sclerosis; MC=mixed cellularity; LD=lymphocyte depleted. In 12 cases the histological subtype was not known.

Table 3 LMP 1 positivity, country, and histological subtype

	All cases LMP 1+ve		*LP LMP 1+ve*		*NS LMP 1+ve*		*MC LMP 1+ve*		*LD LMP 1+ve*	
Country	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*
UK	38/75	(50·7)	5/14	(35·7)	15/37	(40·5)	16/19	(84·2)	2/5	(40·0)
Greece	20/22	(90·9)	2/2	(100)	11/12	(91·7)	6/7	(85·7)	–	
Jordan	8/16	(50·0)	101/1	(100)	2/7	(28·6)	5/8	(62·5)	–	
UAE	6/10	(60·0)	2/5	(40·0)	–		3/4	(75·0)	1/1	(100)
Iran	7/8	(87·5)	–		–		–		–	
Egypt	7/14	(50·0)	1/2	(50·0)	2/3	(66·7)	4/7	(57·1)	0/1	(0·0)
S Africa	9/18	(50·0)	–		2/4	(50·0)	6/10	(60·0)	1/3	(33·3)
Australia	11/16	(68·8)	0/1	(0)	8/11	(72·7)	3/3	(100)	–	
Costa Rica	34/42	(80·9)	1/3	(33·3)	19/24	(79·2)	10/10	(100)	4/5	(80·0)
Kenya	56/56	(100)	3/3	(100)	26/26	(100)	18/18	(100)	8/8	(100)
All cases	196/277	(70·8)	15/31	(48·4)	85/124	(68·6)	71/86	(82·6)	16/23	(69·6)

LP=lymphocyte predominant; LMP 1=latent membrane protein 1; NS=nodular sclerosis; MC=mixed cellularity; LD=lymphocyte depleted.

Table 4 Multivariate logistic regression analysis of LMP 1 status. Significance of the association between LMP 1 positivity and other factors. Relative risk (odds ratio) and 95% confidence interval of levels within the factor

	Seven countries analysis		*Nine countries analysis*	
Factor	*p Value*	*Odds ratio (95% CI)*	*p Value*	*Odds ratio (95% CI)*
Sex	0·108		0·300	
Male		1		1
Female		0·48 (0·22–1·07)		0·60 (0·29–1·24)
Age (per year)	0·208	1·01 (0·90–1·13)	0·621	1·04 (0·93–1·15)
Stage	0·767		–	
I		1		–
II		1·40 (0·48–4·06)		–
III		1·57 (0·64–3·88)		–
IV		1·98 (0·54–7·26)		–
Histological subtype	0·005		0·003	
LP		1		1
NS		1·84 (0·65–5·22)		1·77 (0·67–4·67)
MC		8·40 (2·52–27·8)		6·45 (2·20–18·9)
LD		2·04 (0·46–9·06)		1·27 (0·32–4·96)
Country	0·005		0·001	
UK		1		1
Greece		11·06 (2·20–55·5)		10·6 (2·16–52·1)
Jordan		0·79 (0·21–2·99)		0·75 (0·23–2·52)
UAE		1·67 (0·34–8·18)		1·61 (0·35–7·37)
Iran		–		–
Egypt		–		0·71 (0·18–2·71)
S Africa		0·48 (0·14–1·66)		0·64 (0·20–2·05)
Australia		3·17 (0·84–12·0)		2·81 (0·77–10·2)
Costa Rica		4·96 (1·72–14·3)		4·73 (1·76–12·7)
Kenya		–		49·5 (6·34–386)

LP=lymphocyte predominant; NS=nodular sclerosis; MC=mixed cellularity; LD=lymphocyte depleted.

independently associated with LMP 1 positivity. The results are shown in table 4. In the first analysis, Kenya, Iran, and the UAE were excluded since clinical stage was not known for samples from these countries. No significant association was shown for age, sex and stage, but histological subtype ($p=0·005$) and country of origin ($p<0·001$) were independently associated with LMP 1 positivity. In the second analysis, clinical stage was not analysed, so the data from Kenya and the UAE could be included. The same pattern as before was seen, in that sex and age were not associated with LMP 1 status, while histology ($p=0·004$) and country ($p<0·001$) were independently associated with LMP 1 status. Thus LMP 1 positivity, as indicated by the odds ratio and 95% confidence interval, was significantly higher in the mixed cellularity subtype, and in Greece, Costa Rica, and Kenya.

Table 5 EBV type and country

	EBV type 1		*EBV type 2*		*EBV type 1+2*	
Country (n)	*n*	*(%)*	*n*	*(%)*	*n*	*(%)*
UK (10)	8	(80)	0		2	(20)
Egypt (6)	0		5	(83)	1	(17)
Greece (19)	18	(95)	1	(5·0)	0	
South Africa (12)	12	(100)	0		0	
Australia (6)	6	(100)	0		0	
Costa Rica (27)	12	(44·5)	2	(7·5)	13	(48)
Kenya (25)	7	(28)	9	(36)	9	(36)
All cases (105)	63	(60)	17	(16·2)	25	(23·8)

IN SITU HYBRIDISATION

In 15 cases, in situ hybridisation for both EBER-1 and EBER-2, and immunohistochemical staining for LMP 1, were carried out. In six LMP 1 positive cases, in situ hybridisation showed that EBV was present in both the Reed-Sternberg and the Hodgkin's cells and in a few small lymphocytes. In nine LMP 1 negative cases, no EBV was detected by in situ hybridisation in the Reed-Sternberg cells or Hodgkin's cells, but EBV was present in the small lymphocytes.

EBV STRAIN TYPING

One hundred and five cases from the United Kingdom, Egypt, Greece, South Africa, Australia, Costa Rica, and Kenya were tested with PCR to detect the presence of EB viral DNA and to determine the strain of the virus. In 59% EBV-1 alone was present, in 18% EBV-2 alone was found, and in 23% both virus strains were detected. The distribution of strain types varied between countries (table 5).

Discussion

Geographical or ethnic variation in the incidence of Hodgkin's disease is well described.[20 21] The nature and pathogenesis of Hodgkin's disease is still an enigma and it is not clear whether it is a single entity or a syndrome. Recent studies on the pathogenesis in adults and children have associated it with EBV infection. Published data which used Southern blotting,[22] PCR,[25] in situ hybridisation,[23] or immunohistochemistry[5] reported varying levels of EBV positivity among children with Hodgkin's disease[24–29] (table 6). This may in part be explained by the differences in techniques. Southern blotting and PCR do not identify individual EBV infected cells, while in situ hybridisation and immunohistochemistry can localise EBV to specific cells. LMP 1 has been shown to have an effect on cell growth

Table 6 Reports on EB virus involvement in HD in children from different countries, according to histological subtype

Reference	Technique	Country	Total EBV+		LP EBV+		NS EBV+		MC EBV+		LD EBV+	
			n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Coates *et al*[24]	ISH	UK	5/24	(21)	0		4/19	(21)	1/4	(25)	0/1	
Khan *et al*[25]	ISH, LMP 1	UK	4/24	(25)	0		4/19	(21)	2/4	(50)	0/1	
Jarrett *et al*[22]	Southern blot	UK	7/14	(54)								
Armstrong *et al*[23]	ISH, Southern blot, PCR, LMP 1	UK	3/4	(75)	0		1/2	(50)	2/2	(100)	0	
Armstrong *et al*[26]	ISH, LMP 1	UK	13/22	(59)	0/1		7/7	(100)	4/12	(33)	2/2	(100)
		Brazil	18/25	(72)	0/2		10/12	(83)	7/10	(70)	1/1	(100)
		Saudi Arabia	7/8	(87)	0/1		5/5	(100)	2/2	(50)	0	
Ambinder *et al*[27]	ISH, LMP 1	USA	9/25	(36)	0/2		2/15	(13)	6/7	(85)	1/1	(100)
		Honduras	9/9	(100)	1/1	(100)	3/3	(100)	6/6	(100)	1/1	(100)
Chang *et al*[28]	ISH, LMP 1	Peru	20/20	(100)	0		4/4	(100)	15/15	(100)	1/1	(100)
Preciado *et al*[29]	LMP 1	Argentina	12/49	(41)	1/6	(17)	0/7		10/15	(67)	1/1	(100)

EBV+ =Epstein-Barr virus positive; LP=lymphocyte predominant; NS=nodular sclerosis; MC=mixed cellularity; LD=lymphocyte depleted. ISH=in situ hybridisation; LMP 1=latent membrane protein 1.

and cell survival in experimental systems in vitro[30] and it is likely to be involved in the multistep oncogenic process in vivo. Nevertheless, despite the use of very sensitive techniques, EBV has not been found in a proportion of Hodgkin's disease cases. This has led to the hypothesis that an unknown cofactor is involved in the aetiology of Hodgkin's disease, but it is also possible that EBV negative cases are so diagnosed because of assay insensitivity.

Our aims were to assess the possible role of EBV in the aetiology of Hodgkin's disease in children from different parts of the world. We analysed 277 biopsies from 10 countries with climates ranging from temperate to tropical and with varying socioeconomic conditions. In agreement with previous studies[20 21] we found young age at diagnosis and mixed cellularity subtype were more common in children from developing countries. We note that the geographical distribution of strongly EBV related Hodgkin's disease appears to be very similar to that of EBV related Burkitt's lymphoma. While we showed a strong association between mixed cellularity subtype and EBV this did not account for the differences in LMP 1 positivity between countries.

Recent reports have described the prevalence of EBV strain type 2 in immunocompromised adults,[31] cardiac transplant recipients, and subjects from areas endemic for Burkitt's lymphoma.[32] Because there are known differences in the capability to immortalise B cells in vitro between type 1 and type 2 EBV, there has been much speculation as to their role in malignant proliferation. Sixby *et al* found type 2 virus in 14/34 healthy donors and dual infection in five of 10 HIV positive individuals.[33] Armstrong *et al* found type 2 in 1/25 cases of adult Hodgkin's disease[23] and Bouzid *et al* described dual infection in 14/15 similar Algerian cases.[34] Using a sensitive PCR method we found dual infections in 21% of paediatric cases of Hodgkin's disease. We found EBV type 2 virus in cases from Egypt, Kenya, and Costa Rica and dual infections with type 1 and 2 in the United Kingdom, Kenya, and Costa Rica. However, we do not know the distribution of EBV type 2 in healthy children in Kenya and Costa Rica. It is possible that the dual infections could reflect immune dysfunction, with HIV being a cofactor in the process.

In summary we found that EBV is actively involved in the pathogenesis of Hodgkin's disease in children, but the involvement varies according to the geographical and presumably the socioeconomic conditions. It is of interest that in Kenya all Hodgkin's disease cases had EBV and this resembles the situation in Burkitt's lymphoma.

We showed that dual infections can occur, and futher research is required to determine whether EBV is localised within specific cells. While infection with one strain of EBV might be expected to confer immunity to the other strain, our finding of dual infection in 21% of cases suggests that an underlying immune deficiency may contribute to the high levels of LMP 1 positivity seen particularly in Costa Rica and Kenya.

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