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Hitoshi Jinnai*

*Okayama University,

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Abstract

The incidences, distribution and histopathological findings of N, N'-dimethylnitrosourea (DMNU)-induced brain microtumors in Sprague-Dawley rats were studied. Subcutaneous injections of DMNU in young adult rats one a week resulted in the induction of 122 gliomas in 38 animals with an incidence of 69% after a time lapse of between 157 and 246 days from the first injection. Of these tumors, 66 were classified as microtumors (diameter less than about 1 mm) by detailed light microscopy observation of serial sections. The microtumors were of 3 types: 55 oligodendrogliomas, 8 astrocytomas and 3 mixed gliomas. As the tumors became larger in size, anaplasia appeared, especially in the central part of the tumors. The microtumors developed randomly throughout the brain. It was concluded that, in adult rat brains, the target cells of DMNU were well differentiated glial cells which had already migrated from the matrix layer.

KEYWORDS: dimethylnitrosourea, rat, brain, microtumor, glioma

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STUDIES ON N, N'-DIMETHYLNITROSOUREA-INDUCED BRAIN TUMORS IN RATS —ESPECIALLY, ON MICROTUMORS

Hitoshi JINNAI

Department of Pathology, Okayama University Medical School, Okayama 700, Japan (Director: Prof. K. Ogawa)

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Abstract. The incidences, distribution and histopathological findings of N, N'-dimethylnitrosourea (DMNU)-induced brain microtumors in Sprague-Dawley rats were studied. Subcutaneous injections of DMNU in young adult rats once a week resulted in the induction of 122 gliomas in 38 animals with an incidence of 69% after a time lapse of between 157 and 246 days from the first injection. Of these tumors, 66 were classified as microtumors (diameter less than about 1 mm) by detailed light microscopy observation of serial sections. The microtumors were of 3 types: 55 oligodendrogliomas, 8 astrocytomas and 3 mixed gliomas. As the tumors became larger in size, anaplasia appeared, especially in the central part of the tumors. The microtumors developed randomly throughout the brain. It was concluded that, in adult rat brains, the target cells of DMNU were well differentiated glial cells which had already migrated from the matrix layer.

Key word: dimethylnitrosourea, rat, brain, microtumor, glioma

In 1964, Druckrey *et al.* (1) produced tumors selectively in the nervous system of BD-rats with methylnitrosourea. Since then, accumulating data (2–6) have shown an organotropic affinity of some nitroso compounds for the nervous system. Attention has been mostly paid to tumor induction by transplacental administration of ethylnitrosourea (ENU) (3, 5–10). On the other hand, oncogenic activity of N, N'-dimethylnitrosourea (DMNU) was demonstrated for the first time in BD-rats by Druckrey *et al.* (4) in 1967. Since then, however, there has been only a few reports on DMNU (11–13). In 1976, Ogawa *et al.* (14) produced many brain tumors in adult rats with repeated injections of DMNU and noticed that most of them were well differentiated gliomas in contrast with the infantile type of brain tumors induced by human adenovirus type 12 (Ad12) (15–17). Furthermore it was reported that, in newborn animals the latter developed preferentially in the subventricular zone just beneath the ependymal wall (15–17). The brain tumors induced by transplacental administration of ENU also developed near the ventricles (18). In the present work, the author studied the relationship between the initial sites of tumor development and the cytomorphological differentiation of tumors induced by DMNU in the brains of Sprague-Dawley rats.

MATERIALS AND METHODS

Animals

Seventy-five males Sprague-Dawley rats aged 4 to 6 weeks old were used. The rats had originally been obtained commercially and bred in our laboratory. All the animals were housed in metallic basins and fed with a balanced diet of MF pellets (Oriental Yeast Industrial Company, Tokyo). Water was freely available from suckling bottles. The rats were weighed and inspected every week until death.

N, N'-Dimethylnitrosourea

DMNU (synthesized by Nihon Kankoshikiso Laboratory, Okayama) dissolved in physiological saline solution was freshly prepared every week for subcutaneous injection.

Experimental treatment

The animals were divided into 2 experimental groups and one control group.

Group I. Sixty-one rats were injected subcutaneously in the interscapular region with the solution of DMNU at a dose of 40 mg/kg body weight once a week (totals ranging from 17 to 21 times). The animals were killed between 150 and 246 days. In order to examine the microtumors, most of the animals were killed at an appropriate time before signs of tumor development.

Group II. To examine the stages of DMNU-induced lesions earlier than those of group I, 9 rats were grouped into 3 subgroups, each of which was composed of 3 rats. The first subgroup was treated with DMNU once, the second subgroup 5 times, and the third subgroup 10 times using the same method as group I. All the animals were killed within 24 h of the last injection.

Control group. Five rats were injected subcutaneously with 2 ml of physiological saline solution once a week for a total of 20 times. The animals were killed on the 200th day after the first injection.

Light microscopy

Autopsy was performed and each brain, together with any other lesions, was fixed in 10% formalin. Serial frontal sections of all the brains, from the tractus olfactorius to medulla oblongata, were prepared at intervals of 100 μ. In the present study, spinal cords were not examined unless lesions were evident macroscopically. The sections were routinely stained with hematoxylin and eosin. In addition, silver impregnation method, phosphotungstic acid hematoxylin (PTAH), Klüver-Barrera (KB), Mallory-Azan and periodic acid-Schiff (PAS) stains were used when necessary. Identification of the site of the brain microtumors was achieved by making a comparison with anatomical figures of the rat brain as drawn by Joachin and Renate (19).

RESULTS

Incidences and locations of DMNU-induced brain microtumors. In group I, 55 of 61 rats survived, and 6 rats died of respiratory infection during the early stages. The hair of all the treated rats began to fall out following repeated injections of DMNU. The total dose of DMNU given per rat varied from 174 mg to

236 mg with an average of 209 mg. The total number of injection times was between 17 and 21, and the median was 19.6. In this paper, the term "brain microtumor" means a small brain tumor less than about one mm in diameter, as discussed later. A tumor of this size is recognizable by light microscopy. By scrutinizing serial sections, it was verified that each microtumor was an independent tumor and had no continuation with other tumors. Brain tumors developed in 38 of 55 rats (69%). Of these 38 rats, microtumors were found in 28 rats (Table 1). Fifteen rats had multiple microtumors and the maximum number

TABLE 1. INCIDENCES OF BSAIN TUMORS IN GROUP 1 IN SD RATS WITH N, N'-DIMETHYLNITROSOUREA (DMNU)

No. of experimental animals	Days after the first injection of DMNU	Injection times	Total dose of DMNU (Mg)	Nos. of brain tumors	
				Microtumors	Enlarged tumors
1	150	21	231	0	0
2	157	17	174	1	0
3	157	20	208	0	0
4	158	20	210	0	1
5	160	20	216	0	0
6	165	19	204	0	0
7	170	18	195	0	0
8	172	20	203	0	1
9	176	21	231	0	0
10	179	21	230	1	1
11	180	21	232	0	0
12	185	20	210	0	0
13	186	21	234	4	0
14	187	18	205	7	1
15	187	19	210	5	3
16	187	21	225	0	0
17	188	18	200	1	2
18	190	17	174	0	0
19	190	21	230	0	0
20	192	20	211	2	0
21	193	20	215	0	1
22	193	21	231	2	2
23	196	19	208	4	0
24	196	20	211	0	0
25	200	20	210	4	1
26	200	20	210	0	2
27	200	20	206	0	0
28	200	20	210	0	0
29	201	20	201	1	1
30	203	20	217	4	1
31	205	21	232	1	1
32	205	19	189	0	0
33	206	20	227	3	2
34	209	21	211	4	2

TABLE 1. (Continued)

35	210	18	190	1	1
36	210	20	217	3	2
37	210	21	231	1	1
38	211	19	200	0	4
39	211	19	203	3	0
40	212	20	196	1	1
41	212	21	236	1	2
42	212	19	198	0	0
43	215	20	196	1	1
44	215	20	225	1	3
45	220	20	211	2	1
46	226	21	230	0	1
47	230	20	206	1	2
48	231	17	178	0	4
49	231	20	206	3	1
50	231	20	224	0	1
51	235	18	194	1	3
52	240	20	220	3	4
53	240	17	180	0	0
54	246	17	175	0	1
55	246	17	175	0	1

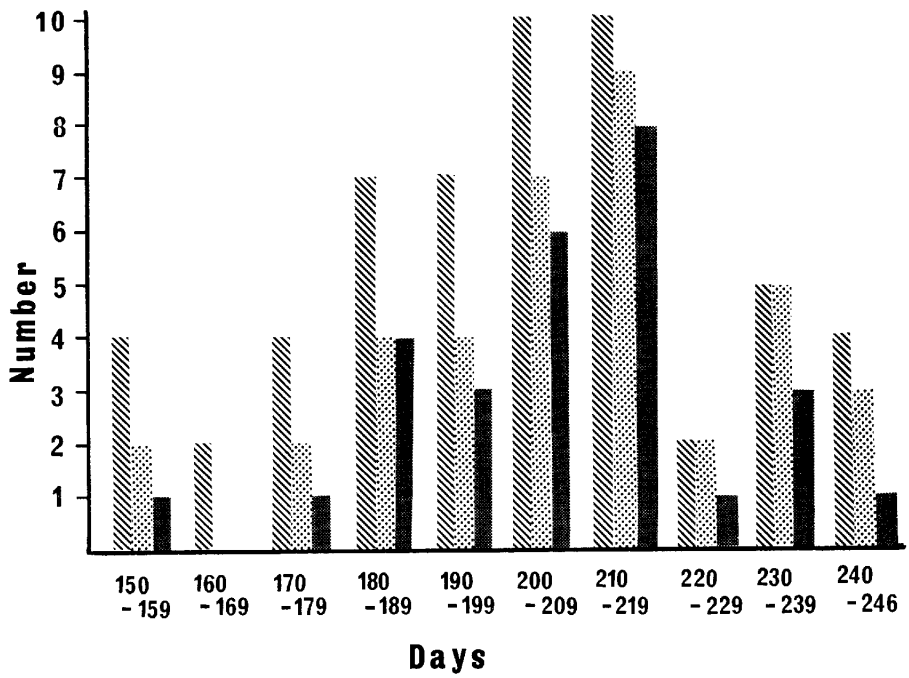

Fig. 1. Incidence of brain tumors after administration of N, N′-dimethylnitrosoura in rats. ▨, number of sacrificed animals; ▩, number of animals developing brain tumors; ■, number of animals developing micro-brain tumors.

per rat was 7. The total number of brain tumors was 122, in which there were 66 (54%) microtumors. The microtumors appeared at between 157 and 240 days after the first injection (Fig. 1). As summarized in Table 2, the microtumors occurred in any part of the brain apart from the cerebellum, although there were some differences in location according to the histological type of the tumor.

TABLE 2. DISTRIBUTION OF MICROTUMORS

Histological type	Site*	Nos. of microtumors
Oligodendroglioma	Cerebral cortex	18
	Corpus callosum	15
	Genu corporis callosi	9
	Splenium corporis callosi	3
	Truncus corporis callosi	2
	Radiatio corporis callosi	1
	Nucleus caudatus putamen	5
	Cingulum	4
	Radiatio thalami superior	2
	Subiculum	1
	Zona incerta	1
	Lamina medullaris interna bulbi olfactorius	1
	Nucleus ventricularis corporis geniculati lateralis	1
	Nucleus dorsalis corporis geniculati lateralis	1
	Commisura fornicis dorsalis	1
	Globus pallidus	1
	Fimbria hippocampi	1
	Capsula interna	1
	Stria terminalis	1
	Hippocampus	1
Astrocytoma	Cerebral cortex	5
	Zona interna	1
	Nucleus ruber	1
	Medulla oblongata	1
Mixed glioma	Cerebral cortex	1
	Nucleus caudatus putamen	1
	Corpus callosum	1

* See reference no. 19.

Oligodendroglioma had a tendency to develop in the corpus callosum (Photo. 1), especially in the genu corporis callosi (Photos. 2, 3). Both oligodendroglioma and astrocytoma occurred throughout in the cerebrum, but showed no particular tendency to develop in subependymal areas.

In group II and the control group, the animals showed no pathological changes in any organs except for depilation in group II.

Histological findings of the microtumors. All the microtumors belonged to the glioma group (Table 3). In 66 microtumors, there were 55 oligodendrogliomas, 8 astrocytomas and 3 mixed gliomas. As shown in Photos. 4, 5, 6, 7, several

TABLE 3. HISTOLOGICAL DIAGNOSIS OF BRAIN TUMORS

Histological diagnosis	Nos. of microtumor	Nos. of enlarged tumor
Oligodendroglioma		
not anaplastic	55	13
anaplastic	0	5
Astrocytoma		
not anaplastic	8	24
Mixed Glioma		
not anaplastic	3	8
anaplastic	0	4
Glioblastoma	0	2
Total Nos.	66	56

microtumors consisted of a group of less than twenty oligodendroglia- or astroglia-like cells. Slight cellular atypism and several mitotic figures were seen in some of these small lesions (Photos. 5, 6). Most of the oligodendrogliomas had comparatively unclear margins and under low power magnification showed monotony of the microscopic field. These fields were usually occupied by fairly compact collections of uniform cells with regular spheroidal central nuclei and relatively clear cytoplasma. Some oligodendroglioma cells had distinct perinuclear halos and showed typical honeycomb structure (Photo. 8). They frequetly formed regularly arranged clusters. Vascular endothelial proliferations were not prominent in general, but small bleeding foci were present occasionally. A few oligodendrogliomas seemed to be composed entirely of tumor oligodendrocytes, however, in most oligodendrogliomas a small number of astrocytes were also present. A small number of mitotic figures were found, but the tumors showed little evidence of cellular pleomorphism such as bizarre giant cells. Areas of calcification and necrosis were not observed. No definite glial fibers were stained with PTAH. With silver staining, intercellular argyrophilic fibers were not noted in the small oligodendrogliomas.

Most of the astrocytomas were considered to be fibrillary astrocytomas. They consisted of relatively small spindle shaped cells with oval or irregularly distorted long nuclei and fibrillated cell processes (Photos. 9, 10–15). The tumor astrocytes had a tendency to proliferate infiiltratively, and the margins of astro-

cytomas were less clear than those of oligodendrogliomas. These tumors were usually attended by a small number of microglia-like cells, inflammatory cells and tumor protoplasmic astrocytes. In some instances, tumor protoplasmic astrocytes proliferated more predominantly than tumor fibrillary astrocytes. Perivascular infiltration of the tumor cells and mild vascular proliferations were noted in some cases (Photo. 16). Mitotic figures were seen variably case by case, but cellular pleomorphism was rare. There was no necrosis or bleeding. With PTAH staining, glial fibers stained in a variable degree (Photos. 17, 18). Argyl-ophilic fibers were noted in the vascular elements.

Although the incidence was not as great as that of other tumors, mixed gliomas also occurred. In these tumors, tumor oligodendrocytes and astrocytes proliferated almost equally (Photo. 19). The tumor oligodendrocytes were morphologically similar to those of the above-mentioned oligodendrogliomas. Most of the tumor astrocytes were of the protoplasmic type. The two types of tumor cells were mixed, but showed no collision picture.

Sequential observations of the brain tumors. The microtumor at the earliest stage (composed of less than twenty neoplastic oligodendrocytes with a few mitotic figures) was observed on the 157th day after the first injection of DMNU. From this time to the 240th day, a large number of microtumors as well as more enlarged tumors developed. Up to the 157th day, however, neither tumor lesions nor any other pathologic changes were noticed in group II by light microscopy.

The enlarged brain tumors without anaplasia showed fundamentally the same histological figures as the microtumors. In the enlarged tumors without anaplasia, there were 24 astrocytomas, 13 oligodendrogliomas and 8 mixed gliomas (Table 3).

Anaplasia occurred as the tumors became larger, and cellular atypism was observed in 11 of 56 enlarged tumors. In these anaplastic tumors there were 5 anaplastic oligodendrogliomas, 4 anaplastic mixed gliomas and 2 glioblastomas. In the central part of the anaplastic oligodendrogliomas, there often appeared areas of pseudopalisading with central necrosis, bleeding foci and bizarre giant cells (Photos. 20, 21). In the outer parts, the tumor oligodendrocytes proliferated showing the same histological figures as those of micro-oligodendrogliomas (Photos. 20, 22). In the intermediate zone there were transitional figures. In the glioblastomas, proliferation of unclassified glial cells with variable cellular atypism, areas of pseudopalisading with central necrosis, bleeding foci and a number of bizarre giant cells were seen (Photos. 23, 24). Vascular endothelial proliferations were not so prominent. With PTAH staining, neuroglial fibers were partially stained to a variable degree. In the anaplastic mixed gliomas, small necrotic areas and several bizarre giant cells were seen in the mixture of tumor oligodendrocytes and tumor astrocytes.

Induction of other tumors. Five schwannomas developed in 5 rats, one in each rat. Two were in the trigeminal nerve fibers (Photo. 25), 2 in the extremities and one in the mediastinum. All the schwannoma were histologically anaplastic. Besides the schwannomas, there were 3 carcinomas considered to have originated from the skin of an extremity, one fibrosarcoma in the mediastinum, one nephroblastoma like neoplasm in the kidney, and one lymphoma in the small intestine.

DISCUSSION

The extensive studies by Druckrey *et al.* (4) on the carcinogenic activity of 65 N-nitroso compounds in BD rats were of great significance. In their report, it was shown that several kinds of N-nitroso compounds, *e. g.* methylnitrosourea, ethylnitrosourea di-and trimethylnitrosourea had a selective affinity for the nervous system. There has, however, only been a small number of reports on the effects of DMNU. Sanders (11) demonstrated production of tumors in several organs including the brain by feeding inactive precursors of DMNU to rats; namely, dimethylurea and sodium nitrate. He suggested that a new carcinogen might be synthesized *in vivo* by simultaneous administration of various inactive compounds. Hiraki (13) produced malignant lymphomas in mice and malignant tumor of the fore stomach, mammary gland and uterus in hamsters with repeated subcutaneous injections of DMNU, but no neurogenic tumors developed. Yanagida (12) showed suppression of the development of cerebellum and retina in hamsters with subcutaneous injections of DMNU in the perinatal period. In the preliminary studies (14), the authors observed that DMNU had a strong affinity for the central nervous system and effectively produced well differentiated brain tumors of the glioma series with repeated subcutaneous injections into adult rats. The tumors by this experimental induction seemed to be a suitable model of adult human brain tumors in cytomorphological differentiation. For the purpose of clarifying the "formal genesis" of adult types of brain tumors experimentally, the cytomorphological differentiation and the initial sites of tumor development of DMNU-induced tumors were intensively investigated on serial sections of early brain tumors. Author's preliminary experiments with approximately 20 S-D rats suggested that it took about 200 days for the brain tumors to reach macroscopic size after at least 20 DMNU-injections. Histopathological changes were not found before 150 days. So the first autopsy in group I was performed on the 150th day after the first injection of DMNU. It was conjectured that some pathological changes should occur in the brain immediately after the first injection of DMNU; however, none of inflammatory, degenerative or precancerous changes were evident in group II using light microscopy.

The first lesion was found at the subiculum of a rat brain on the 157th day.

It was composed of a small group of oligodendroglia-like cells. In order to determine whether lesions are neoplastic, it is generally necessary to examine the transplantability of the cells. But the transplantation of such a small lesion was practically impossible, so the author regarded the following findings as signs indicating a neoplastic nature: [1] no similar lesions were seen in either group II or the control group, [2] many obvious tumors developed within the same time interval, [3] transitional figures were noticed between these early lesions and large obvious tumors, and [4] some cellular atypism and a few mitotic figures were seen in these lesions, although not in all cases. Therefore it seemed as if DMNU-induced brain tumors occurred as a small group of glia cells in the absence of any previous lesion, and proliferated progressively to large tumors within several weeks.

In the present study, the incidence of brain tumors was 69%. But this value was considered to be lower than the true incidence, because most animals were killed before signs of tumor development. Microtumors seemed to be mainly composed of oligodendrocytic cells. The incidence of micro-astrocytomas was lower than that of enlarged astrocytomas (Table 3). This may be due to the peculiar growth pattern of astrocytomas, especially fibrillary types, which infiltrate more sparsely into the surrounding brain tissue than oligodendrogliomas. Most astrocytomas, therefore, had become large enough to be observed.

According to Koestner *et al.* (8, 20), in ENU-induced tumors of the nervous system, 75 of 102 tumors were in the central nervous system and 27 were in the peripheral nervous system. Oligodendrogliomas, the most common type, comprised 31 of 75 tumors, and in this ENU series 10 anaplastic ependymomas, 4 anaplastic glioependymomas and one meningioma were also induced. On the other hand, as shown in Table 3, ependymoma and meningioma were not detected in this experiment. Oligodendroglioma was, however, the most common type in DMNU-induced tumors as well as that in ENU. Wechsler *et al.* (21) commented on the pathology of chemically induced experimental neurogenic tumors, and showed a preliminary classification of the neurogenic tumors transplacentally induced with ENU in BD rats. According to this classification, "mixed glioma" were divided into two types, that is "isomorphous" and "pleomorphous". However, since the term "anaplastic" is widely used, the author preferentially used this epithet rather than "pleomorphous" also for other types of glioma group, when the tumor shows more or less cellular atypism. In the present study, when oligodendroglioma as well as astrocytoma became larger, their central part often showed the histological behavior of multiformed glioblastoma. Therefore, it may be considered that every anaplastic type of glioma can easily change into glioblastoma.

The mechanism of the carcinogenic activity of N-nitroso compounds is not

yet fully elucidated (4, 5, 22, 23, 24). But the effects of ENU in rats appeared to be different from DMNU; namely, ENU showed less affinity for the nervous system in adult rats than DMNU (4), but transplacentally induced neurogenic tumors occurred at a high incidence with a single injection of ENU into mother rats (5, 6, 8, 9). On the contrary, according to the author's preliminary experiment (25) with several Sprague-Dawley rats which received DMNU transplacentally by a single injection of 40 mg/kg on the 21 day of gestation, brain tumors did not develop during the following one year period. Suppression of cerebellar development, however, occurred in all animals. It is difficult to explain such a difference between the effects of these two compounds, but possibly DMNU is more stable than ENU (4) and not decomposed readily into the active alkylating components which may have the affinity for embryonic cells.

In any case, DMNU was thought to be a suitable carcinogen inducing brain tumors of an adult type. Detailed cytological observation in the pretumorous stage should be further investigated by more effective ways than light microscopy.

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Legends to Photographs

In Photos. 2, 4, 6, 9, 11, 13, the left side of each picture shows the ventral side of the brain.

Photo. 1. Micro-oligodendroglioma (arrows) in the radiatio corporis callosi. Examined on the 187th day. H. E. ×10

Photo. 2. Micro-oligodendroglioma (arrows) in the genu corporis callosi. Examined on the 212th day. H. E. ×13

Photo. 3. Higher magnification of Photo. 2. Note the proliferation of small cells with

round or elliptical nuclei. The majority of the cells are tumor oligodendrocytes, but a small number of astrocytic elements are also seen. H. E. ×100

Photo. 4. Micro-oligodendroglioma (arrows) in the subiculum. Examined on the 157th day. H. E. ×10

Photo. 5. Higher magnification of Photo. 4. Several tumor oligodendrocytes with dark spheroidal nuclei and perinuclear cytoplasmic halos are seen (arrows on the lower left side). Note two mitotic figures (arrows on the lower right side). A few original astrocytes are left. (arrow on the upper side) H. E. ×200

Photo. 6. Micro-oligodendroglioma in the cingulum (arrow). Some tumor cells are found in the truncus corporis callosi. Note left lateral ventricle (uper left). Examined on the 201st day. H. E. ×16

Photo. 7. Higher magnification of Photo. 6. Note irregularity of the nuclear size. A small number of astrocytic elements are also seen. H. E. ×130

Photo. 8. Slightly enlarged oligodendroglioma. Note honeycomb structure and vascular stroma (arrows). H. E. ×100

Photo. 9. Micro-astrocytoma in cerebral cortex arrow). Examined on the 205th day. H. E. ×13

Photo. 10. Higher magnification of Photo. 9. The majority of the cells are probably fibrillary astrocytes. Some inflammatory cells are also seen. H. E. ×160

Photo. 11. Micro-astrocytoma in right zona incerta (arrow on the lower side). Note that the left zona incerta is intact (arrow on the upper side). Examined on the 200th day. H. E. ×13

Photo. 12. Higher magnification of Photo. 11. Note proliferation of small oval or spindle cells. Tumor cells are also seen in perivascular area. H. E. ×100

Photo. 13. Micro-astrocytoma in medulla oblongata (arrow). Examined on the 209th day. H. E. ×13

Photo. 14. Higher magnification of Photo. 13. This lesion is slightly larger than that of Photos. 9 and 11. Note the increase of cellular atypism. H. E. ×100

Photo. 15. Higher magnification of other slightly enlarged astrocytoma. Note cellular atypism. H. E. ×200

Photo. 16. Perivascular infiltration of tumor cells in astrocytoma. H. E. ×16

Photo. 17. Part with scanty neuroglial fibers in astrocytoma. P. T. A. H. ×200

Photo. 18. Part with abundant neuroglial fibers in astrocytoma. P. T. A. H. ×300

Photo. 19. Mixed-micro glioma in cerebral cortex (arrows). Note the mixture of oligodendrocytic elements and astrocytic elements. A few original neurons are left. Examined on the 203rd day. H. E. ×66

Photo. 20. Anaplastic oligodendroglioma. Central anaplastic part is seen in the lower half. Peripheral nonanaplastic part is indicated by arrows. H. E. ×25

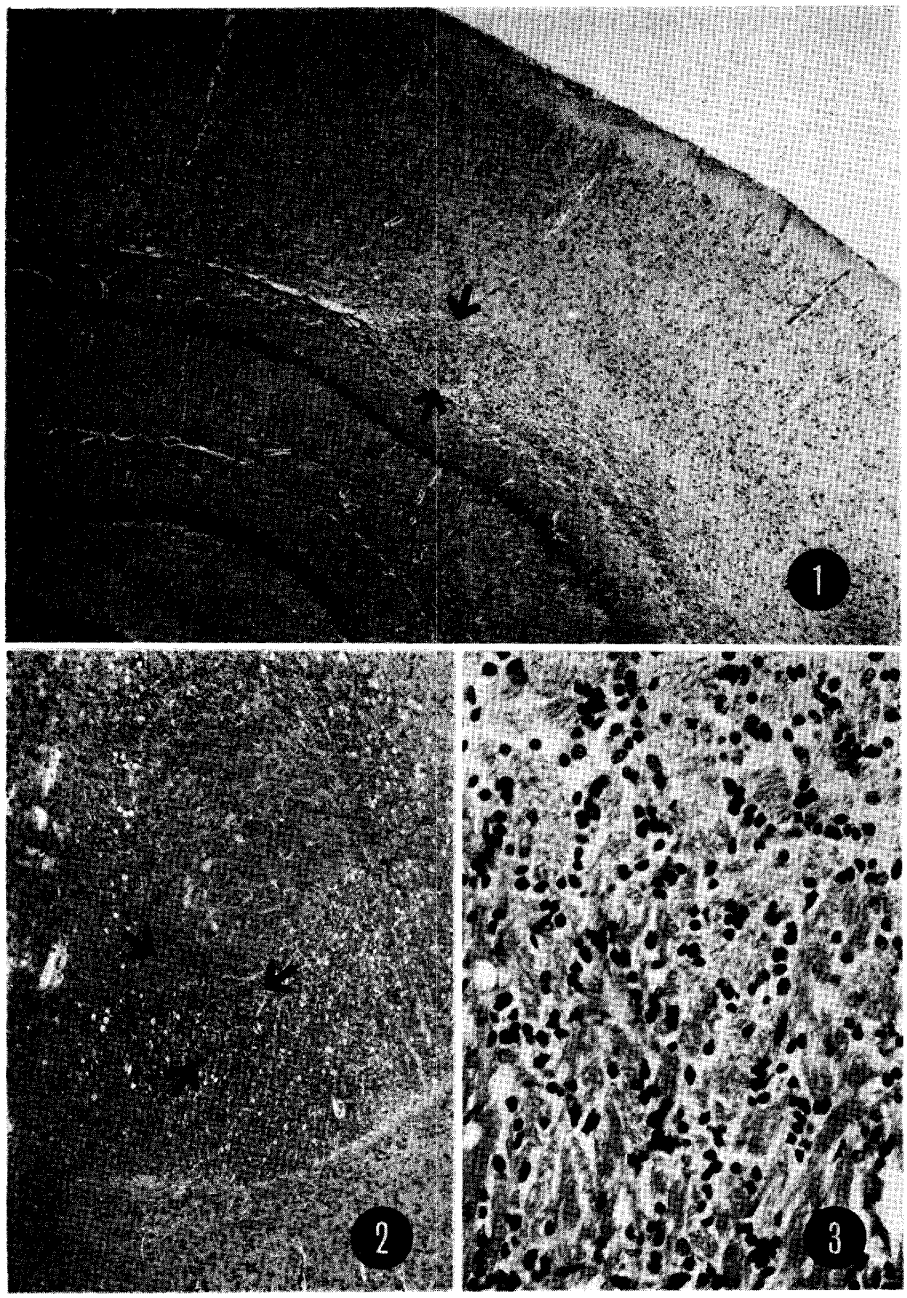
Photo. 21. Higher magnification of the central anaplastic part in Photo. 20. Marked cellular pleomorphism is seen. H. E. ×25

Photo. 22. Higher magnification of the peripheral nonanaplastic part in Photo. 20. This part shows a typical figure of oligodendrocytoma. H. E. ×100

Photo. 23. Glioblastoma. Note areas of pseudopalisading with central necrosis (arrows) and giant cells. Examined on the 200th day. H. E. ×50

Photo. 24. Higher magnification of Photo. 23. Note bizarre giant cells. H. E. ×200

Photo. 25. Schwannoma in right trigeminal nerve is indicated by arrows.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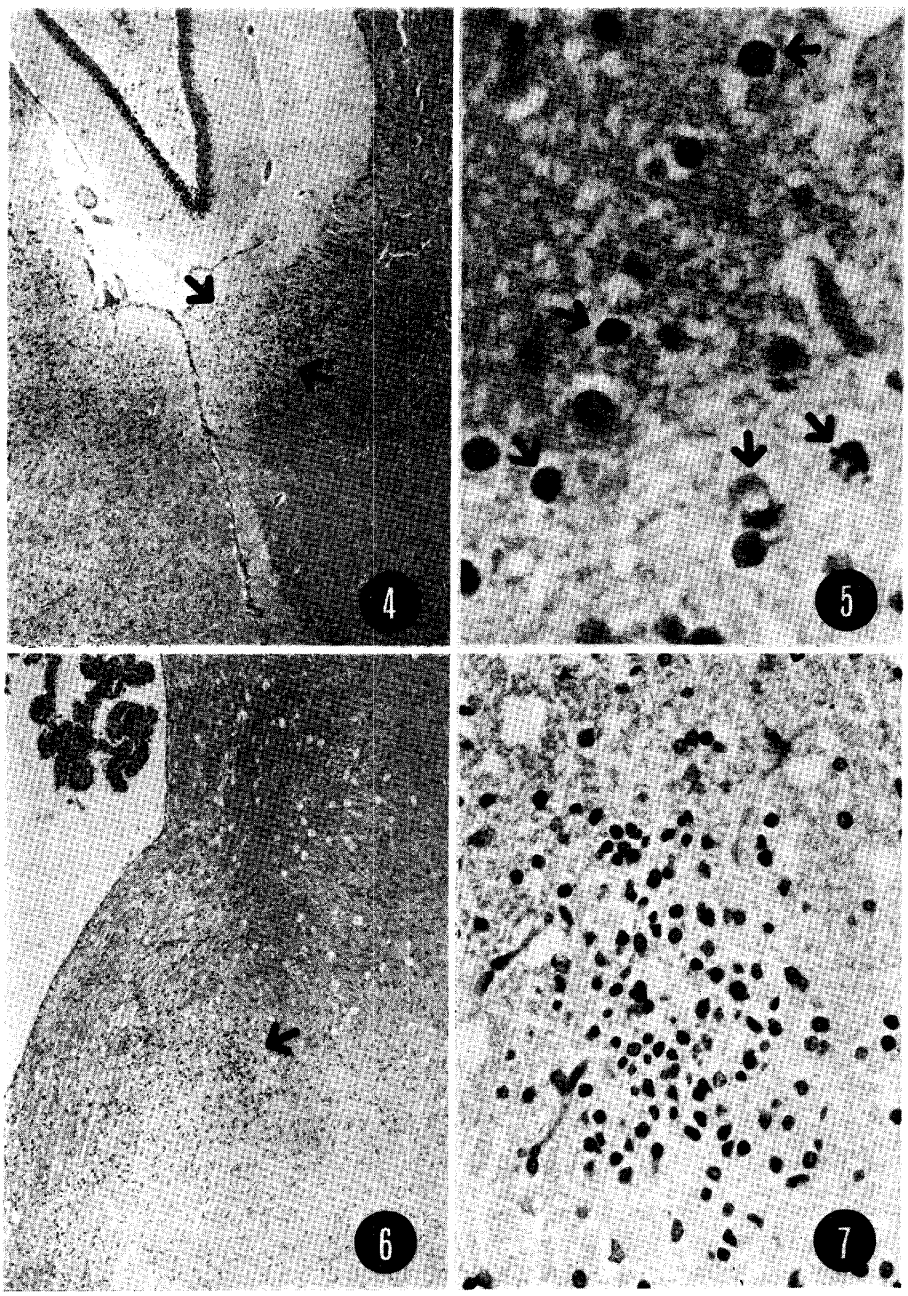
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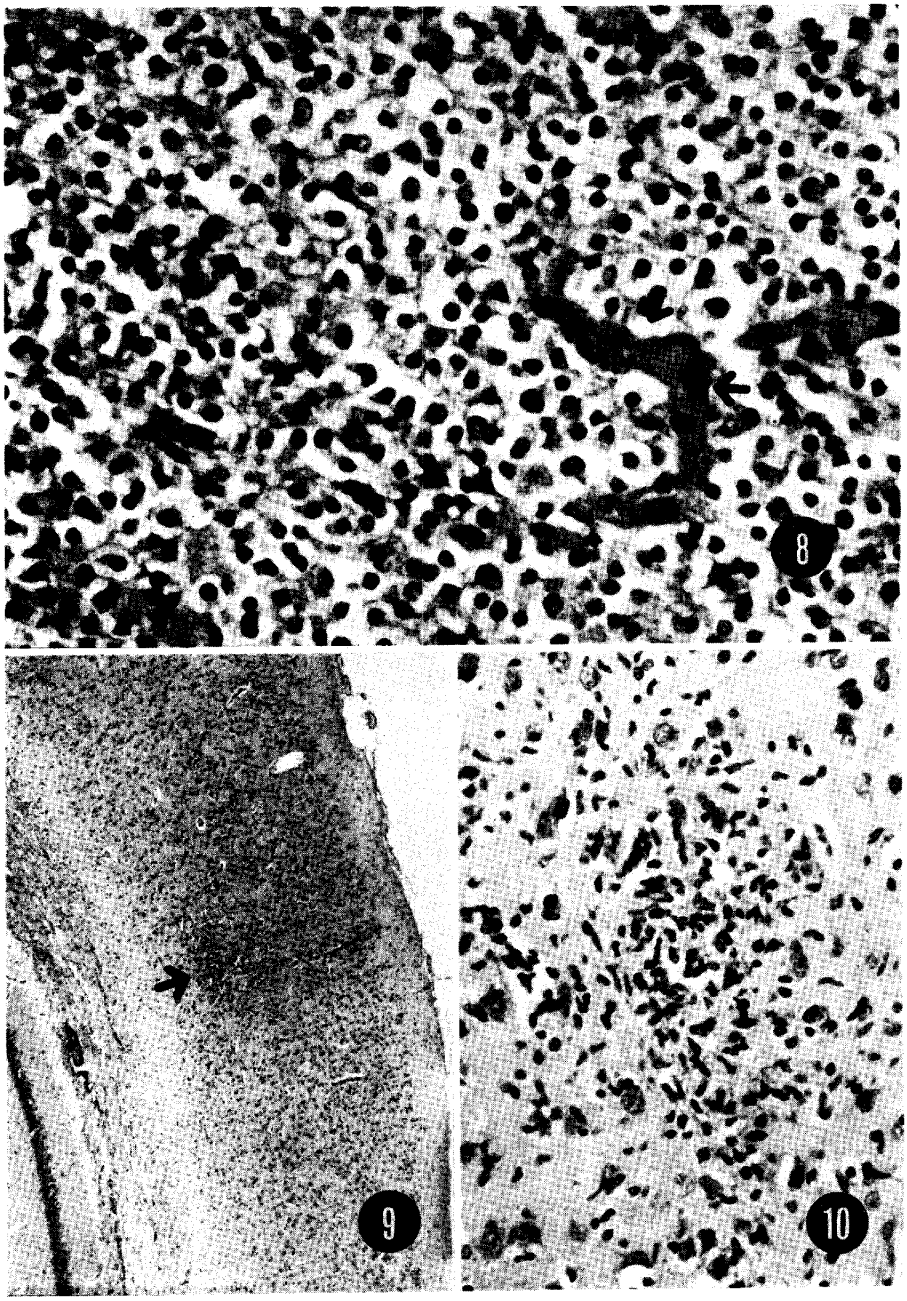
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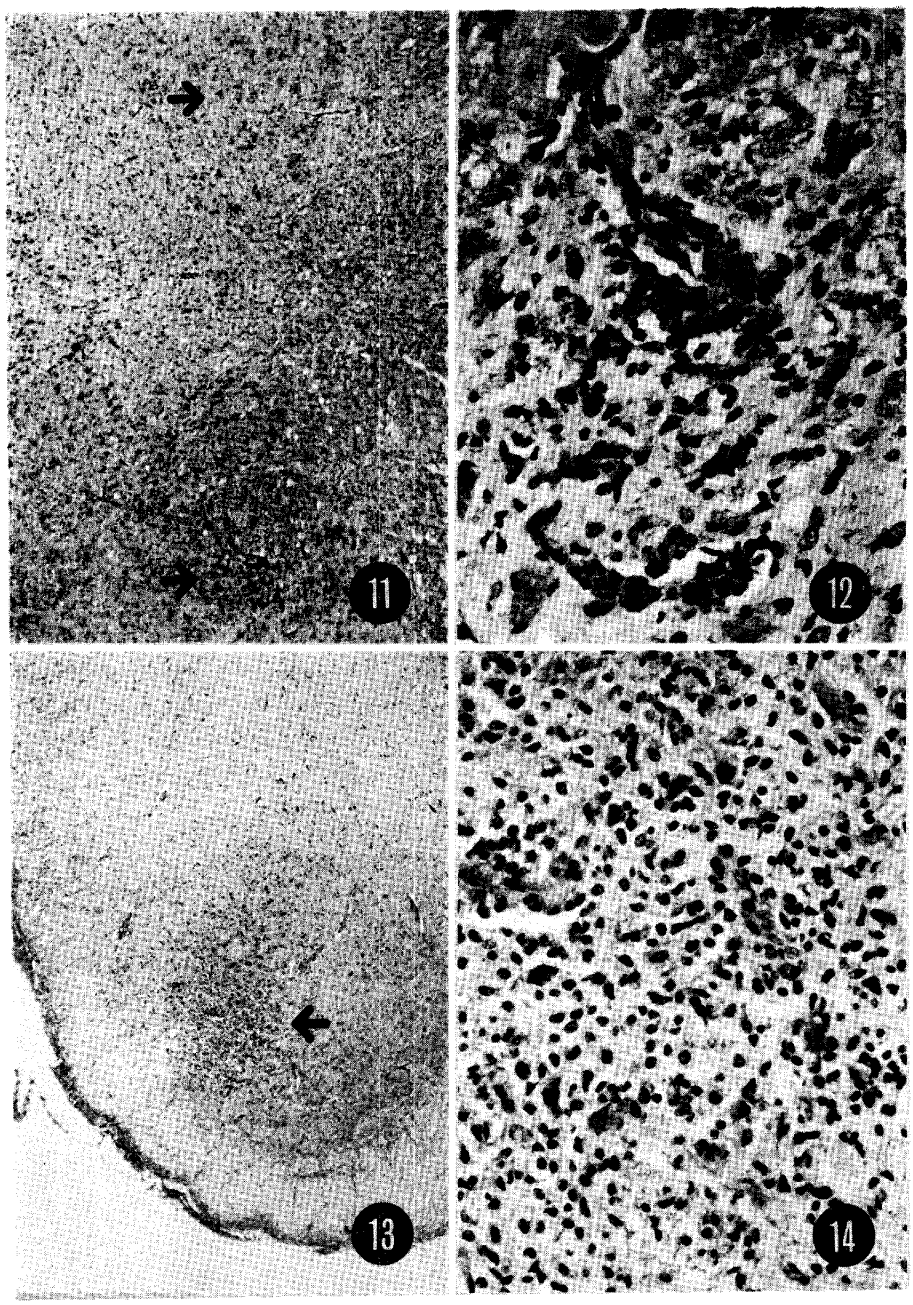
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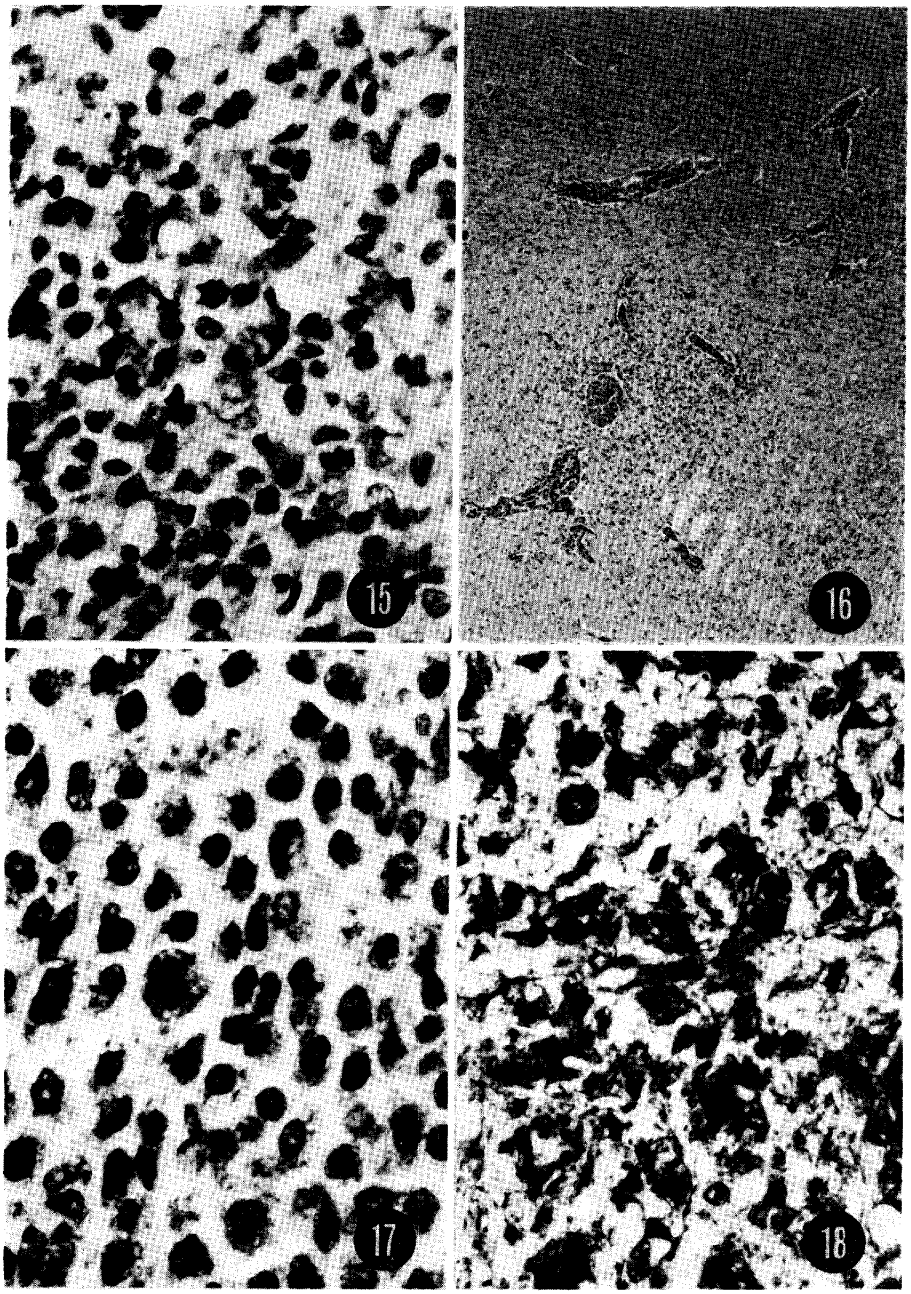
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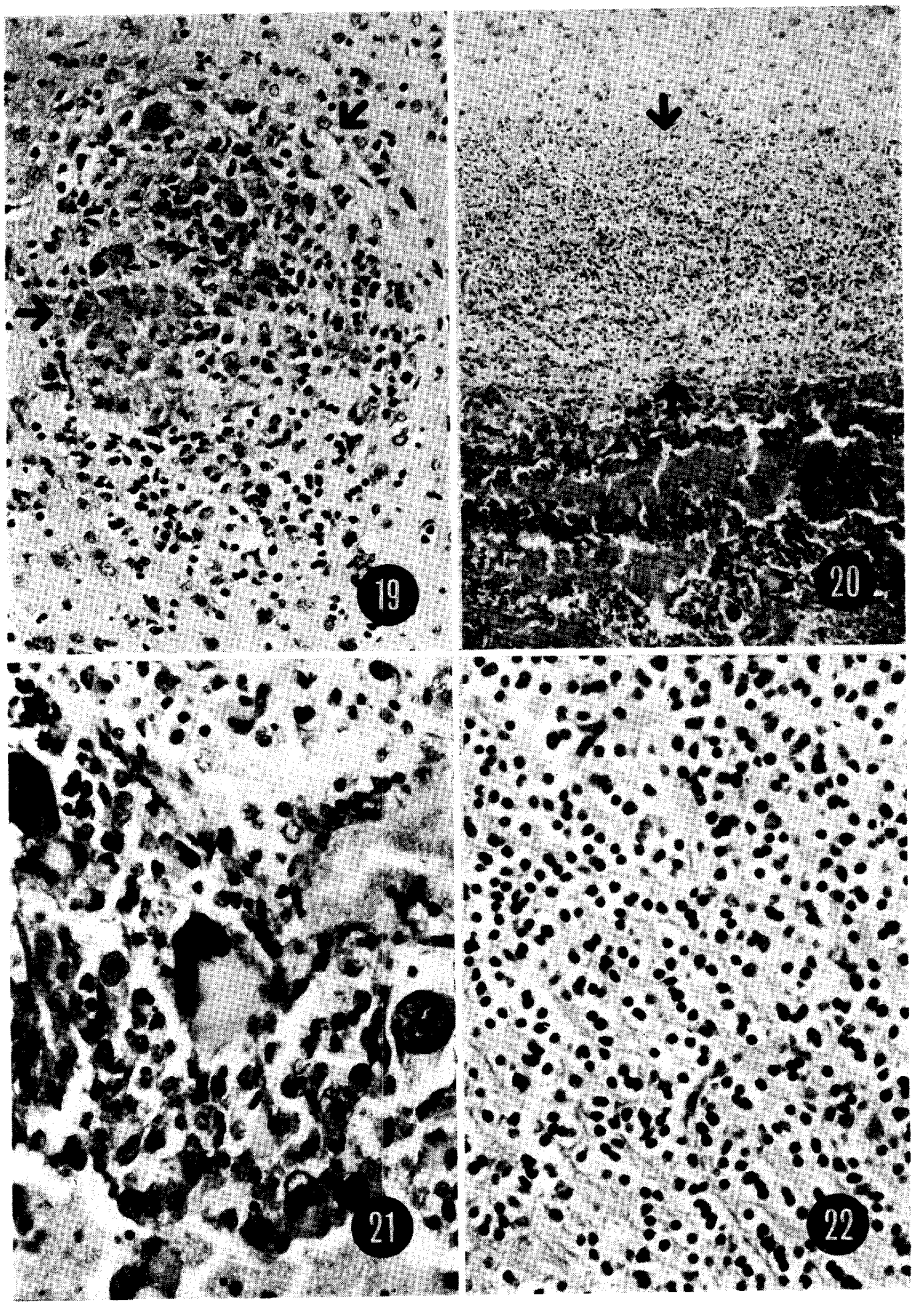
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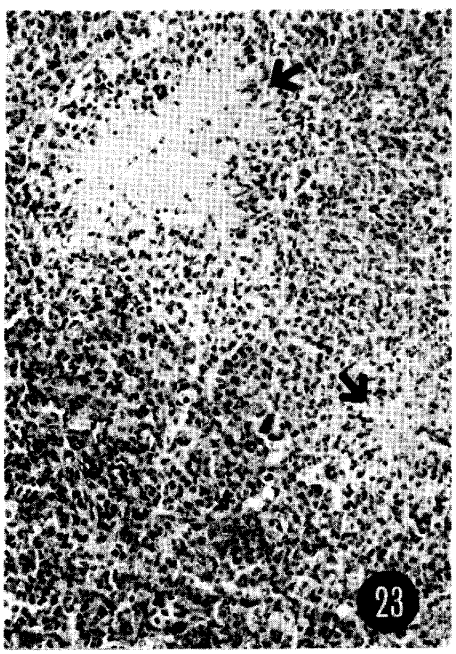

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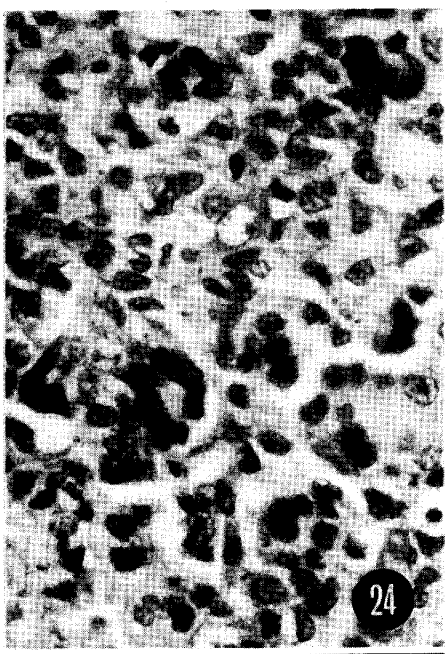

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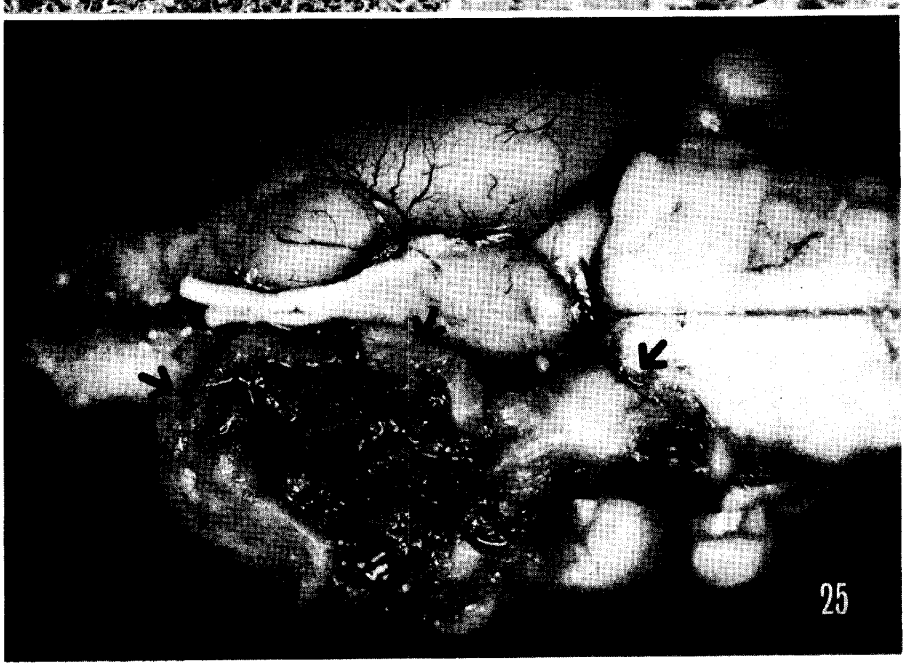

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