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PRESENCE OF EPSTEIN-BARR VIRUS DNA IN TONSILLAR TISSUES

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Summary. — Sera from 48 tonsillar carcinoma (TC) patients, 48 matched controls and 16 recurrent exudative tonsillitis (RET) patients were examined for the presence of Epstein-Barr virus (EBV) associated nuclear antigen (EBNA), early antigen (EA) and virus capsid antigen (VCA). Higher prevalence and significantly higher antibody titres against all three EBV-associated antigens were observed in TC patients in comparison with controls and RET patients. Patients suffering from anaplastic TC had higher titres of antibodies against VCA and EA than TC patients with other histological diagnoses. Five out of 11 TC biopsies obtained from 9 patients were positive for EBV DNA at levels of 0.17, 4 to 5, 15 to 18 and in two cases 3 EBV genome equivalents per cellular genome. Among 16 RET patients, 4 were found positive at levels not exceeding 2.17 EBV genome equivalents per cellular genome. Higher titres of antibody against all EBV antigens were found in TC and RET patients with EBV DNA-positive tonsillar tissue than in those with EBV DNA-negative tonsillar tissue.

Key words: Epstein-Barr virus; Herpesviridae; serology; viral DNA; tonsillar carcinoma; recurrent exudative tonsillitis

Introduction

It has been established that Epstein-Barr virus (EBV) is the causative agent of infectious mononucleosis (Evans *et al.*, 1968; Henle *et al.*, 1968; Henle and Henle, 1973) and a considerable body of evidence has accumulated that it also is involved in the aetiology of two human neoplastic diseases — African Burkitt lymphoma (Henle *et al.*, 1969, 1971; zur Hausen *et al.*, 1970; Nonoyama *et al.*, 1973; Klein, 1975) and nasopharyngeal carcinoma (De Schryver *et al.*, 1969; Henle *et al.*, 1970; Wolf *et al.*, 1973; Andersson-Anvret *et al.*, 1977). Recently we showed that patients suffering from palatine tonsillar carcinoma (TC) had increased titres of antibodies against EBV antigens (Vonka *et al.*, 1977). Since EBV can persist in a letent state in peripheral blood lymphocytes of persons without clinical manifestation

of any EBV-related disease (Henle and Henle, 1970; Gerber *et al.*, 1972), the question arose as to whether EBV genetic information is present in the lymphoid organs, e.g. the palatine tonsils. Indeed, the presence of EBV-associated nuclear antigen (EBNA) in tonsillar B lymphocytes of patients with recurrent exudative tonsillitis (RET) was demonstrated by Veltri *et al.* (1976, 1977) and EBNA-positive cells were detected in a lymph node of a child with severe primary EVB infection (Virelizier *et al.*, 1978). However, Morgan *et al.* (1979), whose findings suggest that salivary glands are the natural sites of EBV replication, failed to demonstrate EBV VCA in tonsillar lymphocytes.

The aim of the present experiments was to obtain more data on the association of EBV with tonsillar tissues. We are reporting serological data on TC patients and the results of reassociation studies for the presence of EBV DNA in biopsy materials from tonsillar tumours. We considered it relevant to complement these investigations with similar tests on sera and tissues from patients tonsillectomized because of RET.

Materials and Methods

Patients. A total of 48 patients with carcinoma of palatine tonsil were included in the study. Their age range was from 19 to 81 years with a majority from 50 to 70 years. Thirty-six patients were taken in the study at the time of their first clinical examination or in the course of treatment, while 12 had been in remission for more than 1 year. The most frequent histological diagnosis was epidermoid cell carcinoma without (17 patients) or with keratinization (13 patients). Anaplastic carcinoma was diagnosed in 9 patients and transistory cell carcinoma in 2. The histological finding was not specified in 7 patients. Biopsy specimens for molecular hybridization tests were obtained from 9 patients. Tonsillar tissues were also obtained from 16 patients in whom tonsillectomy had been performed because of RET. These patients were from 8 to 30 years old (mean 13 years). All TC and RET patients were treated at the Department of Otorhinolarynhology, Bulovka Hospital, Prague.

Cell lines. Human lymphoblastoid cell lines were passed as described by Vonka *et al.* (1972). The Raji and P3HR-1 cells were the same as in experiments of Simonová *et al.* (1977). The EBV-genome-negative Ramos and BJA cells had been kindly provided by Dr. H. zur Hausen. Human diploid lung (LEP) cells were the same as in earlier studies (Břicháček *et al.*, 1980).

Sera and serological tests. Sera were obtained from the 48 TC patients and from 48 controls matched by age and sex. These persons either were in normal health or were suffering from various non-malignant diseases. In addition, sera from 16 tonsillectomized RET patients (see above) were obtained. Sera were examined for the presence of antibodies to EBV-associated nuclear antigen (EBNA) and capsid antigen (VCA) as described by Vonka *et al.* (1977). In the present experiments antibodies against EBV early antigen (EA) were not tested in P3HR-1 virus-infected Raji cells as previously (Vonka *et al.*, 1977) but in Raji cells induced by 3 mM sodium n-butyrate and 0.05% swine anti-human IgM antibody. Antibodies to herpes simplex virus (HSV), cytomegalovirus (CMV) and varicella-zoster virus (VZV) were tested by the complement-fixation (CF) test (Holý *et al.*, 1973). In calculations of geometric mean titres (GMT) sera negative at a 1 : 10 dilution were considered positive at a 1 : 5 dilution.

Biopsy materials. Immediately after removal, each tissue was placed in a rubber-stoppered bottle containing cultivation medium and transported to the laboratory. A portion of each sample, to be used for hybridization tests (see below), was placed at −70 °C.

Extraction and purification of cellular and biopsy-material DNA. DNA from lymphoblastoid cells was extracted and purified as described by Frenkel *et al.* (1976). Tumours and tonsils were minced, homogenized and DNA was extracted and purified in the same way as from the cell lines.

Preparation of labelled EBV DNA. In the experiments, carried out over a period of nearly three years, we used different methods of EBV DNA labelling. This gradually increased the sensitivity

Table 1. EBV antibodies in TC patients and their matched controls and in RET patients

Group	VCA			EA			EBNA		
	No. tested	No. (%) positive	GMT	No. tested	No. (%) positive	GMT	No. tested	No. (%) positive	GMT
TC patients	48	48 (100)	124.1	48	21 (44)	11.4	47	39 (83)	25.6
Matched controls	48	46 (96)	58.7	48	0 (0)	—	43	19 (44)	11.6
RET patients	16	16 (100)	67.5	16	2 (12.5)	6.1	16	8 (50)	12.8

of the hybridization tests for detecting EBV DNA sequences. Originally EBV DNA was prepared from Raji cells superinfected with P3HR-1 EBV (Tanaka *et al.*, 1976) in the presence of 2.22 MBq of ^{3}H-thymidine per ml and purified by two cycles of equilibrium CsCl-gradient centrifugation. The specific activity of this ^{3}H-EBV DNA was approximately 7×10^5 count/min per μg. In later experiments purified viral DNA was labelled with (methyl-^{3}H) thymidine 5'-triphosphate (Radiochemical Centre Amersham, spec. act. 1.74 TBq/mmol) by the nick translation reaction (Rigby *et al.*, 1977). With this procedure, the specific activity of ^{3}H-labelled probe EBV DNA was 8×10^6 count/min per μg. Lastly, EBV DNA was isolated from tumour promoting agent- (TPA)-induced P3HR-1 EBV as described by zur Hausen *et al.* (1978) and labelled by the nick translation reaction with guanosine (5'-^{32}P) triphosphate (Radiochemical Centre Amersham, spec. act. 15.17 TBq/mmol) to a specific activity of 5×10^7 count/min per μg.

Reassociation kinetics of cellular and viral DNAs. The solution in which hybridization experiments were performed contained labelled viral probe DNA and non-labelled test DNA in 0.1 M Tris-HCl pH 8.1, 0.025 M EDTA, 1 M NaCl and 20 % formamide (Frenkel *et al.*, 1976). Portions of 100 μl of this solution were filled into glass capillaries. The mixtures were denatured at 115 °C for 7 min and then incubated at 60 °C for the periods of time indicated below. Fractions of double-stranded probe ^{3}H-EBV DNA were determined by digestion with S1 nuclease (Sigma Chem. Co.) as described by Frenkel *et al.* (1976).

EBV DNA reassociation-data processing. The EBV DNA content in the specimens tested was calculated from the difference between the slopes of reassociation curves for EBV probe DNA in the presence of test DNA and control DNAs. Ramos, BJA or LEP-cell DNAs served as negative controls and Raji cell DNA as a positive one. The calculation was based on the assumption that 50 EBV genomes were present per 1 Raji cell (Nonoyama and Pagano, 1971; zur Hausen *et al.*, 1972; Pritchett *et al.*, 1976). A novel approach was adopted to overcome the main problem of reassociation data evaluation, which is rooted in estimating the sentitivity of the test. This task is tackled in most works on nucleic acid hybridization in a reconstruction experiment. The sensitivity of the test is usually represented by the lowest amount of probe nucleic acid still having a higher reassociation-curve slope than negative control DNA, without explicit respect to the standard deviation of the slope. To involve both the slope and this standard deviation, we used the statistical test of difference between two regression coefficients (Fisher, 1948, pp. 96—97).

The slopes of reassociation curves were calculated by linear regression analysis. The significance of the difference between two reassociation curves was evaluated by testing the difference between the two regression coefficients by means of Student's t-test (Fisher, 1948). A confidence level (α)

Table 2. CF antibodies to HSV, CMV and VZV in TC patients and matched controls

Group	HSV			CMV		VZV	
	No. tested	No. (%)	GMT	No. (%)	GMT	No. (%)	GMT
Patients	20	9 (45)	8.4	15 (75)	12.3	0 (0)	—
Controls	20	12 (60)	12.3	8 (40)	9	5 (25)	7.3

Table 3. EBV antibodies in patients with different histological types of tonsillar carcinoma

Histological diagnosis	No. of patients	EBV antibodies					
		VCA		EA		EBNA	
		No. (%)	GMT	No. (%)	GMT	No. (%)	GMT
Anaplastic carcinoma	9	9 (100)	215.3	8 (88.9)	34.2	7 (77.8)	14.7
Other histological types	39	39 (100)	109.2	13 (33.3)	8.8	32 (84.2)*	29.2

* One serum was not tested for EBNA antibody.

for this difference (two-sided test) was determined for the given degrees of freedom from the critical value of Student's t_α. The value of t_α was calculated according to the formula

$$t_\alpha = \frac{|b_1 - b_2|}{\sqrt{S_{b_1}^2 + S_{b_2}^2}} \quad (1)$$

where b_1 and b_2 are the slopes and S_{b1} and S_{b2} are the standard deviations of the slopes of the respective two curves.

The test of difference between two regression coefficients was also used to estimate the sensitivity of the method. A slope b_1 of a hypothetical curve that differed from the slope b_2 of the negative control at the selected confidence level $\alpha = 0.05$ (one-sided test) was considered as significantly positive; the standard deviation (S_{b1}) of the slope of this hypothetical positive curve was taken as aequal to the standard deviation S_{b2} of the calculated negative control slope. Slope b_1 was calculated according to the following formula derived from equation (1):

$$b_1 = b_2 + t_{0.05} \sqrt{2}\, S_{b2} \quad (2)$$

The number of genomes or the fraction of a genome corresponding to slope b_1 represents the sensitivity of the test.

Results

Serological findings

Sera from 48 TC patients, 48 matched controls and 16 RET patients were examined for the presence of EBV antibodies. (Table 1). All TC patients were VCA-positive, while two of the control subjects lacked this antibody. Antibodies to either non-structural antigen were likewise detected more frequently in TC patients than in controls. The levels of antibodies were significantly higher in patients than in controls ($p < 0.001$ for VCA and EA antibodies; $p < 0.01$ for EBNA antibody). Antibody titres in the RET patients, on the other hand, did not differ markedly from those in the controls to TC patients.

Table 2 shows the prevalence and titres of CF antibodies to three other human herpesviruses in sera from 20 TC patients and the same number of matched controls. At a 1 : 10 dilution HSV and VZV antibodies were detected somewhat more frequently in the controls and CMV antibody in patients. At variance with the results for EBV antibodies, none of these differences was significant ($p > 0.05$).

Table 4. Results of hybridization tests on biopsies from TC patients

Exp. series	Probe DNA spec. act.[1])	Sensitivity of test[2])	Patient No.	Sex	Age (years)	Material code	Histol. diagnosis[3])	No. of EBV genomes per cell
1	7×10^5	2	1	F	64	TC 9	E	3
			2	M	25	TC 11	A	4—5
			3	F	57	TC 13	E	15—18
			4[4])	F	53	TC 14	E	neg.
2	8×10^6	1.45				TC 15	E	neg.
			5	M	81	TC 16	E	neg.
			6[5])	M	77	TC 17_p	E	neg.
						TC 17_m	E	neg.
3	5×10^7	0.1	7	M	68	TC 18	E	neg.
			8	M	65	TC 21	E	0.17
			9	F	78	TC 23	E	3

[1]) Count/min per μg.
[2]) EBV genome equivalents per cell genome.
[3]) E = epidermoid carcinoma without keratinization; A — anaplastic carcinoma.
[4]) In this patient, 2 biopsies (TC 14, 15) were available; the second was obtained one year after the first surgery.
[5]) In this patient, 2 biopsies were available; TC 17_p originated from primary tumour, TC 17_m from a metastase localized in regional lymph node.

In a further analysis, TC patients were divided according to their histological type of tumour and the serological findings were compared. Anaplastic carcinoma patients were found to differ markedly from the other groups (Table 3). Their VCA and especially EA antibody levels were significantly higher than in other patients ($p < 0.01$ and < 0.001, respectively). Paradoxically, the EBNA antibody titres were lower in the anaplastic carcinoma patients than in those with other histological types of the disease. But this difference was not significant ($p > 0.05$).

No marked differences were determined between patients with active disease and those in remission.

Reassociation kinetics in TC tumour biopsies

Eleven biopsy specimens originating from 9 TC patients were examined for the presence of EBV DNA (Table 4). Only one patient was suffering from anaplastic carcinoma; the histological diagnosis in all other patients was epidermoid carcinoma without keratinization. From two patients two specimens each were examined. Since the materials were tested in three different experiments, the sensitivities of which differed considerably, the type of probe DNA and the sensitivity of the assay technique are indicated for each experiment. EBV DNA was detected in five specimens. According to the calculations made, TC9 and TC23 contained approximately 3 EBV genome equivalents, TC14 4 to 5 genome equivalents, TC13 15 to 18 genome equivalents and TC21 0.17 genome equivalent per cell. To illustrate

the method used in these experiments, the reassociation curves of ^{3}H-EBV DNA in the presence of Ramos, Raji and TC13 cell DNA are shown in Fig. 1.

Reassociation kinetics with tonsillar tissues from RET patients

EBV DNA contents were examined in tonsils from 16 RET patients with the same DNA probe as used in Exp. 2 shown in Table 4 (the sensitivity of test was also the same). EBV DNA was detected in 4 out of the 16 materials.

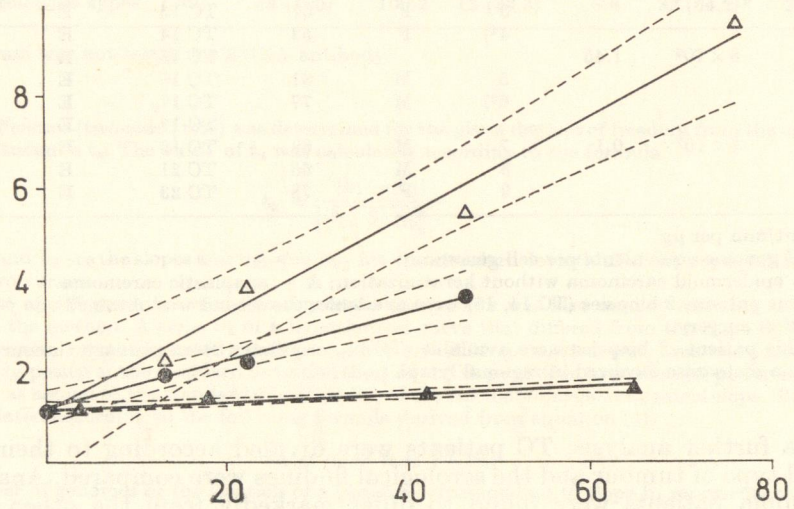

Fig. 1.

Reassociation kinetics of ^{3}H-EBV DNA in the presence of 5 mg of Ramos cell DNA (▲), mixture of Ramos and Raji cell DNA (Raji cell DNA was diluted to obtain a final concentration of EBV DNA corresponding to 10 EBV genome equivalents per cell genome) (●), and TC13 biopsy specimen DNA (△) per ml

Full lines represent respective reassociation curves. Areas between dashed lines represent confidence belts at the confidence level $\alpha = 0.1$. Numerical data were processed by linear regression analysis using a Hewlett-Packard 9810A calculator and graphs were plotted by a Hewlett-Packard 9862A plotter. Values of slopes of reassociation curves of Ramos, Ramos/Raji mixture and TC13 DNA were 0.0073 ± 0.0032, 0.0522 ± 0.0158 and 0.1076 ± 0.0294 hr^{-1}, respectively. Abscissa: reassociation time (in hr); ordinate: reciprocal value of fraction of single-stranded DNA ($1/f_{ss}$).

The maximum EBV DNA content in these tissues was 2.17 EBV genome equivalents per cell genome (the values for the 3 remaining materials were 1.46, 1.47 and 1.97 respectively).

Relationship between presence of EBV DNA and EBV antibodies

The results of hybridization tests were compared with the serological findings in the respective patients (Fig. 2). Although the data were too few to be amenable to statistical analysis, it is apparent that both TC and RET patients

in whose tissues EBV DNA had been detected tended to possess the various EBV antibodies more frequently and in higher titres than those in whom the hybridization tests yielded negative results.

Discussion

The primary aim of the present study was to obtain more data on the relationship between EBV and TC. Since it had recently been demonstrated that EBNA-positive B cells are present in tonsillectomized tissue of RET patients (Veltri *et al.*, 1976, 1977), we also wanted to find out whether detectable amounts of EBV DNA are present in such tissues. The importance of these investigations for the interpretation of hybridization test results in TC patients is obvious.

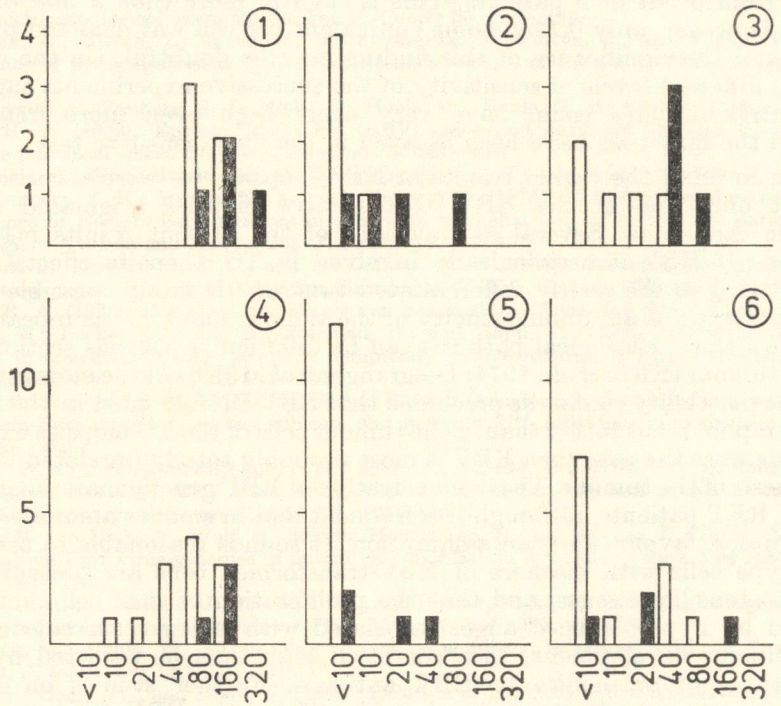

Fig. 2.

Antibody titres against EBV antigens in TC and RET patients. TC patients: 1 — VCA, 2 — EA, 3 — EBNA antibodies. RET patients: 4 — VCA, 5 — EA, 6 — EBNA antibodies
Empty and black columns: patients with EBV DNA negative and positive tonsillar tissues, respectively
Abscissae: antibody titres; ordinates: No. of patients

Our serological findings corroborated and extended our previous findings of a higher prevalence and higher titres of antibodies to the various EBV antigens in TC patients than in matched controls (Vonka *et al.*, 1977). Although the differences between the patients and control subjects are comparable in both of our studies, in the present study patients and control subjects alike displayed EA antibody less frequently and in lower titres than in the previous one. This was probably associated with the induction of EA by butyrate and anti-IgM antibody instead of the use of P3HR-1 virus superinfection as in the previous tests. It is possible that the spectrum of antigens formed was broader in superinfected than in induced cells. It is noteworthy that the highest VCA and EA antibody titres among the TC patients were seen in the anaplastic carcinoma subgroup. This is reminiscent of earlier findings in nasopharyngeal carcinoma (NPC) patients (Henle *et al.*, 1970; Andersson-Anvret *et al.*, 1977; Huang *et al.*, 1978).

Molecular hybridization tests revealed the presence of EBV DNA in tumours from 5 out of 9 patients. This is slightly more than a half of the patients. However, only 0.17 genome equivalent per cell was detected in one tumour, and the significance of this finding may be doubtful. On the other hand, the different levels of sensitivity of the successive experiments suggest that positive findings could have very likely been even more frequent should all the materials have been assayed in the most sensitive test.

Having in mind the earlier results in NPC biopsies, we were surprised to find that only one of the EBV DNA-positive biopsies originated from anaplastic carcinoma. Several explanations of the present results may be considered. If EBV is aetiologically involved in TC, then its effects need not be limited to the poorly differentiated tumours. It is also possible that the findings express an inhomogeneity of the tumour mass; it has repeatedly been shown that histological patterns can be different in various sections of the same tumour (Klein *et al.*, 1974; Desgranges *et al.*, 1975). At the moment, however, the possibility cannot be precluded that EBV DNA resided in the infiltrating lymphoid cells rather than in the tumour cells of the TC biopsies examined. If this were the case then EBV is most probably totally unrelated to the pathogenesis of the tumour. The demonstration of EBV genetic material in the tonsils of RET patients, although less frequent and in smaller amounts than in TC biopsies, favours such an assumption. It sounds reasonable to assume that B type cells with markers of EBV-transformed cells are present frequently in tonsillar tissues, and that the proliferation of such cell clones is supported by hyperplastic changes associated with either recurrent tonsillitis or tumour development. This condition may then be reflected by increased titres of antibodies to EBV antigens. Further studies on EBV DNA-positive tumour biopsies aimed at identifying the EBV DNA-positive cells by cytological in situ hybridization and/or by demonstrating EBNA presence are needed to clarify this point. The rarity of tonsillar carcinomas and the limited amounts of materials available, in addition to technical difficulties, are the main obstacles in these efforts.

Both TC and RET patients with demonstrable EBV DNA in their tonsillar tissues tended to possess higher antibody titres to EBV-associated antigens than those in whose tissues EBV genetic material was not demonstrated. This correlation suggests that the presence of demonstrable amounts of EBV DNA is an indicator of a more active EBV infection.

To summarize, the present data seem to indicate, in line with some previous findings, a rather close association of EBV with tonsillar tissues. This has been revealed by increased levels of EBV antibodies in TC patients and some RET patients and by demonstration of EBV DNA in about one half of TC biopsies and about 25 per cent of RET patients. It remains to be determined in which type of cell the EBV genome resides in TC biopsies.

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