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A SURVEY OF ETHYLNITROSOUREA-INDUCED RAT GLIOMAS FOR THE PRESENCE OF TUMOUR REJECTION ANTIGENS EXPRESSED *IN VIVO*

ALEX M. SPENCE AND GREGORY PRIESTLEY
Departments of Medicine (Neurology) and Pathology (Neuropathology), University of Washington School of Medicine, Seattle, Washington, USA

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A survey of ethylnitrosourea-induced rat gliomas for the presence of tumour rejection antigens expressed *in vivo*

Transplanted lines of seven F-344 (Fischer) rat malignant gliomas induced transplacentally with ethylnitrosourea (ENU) were surveyed by *in vivo* immunoprotection assays for the presence of tumour rejection antigens. These gliomas were representative of commonplace histological types of human primary brain tumours and were analyzed in early transplantation passages. The classical tumour ligation method of immunizing animals was attempted with five glioma lines, but was found unusable in four of these because of a high incidence of local tumour recurrences and distant metastases. In most experiments the animals were immunized by repeated inoculations of heavily-irradiated tumour cells. Two gliomas, a glioblastoma multiforme and a mixed astrocytoma-ependymoma, demonstrated weak but statistically significant tumour rejection responses. Immunization with three other tumours, a mixed oligodendroglioma-astrocytoma and two glioblastomas multiforme, led to enhanced outgrowth of the challenge cell inocula. Neither a rejection nor an enhancement response was observed in assays of the remaining two neoplasms, a glioblastoma multiforme and a mixed astrocytoma-oligodendroglioma. Immunization with a 3-methylcholanthrene-induced urinary bladder carcinoma line, used as a control in assays of six gliomas, had no effect on the outgrowth of transplanted glioma cells. These results suggest that ENU-induced malignant rat gliomas do not uniformly elicit strong tumour-rejection responses *in vivo*.

Introduction

Whether malignant gliomas express tumour-specific antigens that activate tumoricidal anti-glioma immunity *in vivo*, i.e. are immunogenic, is a question of

Address for correspondence: Dr A. M. Spence, RG-20 Neurology, University of Washington, Seattle, WA. 98195, USA.

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fundamental importance to understanding the role of immunosurveillance controlling the development of these neoplasms, the role of the immune privilege of the central nervous system (CNS) in limiting anti-glioma immunity, and the potential role of immunotherapeutic management of these tumours. Although there is now a large body of evidence on the immunobiology of human and experimental brain tumours (Brooks & Roszman, 1978; Cravioto, 1978; 1978b; Wikstrand & Bigner, 1980), it remains uncertain whether gliomas of the common histological types, namely, glioblastoma multiforme, malignant astrocytoma and mixed glioma, uniformly express tumour rejection antigens *in vivo* and, if they do so, whether these antigens are weak or strong.

Several chemically-induced rodent gliomas have been reported to elicit strong tumour rejection responses *in vivo*. These tumours include at least three 3-methylcholanthrene (MCA)-induced C57/B1 mouse ependymal and astrocytic gliomas (Long, O'Conner & Jelsma, 1962; Scheinberg *et al.*, 1962; Wilkins & Ketcham, 1963) and three methylnitrosourea (MNU)-induced F-344 rat astrocytic gliomas (Blume, Wilson & Vasquez, 1974; Cravioto, 1972a; Denlinger *et al.*, 1975; Morantz *et al.*, 1979; Tracey *et al.*, 1978). However, the immunogenicity of most of these tumours was examined after they had been transplanted for many generations so that antigenic changes in the tumour lines or genetic drift in the animal strains of tumour origin may have supervened (Pellis & Kahan, 1976). It is necessary to be cautious, therefore, in concluding from these observations that most gliomas are immunogenic.

We have recently studied seven malignant gliomas induced in F-344 rats with the potent neurotropic carcinogen, ethylnitrosourea (ENU) (Koestner, Swenberg & Wechsler, 1971) using conventional transplantation assays (Heberman, 1977; Sjögren, 1965) to test for tumour rejection antigens. All seven tumours represent histological types of gliomas commonly encountered in human neuro-oncology, and all were investigated early in their transplantation history in order to minimize potential immunological changes due to a long-term serial transplantation maintenance.

Materials and methods

Animals

F-344 (Fischer) rats (Simonsen Laboratories, Gilroy, California) were used. Tumours were induced with ENU in 'foundation line' animals produced by brother-sister matings which had been documented. Limited skin transplantation tests in our laboratory have shown that Simonsen F-344 rats accept grafts from one another.

Tumors

Pregnant rats were injected intravenously with 50 mg/kg of ENU (gift of Dr T. Lloyd Fletcher) between the 17th and 20th days of gestation in order to induce neurogenic neoplasms in the

offspring (Koestner *et al.*, 1971). When these offspring manifested signs of tumour growth between 196 and 405 days of life, they were killed by exsanguination under ether anaesthesia.

Intrinsic brain and spinal cord tumours were isolated under sterile conditions and then divided into portions for routine histology processing and for transplantation or explantation in cell culture. Transplantation was performed in sex-matched syngeneic recipients at the subcutaneous site. Cell cultures were maintained in Waymouth's medium containing 15 or 30% fetal calf serum plus additives as previously described (Spence & Coates, 1978). Seven glioma lines were selected for further study. Four of these were maintained exclusively by subcutaneous transplantation in rats, and three others were initially explanted *in vitro* followed by transplantation in animals as described by Benda *et al.* (1971). Table 1 provides the histological diagnoses of the seven original gliomas from which the transplanted lines were established. It also shows the transplant stage reached by the individual tumour lines at the time they were studied in immunoprotection assays: C_n and A_n refer to culture or animal passage number. For example, C_5A_3 indicates that a tumour, used for either immunization or transplantation challenge, was removed from its parent autochthonous glioma by 3 culture passages followed by three animal passages.

A syngeneic MCA-induced urinary bladder carcinoma line, BC158, donated by Dr Warren Chapman, was used at A_3-A_9 passages in several experiments to immunize control animals against a non-gliogenous neoplasm. This neoplasm expresses tumour-specific transplantation antigens (personal communication).

Immunoprotection assays

To assay for tumour rejection antigens immunization of syngeneic animals was usually performed by ligation or excision of growing tumour grafts, or by inoculation of either radiation-killed tumour cells or live tumour cells at sublethal doses (Heberman, 1977, Sjögren, 1965). Thereafter groups of immunized or unimmunized control animals were challenged with viable tumour cells and tumour outgrowth in experimental and control groups compared.

Preparation of cell suspensions

For both immunization and challenge, cell suspensions were prepared from non-necrotic subcutaneous tumour implants, never directly from material in tissue culture, to avoid any potential

Table 1. The identifying numbers and histological diagnoses of the seven autochthonous gliomas examined, and the passage numbers of their transplanted derivatives used in immunoprotection assays

Tumour number	*Histological diagnosis*	*Passage numbers*
T131B	Spinal mixed oligodendroglioma-astrocytoma	C_9A_1 - - C_9A_2
T161B	Cerebral glioblastoma	C_2A_2 - - C_2A_4
T175A	Cerebral glioblastoma	A_2 - - A_3
T180A	Cerebral mixed astrocytoma-oligodendroglioma	A_1 - - A_4
T181A	Cerebral glioblastoma	A_3 - - A_4
T181B	Cerebral glioblastoma	C_6A_1 - - C_6A_3
T185A	Cerebral mixed astrocytoma-ependymoma	A_3 - - A_5

C = culture, A = animal.

immunologic effects of calf serum components in tissue culture medium. All cell suspensions were prepared by mechanical dispersion methods to avoid trypsinization. Briefly, the tumours were finely minced with iris scissors in chilled serum-free Waymouth's medium. The resulting fragments were gently pressed through 30 or 50 gauge stainless steel screens. This yielded a suspension of single cells mixed with small clumps. For immunization procedures both clumps and single cells were retained and counted together to determine a total cell concentration. For challenge procedures, however, further steps were taken. Clumped cells were removed by allowing the cell suspensions to settle in test tubes for 5 to 10 minutes after which the upper layers were retained. The yield of viable cells was determined by the trypan blue dye exclusion method and found to be generally in the 25 to 40 % range.

Tumour immunization

We first tried to immunize animals by ligation of subcutaneous tumour grafts when they were 5–15 mm in diameter. This was attempted with T131B, T161B, T180A, T181A, and T181B. However, except with T181B this method failed because of local recurrences and the frequent development of lymph node and lung metastases. With T181B, only sixteen of thirty-six animals did not develop recurrences following tumor ligation. Other investigators have reported similar difficulties in working with nitrosourea-induced gliomas (Cravioto, 1978a; Morantz *et al.*, 1979).

Consequently, with all tumors except T181B immunization with radiation-killed tumour cells became the principal method we adopted. Tumour cell suspensions were immediately irradiated with ^{60}Co giving a total dose of 15 000 rad, and were then inoculated in a volume of 1–2 ml at one site per animal. The immunizing dose per animal in each immunization ranged from $2{\cdot}0 \times 10^7$ to $9{\cdot}0 \times 10^7$ cells. The immunizing inocula were given in the dorsal subcutaneous tissue between the shoulder blades or i.p. (Price *et al.*, 1978). Despite treatment with 15 000 rad, these immunizing inocula produced palpable nodules which often attained 5–10 mm in diameter before they disappeared after 7–14 days. From 2 to 6 glioma cell immunizations were performed in all assays. Animals immunized with the BC158 control tumour were similarly treated with irradiated cells subcutaneously.

In initial experiments with T180A, T181A, and T185A, some animals received live cells but did not develop tumours. These animals were incorporated as immunized hosts into new experiments after they had received additional radiation-killed cell inocula.

Tumour challenge

Our aim was to test each glioma at one or two challenge doses close to, but greater than, the cell dose producing tumours in 50% of control animals (referred to as MTD) (Gross, 1943; Pellis & Kahan, 1976). The proper tumour cell dosages were estimated from multiple pilot experiments in which graded doses of tumour cells were injected into untreated control animals. The MTD value for several glioma lines varied widely in early transplant passages (Mennell & Groneck, 1977). This frequently resulted in over- or under-estimations of optimal challenge doses and necessitated repetition of several assays. Challenge of immunized and control animals with viable cells from the respective glioma lines was performed 7–18 days after the last immunization.

All cell challenges were injected s.c. to either the left or right flank. In the majority of experiments each animal was challenged at two sites (Hellström & Hellström, 1978; Vaage, 1972), a lower dose being presented at one site and a higher dose contralaterally. All challenges were administered in a 0·2 ml volume.

Data gathering and statistical evaluation

Tumour challenge sites were monitored weekly to determine the incidence, volume, and latency of tumour outgrowths. All calculations and statistical assessments reported below were performed

on the measurements taken on the day the latest challenge outgrowths appeared, i.e. 6 to 9 weeks following the challenge date. However, the animals were kept under observation 2 to 3 weeks beyond this time to be certain that challenge sites recorded as negative remained so.

Incidence was calculated as the ratio of number of sites showing tumour growth to the total sites challenged with live cells. Growing tumours were measured in their longest (a) and shortest (b) dimensions, and tumour volumes were calculated by means of the formula, $0{\cdot}4ab^2$ in units of mm^3 (Attia & Weiss, 1966). Latency was the number of days elapsed before the first detection of palpable tumour growth, i.e. 4 mm. Challenge sites with no tumour growth were assigned a volume of 0 and were omitted from the calculation of latency. Statistical analysis was by means of the Chi square test with Yates' correction. Mean tumour volumes and latency periods were assessed for significance by means of Student's t tests.

Results

The results on the seven glioma lines are presented below in the following order: two with tumour rejection responses (T181B and T185A), three with enhancement following immunization (T175A, T131B, and T161B), and two with no detectable responses (T180A and T181A).

T181B (Table 2): The incidence of tumour outgrowth in T181B-immunized and control groups was not significantly different. However, the mean volume of outgrowths at both the 1000 and 3000 cell challenge sites in immunized animals was significantly reduced, and the latency period at the 3,000 cell sites was prolonged. No tumour at positive outgrowth sites was observed to regress. Immunization with the bladder carcinoma control tumour produced no significant effects. Thus T181B showed a slight, but statistically significant, tumour rejection response.

Table 2. Tumour rejection response in rats immunized against T181B

	Challenge data†					
	Dosage:	$1{\cdot}0 \times 10^3$ *cells/site*		*Dosage:*	$3{\cdot}0 \times 10^3$ *cells/site*	
*Groups**	*Tumour incidence*	*Tumour volume*	*Latency*	*Tumour incidence*	*Tumour volume*	*Latency*
Control (unimmunized)	14/24(58%)	1290	35	22/24(92%)	5025	30
T181B immunized	6/16(38%)	159‡	31	15/16(94%)	555‡	37§
BC158 immunized	8/12(67%)	3063	33	12/12(100%)	4229	31

* T181B-immunized animals were prepared by ligation of non-irradiated palpable subcutaneous tumour nodules 7 days prior to the challenge date. †Two challenge sites per rat were used. The data shown are from measurements taken on the 55th day following challenge. ‡$P < 0{\cdot}05$ (All footnote P values represent comparisons with unimmunized controls). §$P < 0{\cdot}005$.

T185A (Table 3): Of all the seven gliomas studied, T185A showed the highest MTD and the longest latency. Two groups of T185A-immunized animals were prepared, one immunized twice and the other four times. Statistically significant results were recorded in both groups, but only at challenge sites that received the higher dose of 24 000 cells. The twice immunized and the four-times-immunized groups showed a lower incidence of tumour outgrowth than did the unimmunized controls. Tumour volumes in immunized animals were slightly smaller, but not significantly so. Although we again did not observe regression of any tumours at positive outgrowth sites, the tumour incidence data do denote that immunization with T185A induced a rejection response.

T175A (Table 4): Two extra groups of rats were included in the analysis of this tumour: one that was immunized against T175A and additionally treated with 450 rad total-body irradiation (TBI) one day prior to challenge, and another that was unimmunized, but similarly irradiated prior to challenge (Hellström & Hellström, 1978).

A comparison of the T175A-immunized group to the unimmunized control reveals that immunization led to enhanced tumour growth since the mean volume of tumours at the 3000 cell challenge sites was significantly increased (2025 mm^3 vs. 688 mm^3). The incidence and latency periods demonstrated a trend consistent with this. There was also definite enhancement recorded in the group that was both immunized and subsequently irradiated (third in the table) as compared to the group that received irradiation alone (fourth in the table). That is, at the 12 000 cell sites the volume and latency period values of these two

Table 3. Tumour rejection response in rats immunized against T185A

	Challenge data†					
	Dosage: 8·0 × 10³ cells/site			*Dosage: 24·0 × 10³ cells/site*		
*Groups**	*Tumour incidence*	*Tumour volume*	*Latency*	*Tumour incidence*	*Tumour volume*	*Latency*
Control (unimmunized)	6/24(25%)	951	50	21/24(88%)	4970	44
T185A immunized 2 times	5/23(22%)	275	51	10/23(43%)‡	1500	46
T185A immunized 4 times	7/19(37%)	400	44	10/19(53%)§	3569	39

*Animals were immunized two or four times with live and irradiated cells between 96 and 11 days before the challenge date. †Two challenge sites per rat were used. The data shown are from measurements taken on the 58th day after challenge. No effects from immunization with BC158 were recorded in a previous experiment which included this tumour. ‡$P < 0{\cdot}01$. §$P < 0{\cdot}05$.

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Table 4. Enhanced outgrowth of tumours in rats immunized against T175A

	Challenge data†					
	Dosage: 3·0 × 10³ cells/site			*dosage: 12·0 × 10³ cells/site*		
*Groups**	*Tumour incidence*	*Tumour volume*	*Latency*	*Tumour incidence*	*Tumour volume*	*Latency*
Control (unimmunized)	6/23(26%)	688	24	14/23(61%)	4207	24
T175A immunized	12/25(48%)	2025‡	21	19/25(75%)	4263	22
T175A immunized +TBI	20/23(87%)§	3813§	24	23/23(100%)**	9660**	19††
Unimmunized +TBI	13/21(62%)‡	2367‡	23	21/21(100%)**	5817	22
BC158 immunized	8/21(38%)	2037	24	14/21(67%)	5562	21

*All T175A-immunized animals twice received radiation-killed cell inocula at 27 and 14 days before the challenge date. TBI indicates that rats in these groups received 450r total-body irradiation one day before transplant challenge. †Two challenge sites per rat were used. The data shown are from measurements taken on the 42nd day following challenge. ‡$P < 0.05$ (All footnote P values are comparisons with the unimmunized controls). §$P < 0.001$. **$P < 0.005$. ††$P < 0.02$.

groups were significantly different (volume: 9660 mm³ vs. 5817 mm³, $P < 0.05$; latency: 19 vs. 22, $P < 0.05$). Immunization with BC158 did not influence outgrowth at sites challenged with either dose of T175A.

These data indicate that immunization alone with T175A or in combination with total body irradiation led to significant tumour enhancement. That enhancement did not follow immunization with BC158 further indicates that the capacity to enhance T175A challenge outgrowth was not shared with this non-gliomatous neoplasm.

T131B (Table 5): The incidence of tumour outgrowth in the control animals at both the 1000 and 4000 cell challenge sites was below 50%, indicating that we slightly underestimated the MTD of this tumour. Nevertheless, at the challenge dose of 1000 cells per site the immunized animals showed both a higher incidence and a greater mean tumour volume than did controls, denoting tumour enhancement. A similar but not statistically significant trend was recorded at the higher cell dose sites.

T161B: This glioma line proved difficult to work with because repeated estimates of the MTD value were low, between 100 and 150 cells, and did not reliably predict the outgrowth in the assays. Consequently, repeated experiments were necessary. In one experiment the challenge outgrowths in T161B-

Table 5. Enhanced outgrowth of tumours in rats immunized against T131B

	Challenge data†					
	Dosage: 1·0 × 10⁷ cells/site			*Dosage: 4·0 × 10⁷ cells/site*		
*Groups**	*Tumour incidence*	*Tumour volume*	*Latency*	*Tumour incidence*	*Tumour volume*	*Latency*
Control (unimmunized)	2/18(11%)	170	31	7/19(37%)	2388	28
T131B immunized	9/20(45%)‡	2461‡	28	12/20(60%)	3506	31

*T131B-immunized rats twice received radiation-killed cells at 31 and 18 days before the challenge date. This experiment did not include a bladder carcinoma control group. †Either one or two chellange sites per rat were used. The data shown are from measurements taken on the 48th day after challenge. ‡$P < 0{\cdot}05$.

immunized animals showed a significantly greater mean volume compared to controls, but with no effects on incidence or latency. In a repeat experiment neither rejection nor enhancement effects on tumour challenges resulted from immunization.

T180A: The MTD value was low, between 150 and 300 cells, and also difficult to pinpoint in this glioma line. Animals that failed to develop tumors after live cell challenges in the first and second of three experiments were carried over into a third experiment. This resulted in two groups of animals that were immunized four or six times with both live and radiation-killed cells. These multiple immunizations produced no detectable enhancement or rejection on the challenge outgrowth in any of the three experiments.

T181A: This tumour showed a MTD value of about fifty to 100 cells. Similar to T180A, it was assessed in two experiments which included animals that were immunized two or three times with irradiated and live cells. The challenge outgrowth data revealed neither enhancement nor rejection.

Discussion

The purpose of this investigation was to survey malignant gliomas induced with ENU in F-344 rats for the expression of tumour rejection antigens *in vivo*. Two glioma lines, namely T181B derived from a glioblastoma multiforme and T185A derived from a mixed malignant astrocytoma-ependymoma, showed significant but weak tumour rejection responses. By contrast, immunization with the bladder carcinoma control tumour produced no effect on outgrowth of challenges with T181B or T185A cells. This indicates that the capacity to induce

rejection of these gliomas was not shared by a non-gliomatous neoplasm. However, T181B and T185A were not tested against each other in criss-cross immunization-challenge procedures because these two glioma lines were assessed independently and simultaneously before we had any indication that either would be immunogenic. Our results nevertheless do suggest that the rejection responses observed in these two tumours were tumour-specific. To establish this uncontestably, though, will require further assays which compare these two tumours to each other and include control groups immunized against normal CNS tissue.

In the assays of three tumours (T175A, T131B, and T161B) the immunization procedures used led to enhancement of tumour outgrowth. Immunization with bladder carcinoma cells in the T175A and T161B experiments did not produce such enhancement, and the enhancement effect was not reduced by whole-body irradiation of immunized animals in the T175A experiment. These findings together tend to suggest that immunological processes rather than non-specific feeder effects (Revesz, 1958) were involved in the acceleration of tumour outgrowth. However, a radiosensitive suppressor cell population (Hellström *et al.*, 1978) does not appear to have been responsible for the enhancement.

Enhanced outgrowth of tumour challenges has been observed by some investigators who immunized animals with certain doses of cell free extracts of tumour cells (Pellis & Kahan, 1975; 1976; Tracey *et al.*, 1978) and by others who challenged tumour-immunized animals with live tumour grafts to which radiation-killed tumour cells were purposefully added (Helström & Hellström, 1978). In these studies soluble antigens in the cell free extracts used for immunization and the non-viable cells included in the challenge inocula may have produced enhancement by functioning as blocking factors or by activating suppressor cells. Since we prepared tumour cells for both immunization and challenge purposes by mechanical methods, our cell suspensions undoubtedly contained both soluble antigens and non-viable, mechanically-traumatized tumour cells. The presence of these constituents in the cell suspensions, therefore, may have contributed to the enhancement we observed. However, it remains to be determined whether the enhancement results in our experiments were due to specific cell-mediated immunostimulation (Prehn, 1972; 1977), tumour-enhancing antibodies (Shearer, Philpott & Parker, 1973; Shin *et al.*, 1978), specific blocking factors (Hellström, Hellström & Nepom, 1977), suppressor cells resistant to irradiation, or some other mechanism.

From the evidence we have generated, it appears that some ENU-induced malignant rat gliomas are capable of inducing weak rejection responses as tested by conventional assays. However, only two of our seven tumours showed this capacity. We conclude that the majority of ENU-induced rat gliomas either **a** lack immunogenic determinants, **b** induce nonspecific tumour-stimulatory processes, **c** carry weak determinants that elicit immunostimulation (Prehn, 1977), or **d** express antigens that induce immunosuppression by means of suppressor cells or blocking factors.

There is only a limited amount of recorded data on ENU-induced gliomas with which our results can be compared. Morantz *et al.* (1979) examined a F-344 rat glioma, labelled F, by means of immunoprotection assays in which surgical removal of viable immunizing inocula was attempted. Similar to our experience, 92% of surgically removed tumours recurred at the operative site. There was no protective effect from immunization. Cornain *et al.* (1975) reported that a BD IX rat glioma (GV1A1) demonstrated low but antigenically specific immunogenicity *in vivo*. However, they did not publish any *in vivo* assay data. A further claim that ENU-induced rat gliomas are immunogenic in *in vivo* assays has appeared, but without data to substantiate it (Morantz, Shain & Cravioto, 1978).

The other type of neurogenic tumour that commonly arises in rats treated transplacentally with ENU is the malignant Schwannoma of cranial nerve V, the cauda equina, or other spinal nerve roots (Koestner *et al.*, 1971). Rainbird and Ridley (1977) assessed the antigenicity of six of these tumours *in vivo* and found that only one of the six elicited a tumour-rejection response. These findings, therefore, are consistent with ours on gliomas induced with ENU.

The ENU rat neurogenic tumour model is of great interest for studies of immunosurveillance because there is a long latency period between transplacental tumour induction and the development of symptomatic neoplasms. Several procedures applied during the latency period and aimed at stimulating or counteracting the immune apparatus include **a** treatment with immune stimulating agents, Bacille-Calmette-Guerin (BCG), bovine albumin, and complete Freund's adjuvant (CFA); **b** active specific immunization with glioma or Schwannoma inocula in CFA; **c** administration of immunosuppressive drugs, amethopterin, cyclophosphamide, or hydrocortisone; and **d** treatment with anti-rat lymphocyte serum (ALS) alone or in combination with neonatal thymectomy (Cravioto *et al.*, 1975; Habs & Schmähl, 1976; Morantz *et al.*, 1978; Schmähl, Mundt & Schmidt, 1974; Spence *et al.*, 1979). None of these procedures has been shown to alter the yield or latency period of neurogenic tumours induced with ENU. It can be argued that these procedures were all without effect because the antigens of the target neoplasms were shielded from the immune apparatus by virtue of being located within the immunologically privileged confines of the central nervous system (Morantz *et al.*, 1978). However, drawing upon our tumour rejection assay results and those of Rainbird & Ridley (1977), one can equally plausibly argue that these procedures were ineffective because too few of the target neoplasms expressed tumour rejection antigens, or the supposed antigens were too weak to induce any immunological defense mechanisms.

In contrast to ENU-induced rat gliomas, a small number of rat gliomas induced with the closely related carcinogen, methylnitrosourea (MNU), have been documented to be highly immunogenic *in vivo*. They include glioma 526 reported by Morantz *et al.* (1979), glioma T9 (Benda *et al.*, 1971) investigated by Denlinger *et al.* (1975) and others (Blume *et al.*, 1974; Morantz *et al.*, 1979; Tracey *et al.*, 1978) and an additional glioma produced and reported by Cravioto (1978a).

All three of these originated in Fischer rats. Our findings, indicating that ENU-induced F-344 rat gliomas are weakly or non-immunogenic, therefore, cannot be attributed to an inherent incapacity of glial cells in this rat strain to develop or express strong tumour-rejection antigens, or to strain-related, genetically determined immunological unresponsiveness of F-344 rats.

The reason that MNU-induced rat gliomas are highly immunogenic while those induced with ENU are not is unknown. A conjectural explanation is as follows: although MNU and ENU are both resorptive carcinogens and are similar in chemical structure and action (Goth & Rajewsky, 1974; Kleihues & Cooper, 1976), they are administered at different doses and schedules. MNU is administered at a dose of 5 mg/kg given weekly for 30 or more weeks postnatally (Benda *et al.*, 1971), whereas ENU is administered only once late in gestation at doses in the range of 20 to 50 mg/kg (Cornain *et al.*, 1975; Cravioto, 1978a; Koestner *et al.*, 1971). A conservative rough estimation indicates that MNU-treated animals are exposed to approximately twenty to fifty times greater total carcinogen dosage than are ENU-treated animals. Since the immunogenicity of chemically-induced neoplasms is greater with increasing doses of a given carcinogen (Baldwin, 1973; Prehn, 1975), the substantially larger MNU dosage may well be the reason MNU-induced gliomas are more strongly immunogenic. Alternative speculations are based on the fact that ENU is administered during prenatal development when the target glial cells are not fully differentiated, whereas MNU is administered during a period of the rat's life when glial differentiation is completed. Conceivably, immature glia, deviated prenatally from the normal pathway of differentiation by ENU-induced neoplastic transformation, may not develop the capacity to form strong rejection antigens to the degree that more mature MNU-transformed glial cells do; or the neoantigens in ENU-induced neoplasms are inherently weak but altered embryonic antigens, whereas those generated by MNU are inherently stronger altered differentiation antigens. Only through further experimentation will we be able to understand these interesting possibilities more fully.

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