Acta Medica Okayama

Volume 20, Issue 1 1966 Article 1

February 1966

Oncogenic properties of nucleic acid derived from SV-40 virus

Tetsuo Kimoto* James T. Grace†

*Okayama University,
†Roswell Park Memorial Institute,

Copyright ©1999 OKAYAMA UNIVERSITY MEDICAL SCHOOL. All rights reserved.
Oncogenic properties of nucleic acid derived from SV-40 virus*

Tetsuo Kimoto and James T. Grace

Abstract

The present report describes the findings on the infectivity of DNA partially purified from SV-40 which was propagated in the monkey kidney cells (BSC-1) in vitro and the importance of nucleic acids as oncogenic factors, particularly the induction of tumor by DNA in newborn hamsters. 593 newborn hamsters in total were used in the present experiments, and cannibalism among them posed as a serious problem. On 30 days postinoculation, very remarkable changes occurred in the liver, lung and subcutaneous areas. Cellular responses of the perivascular cells were predominant and they were distributed in the interstitial tissues of the liver (liver cirrhosis in primates) and lung. Three hamsters of those subcutaneously inoculated with nucleic acids developed tumors and two tumors appeared in the subcutaneous tissues on 130 days postinoculation, which were identified to be the ones induced by intact SV-40 virus. Other tumors appeared in the liver, lung, intestinal ducts and abdominal surface at 126 days after subcutaneous injection. The cytological observations revealed multiple hemangiosarcoma combined with proliferation of the perivascular cells. On the other hand, cellular responses to nucleic acids were more marked by inoculation of the cell-free filtrate of BSC-1 infected by DNA than of DNA, and essential histologic findings were similar to the response to infectious DNA. Thirty-nine hamsters (30 per cent) developed tumor within about 200 days postinoculation of the filtrates. Sarcomas were common and they were confined to the subcutaneous tissues in 35 hamsters and to the peritoneum in two others by subcutaneous inoculation of the filtrates. The intestinal gland-cell carcinomas, however, could be induced at 37 and 59 days postinoculation in two hamsters of one litter (7 newborn hamsters) and in the other three newborn hamsters subcutaneous sarcomas were induced by inoculation of the same agent. These results suggest that the observation on the oncogenic capacity of nucleic acids would give us a clue to resolve the course of cancer from the viewpoint of the infectious nucleic acid.

ONCOGENIC PROPERTIES OF NUCLEIC ACID DERIVED FROM SV-40 VIRUS*

Tetsuo KIMOTO** and James T. GRACE***

Roswell Park Memorial Institute, Buffalo, New York, U. S. A.

Received for publication, December 25, 1965

In 1962 it was recognized by EDDY1,2,3, KIRSCHSTEIN and GERBER4 that simian virus (SV-40), which was first described by SWEET and HILLEMAN5 (1960) has oncogenic property of inducing subcutaneous fibrosarcoma and ependymomas in newborn hamsters. Recently SHEIN and ENDERS6,7 described that SV-40 virus is infectious to human embryonic kidney cells and induces cell transformation. KOPROWSKY et al.8 also reported progressive transformation in human tissue cultures by exposure to SV-40 virus. The transformed cell is characterized by the development of cytologic and chromosomal abnormalities usually associated with neoplasia and the question of SV-40 virus as a human cancer virus can be raised along with the problem of human transformed cells by these new reports.

In 1962 GERBER9 described a report that deoxyribonucleic acid also produced a cytopathic effect identical with the one caused by an intact SV-40 virus in vitro.

The present report describes findings on the infectivity of nucleic acids purified partially from SV-40 propagated in the monkey kidney cell (BSC-1) in vitro and the importance of nucleic acids as oncogenic properties, particularly the induction of tumors by nucleic acids in newborn hamsters. The findings of oncogenic capacity of nucleic acids are of a considerable interest for the approach to resolve the course of cancer from the view point of the infectious nucleic acid.

MATERIALS AND METHODS

Original virus and propagation of SV-40 virus in cell cultures:

SV-40 virus was originally obtained from Dr. YOHN at the Roswell Park Memorial Institute (Buffalo, New York, U. S. A.). The source of viral material

* The report was read by Dr. James T. Grace at the Ninth Meeting of Human Cancer Virus Task Force, Airlie House, Warrenton, Virginia, U. S. A. May 24-26, 1964.

** Present address; Department of Pathology, Okayama University Medical School, Okayama Japan.

*** Associate Director; Roswell Park Memorial Institute, Buffalo, New York, U. S. A.
was one pool of the cell free filtrate of BSC-1 preserved in phosphate buffered saline (PBS; pH 7.4) free of magnesium and calcium stored at -42°C.

The virus had an infectivity titer in BSC-1 of $10^3$ to $10^4$/TCID50/0.1 ml and this virus infectivity could be accelerated to $10^8$ TCID50/0.1 ml by several passages through the BSC-1. Then monolayers of BSC-1 cells cultured in test tubes (average cell number $5 \times 10^4$ per one tube) and sometimes monolayers of BSC-1 cells cultured in T type flask 30 (Pyrex) were used for propagation of SV-40. The cell monolayers of these test tubes maintained on one ml, medium 199 supplemented 2 per cent of inactivated calf serum, penicillin 100U/ml but without streptomycin (pH 7.0) were inoculated with 0.1 ml SV-40 virus which had titer of $10^8$ TCID50/0.1 ml. The period of 24 hours was allowed for inoculation when the medium supplemented 2 per cent calf serum was used but sometimes a 3-hour period for adsorption when PBS solution was used for adsorption of SV-40 virus. After the adsorption the fluid was discarded and then the cell sheets of every test tube were washed with PBS and replaced with the medium of 199 supplemented 10 per cent fetal calf serum. As control experiments, BSC-1 cells uninoculated with SV-40 virus were similarly treated with PBS for the same time. Medium was changed every other day according to conditions of the medium pH. Cytopathic effects may occur as early as four days or, occasionally, as late as 14 days after virus was inoculated depending upon the concentration of virus in the inoculum.

The infected cells in test tubes were rinsed with PBS or 0.88 M NaCl. Infected cells were frozen and thawed in acetone-dry ice three times and sometimes infected cells were collected by rubber policeman. Further the medium fluid of virus pool was ultracentrifugated at the harvest time by the model L Spinco centrifuge for 3 hours, at 30,000 rpm in a No. 40 rotor. The centrifugated sediment was resuspended in 1:50 of the original volume with PBS or in 0.88 M NaCl buffered to pH 7.2. The suspension of the BSC-1 cells infected by SV-40 also was ultracentrifuged for 3 hours, at 30,000 rpm and the centrifuged sediment was resuspended in 1:5 of original volume with PBS or in 0.88 M NaCl. This suspension of the BSC-1 cells infected by SV-40 was added to the concentrated virus suspension ultracentrifuged from the medium fluid for preparation of nucleic acids.

**Extraction of nucleic acids from BSC-1 cell infected by SV-40 virus:**

Various extraction methods were tried in attempts to extract infectious nucleic acids from BSC-1 cells inoculated with SV-40 and cold phenol extraction was carried out by the modified method of Gierer, Schramm and Ito. The results with nucleic acids prepared from suspension of infected cells and partially purified virus in PBS and 0.88 M NaCl gave the same as shown in Fig. 1, and the most effective procedure seemed to be the following.
The monolayer cells of BSC-1 inoculated with SV-40 virus in two hundred test tubes were used at least for extraction of nucleic acids routinely each time. On the other hand, nucleic acids from BSC-1 cell non-infected with SV-40 were extracted as the control material. The PBS or 0.88 M NaCl suspension of BSC-1 cell infected with SV-40 were homogenized slightly once using of the Potter homogenizer in ice water. Then two volumes of 88 per cent cold phenol and 1/10 M ethylene-diamine sodium tetracetate (EDTA) (5.6 x 10^{-4} M solution in PBS) were added quickly and the mixer was run for another two minutes. The homogenate was then transferred to a flask placed on a magnetic mixer and ground at 4°C for 1 hour. The homogenate was then transferred to a flask placed in 56°C water bath for 5 minutes and, after immediate cooling, extraction was continued at 4°C for minutes. After centrifugation at low speed, the milky suspension was separated from the tissue debris and was extracted with an equal volume of 80 per cent phenol at 4°C for 30 minutes. The same procedures were repeated on the withdrawn aqueous layer and interphase material. The final aqueous phase was extracted with ether and then the residual ether was evaporated by bubbling nitrogen through the suspension.

**Ultraviolet absorption measurements:**

Absorption curves were obtained with the Beckman spectrophotometer. The extracts containing nucleic acids were diluted 40 times with PBS prior to the measurement by spectrophotometer. The spectrum of the ultraviolet absorption was characteristic of the solution containing nucleic acids, with the maximum adsorption around 265—270 m\(\mu\) as shown in Fig. 1. In addition, calorimetric quantitative determination for DNA was carried by the Burtan’s method^{12} and diphenylamine reaction for DNA was positive in extracted solution.
Infectivity assay of nucleic acid preparations:

The solution containing of nucleic acids was filtered through the Millipore filter (pore size 0.45 μm), monolayer cell cultures of BSC-1 grown in T type flask 30 (Pyrex), also test tubes were available for the susceptible cell, and they were rinsed twice with PBS prior to the use. The monolayer cells grown in the T type flask 30 were inoculated with 0.5 ml of extracted nucleic acid mixed in 4.5 ml of 199 medium containing 2 per cent calf serum, and in the case of the monolayer cells grown in the test tube the cell were inoculated with 0.2 ml of nucleic acids mixed in 0.8 ml of 199 medium containing 2 pen cent calf serum, the mixture being allowed to adsorb for 24 hours at the 37°C incubator. The inoculum was removed and replaced with medium of 199 containing 10 per cent fetal calf serum. For identification of the infectivity of nucleic acids, DNase (concentration of DNase 20—100 μg/ml) was used in the presence of 0.005 M MgSO₄ and RNase (100/μg/ml) diluted in PBS was also treated with infectious nucleic acids. The infectivity of nucleic acids was completely destroyed by exposure for 30 minutes at room temperature to 20 and 100 μg/ml of DNase in the presence of 0.005 M MgSO₄. The treatment with RNase and specific anti-SV-40 rabbit serum had no effect on the infectivity of nucleic acids. On the other hand, the infectious DNA proven to possess the specific infectivity on the susceptible cells was inoculated into newborn hamsters within 24 hours after birth in the following manner.

Fluorescent antibody staining technique:

The indirect fluorescent antibody technique was used for the experiments. Anti-SV-40 serum was obtained from rabbits given intravenous injection of SV-40 PBS suspension containing 10⁸.¹ TCID 50/0.1 ml. About 5 ml virus suspension were injected each time intravenously. The serum was titrated against 100 TCID 50 of SV-40 in culture of BSC-1. The highest dilution preventing the cytopathic effects was 1/1280. Fluorescein-conjugated anti-rabbit goat globulin (Microbiological Associates) was dissolved in PBS according to the directions of the supplier. To prevent non-specific staining, the anti-SV·40 rabbit serum as well as the fluorescein-conjugated anti-rabbit goat globulin solution were adsorbed with powder prepared from the liver of mouse (Microbiological Associates) and BSC-1 cells by shaking for 60 minutes followed by centrifugation at 18,000 rpm for 30 minutes. This procedure was carried out twice. For the first adsorption 300 mg powder of the liver and BSC-1 cell per ml were used. The monolayer cell cultures of BSC-1 cells were set up in Leighton tubes with 2 ml of 199 medium containing 10 per cent fetal calf serum, and when monolayers had been formed, the cell were washed with PBS and then incubated with 1.0 ml PBS containing 0.2 ml nucleic acid preparations, whose contents
Oncogenic Properties of SV-40 DNA

were determined to be, were 0.1 ml cell-free filtrate inoculated by nucleic acids as shown in the following and 0.1 ml intact SV-40 virus (10\(^{8.5}\) TCID 50/0.1 ml) was left as the control. The coverslips were taken from the Leighton tubes at intervals of 4, 24, 48 hours and 3, 5, 7, 10 and 14 days after the inoculation of these agents. They were rinsed in PBS at pH 7.4, dried in air and fixed for 10 minutes in acetone. One or two drops of 3-fold dilution of the anti-SV-40 rabbit serum were placed on the coverslips and incubated in a humidity chamber at 37°C for 30 or 60 minutes. They were then washed for 15 minutes in PBS on the magnetic stirrer, and one or two drops of the 3-fold dilution of fluorescein conjugated globulin solution were added. After the incubation for 40 minutes at 37°C incubator they were washed in PBS for 15 minutes on the magnetic stirrer and mounted in phosphate buffered glycerine on slides.

**Acridine orange staining technique:**

After 5 minutes fixation in Carnoy's fluid, and staining for 8 minutes in 0.001 per cent acridine orange at pH 4.0 in McIlvaine's citric acid disodium phosphate buffer, coverslips were mounted on standard microscope slides, using the buffer as mounting medium, and examined in the fluorescence microscope screened to transmit blue-violet light. Enzyme digestion tests were carried out on similar fixed preparations prior to acridine orange staining and all the specimens treated with enzymes were brought to pH 4.0 in McIlvaine's buffer before staining. The enzymes and their concentration used were as follows: RNase 0.05 per cent 5 x crystallized (Nutritional Biochemical Co.) at pH 7.4 in PBS for 30 minutes: DNase 0.01 per cent 1 x crystallized (Nutritional Biochemical Co.) 0.025 M veronal buffer containing 0.005 M MgSO\(_4\) for 30 minutes.

**Experiments in newborn hamsters:**

As shown at Tables 2, 3 and 4 newborn Syrian golden hamsters were used in an attempt to induce tumors and to observe tumor pathogenesis in vivo. Newborn hamsters were injected with 0.1 and 0.2 ml of nucleic acid preparations proven to have the infectivity of 10\(^{1}\) TCID 50/0.1 ml as shown in Table 1 and 0.1 ml cell-free filtrates of BSC-1 inoculated with nucleic acids (10\(^{6}\) and 10\(^{8}\) TCID 50/0.1 ml) subcutaneously or intracerebrally (Table 3).

As the control groups, newborn hamsters were injected with nucleic acids preparation extracted from the host cell of BSC-1, medium of 199 containing 10 per cent fetal calf serum and intact SV-40 virus (Table 4). On the other hand, untreated newborn hamsters also were observed.

The tissues of hamsters were fixed 10 per cent buffered formalin or absolute ethanol, embedded, sectioned and stained with hematoxylin-eosin, PAS, trichrome phosphotungsten hematoxylin, Feulgen and methylgreen-thionin stainings.
Hemagglutination for agents and hemagglutination inhibition tests for viral antisera:

As the agents used for the tests, SV-40 virus, nucleic acids and cell-free filtrates of BSC-1 cells inoculated with nucleic acids were examined for hemagglutination test. To two-fold dilution of SV-40 virus, nucleic acids, or filtrate of BSC-1 inoculated with nucleic acids and an equal volume of 0.5 ml of washed guinea-pig erythrocytes in 0.85 per cent sodium chloride solution were added and the mixtures were incubated at 4°C until the cells settled in patterns on the bottom of the tubes. Blood for the hemagglutination inhibition tests was collected by decapitation of the hamsters and also anti-SV-40 rabbit serum obtained by cardiac puncture from rabbits was examined. Tumor bearing hamsters induced by infectious nucleic acids, the cell-free filtrates of BSC-1 cell inoculated with nucleic acids, intact SV-40 virus, and non-inoculated normal hamsters were bled and their sera were tested for hemagglutination inhibition.

RESULTS

I. Infectivity of nucleic acid in vitro:

Depending upon the concentration of nucleic acids in the inocula, the magnitude and rapidity of cytopathic changes (CPE) varied and CPE was usually detectable with in 14 days (Table 1). CPE could be detected in the BSC-1 inoculated with nucleic acids in doses of 393, 208, 161, 168, 152, and 106 μg per ml. Generally, the development of CPE in the susceptible cells was prolonged comparing with intact viral effect (Table 1) and various cytochemical changes were observed and distribution of cellular nucleic acids could be detected by acridine orange staining long before the appearance of any CPE in the light microscope. Usually CPE was detectable very slowly 15 or 17 days after incubation of nucleic acids, and nucleic acids proved to produce a cytopathic effect identical with the one caused by an intact virus (Figs. 1 and 2). By 14 days

Table 1  Titration of nucleic acids and cell-free filtrates of BSC-1 inoculated with nucleic acids. TCID 50/0.1 ml.

<table>
<thead>
<tr>
<th>Original intact SV 40 virus</th>
<th>Extracted nucleic acids</th>
<th>Observation period (days)</th>
<th>Cell-free filtrates of BSC-1 passed with nucleic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^10</td>
<td>NA-4</td>
<td>14-21</td>
<td>10^5-10^6</td>
</tr>
<tr>
<td></td>
<td>10^4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NA-3</td>
<td>14-21</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10^5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicator cell: BSC-1 cell ** Medium: 199 containing of 2% calf serum and 199 containing 10% fetal calf serum.
Photos. 1 and 2
Cytopathic effects on BSC-1 inoculated with nucleic acids. Note a typical vacuolation of BSC-1 (Fig. 1), enlargement and clearing of the nuclear chromatin (Fig. 2), which are identified to be the one by intact SV-40 virus, at 15 days postinoculation of nucleic acid. Wright-Giemsa staining. N: Nucleus, C: Cytoplasm
Photos. 3—12 *Newborn Hamsters Injected with Infectious DNA*

Photo 3
Nodular lymphoid cell accumulation (L) in the portal space of the liver, age 39 days old, H-E staining. V: Portal vein, B: Bile duct.

Photo 4
Active proliferation of the bile ducts (B) in the portal space of the liver. 100 days postinoculation, H-E staining. P: Liver parenchymal cells.

Photo 5
Subcutaneous tumor induced by infectious DNA. (N. A-3) 185 days postinoculation.

Photo 6
Histologic appearance of subcutaneous tumor induced by infectious DNA. Note fibrosarcoma and transformed cells (T) from the fibroblastic cells associated with perivascular cells. V: blood vessel. 185 days postinoculation. H-E staining.
and 25 days the nucleus was enlarged, and clearing of the nuclear chromatin could be observed by light microscope (Fig. 2), and there was also an increase in brightness of DNA staining in nucleus and a marked increase in brightness by fluorescence microscope. By 14 and 25 days postinoculation brilliant yellow-green substances filled the whole nucleus, and the first cytoplasmic vacuolation could be observed. By 25 days cytoplasmic vacuolation was well established and a regular tessellated pattern of vacuoles was brilliantly outlined with RNA staining material.

The infectivity of these nucleic acids was completely destroyed by exposure to 20 and 100 μg per ml of DNase in the presence of 0.005 M MgSO₄ for 30 minutes at room temperature but the treatment with RNase had no effect on the infectivity of the nucleic acids as mentioned above. From these findings it has been revealed that infectivity of the nucleic acids was related to viral DNA.

**Immunofluorescence findings:**

The cell-free filtrates of BSC-1 inoculated with nucleic acids was examined for the specific immunofluorescence studies. When the monolayer of BSC-1 cells was inoculated with cell-free filtrates of BSC-1 whose CPE could be observed by inoculation with nucleic acids, the first specific antigen was detectable at 24 hours after the inoculation but at this time less than 2 per cent of the cell exhibited fluorescence. By 24 hours some of the cells revealed fluorescence within the nucleus and the nucleus was completely filled with a brilliant fluorescent mass. These fluorescent mass began to disintegrate later and by 7 days postinoculation both cytoplasmic and nuclear fluorescence could be observed.

The nuclear fluorescent mass had begun to lose its integrity, being located in the nuclear membrane and cytoplasm as brilliant particulate chunks. By 21 day period of the postinoculation there was essentially no nuclear fluorescence detectable but an antigen could be observed in the cytoplasm of cells in which a definite cytopathic effect could be observed at this stage.

It is suggested that the indirect fluorescence technique might be employed not only for the detection of replication of virus developing into complete virus from nucleic acids but also for the differentiation of the specific antigen in the virus susceptible cells.

**II. Infectivity and oncogenic properties of nucleic acids in newborn hamsters**

**A. Induction of tumors in newborn hamsters inoculated with nucleic acid preparation:**

As shown in Table 2, 235 hamsters received a single subcutaneous injection of 0.1 ml or 0.2 ml nucleic acid preparation and 4 hamsters received an intracerebral injection of 0.01 ml nucleic acids within 24 hours of birth. New-
Oncogenic Properties of SV-40 DNA

Table 2 Induction of tumors in newborn hamsters inoculated with nucleic acids

<table>
<thead>
<tr>
<th>Inoculum dose of nucleic acids (ml)</th>
<th>Route</th>
<th>Number of hamsters Injected</th>
<th>Lost</th>
<th>With tumor</th>
<th>Per cent</th>
<th>Appearance of tumors (days)</th>
<th>Localization of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. A.-1 0.1</td>
<td>subcutaneous</td>
<td>51</td>
<td>36</td>
<td>0/15</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. A.-2 0.1</td>
<td>subcutaneous</td>
<td>6</td>
<td>3</td>
<td>0/3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. A.-3 0.1</td>
<td>subcutaneous</td>
<td>123</td>
<td>59</td>
<td>1/64</td>
<td>1.5</td>
<td>*126</td>
<td>Liver, lung intestine subcutaneous tissue</td>
</tr>
<tr>
<td></td>
<td>subcutaneous</td>
<td>3</td>
<td>0</td>
<td>2/3</td>
<td>66</td>
<td>130—185</td>
<td></td>
</tr>
<tr>
<td>0.01 intracerebral</td>
<td></td>
<td>11</td>
<td>7</td>
<td>0/4</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. A.-5 0.1</td>
<td>subcutaneous</td>
<td>41</td>
<td>7</td>
<td>0/34</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>235</td>
<td>112</td>
<td>3/123</td>
<td>2.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Date of death

Born hamsters (123 animals) were used for this experiment where cannibalism was a serious problem. On 30 days after the inoculation, very remarkable changes occurred in the liver, lung and brown fatty tissues along the injected area. Particularly cellular responses of vascular cells were predominant, and these were observable in the interstitial tissues of the liver and lung. At about 14 and 29 days postinoculation, the perivascular foci consisted of lymphoid cells accompanied with many plasma cells appeared in the portal spaces of the livers of all the hamsters inoculated by infectious DNA. At 29 days the foci were increased in size and number with lapse of time and developed into nodular formation. The lymphoid cell foci persisted in the liver for many weeks (Fig. 3). At the early stage of 14 and 30 days, congestion and dilatation of the sinusoid in the liver were remarkable and at the later stage of 50 and 120 days small hemorrhagic lesions and formation of microhemangioma appeared in the liver. However, degeneration of the liver parenchymal cells was relatively inconspicuous and in a few cases peripheral fatty metamorphosis and vacuolation appeared on 32 days postinoculation. On the 100 days following the inoculation of nucleic acids, proliferative activity of the interstitial cell components was combined with inflammatory cell invasion in the portal spaces of the liver (Fig. 4) and these proliferative cellular responses were found predominantly in the newborn hamsters inoculated by cell-free filtrates of BSC-1 inoculated with nucleic acids.

Histological changes of the kidney was inspicuous comparing to changes in the liver and lung. As 30 and 131 days postinoculation, congestion and hemorrhage appeared in the alveolar septum of the lung and subsequent to these changes typical viral pneumonia developed in the lung associated with proliferative...
Oncogenic Properties of SV-40 DNA

Photo 7
Newborn hamsters transplanted with cultured cells ($6 \times 10^6$/ml) of the subcutaneous tumor induced by infectious DNA. Age 40 days old.

Photo 8
40 passage cultured cells from fibrosarcoma induced by infectious DNA. 180 days cultured.

Photo 9
Multiple tumors occurred in the liver, lung and intestine (H). 126 days postinoculation.

Photo 10
Neoplasms of the liver and lung (H) in primates induced by infectious DNA. 126 days postinoculation.
tive delicate connective tissues and inflammatory changes. On the other hand, multinucleate giant cells appeared in the subcutaneous brown fatty tissues accompanied with granulomatous inflammation after nucleic acid inoculation.

Three hamsters of these animals inoculated with nucleic acids subcutaneously developed tumors, and two of them appeared in the subcutaneous area on 130 days postinoculation, which were identified as one induced by intact SV-40 virus (Fig. 5). Characteristics of cellular composition of this tumor were differentiated fibrosarcomas and these were presumably related to proliferative responses of the vascular cells (Fig. 6). It seems to suggest that the perivascular cell is one of the mother cells developing to malignant transformation. There were particularly, numerous new formations of capillary vessels, active proliferation of perivascular cells and connective fibers centering these vascular walls.

Morphologically, in the cellular patterns of tumors there are two types, one of which shows fibroblastic spindle-shaped and the other with reticulum patterns accompanied with multinucleate giant cells. These transformed cells can also be cultured in vitro and they are transplantable in newborn hamsters (Figs. 7 and 8).

The other tumor could be induced in the liver, lung, intestinal ducts and abdominal surface at 126 days postinoculation subcutaneously. The cytological appearance showed multiple hemangiosarcoma and reticulum patterns combined with proliferation of vascular cells (Figs. 9, 10, 11 and 12).

**B. Induction of tumors in newborn hamsters inoculated with cell-free filtrates of BSC-1 infected by nucleic acids:**

Thirty-nine hamsters (Table 3) developed tumors within about 200 days postinoculation. Sarcomas were common and they were confined to the subcutaneous tissues in 35 hamsters and to the peritoneum in 2 others by subcutaneous inoculation of the filtrates. However, the intestinal gland-cell carcinomas could be induced at 37 and 59 days postinoculation in two hamsters of one litter (7 hamsters) and in the other 3 newborn hamsters subcutaneous sarcomas were induced by inoculation of the same agent.

As shown in Table 3 cellular responses to nucleic acids were more stimulated by inoculation of the filtrate than of nucleic acids and essential histologic findings were similar to the response to nucleic acids. The remarkable findings were also seen in the liver, lung, spleen, intestinal ducts, and subcutaneous tissue in the areas injected with this filtrate but the brain, excepting animals inoculated intracerebrally, pancreas and reproductive organs appeared entirely normal.

The most characteristic change was vascular cellular response combined with active proliferation of perivascular cells and connective tissue. On 7 days...
Photo 11
Hemangiosarcoma of the liver induced by infectious DNA (S). 126 days postinoculation. PAS staining.

Photo 12
Pulmonary neoplasm. Note active nodular proliferation of fibroblastic cells (F) and reticulum formation centering around pulmonary blood vessels. H-E staining. 126 days postinoculation.
Table 3 Induction of tumors in newborn hamsters injected by cell-free filtrates of BSC-1 inoculated with nucleic acids

<table>
<thead>
<tr>
<th>No. of nucleic acids</th>
<th>Inoculum dose cell-free filtered passed by nucleic acids</th>
<th>Route</th>
<th>Number of hamsters</th>
<th>Localization of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Injected</td>
<td>Lost</td>
</tr>
<tr>
<td>N. A-3</td>
<td></td>
<td></td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>I</td>
<td>0.1</td>
<td>subcutaneous</td>
<td>43</td>
<td>12</td>
</tr>
<tr>
<td>II</td>
<td>0.1</td>
<td>subcutaneous</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>N. A-4</td>
<td>0.1</td>
<td>subcutaneous</td>
<td>95</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>181</td>
<td>53</td>
</tr>
</tbody>
</table>

* 2 hamsters could be induced in cancer.

postinoculation, congestion, and dilatation of the sinusoid, and portal vein were predominant in the liver, and Kupffer's cells were swollen and regressed at an early stage. By 30 days postinoculation there appeared perivascular foci composed of predominantly lymphoid cells with many plasma cells in the wall of the portal veins and hyperplasia of bile ducts and active proliferation of the connective tissues in the portal spaces (Fig. 13), which were recognizable in 200 day-old tumor bearing hamsters. These interstitial cellular responses, which are associated with active proliferation of vascular cells and connective fibers, represent outstanding characteristic responses to infectious DNA and SV-40 virus in vivo. In this group no hemangiosarcoma developed in the liver but it also was interesting to note that liver cirrhosis was accompanied with active new formation of capillary vessels and proliferation of delicate connective fibers, and cholangiole cells were found on 37, 88, 82, 93, 176 days postinoculation (Fig. 14). As shown in Fig. 15, active fibrosis in the portal spaces of the liver sometimes

Table 4 Induction of tumors in newborn hamsters inoculated with intact SV-40 virus

<table>
<thead>
<tr>
<th>SV 40 Titer TCID 50/0/1</th>
<th>Inoculum dose</th>
<th>Route</th>
<th>Number of hamsters</th>
<th>Localization of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Injected</td>
<td>Lost</td>
</tr>
<tr>
<td>I</td>
<td>10³</td>
<td>0.1 subcutaneous</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>10⁶</td>
<td>0.1 subcutaneous</td>
<td>51</td>
<td>20</td>
</tr>
<tr>
<td>III</td>
<td>10⁷</td>
<td>0.1 subcutaneous</td>
<td>120</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>177</td>
<td>46</td>
</tr>
</tbody>
</table>
Photos. 13—18 Newborn Hamsters Injected by Cell-Free Filtrates of BSC-1 Inoculated with Infectious DNA

Photo 13
Adenomatous proliferation of bile ducts (CH) accompanied with fibrosis (F) in the liver. 176 days postinoculation. H-E staining.
Primary liver cirrhosis without damage of parenchymal cells. Note proliferation of blood capillaries, bile ducts (CH) and connective tissue (F) located in the portal spaces of the liver. 51 days post-inoculation. Azan staining. CV: Central vein. V: Portal vein. P: Liver parenchymal cell.
Photo 15
Predominant fibrosis (F) in the portal spaces of the liver of tumor-bearing hamsters. Note the cellular pattern resembling to pulmonal tumor (Fig. 12) and other fibrosarcoma. H-E staining. V: Portal vein P: Liver parenchymal cells.

Photo 16
Cellular patterns of the subcutaneous tumors. Note giant cells (G) and transformed cells from the blood vessel cells (fibroblastic cells) (T). H-E staining.
resembled the sarcomatous pattern in the lung (Fig. 12).

Including tumor bearing hamsters, at 43 and 198 days postinoculation viral pneumonia as found in all hamsters inoculated with filtrates. In 4 hamsters of 39 tumor bearing hamsters, metastatic foci were seen in the lungs.

Histological changes of the kidney were relatively inconspicuous, and the infiltration of lymphoid cells and plasma cells was not remarkable. In an early stage, slight congestion and hemorrhage of the glomeruli were observed and tumor metastases were found in two hamsters during the observation period of 200 days.

All the tumor bearing animals revealed predominantly perifollicular hemorrhage, proliferations of plasma cells and reticulum cells in the infected spleen.

Within 30 days postinoculation there appeared a striking granulomatous inflammatory consisted of lymphoid cells, plasma cells, and hemorrhage of capillary blood vessels in the subcutaneous tissues inoculated with filtrates. Particularly outstanding proliferation of the perivascular cells was related to developing of malignant transformation, and this proliferative change in the perivascular cells was found not only in the injection areas but also in various other interstitial tissues.

Most of the tumors found in hamsters were fibrosarcomas composed predominantly of well-differentiated spindle-shaped or stellate cells (Fig. 16), and excepting carcinomas induced in primates, some of the lesions had an abundant fibrous matrix in which myxomatoid structure predominated. In some of lesions, anaplastic cells presented a reticular cell pattern. In many cases these cells were combined with multinucleated giant cells, and occasionally delicate fibrils in cytoplasm of these fibrils in cytoplasm of these giant cells were found (Fig. 16).

It was very significant that intestinal carcinoma (Fig. 17) in primates could be induced as one of oncogenic potentialities of this filtrate of BSC-1 inoculated with nucleic acids. Carcinoma cells are well differentiated and these cells invaded into the submucosa and subserosa. Lamina muscularis is destroyed by invasion of cancer cells (Fig. 18).

DISCUSSION

One of the important developments of present day virology is the finding that administration of pure viral nucleic acids produces typical virus-induced cytopathology and histopathology of host tissues from which intact virus can be recovered. GIERER and SCHRAMM reported that RNA from tobacco mosaic virus is infectious, but only at extremely low titer and the studies by FRAENKEL-CNONAT and co-workers have shown the genetic specificity of heterologous
Oncogenic Properties of SV-40 DNA

Photo 17
Well-differentiated gland-cell carcinoma in the small intestine. 59 days postinoculation. H-E staining.

Photo 18
Well-differentiated gland-cell carcinoma in the small intestine. Invasion by cancer cells (G) into the serosa (S) is shown in the central portion and cancer infiltration accompanied with PAS positive cells (PA) into the serosa is observable on the bottom. L: Lamina muscularis. 59 days postinoculation. PAS staining.
combinations of RNA, protein from Holmes Ribgrass virus, and tobacco mosaic virus residues in RNA.

The infectivity of nucleic acid of animal virus was also demonstrated clearly by Colter and his associates. Utilizing the previous method of Gierer and Schramm, the extraction of RNA by using of phenol, they found that extracts of tissue infected with polio and W. Nile encephalitis viruses were infectious (at the titer of 0.1 per cent when inoculated into mouse brain). Although Colter noted cytopathic effects of RNA preparations when they were assayed in tissue culture, these were not always reproducible. However, Alexander and associates demonstrated reproducible plaque formation using polio RNA and that the use of a hypertonic salt medium was necessary to produce extensive infection from which intact normal virus could be recovered. In contrast to the examples cited above, comparable evidence does not exist for the infectivity of nucleic acids of tumor viruses. Although the infective unit of the Rous sarcoma virus has not been prepared as pure RNA (Bryan and Moloney), Bather has shown a strong relationship between infectivity and RNA content of partially purified virus preparations.

On the other hand, findings of infectious DNA have not been substantiated fully. Mayorca, Eddy, Stewart, Hunter, Friend, Bendich have offered evidence that a purified infectious component can be isolated from a crude SE polyoma virus suspension and it is DNase sensitive and RNase resistant, suggesting that it may be DNA. Ito and Evans have succeeded in inducing tumors in domestic rabbits with nucleic acid from partially purified shope papilloma virus and from extracts of the papillomas of domestic and cottontail rabbits. It is also demonstrated clearly by this study that nucleic acid extracted from tumor is related to its oncogenic properties.

Concerning nucleic acids of SV-40, Hsiung and Gaylord, using the Feulgen staining reaction were the first to demonstrate that simian virus 40 was a DNA virus. In 1962, Borisson et al. using SV-40 grown on baban (papio papio) cells with a titer of 10⁸ TCID 50/0.1 ml extracted nucleic acid by Weil's modification of the Kirby method freeing DNA from protein by the use of phenol. More recently Gerber described that infectious DNA derived from SV-40 was extracted by cold phenol method of Gierer and Schramm as modified by Alexander et al. and nucleic acids produced a cytopathic effect identical with the one caused by an intact SV-40 propagated in cercopithecus kidney cell cultures and banded in calcium chloride by the method employed by Weil for extracting polyoma virus DNA. Weil and Eddy reported that infectious virus could be recovered by incubating the nucleic acid with washed and warmed cercopithecus kidney cell cultures and also tumors could be induced in the hamster.
DIDERHOLM et al.\textsuperscript{27} also reported naturally insusceptible cells were infected by DNA of polyoma and SV-40 viruses. Our studies are concerned with the capacity of nucleic acid partially purified from SV-40 virus, to elicit malignant responses in hamsters. The tumor producing capacity of cell-free filtrates of cells inoculated with nucleic acids can be increased by incubation in host cells. These findings suggest that nucleic acid penetrated into the host cell without digestion by nuclease in the blood has oncogenic capacity \textit{in vivo} as HERRIOTT\textsuperscript{28,29} reported in 1961.

For assay of the infectivity of nucleic acid, DNase, RNase and anti-SV-40 rabbit serum were used. Infectivity of nucleic acid was completely destroyed by DNase but treatment with RNase and specific anti-SV-40 rabbit serum had no effect. It is confirmed that infectivity of nucleic acid is contained in DNA. In addition, studies on this infectious DNA by the use of specific immunofluorescence methods have now established that a complete virus may be reproduced from its nucleic acid in a host cell by coating with protein on the surface of the naked nucleic acid.

In newborn hamsters inoculated with nucleic acids subcutaneously (Table 2) cellular responses were observed in the interstitial tissues and particularly these histological changes were found in the vascular tissues in the liver and lung of all the newborn hamsters infected with nucleic acids and cell-free filtrates of BSC-1 inoculated by nucleic acids. It has been verified that SV-40 DNA and intact virus have the active proliferative potentiality for fibroblasts \textit{in vivo}. Tumors could be induced in 2 of 3 hamsters injected with 0.2 ml nucleic acids and in one hamster injected with 0.1 ml nucleic acids subcutaneously (Table 2). In this case of one hamster, multiple hemangiosarcoma, which seemed to be arising from proliferation of perivascular cells was observed in the liver, lung, intestine, and on the peritoneal surfaces. The histological appearance of hemangiosarcoma was identical with hemangiosarcoma which was observed in the hamster induced by mouse tumor agent (polyoma virus) passed in tissue culture by EDDY, STEWART and YOUND et al\textsuperscript{30}.

In general, histological changes and the efficiency of infection in newborn hamsters inoculated with the low titer of infectious DNA are lower in the case of intact virus.

In the newborn hamsters inoculated with cell-free filtrates passed by nucleic acids, oncogenic capacity was markedly accelerated (Tables 2 and 3) and localization of tumors were also extensive in the subcutaneous tissues, peritoneum, and intestine in primates (Table 3). In addition, metastases in the lung and liver from the subcutaneous sarcoma were found. Particularly important was the finding that well differentiated gland-cell carcinoma could be induced in the small intestinal ducts after the inoculation period of 39 and 59 days by cell-free
filtrates of BSC-1 inoculated with nucleic acids (Figs. 17 and 18).

Concerning the information available on spontaneous tumors in Syrian golden hamsters, ASHBEL\textsuperscript{11} reported that only 13 tumors were observed in 1,000 hamsters, HABERMANN\textsuperscript{12} examined 171 hamsters 7 to 11 months old and 342 hamsters 3 to 5 months and found only one neoplasm, a cystic mammary adenocarcinoma among them, and spontaneous tumors were rare in the Syrian golden hamster, until 1957 when a multiplicity of tumors was reported by FORTNER\textsuperscript{33}. According to Fortner's reports, the incidence of adenocarcinoma in the small intestine was 10 out of the total 181 hamsters and the age range of malignant tumors of the small intestine and colon 25 months in female or 32 months in male. The length of the observation period on hamsters was limited to about 250 days in view of the incidence of the spontaneous tumor and contamination of other unknown carcinogenic agents.

In 1963, BLACK and ROWE et al.\textsuperscript{34,35} reported on the cell analysis of SV-40 induced transformation of hamster kidney tissues \textit{in vitro}, and a possible occurrence of tumor arising from some clone of epithelial differentiation by SV-40.

Histologically, cellular responses between nucleic acids and cell-free agents of BSC-1 passed by nucleic acids were identical and in general, interstitial cell responses were highly susceptible for the agents. Liver cirrhosis in primates and extensive proliferation of fibrocytes and connective tissues associated with vascular cells were specific and striking changes \textit{in vivo}. Considering of cellular responses to these agents, various fibrosarcomas of the subcutaneous and hemangiosarcoma of the liver were closely associated with inflammatory proliferation of the vascular cells (Figs. 6, 11, 12 and 16). Microhemangioma and fibrosis of the liver at early stages were also related to potential anaplastic changes. Experimental and untreated hamsters, on the other hand, proved to be not contaminated with any polyoma virus from the negative results of hemagglutination and hemagglutination inhibition tests as mentioned above.

From the experiment concerned with the nucleic acids derived from SV-40 virus it has been demonstrated that SV-40 viral DNA possesses the tumor-induction capacity in hamsters, and fibrosarcoma, multiple hemangiosarcoma and differentiated gland-cell carcinoma in primates can be induced in newborn hamsters by inoculation of SV-40 nucleic acids. According to the results, the initiation of an infection depends on whether at least one DNA can penetrate into a susceptible cell, particularly fibroblast \textit{in vivo} and \textit{in vitro}, where it is safe from the usual host defense mechanisms. Once a cell is infected by infectious DNA and a complete virus is reproducible in a host cell, the spread of the infection in tissue is determined by the same forces plus the added, but little understood, feature of cell-to-cell transmission of the nucleic acids, possibly without its exposure to extracellular insults, for example DNase as a defense mecha-
nisms. It seems to be reasonable to suggest that the nucleic acid possesses a possibility of oncogenic properties in a susceptible cell.

CONCLUSION

The present report describes the findings on the infectivity of DNA partially purified from SV-40 which was propagated in the monkey kidney cells (BSC-1) in vitro and the importance of nucleic acids as oncogenic factors, particularly the induction of tumor by DNA in newborn hamsters. 593 newborn hamsters in total were used in the present experiments, and cannibalism among them posed as a serious problem. On 30 days postinoculation, very remarkable changes occurred in the liver, lung and subcutaneous areas. Cellular responses of the perivascular cells were predominant, and they were distributed in the interstitial tissues of the liver (liver cirrhosis in primates) and lung. Three hamsters of those subcutaneously inoculated with nucleic acids developed tumors and two tumors appeared in the subcutaneous tissues on 130 days postinoculation, which were identified to be the ones induced by intact SV-40 virus. Other tumors appeared in the liver, lung, intestinal ducts and abdominal surface at 126 days after subcutaneous injection. The cytological observations revealed multiple hemangiosarcoma combined with proliferation of the perivascular cells.

On the other hand, cellular responses to nucleic acids were more marked by inoculation of the cell-free filtrate of BSC-1 infected by DNA than of DNA, and essential histologic findings were similar to the response to infectious DNA. Thirty-nine hamsters (30 per cent) developed tumor within about 200 days postinoculation of the filtrates. Sarcomas were common and they were confined to the subcutaneous tissues in 35 hamsters and to the peritoneum in two others by subcutaneous inoculation of the filtrates.

The intestinal gland-cell carcinomas, however, could be induced at 37 and 59 days postinoculation in two hamsters of one litter (7 newborn hamsters) and in the other three newborn hamsters subcutaneous sarcomas were induced by inoculation of the same agent. These results suggest that the observation on the oncogenic capacity of nucleic acids would give us a clue to resolve the course of cancer from the view point of the infectious nucleic acid.

ACKNOWLEDGEMENT

The experiment was supported by Hartford grant (1963 and 1964) of New York State. U. S. A. We wish to thank Drs. Edith SProul, Thomas STim*, Ross H. HALL*, Edwin MIRAND* and Satimaru SENO** for valuable advices and acknowledgement is also due to Blakeslee JIM, Miss. Taggert JOHANNE for technical assistances.

* Roswell Park Memorial Institute, Buffalo, New York, 14203, U. S. A.
** Dept. of Pathology, Okayama University Medical School, 164 Oka, Okayama, Japan
REFERENCES

growth characteristics, and inclusion body formation. J. Exp. Med. 114, 975, 1961
32. HABERMANN: cited from (33)