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DNA of Epstein–Barr Virus Detected in Tissue of Burkitt's Lymphoma and Nasopharyngeal Carcinoma

(herpesvirus DNA/African tumors/complementaryRNA hybridization)

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ABSTRACT **The association of Epstein–Barr virus (EBV) DNA with African tumors was examined by the method of hybridization with complementary RNA. 22 out of 23 biopsies of Burkitt's lymphoma and 18 out of 23 biopsies of nasopharyngeal carcinoma were found to contain EBV DNA. The number of EBV genome equivalents per cell ranged from four to 113. Five out of 24 other African tumors, two adenocarcinomas, and one each of melanoma, reticulum cell sarcoma, and carcinoma of the antrum, also contained EBV DNA.**

The Epstein–Barr virus (EBV) is the only virus that has been so far discovered to be consistently associated with certain malignant tumors in man. The virus was first demonstrated in cell lines established from Burkitt's lymphoma (1). The association of the virus with the disease has been shown by extensive immunological studies. A similar association has been found for nasopharyngeal carcinoma. In 1970 zur Hausen and his collaborators (2) showed by DNA-DNA membrane hybridization that tissues taken from Burkitt's lymphoma and nasopharyngeal carcinoma carried the viral DNA even though these tumors did not show any viral antigen except membrane antigen. These experiments tended to confirm the viral association but needed to be extended because the sensitivity of the hybridization technique was low, and the number of EBV genome equivalents per cell was underevaluated (3). We therefore decided to define more precisely the quantitative relation of the viral DNA to these tumors and also for comparison, to test tissues from other tumors of patients living in the same region of Kenya.

We have recently developed two methods to detect the viral DNA in EBV-carrying cells: cRNA–DNA membrane hybridization (3) and DNA–DNA reassociation kinetics (4). The first method detects more than one genome per cell and requires only small amounts of test DNA (30–50 μg); the second method detects more than one genome in every 50 cells and requires more DNA (500 μg). The number of genome equivalents per cell calculated by these two methods was essentially identical (4). Since most of the tissue obtained at biopsy supplied only a small amount of DNA, we used the cRNA–DNA membrane hybridization method.

Abbreviations: EBV; Epstein–Barr virus; cRNA, RNA complementary to DNA; VCA, virus capsid antigen; EA-D and EA-R, early antigen.

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MATERIALS AND METHODS

Extraction of DNA and Hybridization. The conditions for extraction of DNA and for hybridization with EBV-specific cRNA on a nitrocellulose filter have been described (3).

The tumor tissues obtained from Nairobi, Kenya, were homogenized and treated with Pronase (1 mg/ml) and sodium dodecyl sulfate (1%) followed by extraction of DNA with phenol. The DNA was denatured with alkali and fixed onto nitrocellulose filters which were then baked at 80° under reduced pressure and incubated with EBV-specific radioactive cRNA (1.5×10^5 cpm per filter) in 6 × saline–citrate (0.15 M NaCl–0.015 M Na citrate) at 66° for 20 hr. The filters were washed with 2 × saline–citrate, treated with RNase (20 mg/ml) in 2 × saline–citrate and washed again with 2 × saline–citrate. The hybridized counts were measured in a scintillation counter; the DNA on the filters was measured

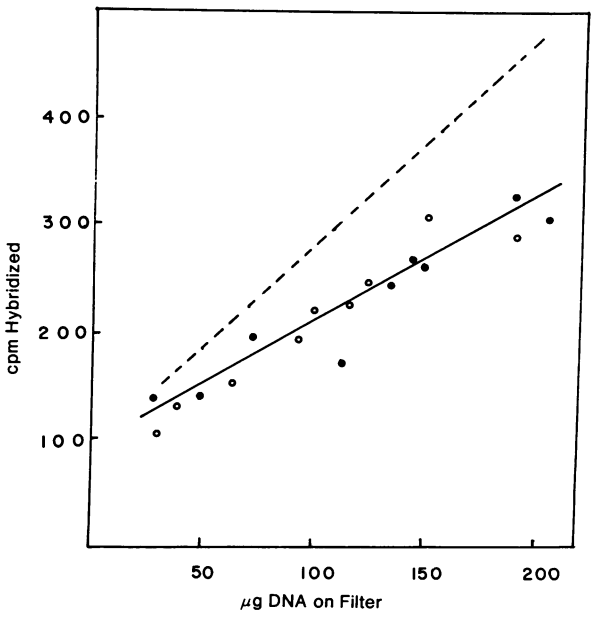

FIG. 1. Comparison of hybridization efficiency between Hep2 DNA and DNA from the single Burkitt's lymphoma without detectable Epstein–Barr virus DNA. Each amount of Hep2 DNA or the lymphoma (FM 1143) DNA was fixed on a nitrocellulose membrane filter and hybridized with 1.5×10^5 cpm of cRNA specific to EBV DBA. O——O Hep2 DNA; ●——● FM DNA (1143); – – – one genome per cell.

TABLE 1. *EBV DNA in African Burkitt's lymphoma**

Patient	KCC†	No. tests	cpm hybridized per 50 μg of DNA	No. of EBV genome equivalents per cell	Serum antibody titers‡		
					VCA	EA-D	EA-R
I.R.	1225	2	4311	113	1280	320	
P.A.	1343	2	3381	92	1280	80	
O.O.	1349	2	2148	57	180	20	320
		1	1054	23			
O.O.		2	1197	27			
K.K.	1464	2	2027	52	160	<10	
N.B.	1315	2	1818	48	640-160§	<10	80
M.C.	1260	2	1877	47	320-160	<10	80
R.W.	1166	2	1761	45	640-160	10-40	40
A.M.		2	1490	38			
N.K.	1393	2	1482	38	40	<10	
K.S.	1364	2	1451	37	320	<10	160
M.N.		2	1413	36			
O.O.	1373	2	1415	36	640	<10	320
M.A.	1367	2	1365	34	1280	320	
		2	910	22			
A.O.	1311	2	1363	34	640	10-20	160-320
M.N.	1223	2	1160	26	640-1280	80-320	
J.C.	1305	2	1055	25	640-80	10-40	160-320
S.O.	1442	2	793	18	160	<10	160
N.K.	1397	2	693	15	320	<10	160
P.N.	1413	4	242	4	160	<10	<10
F.M.	1143	4	152	—	640-2560	20-40	160-320

* Tissue obtained at biopsy.
† KCC is Kenya Cancer Council.
‡ Titers are expressed as reciprocals.
§ Titers given as a range are from sera collected over a period of months.

TABLE 2. *EBV DNA in tissues from nasopharyngeal carcinoma**

Patient	KCC	No. tests	cpm hybridized per 50 μg of DNA	No. of EBV genome equivalents per cell	Serum antibody titers		
					VCA	EA-D	EA-R
R.K.	1444	2	3131	85	160	<10	40
O.R.	1452	2	1813	46			
J.A.		2	1018	24			
K.M.	1434	2	983	23	160	20	20
M.E.		2	901	20	1280	160	
W.M.		2	935	20			
D.K.		2	911	20	640	160	
K.T.	1232	2	801	17	640-1280	320	
K.C.	1278	4	786	16			
K.M.	1268	2	710	14	320-160	40	320-160
N.K.	1262	2	675	13	640-320		
J.A.	1326	2	661	13	2560	1280	
K.A.		4	476	8			
M.K.	1279	4	442	7	160	20-40	
R.K.		2	358	5			
A.O.	1352	4	352	5	320	<10	
N.R.	1433	2	342	5	160	20-40	
M.P.		2	348	5	160	<10	<10
N.M.		2	178	—			
E.W.	1385	2	158	—	160		
B.M.		4	161	—	80	<10	<10
J.N.		4	138	—	40	<10	<10
C.M.		2	141	—			

Conventions as for Table 1.
* Tissues obtained at biopsy.

TABLE 3. *EBV DNA in tissues from African tumors**

Patient	KCC	Disease	No. tests	cpm hybridized per 50 μg of DNA	No. of EBV genome equivalents per cell	Serum antibody titers		
						VCA	EA-D	EA-R
P.W.		Adenocarcinoma (mandible)	2	1782	45			
J.M.	1265	Melanoma (nose)	4	581	11	840	320	
N.M.		Carcinoma (antrum)	2	391	6	320	<10	<10
S.N.	1408	Reticulum cell sarcoma	2	574	11	1280	320	
T.M.	1419	Adenocarcinoma (mandible)	2	399	6			
C.S.	1237	Carcinoma (maxilla)	4	151	—	20	<10	<10
N.M.	1423	Carcinoma (maxilla)	4	161	—	320	<10	80–160
S.N.		Cervical lymph node (tuberculous)	4	143	—	160	<10	<10
M.K.		Carcinoma (antrum)	2	158	—	20	<10	<10
G.M.	1439	Carotid body tumor	2	131	—	20	<10	<10
N.N.		Carcinoma (leg)	2	152	—			
M.M.	1334	Epidermoid carcinoma (nose)	2	128	—	20	<10	<10
N.M.		Carcinoma (maxilla)	2	148	—			
D.W.	1240	Myosarcoma (maxilla)	2	169	—	10–40		
G.M.	1104	Leukemia	2	153	—	80	<10	<10
M.N.		Malignant lymphoma	2	171	—	640	<10	<10
N.N.		Carcinoma (esophagus)	2	132	—	<10	<10	<10
W.W.	1461	Lymphoblastic lymphoma	2	145	—	20	<10	<10
A.M.	1455	Squamous cell carcinoma (mandible)	2	167	—	20	<10	<10
R.S.		Carcinoma (tongue)	2	147	—	320	<10	<10
J.M.	1400	Lymphosarcoma (mandible, abdomen)	2	143	—	160	<10	<10
M.K.	1424	Carcinoma (tonsil)	4	157	—	40	<10	<10
C.M.	1264	Lymphoblastic lymphoma	2	147	—	40		
K.M.		Carcinoma (maxilla)	2	153	—			
Raji		Burkitt lymphoma line, non-virus-producing, established *in vitro*	4	2323	60			
Hep2		Human fibroblastic cells, established *in vitro*	6	152 ± 23	—			

* Tissue obtained at biopsy.

by the diphenylamine test (5). Hybridized cpm were normalized to 50 μg of DNA. The number of genome equivalents per cell was calculated from the values for Raji cells (a non-virus-producing line of Burkitt's lymphoma cells), reported to be 60 genome equivalents per cell, since there is a linear relation between the amount of EBV DNA and the amount of hybridized cRNA (3). Nonspecific background hybridization to Hep2 (human epithelial cell line) DNA was subtracted before this calculation was made.

Immunological Assays. Antibody titers for virus capsid antigen (VCA) and early antigen (EA-D and EA-R) were available for most patients' sera from previous determinations made by W. and G. Henle in the course of other studies (6–8).

RESULTS AND DISCUSSION

We have tested 23 specimens of Burkitt's lymphomas, 23 nasopharyngeal carcinomas, and 24 other tumors from patients in the same African area. 22 of 23 of the Burkitt's lymphomas were clearly positive for EBV DNA, containing viral DNA in genome equivalent amounts ranging between four and 113 (Table 1). 18 of 23 nasopharyngeal carcinomas contained detectable EBV DNA (Table 2), and five of 24 of the other African tumors were also positive for EBV DNA (Table 3).

The single case of Burkitt's lymphoma without detectable EBV DNA was studied further. Different concentrations of control cell DNA (Hep2) and DNA from the lymphoma of F.M. were fixed onto nitrocellulose filters and hybridized with the same amount of cRNA. Fig. 1 shows the result. The background hybridization for Hep2 DNA and the degree of hybridization for the lymphoma did not differ significantly with concentrations of up to 200 μg of DNA. The results indicated clearly that there was less than one genome equivalent per cell.

The tissues from the nasopharyngeal carcinomas that did not have detectable EBV DNA were not examined further because of the poor yield of DNA, and the possibility remains

that these tumors may carry one genome or less per cell, since all the cells obtained at biopsy are probably not tumor cells.

Most of the control tumors were negative for EBV DNA as expected. In five out of 24, however, EBV DNA was found: a melanoma of the nose, a carcinoma of the antrum, a reticulum cell sarcoma, and two adenocarcinomas of the maxilla and mandible.

The antibody titers of the patients to VCA and early antigen, EA-D and EA-R (6–8), are also listed in the tables. The VCA titer was generally high for Burkitt's lymphoma nasopharyngeal carcinoma. The specimen, FM (KCC 1143), that had less than one genome per cell showed a high antibody titer of 1/1280. The EA titer for this biopsy was 1/20 to 1/40 for EA-D, and 1/160 to 1/320 for EA-R. The specimen KCC 1393 had a high number of genome equivalents (38 per cell), but the VCA titer was rather low (1/40) compared with other biopsies of Burkitt's lymphoma. Three positive cases in other African tumors also showed a high VCA titer, corresponding to the hybridization results.

These results strengthen the association of EBV with Burkitt's lymphoma. The histology and clinical findings in the single negative case were typical for Burkitt's lymphoma. The content of viral DNA in this specimen, which was from the ovary, could be below the level that can be detected by RNA–DNA hybridization. The significance of this finding should be assessed after more Burkitt's lymphomas are tested by the use of a new, more sensitive hybridization technique (4).

Most nasopharyngeal carcinomas contained EBV DNA as expected from immunological studies, but with this tumor there were several instances of tissue without detectable EBV DNA. Since EBV can infect only lymphoid cells, at least *in vitro*, this result may be due to the infiltration of EBV-carrying lymphoid cells into the tumor area rather than because the carcinomatous cells contain EBV DNA. Five positive cases in control tumors could also be explained in the same manner. The source for such cells could be the tonsil, since evidence of EBV has been found in throat washings of persons with an EBV antibody titer, presumably following infectious mononucleosis (9, 10). Whether EBV replicates in the tonsil of patients with Burkitt's lymphoma or nasopharyngeal carcinoma is not known. Because the onset of the tumors cannot be ascertained it is also difficult to exclude the possibility that in nasopharyngeal carcinoma and the positive cases of other African tumors, preexisting EBV-transformed lymphocytes that are normally repressed become derepressed and start growing preferentially in the tumor site. In any case quantitative demonstration of viral DNA in nearly all the tissue specimens tightens but does not prove the argument that EBV causes Burkitt's lymphoma.

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