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COMPARATIVE DETECTION OF HERPESVIRUSES IN TISSUE SPECIMENS BY IN SITU HYBRIDIZATION AND IMMUNOFLUORESCENCE

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Summary. — The conditions of in situ hybridization for demonstration of herpesvirus genomes in animal and human tissues were tested using the ORWO(R) K6 emulsion. It was possible to localize herpes simplex virus (HSV) genomes in infected mice organs (brain and liver) as well as Epstein-Barr virus (EBV) genomes in tonsils of patients with infectious mononucleosis and in tumour specimens of patients with nasopharyngeal carcinomas. Immunofluorescence (IF) revealed mostly corresponding results. The in situ hybridization is more favourable due to its higher specifity, but it is more time consuming and expensive. IF seems advantageous for screening of a great number of tissues.

Key words: in situ hybridization; immunofluorescence; herpes simlex virus; Epstein-Barr virus

Introduction

Due to their cell-transforming properties, viruses are discussed as the cause and/or cofactor of tumours in man. From the members of the herpesvirus group EBV is mainly connected with Burkitt's lymphoma and nasopharyngeal carcinoma (de-Thé, 1980). Possibly, also HSV and cytomegalovirus (CMV) are related with certain malignant diseases in man (Fenoglio and Lefkowitsch, 1983).

An essential hint to such virus-tumour relations is given by the detection of viral genetic material in tumour cells with unchanged cytomorphology. For this purpose, the in situ hybridization represents a preferable technique. In the present study we aimed at to demonstrate herpesvirus genomes by a modified in situ hybridization techniques in comparison with the detection of virus-induced antigens by immunofluorescence (IF). The investigations were made in brain and liver tissue specimens of HSV-infected mice, in tonsils of patients with acute infectious mononucleosis, and in tissue specimens of patients with undifferentiated nasopharyngeal carcinoma.

Materials and Methods

Tissue specimens. Four weeks aged F_1 hybrid mice strain ABD2 (Zentralinstitut für Mikrobiologie und Experimentelle Therapie, Jena, G.D.R.) were infected intracerebrally (i.c.) with 10^4 $TCID_{50}$ of virus suspension strain HSV-1 Kupka. Within the following 3—7 days, the animals showed typical symptoms of encephalitis. Immediately after death, the brains were removed under sterile conditions. In addition, mice of the inbred strain ICR/Schö (VEB Versuchstierproduktion Schönewalde, G.D.R.) at the age of 2—3 weeks were infected into the tail vein with HSV-1 strain 511 to induce hepatitis. Animals were killed 4 days after infection and their livers removed.

For demonstration of EBV genomes and nuclear antigens (EBNA) tissue specimens from 10 tonsils of patients with acute infectious mononucleosis, and as well 10 tissue specimens of patients with histologically prooved undifferentiated nasopharyngeal carcinoma were used. In the case of infectious mononucleosis, tonsillectomy was carried out for therapeutic indication within the first week after onset of the disease (Sauerbrei *et al.*, 1983). When nasopharyngeal carcinoma was diagnosed, the primary tumour or the lymph node metastases were examined. In all patients the IgM and IgA antibodies were determined against the capsid and early antigens of EBV. The results confirmed the diagnosis with no exception.

In situ hybridization. In situ hybridization was performed as a modification of methods described by Wolf *et al.* (1973, 1981) and Brahic and Haase (1978). The pretreatment of glass slides, which the tissue sections were placed on, was essential and was carried out as follows: For 3 hr the cleaned slides were incubated at 65 °C in 3 × SSC (Denhardt, 1966) pH 7.0 containing 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin (wt/vol), briefly rinsed in distilled water, and then fixed at room temperature in ethanol/acetic acid (3 : 1) for 20 min. On slides which had been treated in this manner, frozen 7.5 µm sections of the tissue specimens were placed, air dried, and fixed in methanol/acetic acid (ratio 3 : 1) at room temperature for 20 min. The slides were stored at —70 °C until use.

Before hybridization, the sections were treated as follows: 20 min at room temperature in 0.2 mol/l HCl, 30 min at 70 °C in 2 × SSC, 15 min at 37 °C in 20 mmol/l Tris-HCl pH 7.4, 2 mmol/l $CaCl_2$ containing 2 µg of proteinase K per ml, followed by dehydration in graded ethanol. The hybridization of slides was performed with $5—6 \times 10^4$ c.p.m. of ^{3}H-labelled HSV-1 DNA or cloned EBV DNA (Polack *et al.*, 1984) per tissue section. Both DNAs were labelled by nick-translation (Rigby *et al.*, 1977) with ^{3}H-thymidine triphosphate (4 TBq/mmol/l; Amersham) to specific activities of about 5×10^6 c.p.m./µg. The hybridization medium was a mixture of 50% formamide, 10 mmol/l Tris-HCl pH 7.5, 1 mmol/l EDTA, 600 mmol/l NaCl, 100 µg of poly (A) per ml, 0.02% Ficoll, 0,02% polyvinylpyrrolidone (wt/vol), 1 mg of bovine serum albumin per ml, and 1 mg denatured calf thymus DNA per ml. After adding the labelled virus DNA, the hybridization mixture was heated to 100 °C in water-bath for 10 min, quickly cooled to 0 °C, and placed in 10 µl aliquots on tissue sections. The slides were covered with siliconized 10-mm glass coverslips, sealed with an elastic gum, and hybridized at 45 °C in a moist chamber for 48 hr.

After removing the coverslips, the slides were washed extensively. For washings chloroform, SSC, and formamide buffer containing 50% formamide, 10 mmol/l Tris-HCl pH 7.4, 1 mmol/l EDTA and 600 mmol/l NaCl were used. Thereafter the slides were treated with 2 × SSC at 55 °C for 1 hr (Harrison *et al.*, 1973). Finally, the preparations were dehydrated in graded ethanol containing 300 mmol/l ammonium acetate.

After air drying, the slides were dipped twice into the photographic emulsion ORWO$^{(R)}$ K 6 (VEB Filmfabrik Wolfen, G.D.R.) at 40 °C and dried at room temperature for 18 hr. The exposition was performed at 4 °C for 3 weeks in a light-proof box containing silica gel desiccant. The slides were developed for 3—4 min in ORWO$^{(R)}$ M-H 28 developer (VEB Filmfabrik Wolfen, G.D.R.) which had been diluted 1 : 4 with distilled water, followed by washing in 0.05% acetic acid for 1 min, and fixation with fixer A 300 (VEB CKB Bitterfeld, G.D.R.) for 3—4 min. The slides being washed in distilled water were stained with Giemsa for 10 min, and evaluated in the light microscope. Tissue sections of non-infected mice and HSV-infected human thyroid cells or Ramos and P3HR-1 lymphoblastoid cells served as controls for each hybridization experiment.

The evaluation was performed by quantitative analysis of silver grains as granules-like blackenings in non-infected control preparations and by calculating the mean value and mean error

of 100 areas with a size of 100 μm^2. On the basis of this labelling degree which, as a rule, amounted to between 3 and 6 silver grains per 100 μm^2, such cell areas with 18—20 silver grains were regarded as virus genome-positive.

Immunofluorescence. Frozen sections of tissues fixed in acetone at —20 °C for 1 hr were incubated with anti-HSV-positive serum from rabbit for 18 hr (Wutzler *et al.*, 1981). After washing with phosphate-buffered saline pH 7.2, slides were treated with fluorescein isothiocyanate (FITC)--labelled anti-rabbit globulin (Staatliches Institut für Immunopräparate und Nährmedien, Berlin, GDR) for 1 hr. The slides were washed again and air dried. To avoid unspecific reactions tissues from non-infected mice and HSV-infected human thyroid cells served as controls.

Tonsils and tumour tissues were tested for EBNA by anticomplement IF (ACIF) as described by Reedman and Klein (1973) with slight modifications. Briefly, contact preparations of tissues were fixed in methanol/acetone ratio 1 : 1 at —20 °C for 10 min and treated with anti-EBNA--positive and negative human sera for 1 hr. After washing, an anti-EBNA-negative human serum was added as a source of complement. The slides were washed again and incubated with goat anti-human beta-1C/beta-1A-globulin, labelled with FITC (Staatliches Institut für Immunpräparate und Nährmedien, Berlin, DDR/G.D.R) for 1 hr. EBV genome-positive lymphoblastoid cells NC 37 served as controls. The slides were checked for HSV antigen-positive and EBNA--positive cells under the fluorescence microscope FLUOVAL (VEB Carl Zeiss Jena, DDR/G.D.R).

Results

Herpes simplex virus

No doubt, the HSV genome-positive cells showed significantly enhanced number ($\geqq 18$) of silver grains per 100 μm^2 in brain and liver tissues of infected mice (Fig. 1-A, B). On average, the degree of labelling over non--infected cells amounted to 3.4 grains per 100 μm^2 in thyroid cells and brain tissue and 3.8 grains in liver tissue, respectively. Comparative examinations by IF revealed conformable findings. Typical inclusions were observed in the antigen-positive cells (Fig. 1-C) .The corresponding organs of non-infected animals were negative.

Epstein-Barr virus

In 9 of 10 tonsils from patients with infectious mononucleosis EBV genome-positive cells with significantly increased number ($\geqq 20$) of silver grains per 100 μm^2 were found by in situ hybridization (Fig. 2-A), but as well EBNA-positive cells by ACIF (Fig. 2-B). In the control preparations with Ramos cells, the labelling degree amounted to 5.2 grains per 100 μm^2 on average. Using in situ hybridization, the proportion of positive cells in the tonsils ranged between < 1 and 50%, and in ACIF between < 1 and 20%, respectively. In 3 tonsils, the same percentages of positive cells were found by both methods, while only in one case, more EBNA-positive cells could be demonstrated. One tonsil was negative in both examinations.

EBV genomes could be demonstrated in all 10 examined tissue specimens from patients with histologically prooved undifferentiated nasopharyngeal carcinomas by in situ hybridization. The percentage of genome-positive cells ranged between 10 and 50% (Fig. 2-C). Compared to it, EBNA-positive cells (Fig. 2-D) were demonstrated in 9 of the 10 tissue specimens, their portion ranging between < 1 and 50%. In three tissue specimens, more positive cells were obtained by in situ hybridization. In three other tissue specimens

the EBNA demonstration by ACIF revealed more positive cells, and in three tumour tissues a conformity of the results of both methods occurred. It was found by microscopic examination that the EBV genome-positive cells (Fig. 2-C) with vesicular nucleus and prominent nucleolus are no lymphocytes, but — very probably — epithelial tumour cells (Shanmugaratnam *et al.*, 1979).

Discussion

The main principle of the in situ hybridization is that single strands of viral nucleic acids form stable duplexes with radioactively labelled complementary single-stranded nucleic acids. By means of autoradiographic examination techniques, these duplexes are made visible as granules-like blackenings. In comparison with other hybridization methods, — e.g. the reassociation kinetics, filter hybridization or blot hybridization (review: Bornkamm *et al.*, 1983) —, it is of great advantage to localize the viral nucleic acid sequences in single cells which can be examined cytologically. The following conditions are essential to the in situ hybridization: the proper pretreatment of slides, an effective but careful treatment, inclusive denaturation of preparations, the use of denatured viral nucleic acid probes with high specific radioactivity, sufficient washings to remove unspecifically bound nucleic acids, and a suitable method for demonstration of the hybridized virus genome (Brahic and Haase, 1978).

As to the preparation of slides, we employed a mixture of Ficoll, polyvinylpyrrolidone, and bovine serum albumin (Denhardt, 1966; Brahic and Haase, 1978), in order to avoid unspecific adhesion of the labelled viral DNA to the glass. The kind of treatment of the fixed tissue specimens before hybridization was important in order to optimalize on the one hand the hybridization conditions and on the other hand, to minimalize as much as possible alterations in cell morphology. Due to our own experience, the treatment of sections with 0.2 mol/l HCl followed by heating up to 70 °C (which improved the adhesion of tissue sections to the slide, Edwards and Wood, 1983), and a limited proteinase digestion were favourable. Using this method, Brahic and Haase (1978) were able to demonstrate an increasing efficiency of the in situ hybridization, while other authors — owing to the employment of HCl — showed a quantitative reducing of hybridization (Pardue and Gall, 1975; Moar and Klein, 1978). However, in comparison to alcali and heat, the denaturation with HCl seems to be more careful for preserving the morphology of cells (Jones, 1973). After hybridization, the washing procedures are also of great importance. As a rule, a low degree of nonspecific labelling was seen in uninfected cells when sections were washed in SSC solution at 55 °C (Harrison *et al.*, 1973). In our own experiments, the number of granule-like blackenings in non-infected cells always ranged within the normal background limit of 3 to 8 silver grains per 100 μm^2 given for the employed photographic emulsion. The tested photographic emulsion ORWO(R) K 6 was sufficiently sensitive for this purpose.

The results demonstrate a successful detection of herpesvirus genomes in animal and various human tissues by the described in situ hybridization

method. HSV nucleic acids were already demonstrated by in situ hybridization in human and animal sensory ganglion cells (Stevens, 1975; Galloway *et al.*, 1979; Tenser *et al.*, 1982), in brain sections of experimentally infected mice (Sequiera *et al.*, 1979), in cervical tissues undergoing neoplastic changes (McDougall *et al.*, 1980), and human oral squamous cell carcinomas (Eglin *et al.*, 1983), as well as in brain smears from patients with chronic psychiatric disease (Sequiera *et al.*, 1979). EBV DNA could be demonstrated by this method in nasopharyngeal carcinomas (Wolf *et al.*, 1973, 1975), tonsillar carcinomas (Wilmes *et al.*, 1983; Břicháček *et al.*, 1984), supraglottic laryngeal carcinomas (Břicháček *et al.*, 1983), and in human parotid glands (Wilmes and Wolf, 1981). Wolf *et al.* (1973) first demonstrated EBV DNA in the non-lymphoid epithelial tumour cells of nasopharyngeal carcinomas. Meanwhile, similar findings could be achieved for a small fraction of tonsillar carcinomas (Wilmes *et al.*, 1883; Břicháček *et al.*, 1984). The results of this study in regard of 10 nasopharyngeal carcinomas confirm that EBV genomes can regularly be found in the tumour cells of undifferentiated nasopharyngeal carcinomas (Anderson-Anvret *et al.*, 1978). The demonstration of EBNA corresponded to these results apart from one exception.

The demonstration of EBV genomes and antigens in tonsils of patients with infectious mononucleosis and tumour tissues of undifferentiated nasopharyngeal carcinomas reveal and increased number of positive results with the in situ hybridization in comparison to the detection of EBNA by ACIF. These findings demonstrate a higher sensitivity and specifity of the in situ hybridization especially by using cloned viral DNA (Haase *et al.*, 1977). An unspecific binding of the probes to DNA-binding proteins can be excluded by suitable controls. On the other hand IF is more favourable with regard to its economy. Both hybridization technique and IF do not allow an exact quantitative determination of virus genomes, for which reassociation kinetics seems the most suitable.

The importance of demonstrating virus antigens by IF mainly comprehends its use as screening method in order to examine a great number of tissues. Although rapid demonstration of virus-specific antigens in tissues is possible, the presence of viral nucleic acids is not always connected with the expression of virus-specific antigens. Therefore, due to their high expenditure, the hybridization methods should be applied to a smaller number of selected specimens with special scientific tasks.

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Explanation of Micrographs (Plates XX—XXI):

Fig. 1. Demonstration of HSV genomes by in situ hybridization and of HSV antigens by IF in tissues of experimentally infected mice.
A — HSV genomes in the brain (800×)
B — HSV genomes in the liver (800×)
C — HSV-specific antigens in the brain (500×)

Fig. 2. Demonstration of EBV genomes by in situ hybridization and of EBNA by ACIF in tissues of patients with infectious mononucleosis and nasopharyngeal carcinoma, respectively.
A — EBV genomes in the tonsil of a patient with acute infectious mononucleosis (800×)
B — EBNA-positive cells in the tonsil of a patient with acute infectious mononucleosis (250×)
C — EBV genomes in tumour tissue of a patient with nasopharyngeal carcinoma (800×)
D — EBNA-positive cells in the tumour tissue of a patient with nasopharyngeal carcinoma (250×).