EXPRESSION OF THYMOCYTE DIFFERENTIATION ANTIGEN Thy 1 IN MOUSE MAMMARY GLAND CARCINOMA CELLS *IN VITRO*

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UDC 618.19-006.6-097:616.438-018.15]-02:615.357

KEY WORDS: antigen; mammary gland carcinoma.

Numerous investigations have shown that mammary gland carcinoma (MGC) cells, when cultured in hormone-free media, revert phenotypically toward fibroblast-like cells and lose the cell shape and growth type characteristic of epithelium. Addition of certain mammotropic hormones to the medium restores their polygonal shape and their ability to grow in an epithelial sheet [9, 16].

The present writers have raised [1, 2, 4] a clonal cell line of MGC from GR mice, with which it has been shown that the same hormone combination (insulin + dexamethasone) leads to optimal expression of the features of epithelial growth, and to the formation of "domes" — hollow structures detaching themselves from the substrate and formed by epithelial cells in the manner of alveolar sacs, and increases colony formation tenfold in semisolid media, i.e., it makes the cells less dependent on the substrate than the same cells in hormone-free medium.

This phenotypical enhancement of neoplastic features is accompanied by intracellular synthesis of an antigen detectable by allogeneic serum against thymocyte differentiation antigen Thy 1.2 (the allele to which GR mice belong); moreover, the increase in colony formation takes place mainly on account of cells which synthesize an antigen detectable by allogeneic antiserum against Thy 1.2: Treatment of the cells before seeding with antiserum in the presence of complement reduces the number of colonies to the level of colony formation in hormone-free medium.

TABLE 1. Expression of Thymocyte Antigens on Cell Membranes of Clonal Cell Line of MGC in GR Mice (antibodies against Thy 1.2)

Treatment of cells	Method of investigation			
	membrane immunofluorescence (% of cells)		humoral cytotoxic test (cytotoxic index)	
	allogeneic	monoclonal	allogeneic	monoclonal
Without hormones	—	—	—	—
	0	0	0.00	0.00
Insulin + dexamethasone	+	—	+	—
	70-75		0.22-0.27	0.00
Insulin + dexamethasone + antiserum against gp52	+	—	N	N
	70-75	0		
Dexamethasone	—	—	—	—
	0	0	0.00	0.00

Legend. N) Experiment not performed. Here and in Table 2, + denotes positive test, — negative test.

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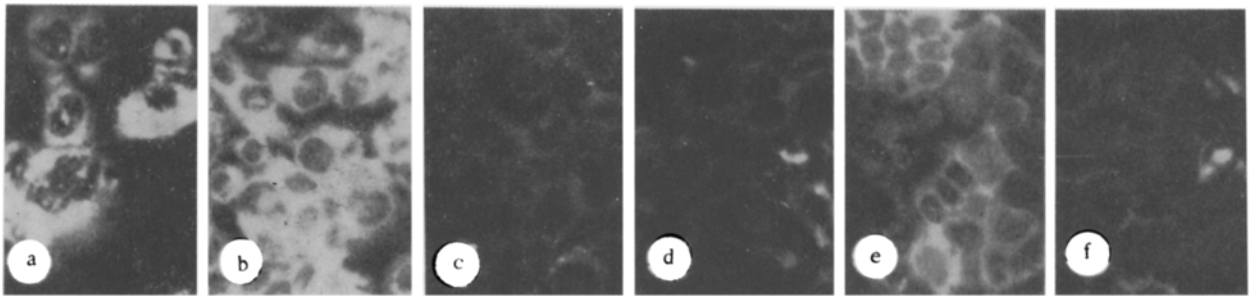

Fig. 1. Indirect immunofluorescence test with mouse MGC cells — clonal line F2 (a-d) and on squash preparations of C3H mouse thymus (e, f). a) Medium with hormones, allogeneic antiserum against Thy 1.2, positive test; b) medium with hormones, monoclonal antibodies against Thy 1.2, positive test; c) medium without hormones, monoclonal antibodies against Thy 1.2, negative test; d) medium with hormones, monoclonal antibodies against Thy 1.1, negative test; e) monoclonal antibodies against Thy 1.2, positive test; f) monoclonal antibodies against Thy 1.1, negative test. 320 ×.

All the morphological and antigenic changes in MGC cells described above are induced by insulin, but not by dexamethasone. The latter specifically activates a system of enzymic "processing" of the ENV (but not the GAG) gene product of mouse mammary tumor virus (MMTV), and according to data in the literature [17], it potentiates transcription of provirus DNA and synthesis of virus proteins. In the presence of dexamethasone the morphological influence of insulin is enhanced.

The molecular basis for the synthesis of thymocyte antigen in MGC cells is not clear. The important role of the thymus in the genesis of MGC is indicated by two groups of facts: The virus inducing them circulates by the lymphogenic route, and structural proteins of the virus have been found [3] in mouse thymus; the percentage of onset of MGC is sharply reduced in thymectomized mice and its latent period lengthened [13, 14].

The study of the conditions of expression of thymocyte differentiation antigen in MGC cells can shed light on the relations between these two processes. However, Thompson [15], using monoclonal antibodies against Thy 1.2 antigen, was unable to find it on MGC cell membranes — hormone-stimulated and containing MMTV. That communication creates doubts about the correlation between the hormone-dependent morphological changes we have described and the increased clonogenicity of MGC cells with activation of synthesis and expression of thymocyte differentation antigen on MGC membranes.

Since in our previous experiments [1, 4] we used allogeneic antiserum of AKR mice against thymocytes of C3H mice, in the present study we used the same cell system to test the presence and conditions of synthesis of Thy 1.2 antigen by means of monoclonal antibodies against this antigen.

EXPERIMENTAL METHOD

The clonal cell line of MGC of GR mice, designated F2, was described previously [1]. The cells were grown on D-MEM medium with 10% neonatal calf serum. Depending on the experimental conditions, insulin was added to the medium in a concentration of 10 μg/ml, dexamethasone up to 10^{-6} M (from Sigma, USA), or the cells were left without hormones.

A stable cell line mm5 and C3H mice (obtained from the USA), growing on medium RPMI 1640 with 10% neonatal calf serum and hormones in the specified concentrations, was used to accumulate MMTV. The cells were grown in roller flasks. The virus was sedimented from the 24-h culture fluid by centrifugation at 27,000 rpm for 1 h (K-32, S-30 centrifuge).

The following antisera were used: allogeneic, obtained from AKR mice against C3H thymocytes (Searle Diagnostic, England); against purified MMTV-gp52 virion membrane glycoprotein (obtained in rabbits in our laboratory); against MMTV-p27 internal membrane polypeptide (obtained in goats sent from the USA under the terms of the International Agreement on Collaboration); antisera conjugated with fluorescein isothiocyanate (FITC), against rabbit (obtained from the N. F. Gamaleya Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR) and goat (USA origin) globulins; monoclonal antibodies — the supernatant of hybridomas of mouse plasmacytoma with AKR mouse lymphocytes, immune to BALB/c thymocytes (anti-Thy 1.2), and with lymphocytes of BALB/c mice, immune to AKR mouse lymphocytes (anti-Thy 1.1).

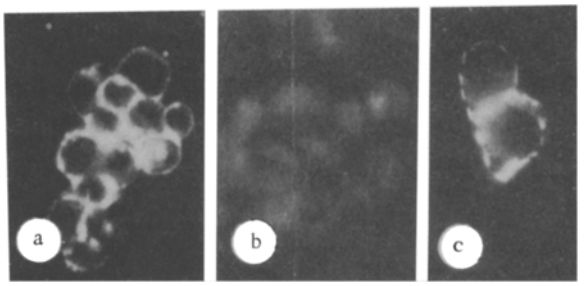

Fig. 2. Indirect immunofluorescence test with unfixed mouse MGC cells (clonal line F2). a) Medium with hormones, allogeneic antiserum against Thy 1.2, positive test; b) medium with hormones, monoclonal antibodies against Thy 1.2, negative test; c) medium with hormones, antiserum against MMTV gp52, then antiserum allogeneic to Thy 1.2, positive test. 320 ×.

TABLE 2. Testing Allogeneic Serum against Thy 1.2 Antigen for the Presence of Antibodies against MMTV Structural Proteins by an Immunoenzymic Method

Substrate	Antibodies			
	anti-Thy 1.2 allogeneic	anti-Thy 1.2 monoclonal	of C3H mice with MGC	of healthy BALB/c mice
MMTV	(1:5—1:50) — — + +	(1:3—1:10) — —	(1:20—1:50) ++ ++	(1:20—1:50) — —

The hybridomas were obtained from the USA under the terms of the International Agreement and were presented by A. V. Chervonskii (Laboratory of Tumor Immunochemistry, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR).

The following methods were used: the indirect immunofluorescence test on fixed (FIF) and unfixed (UIF) cells, according to methods described previously [5]; the humoral complement-dependent cytotoxic test — with seeding of 10^3 cells on the eve of the experiment in "Falcon" No. 3040 plates, with incubation alternately with 50 µl of antiserum and complement for 1 h in each case, and counting the percentage of dying cells as shown by staining with trypan blue. The cytotoxic index (the difference between the percentage of living cells in the control and experiment, divided by the percentage of living cells in the control) was calculated. The immunoperoxidase test on solid phase [7] was used. Sorption of antigen (MMTV) in wells of plastic "Falcon" No. 3040 plates was carried out overnight in carbonate buffer (pH 9.5). Specific antisera were added in different dilutions for 45 min. Antibodies against mouse immunoglobulins, conjugated with peroxidase ("Miles," England) were added for 30 min. The chromogenic substrate was 5-aminosalicylic acid (1 ng/ml, H_2O_2 — 0.05%), pH 6.0. Between all operations the wells were washed three times with phosphate-salt buffer with Tween (0.05%). The reaction (intensity of coloration of the chromogenic substances) was assessed on a 4-cross scale.

EXPERIMENTAL RESULTS

Allogeneic anti-Thy 1.2 serum was first exhausted on the sorbent with soluble lung, liver, and mammary gland proteins of BALB/c mice, cross-linked with 1% glutaraldehyde [6].

In the indirect FIF test both the allogeneic serum and monoclonal antibodies against Thy 1.2 reacted clearly with F2 cells to give fluorescence distributed uniformly in the cytoplasm; synthesis of the antigen was hormone-dependent (Fig. 1). Antibodies against Thy 1.1 did not react with F2 cells. Antibodies against Thy 1.2 but not against Thy 1.1 gave fluorescence on squash preparations of C3H mouse thymus. Antibodies against Thy 1.1 reacted with thymocytes of AKR mice (squash preparations of thymus fixed with acetone).

In the indirect UIF test different results were obtained (Fig. 2): allogeneic serum against Thy 1.2 gave punctate fluorescence on membranes of 70-75% of cells; antigen synthesis

was hormone-dependent. Similar results were obtained in the humoral cytotoxic test (Table 1). Treatment of the F2 cells with dexamethasone alone did not lead to synthesis of an antigen detectable by allogeneic antiserum against Thy 1.2. Monoclonal antibodies did not react in the indirect UIF test.

Since C3H mice — the donors of thymocytes for obtaining the allogeneic serum — contain exogenous MMTV in large quantities, and since the AKR mice used to obtain that serum may contain antibodies against virion proteins, an attempt was made to verify whether antibodies against structural virion proteins take part in reactions of F2 cells with allogeneic serum against Thy 1.2.

Fixed F2 cells were treated in the indirect FIF test with antiserum against MMTV p27 (goat), and then after washing with allogeneic serum against Thy 1.2 and antimouse serum conjugated with FITC. No blocking of fluorescence was observed. In the same way antiserum against gp52 did not block fluorescence in the UIF test with F2 cells (Fig. 2). Hence it follows that antibodies against MMTV structural proteins could not be detected in allogeneic serum against Thy 1.2. The specificity of antisera against MMTV structural proteins was verified previously more than once on the same F2 cells and on preparations of the purified virus; blocking of the reaction by preliminary treatment of the cells with goat, followed by rabbit antiserum (and vice versa) was shown to be possible previously [1].

We verified this conclusion by the more sensitive method of the immunoperoxidase test on a solid phase. MMTV from strain mm5, purified in a stepwise sucrose gradient, was destroyed by NP_{40}, and the preparation was added to the wells in excess (preliminary sorption of about 1-2 μg protein). The reaction was carried out with allogeneic serum and monoclonal antibodies against Thy 1.2. Sera of C3H mice with tumors (MGC) served as the positive control, sera of BALB/c mice as the negative control. It will be clear from Table 2 that antibodies against Thy 1.2 did not react with the virus preparation.

Antigen Thy 1 was one of the first thymocyte differentiation antigens to be described [10, 12]. The presence of two allogeneic determinants — Thy 1.1 (AKR mice) and Thy 1.2 (most known lines of mice) has been demonstrated in mice. Antigens of this complex have been found in brain and MGC cells, and in myoblasts at a certain stage of differentiation [7, 11, 12]. Our experiments showed that Thy 1.2 is in fact synthesized (hormone-dependent) in MGC cells of GR mice, but it could not be found on cell membranes with the aid of monoclonal antibodies.

According to data obtained in Dulbecco's laboratory [7], Thy 1, with a related antigenic determinant in rat lymphocytes, is an antigenic marker of a definite stage of differentiation of mammary gland epithelium, it is found on cell membranes only of myoepithelial cells, and is not found on membranes of cuboidal cells. It may be that the hormonal combination which we used, which induces epithelization of cells and "dome" formation, is not decisive for expression of the Thy 1.2 antigenic determinant on cell membranes. In this respect our data agree with those of Thompson [15].

Antigen found on F2 cell membranes by allogeneic serum against Thy 1.2 in the present experiments is evidently not on MMTV structural protein, and it is unlikely that it is a component of the antigens of the mammary gland epithelium, for absorption of allogeneic serum by a sorbent containing BALB/c mammary gland homogenate did not reduce the activity of the serum.

It must be noted that the antigen which we found is expressed hormone-dependently, and its expression on cell membranes correlates with an increase in their ability to form colonies in semisolid media.

The negative result obtained in the UIF with monoclonal antibodies against Thy 1.2, it can be tentatively suggested, has two causes: 1) only part of the Thy 1.2 molecule is expressed on the cell membrane; moreover, the determinants of this part do not react with monoclonal antibodies; 2) alloantiserum against Thy 1.2 revealed an antigen common to thymocytes and MGC, but different from Thy 1.2, in the cell system described above. This second possibility is currently under investigation.

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PROTECTION OF MICE WITH HEMOBLASTOSIS BY ALLOPURINOL DURING CHEMOTHERAPY WITH 5-FLUOROURACIL

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UDC 616.155.392-085.277.3.015.25:615.254.1:547.828

KEY WORDS: hemoblastosis; allopurinol.

Frequent involvement of normal tissues is one of the most important obstacles to the planned treatment of cancer patients with 5-fluorouracil (FU). For instance, because of the rapid development of toxic complications affecting the gastrointestinal tract, FU is virtually never used in leukemias [4]. It has recently been demonstrated that normal tissues can be protected against the harmful action of FU by means of allopurinol (AP) [5, 6, 8, 9]. Under these circumstances the antitumor effect against certain tumors is maintained [9]. Whereas in an *in vitro* system the cytotoxic action of FU on cells of mouse lymphoblastic leukemias is weakened by AP [10], damage to bone marrow cells still remains [9].

It might be expected that during protection of the animal by AP against the toxic action of FU, damage to malignant cells of nonlymphoid leukemias would be preserved. We give below the results of experiments carried out on a model of hemoblastosis in mice undertaken to test this hypothesis.

EXPERIMENTAL METHOD

Male C57Bl/Gj mice weighting 20-26 g, reared at the "Stolbovaya" Nursery, Academy of Medical Sciences of the USSR, were used (the line will hereafter be abbreviated to B6). A strain of syngeneic hemoblastosis La [2], obtained from the Laboratory of Tumor Strains, All-Union Oncologic Scientific Center, Academy of Medical Sciences of the USSR, was maintained by weekly intraperitoneal passages. In the experiment 10^5 splenic tumor cells were injected intraperitoneally into mice on day 0. AP powder (Hungary) was diluted with physiological saline to a concentration of 1-3 mg/ml and kept in a refrigerator. Before use the suspension was dissolved by heating on a waterbath at 40°C and administered in the necessary volume per os through a gastric tube. A 5% ampul solution of FU (USSR) was diluted immediately before

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(Presented by Academician of the Academy of Medical Sciences of the USSR N. A. Kraevskii.) Translated from Buylleten' Éksperimental'noi Biologii i Meditsiny, Vol. 96, No. 8, pp. 84-86, August, 1983. Original article submitted October 11, 1982.

