Acta Medica Okayama

Volume 30, *Issue* 3 1976 *Article* 3

JUNE 1976

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Abstract

Human adenovirus type 12 (Ad 12) was inoculated through subtentorial route into inbred newborn mice (C3H/BifB/Ki), and sequential changes of the brain and tumor induction were examined by histological and immunofluorescent methods. Two days after virus inoculation, Ad 12 specific tumor antigen (fluorescent T-antigen) appeared in the cells of ependymal and subventricular matrix layers, choroid plexuses and leptomeninges in the subtentorial as well as the supratentorial brains. After 10 days, these fluorescent positive cells decreased gradually in number but still remained focally beneath the ependyma. Sixty days later, early tumor nodules were detected in the same regions in which remained the fluorescent cells. After 107 days, neurological signs and well-developed tumors were noted in 25 of 63 (30.1%) mice examined. In the cerebellum, both of T-antigens and tumors were limited around the IVth ventricle, but not in the granular layers. Histomorphologically, the tumors were of primitive neuroectodermal origin and consisted of the cells resembling immature matrix cells in the subventricular zone. These findings strongly suggest that the virus has a selective affinity to the remaining matrix cells, but not to cerebellar granular cells, at least, in newborn mice.

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*PMID: 136869 [PubMed - indexed for MEDLINE]

Acta Med. Okayama 30, 163—179 (1976)

TARGET CELLS OF HUMAN ADENOVIRUS TYPE 12 IN SUBTENTORIAL BRAIN TISSUE OF NEWBORN MICE
I. CYTO-HISTOMORPHOLOGIC AND IMMUNOFLUORESCENT MICROSCOPIC STUDIES *IN VIVO*

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Received for publication, June 15, 1976

Abstract: Human adenovirus type 12 (Ad 12) was inoculated through subtentorial route into inbred newborn mice (C_3H/BifB/Ki), and sequential changes of the brain and tumor induction were examined by histological and immunofluorescent methods. Two days after virus inoculation, Ad 12 specific tumor antigen (fluorescent T-antigen) appeared in the cells of ependymal and subventricular matrix layers, choroid plexuses and leptomeninges in the subtentorial as well as the supratentorial brains. After 10 days, these fluorescent positive cells decreased gradually in number but still remained focally beneath the ependyma. Sixty days later, early tumor nodules were detected in the same regions in which remained the fluorescent cells. After 107 days, neurological signs and well-developed tumors were noted in 25 of 83 (30.1%) mice examined. In the cerebellum, both of T-antigens and tumors were limited around the IVth ventricle, but not in the granular layers. Histomorphologically, the tumors were of primitive neuroectodermal origin and consisted of the cells resembling immature matrix cells in the subventricular zone. These findings strongly suggest that the virus has a selective affinity to the remaining matrix cells, but not to cerebellar granular cells, at least, in newborn mice.

Brain tumors of various types have been induced experimentally with several kinds of viruses (1). The intracranial tumors induced by human adenovirus type 12 (Ad 12) show histologically very undifferentiated characters of neuroectodermal tumors and are considered to be morphologically compatible with the human infantile brain tumors such as medulloblastoma, ependymoblastoma, and embryonic glioma (2, 3, 4, 5, 6). It is well known that the brain tumors in early human life occur likely in the subtentorial tissues including the cerebellum (7, 8). However, it is still disputed whether medulloblastoma, one of the subtentorial tumors, originates from external granular cells in the cerebellar cortex (9, 10) or from embryonic resting cells in the medullary verum (11, 12, 13).

In previous reports on the oncogenesis of Ad 12, it remains still unclarified how this virus has the affinity to the cerebellum. For the purpose to elucidate the relation between the infection by Ad 12 and the tumor development in the subtentorial brain, the author inoculated Ad 12 into newborn mice through subtentorial route and then examined sequential changes of the brain by histological method and fluorescent antibody technique for the detection of T-antigen (14).

MATERIALS AND METHODS

Virus preparation and titration. The prototype "Huie" strain of Ad 12 which was kindly supplied by Prof. Y. Yabe, the Institute for Cancer Research, Okayama University Medical School, were serially cultured in HeLa cells in our laboratory. After the appearance of complete cytopathic effect, the infected HeLa cells were scraped off in one-tenth of the original medium volume, frozen and thawed 5 times and centrifuged at 1000 r.p.m. for 5 min. The supernatant fluid were tentatively titrated with HeLa cells by 50% end point ($TCID_{50}$) 5 days after infection. The virus stock with titer of 10^3 $TCID_{50}$/0.1 ml was used.

Preparation of anti-T conjugate. Anti-sera for T-antigen were obtained from hamsters carrying a transplanted tumor originally induced by Ad 12. After blood-letting from the retroorbital plexus of the hamsters, the sera were separated from the pooled blood by centrifugration and stocked at −20°C. Crude γ-globulin was precipitated with cold, one third saturated ammonium sulfate and labeled with fluorescein isothiocyanate (FITC) at pH 9.0 for 6 hr at 4°C. The conjugates were passed through a column of Sephadex G-25 to remove unbound FITC and then purified by passing over a DEAE cellulose column. Finally, the samples were filtered with a millipore filter and stocked at 10°C till use. The conjugates were absorbed twice with 100 mg/ml of acetone-extracted hamster liver powder immediately before staining.

Animals. Inbred newborn mice of C_3H/Bif B/Ki strain were obtained from Mouse Colony of Okayama University Medical School.

Virus Inoculation. Newborn mice within 24 hr after birth were inoculated subtentorially with 0.005 to 0.01 ml of the virus fluid at the occipital region. Control mice were similarly inoculated with the supernatant from uninfected HeLa cells. One hundred and sixty-three animals were divided into two groups. In one group, two or three of 51 mice were sacrificed every 2 or 3 days to examine the sequential changes of the brain after the virus inoculation and to detect the T-antigen and early tumors. In the other group, 25 mice showing distinct neurological signs were sacrificed to examine the pathomorphological characteristics of the tumors. Fifty-eight mice without any signs and 29 control mice were sacrificed over 280 days after the virus inoculation.

Detection of T-antigen. The brains with cranium of mice younger than one month and those of without cranium of adult mice were quickly frozen in n-hexane cooled with dry-ice-acetone mixture. The brain tissues were sectioned by a cryostat at 6 to 10 μ in sagittal direction, treated with cold acetone for 10 min

and then stained with the conjugate for 60 min at 37°C in a moist chamber. For blocking tests, the specimens were covered with unconjugated serum including anti-T globulin for 30 min before staining. The stained sections were mounted with buffered glycerin (pH 9.0) after washing in phosphate buffered saline (PBS) and were observed by a fluorescent microscope. Thereafter, they were stained with hematoxylin-eosin (H-E) to identify the fluorescent cells.

Histological examination. Most brains and spinal cords of animals were fixed in 10% buffered formalin, embedded in paraffin and sectioned serially on the sagittal or frontal plane. The sections were stained with hematoxyline-eosin (H-E) and, if necessary, with phosphotungstic acid-hematoxylin (PTAH), silver impregnation, Bodian's nerve fiber stain, Mallory's azan stain and Penfield's or Cajal's method for glial fiber.

RESULTS

A. *Sequential changes of the brain from virus inoculation to tumor development.*

1) *Histological findings*

Mice inoculated with Ad 12 and control mice were sacrificed sequentially from one day to 80 days. No differences in growth patterns of the brain were observed between these two groups.

From the first to the fifth day, walls of the olfactory, lateral and IVth ventricles were composed of inner single-layer of ependyma and outer thick layer of immature matrix cells (Fig. 1). On the seventh or the tenth day, the number of immature cells decreased rapidly, but later some of them forming a small group remained scatteringly beneath the ependyma (Fig. 2). After the sixtieth day, such cell rests grew scarce in the ventricular wall.

In the cerebellar hemisphere, from the first to the seventh day, small neuronal cells were arranged to form the external granular layer of the cortex (Figs. 3 and 4). Thereafter, these cells migrated inward to form the inner granular layer. The external granular layer gradually decreased in thickness (Fig. 5) and almost disappeared on the 14th day. Then, the cerebellar cortex became to be composed of outer molecular layer and newly-formed inner granular layer and revealed mature architecture till the 21st day (Fig. 6).

From 60 days after the virus inoculation, out of four mice, five tumors appeared in the olfactory bulb, anterior horn of the lateral ventricle and the wall of the IVth ventricle (Fig. 15).

2) *Fluorescent microscopic findings*

The production sites and sequential fluctuations of T-antigen are summarized in Table 1. Two days after the virus inoculation, many fluorescent cells first appeared in the ependymal and subependymal matrix layers of all ventricular walls, epithelium of the choroid plexuses and leptomeninges. (Figs. 7, 9 and 10). The fluorescent T-antigen was detected as crescent-shaped flecks,

TABLE 1. SEQUENTIAL CHANGES IN PRODUCTION OF FLUORESCENT T-ANTIGEN IN MOUSE BRAIN INOCULATED SUBTENTORIALLY WITH AD 12

Location	Cerebrum		Ventricular System			Cerebellum including granular cell layers	Leptomeninx
After inoculation	Cortex &	White matter	Ependyma	Choroid plexus	Matrix layer		
6 hours	−	−	−	−	−	−	−
12 hours	−	−	−	−	−	−	−
24 hours	−	−	−	−	−	−	−
2 days	−	−	++	+++	++	−	+++
3 days	−	−	+++	+++	+++	−	+++
5 days	−	−	++	++	++	−	+++
7 days	−	−	+	++	+	−	++
10 days	−	−	+ scanty	+	+ focal	−	+
14 days	−	−	+ scanty	+ scanty	+ focal	−	+ scanty
21 days	−	−	+ scanty	+ scanty	+ focal	−	+ scanty
30 days	−	−	± rare	+ scanty	+ focal	−	+ scanty
60 days	−	−	−	± rare	± * rare	−	± rare
90 days	−	−	−	−	− *	−	−
120 days	−	−	−	−	− *	−	−
150 days	−	−	−	−	− *	−	−

Note: * − Tumor tissues developing in some mice were strongly flurescent.

slender irregular threads, granules or dots in the nucleus and/or in the cytoplasm of individual cells. Fluorescent cells reached maximum in number on the third day and gradually decreased from the fifth day. In the subventricular areas, the fluorescent cells identified as immature cells by the post-staining with H-E. They rapidly decreased in number from the 7th to the 10th day, but some of them still remained in a focal accumulation which scattered beneath the ependyma (Fig. 12). These findings continued till the 30th day. After the 60th to the 150th day, T-antigen was limited to the tumor nodule. In the white and gray matters excepting subventricular regions contained no fluorescent cells throughout the periods. In the subtentorial brain, T-antigen was found in the ependymal layer, matrix layer, choroid plexus of the IVth ventricule and leptomeninges (Fig. 7), but they could not be detected in the external and internal granular cells of the cerebellar hemisphere (Fig. 8).

B. *Characteristics of intracranial tumors*

1) *Latency, incidence, location and macroscopic findings of the tumors*

As indicated in Tables 2 and 3, visible tumors were detected one or more in each brain of 25 of 83 mice examined (30.1%) from 107 to 252 days after

TABLE 2. INCIDENCE OF INTRACRANIAL TUMORS IN MICE INOCULATED SUBTENTORIALLY WITH AD 12

No. of Mice Tested	No. of Mice Developing Tumor (%)	Average of Latent Period (days)
I.* 83	25 (30.1)	
♂ 38	♂ 11 (28.9)	♂ 169
♀ 45	♀ 14 (31.1)	♀ 134
II.* 29	0	
♂ 13	♂ 0	
♀ 16	♀ 0	

Note: ♂ : male ♀ : female
* Mice observed over 60 days after subtentorial inoculation with Ad 12
** Control Mice observed over 280 days

the virus inoculation. The incidence of the tumor induction was almost the same in both sexes, but the latent period was shorter in female mice. No tumor was found in 29 of the control mice despite observation over 280 days. Macroscopically, each tumor was soft in consistency and greyish-white or whitish-yellow in color. The larger ones were more necrotic and hemorrhagic in the central part. The tuomrs were fairly well defined without any clear encapsulation and compressed surrounding normal tissues. Some mice with tumors showed various degrees of hydrocephalus.

The primary location and the size of all tumors are shown in Table 3 and Text-fig. I. The tumors developed in the areas related to the ventricular system, though some of them developed so extensively that the primary sites could hardly be decided. The predilection sites were the walls of the olfactory ventricles, inferior, anterior or posterior horns of the lateral ventricles and the IVth ventricles (Figs. 16, 17 and 18). In the subtentorial brain, the tumors were limited in the medial part around the IVth ventricle. No tumor developed in the cerebellar parenchyma including the external and internal granular layers and the white matter. No tumor could be detected in the spinal cords and leptomeninges.

2) *Histomorphological observation of the tumors*

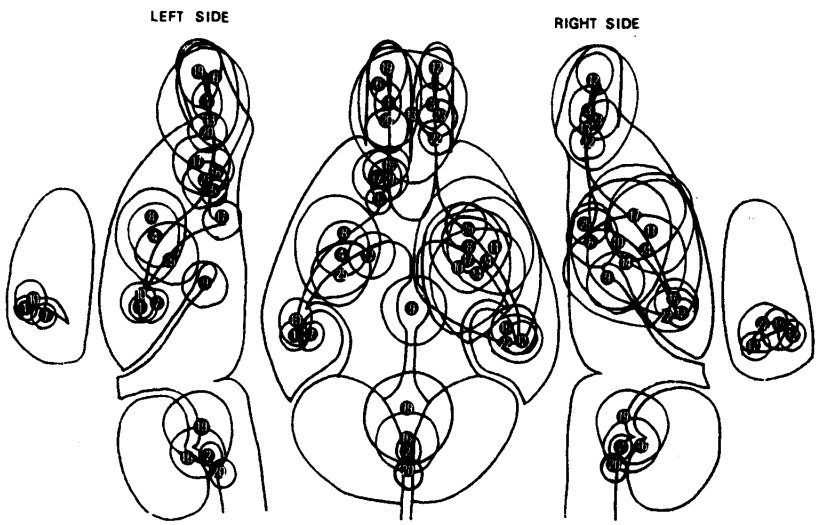
The tumors were composed of small, round or oval cells having scanty cytoplasm which contained a round dark nucleus with fine chromatin granules. In most areas, the tumor cells were closely packed and showed no particular arrangements corresponding to the subventricular zone type (type S) (Fig. 19). There could be seen frequent mitoses. In some areas, they were small spindle or tadpole in shape having uni- or bipolar cytoplasmic processes faintly stained with PTAH, and revealed pseudorosettes (Fig. 20) and pseudostriated or ribon-like trabecular patterns (type V). No true rosettes and blephaloplasts,

however, could be demonstrated with PTAH. In other areas, the tumor cells were somewhat elongated with slender bipolar cytoplasmic processes and contained an oval nucleus (Fig. 21). They proliferated rather loosely to from irregular interlacing bandles, indicating a little tendency to glial differentiation (type G), and a few of the processes were stained blue by PTAH. Mallory's azan and silver impregnation revealed scanty stromas in the tumors. The tumor

TABLE 3. LOCALIZATION AND DISTRIBUTION OF TUMORS

Case No.	Sex	Latent Periods	Location of Tumors	(Multiplicity)
1	F	107	left olfactory, left lateral ventricle (posterior horn)	(2)
2	F	109	IV ventricle	(1)
3	F	120	right olfactory	(1)
4	F	127	left lateral ventricle	(1)
5	M	127	left lateral ventricle (anterior horn)	(1)
6	F	129	left olfactory	(1)
7	F	129	left lateral ventricle (posterior horn)	(1)
8	F	131	right lateral ventricle (inferior horn), left lateral ventricle*	(2)
9	M	134	III ventricle	(1)
10	F	139	left lateral ventricle (posterior horn), right lateral ventricle*	(2)
11	F	139	right lateral ventricle*	(1)
12	M	143	right olfactory, right lateral ventricle (posterior horn)	(2)
13	F	150	both olfactory	(1)
14	M	151	right lateral ventricle*	(1)
15	F	153	right lateral ventricle (posterior horn)	(1)
16	F	158	IV ventricle	(1)
17	F	165	left lateral ventricle (anterior and inferior horn), right lateral ventricle	(3)
18	M	165	left lateral ventricle (anterior horn)	(1)
19	M	169	IV ventricle, left olfactory	(2)
20	M	169	left olfactory, IV ventricle	(2)
21	M	169	left lateral ventricle (anterior horn)	(1)
22	M	171	right lateral ventricle (posterior horn), right olfactory	(2)
23	F	186	right olfactory	(1)
24	M	212	right lateral ventricle*	(1)
25	M	252	right lateral ventricle (inferior horn), left lateral ventricle	(2)

Note : * too extensive to decide the precise site of tumor development
M : male F : female



Text-fig. I. Localization and Size of Tumors Induced by Subtentorial Inoculation with Ad 12 into Mice from No. 1 to No. 25. The Arabic numerals correspond to case No. in Table III, respectively.

revealed often anaplastic configuration (type A), and the tumor cells showed marked pleomorphism and had one or more disorderly arranged fibrillary processes, especially in some degenerated areas. In such a histological type, many bizarre multinucleated giant cells and necrotic foci were scattered (Fig. 22). The histology of the subtentorial tumors is the same as that of the supratentorial tumors.

3) *Fluorescent T-antigens of the tumors*

Several tumors were examined to detect T-antigen by fluorescent microscopic method. In most tumor cells, fluorescent particles of thick, crescent-shaped flecks, irregular filamentous threads or granules were noted more abundantly in the cytoplasm than in the nucleus. Moreover, ependymal and epithelial cells of the choroid plexus, which covered the subependymal tumor, contained fluorescent T-antigen as flecks in a small number (Figs. 13 and 14). But, no T-antigen was demonstrated in the other parts of ependyma and choroid plexus.

DISCUSSIONS

Huebner *et al.* (15) in 1962 first described the induction of intracranial tumors by Ad 12 in hamsters. From the detailed pathoanatomical investigations with hamsters and mice, Ogawa *et al.* (2) in 1969 postulated that the histogenesis of this tumor may be of undifferentiated neuroectodermal origin.

Later, Mukai *et al.* (3, 4) supported this opinion on the basis of similar experiments using hamsters and rats. Murao *et al.* (6) also reported the histological characteristics of the brain tumor in rats and its higher incidence than that in murine and hamster tumors. The tumors of these three species of animals have revealed immature features histomorphologically corresponding to human medulloblastoma, ependymoblastoma and glioblastoma in early life. Except for the areas in which peripheral nerves are distributed, the tumors of the central nervous system have developed predilectively in the regions related to the ventricular system. Ogawa (16) reported that the embryonic matrix cells remaining until the postnatal period might be the primorida of the Ad 12-induced tumors. But the relation of Ad 12 to the cerebellum has not fully been examined. In this experiment, the virus was inoculated subtentorially.

It is well known that Ad 12-specific tumor antigen is produced not only in the tumor cells or *in vitro* transformed cells but also in the cells infected with this virus in more early stage (14), and T-antigen is important as a marker suggesting the presence of virus genome. From the results of the present immunofluorescent studies, which coincide with those reported by Murao (5) on the supratentorial virus inoculation, it was considered that the virus fluid injected into the subtentorial space extended widely to all of the central nervous tissues, and the virus selectively infected the cells of the ventricular walls, choroid plexuses and meninges. The tumor, however, developed in the sites in which T-antigen was previously detected, but limited in the peculiar parts of the ventricular wall where immature cells remained abundantly and the virus fluid could be well retained for a time. Therefore, from these findings it might be suggested that (a) the presence of a certain amount of target cells, perhaps in some mitotic phase adequate to integrate virus genome, and (b) the retention of sufficient volume of effective virus fluid for a period are necessary for the acutual malignant change.

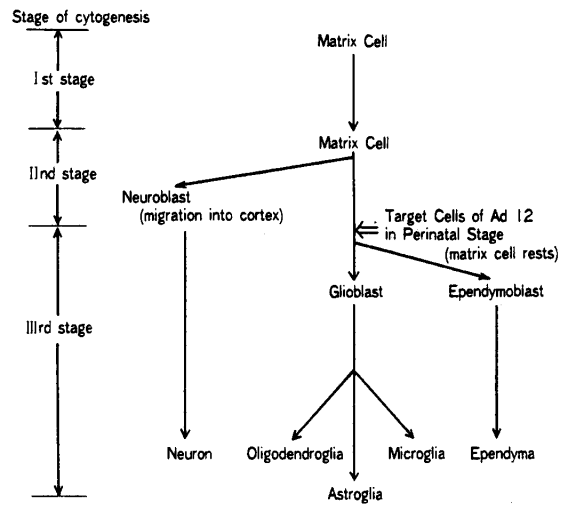
Murao (5) reported that the brain tumors developed in 21 of 45 (46.7%) mice with the supratentorial injection of Ad 12 of 0.015 ml. In the prerent experiment with the same titer of Ad 12 of 0.005 to 0.01 ml, tumors developed in a lower incidence (30.1%). This difference may be mainly based on the volume of the inoculated virus.

The histological features of the tumor were similar to those reported by Ogawa *et al.* (2, 16). Namely, the tumor was divided into 4 types; the type V tumor showed rosettelike arrangements (pseudorosette), the type S was composed of irregularly arranged immature cells, the type G consisted of somewhat enlongated cells with glial fibers faintly stained with PTAH, and the type A was characterized by appearances of anaplastic bizarre cells. From these findings, the tumor induced by Ad 12 in this field should be considered to be

a primitive neuroectodermal tumor morphologically which has derived mainly from subventricular zone and has some ependymal and glial differentiation.

Fujita (17, 18, 19) resently postulated a new consideration of the cytogenesis of the central nervous system (Table 4) based on autoradiographic

TABLE 4 CYTOGENESIS IN CENTRAL NERVOUS SYSTEM (FUJITA)



examinations. According to this theory, the neural tube is composed of only one kind of "matrix cells" which later differentiate into nuronal and glial cells through three stages. Soon after the second stage in which neuroblasts have migrated into the cortical plate, "matrix cells" change themselves to "ependymoblasts" or "glioblasts" in the third stage. It may also be considered in the view of this theory and the results of the present experiment that the target cells of Ad 12 mainly correspond to the embryonic resting cells in the third stage having some potentiality of ependymal or glial differentiation (Table IV). At least, in the cerebellum of newborn mice, Ad 12 have no affinity to the neuronal cells which have migrated to form the external granular layer, because fluorescent T-antigen could be detected in the meninges in which immature peripheral nerves are distributed, but not in the adjacent granular layer through the entire period after inoculation. However, further experiments with virus inoculation in all developing stages of cerebellum, i.e., from embryo to young animals, will be necessary to confirm whether the Ad 12 cannot certainly produce tumor, such as human medulloblastoma which is regarded as a neuronal tumor of external granular cell origin.

Acknowledgment. The author is much indebted to Prof. K. Ogawa for his painstaking guidance, to Dr. M. Motoi for his valuable advices and suggestions, and to Dr. S. Kobayashi for his advices. The excellent technical assistance of Mrs. Y. Sakamoto, Miss M. Kagawa, Miss E. Nagai, Mrs. K. Koyama and Mrs. H. Tsuda is gratefully acknowledged. The author is also much thankful to Mrs. S. Ariyoshi and Mrs. S. Yoshioka, Mouse Colony of Okayama University Medical School, for kindly supplying experimental animals. This work was supported in part by the Grant-in-Aid for Scientific Research from Japanese Ministry of Education.

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Fig. 1. A sagittal section of the brain of a 2-day-old mouse. The wall of the lateral ventricle is composed of the inner single-layer of ependyma and outer thick layer of immature matrix cells. H-E. ×400.

Fig. 2. A sagittal section of the brain of a 10-day-old mouse. Small groups of immature matrix cells remaining beneath the ependyma of the lateral ventricle. H-E. ×400.

Fig. 3. A sagittal section of the brain of a one-day-old newborn mouse. The external layer of the cerebellum composed of small neuronal cells. The subventricular zone and choroid plexus of the IVth ventricle (below). H-E. ×100

Fig. 4. A sagittal section of the cerebellum of a 7-day-old mouse. The external granular layer is seen distinctly. H-E. ×100.

Fig. 5. The cerebellar cortex of a 12-day-old mouse, showing well-developed inner granular layer. The external layer still remains. H-E. ×200.

Fig. 6. The cerebellar cortex of a 30-day-old mouse. The external granular layer has disappeared. H-E. ×200.

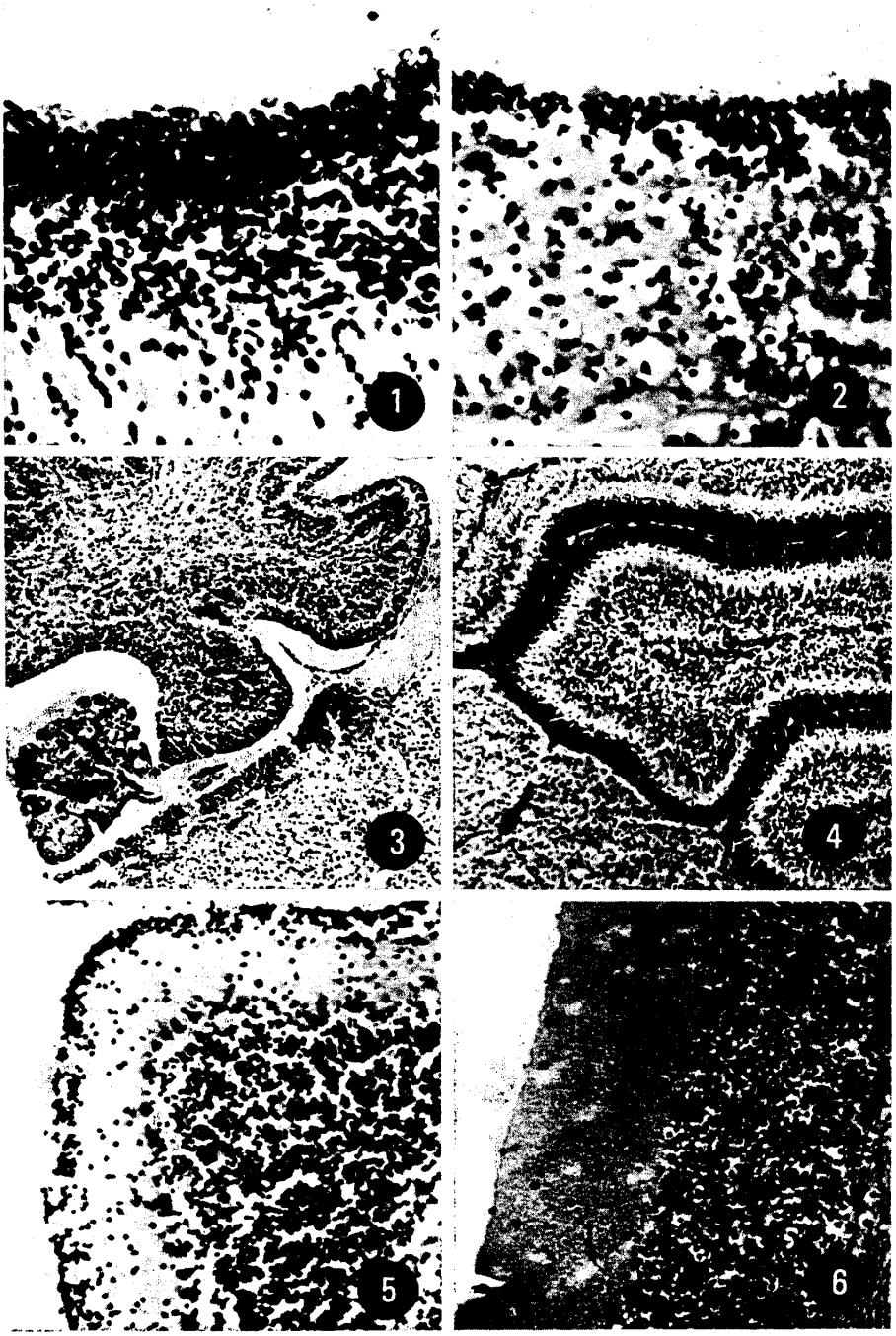
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Fig. 7. Many fluorescent T-antigens in the ependymal (E) and subependymal (S) layers of the IVth ventricular wall. Two days after virus inoculation. Stained with anti-T conjugate. ×200.

Fig. 8. No fluorescent cells are noted in the cerebellar cortex, but fluorescent T-antigens are seen in the leptomeninx (arrow). Two days after virus inoculation. Stained with anti-T conjugate. ×200.

Fig. 9. Many fluorescent T-antigens in the ependymal (E) and subependymal matrix (S) layers of the lateral ventricle (LV). Two days after virus inoculation. Stained with anti-T conjugate. ×200.

Fig. 10. Many fluorescent T-antigens in the choroid plexus of the lateral ventricle (LV). Two days after virus inoculation. Stained with anti-T conjugate. ×200.

Fig. 11. Many fluorescent T-antigens in the leptomeninx at the basal part of the brain. Two days after virus inoculation. Cerebrum (C). Stained with anti-T conjugate. ×100.

Fig. 12. Fluorescent T-antigens in immature matrix cells focally remaining beneath the ependyma of the lateral ventricle. Fourteen days after virus inoculation. Ependyma (E). Choroid plexus (C). Stained with anti-T conjugate. ×100.

Fig. 13. Section of a well-developed tumor in the lateral ventricle. Animal No. 8 in Table III. Many fluorescent T-antigens in the tumor. Choroid plexus (C). Stained with anti-T conjugate. ×40.

Fig. 14. Higher magnification of Fig. 13. Many fluorescent flecks and dots are found mainly in the cytoplasm of the tumor cells. ×400.

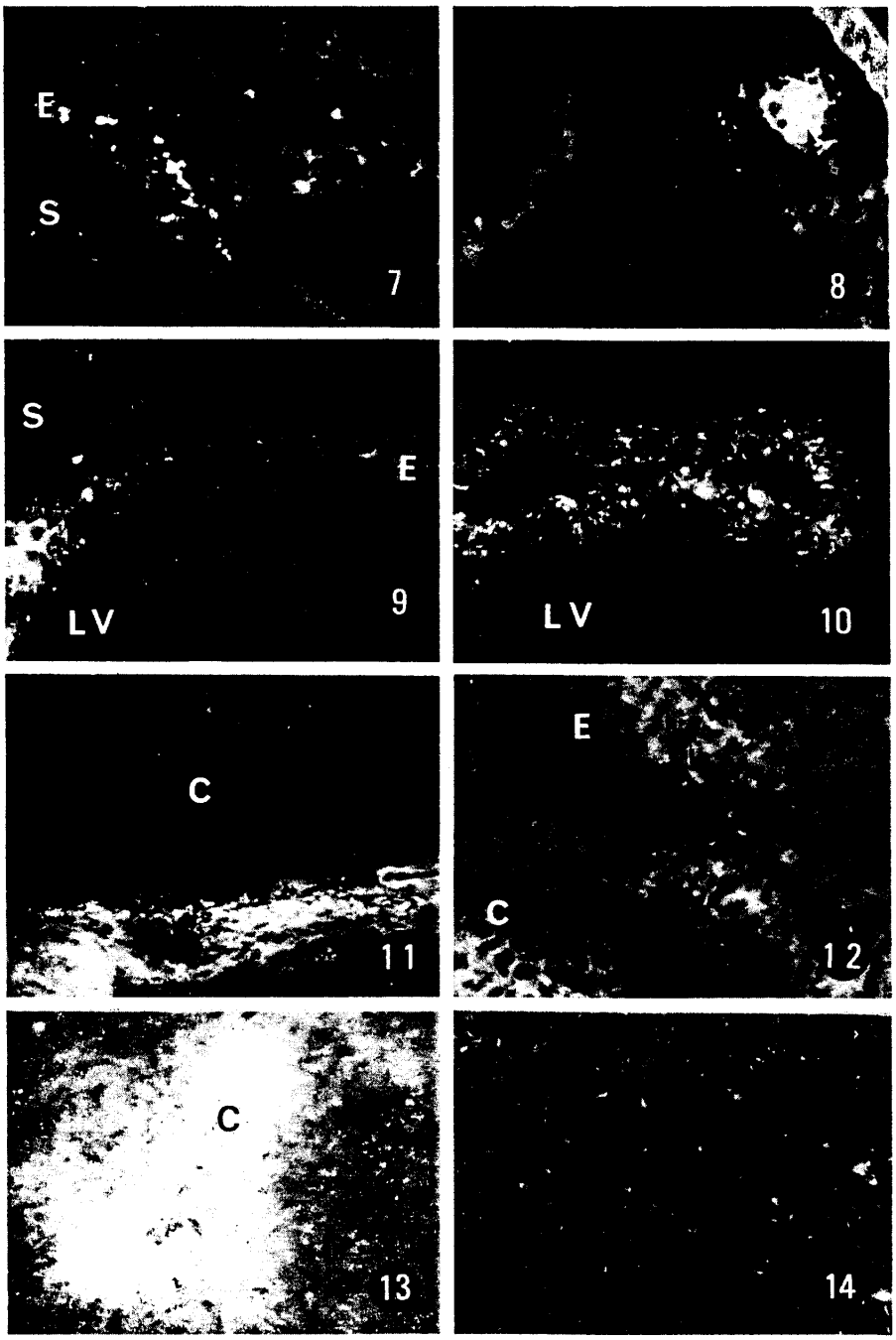
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Fig. 15. A sagittal section of the brain, showing small tumor nodules in the subependymal area of the anterior horn of the lateral ventricle (T1) and of the IVth ventricle (T2). Killed 60 days after virus inoculation. H-E. ×5.

Fig. 16. A sagittal section of the brain, showing tumor nodule (T) in the olfactory and hippocampal regions. Animal No. 22 in Table III. H-E. ×5.

Fig. 17. A sagittal section of the brain, showing a well-developed tumor in the wall of the lateral ventricle. Animal No. 5 in Table III. H-E. ×5.

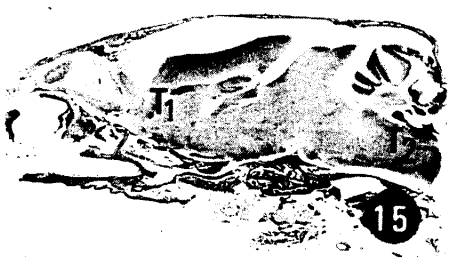
Fig. 18. A sagittal section of the brain, showing well-developed tumors in the olfactory bulb and the IVth ventricle. Animal No. 19 in Table III. H-E. ×5.

Fig. 19. Tumor developing in the IVth ventricle is composed of small round or polygonal cells with scanty cytoplasm and a dark nucleus, and shows no characteristic architecture. Type S. H-E. ×200.

Fig. 20. Tumor developing in the lateral ventricle. Pseudorosetts formed by spindle- and tadpole-shaped tumor cells. Type V. H-E. ×200.

Fig. 21. Tumor developing in the lateral ventricle. Tumor cells are somewhat elongated, showing a fascicular arrangement. Type G. H-E. ×200.

Fig. 22. Tumor developing in the lateral ventricle. Anaplastic tumor cells including some bizarre giant cells. Type A. H-E. ×200.


T1
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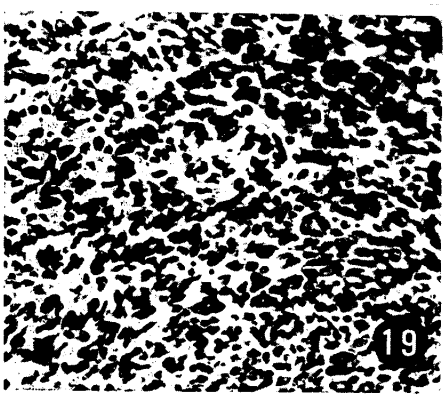
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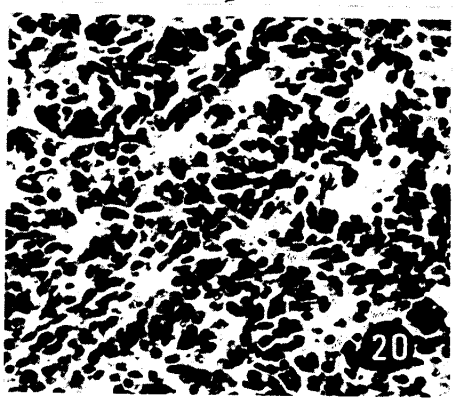
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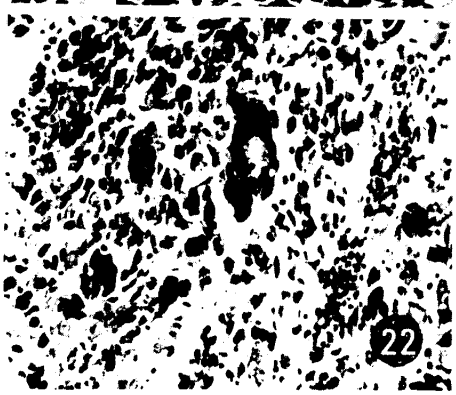
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