Synopsis

Bovine adenovirus type-3 was inoculated subcutaneously in hamsters of various ages from 1 to 95 days. Tumors developed in many hamsters of every age group. When the virus was inoculated into animals younger than 14 days, two types of tumor, differentiated and undifferentiated, developed at the site of injection, which grew non-progressively and progressively, respectively. In animals older than 21 days, undifferentiated type of tumor developed and its growth was of non-progressive type. Such growth behavior of the tumors seemed to depend on the age-dependent resistance of the host to tumor growth.

INTRODUCTION

In 1966, Darbyshire reported that the WBR-1 strain of bovine adenovirus type-3 had a high oncogenicity in newborn hamsters. The tumor did not grow further when it reached a size of approximately 2-3 cm in diameter, being non-progressive, but when such a tumor was transplanted, it grew progressively, finally leading the animals to death. It is also reported that high doses of the virus produced cystic tumors, whereas low doses solid tumors. Nishibe et al. suggested that the peculiar tumor growth may be due to the heterogeneous population of the virus particles, each having different tumorigenicity.

To elucidate the mode of tumor growth, the virus was inoculated into hamsters of various ages and the tumor growth was observed, comparing with histological pictures.

MATERIALS AND METHODS

Virus The WBR-1 strain of bovine adenovirus type-3 was kindly supplied by Dr. Y. K. Inoue, Institute for Virus Research, Kyoto University. The virus was propagated in the primary calf kidney cells, which were maintained in the Eagle’s minimum essential medium containing 2% calf serum. The titer of the virus was tentatively determined with the same cells by 50% end point 7 days after infection. The virus fluid with titers of $10^3.5$, $10^4.5$, and $10^5.5$ TCID$_{50}$/0.1 ml was used for the following experiments.

Animals Golden hamsters bred in our laboratory, ranging in age from 1 to 95 days, were used and the virus was inoculated subcutaneously on the back.

*1 This constitutes Part I of a series entitled “Pathological Studies on Carcinogenesis by Bovine Adenovirus Type-3”.

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Observation After virus inoculation, the growth of tumors was observed by palpation once every week and the size of tumors was measured.

Transplantation The extirpated tumor was cut into small pieces with scissors in physiological saline solution, and a piece of tissue, about $2 \times 2 \times 2$ mm, was transplanted with trocar into 4- and 30-day-old hamsters. The remaining tissues were examined histologically.

Histological Examination A complete autopsy was performed on all the animals sacrificed. The tumor tissues were fixed in 10% Formalin solution, dehydrated, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin. Some sections were also stained with phosphotungstic acid–Hematoxylin, Azan's stain, and silver impregnation.

Detection of T-Antigen Following the method of Nishibe et al.,7,12) 1 ml of the 1:1 mixture of the virus-induced tumor homogenate (20% w/v, in buffered saline) and Freund's complete adjuvant was intraperitoneally inoculated into young adult hamsters once every week. Seven days after the 6th inoculation, blood was obtained from the orbital plexus, the serum was separated, and $\gamma$-globulin fraction of the serum was conjugated with fluorescein isothiocyanate. For direct immunofluorescence tests, cultured tumor cells were treated with CCl$_4$ for 2 hr at 37° and stained with the conjugate in a moist chamber for 24 hr at 4°. The specificity of the fluorescence was examined by the blocking test and by staining the cultured normal whole embryo cells of hamsters with the conjugate.

RESULTS

Induction of Tumors by Bovine Adenovirus Type-3 in Hamsters of Various Ages

As shown in Table I, tumors developed similarly in every group of animals inoculated with the virus at birth (Expt. 1, 2, and 3) after a latent period of 28~32 days, showing no difference according to the virus dose. In the animals as young as 7 to 14 days old (Expt. 4 and 5), the tumors also developed after a latent period of 30 to 43 days. Throughout the five experiments, tumors developed multicentrically at the site of injection and some of them grew progressively in the animals less than 14 days old. In contrast, in the animals older than 21 days, no progressive growth of tumors was observed, and the incidence of tumors was 5/5 at the age of 21 days (Expt. 6), 5/7 at 35 days (Expt. 7), and 6/7 at 95 days (Expt. 8), revealing the prolongation of the latent period.

Observation of Tumor Growth

The growth of the tumors in hamsters of various ages is illustrated in Fig. 1. In newborn hamsters (Fig. 1, A) the tumors reached the size of 4 cm in diameter by 8 to 21 weeks after the virus inoculation, and all the animals died of the tumor. One hamster (No. 180) had a tumor of 0.2 cm in diameter up to 27 weeks, and thereafter showed a progressive growth until death at the 34th week. Generally, the tumors grew more progressively in females than in males. In the group inoculated with the virus at the age of 7 days (Fig. 1, B), 3 out of 4 animals showed a progressive tumor growth and they died respectively at the 37th, 46th, and 62nd week. In the group of 14-day-old animals (Fig. 1, C), 2 out of 3 died of tumor after 24 and 39 weeks, respectively. The remaining one (No. 380) survived 80 weeks with a tumor of 0.2 cm in diameter. In the group of animals older...
### Table 1. Development of Tumors by Bovine Adenovirus Type-3 in Hamsters of Various Ages

<table>
<thead>
<tr>
<th>Age of</th>
<th>Total No. of animals inoculated</th>
<th>Virus dose per hamster</th>
<th>Latent period with tumor</th>
<th>Observation period (days)</th>
<th>Growth pattern of tumors</th>
<th>Histological type of tumors</th>
<th>Differentiated and undifferentiated</th>
<th>Expt. No.</th>
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<td>(log₁₀ TCID₅₀)</td>
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</tbody>
</table>

(1) No. of virus-inoculated hamsters which survived longer than 35 days.

(2) Up to 0.5 cm.
GROWTH OF BOVINE ADENOVIRUS TYPE-3 TUMOR

than 21 days (Fig. 1, D, E, and F), no progressive growth to lead animals to death was observed. As an exception, one animal (No. 295) of this group tended to show a slight progressive growth of tumor about 72 weeks after the virus inoculation and died in the 78th week, but the tumor was only 1.5 cm in diameter and the death was due to pneumonia.

Pathological Findings of Tumors

(A) Tumors Developed by Inoculation with the Virus into Hamsters less than 14 Days Old

Macroscopic Appearance: The subcutaneous tumors induced by bovine adenovirus type-3 were solid tumors and not of cystic type. Tumors produced by the virus inoculation into animals younger than 14 days old were classified into three types, a non-progressive, slow-progressive, and progressive types. The tumor of the non-progressive type was a flat, firm tumor less than 0.5 cm in diameter and grayish white. The slow-progressive type was somewhat protuberant, pinkish, and elastic. In contrast, the progressive type had a larger and globular appearance revealing manifold colors due to hemorrhage and necrosis, and was soft in consistency (Photo 1).

Every tumor of these types was attached firmly to the skin but did not infiltrate so intensely to the deeper tissue.

Microscopic Appearance: Histological findings of each type of tumors coincided well with the macroscopic findings and the tumor growth. Tumors of the non-progressive type showed histologically differentiated characteristics and the slow-progressive and progressive types were of undifferentiated ones. The non-progressive type grew along the subcutaneous skin-muscle layer (Photo 2), and tumor cells were loosely distributed between abundant collagen fibers (Photo 14). Tumor cells were spindle shaped having slender protoplasmic projections and their nuclei were oval or spindle shaped showing no atypism. Nuclear chromatin was abundant and intranuclear structures were indiscernible. Such tumor cells were irregularly arranged, partially running longitudinally in a relatively regular array. A small number of lymphoid cells were scattered between tumor cells (Photos 3 and 4). Hyalinization of the stroma was observed in the old tumor. Silver impregnation revealed no characteristic architecture, generally yielding a small amount of argyrophil fibers. These small tumors indicated an intimate relation to the nerve fibers immediately distributed along the subcutaneous skin-muscle layer. In the non-progressive tumor tissue, a small tumor nodule of progressive type doubly developed in some instances (Photos 13 and 14).

The slow-progressive type showed a higher cellularity than the non-progressive type. The tumor cells were of short spindle or ovoid shape and arranged in strands exhibiting a slight atypism and some mitotic figures (Photos 5 and 6).

The progressive type revealed a highly undifferentiated characteristics with much cellularity and less stroma. The tumor cells were round, ovoid, or polygonal in shape with a large nucleus, having 2~3 distinct nucleoli, and revealed a strong atypism forming numerous giant cells. The cells were arranged in strands or in irregular sheet-like patterns resembling a type of human adenovirus type-12 induced tumors. The secondary changes, hemorrhage, necrosis, and mucoid degeneration, were observed (Photos 7 and 8).
Table II. Transplantation of Tumors Induced by Bovine Adenovirus Type-3 in Hamsters

<table>
<thead>
<tr>
<th>Expt. No.</th>
<th>Source of tumor</th>
<th>Type of tumors transplanted</th>
<th>Age of hamsters at transplantation (days)</th>
<th>No. of animals transplanted</th>
<th>Animals with tumors</th>
<th>Average survival (days)</th>
<th>Type of tumors developed by transplantation</th>
<th>Type of tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tumors from virus inoculation at birth</td>
<td>Non-progr.</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>76</td>
<td>Progr.</td>
<td>Undiff.</td>
</tr>
<tr>
<td>2</td>
<td>Tumors from virus inoculation at birth</td>
<td>Non-progr.</td>
<td>30</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Tumors from virus inoculation at 14 days after birth</td>
<td>Slow-progr.</td>
<td>30</td>
<td>9</td>
<td>9</td>
<td>91</td>
<td>Progr.</td>
<td>Undiff.</td>
</tr>
<tr>
<td>4</td>
<td>Tumors from virus inoculation at 14 days after birth</td>
<td>Progr.</td>
<td>30</td>
<td>5</td>
<td>5</td>
<td>54</td>
<td>Progr.</td>
<td>Undiff.</td>
</tr>
<tr>
<td>5</td>
<td>Tumors from virus inoculation at 95 days after birth</td>
<td>Progr.</td>
<td>30</td>
<td>4</td>
<td>4</td>
<td>67</td>
<td>Progr.</td>
<td>Undiff.</td>
</tr>
<tr>
<td>6</td>
<td>Tumors from virus inoculation at 95 days after birth</td>
<td>Non-progr.</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>142</td>
<td>Progr.</td>
<td>Undiff.</td>
</tr>
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</table>


\(b\) Diff.: Differentiated tumor, Undiff.: Undifferentiated tumor.
GROWTH OF BOVINE ADENOVIRUS TYPE-3 TUMOR

(B) Tumors Developed by Inoculation with the Virus into Hamsters Older than 21 Days

In animals older than 21 days, the tumors were macroscopically of non-progressive type resembling that of younger animals described above, but the histological picture was an undifferentiated type (Photos 11 and 12).

Transplantation of Tumors

The results of the transplantation of tumors induced by bovine adenovirus type-3 are shown in Table II. The tumors of slow-progressive and progressive types were transplantable in adult hamsters, and the transplanted tumors grew progressively. The survival time of the adult animals after tumor transplantation was somewhat prolonged in the slow-progressive type. The tumor of non-progressive type and the tumors developing in adult animals were transplantable only in baby hamsters, and the resulting tumors were also of progressive type.

The transplanted tumor revealed invariably an undifferentiated histological type (Photos 9 and 10) and had a strong tendency to become necrotic. When transplantation was repeated from generation to generation, the growth of tumors became progressively more rapid, and survival time of the animals seemed to be gradually shortened. The histological pictures of transplanted tumors did not show appreciable changes, at least within 4 generations.

Detection of T-Antigen in Tumors

The direct immunofluorescence technique to detect T-antigen in the bovine adenovirus type 3-induced tumors and transplanted tumors indicated that in every type of tumors, T-antigen was detected in about 10~20% of the cells as fluorescent spots of granular or fleck shape, both in the nucleus and cytoplasm or in the nucleus alone (Photos 15~18).

DISCUSSION

According to available reports,3,4,5,11) bovine adenovirus type-3 among the oncogenic adenoviruses14) appears peculiar in that the primary tumor produced by its inoculation into newborn hamsters is non-progressive and that two types of tumor, cystic and solid ones, develop in hamsters.

However, contrary to the findings of preceding reports, we observed that the primary tumor become progressive if observed for a sufficient length of time. Moreover, in spite of using the virus of high titer, none of the hamsters developed a cystic tumor. The reason for these differences is obscure, but the strain difference of animals used might be responsible.

The histology of bovine adenovirus type-3 tumor is controversial. Berman3) mentioned that the fast-growing bovine adenovirus tumor resembled the characteristic primate adenovirus tumor in histology but that the slow-growing one did not. Levenbooks16) suggested that the tumor bore evidence of vascular origin. In our observation, differentiated and undifferentiated tumor appeared to be of the same origin with different stage of differentiation on the following grounds. First, morphological transition among the tumor cells of each type and the occurrence of undifferentiated tumor in the differentiated type tumor (Photo 13) were observed. Second, when differentiated tumor was transplanted, undifferentiated tumor developed. In addition, undifferentiated tumor resembled histologically the human adenovirus type-12 tumor13) with some differences. The greater
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cellular pleomorphism, more abundant collagen fibers, and absence of characteristic rosetting were observed in bovine adenovirus tumor. A small tumor was located in intimate relation to the nerve fibers of subcutaneous skin–muscle layer. Histogenesis of this tumor is yet obscure, but from these findings we assume that the tumor is of neurogenetic origin.

Oncogenic DNA viruses, for example polyoma virus,9) SV40,8) and human adenovirus type-12,15,16) induce tumor in adult animals but the incidence of tumor development is very low. Bovine adenovirus type-3 has a high oncogenicity not only in newborns but also in adult hamsters. In this case, tumor development and its growth were influenced by the age of animals; in young hamsters, some of tumor nodules grew progressively, and in adult hamsters, tumor growth was non-progressive and the tumor was less than 1.5 cm in diameter, and remained static, which might be termed a "dormant" tumor, but it was histologically undifferentiated. As for no occurrence of differentiated type tumors in adult animals, it may be assumed that in adult animals with a strong host resistance, undifferentiated type of tumor having strong growth potentiality can grow only to a small size.

Although many factors involving the growth of tumors should be taken into consideration, much evidence1,2,6) accumulated recently suggests that the immunological function of the host may be involved in the course of tumor development. In the present experimental system, small tumor nodules developed well but the growth was suppressed in adult hamsters. This fact strongly indicates that the immunological effect in the age-dependent resistance of the host plays an important rôle in the process of promotion of carcinogenesis.

The authors are greatly indebted to Dr. Y. K. Inoue, Institute for Virus Research, Kyoto University, for kindly supplying the virus materials. The technical assistance of Miss M. Sano and Miss K. Nogami is gratefully acknowledged.

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REFERENCES

GROWTH OF BOVINE ADENOVIRUS TYPE-3 TUMOR

EXPLANATION OF PLATES LXXVIII~LXXXII

Photo 1. Macroscopic findings of tumors induced by bovine adenovirus type-3. The tumor of non-progressive type is a small flat tumor, the slow-progressive type is somewhat protuberant, and the progressive type gives a larger and irregular globular appearance.

Photo 2. A small tumor (T) is located along the subcutaneous skin-muscle layer. Hematoxylin-Eosin. ×40.

Photo 3. Histological picture of the non-progressive type. The tumor cells are distributed loosely in abundant collagen fibers, spindle shaped, showing no atypism. In the stroma a small number of lymphoid cells are scattered. Hematoxylin-Eosin. ×100.


Photo 5. Histological picture of the slow-progressive type. The tumor shows a higher cellularity than the non-progressive type, is of short spindle or ovoid shape, exhibiting a slight atypism, and arrayed in strands. Hematoxylin-Eosin. ×100.


Photo 7. Histological picture of the progressive type. The tumor reveals an exclusively high cellularity with a less amount of stroma. The tumor cells are round, ovoid, or polygonal in shape and reveal a strong atypism forming giant cells. The cells are arrayed in straods or in irregular sheet-like patterns. Hematoxylin-Eosin. ×100.


Photo 9. Histological picture of the tumor that developed by transplantation of primary tumors. The picture is similar to that of the progressive type tumor induced by the virus. Hematoxylin-Eosin. ×100.


Photo 11. Histological picture of the tumor in a hamster inoculated with the virus at 35 days after birth. The tumor shows a picture similar to the slow-progressive or progressive type. Hematoxylin-Eosin. ×100.


Photo 13. A small tumor nodule of the progressive type (P) is found in the non-progressive tumor tissue (N).

Photo 14. This picture shows the difference in the amount of collagen fibers in the non-progressive and progressive tumor of Photo 13. Azan. ×100.

Photo 15. T-antigen in the non-progressive type tumor; a small number of cells have nuclear immunofluorescent spots. ×100.

Photo 16. T-antigen in the progressive type tumor; immunofluorescent spots are noted in the nucleus and/or cytoplasm. ×100.

Photos 17 and 18. T-antigen in a tumor cell of the progressive type tumor; immunofluorescent spots of dot or fleck shape in the nucleus. ×200.