INDUCTION OF PAPILLARY EPENDYMOMAS AND INSULINOMAS IN THE SYRIAN GOLDEN HAMSTER BY BK VIRUS, A HUMAN PAPOVAVIRUS*1

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Newborn hamsters were inoculated intracerebrally with BK virus. Between 3 and 6 months after inoculation, they developed papillary ependymoma (8 hamsters, 42%) or functional malignant islet cell tumors of the pancreas (insulinoma, 8 hamsters), or both (1 hamster). Both tumors contained an antigen reactive to SV40 T-antibody, suggesting that at least a part of BK virus genome has been integrated into the tumor cells. No infectious virus was detected in the extract of these tumors.

BK virus was first isolated by Gardner et al.4) from the urine of a renal transplant recipient who had undergone immunosuppression therapy. BK virus induces the synthesis of T-antigen, which is serologically indistinguishable from that of SV40, in lytic17) and transformed cells.8,10,14) Seroepidemiological studies have shown that infection with BK virus in man is very common and the primary infection occurs in early childhood,11) but BK virus has not yet been related to any illness. Although a BK virus was isolated from a reticulum cell sarcoma of the brain of a patient with Wiskott-Aldrich syndrome, a direct relationship of the virus to the tumor was not established.18)

The oncogenicity of BK virus for hamsters was studied by Shah et al.14) They reported that an undifferentiated fibrosarcoma occurred in 1 of 52 hamsters when newborns were inoculated subcutaneously with BK virus. Similar results were obtained by Näsé et al.8) and by van der Noordaa.23) Since it has been previously shown that newborn hamsters are susceptible to the induction of tumors after intracerebral inoculation of polyoma virus,11) SV40,5) and JC virus,24) another human papovavirus, studies were undertaken to determine the effect of intracerebral inoculation of large doses of purified BK virus into newborn hamsters. This paper deals with its general results, and details of the histopathological findings will be reported separately.

MATERIALS AND METHODS

Cells Primary human embryo kidney cell (HuEK) cultures were used to propagate and isolate BK virus. Kidneys obtained from fetuses of 5-month gestation were minced, and the cells were dispersed with 0.25% trypsin and grown in a YLM medium supplemented with 10% calf serum. The YLM medium consists of equal parts of Eagle's minimum essential medium (MEM) and YLE (0.5% lactalbumin hydrolyzate and 0.1% yeast extract in Earle's salt solution). Human embryo fibroblast (HuEF) cultures were used to titrate and isolate BK virus. Whole embryos (3-month gestation) were cultured as described above except that they were grown in MEM containing 10% calf serum. After infection, the cells were maintained in YLM (HuEK) or MEM containing 2% calf serum (HuEF).

Virus BK virus isolated by Gardner was kindly provided by Dr. K. K. Takemoto (N.I.H., Bethesda, U.S.A.). The virus was propagated in

*1 This constitutes Part I of a series entitled “Pathogenicity of BK Virus.” This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture (No. 001026).

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HuEK cultures and purified by the method used for SV40. The infected cells at the stage of maximum cytopathic effect were suspended in a small volume of the medium, sonicated for 5 min, treated with 1% sodium deoxycholate at 36° for 30 min, and centrifuged at 17,000g for 20 min. The supernatant was layered over 9 ml of KBr solution (55%, w/v KBr in 0.01M Tris buffer at pH 7.4) and spun in an SW 25.1 rotor at 23,000 rpm for 2.5 hr. The virus band was collected, dialyzed against 0.01M Tris buffer, and spun to equilibrium in CsCl in an SW 50.1 rotor at 35,000 rpm for 18 hr. The full particle band (1.34 g/cm³) was collected and dialyzed against phosphate-buffered saline at pH 7.4. The purified sample contained 2.4 × 10¹¹ virus particles and 1.4 × 10⁶ T-antigen-forming units per 0.02 ml. The virus particle concentration was estimated from the optical density (1 OD 258 nm unit = 6.4 × 10¹² virions) and also from particle count by the loop drop method. The values obtained by both methods agreed well. The T-antigen forming ability was assayed by the indirect immunofluorescence method. HuEF cells 3 days after infection were stained with fluorescent SV40 T-antibody and positive cells were counted. That the virus sample did not contain SV40 was checked by inoculation onto green monkey kidney cell cultures.

Animals Pregnant Syrian golden hamsters (Mesocricetus auratus) were obtained from a colony maintained in the Department of Veterinary Science of our Institute. It had been introduced from the Harvard Cancer Commission in 1959. The newborn animals were inoculated with 0.02 ml of the virus into the midportion of the right cerebral hemisphere using a tuberculin syringe and 1/5 vein needle. The animals were observed daily or every other day. At the time of death, blood was collected whenever possible before autopsy.

Blood Glucose, and Blood and Tumor Immunoreactive Insulin Blood glucose was determined by the method of Sasaki (o-toluidine-boric acid method) using an autoanalyzer. Blood or tumor immunoreactive insulin was measured by the method of Yalow and Berson. Insulin was extracted from the intra-abdominal tumors or normal pancreas by the acid ethanol procedure.

Indirect Immunofluorescence Test Coverslips with tumor imprints were fixed with cold acetone for 10 min and reacted with a hamster anti-SV40 T-serum at 36° for 45 min. Complement-fixing antibody titers of the serum were 1:2560 against SV40 T-antigen and 1:<10 against SV40 V-antigen. The serum was used at a dilution of 1:40. After washing 3 times with phosphate-buffered saline, they were reacted with fluorescent rabbit anti-hamster globulin at 36° for 30 min.

Complement Fixation Test Tumors and normal hamster organs were homogenized in 10 volumes of MEM, frozen and thawed 3 times, sonicated for 3 min, and clarified by centrifugation at 17,000g for 30 min. The antigenicity of the supernatants was examined by a microcomplement fixation test using the anti-SV40 T-serum described above.

Virus Isolation Each supernatant of tumor extracts described above was layered over cultures of HuEF or HuEK and adsorbed at 36° for 90 min. The cultures were maintained with weekly changes of the medium and examined for cytopathic effect after 2 weeks of incubation. The cells were then sonicated for 3 min and inoculated on new cultures grown on coverslips. After 2 weeks of incubation, the cultures were examined for cytopathic effect and then for T-antigen by the indirect immunofluorescence method.

RESULTS

Eye Illness Twenty-two hamsters (3 litters) less than 24 hr of age were inoculated intracerebrally with BK virus. A litter of 10 control hamsters received phosphate-buffered saline. Between 13 and 31 days after inoculation, 16 virus-inoculated animals (73%) suffered from an eye illness, apparently conjunctivitis, of one or both eyes. Though many of them recovered within a few days, 3 animals became blind in the right eye and one in both eyes between 15 and 26 days after inoculation. A few swab samples were collected from the eyes with conjunctivitis for virological examination. Two animals suffering from conjunctivitis, one blind, and 2 control normal animals were killed for virological and pathological examinations. The remaining 19 hamsters inoculated with BK virus, including the blind ones, as well as the control 8 hamsters survived till the signs of tumor in the virus group appeared.

Brain Tumors Between 3 and 6 months after inoculation, 17 virus-inoculated hamsters died or became ill, while the controls were all well (Table I). Three of these ani-
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Table I. Relationship between Signs and Macroscopic Tumors Induced after Intracerebral Inoculation of BK Virus

<table>
<thead>
<tr>
<th>Sign</th>
<th>No. of hamsters</th>
<th>No. of hamsters with (autopsy: days after inoculation)</th>
<th>No. of hamsters with intra-abdominal tumor</th>
<th>Tumor undetected</th>
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<tbody>
<tr>
<td>Sudden death</td>
<td>3</td>
<td>2(98, 180)</td>
<td>1(180)</td>
<td>1(85)</td>
</tr>
<tr>
<td>Paralysis</td>
<td>5</td>
<td>5(113, 125, 125, 146, 166)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Subacute or chronic weakness</td>
<td>9</td>
<td>1(169)</td>
<td>8(154, 157, 166, 166, 171, 181, 189)</td>
<td>0</td>
</tr>
<tr>
<td>Apparently healthy</td>
<td>2</td>
<td>1(181)</td>
<td>0</td>
<td>1(181)</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>9</td>
<td>9</td>
<td>2</td>
</tr>
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</table>

a) Both types of tumors were detected in this animal.

b) Cerebral hemorrhage and severe lung hyperemia due to unknown etiology.

c) Brain tumors were detected histologically.

mals were found dead. Five showed paralysis, among which 4 appeared moribund, and were immediately killed. Nine hamsters became debilitated gradually and many of them were killed before becoming moribund. Six months after inoculation, the remaining 2 apparently healthy hamsters and the 8 controls were killed.

The brain tumors were mainly found in the animals which showed paralysis or died suddenly (Table I). These brain tumors were light red-greyish, soft masses of about 1~7 mm in diameter, filling the lateral (rarely the third or fourth) ventricles (Photo 1). In several instances, this resulted in severe hydrocephalus. Histologically, they were diagnosed as papillary ependymomas including plexus papillomas. Their details will be reported separately.

In one of these brain tumor-bearing hamsters, the intra-abdominal tumor described below was detected. Microscopic brain tumors were found in 2 hamsters in which intra-abdominal tumors were detected. The overall brain tumor incidence was 58% (11/19 hamsters).

**Intra-abdominal Tumors** In the 6th month (150~180 days) after inoculation, 9 hamsters showed subacute or chronic signs, such as wasting, decreased activity, unsteady gait, or somnolence, and one of them died after a severe attack of tonic cramp of the extremities and flooding of saliva. Intra-abdominal tumors were mainly detected in these animals (Table I).

The tumors were found in the pancreas, especially at the tail, singly (3 cases) or multiply (6 cases) (Photo 2). They were dark red, soft masses of about 1~15 mm in diameter, covered with a thin fibrous membrane. The cut surface was light red or light yellowish white. In several cases, metastasis was found in the liver, macroscopically (2 cases) or histologically (4 cases). The results of histocytological examinations will be reported separately. Briefly, the tumors contained ribbons of several rows or rosettes of columnar cells, intimately related to capillaries. Some tumor cells contained a large amount of $\beta$ granules (Ivic's staining method or electron microscopy). The tumors were diagnosed as malignant islet cell tumor of the pancreas.

Gross lesions other than these tumors were light or moderate degeneration of the liver in many cases, severe lung hyperemia in one case, and severe hydrocephalus in another case.
Blood Glucose, and Blood and Tumor Immunoreactive Insulin  Since a severe attack was observed and the tumors were present in the pancreas, blood glucose and insulin were assayed. In 4 out of 6 animals with intra-abdominal tumor, the concentration of blood glucose was 26–59 mg/100 ml, which was significantly lower than that of 6 animals with brain tumor only, one virus-inoculated animal without tumor, or 5 control animals, which ranged from 86 to 302 mg/100 ml. The concentration of blood immunoreactive insulin was very high (81–334 μU/ml) in 5 hamsters with intra-abdominal tumor, including 2 animals which showed normal glucose content in blood, compared with that in 3 hamsters with brain tumor only, one virus-inoculated animal without tumor, and 4 control animals, which showed a value of <5–30 μU/ml. Immunoreactive insulin contents of 3 intra-abdominal tumors were 2.1, 3.6, and 5.4 U/g, and that of a normal pancreas was 1.0 U/g. These data indicate that the intra-abdominal tumors were functional insulinomas.

Presence of SV40-related T-Antigen in the Tumors  Tumor imprints were examined for the presence of SV40-related T-antigen by the indirect immunofluorescence test using an anti-SV40 T-serum. A positive fluorescence reaction was found in nuclei of cells from 6 brain tumors tested (Photo 3), while no specific staining was found in imprints of normal cerebral tissues of 3 hamsters with intra-abdominal tumor and 3 controls. Though the same specific intranuclear fluorescence was also observed in 7 intra-abdominal tumors tested (Photo 4), the observation was often difficult, because of apparently nonspecific brilliant fluorescent flecks scattering over the imprints. Similar fluorescent flecks were observed in some areas in normal pancreatic tissue imprints, but not in imprints of normal brain, liver, spleen, kidneys, and adrenal gland.

As the results of immunofluorescence test of the intra-abdominal tumors were often obscure, presence of the antigen was examined by the complement fixation test. Both types of tumors contained the antigen reactive with SV40 T-antibody (Table II), while none of the normal organs (brain, pancreas, liver, spleen, kidney) contained the antigen. The antigen titers of 2 brain and 3 intra-abdominal tumor extracts (10%) other than those shown in the table were assayed using 8 units of antibody (1:40 dilution of serum), and the titers of 1:4 and 1:16 in the former and

<table>
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<th>Table II. “Chessboard” Complement Fixation Tests of BK Virus-induced Tumors with Serum from an SV40 Tumor-bearing Hamster</th>
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<tbody>
<tr>
<td><strong>BK virus-induced</strong></td>
</tr>
<tr>
<td>brain tumor-antigen</td>
</tr>
<tr>
<td>2 4 8 16</td>
</tr>
<tr>
<td>Anti-SV40</td>
</tr>
<tr>
<td>T-serum&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
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<sup>a</sup> Figures are reciprocal of dilution.
<sup>b</sup> 4 plus on a scale of 0 (complete hemolysis) to 4 (no hemolysis).
<sup>c</sup> Supernatants of 10% homogenates in MEM. Figures are reciprocal of dilution.
<sup>d</sup> Prepared from cultures 3 days after infection.
<sup>e</sup> Prepared from an SV40-transformed 3T3 cell line, M<sup>27</sup>
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1:8, 1:16, and 1:16 in the latter were obtained. As indicated in Table II, the anti-SV40 T-serum had 8-fold higher antibody titer against SV40 T-antigen than against BK virus T-antigen. This relationship is similar to that obtained by the immunofluorescence test reported by Shah et al. These results suggest that at least a part of BK virus genome has been integrated into the cells of both tumors.

Virus Isolation. The results of virus isolation from tumor extracts and eye lesions were negative. The materials used were as follows: 10% homogenates of 3 brain, 3 intra-abdominal, and one metastatic liver tumors, the ocular bulbus and an apparently normal brain of an 18-day-old hamster which became blind 15 days after inoculation, and 2 swabs from eyes with conjunctivitis.

DISCUSSION

We inoculated a concentrated and purified BK virus sample intracerebrally into newborn hamsters and found that 90% of the animals developed tumor (ependymoma or islet cell tumor) during a 6-month observation period. Evidence for an etiological role of BK virus was provided by the following findings: (1) High development rates of both tumors, (2) demonstration of antigen reactive to SV40 T-antibody in the tumors, as in other BK virus-induced tumors and transformants, (3) failure to detect C-type particles by electron microscopy, which will be reported later, and (4) no incidence of spontaneous tumor development within 1 year after birth in a long-term observation on our hamster colony. Most animals in our colony died between 1.5 and 3 years, mainly by intestinal adenocarcinomas and lymphomas, and presumed islet cell tumors of the pancreas were found in 2% (Muto and Kutsukake, unpublished). These results were not so different from those reported by Fortner.

The oncogenicity of BK virus observed in this experiment was not low. However, hitherto reports have shown that BK virus is weakly oncogenic for hamsters. In order to elucidate whether the difference is due to virus doses inoculated or the route of inoculation, or a mutant virus which might have developed during 3 passages in human embryo cells in our laboratory, an experiment using another virus sample prepared in different batches of cells is now under progress.

A temporary eye disease occurred between 15 and 30 days after inoculation. It has not been observed in intracerebral inoculation of hamsters with SV40 (Uchida, Watanabe, and Muto, unpublished data), polyoma, and JC viruses. It may be due to virus doses (10^10 and 2 × 10^11 virions per hamster in our SV40 and the present BK virus experiments, respectively), or a specific activity of BK virus, although the results of virus isolation from the lesions were negative. Histopathological examination is now under way.

In intracerebral inoculation into newborn hamsters, BK virus induced papillary ependymomas like SV40 (our unpublished results). In contrast, polyoma virus and JC virus induced mesenchymal tumors and malignant gliomas, respectively. Tanaka et al. reported, however, that BK virus induced selective transformation of neuroglial cells in hamster brain cell cultures, unlike SV40 which induced transformation of both neuroglial cells and mesenchymal cells in these cultures.

Majority of hamsters which escaped early death (3 to 5 months after inoculation) due to brain tumors developed malignant islet cell tumors of the pancreas in the 6th month. The tumors closely resembled human functional insulinomas with regard to the frequent development of tumors at the pancreas tail, metastasis to the liver, signs such as somnolence and tonic cramp, hypoglycemia, high concentration of blood and tumor insulin, and the histopathological findings, which will be reported later, but further morphological and endocrinological studies should be done to characterize these tumors.
Since the tumor cells contained an antigen reactive to SV40 T-antibody, BK virus must have reached β-cells of the islets. Elucidation of migration route of the virus from the brain to the pancreas is an interesting problem. SV40 and JC virus do not seem to induce intra-abdominal tumors, for the tumors were not found in spite of careful autopsy of the virus-inoculated animals surviving 6 or 7 months after inoculation.\(^5,24\) This is probably the first report of an induction of hormone-producing tumors due to viruses. The establishment of transplantation and in vitro culture of tumor cells from BK virus-inoculated hamsters may provide a useful tool for biological studies as shown in a transplantable islet cell tumor of the golden hamster obtained from a spontaneous tumor.\(^6,16\) Since BK virus is ubiquitous in the human population, the virus should be considered seriously as an agent that could be related etiologically to the analogous or other various neoplastic diseases of man, although the results presented here were obtained in hamsters.

We are grateful to Prof. F. Ikuta and Dr. K. Yamazaki of the Department of Neuropathology, Brain Research Institute, Niigata University, for autopsy and histopathological examination of a part of the animals, and to Dr. Isamu Tagaya for critical review of the manuscript.

**Addendum** Costa, Yee, Tralka, and Rabson have recently demonstrated that ependymomas were produced in 3 of 11 newborn hamsters inoculated intracerebrally with a human papovavirus (MMV) isolated from a malignant lymphoma of the brain of a child with Wiskott-Aldrich syndrome [*J. Natl. Cancer Inst.*, 56, 863−864 (1976)].

(Received July 7, 1976)

**REFERENCES**

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EXPLANATION OF PLATE

Photo 1. Tumor mass in the lateral ventricle which developed 115 days after inoculation of BK virus soon after birth.

Photo 2. A tumor (arrow) at head portion in the pancreas of a hamster which was autopsied 180 days after inoculation of BK virus.

Photo 3. Brain tumor imprint stained with an anti-SV40 T-serum by indirect immunofluorescence method. ×800.

Photo 4. Intra-abdominal tumor imprint stained with an anti-SV40 T-serum by indirect immunofluorescence method. ×800.
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