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A Possibility of Predicting Metastatic Ability of Tumours by Implantation into Chick Embryos

Epigenetic Grading of Metastasizing and Non-Metastasizing Forms of a Hamster Lymphosarcoma*

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Abstract—*The degrees of malignancy of hamster TRES fibrosarcoma and a metastasizing (ML) and a non-metastasizing (NML) form of a hamster lymphosarcoma were investigated by the epigenetic grading (EGG) test. The test involved the implantation of the tumours into chick embryo blastoderms and examination of the responses produced by the embryonic cells after the tumours are implanted. Hamster kidney tissue was found to be EGG 3, the low grade indicating its normality. The TRES fibrosarcoma was found to be EGG 8, and only marginally higher than the NML tumour. This is consistent with the observation that the fibrosarcoma is only moderately malignant* in vivo, *and that the NML tumour does not invade and form distant metastases. The two forms of the lymphosarcoma possessed similar histology and comparable growth rates, but the EGG tests clearly distinguished between them. Their epigenetic grades were strikingly different, EGG 7 and 15 for purified cell preparations of NML and ML tumours respectively, and EGG 6 and 12 for the undissociated NML and ML tumours. The high EGG of the ML tumours is compatible with the observation that these tumours are highly malignant* in vivo.

INTRODUCTION

THE CLASSICAL distinction between benign and malignant tumours is based on the predominantly localised slow growth and the greater degree of differentiation of the former and the more rapid and anaplastic growth of the latter accompanied by the ability to invade and to form distant metastases. In the clinical management of patients, histological grading of the tumours and clinical staging are used for determining the degree of malignancy and progression of tumours, but neither method can predict from an examination of the primary tumour whether it has the potential to invade and form metastases.

In previous publications, we described an embryological method by which it is possible to distinguish between normal and neoplastic cells and also to determine the degree of malignancy of tumours [1–3]. This method is based on the cellular responses made by relatively undifferentiated chick embryo blastoderms after implantation of tumour cells. Three main cellular responses are evaluated, of which the ectodermal and endodermal proliferative and morphogenetic responses (EctR and EndR) increase with increase in malignancy of the tumours, while a third response, viz. the mesodermal response, decreases with increase in malignancy of the implanted tumour cells [1, 4, 5]. These cellular responses are scored for the frequency and intensity of the responses to arrive at a numerical grading called the epigenetic grade (EGG). In a spectrum of minimum-deviation rat hepatomas, the EGG was found to be related to the malignancy of the tumour as judged by their metastatic ability and survival times of the tumour-bearing host animals [1]. In a series of human astrocytomas, the EGG was found to be closely correlated with the post-

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operative survival times of the patients [2]. It can therefore be concluded that the EGG is related to the malignancy of the tumour, the higher the malignancy the greater being the EGG. In this paper, we examine the ability of the EGG test to assess the metastatic potential of tumours and show that it can distinguish between metastasizing and non-metastasizing forms of a hamster lymphosarcoma.

MATERIALS AND METHODS

Lymphosarcoma

Metastasizing (ML) and non-metastasizing (NML) forms of a homotransplantable lymphosarcoma [6] were used. The tumours were maintained by serial transplantation in 2–4-month old inbred Syrian cream hamsters (Wrights of Essex) [7]. Animals were injected subcutaneously with 0.2 g of finely chopped tumour tissue in 0.5 ml of medium 199.

Differences in biological behaviour

The differences in malignancy of the two tumours were established by investigation of tumour bearing animals for the presence of metastases in the lung, liver and kidney, both by visual assessment for macroscopic deposits and histologically for microscopic secondary deposits.

The differences in the metastatic ability were also tested by implanting liver tissue from tumour-bearing hamsters into new hosts, and recording the number of animals developing tumours. The liver was excised from tumour-bearing hamsters, washed in saline, minced, and 0.2 g of suspension in 0.5 ml of medium 199 was injected subcutaneously into new hosts. These experiments were based on the reasoning that if secondary tumour foci were present in the liver, they would be able to form tumour in the new hosts. Twenty animals each were used for testing the NML and ML tumours.

Survival times of animals bearing ML and NML tumours were also recorded. Twenty animals each were used for ML and NML tumours.

The growth rates of the tumours were measured as follows. Ten animals were each injected with 0.2 g of the NML tumour and 17 animals with the ML tumour. After the tumours became palpable, two perpendicular dimensions of the tumours were recorded, the sum of which was regarded as indicating tumour size [8]. These measurements were made until day 20 after inoculation. The slope was calculated for each growth curve by regression analysis.

Preparation of cells

Unicellular suspensions of the tumours were prepared by collagenase disaggregation as described by Guy *et al.* [7]. The yield was approximately 50×10^6 cells/g for both tumours. The viability of the cell suspensions before purification was approx. 50%. Viable cells were separated from non-viable ones and erythrocytes by sedimenting the cells at 1500 ***g*** for 15 min in a 10 ml mixture of Ficoll 400 (6.35% w/v, Pharmacia)-Hypaque (9.97% w/v, Winthrop Laboratories, Newcastle upon Tyne [9]. After purification, viabilities were 94 and 92% respectively for NML and ML cells.

Tumour cell content

The cells (10^6) were sedimented at 600 rev/min for 5 min in a cytocentrifuge (Shandon Southern, Camberley, Surrey), fixed and stained by Wright's stain as described by Baker *et al.* [10]. Tumour cell content was 91 and 94% for ML and NML cell preparations.

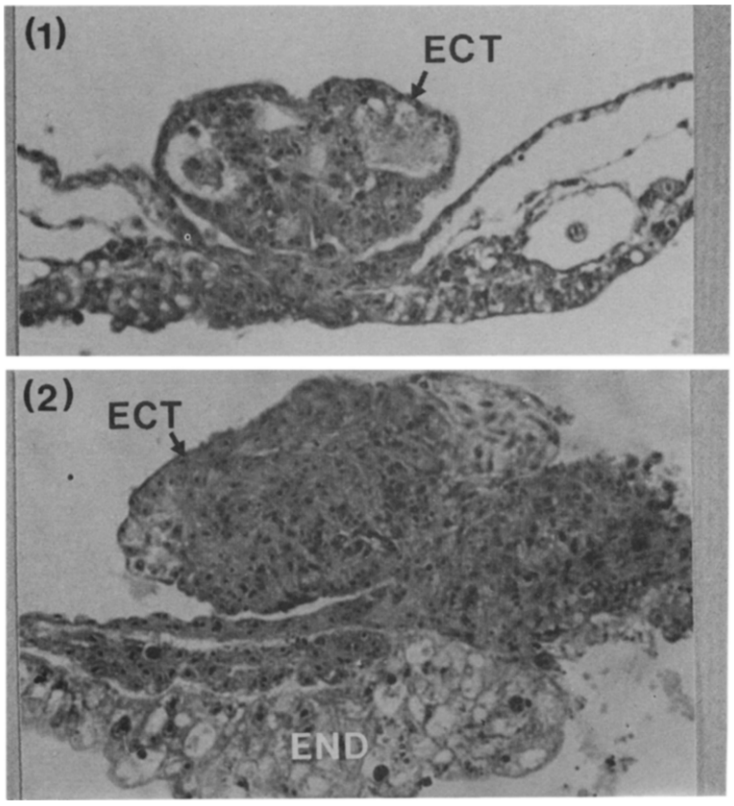
Cell preparations for malignancy tests were made from two ML and two NML tumours. In each case the tumours were separated by 5 generations. Undissociated tumour tissue was also tested. Sections of tumour free of necrosis were chosen for implantation.

TRES fibrosarcoma cells

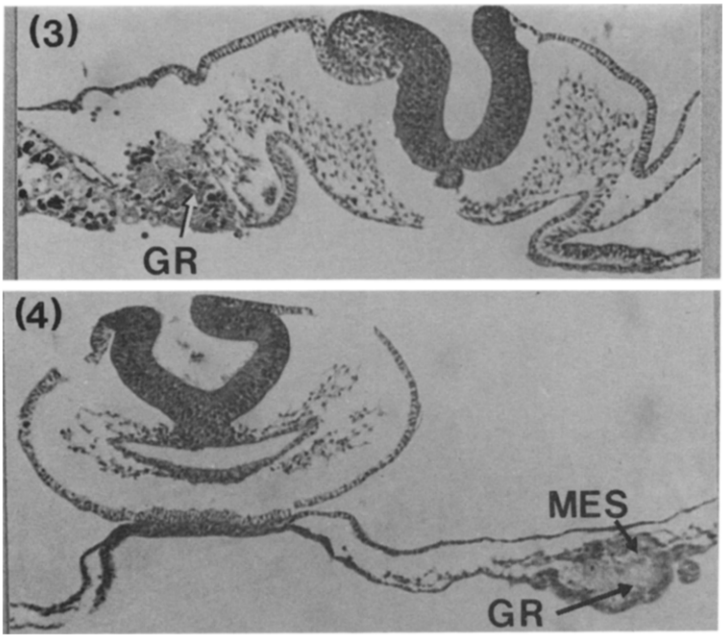
These cells are a malignant cell line recovered from primary tumours produced by BHK21/C13 cells transofmred *in vitro* by crude rat liver histone [11]. These cells were grown in Dulbecco's minimum essential medium with 10% newborn calf serum, supplemented with 3.75 mM glutamine and antibiotics (Penicillin G 500 units, streptomycin sulphate 0.25 mg and Mycostatin 600 units/ml) and equilibrated with 20% CO_2.

Malignancy tests (EGG tests)

The malignancy of the TRES fibrosarcoma cells and the purified ML and NML cells as well as the ML and NML tumour tissue was determined by epigenetic grading (EGG) tests developed by Sherbet and Lakshmi [1, 3]. The cell pellets or the tumour tissue were implanted into 16–18-hr old chicken blastoderms [12, 13]. The blastoderms with the implanted tumour cells were incubated for a further 18 hr, when they were fixed, sectioned, stained and examined histologically. The cel-



Figs. 1 *and* 2. *The ectodermal response* (*ECT*) *induced by the metastasizing lymphosarcoma. END is the endodermal layer of the blastoderm. H & E* × 250.



Figs. 3 *and* 4. *Implants of NML cells* (*GR*) *showing mesodermal effects. In Fig.* 3, *the mesenchymal cells are converging on the implant, while in Fig.* 4 *the implant can be seen covered over by mesoderm* (*MES*). *H & E* × 150.

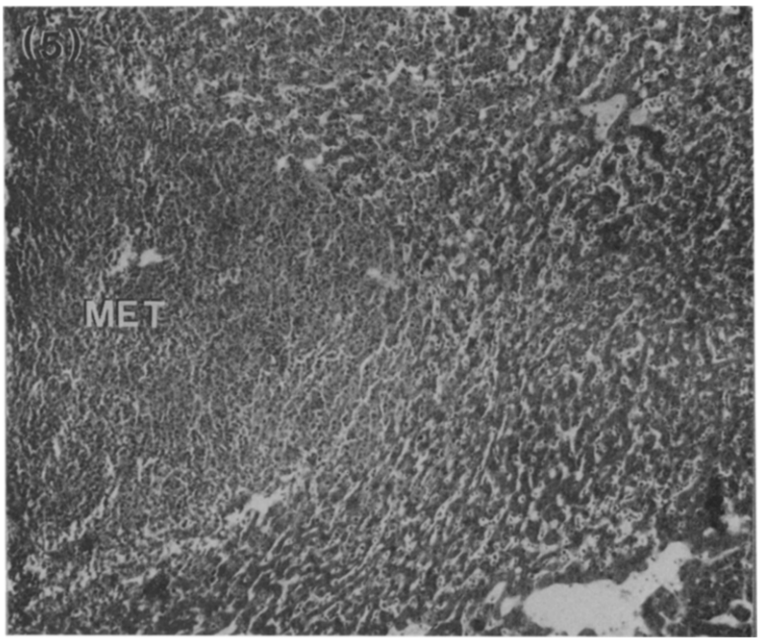
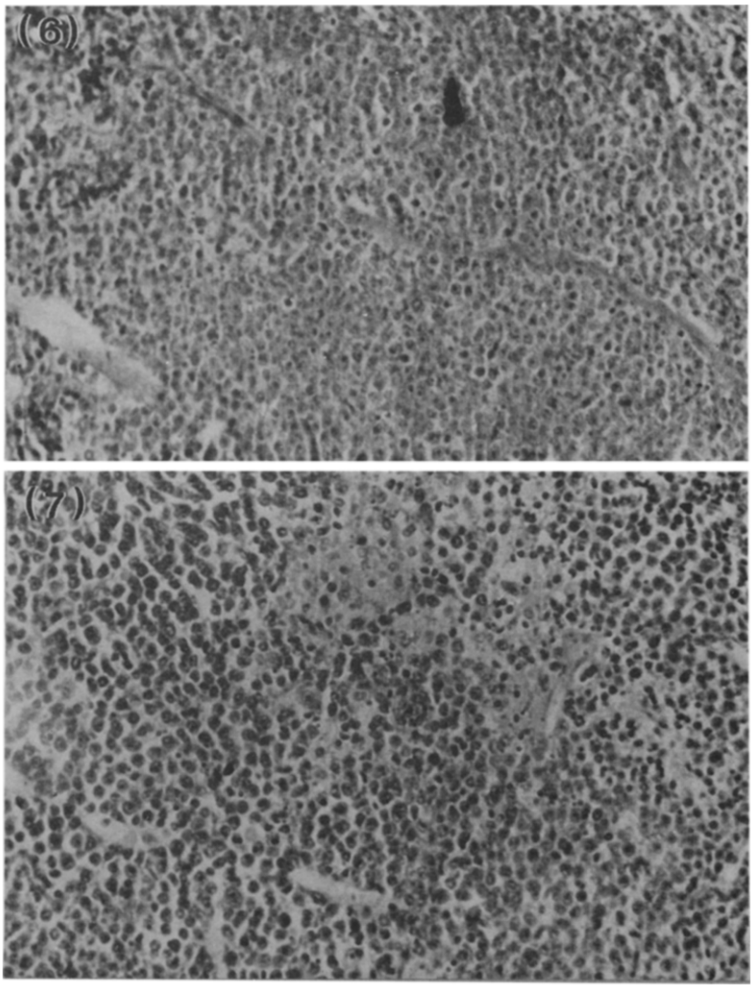


Fig. 5. *Histology of liver of ML bearing hamster showing metastatic deposit (MET). H & E* × 150.



Figs. 6 *and* 7. *Histology of the metastasizing and non-metastasizing tumours. H & E* × 250.

lular responses from the embryo were scored and the tumours embryologically graded as described previously [1].

RESULTS

EGG of lymphosarcomas

The EGG tests have revealed marked differences between the NML and ML tumours. The data are summarised in Table 1. The tumour tissues were found to be EGG 6 and 12 respectively; while the purified cell preparations from NML and ML tumours gave EGG values of 7 and 15. The differences in EGG are due to three major differences in the cellular responses: (a) the ectodermal responses were low or absent in NML but occurred in 75 and 80% of the cases in the ML tumours (Figs. 1 and 2). These responses were also more intense; (b) The NML tumour elicited the mesodermal response (Figs. 3 and 4) in 57 and 60% of the cases, while this response was produced only by 22% of the purified cell implants and 44% of solid tumour tissue implants of the ML tumour; (c) The morphogenetic displacement of ML implants was considerably greater than that of the NML tumour. The endodermal response produced by the two tumours was comparable both in the frequency and intensity of the response.

Biological behaviour of the lymphosarcomas

The biological tests on the behaviour of the tumours on implantation into Wright hamsters clearly confirmed the differences in the malignancy of NML and ML tumours (Table 2). Of thirty animals examined for each tumour type, none of the animals bearing the NML tumour showed metastases in the lungs, liver or the kidney. But 7% and 100% of animals with ML tumour showed metastases into the lungs and liver (Fig. 5) respectively. About 3% of the animals developed metastases in the kidney. This was further confirmed by injecting liver tissues from animals bearing the NML and ML tumours into 20 hamsters for each tumour type. None of the animals which had been injected with liver tissue derived from NML-bearing hamsters developed tumours. In contradistinction, all of those injected with liver tissue from ML-bearing animals developed tumours, indicating that the latter tissue contained secondary foci of the tumour. The survival times of hamsters bearing the NML and ML tumours were different. The average survival times were 24 days and 18 days respectively for NML and ML-bearing animals. The differences were found to be statistically significant. The overall growth rate of the NML was slightly higher than that of ML cells. However, in the first 4 days after tu-

Table 1. *Data on EGG tests relating to TRES, NML and ML tumours*

EGG parameter	Non-metastasizing lymphosarcoma		Metastasizing lymphosarcoma		Normal hamster kidney	TRES hamster fibrosarcoma
	Purified cell prep.	Tumour tissue	Purified cell prep.	Tumour tissue		
No. of implants	10	17	10	13	14	12
Ect R_f%	0	18	80	75	14	45
Ect R^0	0	+	2+	+	+	+
End R_f%	50	41	50	62	36	82
End R^0	2+	+	2+	+	+	+
HMR %	60	57	22	44	91	57
MD%	22	47	70	70	0	27
EGG	7	6	15	12	3	8

Ect R_f and End R_f: frequency of ectodermal and endodermal responses; Ect R^0 and End R^0: intensity grade of ectodermal and endodermal responses; HMR and MD: frequency of occurrence of mesodermal responses and morphogenetic displacement of the implants.

The tumours were graded according to the following method of scoring: ectodermal and endodermal response (frequency) 75–100%, 3 points; 50–74%, 2 points; 25–49%, 1 point; 0–25%, 0 point; ectodermal and endodermal response (degree)+ 1 point, 2+ 2 points; host mesodermal response (frequency) 75–100%, 0 point; 50–74%, 1 point; 25–49%, 2 points, 0–24%, 3 points; morphogenetic displacement (frequency) 75–100%, 3 points; 50–74%, 2 points; 30–49%, 1 point, 0–29%, 0 point.

Table 2. Differences in the biological behaviour of NML and ML tumours implanted into Wright's hamsters

Parameter	NML*	ML*
Tumour incidence (%)	100 (30)	100 (30)
Local invasion	None	Highly invasive
Metastasis in lungs (%)	0 (30)	7 (30)
Kidney	0 (30)	3 (30)
Liver	0 (30)	100 (30)
Animals forming tumour after inoculation of tumour-bearer liver (%)	0 (20)	100 (20)
Survival time (days)	24 ±3 (20)	18 ±2 (20)
Growth rate† (arbitrary units)	4.9±1.1 (10)	4.0±0.7 (17)

*Figures in parentheses refer to the number of animals used or the number of experiments.
†The method adopted for measuring growth rate is described in Materials and Methods. Although the overall growth rate was higher for NML than for the ML tumour, ML tumours grew faster than the NML in the first 4 days after the tumours became palpable.

mours became palpable, the ML tumours grew faster than the NML.

EGG of normal hamster cells and hamster TRES fibrosarcoma

For comparison with the lymphosarcomas, hamster kidney tissue and a hamster fibrosarcoma, viz. the TRES fibrosarcoma, were also epigenetically graded. The kidney tissue produced a low level of response from the embryonic tissue and was found to be EGG 3. The TRES fibrosarcoma was found to be EGG 8 (Table 1).

DISCUSSION

Previous work in the authors' laboratory [4, 5, 14, 15] and in other laboratories [16, 17] has shown that it is possible not only to distinguish between normal and neoplastic cells, but also to determine the degree of tumour malignancy [1] by implanting the cells into chick embryos.

Compatible with these earlier observations are the data obtained in the present series of experiments. As expected, normal hamster tissue was found to be of low EGG. The TRES fibrosarcoma was EGG 8, and only marginally higher than the non-metastasizing lymphosarcoma. In other words, the TRES fibrosarcoma seems to be only moderately malignant. This view conforms with the findings of Latner *et al.* [11] that although TRES fibrosarcoma cells, which are BHK-21 cells transformed by histone treatment, were highly tumorigenic, producing tumours in 75% of animals inoculated, invasive behaviour and metastatic deposits were seen only in about 30% of the animals. The slightly higher EGG rating of the fibrosarcoma than the NML tumour is consistent with the fact that although the NML cells are also highly tumorigenic, the tumours do not show invasiveness or form distant metastases (Table 2).

The EGG tests demonstrate that considerable differences exist between the metastasizing and non-metastasizing forms of the lymphosarcoma. This is compatible with the differences in their behaviour *in vivo*. In malignancy rating, the non-metastasizing form was comparable with that of human meningiomas [3] and mammary fibroadenomas (Sherbet and Lakshmi, unpublished work), but slightly higher than cystic hyperplastic tissue of the breast [3]. However, the metastasizing lymphosarcoma was on a par with the highly malignant glioblastoma multiforme [2] and carcinomas of the breast [3]. Compatible with the differences in the malignancy of the tumours also are the higher ectodermal responses and the lower mesodermal responses induced by the ML tumour.

The two forms of lymphosarcoma used in the present investigation are histologically similar in appearance (Figs. 6 and 7), and possess roughly comparable rates of growth, but they show wide differences in their malignancy. The NML tumour grows fully encapsulated and not fixed rigidly to the body wall. This tumour is neither locally invasive nor able to form distant metastases. In contradistinction, the ML tumour is highly invasive and metastasizes extensively into the liver. Not only has this been demonstrated by Carter and Gershon [6, 18] and Gershon *et al.* [19, 20] but also conclusively proved by our

own investigation (Table 2). The survival times of the tumour-bearing animals are different, being significantly lower in the case of the ML tumour. The present investigation shows that the EGG test is able to distinguish unequivocally between the non-metastasizing and the metastasizing lymphosarcoma. Although histological grading and clinical staging systems provide adequate guidelines for cancer treatment, neither method can provide clues as to the malignancy potential of a given tumour. Therefore, the epigenetic test could be of considerable clinical use.

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