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Direct Evidence for the Presence of Epstein–Barr Virus DNA and Nuclear Antigen in Malignant Epithelial Cells from Patients with Poorly Differentiated Carcinoma of the Nasopharynx

(*nude* mice/transplantation/nucleic acid hybridization/isozyme)

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ABSTRACT **A well-differentiated squamous cell carcinoma and three poorly differentiated carcinomas of the nasopharynx were analyzed for the presence of Epstein–Barr virus DNA by hybridization with radioactive complementary RNA. The well-differentiated carcinoma contained no detectable Epstein–Barr virus DNA, whereas the three anaplastic carcinomas contained 41, 16, and 14 viral genome equivalents per cell. The anaplastic carcinomas were heavily infiltrated with lymphocytes and other nonneoplastic cells. All four tumors were successfully passaged in *nude* (thymusless) mice. Mouse cell admixture in the heterotransplanted tumors was estimated by analysis of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and varied between 25% and 80%. After two passages in *nude* mice, the carcinoma that was negative for Epstein–Barr virus DNA remained negative, while the three anaplastic carcinomas retained their viral DNA. After correction for mouse cell admixture, the latter tumors were found to contain about 80, 55, and 160 Epstein–Barr virus genome equivalents per human cell. The virus-determined nuclear antigen was localized in the large carcinoma cell clusters in all three mouse-passaged tumors positive for the viral DNA, but other virus-determined antigens were not detected, indicating the absence of virus induction. In contrast to the original tumor biopsies, the *nude*-mouse-passaged tumors showed virtually no lymphocytic infiltration. It is concluded that the Epstein–Barr virus DNA found in biopsies of human nasopharyngeal carcinomas is localized in the neoplastic cells.**

DNA of Epstein–Barr virus (EBV) has been regularly detected by nucleic acid hybridization in African Burkitt lymphoma cells (1–3), which also contain a characteristic EBV-determined nuclear antigen (EBNA) (3–5). Since Burkitt lymphoma is known to have a single cell origin (6), this disease can be regarded as a malignant proliferation of a clone carrying the EBV genome. In contrast to the African form of the disease, EBV DNA has not been detected in tumors with histological and cytological appearance indistinguishable from African Burkitt lymphoma, which occur rarely in other parts of the world (7, 8).

The only other tumor in which EBV DNA has been consistently detected (1, 2) is poorly differentiated nasopharyngeal carcinoma (NPC), a disease that is common (9) in certain areas (e.g., China) and rare in others (e.g., Western Europe). While nucleic acid hybridization tests have mainly been performed on biopsies from East African patients, preliminary hybridization studies on biopsies from non-African patients (10) and extensive serological investigations of Chinese, African, American, and European patients suggest (11, 12) that, in contrast to Burkitt lymphoma, the association between EBV and NPC is universal and independent of the disease frequency. Subjects with other tumors of the nasopharynx and those with neoplasms involving the oro- and hypopharynx do not differ from control populations with respect to the distribution of EBV-associated antibodies.

Anaplastic nasopharyngeal carcinomas are generally heavily infiltrated with lymphocytes. Reticulum cells, resembling the tumor cells, are also found scattered among the epithelial cells (13). As a group, NPC biopsies contain less EBV DNA than Burkitt lymphoma biopsies (2). This may be related to the heterogeneity of the histological picture. Due to the variety of cell types present in NPC, together with the difficulty of obtaining technically satisfactory cell suspensions of good viability, we were unable to perform reliable EBNA tests directly on human NPC biopsies. Since EBV is specifically associated with human bone-marrow-derived (B-type) lymphocytes, it appeared likely that the EBV DNA found in the NPC biopsies would be localized in the infiltrating lymphoid cells rather than in the carcinoma cells. The fact that EBV-carrying lymphoblastoid cell lines could be readily established from NPC biopsies (14) appeared to support such a notion. Such cell lines have been established from the peripheral blood (15) and the lymph nodes (16) of normal individuals seropositive for EBV as well, however. They can be also grown from tumors that do not contain the EBV genome, e.g., myelomas or leukemias (17), where they are clearly not representative of the tumor cell population; only in cell lines containing EBV DNA, established from African Burkitt lymphoma biopsies, has it been conclusively shown that the lines are really derived from the tumor cells (18, 19).

One obstacle to providing a definitive answer to the question of whether the EBV genomes are associated with neoplastic epithelial cells or with infiltrating lymphocytes in anaplastic nasopharyngeal carcinoma is the inability to grow

Abbreviations: EBV, Epstein–Barr virus; NPC, anaplastic nasopharyngeal carcinoma; EBNA, nuclear antigen determined by presence of EBV; EA, intracellular antigen determined by EBV; cRNA, RNA complementary to DNA; G-6-*P* dehydrogenase, glucose-6-phosphate dehydrogenase.

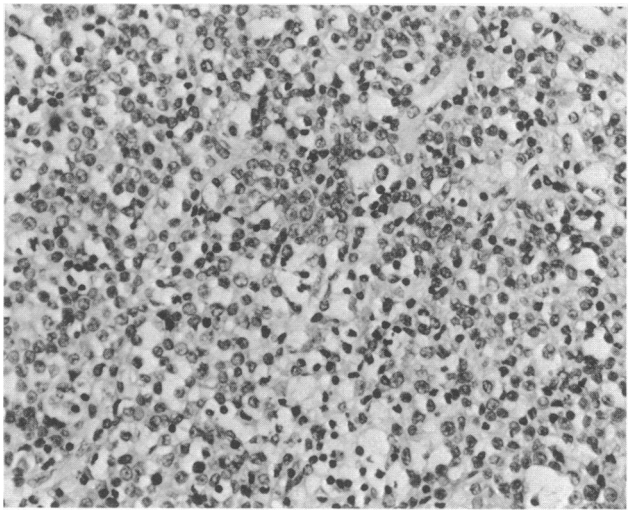


FIG. 1. Histologic appearance of the original J.G. tumor. Hematoxylin and eosin staining, 90×. Note the lymphocytic infiltration.

the malignant cells in long-term culture *in vitro*. This difficulty can be circumvented by passaging the tumors in *nude* mice. These animals have thymic aplasia, with consequent deficiency in thymus-dependent immunologic reactions. They can be maintained for prolonged periods under strict pathogen-free conditions (20). They accept grafts of foreign tumors. Human carcinoma cells grown in *nude* mice maintain their original histological appearance, but the stroma and infiltrating cells are replaced by mouse stroma (21). We have passaged anaplastic nasopharyngeal carcinomas in *nude* mice, estimated the proportion of mouse and human cells by isoenzyme analysis, determined the number of EBV genome equivalents per cell by hybridization with EBV complementary RNA (cRNA), and localized the cells that carry the EBV genome with the EBNA antigen test. The results demonstrate that the EBV provirus is carried by the malignant epithelial cells.

MATERIALS AND METHODS

Tumors. Biopsies of nasopharyngeal carcinomas were obtained in Nairobi, immersed in tissue culture medium, and shipped to Stockholm on wet ice by a direct flight, such that they reached the Stockholm laboratory within 24 hr. After arrival, the material was subdivided into aliquots for nucleic acid hybridization, for isozyme analysis, and for inoculation into *nude* mice. Viable pieces in cold tissue culture medium were shipped to Houston for inoculation into *nude* mice. They arrived 77–81 hr after removal from the patient and were transplanted into *nude* mice within 2 hr. Frozen pieces were sent from Stockholm to Seattle, where their isoenzyme patterns were determined. Other frozen pieces were kept in Stockholm for nucleic acid hybridization.

Tumor biopsies from four patients were established as serially propagated carcinomas in *nude* mice. Patient K.K. (Unit no. 6132/73) had an epidermoid carcinoma containing numerous epithelial pearls. There was no lymphocytic infiltration of the neoplastic tissue. Lymphocytes were present in moderate amounts in the surrounding normal tissues. Patients M.M. (Unit no. 5442/73), N.M. (Unit no. 7241/73), and J.G. (Unit no. 81457/74) had poorly differentiated carcinomas infiltrated by lymphocytes, which were also present in large numbers in the neighboring normal tissues (Fig. 1).

Transplantation to nude Mice. Each biopsy specimen was washed with sterile minimal essential medium and minced finely with iridectomy scissors. The mince was suspended in 2–3 ml of the same medium and injected subcutaneously in two to four adult *nude* mice with a syringe equipped with a 16-gauge needle.

Isoenzyme Analysis. The original tumor biopsies and the tumors passaged in *nude* mice were divided into multiple samples, each about 3–6 mm in thickness and 3–8 mm in diameter. The pieces were homogenized; after centrifugation the supernatant fractions were analyzed for glucose-6-phosphate dehydrogenase (G-6-*P* dehydrogenase; EC 1.1.1.49; D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase) activity after starch-gel electrophoresis (22). Mouse G-6-*P* dehydrogenase migrates more rapidly than either the usual B type human enzyme or the variant types A and A^-. The proportion of mouse-to-human-enzyme activity was estimated visually.

Nucleic Acid Hybridization. DNA·cRNA hybridizations were performed as described (3). Briefly, 10-μg aliquots of cellular DNA from biopsies were denatured, fixed to 13-mm nitrocellulose filters, and incubated with [^{3}H]cytidine-labeled EBV cRNA (1.5×10^7 cpm/μg) or ^{32}P-labeled EBV cRNA (7×10^7 cpm/μg), 50,000–150,000 cpm, for 96 hr at 45° in 0.3 ml of 0.9 M NaCl–0.09 M Na_3-citrate (pH 7.5), containing 50% formamide. The filters were then washed, treated with ribonuclease, and analyzed for radioactivity and DNA content. The average number of EBV genome equivalents per cell in a biopsy was obtained by comparison with a standard curve, constructed with filters containing 10 μg of salmon sperm DNA and known amounts (1–70 ng) of EBV DNA. The molecular weight of EBV DNA was taken as 1×10^8, and that of human cell DNA as 4×10^{12}. Controls positive (Raji) and negative (HeLa, salmon sperm) for EBV DNA were always included in hybridization experiments.

Antigens. Tests for the EBV-determined nuclear antigen (EBNA) were carried out on smear preparations, fixed in methanol–acetone, as described (4, 5). Three types of cell suspensions were used: mechanically minced and shaken suspensions, trypsinized suspensions, and trypsinized suspensions separated into living and dead cell fractions by centrifugation at $700 \times g$ for 10 min through a mixture of 7% Ficoll and 8% Isopaque. Fixed smears were reacted with a reference serum containing antibodies against EBNA (00), diluted 1:8 with balanced salt solution, followed by 1:5 diluted serum of an EBV-negative donor, used as complement source, and subsequently exposed to a fluorescein isothiocyanate-conjugated goat antiserum to human complement (β_1C/β_1A) diluted 1:40 (Hyland Laboratories, Los Angeles, Calif.). As a negative control, an EBNA-negative reference serum was used (IE). As a further check on the specificity of the reactions obtained, a series of known EBNA-positive and EBNA-negative sera were tested against selected specimens, as in a previous study (4). As a positive cell control, a lymphoblastoid cell line (Raji) carrying EBV DNA was stained with the same sera and reagents in parallel in every experiment. All smears were examined with oil immersion in a Leitz Labolux microscope (540×). Tests for the EBV-determined early antigen (EA) were performed as described (23).

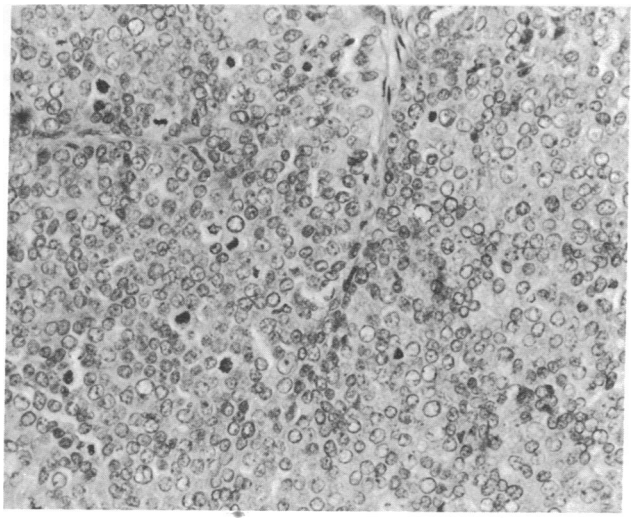


FIG. 2. Histologic appearance of a nasopharyngeal carcinoma (J.G.) growing in *nude* mice. Hematoxylin and eosin staining, 90×. Note the lack of lymphocytes.

RESULTS

Transplantability. The original K.K. tumor biopsy was transplanted into two *nude* mice. After 25 days, a nodule of 1-cm diameter was excised from one of the animals and retransplanted into four *nude* mice. All of them developed rapidly growing tumors. Histologically, these are epidermoid carcinomas with squamous cell differentiation, identical to the tumor of origin.

The original N.M. biopsy was transplanted into four *nude* mice. One of them developed a nodule that became visible after 45 days and reached the size of 2.5 × 1.5 × 1.0 cm after 65 more days. The other three animals were negative after 240 days. The first tumor was injected into eight *nude* mice. All of them developed rapidly growing tumors. This carcinoma has now been passed three times, and 16 of 16 mice have shown positive takes. Histologically, the tumors are poorly differentiated carcinomas. They differed from the original neoplasm by the lack of lymphocytic infiltration.

The original M.M. tumor biopsy was transplanted to two *nude* mice. A nodule became visible in one of the two mice after 25 days, and grew into a 2-cm diameter tumor in 85 days. The other mouse was negative after more than 300 days. The tumor is now in its third passage, in which 10 of 12 animals developed tumors. Histologically, they were all poorly differentiated carcinomas. There was no detectable lymphocyte infiltration.

The original J.G. biopsy was transplanted into two *nude* mice. A nodule became visible in both animals after 30 days. After 41 more days, one of these tumors was excised and retransplanted in eight *nude* mice, which all developed rapidly growing tumors. The original biopsy was a very poorly differentiated carcinoma, heavily infiltrated with lymphocytes. After one mouse passage all lymphocytes had already disappeared, leaving only epithelial cells growing in clusters (Fig. 2).

Isoenzyme Analysis. These measurements were performed on hetero-transplanted M.M., N.M., and J.G. tumors. Nucleic acid hybridization tests were made on the same tumor material. All three patients had type B G-6-*P* dehydrogenase in their normal tissues and tumors. In the heterotransplanted tumors, the estimated proportion of human type G-6-*P* dehydrogenase activity in the tumor specimens varied from 20 to 75% (Table 1). There was a direct relationship between the estimated proportions of human type enzyme and the numbers of EBV genome equivalents per cell in the tumor biopsies.

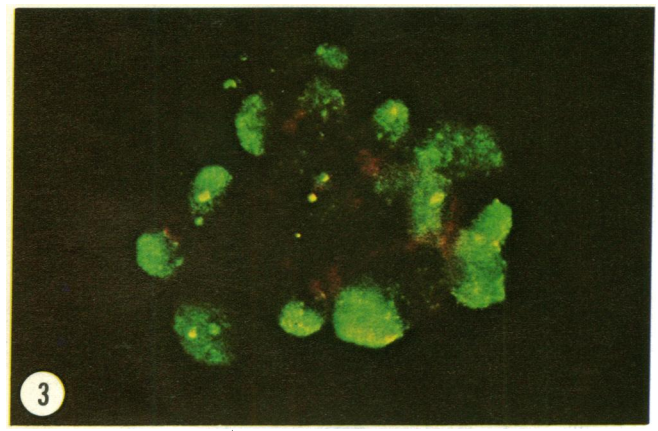
TABLE 1. *EBV DNA content of human nasopharyngeal carcinomas before and after heterotransplantation into nude mice, determined by nucleic acid hybridization*

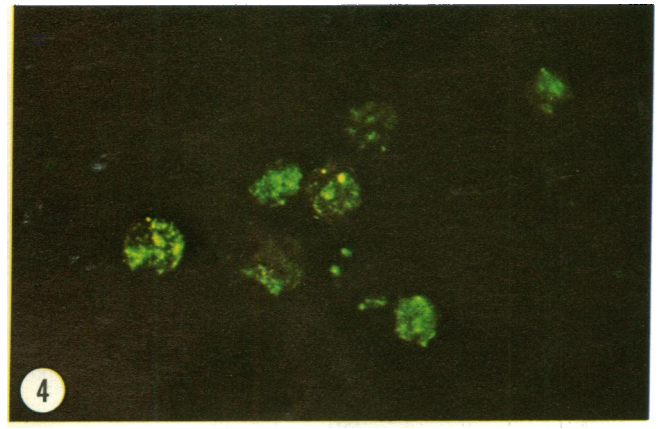
Source of tumor DNA	No. of hybridization determinations	[^{3}H]cRNA, cpm bound per 10 μg of DNA	EBV genome equivalents per cell in tumor	% Human G-6-*P* dehydrogenase activity*	EBV genome equivalents per human cell†
M.M., primary tumor	5	2098	41	100	41
M.M., mouse no. 1	4	1884	37	60	62
M.M., mouse no. 2	3	1059	19	20	95
M.M., mouse no. 6	3	1301	24	30	80
M.M., mouse no. 7	2	1192	22	25	88
N.M., primary tumor	4	912	16	100	16
N.M., mouse no. 1	2	2034	40	75	53
N.M., mouse no. 2	2	1517	29	50	58
J.G., primary tumor	3	831	14	100	14
J.G., mouse no. 1	3	3144	64	40	160
J.G., mouse no. 2	2	1985	39	—	—
J.G., mouse no. 4	2	2036	40	30	130
K.K., primary tumor	2	189	< 2	—	<2
K.K., mouse no. 1	2	217	< 2	—	—
HeLa cells	6	178	0	—	—
Raji cells	6	2683	54	—	—

* % Human G-6-*P* dehydrogenase activity is the mean of two to three determinations per biopsy.

† For the purpose of obtaining rough estimates of the number of EBV genome equivalents per human cell in the mouse-passaged tumors, it was assumed that the percent of human G-6-*P* dehydrogenase activity was equal to the percent of human cells in the biopsy.

Nucleic Acid Hybridization. The original human tumor biopsies were analyzed for EBV DNA by hybridization with radioactive EBV cRNA. The K.K. tumor did not contain detectable EBV DNA, whereas M.M. tumor contained an average of 41, N.M. tumor 16, and J.G. tumor 14 EBV genome





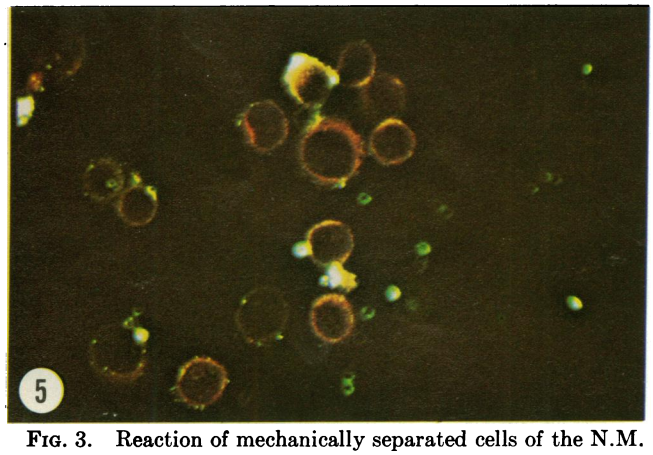


FIG. 3. Reaction of mechanically separated cells of the N.M. tumor passaged in *nude* mice with the EBNA-positive reference serum in the anti-complement fluorescence test, 345×.

FIG. 4. Trypsinized cells of the M.M. tumor passaged in *nude* mice. EBNA-positive reference system, 345×.

FIG. 5. Same cells as in Fig. 4, exposed to EBNA-negative serum, 345×.

equivalents per cell. It should be noted that the calculations of EBV DNA content were done with the assumption that all cells in a biopsy contain the same amount of virus DNA. This is obviously not correct, since these human tumor biopsies contained several types of cells, some of which presumably did not carry EBV DNA at all. The amount of viral DNA in the actual cells carrying EBV DNA, therefore, must be higher than these estimates.

All four mouse-passaged tumors were also tested for EBV DNA. The K.K. tumor, derived from the biopsy that was negative for EBV DNA, was still negative after *nude* mouse passage. Four tumors were tested of the M.M., two of the N.M., and three of the J.G. heterotransplanted tumors (Table 1). All nine tumors retained large amounts of EBV DNA after two consecutive passages in *nude* mice, in spite of considerable infiltration by mouse stroma. When the relative amounts of human compared to mouse G-6-*P* dehydrogenase were used to estimate the approximate proportion of human cells, the calculated amount of EBV DNA per human cell was 2 to 12 times higher than in the original biopsies. Thus, the N.M. heterotransplanted tumor contained 50 to 60 EBV genome equivalents per human cell, compared to 16 in the human biopsy. Similarly, the M.M. mouse-passaged tumor contained 80 ± 20 EBV genome equivalents per human cell, compared to 41 in the original tumor biopsy. With the J.G. material, the heterotransplanted tumor had 130 to 160 EBV genome equivalents per human cell, compared to only 14 in the original biopsy. These data indicate that 50–90% of the cells in the original biopsies were infiltrating nontumor cells that did not carry EBV DNA, and that these cells were eliminated during the mouse passage. The alternative explanation of induction of EBV DNA replication in a fraction of the human tumor cells during their growth in *nude* mice is unlikely, because the heterotransplanted tumor cells did not contain any detectable EBV-associated "early antigen" (EA), which is known to be expressed before virus DNA replication during the viral lytic cycle (24).

EBNA Tests. With the EBNA-positive reference sera, fine granular nuclear staining was obtained with mechanically dispersed or trypsinized cells from the N.M., M.M., and J.G. heterotransplanted tumors, while no staining was observed with the EBV-negative, mouse-passaged K.K. tumor. It was quite clear that the large carcinoma cells were stained, often in adherent complexes (Figs. 3–5). There were no stained lymphocytes or lymphoblasts.

As a check on the specificity of the reaction, 12 EBNA-antibody-positive sera and five negative sera were tested against the trypsinized M.M., N.M., and J.G. cell suspensions. None of the five negative sera gave any staining. All 12 positive sera stained the nuclei of the three heterotransplanted tumors containing EBV DNA, although with varying intensity. It is concluded that the nuclear antigen detected in the carcinoma cells is identical to EBNA.

DISCUSSION

It is of fundamental importance for the interpretation of the well-documented association between EBV and nasopharyngeal carcinoma to establish whether the EBV genome resides in the neoplastic cells or in contaminating lymphocytes. The present study strongly supports the notion that the EBV DNA is found in the carcinoma cells in NPC. Thus, in contrast to the abundance of lymphocytes in the original biopsies, the tumors passaged in *nude* mice showed virtually no lymphocyte admixture, but they did retain the appearance of undifferentiated carcinoma. The tumors still contained large amounts of EBV DNA (0.1–0.3% of the total DNA in the carcinoma cells), and the nuclear antigen, EBNA, was entirely restricted to the large carcinoma cell clusters. It is, therefore, quite unlikely that the EBV provirus is carried only by infiltrating lymphoid cells in human NPC tumors.

Wolf *et al.* (25) first proposed that the EBV DNA in NPC biopsies was present in the carcinoma cells themselves,

rather than in infiltrating lymphoid cells. They performed *in situ* cytohybridization with ^{3}H-labeled EBV cRNA on sections of two biopsies containing EBV DNA. The grains were predominantly localized over foci of large pale nuclei, apparently corresponding to clusters of epithelial cells, while lymphocyte nuclei seemed to be unlabeled. Further, tumors with a large proportion of infiltrating lymphocytes contained less EBV DNA than tumors with many epithelial cells (Wolf, H., zur Hausen, H., Klein, G., Becker, V., Henle, G. & Henle, W., *Int. J. Cancer*, submitted). The latter point does not prove the association between EBV and epithelial cells, however, because the much more homogeneous biopsies of Burkitt lymphoma are known to contain widely different amounts of EBV DNA in different biopsies (1–3).

What relevance do these findings have for understanding the etiology of NPC? Poorly differentiated nasopharyngeal carcinoma in all geographical localizations thus far studied has a striking serological association with EBV. This association is paralleled only by African Burkitt lymphoma (11, 12). In anticipation of human tumor studies, extrapolation of the work on virally determined antigens in experimental tumor systems has led to the conclusion (26) that consistency and regularity of a given association is the only helpful criterion for the distinction between a causative and a passenger relationship. Since EBV is not only regularly associated with NPC (in contrast to other tumors of the naso-, oro-, or hypopharyngeal area), but is actually carried in a provirus form by the carcinoma cell, the previously prevalent view that the virus is merely a passenger in NPC requires reexamination. The fact that NPC is a very rare tumor in the West, but relatively frequent in certain ethnic groups of Chinese (9), will have to be reconciled with the ubiquitous occurrence of EBV in all human populations. Conceivably, different EBV strains may exist and NPC could be associated with a particular variant, not yet distinguished from other strains of EBV. From an analysis of the DNA reassociation kinetics, Kawai *et al.* (27) concluded that the EBV DNA of NPC biopsies is present as complete or nearly complete viral genomes, and that it is indistinguishable from the EBV DNA found in Burkitt lymphoma biopsies and in cells transformed by infectious mononucleosis virus. This method cannot distinguish between a 90% and 100% homology, however. Biological differences in transforming compared to lytic activity have been recently demonstrated among different EBV strains (28; Klein, G., Sugden, B., Leibold, W. & Menezes, J., *Intervirology*, submitted). As another alternative, the genetic constitution of the host may be responsible for the different virus–target cell interactions. It is known that a strong hereditary influence is involved in NPC (9).

An interesting implication of the association between EBV and the nasopharyngeal carcinoma cell is that B-type lymphocytes can no longer be regarded as the only cells in the human body capable of carrying the EBV genome. The poorly differentiated carcinoma cell is presumably derived from the surface epithelium of the nasopharynx. Since throat washings of acute mononucleosis patients are the only known natural source of infectious EBV (29, 30), the cell populations of the oro- and nasopharyngeal cavity are in need of closer examination with regard to their EBV susceptibility.

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