CLINICAL STUDIES

Epstein-Barr Virus in Biopsies from Patients with Hodgkin and Non-Hodgkin Lymphoma at the University of Puerto Rico Immunohistochemistry Laboratory

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Objectives. **We aimed to determine the Epstein-Barr Virus (EBV) presence rate in our laboratory's lymphoma tissue biopsies for comparison with that reported in literature.**

Background. **The presence of EBV has been established in Hodgkin lymphoma (HL), endemic Burkitt Lymphoma and some non-Hodgkin lymphomas (NHL). It has been linked to geographic, ethnic and socioeconomic factors, with a lower rate in developed countries.**

Methods. **We used the immunoperoxidase technique to determine the rate of the EBV LMP-1 in eighty-seven biopsies diagnosed as lymphoma. Tissue slides were stained using the Ventana® Automated Slide Stainer with the DAKO® EBV LMP-1 primary antibody and the results were analyzed with the SYSTAT® program.**

Results. **We found an LMP-1 positive rate of 50% for 22 cases of HL and 35% for 63 cases of NHL. Among HL, 5 were children and 16 were adults, with LMP-1 positive rates of 60% and 50% respectively. Among NHL, 3 were children and 59 were adults, with equal LMP-1 positive rates of 33%. The sex LMP-1 positive rates for HL were 42% for 12 males and 60% for 10 females. Among NHL, the sex LMP-1 positive rates were 39% for 38 males and 28% for 27 females. NHL was further subdivided into subtypes and LMP-1 primary antibody positive rates were reported.**

Conclusions. **We found a similar presence rate of EBV in the HL biopsies to that of developed countries, but a similar presence rate of EBV in NHL biopsies to that of developing countries.**

Key words: Epstein-Barr virus, Lymphoma, Hodgkin lymphoma, Non-Hodgkin lymphoma, Puerto Rico.

The Epstein - Barr virus (EBV) belongs to the Herpes virus family and has been found to be ubiquitous in humans (1). It has been identified as the causative agent of infectious mononucleosis and endemic Burkitt lymphoma (BL) (2). A symbiotic presence of the virus in normal individuals, in patients with infectious mononucleosis, with B-cell lymphomas, and with squamous cell cancer has also been described (1). EBV may also be found in other non-Hodgkin's lymphomas (NHL) such as peripheral T-cell lymphomas and in sporadic Burkitt lymphoma (3). An unusual predominant association with T-NHL over B-NHL has been found in studies from Western Europe and Asia (3). In recent years, molecular and serological assays have demonstrated EBV association with several other malignancies, and EBV is now suspected as a pathogenic agent in other NHL (e.g., acquired immune deficiency syndrome [AIDS]-associated, post transplant, and nasal T/natural killer cell), nasopharyngeal carcinoma, lymphoepithelioma-like carcinoma, and gastric adenocarcinoma, as well as leiomyosarcoma and leiomyoma associated with immunosuppression (4). EBV genomes have also been reported on cases of Hodgkin lymphomas (HL) (2, 4) with various studies suggesting strong linkage between EBV and the pathogenesis of HL (5, 6). Evidence for this association includes the localization of EBV DNA and the latent gene products, including the latent membrane protein-1 (LMP-1) and LMP-2 in the malignant Reed-Sternberg and Hodgkin cells of HL (2, 6). The frequency at which EBV can be detected in lymphomas depends not only on the histological subtype, but is influenced also by other tumor-specific, as well as epidemiological factors (3). The frequency of EBV-associated HL has been found to vary with age, sex, cellular subtype, ethnicity and country of residency (6).

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Our study aims to determine the EBV-lymphoma association of HL and NHL cases referred to our laboratory during the year 2001 and to correlate our findings with those reported in literature. According to the International Agency for Research in Cancer (IARC) last figures (1991), there were 38 female cases and 45 male cases of HL reported in Puerto Rico. That same year, 139 female cases and 166 male cases of NHL were reported. We used the immunoperoxidase technique to stain for EBV LMP-1 in 87 consecutive lymphoma cases obtained from the accession book of the Immunohistochemistry Laboratory of the University of Puerto Rico School of Medicine. We used the immunoperoxidase technique for EBV LMP-1 as a marker of EBV positivity because of the high sensitivity and correlation (90-100%) with *in situ* hybridization for EBERs reported in literature (4, 5, 7, 8).

Materials and Methods

Eighty-seven consecutive lymphoma cases were sequentially selected from the accession book of the Immunohistochemistry Laboratory of the University of Puerto Rico School of Medicine from January 2001 until December 2001. Identifying data was extracted from the immunohistochemistry reports and cases were identified only by laboratory immunoperoxidase case numbers (IP#) and were classified by age, sex, and type of lymphoma using the WHO classification method.

Paraffin embedded tissue blocks were received from different hospitals and laboratories located through the island for immunohistotyping for lymphomas. A spare section of the formalin fixed paraffin embedded tissue blocks were cut and the slides were placed in the oven for two hours at 75 °C. They were deparaffinized in Xylol and rehydrated in graded ethanol and water. Each slide was properly labeled and placed in the Ventana Automated Slide Stainer® (Ventana Medical System, Inc. Tucson, AZ) and the protocol for EBV primary antibody was conducted. The DAKO® Monoclonal Mouse Anti-Epstein-Barr Virus LMP1, Clones CS 1-4 (Code No.M0897, Lot 013) was used as the primary antibody. DAKO® positive control slides for LMP1 were processed along with the case slides. Two different pathologists independently analyzed the processed slides to classify them according to the reaction. Cases were classified as either positive for EBV LMP-1, when the malignant cells were immune reacting, (LMP-1+) or negative for LMP-1, when the malignant cells were not reactive, (LMP-1 -). In cases where there was disagreement, both pathologists viewed the slides together and reached a consensus. Cases where the results were not satisfactory were so categorized, using ns. The results were tabulated according to the type of lymphoma, as described above, age and sex. Statistical analysis was performed with the SYSTAT® program for Yates corrected Chi-square or Fisher exact test (two tail) when appropriate.

Results

All 22 HL cases were satisfactory for evaluation. Eleven of the 22 (50%) HL cases had tumor cells with positive LMP-1 staining at the cell membrane and within the cytoplasm (Figure 1). Two of the 65 NHL cases were non satisfactory for EBV LMP-1 evaluation (ns), twenty two of the remaining 63 (35%) NHL cases were LMP-1 positive

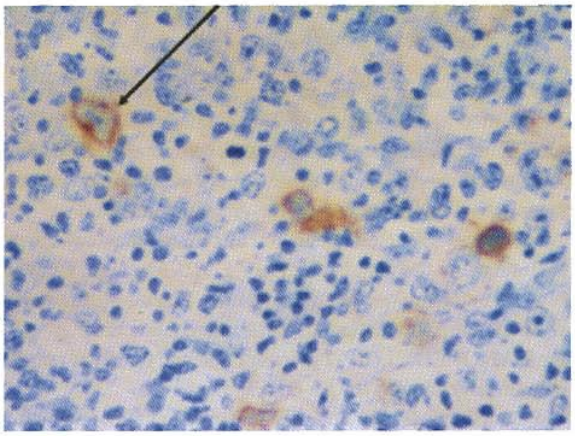

Figure 1. EBV* + LMP-1† in Hodgkin lymphoma. Arrow indicates characteristic + Reed-Sternberg cell, *EBV=Epstein-Barr virus, †LMP-1=Latent membrane protein.

(Figure 2). Only these last 63 NHL cases were used for statistical analysis. No significant statistical difference was found between HL and NHL LMP-1 positive rate (P= 0.32, Yates corrected Chi-square). Positive cases in the NHL

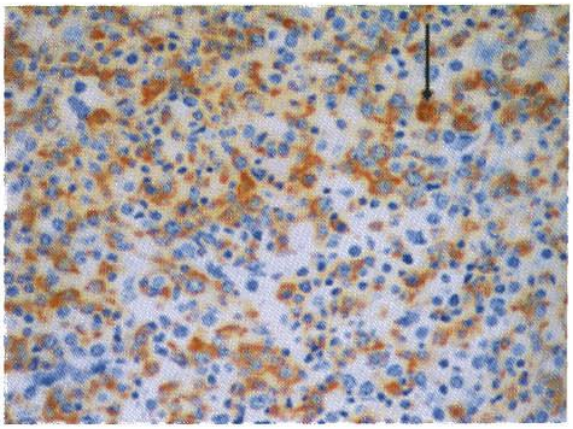

Figure 2. EBV* + LMP-1 † in non-Hodgkin lymphoma. Arrow indicates diffuse large B-cell non-Hodgkin lymphoma, *EBV=Epstein-Barr virus, †LMP/1=latent membrane protein.

were widely distributed including, among different subtypes, 11 large B cell, 2 large T cell, 5 small B cell, 1 B mixed cell, 1 B Marginal zone, 1 large null type and 1 T peripheral NHL. Overall, we found 18 (33%) positive B cell, 3 (50%) positive T cell and one (50%) positive null cell NHL cases for EBV LMP-1 in our study. The small number of T cell NHL precludes reaching conclusions regarding T versus B cell EBV presence rate.

Among the HL cases, 5 were pediatric, of which 3 (60%) were LMP-1 positive. The remaining 16 HL cases were from adults, 8 (50%) of which were LMP-1 positive. We did not have information on the age of one HL patient, who was LMP-1 negative. No significant statistical difference was found between adult and pediatric HL LMP-1 positive rate (P= 1.0, Fisher exact test [two tail]).

Of the NHL cases, 3 were pediatric, of which 1 (33%) was found to be LMP-1 positive. Of the remaining NHL cases 59 were from adults, of which 18 (33%) were LMP-1 positive and 2 were ns for LMP-1. The remaining 3 positive LMP-1 NHL cases were of unknown age. No significant statistical difference was found between adult and pediatric NHL LMP-1 positive rate (P= 1.0, Fisher exact test [two tail]).

Among the HL cases, 12 were males, of which 5 (42%) were positive for LMP-1. The remaining 10 HL cases were from females of which 6 (60%) were LMP-1 positive. No significant statistical difference was found between LMP-1 positive male and female HL cases (P= 0.67, Yates corrected Chi-square). Of the NHL cases, 38 were males, 15 (39%) of which were LMP-1 positive. The remaining 27 NHL cases were from females, of which 7 (28%) were LMP-1 positive. No significant statistical difference was found between LMP-1 positive male and female NHL cases (P= 0.51, Yates corrected Chi-square).

Discussion

We used the immunoperoxidase technique to identify EBV LMP-1 as a marker of EBV positivity because it is a non expensive, fast and practical method, which also has been proved to be highly sensitive and to correlate well (90-100%) with *in situ* hybridization for EBERs reported in literature (4, 5, 7, 8). This study shows an overall EBV-lymphoma presence of 50% (11/22) for the HL cases and of 35% for the NHL (22/63) cases. Only 63 of the 65 NHL cases were used during statistical calculation, the two NHL cases that were non satisfactory (ns) for EBV LMP-1 were not included. Two pathologists confirmed independently the immunohistochemistry reactions. Our overall EBV positive HL rate correlates well with the overall EBV positive HL rates reported in the literature (Table 1), which ranges from 30 to 75% (5-10). EBV positive HL rates reported for Kenya ranges from 87 to 100% (9, 10) and are higher than our findings. This could be accounted for what some authors describe as socioeconomic relationships between EBV and HL association, with lower rates in developed countries than in developing countries (6). We found no significant statistical differences between our pediatric and adult HL EBV rates (P= 1.0), differing from the literature, which reports differences in age (6). Our pediatric HL cases had an EBV rate of 60% (3/5) (Table 1) correlating with pediatric HL in British children (50%) (9), but much higher than reported in Italy (14%) (10), and lower than reported for Kenyan pediatric HL cases, where it reaches 98 to 100% (9, 10). Marked differences in pediatric EBV rates are attributed to socio-economic differences and to HL incidence pattern, which has been reported to be bimodal, with a first peak at a younger age in developing countries, suggesting a possible infectious etiology (6), but our small sample size precludes reaching definitive conclusions. Our HL adult cases had an EBV rate of 50% (8/16) (Table 1), similar to HL-EBV adult rates reported for Italy (50%) (10) and one series in Kenya (66%) (9). Leoncini *et al* reported an 88% HL-EBV adult rate in Kenya (10), which is attributed to the socioeconomic relationships described before (6). Our EBV HL rates showed no significant statistical difference in gender, with rates of 42% (5/12) and 60% (6/10) in males and females, respectively (P= 0.67). These results differ with what is reported in the literature, that EBV-HL rates differ with sex (6). However, our sample size is too small to reach conclusions.

We found an overall EBV rate of 35% (22/63) for our NHL cases (Table 2), which correlate with overall EBV rates for NHL documented in the literature (5-44%) (4, 9,

Table I. EBV* presence rate in HL † previosly reported in studies from different countries

Country of study (Reference)	Total seires EBV presence rate‡ (%)	Children EBV presence rate **(%)
Puerto Rico	11/22 (50)	3/5 (60)
Great Britain (6)	34/55 (62)	34/55 (62)
(9)	37/74 (50)	37/74 (50)
Kenya (9)	85/101 (84)	53/53 (100)
(10)	85/92 (92)	
Italy (7)	42/57 (74)	
(10)	31/65 (48)	
Japan (5)	43/106 (41)	
(13)	17/32 (53)	
Czech Republic (8)	47/142 (33)	

*EBV = Epstein/barr virus, †HL = Hodgkin lymphoma, ‡Total series EBV presence rate = EBV + cases/Total cases, **Children EBV presence rate = children EBV+cases/total children cases.

11-13). Differences encountered between studies can be accounted by socioeconomic differences, with developing countries having higher EBV-NHL association and by immunosuppression in NHL patients (4). Large B cell NHL has typically been the most common malignancy of immunosuppressed patients, whether being secondary to HIV, prior organ transplantation, or congenital immunodeficiency (4).

Table 2. EBV* presence rate in NHL † previiosly reported in studies from differemt countries

Country of study (Reference)	Total series EBV presence rate‡ (%)	Children EBV presence rate**(%)
Puerto Rico	22/63 (35)	1/3 (33)
Argentina (4)	8/32 (25)	8/32 (25)
Denmark (1)	66/250 (13)	
Korea (12)	22/50 (44)	
Malaysia (11)	20/148 (14)	

*EBV=Epstein-Barr virus, †NHI=non-Hodgkin lymphoma, ‡Total sries EBV presence rate=
EBV + cases/Total cases, **Children EBV presence rate = children EBV + cases/total children cases.

We had no access to the immune status in our NHL cases, so we were not able to establish any association between EBV rate and immunosuppression in our NHL cases. This would be a worthy area of future study, because EBV-T cell NHL patients have a worse prognosis (1). Since we only used LMP-1 as a marker for EBV presence, we could not establish patterns of latency in our cases. Three different types of latency (I, II and III) have been identified in EBV-associated malignancies (4). Burkitt lymphoma usually displays latency type I, in which only EBERs and EBNA-1 are expressed. However, we found several series in which endemic (usually regarded as EBV-associated) as well as non-endemic Burkitt lymphoma have expressed LMP-1 in tumor cells (4). None of our 3 Burkitt lymphoma cases expressed LMP-1, but still, EBV-association cannot be ruled out, unless EBERs and EBNA-1 studies are performed. Hodgkin disease and nasopharyngeal carcinoma are among those malignancies that express type II latency, in which EBERs, EBNA-1, LMP-1 and LMP-2 are expressed. Type III latency, with full complement of latency antigens expression (EBNAs and LMPs) is only seen in immunocompromised individuals.

Overall, we found 18 (33%) positive B cell and 3 (50%) positive T cell NHL cases for EBV in our study. These findings are similar with those reported in literature for B and T cell NHL of 7 to 24% and 31 to 44%, respectively (9, 11, 13). Our pediatric NHL population had an EBV rate of 33% (1/3), correlating with pediatric cases from Argentina, 25% (4). However, in this same series from Argentina EBV-NHL presence rose to 100% in immunocompromised pediatric patients. Since our sample size was small and we do not have the immune status of our patients, we cannot compare our results to this variant. We found no significant statistical differences in gender for our EBV-NHL cases, with 39% (15/38) and 28% (7/25) rates in males and females, respectively (P= 0.51).

Our results support the ongoing argument of possible EBV involvement in the etiology of malignant lymphomas. Our HL tissue biopsies had an EBV presence rate similar to that of developed countries, both in the adult and pediatric biopsies, although this later sample was small. However, NHL tissue biopsies had an EBV presence rate similar to that of developing countries. Since our sample size is small and we did not have information on the immune status of patients in our NHL cases, we are not able to establish a direct association between EBV presence and the pathogenesis in our NHL cases.

Resumen

El virus de Epstein-Barr (VEB) ha sido ligado al linfoma de Hodgkin (LH), el linfoma de Burkitt tipo endémico y algunos linfomas tipo no Hodgkin (LNH), presentando una menor asociación en países desarrollados. Se intentó determinar la razón de presencia del VEB en biopsias de tejido de linfomas en el laboratorio para comparar con lo reportado en la literatura. Se utilizó la técnica de immunoperoxidasas en ochenta y siete casos consecutivos diagnosticados con LH o LNH en el laboratorio durante el año 2001. Se utilizó la máquina "Ventana® Automated Slide Stainer" con el anticuerpo primario (monoclonal) DAKO® EBV LMP-1. Los resultados fueron analizados utilizando el programa "SYSTAT®". Se encontró una presencia del VEB en biopsias de LH similar a la reportada en países desarrollados, pero una presencia del VEB en biopsias de LNH similar a la reportada en países en desarrollo.

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