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Abstract

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KEYWORDS: experimental brain tumor, BK virus, human papovavirus, T antigen, immunocytochemistry

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IMMUNOCYTOCHEMICAL LOCALIZATION OF T ANTIGEN IN CELLS OF BK VIRUS-INDUCED HAMSTER BRAIN TUMOR

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Abstract. The localization of both the large T and small t tumor (T) antigens in cultured cells (Vn 12 cells) of hamster brain tumors induced with BK virus (BKV), a new human papovavirus, was studied by an enzyme labelled antibody method at both the light and electron microscopic levels. Under the light microscope, BKV T antigen was observed in the nucleus, except for the nucleoli, of cells in interphase, and under the electron microscope it was observed in the nucleus except for the nucleoli and nuclear membrane. BKV T antigen appears to be closely associated with nuclear chromatin as previously reported for simian virus 40 tumor antigen (SV40 T antigen). The intracellular localization of BKV T antigen was the same as that of SV40 T antigen. In metaphase, BKV T antigen seems to be distributed diffusely throughout the cytoplasm except for the chromosomes. In telophase, BKV T antigen transfers from the cytoplasm to the nucleus. The migration of BKV T antigen during the cell cycle is thought to be related to the function of T antigen.

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BK virus (BKV), JC virus (JCV) and simian virus 40 (SV40) are well-known papovaviruses. BKV was isolated from the urine of a patient with a renal allograft (1). JCV and SV40-PML viruses were isolated from the brain tissue of patients with progressive multifocal leukoencephalopathy (PML) (2, 3). MM virus (MMV) was isolated from brain tumor tissue of a patient with Wiskott-Aldrich syndrome (4). A high percentage of infection of humans by these papovaviruses has been reported in etiological studies (5, 6).

The oncogenic potential of these papovaviruses in hamsters has been established. BKV and SV40 induce ependymoma and choroid plexus papilloma by intravenous or intracerebral inoculations (7-9), while JCV induces medulloblastoma, ependymoma, glioblastoma and pineocytoma (10-13). These facts suggest that chronic infection by papovaviruses could be one cause of human brain tumors. In fact, the possible causal relationship between papovavirus infection and certain human brain tumors, such as meningioma, ependymoma, choroid plexus papilloma, glioblastoma and cerebellar spongiosis, has been suggested in several reports (14-19). The study of papovavirus T antigens in experimental
brain tumors may prove useful in investigating the relationship between papova-
viruses and brain tumors.

In the present study the author describes the immunocytochemical localization
of BKV T antigen in cells of hamster brain tumors induced with BKV, and dis-
cusses differences in the localization of BKV T and SV40 T antigens.

MATERIALS AND METHODS

Cells. Vn 12 cells of an established cell line from a brain tumor induced in a Syrian
golden hamster (Mesocricetus auratus) by the intracerebral inoculation of BKV were given
by Prof. S. Uchida (Inst. of Medical Science, Univ. of Tokyo, Tokyo, Japan). The Vn 12 cells
were grown on Falcon plastic cell culture dishes (10 cm in diameter) with Eagle's minimum
essential medium (GIBCO, USA) supplemented with 10% fetal calf serum (GIBCO), 50 units/
ml penicillin and 500 μg/ml streptomycin (Meiji Seika, Co., Japan) at 37°C in a humidified
incubator under 5 % CO₂.

The SV40-induced subcutaneous tumor of a hamster was given by Prof. T. Oda (Cancer
Institute, Okayama University Medical School, Okayama, Japan). The SV40-induced tumor
cells were cultured in TD-40 glass culture bottles (13 × 4.5 × 2 cm, Ikemoto Co., Tokyo) with
the same medium and at the same temperature described above.

Preparation of antisera for immunocytochemistry. Syrian golden hamsters bearing subcutaneous

![Graph](image)

Fig. 1. Elution pattern of 5 mg hamster anti-BKV Fab' conjugated with 4 mg HRPO. The
conjugate was chromatographed on a column of Sephadex G-100. Flow rate, approximately 12 ml/
hr. Eluant, PBS. Optical densities (O.D.) at 403 and 280 nm were measured.
Tumors from inoculation of either Vn 12-or SV40-transformed cells were anesthetized 4 to 8 weeks after the inoculation. The blood of tumor bearing hamsters and normal hamsters was collected by direct puncture of the heart. Hamster anti-BKV T and anti-SV40 T antisera, and normal hamster serum were obtained from the blood. IgG fractions were isolated from hamster normal or anti-BKV T and SV40 T antisera by precipitation with ammonium sulfate. The precipitates were washed twice in cold 33 % saturated ammonium sulfate and were desalted by gel filtration on a column of Sephadex G-25 (Pharmacia Fine Chemicals, Sweden) (20).

Preparation of conjugate. Fab' fragments prepared from isolated IgG were coupled to horseradish peroxidase (HRPO) (Sigma, type VI) according to the method of Nakane, et al. (21). The conjugate mixture was chromatographed on a column of Sephadex G-100 equilibrated with PBS. The optical absorbances of each fraction at 280 nm and 403 nm were measured (Fig. 1).

Direct immunoperoxidase staining. For light microscopy, BKV- and SV40-transformed hamster cells cultured in chamber slides (Lab-Tek, USA) were fixed with cold acetone for 5 min at 4°C. After washing in cold PBS, the fixed cells were exposed to a peroxidase-labelled IgG conjugate solution for 30 min at room temperature. After washing in PBS, the cells were incubated in a freshly prepared substrate of 0.05 M Tris-chloride buffer, pH 7.6, containing 0.03 % 3, 3'-diaminobenzidine tetrachloride (Sigma) and 0.005 % hydrogen peroxide for 10 min at room temperature, washed in PBS, dehydrated in a graded ethanol series and mounted permanently (22).

For immunoelectron microscopy, cultured cells in chamber slides were fixed with periodate-lysine-paraformaldehyde (PLP) solution (23) for 30 min at 4°C. After fixation, the cells were washed with 3 % sucrose in PBS overnight at 4°C and incubated with the peroxidase-labelled Fab' fragments for 4 h at room temperature. The treated cells were postfixed with 2 % glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. Subsequently the cells were immersed in the freshly prepared substrate described above for 5 min at room temperature, and postfixed with 2 % glutaraldehyde and with 1 % osmium tetroxide (Merck, West Germany) in 0.05 M cacodylate buffer, pH 7.4, for 30 min at 4°C. Dehydration was carried out in a graded ethanol series after washing with PBS, and the cells were embedded in Epon (Shell Chemical Co. West Germany). Ultrathin sections were cut with a Porter-Blum I ultramicrotome and observed under a Hitachi H-700 or H-300 electron microscope.

Control experiments. Cultured cells in chamber slides were incubated with the normal hamster IgG or Fab' conjugates as described above. The cells were also treated with the hamster anti-BKV T or anti-SV40 T IgG conjugate for 30 min after inactivation of T antigen by heat treatment at 55°C for 30 min (25).

RESULTS

Cross-reactivity and specificity of anti-T antiserum. Vn 12 cells showed similar intranuclear staining whether by either anti-BKV T or anti-SV40 T conjugates. Also, SV40-transformed cells revealed the same intranuclear staining whether by either anti-BKV T or anti-SV40 T IgG conjugates. Thus, the crossreactivity between BKV and SV40 T antigens was immunocytochemically confirmed.

Vn 12 cells treated with normal hamster IgG conjugate showed no specific enzyme reaction (Photo. 1). When Vn 12 cells pretreated with heat at 55°C for...
Photo 1. No positive enzymic reaction is observed in the control study of Vn 12 cells. × 200

Photo 2. Vn 12 cells treated with either anti-BKV T (a) or SV40 T (b) IgG conjugates have the mosaic pattern which indicates positive immunoperoxidase staining in the nucleus except for the nucleoli. The cross-reactivity between BKV T and SV40 T antigens is shown. × 400

Photo 3. SV40-transformed cells incubated with either anti-SV40 T (a) or BKV T (b) IgG conjugates reveal positive immunoperoxidase staining in the nucleus except for the nucleoli. × 200
Photo 4. A cellular difference in the intensity of positive nuclear staining of BKV T antigen is revealed. (a) × 200 (b) × 1,000
30 min were reacted with hamster anti-BKV T IgG conjugate, no specific staining of BKV T antigen was observed in any part of the cells. The specificity of the immunocytochemical reaction for both BKV T and SV40 T antigens was confirmed by these two control experiments.

Light microscopy. When Vn 12 cells were reacted with either anti-BKV T or anti-SV40 T IgG conjugates, brownish positive staining was observed in the nucleus except for the nucleoli of cells in interphase (Photo 2). The SV40-transformed cells reacted with either anti-SV40 T or anti-BKV T IgG conjugate showed a staining pattern similar to that shown in Photo 3. The localization of BKV T antigen and that of SV40 T antigen were the same.

The distribution of positive staining for BKV T antigen was observed in Vn 12 cells at various cell cycle phases. A difference in the intensity of positive nuclear staining was recognized among nuclei of cells at interphase (Photo 4). At metaphase BKV T antigen seemed to be distributed diffusely throughout the cytoplasm. At telophase BKV T antigen, which had been distributed throughout the cytoplasm, was subsequently transferred to the nucleus (Photo 5). This migration of BKV T antigen during the cell cycle was also observed with SV40 T antigen (24).

Immunoelectron microscopy. The positive immunoperoxidase reaction for BKV T antigen was seen as electron dense deposits under the electron microscope. When Vn 12 cells were treated with the hamster Fab’ conjugate against BKV T antigen, the dense deposits were distributed like a mosaic in the nucleoplasm except for the nucleoli and nuclear membrane. The dense deposits appeared to be associated with the nuclear chromatin. These electron dense deposits were more often associated with euchromatin than heterochromatin (Photo 6).

When SV40-transformed cells were reacted with the hamster Fab’ conjugate against SV40 T antigen, positive dense deposits were distributed similarly as in the case of BKV T antigen (Photo 7). The ultrastructural localization of BKV T and SV40 T antigen seems to be identical.

As a control, the cells were treated with the normal hamster Fab’ conjugate, and no positive dense deposits were seen in the nucleoplasm of Vn 12 cells (Photo 8).
DISCUSSION

The specificity of the immunocytochemical reaction of BKV T antigen was confirmed by two independent experiments in this study. Neither Vn 12 cells exposed to the IgG conjugate from normal hamster serum nor those which had been treated by heating at 55°C for 30min in order to inactive T antigen revealed any positive staining in the nuclei.

As revealed by light microscopy, the pattern of positive staining in Vn 12 cells treated with either the hamster IgG conjugate against SV40 T antigen or that against BKV T antigen was similar to that of SV40-induced tumor cells treated with either anti-BKV T or anti-SV40 T antibody. The cross-reactivity between BKV T and SV40 T antigens was thus verified in this study. A close antigenic relationship of human papovaviruses, such as BKV, SV40 and JCV, and cross-reactivity among T antigens of three human papovaviruses have been suggested (26, 27). Simmons et al. (28) indicated that T antigens induced by BKV, JCV and SV40 consisted of two peptide subunits, large T and small t antigen. Yong et al. reported that the DNA sequence homology between SV40- and BKV-DNA was 70% (29); and that the amino acid sequence homology between SV40 T and BKV T antigens was 87% (30). The high homology of early genes of BKV and SV40 explains the cross-reactivity between BKV T and SV40 T antigens because T antigen is known to be an early gene product of these viruses.

The light microscopic localization of BKV T and SV40 T antigens has been reported by several investigators (31-36). Pope et al. (35) first determined that SV40 T antigen was localized in the nucleus by an immunofluorescent (IF) method. Dougherty (36) observed BKV T antigen only in the nucleus by an immunoperoxidase (IP) method. Both IF and IP methods have been used for immunocytochemical studies, but the author considers the IP method preferable because of less nonspecific staining and the permanent preservation of specimens.

The ultrastructural localization of SV40 T antigen has also been studied by several investigators (37-41). Oshiro et al. (37) first reported that SV40 T antigen was localized in the nucleus except for the nucleolus by the direct immunoferritin technique. Levinthal et al. (40) also studied the localization of T antigen by the indirect immunoferritin technique and found that closely packed clusters of ferritin were in parts of the nucleus, but that the nucleoplasm was poorly stained. The molecular weight of ferritin-labelled antibody is thought to be too large to penetrate the cellular membranes of fixed cells; therefore, the specific staining

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Photo 7. SV40-transformed cells treated with anti-BKV T Fab' conjugate show a similar distribution of electron dense deposits in the nucleoplasm as Vn 12 cells. N: nucleus, n: nucleolus. Bar = 1 μm.

Photo 8. Specific electron dense deposits are not seen in the nucleoplasm of Vn 12 cells in the control study. n: nucleolus. Bar = 1 μm.
of the antigens in the nucleus would be expected to be poor and the precise localization of T antigen undiscernible. Leduc et al. (38) studied the localization of SV40 T antigen by the direct immunoperoxidase technique with anti-SV40 T IgG conjugate. The intranuclear staining of T antigen was poor and the cytoplasm was stained non-specifically. When they treated cells by the freeze and thaw method during the reaction with the antiserum, the cytoplasm was destroyed and associated with non-specific staining, though the nucleoplasm was heavily stained except for the nucleolus. Baba et al. (41) examined the ultrastructural localization of T antigen by the indirect immunohistochemical method using peroxidase-labelled IgG and found that the nucleoplasm was poorly stained, the cytoplasm was stained non-specifically and the cellular membrane was heavily stained. In order to get better penetration of the tracer into cells, Leduc also used peroxidase as a marker instead of ferritin because of its smaller molecular weight, but comparing the peroxidase-labelled IgG technique with the peroxidase-labelled Fab' technique, positive staining in the nucleus was still poor.

Tabuchi et al. (39) detailed the localization of SV40 T antigen using Fab'-HRPO conjugate instead of IgG-HRPO conjugate. The author believes that at the present time the most suitable method for analyzing ultrastructural localization of T antigen is the peroxidase-labelled antibody fragment (Fab') technique.

The localization of BKV T antigen changes according to the cell cycle phase. BKV T antigen was localized primarily in the nucleoplasm in a mosaic pattern at interphase, but it was seen throughout the cytoplasm except for the chromosomes at metaphase. T antigen began to migrate gradually into the nucleoplasm from the cytoplasm during telophase (Fig. 2). BKV T antigen appeared to be localized near or on the nuclear chromatin at interphase by electron microscopy. Tabuchi et al. (24) observed that the distribution of SV40 T antigen changed according to the cell cycle phase and that T antigen seemed to be associated with nuclear chromatin at interphase and was scattered throughout the cytoplasm at metaphase. D'Alisa et al. (42) found using the immunofluorescent technique that SV40 T antigen bound to the host cell chromosome. Griffith et al. (43) examined the binding site of SV40 DNA under the electron microscope. Jessel et al. (44) studied SV40 T antigen-binding sites using the restriction endonuclease fragments and the nitrocellulose filter DNA-binding technique. T antigen is known to bind to chromatin two to three times (45). As a result, of such studies, T antigen is thought to be localized on the nuclear chromatin at interphase. The intensity of the positive staining in the nuclei was not uniform from cell to cell at interphase. This difference in staining intensity appears to be due to the change in the amount of T antigen during the cell cycle (46). Stenman et al. (47) reported that SV40 T antigen was dissociated from the chromosomes and was distributed throughout the cytoplasm during mitosis. Tabuchi et al. (39) reported that SV40 T antigen was probably synthesized at cytoplasmic ribosomes and transferred into the nucleus. Both BKV T and SV40 T antigens are cross-reactive and the function of BKV
T antigen is considered to be same as that of SV40 T antigen.

It is well-known that SV40 T antigen binds at the 0.65 to 0.75 portion of the SV40 DNA physical map (43) and initiates DNA replication of transformed cells (48). It is supposed in the present study that BKV T antigen also binds near or at the replication origin of BKV DNA and might regulate the initiation of DNA replication of transformed cells or maintain the transformed state.

The function of T antigen is not completely clear yet. But the author believes that the investigation of T antigen in experimental brain tumors is important to clarify the possible causal relationship between human papovaviruses and human brain tumors.

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364  M. Tsuboi

T Antigen in BK Virus-induced Tumor Cells


366

M. TSUBOI


