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Persistence of Epstein-Barr Virus in the Parotid Gland

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Two independent techniques, in situ hybridization on frozen sections and reassociation kinetics, have been used to localize Epstein-Barr virus genomes in tissue samples from healthy human adults. Whereas specimens taken from the palatine tonsils were invariably negative, all samples from the parotid gland were positive when tested with either technique. This observation suggests that the parotid gland is, besides the peripheral lymphocytes, a site of lifelong persistence of Epstein-Barr virus and probably the site of low-level virus production which may be the source of virus found in the oropharynx.

Epstein-Barr virus (EBV) causes infectious mononucleosis as a primary disease, persists lifelong in peripheral B lymphocytes (7), and seems to be an essential factor in the development of nasopharyngeal carcinoma and the African Burkitt's lymphoma (for a review, see references 4 and 11).

Nevertheless, it is not known where this virus is produced during acute or persistent infections, and the site of its persistence has not been identified. Virus can be regularly detected in saliva of infectious mononucleosis patients (5) and seropositive healthy persons (6). The site of virus production could be B lymphocytes of the oropharynx, because only these cells are known to have EBV receptors. Alternatively, specialized cells at specific sites might provide the suitable conditions for the persistence and low-level production of EBV, resembling a situation found with Marek's disease virus (3).

We looked for evidence of persistence of EBV in tissues of two origins: tonsils of the palate, which represent lymphocyte-rich organs in the oropharynx, and parotid glands, which are the major salivary glands and are known to be the site of replication for other viruses exemplified by mumps virus (12) and cytomegalovirus (10). Because one cannot assume that virus production will be frequent in a given specimen of a healthy individual, all techniques for detecting only late viral products or even virus particles seemed to be inappropriate, and we decided to use in situ hybridization to detect viral nucleic acid sequences in human tissues. This technique was used with materials from human tumors in an attempt to localize EBV genomes in the epithelial tumor cells of nasopharyngeal carcinomas (14).

MATERIALS AND METHODS

Biopsy materials. All tissue donors were adults ranging from 25 to 45 years of age without clinical or serological evidence for acute EBV infection. The antibody titers to EBV capsid antigen ranged between 1:32 and 1:126, no antibody titers to EBV early antigen could be detected, and antibodies to EBV nuclear antigen were present. Parotid glands from three persons (victims of traffic accidents and patients who had undergone operations for unrelated reasons) were used for in situ hybridization tests (see Fig. 1).

^{3}H-labeled EBV DNA. ^{3}H-labeled EBV DNA was prepared from EBV virion DNA, which was rebanded on CsCl (15), used for nick translation, and has been described elsewhere (9).

^{3}H-labeled herpes simplex type 1 DNA. ^{3}H-labeled herpes simplex virus type 1 DNA was purified from virions of the F strain, rebanded in CsCl, labeled as described for EBV DNA, and used in all experiments as control in consecutive sections.

In situ hybridization. The method used was based on procedures described before (2, 14, 15) with modifications. Briefly, 8-μm-thick frozen sections were spread on pretreated slides (2), fixed 20 min in 25% acetic acid–75% methanol, incubated 30 min at 70°C in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), and treated 15 min at 37°C with 1 μg of proteinase K per ml. Immediately preceding hybridization, the preparations were denaturated by immersion into 0.1× SSC for 30 s at 98°C and transferred to the same buffer at 0°C. A 10-μl portion of hybridization solution containing 100,000 cpm of denatured ^{3}H-labeled EBV DNA (specific activity, 5×10^6 cpm/μg in 50% formamide, 0.6 M NaCl, 10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA, 100 μl of polyadenylate, 0.02% [wt/vol] polyvinylpyrrolidone, 0.02% Ficoll, and 1 mg each of bovine serum albumin and tRNA per ml) was placed under siliconized cover slips and sealed with rubber cement. After hybridization at 45°C for 48 h, the cover slips were removed, and the slides were washed with 5 changes of formamide buffer (as above, without polyanions) altogether for 15 h, rinsed with 2× SSC, rinsed for 5 min in 70% ethanol–300 mM ammonium acetate and 5 min in 90% ethanol–300 mM ammonium acetate, and air dried. The preparations were dipped in Kodak NTB emulsion diluted 1:1 with 600 mM ammonium acetate at 40°C, dried, and exposed for 3 weeks at 4°C. After development with Kodak D-19 X-ray developer, the preparations were stained with Giemsa stain.

Reassociation kinetics. The DNA mixtures were denatured and fragmented by treatment with 0.27 N NaOH for 10 min at 100°C and subsequently neutralized with HCl. The end concentration in the hybridization reaction was 0.5 M NaCl–0.2 M Tris-hydrochloride–10 mM EDTA–0.05% Sarkosyl (pH 8.0), and the temperature was 68°C. Double-stranded DNA was separated from single-stranded DNA by hydroxylapatite chromatography at 60°C. Single strands were eluted with 0.14 M and double strands with 0.4 M phosphate buffer, and the whole fractions were counted.

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RESULTS

Frozen sections from five tonsils and three parotid glands were hybridized with a probe of nick-translated ^{3}H-labeled EBV DNA. As controls, we used cell lines containing no EBV genomes (BJA-B) (not shown) and P3HR1 cultures with few genomes per cell but containing virus-producing cells having a heavy genome load. BJA cells were essentially free of grains (similar to Fig. 1B and H). P3HR1 cells (Fig. 1A) contained several cells heavily labeled with grains representing virus-producing cells, whereas the other cells were devoid of grains. As additional controls, consecutive sections of all samples tested were hybridized with ^{3}H-labeled herpes simplex viral DNA of comparable specific activity. Careful analysis of these preparations did not reveal any significant hybridization signals. One area demonstrating the average background is shown in Fig. 1B. The frozen sections from parotid gland biopsies (Fig. 1B through G) obtained from different individuals contained variable numbers of cells containing variable numbers of grains. Very few cells contained as heavy a load of grains as that seen in the virus-producing cells of the P3HR1 line (Fig. 1A). In some instances, the distribution of these grains was restricted to the outer areas of the nucleus (Fig. 1F) and was similar to the

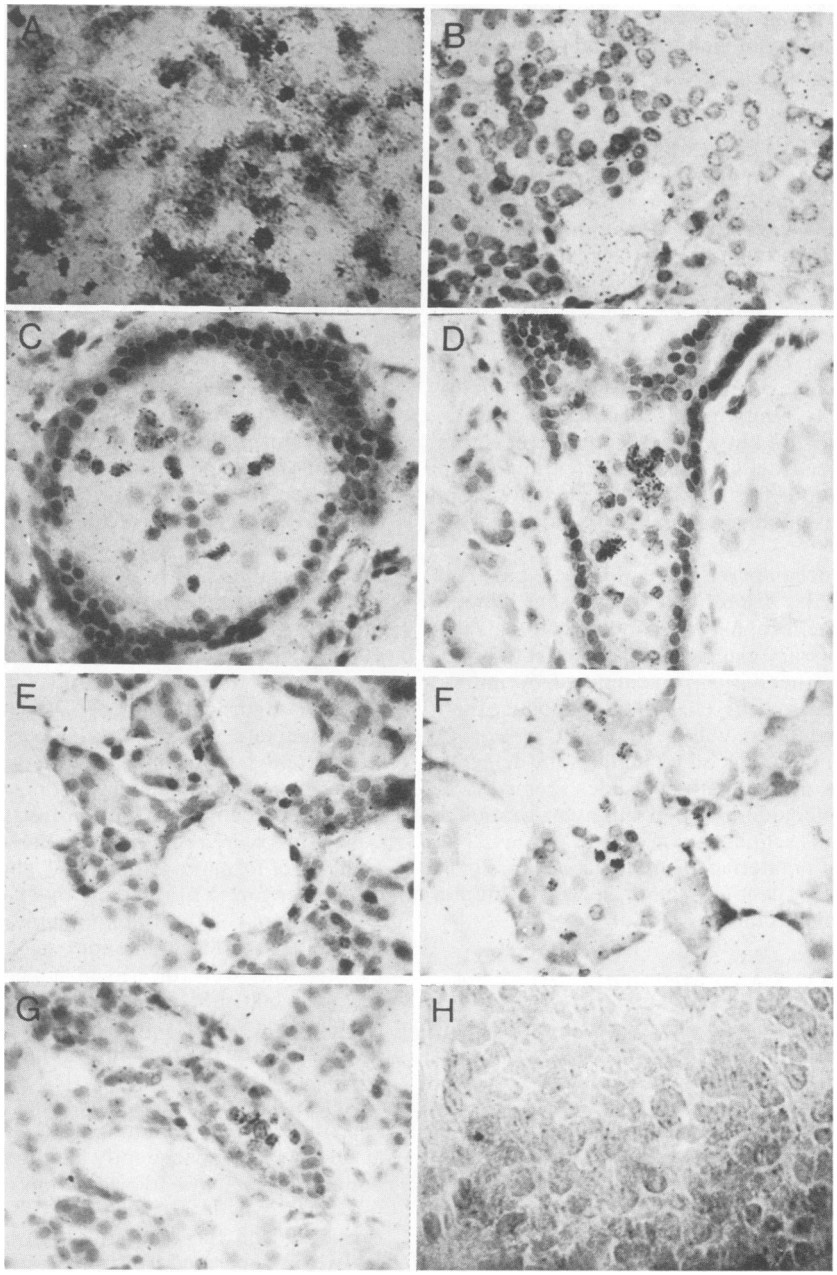

FIG. 1. Autoradiograms after in situ hybridization are shown for the following materials: A, cell smear from P3HR1 cells; B through G, frozen sections from parotid glands; and H, frozen section from a tonsil. Preparation B was hybridized with ^{3}H-labeled DNA from herpes simplex virus; for all other preparations, ^{3}H-labeled EBV DNA was used.

distribution of total chromatin in herpes-infected cells. The fact that virus genome-containing cells were rather loosely scattered did not permit the use of consecutive sections for an identification of the cell types. Because cytoplasmic staining methods did not work after the in situ hybridization procedure, the question remains unanswered for the cell types carrying the EBV genome. However, the analysis of a series of sections (Fig. 1C through G) suggests that the EBV genome-carrying cells are not infiltrating lymphocytes. The most typical location of cells with grains representing EBV DNA was within the lumen of duct-like structures, and usually a good contrast to the background grains present in adjacent cells was evident (Fig. 1C through G).

Further up in the duct (Fig. 1E), the EBV genome-containing cells seem to be in the lining epithelium of the duct, suggesting that during the lytic cycle of virus production these cells may lose their contact with neighboring cells and appear in the lumen of more distal sections (Fig. 1C and D). In contrast, similar sections from tonsils were essentially free of grains and therefore do not contain detectable numbers of EBV genome-containing or EBV-producing cells (Fig. 1H).

Due to inherent problems in the technique of in situ hybridization, in which partial evaporation of solvent or nonspecific binding of the radioactive probe DNA might in some experiments mimic positive hybridization, it seemed desirable that these results be confirmed with an independent technique which allows the application of stringent conditions of hybridization. This was done by using DNA extracted from six tonsils and three parotid glands and hybridized in liquid with nick-translated EBV virion DNA. Figure 2 shows the kinetics of the reassociation reaction. DNA from one parotid gland contained almost as much viral DNA as a DNA preparation from Raji cells, which was mixed with a 10-fold excess of calf thymus DNA and thus was representative of a DNA containing about six viral genomes per cell. The other DNA preparations from parotid glands contained approximately one-tenth that amount of viral DNA and hybridized similarly to Raji DNA mixed with a 100-fold excess of calf thymus DNA. The reassociation kinetics from the tonsils were indistinguishable from those of pure calf thymus DNA and, therefore (within the limits of the test used), excluded the presence of EBV DNA in DNA extracted from tonsils.

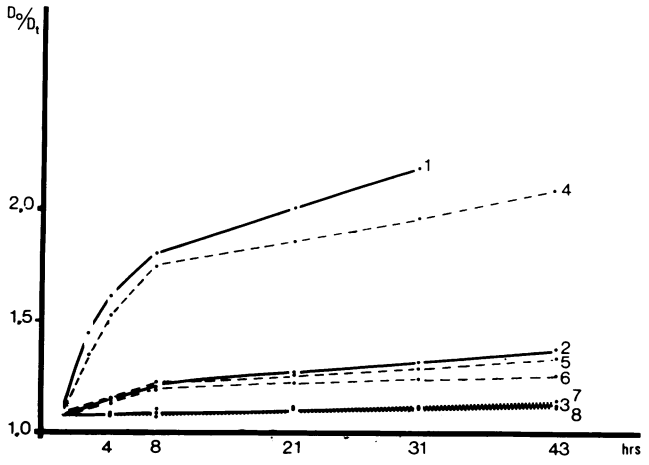

FIG. 2. Reassociation kinetics with DNA from EBV (5×10^6 cpm/μg) as tracer, DNA from calf thymus (3, negative control) as driver, and Raji cells with a 10-fold (1) and 100-fold (2) excess of calf thymus DNA as positive controls. Test DNA was obtained from tonsils (7, 8) and parotid glands (4, 5, 6).

DISCUSSION

Under the conditions used, the sensitivity of the in situ hybridization did not detect single copies or small groups of copies of EBV DNA within a cell. It may be that more cells within the sections shown in Fig. 1 contain EBV DNA. Only the producer cells of the P3HR1 line are clearly labeled, whereas the other cells which also have few EBV genomes are negative (Fig. 1A). The positive cells from biopsy materials might be a subset of EBV genome-carrying cells with a higher genome load.

Because all tissues were taken from normal adults without acute EBV infection, our data suggest that the parotid gland is an organ in which EBV is produced and released into the saliva. The isolation of EBV from the efferent duct of the salivary gland (8) supports this hypothesis. Conversely, the direct evidence of EBV DNA-containing cells adds confidence to the assumption that the virus isolated was indeed produced in the salivary gland and did not ascend through the duct. The fact that antibodies to major proteins synthesized during virus replication (1) which are serologically defined as the EA complex are usually absent in immune patients, despite an evident virus production, might find its explanation in the localization of the virus-producing cells. Early antigens may be shed together with virus upon lysis of the producer cells with the saliva and may therefore not be available as an antigen within the body. The lifelong antibody titers to virus capsid antigen might be maintained because virus from the saliva is absorbed by receptor-bearing cells or macrophages of the oropharynx and continuously presented as antigen to the immune system of the body (13). Virus shed from the parotid gland may also constantly convert new B lymphocytes to contain EB nuclear antigen and EBV DNA.

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