EB Viral Genomes in Epithelial Nasopharyngeal Carcinoma Cells

PATIENTS with nasopharyngeal carcinomas regularly show high antibody titres directed against Epstein-Barr virus (EBV)-specific antigens[1,2]. The incidence of tumours is high in certain populations of South-east Asia, central Africa and Tunisia, but low in most other parts of the world[3,4].

We have reported the presence of EB viral DNA in biopsy material derived from Burkitt lymphomas and nasopharyngeal carcinomas[5]. Although our data indicated that the tumour cells in Burkitt lymphomas contained the viral DNA, the localization of virus-specific nucleic acids within nasopharyngeal carcinomas remained obscure. The latter tumours consist of epithelial tumour cells and varying numbers of infiltrating lymphocytes[6]. Until now, infections with EBV have only been demonstrated in cells of lymphatic origin, so it was tempting to speculate that the infiltrating lymphocytes would harbour the viral DNA. The serological reactions to EBV would thus be due to the stimulation of these (viral antigen-synthesizing) lymphocytes, but not to transformation of epithelial cells by this virus. By combining biochemical, histological, and immunoserological techniques we have attempted to localize and identify EBV genome-harbouring cells within nasopharyngeal carcinomas. The results indicate the presence of EBV genomes within epithelial tumour cells.

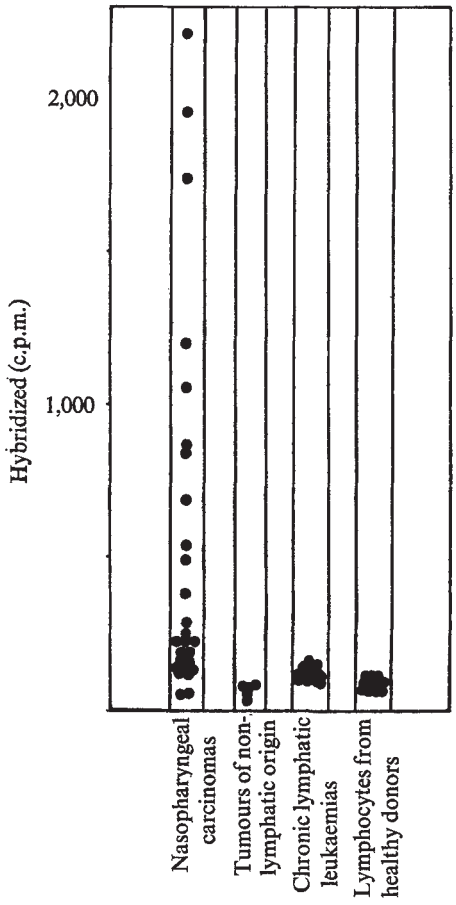

Fig. 1 Hybridization of DNA derived from various biopsies and from lymphocytes of healthy donors with EBV-cRNA. Fifty μg of heat-denatured DNA was bound to nitrocellulose filters and annealed with 45,000 c.p.m. of EBV-cRNA (specific activity 1.8×10^7 c.p.m. μg^{-1}) in 50% formamide–×2.5 SSC as described before[9]. The solution was incubated for 6 d at 45° C. In these conditions, filters with DNA from EB virus-synthesizing P3HR-1 cells bound about 20% of the cRNA input, the negative controls (DNA from umbilical cord leukocytes) approximately 0.1%.

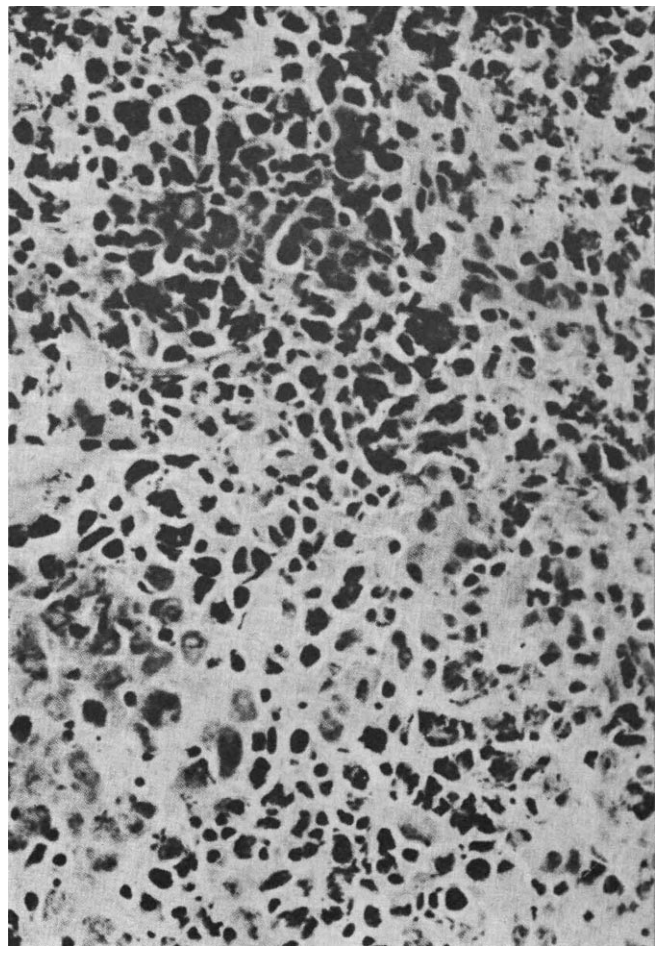

Fig. 2 Frozen section of a nasopharyngeal carcinoma biopsy (No. 34621) containing predominantly epithelial cells. DNA derived from this tumour annealed with 1,843 c.p.m. of EBV-cRNA. Magnification ×288.

Fresh biopsy material was removed surgically from patients with nasopharyngeal carcinomas and was shipped to Erlangen from Kenya via Stockholm at 0° C in tissue culture medium. Immediately on arrival, part of the tumour was DNA-extracted as described previously[5], and the remaining part was frozen sectioned after storage in liquid nitrogen. DNA of twenty-eight tumours was annealed with EBV-specific complementary RNA (cRNA) which was prepared as described before[7]. DNA derived from other carcinomas, chronic lymphatic leukaemias, and lymphocytes from healthy adult donors was also included in the test (Fig. 1). Most of the nasopharyngeal carcinoma specimens hybridized clearly above the level of other DNA preparations with EBV-cRNA. Those tumours which annealed in the highest range were predominantly epithelial when examined histologically (Fig. 2). Low hybridizing preparations consisted mainly of lymphatic cells or paratumorous tissue.

Biopsy pieces of two nasopharyngeal carcinomas and three other issues (one adenoma and two salivary glands) were frozen sectioned, denatured and subjected to *in situ* hybridizations with EBV-cRNA as described[7]. After 3 weeks of exposure to autoradiography they were developed and stained with a modified Giemsa solution[7].

Whereas the adenoma and salivary gland preparations did not reveal any clustering of label or accumulation of grains over nuclei, this was clearly the case in nasopharyngeal carcinomas. Sections of one tumour which annealed in filter hybridizations with 1,843 c.p.m. were rather heavily labelled. The heavy labelling of the majority of nuclei made their definite identification difficult, especially in view of the deteriorated morphology after NaOH-denaturation. In the

second tumour which annealed with 836 c.p.m., the situation was better; numerous foci of large pale nuclei were clearly labelled within those sections (Fig. 3), whereas nuclei of the surrounding tissue were not. The arrangement of labelled clusters of nuclei corresponded to similar clusters of epithelial cells in non-denatured sections of the same tumour. Nuclei of lymphocytes seemed to be unlabelled. It could not be excluded, however, whether or not nuclei of reticular cells were also labelled.

The technique of Reedman and Klein (to be published) to demonstrate EBV-specific complement-fixing antigen-antibody complexes by anticomplement immunofluorescence was applied to frozen sections of biopsy material from a nasopharyngeal carcinoma and three other tumours. Cells of the Raji line, derived from a Burkitt lymphoma[8], served as positive control. No anticomplement fluorescence was observed in the control tumours, whereas Raji cells and apparently epithelial cells of the nasopharyngeal carcinoma revealed a clearly positive fluorescence reaction (Fig. 4). In Raji cells this fluorescence was mainly confined to the nuclear regions, whereas in the nasopharyngeal carcinoma cells it appeared to be more granular and was also observed in the cytoplasm. The brilliant staining depended on the presence of antibodies against EBV-associated antigens. No EBV-specific viral capsid antigen (VCA) or early antigen (EA) fluorescence was detected within the same sections.

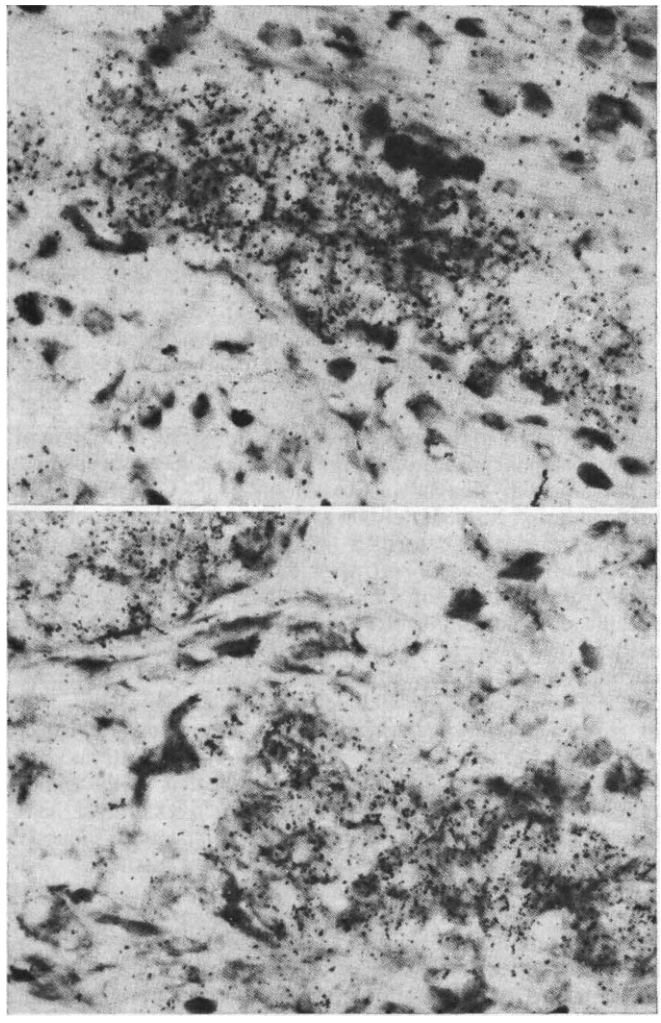

Fig. 3 *In situ* hybridization of frozen sections of a nasopharyngeal carcinoma (No. 1497) with EBV-cRNA. The frozen sections were fixed in methanol–acetic acid (3 : 1), and treated for 2 min with 0.07 N NaOH. After thorough washing they were annealed under a coverslip with 40 µl (48,000 c.p.m.) of EBV-cRNA, incubated for 1 week at 45° C, treated with RNase as described[7] and exposed for 3 weeks to autoradiography (Ilford G5 emulsion). After developing they were stained with Giemsa solution. Magnification ×900.

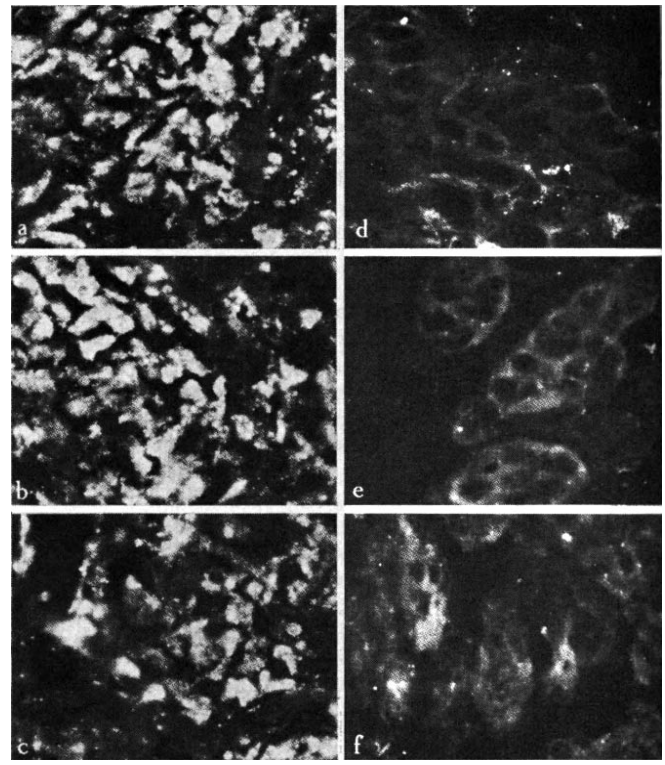

Fig. 4 Cellular localization of complement-fixing antigens in nasopharyngeal carcinoma cells. Frozen sections (a–c) of a nasopharyngeal carcinoma (No. 47472) were fixed for 10 min in acetone at −20° C. They were then treated with EBV-reactive test serum (VCA-titre 1 : 320, EA-titre 1 : 20) at a 1 : 8 dilution, containing a 1 : 10 dilution of human complement. Incubation was carried out at 37° C for 30 min in a moist chamber. The slides were washed by stirring for 10 min in PBS, and stained with a 1 : 30 dilution of FITC-conjugated anti-human β_{1C}/β_{1A} globulin (Hyland Laboratories, Los Angeles, Calif.) for 90 min at 4° C. d–f, Frozen sections of a human adenoma treated and photographed in identical conditions. Magnification ×525.

Our data present evidence that the epithelial cells of nasopharyngeal carcinomas harbour the EB viral genomes which were demonstrated by nucleic acid hybridizations. This tumour represents the first example of an infection of human non-lymphatic cells with EBV. The role of the infiltrating lymphocytes remains to be clarified. It is possible that they might be involved in the immune response against antigens specified by the virally transformed epithelial cells. The presence of EBV DNA within the latter substantially supports the circumstantial evidence for a causal role of EBV in this malignancy.

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Relation of Lysosomal Fragility in CLL Lymphocytes to PHA Reactivity

AFTER addition of various mitogens such as phytohaemagglutinin (PHA) *in vitro* lymphocytes shift from a "resting" state to one of rapid enlargement, culminating in DNA synthesis and mitosis. The mechanism initiating transformation and proliferation is not yet fully understood. Some observations, however, suggest that alterations of cellular membranes might be involved in these processes. In their cytochemical studies, Allison and Mallucci[1] observed increased permeability of lysosomes in normal human peripheral blood lymphocytes after exposure to PHA for 5 h. Hirschhorn *et al.*[2] demonstrated that during the early phase of stimulation 30–120 min after addition of PHA in normal human blood lymphocytes a redistribution of lysosomal acid hydrolases from a sedimentable into a non-sedimentable form as well as an increased release by agents which disrupt membranes, such as streptolysin S and filipin, of lysosomal enzymes from a subcellular fraction rich in lysosomes are taking place. It has been suggested that these alterations, both reflecting enhanced fragility of lysosomal membranes, might be due to increased endocytosis induced by the mitogen, and the hypothesis was raised that lysosomal hydrolases might be involved in intracellular processes leading to lymphocyte activation[2]. We have therefore studied the fragility of lysosomes in unstimulated and PHA-treated lymphocytes from patients with chronic lymphocytic leukaemia (CLL), because lymphocytes in most patients with this disease show a diminished and/or delayed or even no response to PHA[3] and other mitogens[4,5].

Column-purified peripheral blood lymphocytes from normal donors and patients with CLL were incubated *in vitro* with and without PHA (PHA-P; Difco Lab., Detroit, Michigan) for 2 h. Cell suspensions were then cooled, centrifuged, subjected to hypotonic lysis of erythrocytes and resuspended in 0.34 M sucrose containing 0.01 M EDTA, adjusted to *p*H 7.0 by addition of 1 N NaOH, with 50 IU heparin ml^{-1} suspension medium. After homogenization with an all-glass tissue grinder, subcellular fractions were obtained by differential centrifugation. It was not possible to resuspend the lysosomes present in a 20,000*g* × 20 min pellet without vigorous measures injuring the particles, so a 500*g* × 10 min (postnuclear) supernatant containing the majority of suspended lysosomes was used for the assays. Aliquots of this preparation were incubated with increasing concentrations of the membrane-disruptive agent lysolecithin (Koch-Light Lab. Ltd, Colnbrook, Bucks) dissolved in 50% (v/v) ethanol for 1 h at 37° C. Controls were performed with ethanol and 'Triton' X-100 at a final concentration of 0.1%. Blanks were run by adding adequate volumes of suspension medium. After centrifugation the activities of β-glucuronidase and acid phosphatase rendered non-sedimentable at 20,000*g* × 20 min and the enzyme activities remaining in the corresponding pellet were determined. 'Triton' X-100 released more than 98% of total sedimentable hydrolases, as revealed by assays of β-glucuronidase and acid phosphatase in both the 20,000*g* × 20 min supernatant and the corresponding pellet, so all enzyme activities released by lysolecithin were calculated as a percentage of the activities released by 'Triton' X-100. In order to test lymphocyte reactivity to PHA, lymphocytes from both normal individuals and patients with CLL were suspended in Eagle's Minimum Essential Medium, Spinner modification, completed with 20% foetal calf serum, 0.2 mmol L-glutamine, 10,000 IU penicillin and 10,000 μg streptomycin per 100 ml medium (all substances from Grand Island Biol. Co., Grand Island, New York), and incubated with and without PHA over a 7 d period. At days 3, 5 and 7, 20 μCi of ^{3}H-methylthymidine (New England Nuclear Corp., Boston, Massachusetts; specific activity 2.0 Ci $mmol^{-1}$) was added to triplicate cultures 4 h before collection as described by Naspitz and Richter[6], and DNA synthesis, as measured by thymidine incorporation, was determined.

Incubation of postnuclear supernatant rich in lysosomes from both unstimulated normal and CLL lymphocytes with various concentrations of lysolecithin revealed a dose-dependent increase of non-sedimentable β-glucuronidase and acid phosphatase activities (Fig. 1) accompanied by a corresponding decrease of hydrolase activities in the 20,000*g* × 20 min pellet (not shown in the figure). Thus, as in normal lymphocytes[7], acid hydrolases present in CLL lymphocytes not only showed sedimentability but also latency, a further characteristic of lysosomes[8]. It has been found that lymphocytes from most patients with CLL contain lower total activities of acid hydrolases than normal lymphocytes[9] which, as demonstrated by semiquantitative electron microscopy, are due, at least in part, to a reduced number of lysosomes per cell[10]. Some dilution of lysosomes therefore had to be considered in postnuclear supernatant derived from CLL lymphocytes. But the percentage of total enzyme released by the various concentrations of lysolecithin used in this study was not altered by comparison with the hydrolase release from the more concentrated lysosome suspension derived from normal lymphocytes (Fig. 1).

Figure 2 summarizes the activities of β-glucuronidase and acid phosphatase rendered non-sedimentable by the addition of lysolecithin to postnuclear supernatant derived from both

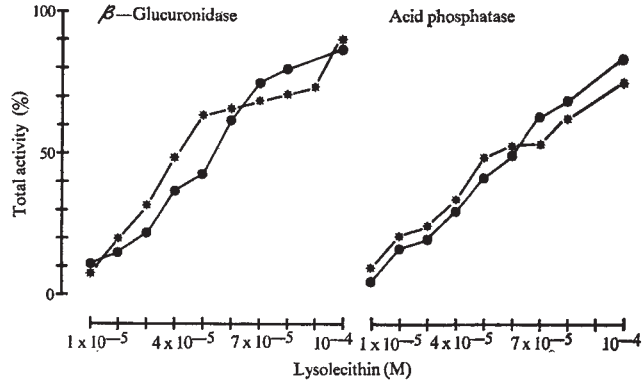

Fig. 1 Release of β-glucuronidase and acid phosphatase activities by various concentrations of lysolecithin from lysosomes present in 500*g* × 10 min (postnuclear) supernatant derived from unstimulated normal (●—●; number of individuals tested, 12) and CLL (★—★; number of patients tested, 18) peripheral blood lymphocytes. Postnuclear supernatant was prepared by homogenization of 60–100 × 10^6 lymphocytes suspended in 3.7 ml of 0.34 M sucrose containing 0.01 M EDTA and 50 IU ml^{-1} of heparin and centrifugation of the homogenate at 500*g* for 10 min. Samples (0.05 ml) of an ethanol solution (50%, v/v) containing the concentration of lysolecithin to be tested were added to 0.95 ml aliquots of postnuclear supernatant. After incubation for 1 h at 37° C, the mixture was centrifuged at 20,000*g* × 20 min, and the final supernatant was assayed for β-glucuronidase and acid phosphatase. The 20,000*g* × 20 min pellets were reconstituted with 1 ml EDTA-sucrose-heparin solution. After addition of 'Triton' X-100 at a final concentration of 0.1% and centrifugation at 20,000*g* × 10 min, enzyme activities were determined in the resulting supernatant representing sedimentable lysosomal hydrolase activities. β-Glucuronidase was assayed using phenolphthalein-glucuronic acid (Sigma Chemical Co., St Louis, Missouri) as substrate; for acid phosphatase assay the substrate D,L-β-glycerophosphate (Sigma Chemical Co.) was used; details of the methods are given in ref. 7. Enzyme activity released by lysolecithin was calculated as a percentage of enzyme activity released by 'Triton' X-100.