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Occurrence of Epstein–Barr Virus Genomes in Human Lymphoblastoid Cell Lines

DURING the past two years, work in this and other laboratories has demonstrated viral genomes characteristic of Epstein–Barr (EB) virus in cell lines and biopsy cells derived from Burkitt lymphomas[1–4], and anaplastic carcinomas of the nasopharynx[5]. Our technique consists of hybridizing DNA from the cells concerned with the complementary RNA (cRNA) obtained by the transcription of EB virus DNA *in vitro* with *E. coli* RNA polymerase. We have now applied the technique to various lymphoblastoid cell lines which lack virus particle and structural viral antigens and found that they all seem to carry EB virus genomes. DNA from human umbilical cord leucocytes, however, seems to be free of such genomes.

DNA from various lymphoblastoid cell lines, apparently free of EB virus-particle synthesis, as well as DNA derived from umbilical cord leucocytes, and from human and hamster control preparations was hybridized with EBV–cRNA (Table 1). DNA from all lymphoblastoid cell lines, regardless of origin, hybridizes with EBV–cRNA. If complete copies of viral genomes were present within the lymphoblastoid cells and if the cRNA were equivalent to a copy of the whole EBV-genome[1–2], the lymphoblastoid cells would contain between three and 121 viral genomes per cell. Because part of the perfectly hybridized DNA–cRNA might be lost from the filters (M. Haas, M. Vogt, R. Weinberg and R. Dulbecco, personal communication), however, genome equivalents based on reconstruction tests may be overestimates[2]. Even so, the hybridization data show that the lymphoblastoid cell lines tested contain EBV-specific DNA and that the EBV-genome "load" varies considerably from one cell line to another. The high EBV–DNA content of the NC-37 line, derived from a healthy donor with antibodies to EBV[9], is especially surprising. Theoretically, the 121 viral genomes calculated would correspond roughly to 0.25% of the total cellular DNA. *In situ* hybridizations[12–14] of cytological preparations of Raji cells with EBV–cRNA revealed the presence of many labelled loci, associated with metaphase chromosomes[1,2]. The absence of such loci in preparations of human KB and hamster cell origin is further support for the view that there are several viral genome copies in each cell, at least for the Raji line. The DNA of leucocytes derived from the umbilical cord of newborn babies bound approximately the same amount of EBV–cRNA as the other human and hamster cell DNA controls, and can be regarded as negative.

When DNA preparations from the Raji line, extracted before and after an interval of one year of serial propagation were annealed with EBV–cRNA, 1,483 and 1,542 c.p.m. were bound, respectively. This remarkable similarity of hybridized counts, even after prolonged periods of cultivation *in vitro*, suggests a genetically stable association of viral DNA with host cell chromosomes.

Table 1 Hybridization of Cellular DNA with EBV-specific cRNA

Cells	Donor	Hybridized c.p.m.	Genome equivalents*	Input hybridized (%)	Cells positive for EBV capsid antigen (%)†
Raji	Burkitt's lymphoma	1,433, 1,580	50	1.80	<0.001
RPMI 64–10	Chronic myeloblastic leukaemia	776, 768	23	0.92	<0.001
SK-L 1	Monocytic leukaemia	216, 198	3–4?	0.25	<0.001
HKLY-1	Nasopharyngeal carcinoma	781, 870	26	0.98	<0.001
HKLY-2	Nasopharyngeal carcinoma	724, 736	20	0.87	<0.001
NC-37	Healthy donor	3,183, 2,989	121	3.68	<0.001
S 84	Hodgkin's disease	940, 957	31	1.14	<0.001
S 95	Hodgkin's disease	771, 787	23	0.93	<0.001
P3HR-1	Burkitt's lymphoma	11,184, 10,838	370	13.10	8.7
D 75	EBV-transformed line	6,121, 5,208	188	6.74	0.3
Ri	Umbilical cord leucocytes	86, 81	0	0.10	—
Mü	Umbilical cord leucocytes	124, 128	0	0.15	—
Sa	Umbilical cord leucocytes	96, 79	0	0.105	—
Kl	Umbilical cord leucocytes	101, 151	0	0.15	—
Kr	Umbilical cord leucocytes	102, 112	0	0.13	—
HESM	Human embryonic fibroblasts	109, 121	0	0.14	—
KB	Human carcinoma line	125, 105	0	0.14	—
H-A12-7	Adenovirus 12-transformed hamster cells	138, 112	0	0.15	—

* Calculated merely to compare the hybridized counts with each other.

† Determined by direct immunofluorescence.

Cellular DNA (50 μg) was annealed with 84,000 c.p.m. of EBV-cRNA. The RPMI 64–10 line derived from a patient with chronic myeloblastic leukaemia[6] and the SK-L 1 line (monocytic leukaemia)[7] were both obtained through the courtesy of Dr Werner Henle, Philadelphia. The HKLY-1 and HKLY-2 lines (both isolated from nasopharyngeal carcinomas)[8] were provided by Dr G. de Thé. The NC-37 line (derived from a healthy donor)[9] was provided by Dr G. Klein. The S 84 and S 95 lines were isolated from the peripheral blood and from pleural effusions of patients with Hodgkin's disease by one of of us (V. D.). DNA from EBV-synthesizing P3HR-1 (ref. 10) and D 75 (ref. 11) cells served as positive controls. DNA from leucocytes isolated from the umbilical cord of newborn babies, as well as DNA from human KB cells, human embryonic skin and muscle fibroblasts, and adenovirus type 12-transformed hamster cells were used as additional controls.

Our results confirm and extend previous studies[1,2] and the data of Nonoyama and Pagano[4] who also reported EBV-specific DNA in the RPMI 64–10 and NC–37 cells. The latter authors found, in addition, EBV–DNA within a "virus-free" lymphoblastoid cell line, F-265, derived from a healthy donor. Thus, cells of all nine "virus-free" lymphoblastoid lines tested so far contain EBV-specific DNA sequences. This raises the question whether or not the presence of the EBV genome is responsible for the continuous proliferation of these cells *in vitro*. Lymphocytes derived from human embryos fail to grow unless infected by EBV[15–17] and apparently EBV-specific complement-fixing antigens have been described in lymphoblastoid cell lines[18,19]. The results reported here provide further evidence for a role of EBV in the altered growth properties of these tissue culture cells, and suggest

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that the cells examined carry a considerable load of foreign genetic material. The physical state of this DNA remains to be elucidated.

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Defects in the Blood-Nerve Barrier in Mice with Leprosy Neuropathy

PERIPHERAL nerve damage is the most common and the most serious complication in patients with leprosy[1]. The pathogenesis of leprosy neuropathy has not been studied systematically because of the impossibility of taking serial biopsies from proximal and distal parts of infected nerves in a series of patients, so that investigations have to be confined to chance biopsies from patients with early forms of the disease, limb amputation or post-mortem material.

Histological examination of such material shows that the leprosy bacillus (*Mycobacterium leprae*) causes severe damage to Schwann and perineurial cells, and leads to a loss of axons and to an increase in the amount of endoneurial collagen. These findings, however, cannot explain all physiological changes taking place in infected nerves, particularly the early diminution in conduction velocity occurring in major nerve trunks in patients with minimal signs of clinical peripheral nerve involvement. This early impairment of neural function may, in any neuropathy, involve changes in the endoneurial environment of the axons, since it is known that changes in the nerve blood supply can lead to the generation of spontaneous neural activity, and to a block in the passage of impulses[2]. A balanced endoneurial environment in healthy nerves is probably maintained by the blood-nerve barrier whose existence in mice is known[3], and by the integrity of the perineurium[4].

In leprosy, there is histological evidence that the perineurium is affected, but data on the morphology and especially on the integrity of the blood-nerve barrier are not available, the latter requiring experimental studies which cannot be carried out with patients. Mice infected with *M. leprae* can, however, be used for this purpose as it is known that this experimental model also replicates many histopathological features of human leprosy, including neuropathy of peripheral nerves, for example, sciatic nerves, the clinical involvement of which is presented first as weakness ("foot-drop"), and later as complete paralysis of the hind-limbs within 2–2.5 yr from the date of infection[5,6]. Such mice and their uninfected littermate controls were used in this study on the histological and histophysiological properties of endoneurial blood vessels.

To test the permeability of these blood vessels, trypan blue and ferritin were given intravenously to a proportion of the animals (trypan blue readily combines with plasma proteins and can be considered, like ferritin, a protein). Some mice received two intravenous injections of 0.2 ml. 0.5% trypan blue in saline, given 4 days and 24 h respectively, before the mice were killed. Other mice were given similar injections of trypan blue at comparable intervals, but with 0.18 ml. 10% ferritin (twice crystallized, "cadmium-free") added to the second injection. The remaining mice did not receive markers.

After anaesthesia, both sciatic nerves (one for light microscopy and the other for electron microscopy) were prefixed *in situ*, following which various tissues were exposed and their colour noted. After postfixation and processing of the sciatic nerves, longitudinal paraffin sections and transverse semithin and ultrathin 'Araldite' sections were used to examine the histology of the endoneurial capillaries, and to look for trypan blue and ferritin deposits in or around the nerve (ferritin accumulations being demonstrated for light microscopy by the Prussian blue reaction).

In uninfected mice treated with trypan blue, alone or combined with ferritin, the peripheral nerves and the brain were grossly white against a background of blue stained tissues. Within the sciatic nerve neither trypan blue nor ferritin were found by light or electron microscopy in endoneurial macrophages, fibroblasts, Schwann cells or endothelial cells of endoneurial blood capillaries. The structure of the endothelium in these capillaries was comparable with that of brain capillaries which constitute the blood-brain barrier[7]. Trypan blue and ferritin, however, were found in epineurial macrophages and in the occasional macrophage situated between the outer layers of the perineurium.

By contrast, in the most severely infected mouse with bilateral paralysis of its hind legs, after treatment with the markers both sciatic nerves were macroscopically blue while other peripheral nerves and the brain were white. Histologically, trypan blue globules and ferritin accumulations were found within the sciatic nerves which were severely affected, both markers being situated in lysosomes of bacilli-containing macrophages which were scattered throughout the endoneurium and concentrated directly beneath the perineurium. Trypan blue and ferritin were also present within endothelial cells of endoneurial capillaries, and ferritin was on one occasion found in a blood monocyte penetrating a capillary wall. Furthermore, the structure of the endothelium was found to be abnormal, the cells being either locally fenestrated and attenuated, or possessing thick marginal protrusions extending into the vessel lumen and into the endoneurium. On the other

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