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Influence of host sex on the growth of a human glioblastoma line in athymic mice

C. VERZAT*†, M.-B. DELISLE‡, P. COURRIERE† AND E. HOLLANDE*

** Laboratoire de Biologie Cellulaire, Université Paul Sabatier, 38 rue des Trente Six Ponts · 31400 Toulouse, France, † Groupe de Recherche du Centre Claudius Régaud, 24 rue du Pont Saint-Pierre, 31052 Toulouse, France and ‡ Centre Hospitalier Universitaire de Toulouse-Rangueil, Service d'Anatomie et Cytologie Pathologique, 1 avenue Jean Pouilhas, 31052 Toulouse, France*

Verzat C., Delisle M.-B., Courriere P. & Hollande E. (1990) *Neuropathology and Applied Neurobiology* **16**, 141–151. **Influence of host sex on the growth of a human glioblastoma line in athymic mice.** Glioblastomas are generally thought to be more common in men than in women. In order to investigate the hormone-dependence of these tumours, we established a human glioblastoma line in athymic mice. The tumour cell type was characterized using immunocytochemical methods. The influence of host sex on growth was evaluated, and hormone receptors were characterized biochemically.

The histological features of the initial tumour were conserved in the heterotransplanted tumours, which consisted of vimentin and GFAP immunoreactive astrocytes. There was a highly significant difference in tumour growth between the two sexes ($P < 0.01$). In the male mice, tumours were from 2.5 to 10 times larger than in the females, the latency periods were 30% shorter, and the growth phases were characterized by periods of slow or zero growth. In addition, androgen and oestrogen receptors were detected at low levels (80–270 fmol/g tumour) in the heterotransplanted tumours especially in the males. The fact that the male tumour growth profiles resembled those of some hormone-dependent lines, and that androgen receptors were found preferentially in the male rather than in female tumours would tend to indicate that there is a hormonal influence on the growth of the heterotransplanted tumours. These results provide further evidence for an influence of sex-steroid hormones on the growth of glioblastomas.

Keywords: human glioblastoma, sex-steroid receptors, hormone-dependence, GFAP, vimentin, heterotransplantation

Introduction

The sex distribution of glioblastomas with a predominance in males has been reported by several authors (Zülch & Wolff, 1963; Lumenta & Schirmer, 1984; Roth & Elvidge reviewed by Kelly, Kirkwood & Kapp, 1984). Walker, Robins and Weinfeld (1985) only found a slight male predominance, although they noted that this increased after the age of 45. Likewise, the highest sex-ratios, 2.3 for Zülch and Wolff (1963) or 1.6 for Lumenta and Schirmer (1984) are found between the ages of 40 and 60 years.

Correspondence to: Professeur Etienne Hollande, Laboratoire de Biologie Cellulaire, Université Paul Sabatier, 38 rue des Trente Six Ponts, 31400 Toulouse, France.

The hormone-dependency of rodent gliomas was initially investigated after castration. In two studies, a lower level of gliomas was observed in the castrated animals (Avtsyn and Yablonovskayan, 1964; Hopewell, 1975). However, in another report, castration was found to have no effect (Perese, 1964). More recent *in vivo* experiments on gliomas have found a significant influence of host sex on both tumour incidence and host survival: in a survey of 75 000 mouse brains, Fraser (1986) found that the incidence of spontaneous astrocytomas in males was almost twice that in females. Moreover, Lee *et al.* (1988) in a study of the therapeutic efficiency of antitenascin monoclonal antibodies noticed that male rats with intracranial xenografts had a significantly lower survival rate than the females.

Sex-steroid hormone receptors have been detected in gliomas. In the few glioblastomas studied (approximately 20), both oestrogen (Pearl *et al.*, 1982; Poisson *et al.*, 1983) and androgen receptors (Poisson *et al.*, 1983; Brentani *et al.*, 1984) have been detected, although progestin receptors seemed rare.

To investigate the hormone dependency of glioblastoma cells, we heterotransplanted a human glioblastoma into *nude* mice and compared tumour growth between the two sexes. We also assayed oestrogen, androgen and progestin receptors in the heterotransplanted tumours. The tumours were characterized histologically and immunocytochemically with respect to both the initial tumour and host sex.

Materials and methods

Initial tumour

The tumour was obtained from the right frontal lobe of a 74-year-old Caucasian woman. A tumour sample was immediately placed in Dulbecco's medium (Gibco, New York, USA) supplemented with 10% Fetal Calf Serum (Biopro, West Germany), and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml and amphotericin B (fungizone) 0.25 μg/ml).

This primary tumour was divided into three samples: one sample was reserved for histological examination, one for hormone-receptor determination, and the other for serial heterotransplantation into *nude* mice.

Nude mice

Male and female, genetically athymic nu/nu mice (Swiss, IFFA-CREDO, France) were maintained in our laboratory under pathogen-limited conditions.

Heterotransplantations

Two successive heterotransplantations were carried out. The first was performed on 4-month-old animals (5 males and 5 females) and lasted one year. The second was performed on 6-week-old animals (10 males and 10 females) and lasted 6 months. The tumours were minced with scissors in Dulbecco's medium, and 0.4 ml of this suspension were inoculated in the mid-dorsal region of the mice.

Growth

Tumours were measured in three dimensions every 5 days with slide calipers, and the volumes were calculated on the assumption of an ellipsoid shape:

$$V = 4\pi/3(L/2 \times W/2 \times H/2)$$

(L = Length, W = Width, H = Height).

Growth curves were drawn on semi-logarithmic paper. The latency was taken as the interval between the inoculation day and the onset of the exponential growth phase. Student's *t*-test was used to compare the mean tumour volumes, the mean latency period and the tumour doubling time between the two sexes.

Histological and immunocytochemical studies

Initial tumour and tumour line fragments were fixed in Bouin's solution, dehydrated and embedded in paraffin. The morphological features of the tumour tissue were studied after hemalum-eosin and Mallory trichrome staining.

The immunocytochemical study was based on the detection of two antigens: glial fibrillary acidic protein (GFAP) which is an astrocyte cytoskeleton-associated protein (Eng *et al.*, 1971) and vimentin (VIM), an intermediate filament protein which has been found in cells of mesenchymal origin (Franke *et al.*, 1979); vimentin is also present in immature and neoplastic astrocytes (Dhal, Rueger & Bigmani, 1981; Schiffer *et al.*, 1986). Deparaffinized sections were incubated with anti-GFAP polyclonal antibody (DAKO, 1/200, 30 min) and with anti-VIM monoclonal antibody (DAKO, 1/10, 30 min). The anti-human-GFAP staining was developed by the PAP technique (Sternberger *et al.*, 1970). VIM was also visualized by a peroxidase staining method using: (1) mouse anti-human-VIM; (2) rabbit anti-mouse immunoglobulins labelled with peroxidase (DAKO, 1/15) with normal human serum 1/15; (3) swine anti-rabbit immunoglobulins labelled with peroxidase (DAKO, 1/15) with normal human serum 1/15; (4) peroxidase was revealed by 3-3'-diaminobenzidine.

Determination of cytosolic sex-steroid binding sites

Tissues were rapidly thawed and homogenized at 4°C using an Ultraturrax in five volumes of ice-cold buffer pH 7.4 (10 mM tris-HCl, 250 mM sucrose, 12 mM monothioglycerol and 20 mM sodium molybdate). Cytosolic binding sites were determined by incubating cytosol (105 000 g, 1 h, supernatant) (5.17 ± 0.52 g protein/l) with six increasing concentrations of radioligands (0.5–1–2.5–5–10–15 nM) in the absence (total binding) or presence (non-specific binding) of a 100-fold excess of the corresponding unlabelled steroid (Table 1). [^{3}H]-Organon 2058 and [^{3}H]-Mibolerone were chosen for their high affinities (Blankenstein, Blaauw & Lamberts,

Table 1. Receptor assays: experimental conditions for sex-steroid receptors

Hormone sex receptors	*Tritiated ligand Specific activity*	*Radio-inert ligands*	*Incubation-conditions*
Androgen receptor	Mibolerone 85 Ci/mmol	Mibolerone + Organon	24 h at 0–4°C
Oestrogen receptor	17β-Oestradiol 110 Ci/mmol	17β-Oestradiol + Dihydrotestosterone	♂: 24 h at 0–4°C ♀*: 1 h at 25°C and 24 h at 0–4°C
Progestin receptor	Organon 2058 58 Ci/mmol	Organon 2058 + Hydrocortisone	24 h at 0–4°C

* Cytosols extracted from female tumours were incubated at 25°C in the presence of an antiprotease (0.02 mM Leupeptine).

1984) and specificity (Winters, Keeping & Troen, 1986). Unbound hormones were eliminated by a dextran-coated charcoal adsorption technique.

The concentration of binding sites and the dissociation constant (*K*d) were evaluated from Scatchard plots (Scatchard, 1949). The concentrations of receptors were expressed in femtomoles per gram of tumour tissue (fmol/gT). Three criteria were used to establish receptor activity: a concentration over 80 fmol/gT, a *K*d value under 5 nM and an alignment of at least three measures with a correlation coefficient of at least 0.75. One gram of tumour contained on average 25.8 ± 2.6 mg of cytosolic proteins.

Results

Morphological study of initial and heterotransplanted tumours

No significant morphological differences were observed between the initial tumour and the *in vivo* tumours.

The predominant histological characteristics of the initial tumour (Figures 1 and 2) were those of a highly malignant neoplasm with a dense cellularity, marked polymorphism, a rich proliferating capillary network and large foci of necrosis.

The transplanted tumours (Figures 3 and 4) displayed the same histological features. Most of them had poorly developed connective tissue which remained peripheral or was found inside the tumour along vascular septa. These features were observed in both males and females. The fibroblastic component represented between 0 and 5% of the tumour tissue depending on the particular section.

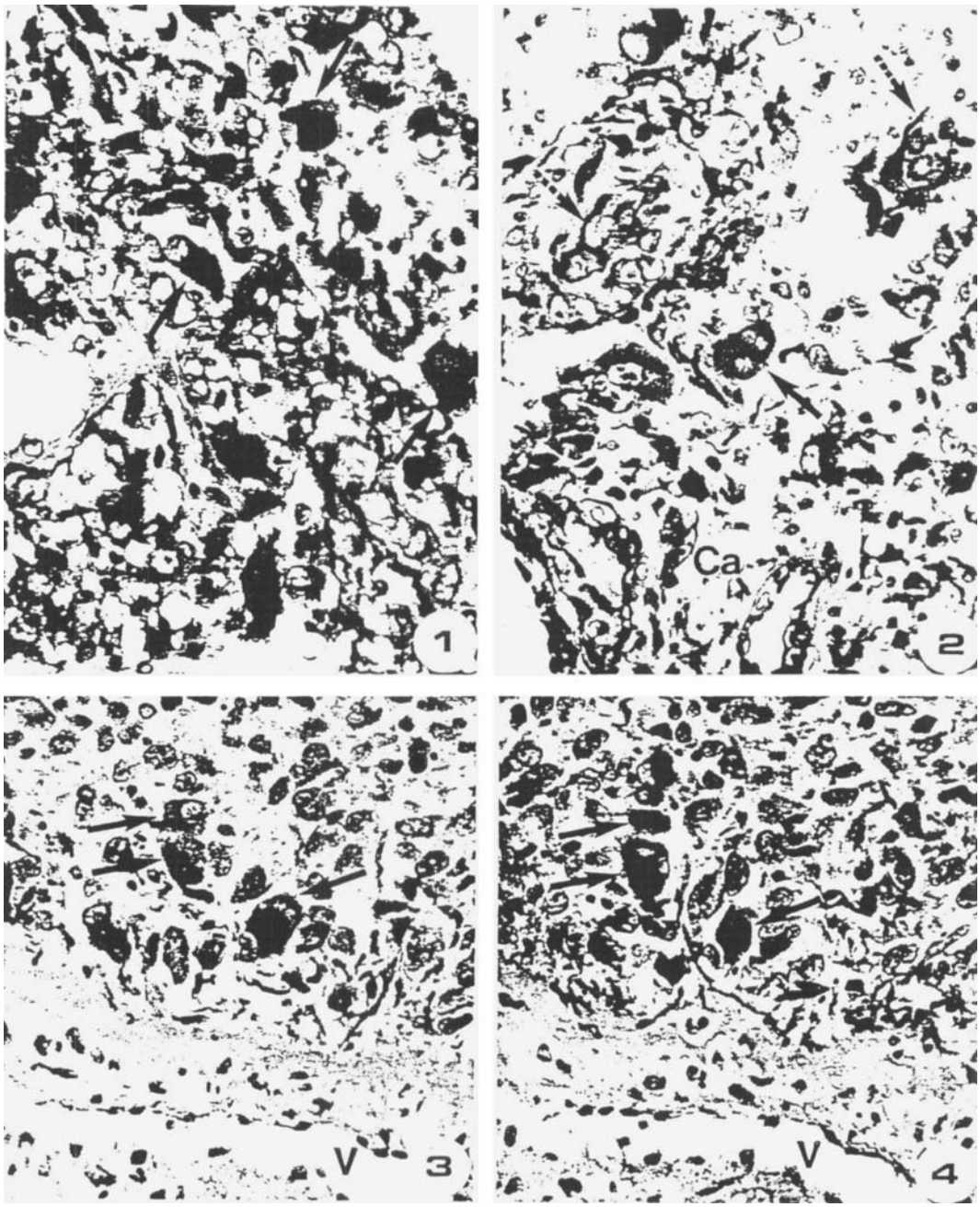
A similar cellular morphology was observed in the initial and heterotransplanted tumours. The cells were round, asteriform or fusiform, with diameters ranging from 10 to 30 μm. The nuclei were pleomorphic with irregular shapes, dense chromatin and occasional pseudo-inclusions. In both the initial and heterotransplanted tumours, the cells were immunoreactive for GFAP and VIM. On adjacent sections (Figures 3 and 4), the same cells appeared to be stained by both antibodies. However, a difference was noted in the immunoreactivity of endothelial cells which were VIM-positive in the initial tumour and VIM-negative in the heterotransplanted tumours. No differences were noted between male or female mice in the morphology and staining patterns of tumours.

In conclusion, the cell features of all the tumours were characteristic of astrocytes, indicating that the tumour line was a glioblastoma.

Tumour line growth

Growth was measured during two successive heterotransplantations. At the first heterotransplantation, the number of tumour takes was higher in the male (3/5) than in the female mice (1/5). For the male mice, the tumour growth curves were irregular: after 5 months of latency, the exponential growth phase was interrupted intermittently when the mice were 6 or 10 months old (Figure 5). The only tumour developing in a female mouse showed a 1 month longer latency followed by a regular exponential growth phase (Figure 5).

At the second heterotransplantation, the four parameters: incidence of takes, latency, growth profile and tumour volume were measured and compared between the two sexes, (Table 2 and Figure 6); (1) The incidence of takes was higher than in the first heterotransplantation, but did not differ between males and females (9/9 versus 9/10); (2) For both sexes, the mean latency was lower than that observed in the first heterotransplantation, and remained one month shorter in males (2 months) than in females (3 months). This difference was



Figures 1 and 2. Initial tumour: morphological and immunocytochemical characteristics. Figure 1, Large cancerous polymorphic GFAP-positive cells (arrows), with eccentric nuclei surrounded by small negative cells. GFAP, × 350. Figure 2, VIM-positive tumour cells. Note: (i) the giant multinucleated cell (arrow) and the numerous darkly stained cytoplasmic processes (dotted arrows); (ii) the positive capillary (Ca) endothelial cells. VIM, × 350. **Figures 3 and 4.** Second heterotransplantation: juxtaposed sections showing that the GFAP-immunoreactive cells (Figure 3, arrows) are also VIM-immunoreactive (Figure 4, arrows). Figure 3, note the intense staining of GFAP in large cells and the weak staining in small cells. Vessel (V). GFAP, × 450. Figure 4, note the intense staining of VIM in large cells and weak staining in small cells, with an absence of VIM in the endothelial cells of the vessel (V). VIM, × 450.

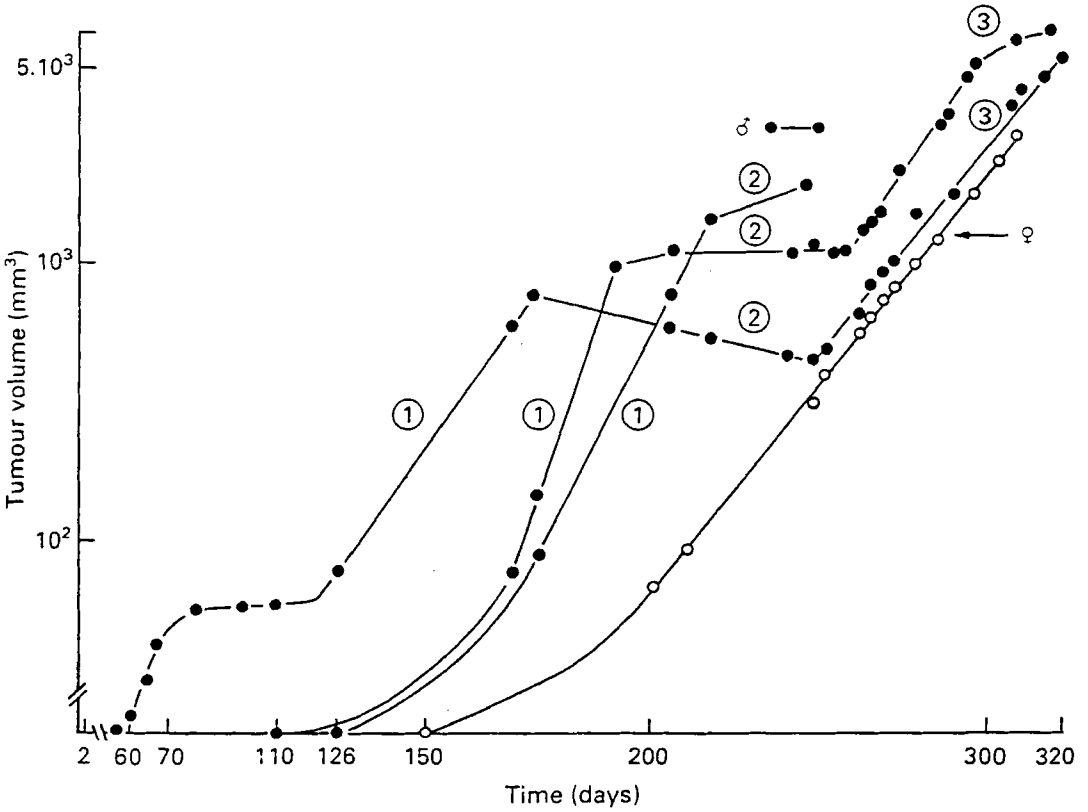


Figure 5. Glioblastoma line. Individual growth curves of the first heterotransplantation established during 320 days in *nude* mice. For males the curves show sequences of exponential growth (1 and 3) interrupted by slow, zero or negative growth periods (2). In the female (♀) mice, the tumours grew continuously.

statistically significant ($P < 0.01$). (3) As for the first heterotransplantation, the tumour growth profiles in males and females differed (Figure 6). The slope of male mouse tumour growth curve flattened for a period of around one month when the male mice were 6 months old. In contrast, the female mice tumour growth curves were regular. (4) During the whole growth period, the mean female tumour volumes were 2.5 to 5 times less than the male ones ($P < 0.01$). At the time of sacrifice, male tumours reached an average volume of 7.2 cm^3 (versus 3 cm^3 for the female tumours). However, the volume doubling time was similar in both sexes.

Table 2. Glioblastoma growth in male and female mice during the second heterotransplantation. The mean latencies and doubling times are expressed in days ($n = 9$). In the male mice the exponential growth phase could be divided into three different periods.

Mice sex	*Take number*	*Latency period*	*Doubling time*		
Female	9/10	90.6±22.6		11.7±3.2	
			Period 1	Period 2	Period 3
Male	9/9	58.3±19.6	13.9±1.8	30.2±11.2	10.1±1.9

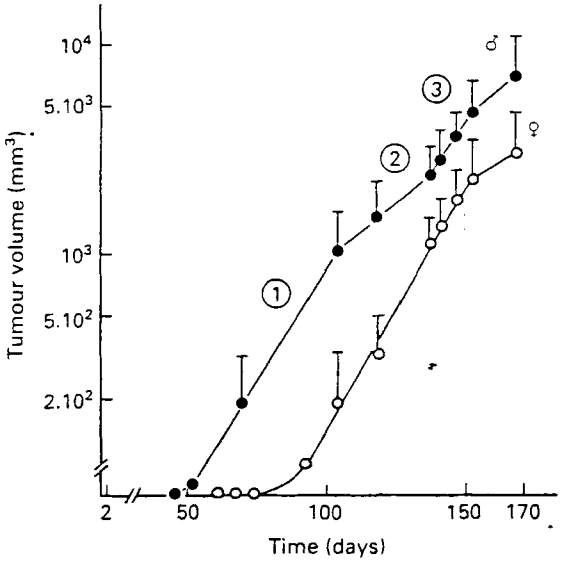


Figure 6. Mean growth curves of the glioblastoma line in *nude* mice of both sexes, established during 170 days (2nd heterotransplantation). For the male (♂) mice, the exponential growth phase can be divided into three different periods (1–3), whereas for the female (♀) mice the slope is regular. Each point represents the mean for nine tumours. Bars indicate SD.

Sex-steroid receptors (cf., Table 3)

Androgen, oestrogen and progestin receptors were not detected in the initial tumour.

In heterotransplanted tumours, androgen and oestrogen receptors were detected, and, as indicated by the 'saturation' curves and the Scatchard plots, these sites were saturable, and had high affinity for the radioligands.

Androgen receptors. [^{3}H]-Mibolerone binding sites were detected in all the five tumours from the male mice at a mean level of 150 ± 76 fmol/gT, with a mean dissociation constant (*Kd*) of 1 nM. In contrast, no androgen receptors were detected in the four female tumours.

Oestrogen receptors. [^{3}H]-17β-oestradiol binding sites were detected in four out of five tumours from the male mice at a mean level of 216 fmol/gT with a mean *Kd* of 0.49 nM. In the

Table 3. Sex steroid receptor concentrations in heterotransplanted glioblastomas.

Mice sex	*Heterotransplantation number*	*Androgen receptors*				*Oestrogen receptors*				*Progestin receptors*			
		C	*Kd*	*n*	*r*	*C*	*Kd*	*n*	*r*	*C*	*Kd*	*n*	*r*
Male	1st	88	−0.41	5	0.9	142	−0.38	4	0.95	0	—	—	—
		136	−0.9	5	0.83	258	−0.4	4	0.97	0	—	—	—
	2nd	88	−0.2	5	0.97	0	—	—	—	0	—	—	—
		235	−2.7	4	0.89	271	−0.4	4	0.75	0	—	—	—
		220	−0.6	5	0.98	200	−0.77	5	0.93	0	—	—	—
Female	1st	0	—	—	—	85	−0.09	3	0.95	0	—	—	—
	2nd	0	—	—	—	0	—	—	—	0	—	—	—
		0	—	—	—	0	—	—	—	0	—	—	—
		0	—	—	—	110	−0.1	3	1	0	—	—	—

C, concentration (expressed in fmol/g of tumour). *Kd*, dissociation constant (expressed in nmol/l). *n*, number of measurements. *r*, correlation coefficient between the experimental points and points on the calculated regression line.

females, two out of four tumours contained low levels of oestrogen receptors (mean = 97.5 fmol/gT, 4 fmol/mg protein; mean Kd = 0.5 nM/l).

Progestin receptors were not detected in any tumours from either sex.

Discussion

The *nude* mouse is a useful host for the production of hormone-dependent prostate and breast tumour cell lines (Leung & Shiu, 1981; Ito *et al.*, 1985). Tumours transplanted into *nude* mice generally exhibit similar characteristics to those of the original specimen from which they were derived. However, Jones *et al.* (1981) have reported that GFAP expression may be lost on numerous repeated heterotransplantations. In our experiments with two successive heterotransplantations over a period of 18 months, we found that the morphological and immunocytochemical features (GFAP and VIM immunoreactivity) of the initial glioblastoma were conserved. The connective tissue cells observed on sections of the tumour line were probably derived from the host mice since the cells were not bound by the human anti-vimentin antibodies. There was no difference in abundance of these cells between the sexes, although there was an increase in this connective tissue at the third passage (data not shown).

Taken together, these observations indicate a hormonal influence on the growth of the glioblastoma line: for the 22 tumours studied (12 in male mice, and 10 in females), we observed that tumour volumes were 2.5 to 5 times greater, and latency periods were 30% shorter in the males than in the females. Over the nine tumours assayed for the three sex steroid receptors, six were positive for at least one receptor type. Furthermore, irregular growth profiles were only observed for tumours in the male mice.

The enhanced growth in the male mice is in agreement with human epidemiological data. Analysis of the growth curves showed that the larger tumours obtained in the males was due to a shorter latency rather than a shorter doubling time. Examination of the factors affecting the initiation of tumour growth in the *nude* mouse has indicated that two main parameters influence the latency time (Reid & Shin, 1978): the specific cell type inoculated and the regional vascular supply for inoculation. In our experiments, the inoculated fragments all came from the same initial tumour. The same immunocytochemical features were observed in all the various heterotransplanted tumours. The thoracic region of the back was chosen because it is a weakly-vascularized region. This was to circumvent individual differences in vascularization of the inoculation site. Under these experimental conditions, the results were sufficiently homogeneous within each group to allow statistical analysis, and highly significant differences in mean latencies were observed between the males and females. The only parameter which differed between the two tumour groups was the sex of the host mice. A shorter latency in males has not previously been reported. In other experimental studies on the incidence of gliomas with respect to sex or on the influence of castration, latencies and growth curves were not reported, and only tumour take rates were presented (Avtsyn & Yablonovskayan, 1964; Hopewell, 1975; Perese, 1964; Fraser, 1986). In the present study, a slight but not statistically significant difference in tumour take rates between the sexes was noted. However, our experiments are not strictly comparable to other studies since we used a human tumour, while the above mentioned authors studied murine tumours.

Another indication of a hormonal influence is the presence of hormone receptors in the tumours of our line. Although receptors were detected at low levels, it is noteworthy that the binding affinity for the radioligands was the same as that reported for receptors in endome-

trium or prostate (Clark & Peck, 1979; Winters *et al.*, 1986). In agreement with the findings of Poisson *et al.* (1983), progestin receptors were rarely detectable in our tumours. The low concentrations may indicate a low proportion of hormone-dependent cells as for healthy breast tissue, or the presence of few sites per cell. Unlike the classical sex steroid target tissues where there is a relatively rapid and dramatic response to the relevant hormones, regulation of glial cancer cells by sex steroids may be characterized by a muted response over a longer period of time. Nevertheless, the presence and the quantity of androgen receptors appeared to be related to host sex. Androgen receptors were only detected in the tumours from the male mice (cf, Table 3). Although our data do not definitively establish that the receptor distribution is sex-related, it is noteworthy that no androgen receptors were detected in the tumours in the female mice nor in the initial tumour obtained from a female patient. It is perhaps relevant that male sex hormones have been reported to increase androgen receptor concentrations in the prostate (Ichii, 1980). In another point of view, some brain tumours like glioblastoma (Libermann *et al.*, 1984) and meningioma (Grimaux, Magdelenat & Poisson, 1988) present very high epidermal growth factor receptors (EGFR) levels; a correlation between EGFR and steroid receptor could be investigated in glioblastoma.

The last indication of a hormonal influence was the marked difference in growth curves between the sexes. The irregular tumoral growth observed in the male *nude* mice is in agreement with observations of two other carcinoma lines established in *nude* mice (Reid & Shin, 1978). In the present study, the female tumour growth profiles were consistent with those normally observed for non-hormone-dependent lines in *nude* mice (Freedman & Shin, 1978). If there is a hormonal regulation of growth, it only occurs in the male mice. The irregular growth curves in the male animals may thus be due to an antagonistic action of sex hormones. The presence of two types of receptor : androgen and oestrogen receptors in the male tumour would provide a biochemical substrate for an action of sex hormones on glioblastoma growth. This may be similar to the antagonist effects of these hormones during embryonic sexual differentiation, or to the effectiveness of oestrogens in the treatment of androgen-dependent prostatic tumours.

Differences in tumour growth between 14 males and 15 females determined over a further 18 month period observation support a sex-related growth of this human glioblastoma in *nude* mice. This is in agreement with the recent reports on the influence of host sex on the incidence of glial tumours and host survival in mice (Fraser, 1986; Lee *et al.*, 1988).

The presence of androgen and oestrogen receptors in male tumours would be a prerequisite for an influence of sex steroid hormones, although direct hormonal manipulation will be required before a hormonal action on glioblastoma proliferation can be established beyond doubt.

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