Epstein-Barr Virus: Its Site of Persistence and Its Role in the Development of Carcinomas

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A. Introduction

Epstein-Barr virus (EBV) causes in man infectious mononucleosis as a "primary" disease. Burkitt's Lymphoma and Nasopharyngeal Carcinoma may be considered as "secondary" diseases developing only in persons with long preceeding EBV infection.

Following the primary infection with EBV, virus can be isolated from the salivary duct (Morgan et al. 1979) and from throat swabs for months or years (Gerber et al. 1972; Chang and Golden 1971), and B-lymphocytes are lifelong carriers of EBV genomes (Nilsson et al. 1971) and can be used to establish lymphoblastoid cell lines (Pope 1967; Rickinson et al. 1980). For the nasopharyngeal carcinoma, where serology (for review see Henle and Henle 1979) suggested that EBV is regularly associated with the disease, it has been shown by in situ hybridization (Wolf et al. 1973) and other unrelated techniques involving cell separation (Desgranges et al. 1975) or passage through nude mice (Klein et al. 1974) that EBV is indeed regularly present in the epithelioid tumor cells. The ability of EBV to induce tumors in nonhuman primates (Shope et al. 1973; Wolfe and Deinhardt 1978) and the proof of EBV DNA in tumors from these animals (Wolf et al. 1975b) have added further evidence for an etiologic relationship of EBV to the development of neoplasia in man.

B. Results

I. Site of EBV Persistence in the Body

Epstein-Barr virus is associated with B-lymphocytes (Pope 1967). It is, however, not clear whether spontaneous activation of EBV genomes in carrier lymphocytes, which are present in the lymphocyte rich area of the oropharynx, is responsible for lifelong persistence of antibody titers directed against EBV-related antigens and the shedding of virus into the oropharynx or whether EBV resides in addition in specific sites of the body and is produced there, resembling somehow the situation of Marek's disease virus of chicken. That virus is found in T-lymphocytes and produced and spread from the epithelium of feather follicles (Calnek and Hitchner 1969). In situ hybridizations using sections from the parotic gland as well as from tonsils and other tissues of the ear-nose-throat area were performed in an attempt to find cells which harbor EBV genomes. EBV producing cells (P3HR1), EBV carrier Raji cells containing about 50 EBV genomes/cell, and EBV genome negative BJA-B cells were included as controls.

Figure 1 shows that only cells in the sections from the parotic gland contained detectable numbers of grains indicating the presence of EBV DNA. The number of grains per cell seems to be in the range of or higher than that seen in Raji cells which carry 50 viral genomes per cell, though clearly lower than that seen in the virus producing P3HR1 cells. The fact that no producer-type cells were found had to be expected, as the biopsies were taken from healthy adults for reasons not related to EBV infections. The induction of a lytic cycle has to be a rare event, since it leads to cell destruction and would, if it happened on a larger scale, cause necrosis.

Because in situ hybridization, in some cases, may give artifactual results, a confirmation of the results with an independent method would
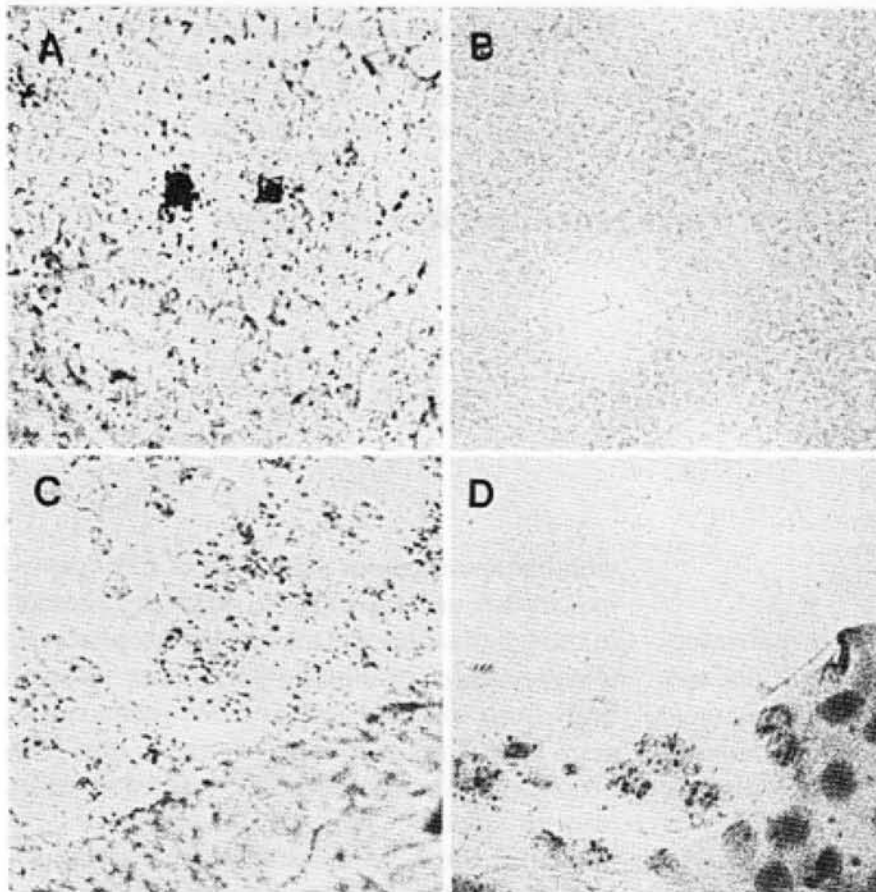
Fig. 1. P3HR1(**A**), frozen sections from tonsils (**B**), and parotid glands (**C, D**) were hybridized with 50,000 cpm H³-labeled, nick-translated EBV DNA and exposed with photoemulsion (Wolf and Wilmes 1981)

add confidence. Reassociation kinetics experiments under stringent conditions gave results in agreement with those from in situ hybridization and substantiate the presence of EBV genomes in parotic gland tissue (Fig. 2).

It is suggested that spontaneous activation of EBV in peripheral lymphocytes is a very rare event and neither the source of virus in the saliva nor the reason for the lifelong persistence of antibodies to EBV-related antigens.

II. Antibodies to EBV Specified Antigens in Carcinomas of the Postnasal Space and Other Locations Within the Waldeyer's Ring

During acute infection with EBV IgM and IgG antibodies directed against EBV-specified antigens are regulary observed, and in the case of IgG antibodies against Virus Capsid Antigen (VCA) and the EBV-specified nuclear antigen (EBNA) persist lifelong after primary exposure to EBV. It was first described by Henle and Henle (1976) that serum-IgA antibodies to VCA and early antigen (EA) were significantly associated with the EBV-linked neoplasias Burkitt's Lymphoma and Nasopharyngeal Carcinoma. We were able to confirm the data

obtained with a Chinese population with 62 cases collected within 2 years in Munich and found that IgA antibodies against VCA are closely associated with NPC and are therefore already of high diagnostic value in a single antibody determination (Wilmes et al. 1979). Successful therapy (radiation) was regularly followed by a decrease in antibody titers (Fig. 3a). In seven cases the VCA-IgA antibody level rose after initial decrease.

In three of these cases careful examination and sample biopsies revealed that new tumors had developed. Thus regular determination of anti-VCA IgA antibodies seem to be of high value as an early marker for relapse. Similar high antibodies levels, including IgA antibodies for EBV antigens, have been found recently in tumors of other locations than the nasopharynx, namely, three lymphoepithelial carcinomas and one undifferentiated carcinoma of the tonsil, two undifferentiated carcinomas at the root of the tongue, and one lymphoepithelial carcinoma of the soft palate. All these locations fall within the Waldeyer's ring. Although these observations are based on serology only and need confirmation by the demonstration of EBV DNA in tumor biopsies, they seem to be of interest because the
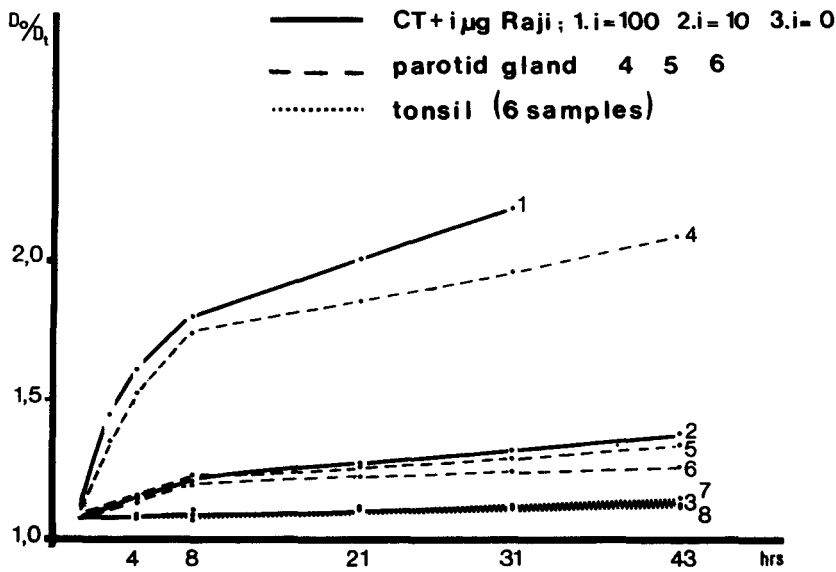
D_o/D_t

CT + i µg Raji ; 1.i = 100 2.i = 10 3.i - 0

— — — parotid gland 4 5 6

·············· tonsil (6 samples)

2,0 ·1

·4

1,5

:2
:5
:6
:7
:3 8

1,0

4 8 21 31 43 hrs

Fig. 2. Each DNA was hybridized in a DNA concentration of 2 mg/ml with 60,000 cmp/ml nick-translated EBV DNA (10^7 cpm/ug). Hydroxylapatite columns were used to separate double stranded from single stranded DNA (for details see Wolf and Wilmes 1981)

Waldeyer's ring is a histologically unique site with respect to the close contact between lymphocytes and epithelial cells (Döhnert 1977).

III. Cell Fusion Ability of EBV-Infected Cells

The EBV genomes containing lymphocytes can be found in the peripheral blood throughout the life of every human following primary infection with EBV. From peripheral blood of persons with prior EBV infection spontaneously growing lymphoblastoid cell lines of the B type, which contain EBV genomes, can be established (Nilsson et al. 1971; Pope 1967; Rickinson et al. 1980). The majority of these permanently growing cell lines do not produce

EBV or viral antigens other than EBNA. Superinfection with EBV, however, leads to the synthesis of viral proteins (Bayliss and Wolf, to be published) and, dependent on the multiplicity of infection, to the synthesis of progeny virus. When such viable lymphoblastoid cells are immobilized on anti-lymphocyte globulin (ALG) coated surfaces to yield a dense monolayer (Bayliss and Wolf 1979), they allow the observation of the effects of virus infection in specific cells. As early as 2 h post infection with EBV the formation of polycaryocytes could be observed (Fig. 4a).

This fusion could be blocked by UV irradiation of the virus, by treatment with neutralizing antibodies, or by the addition of sodium azide or cycloheximide to the cultures. Amino acid
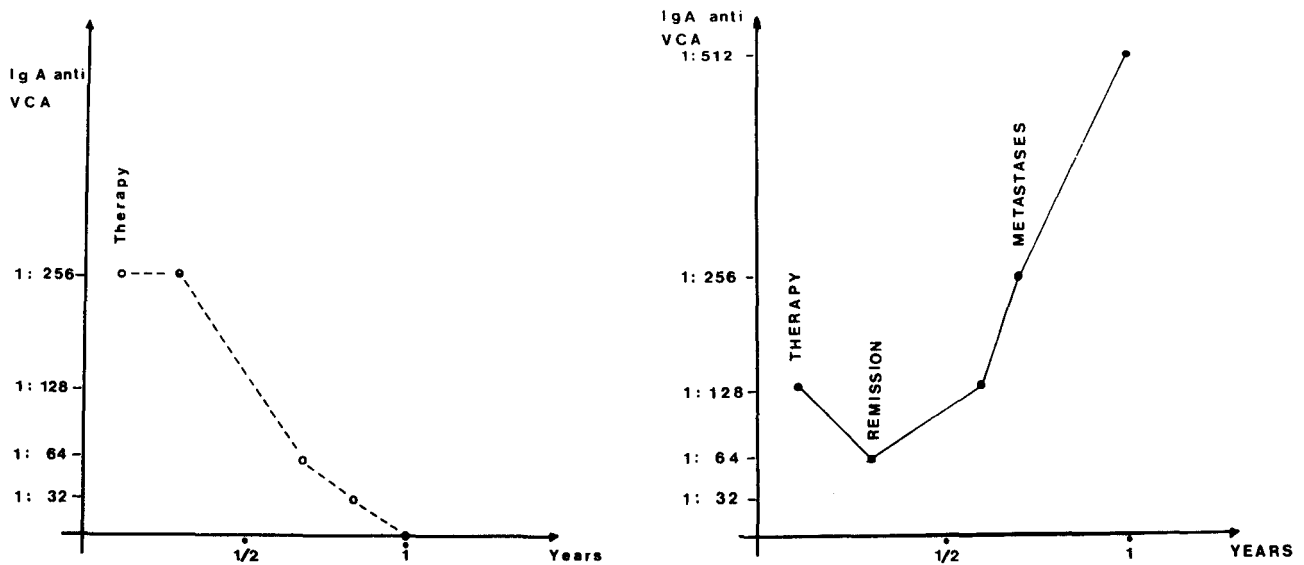
Fig. 3. IgA anti-VCA (virus capsid antigen) titers of two patients determined during an observation of 1 year
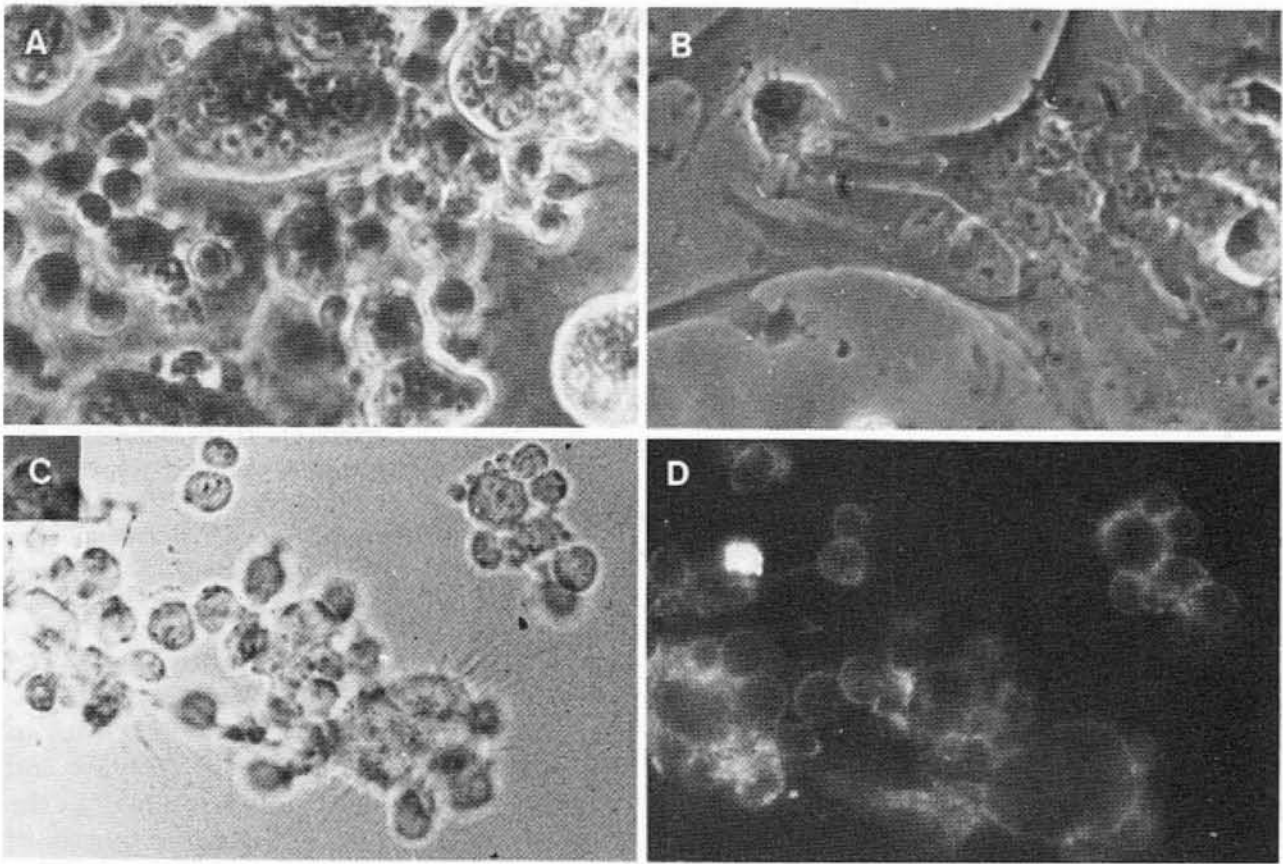
Fig. 4. A Phase contrast picture of a polycaryocyte from Raji cells which were immobilized and superinfected with EBV (8 h. P.I.) **B** Polycaryocyte (seven nuclei) between superinfected Raji cells and a human fibroblast. **C** and **D** polycaryocyte formed in a mixture of 10% superinfected Raji cells and 90% T-cells immooilized on plates. **D** shows areas of clear membrane fluorescence after staining with an anti-T-cell serum (a gift from H Rodt)

analogues (L-canavanine, L-azetidine) or inhibitors of DNA replication (hydroxyurea, phophonoacetic acid) had no such inhibitory effect. The latter drugs have been shown to permit the synthesis of at least a subset of virus-induced proteins in EBV-superinfected Raji cells. The fusion-inducing viral proteins in lymphoblastoid cells can also be produced by the resident EBV genomes of Raji cells, as treatment of these cells with 50 µg/ml of iododesoxyuridine or 20 ng/ml 12-0-tetradecanylphorbol-13-acetate (TPA) leads to the partial activation of the resident EBV genes (zur Hausen et al. 1979). EA can be detected within these cells and they have the ability to fuse to recipient cells. It is not mandatory for the recipient cells to carry EBV receptors on their surface. When superinfected Raji cells are mixed with human fibroblasts or lymphoblastoid cells of the T type (Jurkat, devoid of EBV genomes or EBV receptors) they form polycaryocytes with these cells (Fig. 4b,c). The participation of these T cells in the polycaryocyte can be clearly demonstrated by membrane fluorescence with specific anti-T-cell sera (Fig. 4d) (Bayliss and Wolf 1980). When monolayers of Raji cells on ALG-coated surfaces are subconfluent, spontaneous synthesis of EA can be observed in up to 5% of the cells (Bayliss and Wolf 1979). When treated with ALG in suspension, no EA synthesis is observed in Raji cells. Anti-IgM sera on the other hand have been shown (Tovey et al. 1978) to have the potential to induce EA in Raji cells in suspension. The differential effect of ALG in suspension as compared to the effect when ALG is fixed on surfaces upon EA induction in Raji cells may be due to the inhibitory effect the ALG serum had on cell growth. ALG at concentrations of 1.6 to 16 µg/ml even depressed the induction of EA in Raji cells by IUdR. It has been postulated earlier (Hampar et al. 1976) that active cell growth is required for activation of EBV. From these observations one might conclude that the presence of autoantibodies in patients with EBV-carrying lymphocytes could induce the synthesis of EBV-spcified antigens which would, upon cell death, stimulate the production of antibodies.

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C. Discussion

During infectious mononucleosis and the following lifelong carrier state EBV seems to be restricted to B-lymphocytes and the carrier-producer cells in the parotic gland. In case of the nasopharyngeal carcinoma, however, it has been shown that EBV genomes reside in the epithelioid tumor cells (Wolf et al. 1973; Desgranges et al. 1975; Klein et al. 1974; Wolf et al. 1975). This finding prompted the question how these EBV genomes came into the tumor cells. Epithelial cells are believed to be devoid of receptors for EBV. All attempts to infect other cells than B-lymphocytes with EBV in vitro failed. The negative results from binding studies using labeled EBV and the failure of induce viral antigens in primary cultures of nasopharyngeal tissue (Wolf, unplublished work) do not support the presence of EBV receptors in these cells. This conflict led to the suggestion that other agents like paramyxoviruses may induce fusion of EBV genome containing lymphocytes and epithelial cells (Gazzolo et al. 1972). Under the condition of close cell to cell contact (Döhnert 1977) we were able to demonstrate in vitro that EBV is able to induce cell fusion. One might speculate that similar events take place in vivo. The exceptional close contact of lymphocytes and epithelial cells found in the Waldeyer's ring of the throat may provide the necessary conditions for cell fusion. The activation of EBV genes in EBV genome carrying lymphocytes may be induced by a variety of influences, including drugs like IUdR or tumor promoters like TPA. If fusion occurs in nature one should be able to observe cells with more than the normal set of chromosomes. This is, however, not necessarily so because induction of the lytic cycle of EBV replication leads to the pulverization of the cellular genome (Seigneurin et al. 1977). Thus only fusion soon after induction of a lymphocyte would lead to the transfer of both viral and cellular DNA, whereas fusion late after induction may not lead to the transfer of cellular chromosomes but only to the transfer of possibly protected circular EB viral DNA.

Finerty et al. (1978) studied the karyotypes of cells from nasopharyngeal carcinomas and found that indeed three out of five had near triploid and two near diploid karyotypes. The postulated interaction of several factors may explain the location of the tumor in the body as well as the increased risk for the development of this tumor in certain areas of the world, especially in Cantonese China. The geographic distribution might be due to the spread of certain substances activating EBV with high efficiency in carrier lymphocytes. Alternatively, endemic virus strains may vary in their fusion inducing ability. Some observations may point in that direction but need further confirmation. The age of first exposure may play an important additional role as early transfer of EBV genomes to epithelial cells could be esential for the evolution of proliferating malignant cells.

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