

Antigenic Cross-Reactivities of Rat Neurogenic Tumors Induced by Ethylnitrosourea Tested by Capillary Migration Inhibition Test*

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Summary

Antigenic cross-reactivities of rat neurogenic tumors induced by ethylnitrosourea (ENU) were examined by capillary migration inhibition test.

Sensitized lymphoid cells were obtained from the rats sensitized by subcutaneous injection of 1×10^7 mitomycin (MMC)-treated tumor cells at weekly intervals for 5 weeks, and from rats bearing transplantable tumors originated from ENU-induced neurogenic tumors. A mixture of thymus and lymph node cells was used as migration cell.

Most of the tumors showed similar reactivities, but quite different intensities. Even tumors with the same histological appearance, showed uncorrelated antigenic patterns.

The reactions of sensitized lymphoid cells with adult rat brain and liver, normal fetal tissue of two weeks' gestation excluding brain and spinal cord, normal fetal brain of two weeks' gestation, normal whole fetal tissues of one week's gestation and spontaneous rat mammary adenocarcinoma were also examined.

It was confirmed that the lymphoid cells sensitized by T1 tumor (peripheral nerve neurinoma) reacted with all ENU-induced neurogenic tumors examined in, 1-week fetal tissue and adult brain, but not with extract of normal adult liver, spontaneous mammary cancer, nor 2-weeks fetal tissue. On the other hand, the lymphoid cells sensitized by T3 tumor (trigeminal nerve neurinoma) reacted with T1, T3 and T4 tumors, fetal tissues and spontaneous mammary cancer, but not with adult brain and liver. T2 tumor (trigeminal nerve neurinoma) was not sufficiently antigenic to induce positive reactions in the migration inhibition test with lymphoid cells sensitized by MMC-treated cells.

The present study suggested that these neurogenic tumors may share a common antigenic

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enicity which is independent from normal brain, and 2-weeks fetal tissue.

Introduction

Since the DRUCKREY's report⁵⁾ of the induction of neurogenic tumors by nitroso derivatives, many types of tumors have been induced by many different nitroso-compounds¹³⁾¹⁷⁾ in various strains of animals.

Oncogenic properties and the neurotropic nature of ENU have been examined extensively, and immunological examination as well as biochemical analysis⁷⁾ have become very important in the oncogenic study of ENU.

In general, a chemically induced tumor has an individual antigenicity. However, there are conflicting opinions on the nature of antigenicity of rat neurogenic tumors induced by ENU. FIELDS⁶⁾ has reported a common antigenicity, while CORNAIN³⁾ and TOH¹⁵⁾ have insisted on individual antigenicities. DENLINGER's report⁴⁾ on the presence of viral particles and the numerous reports on the presence of S-100 protein¹⁾¹⁴⁾¹⁹⁾ in these tumors suggest that they have a common antigenicity.

TROUILLAS¹⁶⁾ and we⁸⁾ have studied the antigenic natures of human nervous tumors, and reported common glial antigenicities and common reactivities with fetal tissues. In this experiment we have tested the similarity of these rat neurogenic tumors and human neurogenic tumors in vitro.

Materials and Methods

Rats : Inbred Wistar/AfHanMolFib. (Wistar/Fib) rats were obtained from our animal colony maintained under 'minimal disease' conditions at the Fibiger Laboratory.

Methods of induction and transplantation were as described previously⁹⁾.

3M-KCl extracts of each tissue were obtained by BODDIE's method²⁾. Brain, liver, 2-week fetal brain, 2-week fetal tissue excluding brain and spinal cord, and 1-week whole fetal tissue were obtained from syngeneic Wistar rats. 3M-KCl extracts were kept at -80°C and thawed immediately before use. No sample was subjected no more than one cycle of freezing and thawing.

Sensitization of rats : Tumor cell suspensions were made from transplanted solid tumors with a Daunce glass homogenizer after cutting into small pieces with scissors and filtering through a monolayer nylon mesh of 80 μ pore size. 1×10^7 of these MMC-treated tumor cells were injected subcutaneously 5 times at weekly intervals. Sensitized rats were used 7 to 10 days after the last sensitization.

Migration Inhibition Test (MIT) : Lymphoid cells were obtained from the thymuses and lymph nodes in the axillar, brachial, inguinal, parathymic and submandibular regions of sensitized and non-sensitized rats. The lymphoid cell suspensions were made in the same way as the tumor cell suspensions. Then the cells were washed 5 times with phosphate buffer solution (PBS) and once with Parker 199 medium containig 15% heat-inactivated horse serum, 100 μ g/ml of penicillin and 100 μ g/ml of streptomycin. This medium

also served as migration medium. After the last centrifugation, the packed lymphoid cells were resuspended in 6 volumes of the migration medium and sucked into 20 μ l Drummond capillary tubes with a vacuum pump. The capillary tubes were presealed at the upper end by heating. The filled capillary tubes were inverted and centrifuged at 750 g for 3 minutes. Finally they were cut at the cell-fluid interphase and the part containing lymphoid cells was fixed to the bottom of the migration wells of a Sterilin No 308 migration plate (Richmond, U. K.) with silicon grease.

Each well received 0.4 ml of migration medium. Antigen (ag) was added at a concentration of 100 μ g/ml protein. After sealing the wells with coverslips, the migration plates were incubated for 22-24 hours at 37°C in a CO₂ incubator. Then the migration plates were placed in a Liesegang projector, and the circumferences of the projected migration zones were drawn on paper and cut out. The migration area (MA) were quantified by weighing and the average results in each experimental group were calculated.

In each group, 6 migration cultures were studied. In another study (unpublished), we found that 95% of individual measurements divided by the average of all 6 migration cultures showed values between 0.72 and 1.28. These values were applied to the cultures without antigen, and results showing values or more than 1.30 or 0.70 were excluded as extraneous values. After exclusion of the extraneous values, the migration inhibition index (MII) for each migration culture was calculated as :

$$\text{MII} = \frac{\text{MA with ag}}{\text{mean MA without ag}}$$

A significant difference between the average MII with the lymphoid cells from sensitized and non-sensitized rats was established by p values ≤ 0.05 by Student's two tailed t-test.

Results

In a preliminary experiment, we examined the reaction of the lymphoid cells of rats to which ENU had been given transplacentally, and observed some neurological signs [in the 'original tumor bearers'] (G-group). The average age of these rats was about 8 months, and their thymuses were usually small, being just enough to test against one of the antigens. The preliminary tumor extract from the pooled ENU neurogenic tumors (T_{mix}) consisted mainly of peripheral nerve neurinoma of the same origin as T₁ tumor, with other neurinomas and spinal ependymomas. Further extracts were prepared from various normal adult and fetal tissues as previously mentioned.

The lymphoid cells derived from 39 primary hosts carrying various types of neurogenic tumors were tested against 3M-KCl extracts of transplantable neurogenic tumors (T₁, T₂, T₃), T_{mix}, and of normal adult brain. In 19 cases a significant inhibition (MA with ag < MA without ag) was obtained with at least one extract (Table I). All of the extracts examined showed some percentage of responses against the tumor bearer, varying from 36% (T₃) to 60% (brain).

The results of these pilot studies might suggest the presence of an antigen common

Table. I. Lymphoid cell reaction of the original tumor-bearing rats

lymph. cell	antigen				
	T-mix.	T1	T2	T3	Brain
G-group* rats	4/7 cases (57%)	8/20 (40%)	4/7 (57%)	5/14 (36%)	3/5 (60%)

(no. of positive cases/total cases)

* 19/39 rats showed positive migration inhibition against at least one of the tumors.

to a number of ENU-induced tumors and normal brain.

In the next step, the reactivities of lymphoid tissues of the recipients of transplantable tumors were examined. Most of these rats developed huge subcutaneous tumors [(T1; 1-2 passage), (T2; 1-6 passage), (T3; 1-3 passage)] of 1-3 passage and were in the preterminal stages. In this study, the cases which showed migration enhancement were excluded for the reason mentioned elsewhere¹⁰⁾.

T1 tumor bearers reacted with the extract of all three tumors (Table II), but T2 bearers reacted with T1 tumor extract alone. T3 tumor bearers reacted with the extracts of T1 and T3 tumors. These findings suggested that T1 tumor had a common antigenicity with T3 tumor and the antigenic intensities of T1, T3 and T2 tumors decreased in this order.

Table. II. Reaction of transplanted tumor-bearing rats

antigen	T-1		T-2		T-3	
	anti-T1	control	anti-T1	control	anti-T1	control
lymphoid cells						
MII	<u>0.92±0.06</u>	1.06±0.08	<u>0.98±0.07</u>	1.16±0.11	<u>0.81±0.06</u>	0.92±0.08
p-value	0.01--0.005		0.025--0.0125		0.05--0.025	
lymphoid cells						
MII	<u>0.90±0.07</u>	1.05±0.06	<u>0.89±0.13</u>	1.13±0.16	<u>0.79±0.15</u>	0.83±0.11
p-value	0.05--0.025		0.10--0.05		0.40--0.35	
lymphoid cells						
MII	<u>0.92±0.10</u>	1.01±0.05	<u>0.84±0.19</u>	0.99±0.19	<u>0.71±0.12</u>	0.87±0.13
p-value	0.05--0.025		0.15--0.10		0.0125--0.01	

Underlined values differ significantly from the MII of sensitized lymphoid cells without antigen.

To further elucidate these antigenicities, the rats were hypersensitized with MMC-treated tumor cells. The extracts were prepared from transplantable tumors, normal adult and fetal tissues, as mentioned before. T4 tumor originated from a mixed glioma of the brain.

The lymphoid cells sensitized by T1 tumor reacted with the extracts of all tumors, 1-week fetus and adult brain, but not with 2-week fetal tissues, spontaneous mammary

cancer or adult liver. Moreover, the reaction of T1-sensitized lymphoid cells against T1 tumor itself was the strongest and the reactions against T2 and T4 were weaker than that against T3 (Table III).

The lymphoid cells sensitized by T2 tumor reacted with T1 extract only (Table IV). On the other hand, the lymphoid cells sensitized by T3 tumor reacted with the extracts of T1, T3, T4, spontaneous mammary cancer and all fetal tissue, but not with T2, adult brain or liver (Table V).

The cross-reaction observed in these experiments are summarized in Table VI. From the results of these experiments, it appeared that T1 and T3 share a common antigen which is also present in T4 and mixed fetal tissues of 1 week of gestation. The extracts of spontan-

Table. III. Reaction of T1-sensitized lymphoid cells

antigen	MII		p-value
	sensitized lymph.	control lymph.	
(T-mix.	0.80 ± 0.27	0.99 ± 0.30	0.30 -- 0.25)
T-1	0.81 ± 0.07	0.99 ± 0.09	0.0005 --
T-2	0.88 ± 0.12	1.01 ± 0.03	0.0125 -- 0.01
T-3	0.75 ± 0.08	0.87 ± 0.11	0.0025 -- 0.0005
T-4	0.85 ± 0.08	0.97 ± 0.04	0.025 -- 0.0125
spont. mammary cancer (T-9)	0.69 ± 0.17	0.85 ± 0.08	0.10 -- 0.05
2-wk fetus	0.71 ± 0.33	0.96 ± 0.04	0.15 -- 0.10
2-wk f. brain	0.87 ± 0.13	1.02 ± 0.11	0.10 -- 0.05
1-wk fetus	0.69 ± 0.10	0.90 ± 0.06	0.025 -- 0.0125
adult brain	0.82 ± 0.12	0.95 ± 0.09	0.025 -- 0.0125
adult liver	0.84 ± 0.20	0.91 ± 0.18	0.30 -- 0.25

Cf. the footnote of Table II.

Table. IV. Reaction of T2-sensitized lymphoid cells

antigen	MII		p-value
	sensitized lymph.	control lymph.	
T-1	0.80 ± 0.08	0.96 ± 0.05	0.025 -- 0.0125
T-2	0.77 ± 0.15	0.92 ± 0.14	0.10 -- 0.05
T-3	0.77 ± 0.13	0.96 ± 0.24	0.10 -- 0.05
T-4	0.88 ± 0.14	1.07 ± 0.16	0.10 -- 0.05
spont. mammary cancer (T-9)	0.67 ± 0.20	0.83 ± 0.11	0.15 -- 0.10
2-wk fetus	0.74 ± 0.19	0.86 ± 0.16	0.25 -- 0.20
2-wk f. brain	0.87 ± 0.03	1.10 ± 0.01	0.005 -- 0.0025
1-wk fetus	0.67 ± 0.17	0.81 ± 0.17	0.20 -- 0.15
adult brain	0.75 ± 0.11	0.92 ± 0.20	0.25 -- 0.20
adult liver	0.64 ± 0.19	0.84 ± 0.13	0.15 -- 0.10

Cf. the footnote of Table II

Table V. Reaction of T3-Sensitized Lymphoid cells

antigen	MII		p-value
	sensitized lymph.	control lymph.	
T-1	$\underline{0.88} \pm 0.10$	0.98 ± 0.05	0.025--0.01
T-2	$\underline{0.87} \pm 0.14$	0.97 ± 0.14	0.15--0.10
T-3	$\underline{0.78} \pm 0.11$	0.93 ± 0.11	0.01--0.005
T-4	$\underline{0.86} \pm 0.11$	1.01 ± 0.08	0.025--0.0125
spont. mammary cancer (T-9)	$\underline{0.61} \pm 0.12$	0.83 ± 0.10	0.01--0.005
2-wk fetus	$\underline{0.77} \pm 0.10$	0.91 ± 0.04	0.025--0.01
2-wk f. brjin	$\underline{0.84} \pm 0.05$	1.06 ± 0.07	0.01--0.005
1-wk fetus	$\underline{0.65} \pm 0.0005$	0.91 ± 0.08	0.05--0.025
adult brain	$\underline{0.70} \pm 0.18$	0.77 ± 0.20	0.30--0.20
adult liver	$\underline{0.77} \pm 0.18$	0.88 ± 0.13	0.20--0.15

Cf. the foot note of Table II

eous mammary carcinoma (T9) and fetal brain of 2 weeks of gestation caused a specific inhibition of anti-T3 lymphoid cells, while the difference between non-sensitized and anti-T1 lymphoid cells was only on the borderline of statistical significance.

The extracts of T1 and fetal brain tissue showed cross reaction with anti-T2 lymphoid cells, while T2 extract reacted only with anti-T1 lymphoid cells. The antigenic strength of this tumor seemed rather low.

Table VI. Cross-reactions of sensitized lymphoid cells

with extracts from ENU-induced neurinomas (T1, T2, T3) mixed glioma (T4), spontaneous mammary carcinoma (T9) and various fetal and adult rat tissues

Lymphoid cells sensitized to :	Extract										
	T-1	T-2	T-3	T-4	T-9	1-wk fetus	2-wk fetus*	2-wk fetal brain	Adult brain	Adult liver	
T-1	+	+	+	+	-	+	-	-	+	-	
T-2	+	-	-	-	-	-	-	+	-	-	
T-3	+	-	+	+	+	+	+	+	-	-	

* without brain and spinal cord

Discussion

The nature of ENU-induced tumor has been examined immunologically by many investigators. Under the in vivo transplantation test, young recipients have a higher take-ratio than older ones¹⁸⁾ and the success rate is higher for intracerebral transplantation than subcutaneous inoculation¹¹⁾. These observations indirectly support the presence of the transplantation antigens on these tumor cells. ToH¹⁵⁾ failed to show cross-reactivity between schwannoma and glioma by a direct mixed culture method, and stated that the immunological intensities must be weak in schwannoma. He used lymphoid cells from tumor bearing

rats. Therefore, serum factors have masked the real reaction. Moreover, his technique might not be sensitive enough to detect these weak tumor antigens.

The presence of S-100 protein, as mentioned before, suggests the presence of cross-reactivity between neurogenic tumors and normal brain. FIELDS⁶⁾ reported a study on the cross-reactivity between ENU tumors, normal brain and fetal tissues, by making anti-tumor serum and absorbing it with various tissues. CORNAIN³⁾ used splenocytes to study the cross-reactivity of ENU tumors by the cytotoxicity test. The unpurified splenocytes showed a wide cross-reaction, although purified ones showed distinctive antigenicities. Although it is better to purify the cells to examine the exact antigenicity of the tumors, the unpurified cells act cooperatively *in vivo*. Therefore, we feel the purification of the splenocytes should be adjusted to the stage in which they show the same responses as *in vivo* study. We have performed *in vivo* studies, but so far have not obtained distinctive results by active protection and tumor neutralization test.

In the present study we exclusively used thymocytes and lymph node cells as the source of lymphoid cells, because the splenocytes gave variable results in MIT.¹⁰⁾

From our results, all tumor cells tested showed some cross-reactivities, though the intensities of immunity were different. Anti-T1 lymphoid cells also reacted with the extract of normal adult brain, but anti-T3 lymphoid cells did not. Therefore, the common part of the tumor antigenicity can not be the antigen present in normal adult brain.

It was reported that the expression of fetal antigenicity is most intense in the 2-week fetus¹²⁾. However, both of the sensitized lymphoid cells reacted with the extract of 1-week fetus, but not with 2-week fetus in our study. Therefore, the cross-reactivity with fetal tissues should be reexamined later. We are planning to sensitize rats with fetal tissue extracts, and these sensitized lymphoid cells will be tested with the extracts of each tumor.

Studies on ⁵¹Cr-release cytotoxicity and inhibition of DNA synthesis are also in progress, and will give extensive reconfirmations of the results of migration inhibition test.

Finally, the *in vivo* transplantation test is expected to become the standard for these *in vitro* studies.

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和文抄録

遊走阻止反応でみたエチルニトロソウレア
誘発神経系腫瘍の免疫学的交叉反応

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ethylnitrosourea (ENU) で誘発された各神経系腫瘍の抗原性を, 毛細管による遊走阻止反応で調べた.

感作リンパ球は, 移植腫瘍の host 又は, mitomycin で処理した腫瘍細胞で5回感作した rat の胸腺, リンパ腺より得た. 組織学的に同様の腫瘍でも抗原性には相関々係はなかった.

T₁ (末梢神経 neurinoma) は, すべての ENU 神経系腫瘍, 1週胎児, 正常脳と反応したが, 肝, 乳癌, 2週胎児などとは反応しなかった.

T₃ (三叉神経 neurinoma) は一部の ENU 神経系腫瘍, 全胎児組織, 乳癌と反応したが, 正常脳や肝臓とは反応しなかった.

T₂ (三叉神経 neurinoma) は抗原性が極少であった.

以上より ENU 神経系腫瘍に共通する抗原は乳癌, 脳, 2週胎児などには存在しない性質のものであろうと考えられた.