Antigenicity of Two Established Tumorogenic Cell Lines Estimated by the Migration Inhibition Test[1] (35528)

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The aim of the present study was the analysis of the antigenic properties of two cultured transformed cell lines derived from lung tissue from normal adult C3H mice. The methodological approach used in this work was mainly the migration inhibition test. This technique is attracting the interest of an increasing number of investigators working in the field of transplantation and cancer research (1–3). For comparison, the results of some transplantation experiments are included.

Materials and Methods. Two cultured cell lines and tumors developed after inoculation of these cells into the original inbred strain of C3H mice were used. The two cell lines were established in 1964 (L-1) and 1969 (L-2c). Both underwent apparently spontaneous malignant conversion during propagation *in vitro,* the L-1 line after 6 months and the L-2c line after 10 months of cultivation. Composition of media and methods of cultivation have been described elsewhere (4), together with the morphologic characteristics, growth pattern, metabolism, and tumorogenicity of the L-1 line. The morphologic alterations which preceded the malignant conversion of the L-2c culture resembled those observed in the L-1 line. In the course of the present experimental work the cell cultures were controlled twice for contamination by mycoplasm with negative results. After subcutaneous (sc) inoculation of the cultured cells in newborn C3H mice, but not in adult mice, anaplastic sarcomas developed as previously described (4). From these sarcomas two tumor lines (C3H-L1 and C3H-L2c) were established in adult inbred C3H mice by sc transplantation.

Immunization. Inbred strains of C3H and DBA/2 mice were immunized by sc injections of 10^7 cells in Dulbecco's phosphate buffered saline (PBS), and reimmunized after 1–6 weeks. In most experiments, reimmunization was performed after 2 weeks. In some experiments, minced tumor tissue from tumors propagated *in vivo* was used for preimmunization. Animals showing regressive tumor growth were selected for reimmunization.

The immunological response was checked *in vivo* and *in vitro,* partly by the sc transplantation of 0.2 ml of minced C3H-L1 and C3H-L2c tumor tissue into preimmunized animals, and partly by the migration inhibition test (MIT) in the modification described by Haskova *et al.* (5).

Migration inhibition test (MIT). In Haskova and co-workers' modification, spleen fragments rather than peritoneal exudate cells packed in capillary tubes, are used as the reacting tissue. The test was performed 10–20 days after reimmunization.

Fragments of spleen pulp with a diameter of ca. 1 mm were cultured in plastic trays (Linbro FB 48 clear), containing wells of 6-mm diameter, for 18 hr at 37°. Most of the cultures were incubated in a CO_2 (5%) incubator. Some of them were cultured in the normal atmosphere, in those cases namely when the addition of ag-homogenate lowered the pH beyond the control of the buffer system of the medium. Therefore, as a rule, pH of the medium was checked after incubation. Cultures showing pH values exceeding a range of 6.8–7.4 were discarded as technical failures.

Ten to 12 fragments of each spleen were

[1] Supported by grants from the Daell Foundation, the Schepler Foundation, and the Pedersholm Foundation.

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[3] Sponsored by the Danish Cancer Society.

TABLE I. Protection and Cross-Protection of C3H Mice Preimmunized with Cultured L-1 and L-2c Cells[a] Against Their Corresponding Tumors Propagated *in Vivo*.

Tumor challenge	Preimmunization:	No. of mice with progressively growing tumor/total no. of mice			
		L-1	Control	L-2c	Control
C3H-L1 tumor	I[b]	9/9	10/10	2/12	8/10
	II	0/10	10/10		
C3H-L2c tumor	I	10/10	10/10	10/10	10/10
	II	10/10	10/10	10/10	10/10

[a] 10^7 cells injected sc.

[b] I, one preimmunizing injection; II, three preimmunizing injections given at weekly intervals.

cultured independently, *i.e.*, each animal spleen was measured 10–12 times. Half of the cultures contained no antigen (ag).

Antigens were introduced into the reacting system as homogenates of cells diluted by the culture medium. The concentration of ag was controlled by protein determination.

Preparation of ag for the in vitro, *challenge.* Established cell lines or spleen cells, respectively, were suspended in PBS, and centrifuged for 10 min at 800*g* at room temperature. The cells were then resuspended in PBS in concentration of 10^9 cells/ml and homogenized in a glass homogenizer at 0°.

Fifty to 100 mg of minced tissue were homogenized in 1 ml of PBS in a glass homogenizer at 0°.

Homogenates were diluted in the Fib 14B culture medium (4). Final concentration of the ag added to the culture medium varied from 1–0.2 mg of protein/ml. In few experiments, concentrations as high as 7 mg/ml and as low as 0.06 mg were used.

Planimetry and calculation of the results. Planimetry was performed under linear magnification of 12.5×, and migration index (MI) was calculated. MI is defined by the formula:

$$MI = r_2 - r_1,$$

where r_2 is the radius of the area of migration of macrophages and r_1 is the radius of the central fragment. Therefore:

$$MI = \frac{1}{(\pi)^{1/2}} \cdot [(P_2)^{1/2} - (P_1)^{1/2}],$$

where P_2 and P_1 are the measured areas of migration and the central fragment, respectively.

The MI values were used for calculation of inhibition index (II), which shows the inhibition in the experimental system by comparison of the MI values in presence and absence of ag in the medium.

II is therefore by definition = (MI with ag)/(MI without ag) in culture medium, and may vary theororetically from 1 to 0.

As some unspecific inhibition might occur when ag is applied in the form of the crude homogenate, controls on unsensitized animals were performed. The proper concentration of ag in medium was found by choosing the concentrations showing none or minimal unspecific inhibition when tried on spleens of nonsensitized animals.

The mean value of II was used for statistical evaluation of the results obtained with sensitized animals in relation to unsensitized ones. The alternative possibility of evaluation by comparing with the value of 1, is discussed below.

Results. Table I shows the influence of preimmunization with the two cultured cell lines on the susceptibility of inbred C3H mice to the C3H-L1 and the C3H-L2c tumors propagated *in vivo*.

The systems studied with MIT in this work are shown in Table II. The results of the measurements are given in the Tables III, IV, and V.

Two small groups of animals (Table V, 3 and 4) containing three animals each were evaluated statistically as one group, because

TABLE II. Experimental Systems Used in the Migration Inhibition Test (MIT).

Mouse strain	Material used for: Preimmunization	Challenge	Problem
C3H	L-1	L-1	Sensitization
	L-1	L-2c	Cross-reaction
	L-1	C3H spleen	Autoimmunization
	L-2c	L-2c	Sensitization
	L-2c	L-1	Cross-reaction
	L-2c	C3H spleen	Autoimmunization
DBA	L-1	L-1	Sensitization
	L-2c	L-2c	Sensitization
	L-1	C3H spleen	H-2^k
	L-2c	C3H spleen	H-2^k
C3H	C3H-L1 tumor and cells	L-1	Cross-reaction
	L-2c	C3H-L2c tumor	Cross-reaction
	C3H-L2c tumor	L-2c	Cross-reaction
C3H	No	L-1	
		L-2c	Controls
DBA	No	C3H spleen	

of the similarity of the immunological factors involved.

Discussion. 1. Preimmunization of C3H mice with cultured L-1 and L-2c cells protected the recipients against subsequent transplantation with the *in vivo* propagated C3H-L1 tumor, indicating the presence of common isoimmunizing antigens in the two cultured cell lines and the C3H-L1 tumor. The absence of a similar protection against the C3H-L2c tumor suggests that this tumor does not share this or these antigens. However, cross-reaction between the cultured L-2c cells and the C3H-L2c tumor was revealed by the MIT *in vitro* (Table V, 3 and 4). Thus, the negative result of the transplantation test may rather be interpreted as a manifestation of high virulence enabling the C3H-L2c tumor to overcome the immunological barrier even in hyperimmunized C3H mice.

2. Evaluation of MIT was based on the calculation of II which theoretically varies between 0 and 1 value. The experimental results might therefore be compared with respect to their significance to the value of 1. This is true only when ag introduced into the medium does not influence at all the unsen-

TABLE III. Isoimmunization, Cross Immunization, and Autoimmunization by L-1 and L-2c Mouse Cells as Measured by Migration Inhibition Index (II).

No.	Mouse strain	Preimmunization	Challenge	II	n	s	t comp. to:	Expt. no.
1	C3H	L-1	L-1	0.72	11	0.08	3.46	7
2		L-1	L-2c	1.11	8	0.11	5.57	8
3		L-1	C3H spleen	0.92	6	0.07	2.00	9
4		L-2c	L-2c	0.55	12	0.09	6.32	8
5		L-2c	L-1	0.83	12	0.17	0.95	7
6		L-2c	C3H spleen	0.90	8	0.05	2.78	9
7	C3H	No	L-1	0.90	9	0.14		
8		No	L-2c	0.82	8	0.08		
9		No	C3H spleen	1.05	5	0.12		

TABLE IV. Response of DBA/2 Mice Sensitized by the Malignant L-1 and L-2c Cells to the Challenge by Nonmalignant C3H Spleen Cells as Measured by the Malignant Inhibition Index (II).

No.	Mouse strain	Preimmunization	Challenge	II	*n*	*s*	*t* comp. to:	Expt. no.
1	DBA	L-1	L-1	0.55	5	0.05	3.23	5
2		L-2c	L-2c	0.50	5	0.05	2.57	6
3		L-1	C3H spleen	0.92	8	0.17	1.79	7
4		L-2c	C3H spleen	0.91	6	0.12	2.12	7
5	DBA	No	L-1	0.86	5	0.18		
6		No	L-2c	0.86	5	0.12		
7		No	C3H spleen	1.10	5	0.15		

sitized controls. In our experiment, this was not always the case. The unwanted unspecific toxicity was observed for as low concentrations of ag (estimated by protein conc) as 0.05 mg/ml. As it might be useless to use lower concentrations of ag at the risk of not releasing the migration inhibition factor (MIF), the significance of the results was calculated in relation to the II of unsensitized controls.

3. It was shown that not only the L-1 but also the L-2c cell line has isoimmunogenic properties (Table III, 1 and 4). However, the MIT did not reveal any cross-reaction between these two lines (Table III, 2 and 5). This might be explained by the unspecific toxicity discussed above, which influences the unsensitized controls (Table III, 7 and 8) used for the statistical evaluation of the significance, thus lowering the sensitivity of the test.

4. The antigenic similarity of the transformed cell lines to the original tissues allowed for theoretical assumption of the induction of autoimmunization after sensitization. The mechanism of the termination of tolerance by the use of antigens closely similar to the tolerogenic ones, could be responsible for this phenomenon. Slight signs of autoimmunization were shown in our systems (Table III, 3 and 6).

5. The unexpected result of the experiments was that although both cell lines derived from the C3H strain sensitized the DBA mice across the H-2 barrier (Table IV, 1 and 2) the sensitized animals do not cross-react readily with the normal C3H tissues (Table IV, 3 and 4).

Until more fundamental data on the nature of the tumor ag become available the simplest way to discuss the results is to assume that antigenicity of malignant cells based on a mosaic pattern of normal and tumor antigens is easier recognized by the sensitized animals when the complete pattern rather than its single component is presented (6). The decreased concentration of the H-2^k surface ag by the dilution caused by the newly acquired tumor ag also might be taken into account (7, 8).

6. In the experiment on the cross-reaction, discussed above, between the two cultured cell lines unexpected promotion of migration was seen in the system in which spleens from mice sensitized by the L-1 cells were challenged by L-2c ag (Table III, 2). The results of the present experiments do not offer any explanation of this observation which might be related to the promotion of migration by very small concentrations of ag observed by other authors (9–11).

7. Cross-reaction of the L-1 cell line with the tumors originating from the same cell line (Table V, 1 and 2) was observed in animals sensitized by regressed tumors and challenged by cultured cells. Similar findings were made for L-2c cells and tumors derived from this cell line (Table V, 3 and 4) in contrast to the negative transplantation test discussed above.

8. The present results indicate the usefulness of the MIT in experimental oncology in particular in cases where transplantation systems fail to reveal the immunogenicity of transplanted tumors of high virulence.

Summary. a. MIT used in the present work was found to be a useful method for the evaluation of sensitization occurring in on-

TABLE V. Cross-Reaction Between Cultured Cell Lines (L-1 and L-2c) and Their Corresponding Tumors (C3H-L1 and C3H-L2c) as Measured by the Migration Inhibition Index (II).

No.	Mouse strain	Preimmunization	Challenge	II	n	s	t comp. to:	Expt. no.
1	C3H	C3H-L1 tumor and L-1 cells	L-1	0.76	9	0.07	5.12	2
2	C3H	No	L-1	0.94	6	0.07		
3	C3H	C3H-L-2c tumor	L-2c	0.77	1			
		C3H-L-2c tumor	L-2c	0.68	1			
		C3H-L-2c tumor	L-2c	0.78	1			
4	C3H	L-2c	L3H-L2c tumor	0.64	1			
		L-2c	C3H-L2c tumor	0.57	1			
		L-2c	C3H-L2c tumor	0.77	1			
3 + 4	C3H		$\bar{x}$	0.70	6	0.08	2.49 (Table III, 7)	

cological experimental systems, where transplantation tests may fail due to high virulence of the tumor.

b. Both cell lines used, L-1 and L-2c, which are established tumorogenic cell lines derived from the C3H strain, sensitize isogenic hosts when injected sc.

c. No cross-reaction between the two cell lines could be demonstrated by the MIT as used in this work.

d. Both cell lines sensitize hosts of DBA strain across H-2. However, only slight signs of cross-reaction were seen, when DBA spleen sensitized this way was challenged by C3H spleen cell homogenate.

e. Experiments on tumors derived from the tumorogenic cell lines show cross-reactions with the original cell lines.

f. Slight signs of autoimmunization were observed in C3H mice after sensitization by the transformed cell lines, when challenged by the normal C3H spleen homogenate.

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Received Dec. 8, 1970. P.S.E.B.M., 1971, Vol. 137.