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Metastasis from the Brain of Transplanted *N*-Ethyl-*N*-Nitrosourea-Induced Central Nervous System Tumors in Rats

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Abstract—*Two transplantable rat central nervous system (CNS) tumors induced by N-ethyl-N-nitrosourea (ENU) were employed to study the mechanisms controlling extracranial metastasis from intracranial tumors. Cells derived from a serially passaged anaplastic astrocytoma and a malignant glioma were injected intracerebrally at doses of* 10^4, 10^5 *and* 10^6 *cells per rat (Sprague–Dawley and WAG/Rij rats). As soon as neurological dysfunction appeared, animals were sacrificed and examined histologically for* (1) *extracerebral outgrowth of the intracerebral tumor,* (2) *the presence of distant metastases within the CNS and* (3) *remote metastases outside the CNS. In addition to histology, a bioassay procedure was performed. Metastases were found in cervical lymph nodes* (74%), *lung* (21%) *and liver* (5%). *For both tumor groups, rats with both distant CNS metastases and local extracerebral outgrowth developed remote metastases more frequently* ($P<0.05$) *than animals with intracerebral growth only. The data indicate that both local extracranial spread due to surgical intervention as well as local and distant invasion of leptomeninges promote extracranial metastatic spread.*

INTRODUCTION

THE APPEARANCE of a remote metastasis from a primary CNS tumor is a rare but recognized phenomenon in man. To date more than 200 of such cases have been reported in the literature [1–4]. When metastasis does occur, it is generally related to prior surgical intervention or to tumor growth into the leptomeninges. The rarity of extracranial metastasis from brain tumors has been ascribed to immunological properties and the absence of lymphatics in the brain, the biological characteristics of malignant glioma cells, the blood–brain barrier and quantitative factors related to short survival of the patient due to the limited space within the cranial cavity [5–7]. To our knowledge, no experimental studies have been performed to test these hypotheses.

In general, chemically induced brain tumors do not metastasize from their intracranial site of origin even though dissemination has been observed following subcutaneous transplantation [8, 9]. In this study ENU-induced primary brain tumors were transplanted into the cerebral tissue of syngeneic rats in order to study metastasis from the brain. The rat was chosen as an experimental model since several transplantable CNS tumors from a previous study [9] were available.

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MATERIALS AND METHODS

Animals

Partially inbred Sprague–Dawley (SD) and inbred WAG/Rij rats were used. The animals were housed three to five per cage and were given tap water and standard laboratory chow *ad libitum*. Details on the husbandry conditions in our colony have been published [10].

Tumors

The methods employed for inducing the primary CNS tumors have been reported [9]. Briefly, ENU was administered i.m. at a dose of 10 mg per kg body weight to new-born rats. When neurological dysfunction appeared, the

animals were killed, and the tumors removed and transplanted subcutaneously into 2- to 3-month old male rats of the same strain. From a series of 77 ENU-induced central nervous system tumors two tumor lines with proven metastatic potential were chosen. One tumor line, histologically characterized as an anaplastic astrocytoma, originated in an SD rat and was serially transplanted subcutaneously for approximately 70 passages without apparent histological change until the current intracerebral implantations. A second tumor line appearing initially as an astrocytoma and originating in a WAG/Rij rat was transplanted subcutaneously for 10 passages during which time this tumor changed histologically into a malignant glioma. Such a histological change has also been observed by others [11, 12]. During a period of three years this tumor was kept frozen in dimethylsulfoxide (DMSO) at −190°C. After rapid thawing, the tumor was transplanted subcutaneously for two passages, followed by intracerebral implantation.

The tumor cell suspension was prepared by finely mincing fragments of tumor tissue from which necrotic areas had been removed, followed by mixing with Hank's balanced salt solution, addition of collagenase (Sigma) and shaking in a water bath at 37°C. The suspension was centrifuged at 400 *g* for 10 min, the fluid decanted and the cell pellet resuspended in Hank's solution. In order to achieve a monocellular suspension, it was filtered through four layers of nylon gauze. Viable cells were counted using trypan blue and the cell suspension was adjusted to the required concentration: 10^4, 10^5 or 10^6 cells per 0.05 ml.

Intracerebral inoculation

The animals were anesthetized with ether and the head was immobilized. The skin was incised on the midline of the skull and the calvarium was cleared of subcutaneous tissue. In the right parietal bone, 2.5 mm rostral to the sutura lambdoidea and 2.5 mm dextrad to the sutura sagitallis, a small burr hole was made using care to not damage the dura mater. The cell suspension was injected by means of a 25 g needle, which was inserted vertically into the brain through the burr hole. The suspension was slowly injected at a depth of 4.5 mm from the calvarium by means of a needle fitted with a special collar. Then the needle was withdrawn for 2 mm, followed by electrocautery upon the needle during further withdrawal in order to sear the needle tract preventing egression of tumor cells. The burr hole and its direct surroundings on the calvarium were cauterized to kill escaped tumor cells. The skin was closed with autoclips. The whole procedure lasted about 15 min.

Follow-up and necropsy

The animals were inspected daily. As soon as neurological signs appeared (i.e., severe apathy, hypotonia, paralysis), animals were killed and a necropsy was done. The following tissues were fixed in 4% formaldehyde, stained with hematoxylin–phloxine–saffron (HPS) and examined histologically: skin of the skull, calvarium surrounding burr hole, brain, base of skull, thoracic vertebral column with spinal cord, lung, heart, sternum, liver, spleen and submandibular, cervical, para-aortic, mesenteric and axillary lymph nodes.

When the neoplasm was confined to the injection site with involvement of the overlying leptomeninges, it was designated *intracerebral growth.* When clumps of tumor cells were observed adherent to the dura or invading the calvarium around the burr hole or even more outward in the subcutaneous tissue of this area, the growth was termed *local (extracerebral) outgrowth.* When tumor cells were encountered in the meninges at the level of the anterior, middle and posterior cranial fossa and/or in the meninges of the spinal cord, these were scored as *distant CNS metastases.* When tumor was found in extraneural organs, it was called *remote metastasis.*

Bioassay

In addition to sampling for histology, a bioassay was performed in 19 and 22 rats from the anaplastic astrocytoma and malignant glioma groups, respectively; histological examination alone was done in two and six rats from the two respective groups. The bioassay was performed by transplanting parts of lung, liver, cervical lymph nodes and blood clots from a tumor-bearing rat to a syngeneic recipient. These tissues were transplanted subcutaneously on both sides of the back in a fixed sequence. The recipient rats were kept under observation until palpable tumors appeared which were examined histologically. When no palpable lesions were found by three months after transplantation, the rats were sacrificed and the subcutaneous sites examined for neoplastic growth.

Student's *t* test and Fischer's exact probability test were used for statistical analysis.

RESULTS

Anaplastic astrocytoma

This tumor showed a solid growth pattern with extension along intracerebral vessels and

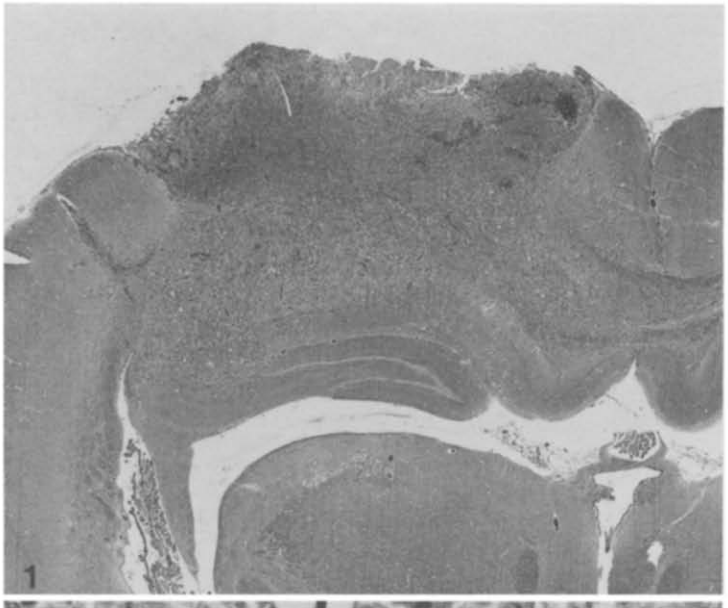


Fig. 1. Photomicrograph of cerebral cortex of a male Sprague–Dawley rat injected intracerebrally with 1×10^6 *astrocytoma cells* 24 days *before death. Note infiltration by the tumor of the hippocampus and corpus callosum, and extension along intracerebral blood vessels. Hematoxylin–phloxine–saffron (HPS)* × 14.

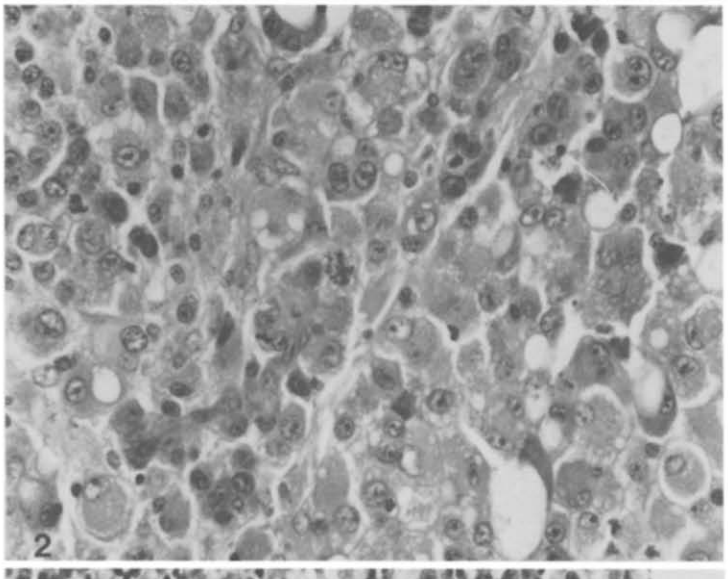


Fig. 2. Higher magnification of tumor in Fig. 1. The tumor cells are moderately pleomorphic and possess abundant cytoplasm which is often vacuolated. The nuclei contain one or two prominent nucleoli. Note the atypical mitosis in the center of the photomicrograph. HPS × 340.

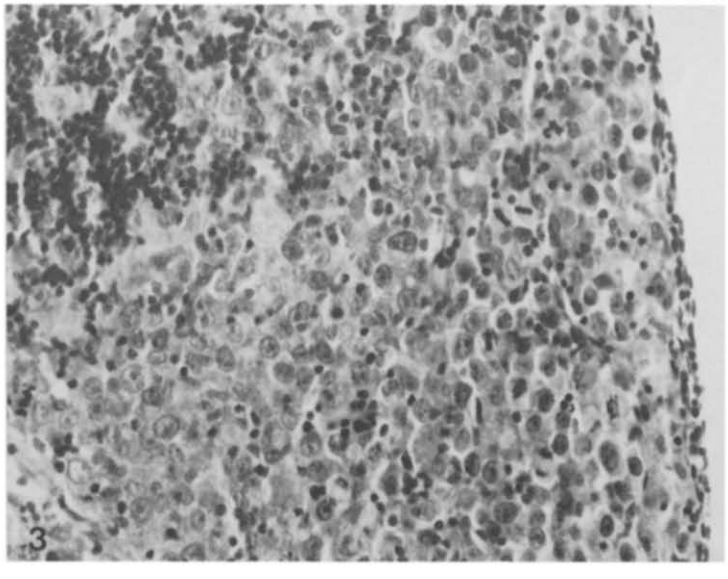
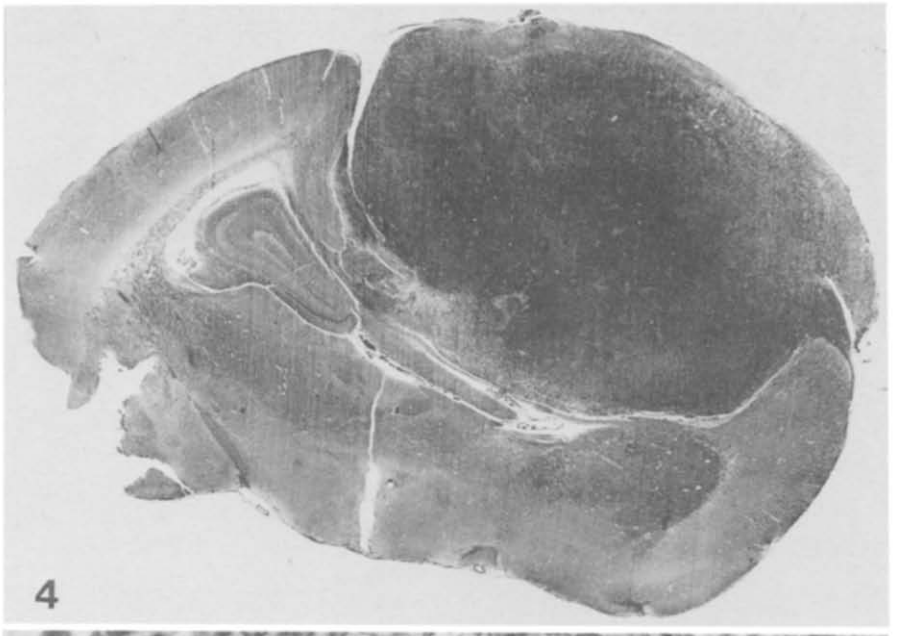


Fig. 3. Cervical lymph node with metastatic astrocytoma from a male Sprague–Dawley rat injected with 1×10^4 *tumor cells. The subcapsular sinus (right hand side of photomicrograph) and the adjacent cortex are diffusely infiltrated by tumor cells. Note the numerous mitotic figures. HPS* × 340.



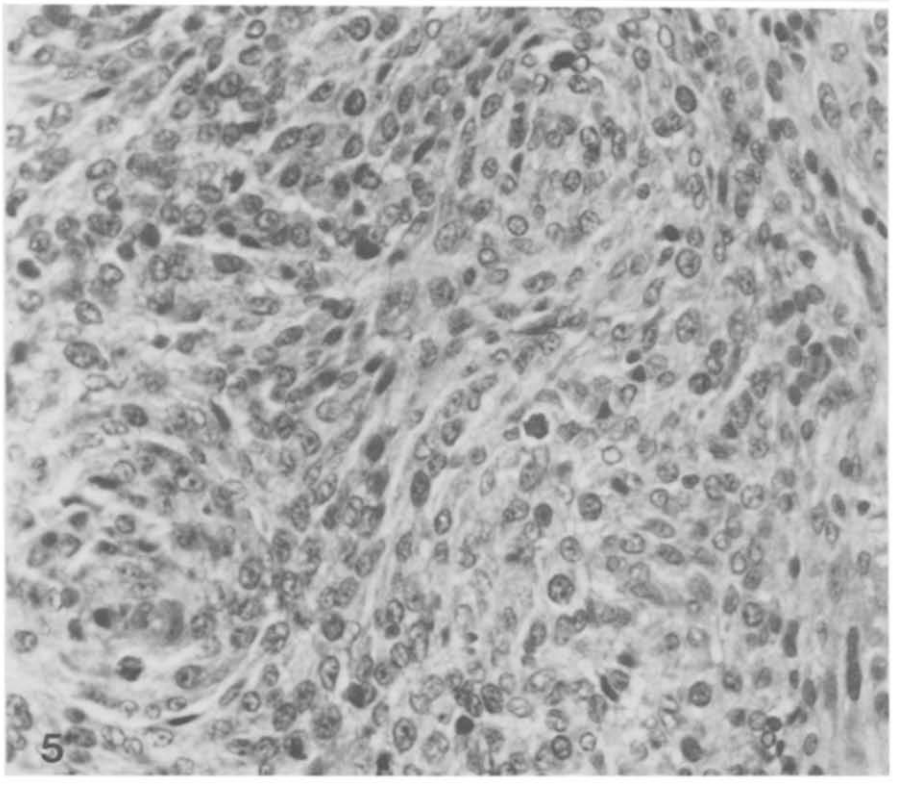


Fig. 4. *Coronal section of brain from a male WAG/Rij rat injected intracerebrally* 18 days *previously with* 1×10^6 *malignant glioma cells. Note infiltration of subarachnoid space at the periphery of the tumor. HPS* × 7.

Fig. 5. *Higher magnification of malignant glioma shown in Fig.* 4. *The tumor cells are fusiform or stellate with indistinct cytoplasmic borders, and are compactly arranged in fascicles and whorls. HPS* × 400.

often diffuse infiltration of the subarachnoid space (Fig. 1). Direct extension into the ventricular cavities was also a common feature. In the center of this tumor, areas of necrosis were found regularly. The tumor cells varied in size and were generally round or polygonal (Fig. 2). The nuclei were round or oval with usually one or two prominent nucleoli. Multinucleated cells were commonly seen. The cytoplasm was abundant, vacuolated and acidophilic. Mitoses were relatively common including many atypical mitoses. The overall histological appearance most resembled a pleomorphic gemistocytic astrocytoma.

In all 21 cases the tumor had invaded the leptomeninges at the convexity of the cerebral hemisphere. Sometimes it seemed as if the outward growth was facilitated via the needle tract. Distant CNS metastases were found in seven animals. Remote metastases outside the CNS were recognized in eight cases in cervical lymph nodes only (Fig. 3). Large, occasionally bizarre, cells with large nuclei and abundant cytoplasm in the subcapsular and cortical sinuses of the lymph node were readily visible.

Malignant glioma

The tumor was generally solid and expansive. Diffuse infiltration of the subarachnoid space at the convexity of the cerebral hemispheres was always present and direct extension into the ventricular cavities was frequently seen (Fig. 4). This tumor was characterized primarily by two histological patterns. The predominant type consisted of interwoven fascicles and whorls composed of compactly arranged spindle-shaped or stellate cells with uniformly appearing round to oval nuclei (Fig. 5). The cells possessed a moderate amount of acidophilic cytoplasm which was often finely vacuolated. The second, less common pattern consisted of loosely arranged stellate or fusiform cells with round nuclei and scanty acidophilic cytoplasm. In ten cases distant CNS metastases were found. Metastasis to lymph nodes was often difficult to assess in routine histological sections due to the small number of metastatic cells in lymph nodes when present, and also due to their resemblance to histiocytes. For that reason the assessment of metastasis was based on positive bioassay. In the seven cases in which remote metastases were found, they occurred in cervical lymph nodes (three cases), lung (one case), both cervical nodes and lung (two cases) and cervical node, lung and liver (one case).

For both the anaplastic astrocytoma and malignant glioma, the incidence of remote metastasis in rats with (a) intracerebral growth only, (b) distant CNS metastasis, (c) local extracerebral outgrowth or (d) both distant CNS metastasis and local extracerebral outgrowth are given in Table 1, for 10^4, 10^5 and 10^6 injected tumor cells separately. Table 2 shows the combined data and survival times. In both the anaplastic astrocytoma and malignant glioma groups the rats with both distant CNS metastasis and local extracerebral outgrowth had remote metastases more frequently ($P<0.05$) than did the rats with intracerebral growth only: three out of three (100%) versus three out of thirteen (23%), respectively, for the astrocytoma-bearing rats, and five out of eight (63%) versus one out of nine (11%), respectively, for the glioma-bearing rats. Survival times did not differ significantly among the various groups, except for the total number of glioma-bearing rats with remote metastases (28.9 ± 4.8 days) as compared to the total number without remote metastases (22.1 ± 4.8 days, $P<0.001$). Table 3 shows the assessment of metastases by either histology or bioassay.

DISCUSSION

This study was performed in order to examine whether metastasis from primary brain tumors could be produced and, if so, to study the underlying mechanism. We could identify three factors that play a role in the development of remote metastasis. The first is local extracerebral outgrowth from the intracerebrally implanted tumor. The local outgrowth invariably appeared at the site of burr hole and needle tract through which injection of tumor cells took place. We assume that the tumor cells, once outside the CNS, can invade lymphatic channels and/or blood vessels and that this readily explains remote metastasis, particularly since most metastases were found in the cervical lymph nodes.

A second factor was the presence of the leptomeningeal spread. In human pathology, local involvement of the subarachnoid space is well known with glioblastomas and wider dissemination even into the spinal canal is common and well documented [13–15]. Distant CNS metastasis to the base of the skull and/or the spinal cord is the second most common factor associated with remote metastasis outside the CNS in man (the first is tumor at the site of the operation flap) [3]. On theoretical grounds, the transdural exten-

Table 1

No. of cells injected	Intracerebral	Distant CNS metastasis	Local outgrowth	Both distant CNS metastasis and local outgrowth
		Anaplastic astrocytoma		
10^4 cells				
with remote met.	1	0	1	0
without remote met.	1	1	0	0
(no growth: $n=6$)				
10^5 cells:				
with remote met.	0	1	0	2
without remote met.	3	1	0	0
(no growth: $n=1$)				
10^6 cells:				
with remote met.	1	1	0	1
without remote met.	6	0	1	0
(no growth: $n=0$)				
		Malignant glioma		
10^4 cells*				
with remote met.	0	0	1	4
without remote met.	0	0	0	2
(no growth: $n=0$)				
10^5 cells				
with remote met.	0	0	0	0
without remote met.	5	1	1	1
(no growth: $n=1$)				
10^6 cells†				
with remote met.	1	0	0	1
without remote met.	3	1	1	0
(no growth: $n=0$)				

* In four animals no bioassay was performed and these were thus not included (see also text in Results section).
† In two animals no bioassay was performed and these were thus not included.

sion of glioma cells through the pacchionian granulations of the arachnoid has been suggested as a mode of exit [16]. The leptomeninges of rats with intracerebral growth only of both tumor lines were found invariably to be locally invaded at the site of inoculation. In patients with metastases from glioblastomas without prior surgery, the tumors invaded meninges or veins and sinuses in most cases [5, 17–19]. Thus, remote metastasis could also occur along this route. However, in our rats, it was only when both local outgrowth and distant CNS metastases were present that remote metastasis became significantly more likely ($P<0.05$). Occurring simultaneously, these factors probably simply increase the chance of extracranial spread and in this way facilitate metastasis. Admittedly, in either the local extracerebral outgrowth group or the distant CNS metastasis group, the incidence of remote metastasis is not as high as one would have expected in line with this explanation. Yet, the group size was small and the observed distribution of metastasis uneven and, therefore, argues neither for nor against it.

A third factor may be a prolonged survival time necessary to develop extraneural metastasis [3]. The significantly longer survival time in rats with remote metastasis of malignant glioma might very well be explained as such.

The remote metastasis observed in this study is in contrast to the lack of metastasis from experimentally induced primary brain tumors in previous studies [8, 12, 14]. We did not observe any metastasis in the 77 ENU-induced rat primary CNS tumors of which two cell lines were used in this study [9]. There are, however, two major differences between those observations and our present results. Firstly, in this study a traumatic intervention with interruption of the skull, meninges and cerebral tissue took place. Although precautions were taken to prevent this, spread of tumor cells during the injection procedure or later from this site may have taken place. In this respect, it is of interest that in the case of clinical remote metastasis, 87–92% of all cases were subjected to prior surgical operation. Thus, for obvious reasons many authors have put much emphasis on the role of surgery in explaining metastasis from the brain [1, 4, 5]. Secondly, as already mentioned, all tumors observed on microscopic examination showed growth at or over the convexity of the

Table 2

	With remote metastasis		Without remote metastasis	
	No. (%)	Survival* (days: $n \pm$S.D.)	No. (%)	Survival* (days: $n \pm$S.D.)
			Anaplastic astrocytoma	
Intracerebral ($n=13$)	3(23%)	61.3±33.9	10(77%)	42.0±23.5
Distant CNS met. ($n=4$)	2(50%)	35.0±15.6	2(50%)	45.5±36.1
Local outgrowth ($n=1$)	0(0%)	—	1(100%)	33.0
Distant CNS met. & local outgrowth ($n=3$)	3(100%)†	27.0± 9.6	0(0%)	—
Total ($n=21$)	8(38%)	41.9±25.4	13(62%)	40.5±22.5
			Malignant glioma	
Intracerebral ($n=9$)	1(11%)	21.0	8(89%)	20.0± 1.7
Distant CNS met. ($n=2$)	0(0%)	—	2(100%)	20.0± 0.0
Local outgrowth ($n=3$)	1(33%)	29.0	2(67%)	24.0± 4.2
Distant CNS met. & local outgrowth ($n=8$)	5(63%)†	30.4± 4.0	3(37%)	28.7± 4.0
Total ($n=22$)	7(32%)	28.9± 4.8‡	15(68%)	22.1± 4.8

*All animals were killed when neurological signs were first seen.
†Significantly different ($P<0.05$) from animals with intracerebral growth.
‡Significantly different ($P<0.001$) from survival of total number of rats without remote metastases (malignant glioma group).

Table 3. *Frequency of remote metastasis*

	Anaplastic astrocytoma	Malignant glioma
Total cases	8*	7†
As assessed by histology	4	0‡
As assessed by bioassay	2	7
As assessed by both histology and bioassay	2	0

*Metastases were found in cervical lymph nodes only.
†Metastases were found in cervical lymph nodes, lung and liver; for further description, see Results.
‡Histology was regarded as unreliable in determining metastasis in cervical lymph nodes and conclusions were based on bioassays only; for further explanation, see Results.

brain with growth into the subarachnoid space.

In the anaplastic astrocytoma group all remote metastases were found in cervical lymph nodes. In the malignant glioma-bearing rats, metastases were also mainly cervical. The lung and liver metastases in the latter group could be either the result of hematogenous spread secondary to lymphogenous metastasis or, of course, due to primary hematogenous spread. It has been postulated that the CNS must have lymphatics, based on the mere presence of cervical lymph node metastases in unoperated glioma patients [1]. This view is contrary to the general opinion regarding the presence of lymphatics in the CNS [20]. In this context it is of interest that within the Virchow–Robin spaces thin-walled channels resembling lymphatic capillaries have been found recently in neurological patients [21]. Our results do not shed new light on this question because the cervical metastases may

be fully explained by surgical intervention and the other metastatic sites (lung, liver) either as a secondary hematogenous metastasis or primary due to invasion of leptomeningeal vessels and veins.

Since surgery for brain tumors is common, meningeal spread is not rare [15], and patients being treated are living longer in general, one might question why extracranial metastasis is not found more frequently. Properties related to the glial tumor cell are unlikely because these tumors grow easily outside the CNS and are able to metastasize from a subcutaneous site [8, 9, 12]. For the same reason, immunological reasons seem improbable as a preventive factor for growth and metastasis outside the CNS. What remains are features associated with the CNS itself. In particular, factors related to a barrier for glial cells to invade blood vessels (blood–brain barrier) and quantitative factors due to the limited space within the cranial cavity leading to short survival time [7]. It seems that these quantitative factors combined with the difficulty of introducing brain tumors without surgery, makes it difficult to elucidate the problem further.

We conclude that the observed metastasis from transplanted ENU-induced CNS tumors is probably related to unavoidable surgical intervention and/or to invasion of local and distant leptomeninges and blood vessels by the tumor. In this way it confirms considerations put forward to explain metastasis from the brain.

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