Surface Antigens Common to Mouse Cleavage Embryos and Primitive Teratocarcinoma Cells in Culture

(cytotoxic test/peroxidase-coupled antibody against mouse immunoglobulin/cell differentiation)

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ABSTRACT Syngeneic antisera have been produced in mouse strain 129/Sv-CP males against the primitive cells of teratocarcinoma. These sera react specifically with the primitive cells and are negative on various types of differentiated teratoma cells derived from the same original tumor. They are negative on all other mouse cells tested, with the exception of male germ cells and cleavage-stage embryos. Thus, teratoma cells possess cell-surface antigens in common with normal cleavage-stage embryos.

Mouse teratocarcinomas arise from abnormally proliferating primordial germ cells in male embryos (1). Some can be serially transplanted either subcutaneously or intraperitoneally. In the latter case, tumor-bearing mice exhibit ascites-containing "embryoid bodies" which resemble normal morulae and blastulae in morphology and potential for differentiation. When grown in culture, embryoid bodies give rise not only to primitive teratocarcinoma (PTC) cells, but also to various differentiated cell types (DTC). From PTC cells, lines can be established in culture that remain "undifferentiated." Some of these, when injected into mice, give rise to tumors containing various tissues (2, 3). Such a system should provide valuable material for studying differentiation, provided PTC cells in culture can be shown to share some properties with early embryo cells.

We have investigated this point according to the following premises. (1) Early-embryo cells may be expected to possess some specific antigens that are involved in development and disappear later from the organism. These antigens should, therefore, evoke a specific immune response in the syngeneic adult organism. (2) If PTC cells possess such determinants, hyperimmunization of syngeneic adult mice should elicit the formation of antibodies reacting, not only with PTC cells, but also with early-embryo cells.

MATERIAL AND METHODS

Mice. All strains used are standard inbred strains from the Jackson Laboratory stocks, maintained at the Pasteur Institute.

Tumor Lines. The tumor we received from Dr. B. Ephrussi originated from tumor OTT6050 of Stevens (4). It was maintained by serial transfer into 129/Sv-CP (129) mice.

Abbreviations: PTC and DTC, primitive and differentiated teratocarcinoma cells, respectively; PPLO, pleuropneumonia-like organisms; C', complement; IPT, Immunoprecipitin-tested fetal-calf serum.

One derivative resembled the yolk-sac carcinomas described by Pierce (5).

Cell Lines. Several lines were derived from embryoid bodies of tumor OTT6050. They are listed in Table 1, together with other teratoma cells used. We took as PTC prototype F9, isolated by Mrs. Grandchamp in Dr. Ephrussi's laboratory. *In vivo*, some cell lines (such as PCC3 and PCC4) retain the capacity to form derivatives of all three germ layers. Others have partially (such as PTE) or totally (such as F9 or T17) lost that capacity. Nonteratoma cell lines are listed in Table 2.

Cell Cultures. F9 and G26 cells were cultivated in tissue-culture dishes coated with gelatin in Dulbecco's modified Eagle's medium containing 15% fetal-calf serum in an atmosphere of 12% CO₂. The other cell lines used do not require gelatin. All cells were free of pleuropneumonia-like organisms (PPLO) except F9, which contained rat-type PPLO. Subsequently, a PPLO-free derivative of F9, reisolated by passage of the cells in mice, was found to behave exactly as the original line.

Antisera. We prepared antisera against PTC by hyperimmunizing 129 male mice every 2 weeks (Table 3) with F9 cells grown in tissue culture, harvested in EDTA, and suspended in Hanks' balanced salt solution. The cells were irradiated with a cesium source. Mice were bled from the tail 7 and 9 days after each immunization. Sera from individual mice and different bleedings were not pooled.

Cytotoxicity Test. (a) Cells were prepared by washing cultures in petri dishes once with Earle's balanced salt solution without Ca⁺⁺ and Mg⁺⁺ and incubating them at 37° for 7 min with 2 mM EDTA (in Ca⁺⁺- and Mg⁺⁺-free saline) with 2% chicken serum. An equal volume of Earle's solution without Ca⁺⁺ and Mg⁺⁺ was added. The cells were centrifuged at 500 × *g* for 8 min at 4° and suspended in cold test medium [Hanks' medium containing 4% immunoprecipitin-tested fetal-calf serum (IPT)(Gibco)] before final adjustment of cell concentration. Aggregation of isolated teratocarcinoma cells was avoided by use of a very dilute cell concentration. The cell concentration used before testing was 1.4 × 10⁵/ml.

(b) **Complement.** Selected rabbit serum was used as a source of complement (C'). PTC cells (F9) were particularly sensitive to C' toxicity even after absorption with adult mouse

TABLE 1. *Cells of teratoma origin*

Cell line	Original teratoma	<i>In vitro</i> cell type(s)	<i>In vivo</i> cell type(s)	References	Reaction with anti-F9 sera ^a	
					Cyto-toxic test	Absorption
F9	OTT6050	PTC	PTC	Mrs. Grandchamps in B. Ephrussi lab.	++ ^b	pos ^c
T17	LS 402 A III	PTC	PTC	(2) ^d	++	nt ^e
PCC3	OTT6050	PTC in exponential growth	PTC and DTC derivatives of all three germ layers	H. Jakob, Pasteur Institute	+ ^f	nt
PCC3-neural	OTT6050	DTC selected neural-type population	nt	H. Jakob, Pasteur Institute	—	nt
PCC4 and clones derived from it	OTT6050	PTC in exponential growth	PTC and DTC derivatives of all three germ layers	H. Jakob, Pasteur Institute	+	pos
PCD1	OTT6050	DTC homogeneous epithelial, contains myosin, contact inhibited	No growth	T. Boon, Pasteur Institute	—	neg ^g
PCD2	OTT6050	DTC myoblast, fuses to form striated myotubes containing myosin	No growth	T. Boon, Pasteur Institute	—	neg
PTE	OTT6050	No growth	DTC: ascites containing many single cells, endodermal cysts, solid tumor containing only endoderm and trophoblast	Derived from OTT6050 by passaging ascitic single cells	—	neg
—	LS 4799-508	DTC neural-type population (after separation from PTC)	PTC and DTC neural only	(14)	—	nt

^a For all tests (except PTE), cells from cultures were used. ^b 95% lysis. ^c Removes all activity. ^d Given to us ready to test by Dr. J. Jami. ^e Not tested. ^f 40-60% lysis. ^g Removes no activity.

TABLE 2. *Nonteratoma cells exhibiting no reaction with anti-F9 sera*

Cell	Description	Source of cells for test	Reaction with anti-F9		
			Cytotoxic test	Absorption	Origin (ref.)
C1300	A/J Neuroblastoma clone N18	Culture	neg ^a	neg	(15)
G26	C57BL/6 Glioblastoma	Culture	neg	nt ^b	(16)
3T3	Swiss embryo fibroblast	Culture	nt	neg	(17)
Py 3T3	Polyoma-transformed Swiss embryo fibroblast	Culture	neg	neg	(18)
3T6	Swiss embryo fibroblast	Culture	neg	rt	(17)
SV 3T3	SV40-transformed Swiss embryo fibroblast	Culture	neg	nt	(19)
E σ G2	C57BL/6 Gross virus-induced leukemia	Spleen	neg	nt	(20)
EAKR	C57BL/6 Gross virus-induced leukemia	Ascites	neg	neg	(21)
GiL4	C57BL/6 FMR leukemia	Ascites	neg	neg	(22)
—	129/Sv thymocytes	Thymus	nt	neg	—
—	AKR thymocytes	Thymus	neg	nt	—
—	AKR lymphocytes	Lymph node	neg	nt	—
CCL 51	(C57BL $\times$ Af) f ₁ spontaneous mammary tumor containing MTV	Culture	neg	neg	(23)

^a Tested and negative for crossreaction. ^b Not tested. SV, simian virus; MTV, mammary tumor virus.

TABLE 3. Immunization schedule

Immunization number	Dose of cells ($\times 10^{-6}$)	Route	Dose of cell irradiation (R)
1	5	sq ^a	10,000
2	10	ip ^b	5,000
3	20-25	ip	3,000
4	20-25	ip	3,000
5	25	ip	3,000
6-11	1-4	ip	None

^a Subcutaneous. ^b Intraperitoneal.

lymphoid and testicular cells. 12-ml Batches were absorbed in the presence of EDTA (6) with adult lymphoid cells (10 volumes of C':1 volume of cells) and subsequently with teratoma embryoid bodies (4 volumes of C':1 volume of cells); the absorbed rabbit serum was then relatively non-toxic to F9 at volume ratios of 1:4 or 1:12 (C' of control averaged 10-20% dead), depending on the batch.

(c) *Antisera*. Nonimmune, noninactivated normal sera exhibited relatively high C'-dependent cytotoxic activity against F9. There was no abrupt decrease in activity with increasing dilution of serum and all sera had activity up to a dilution of at least 1:160. This level was independent of sex, age, parity, H-2 type, and pathogen-free status. In certain strains, such as 129, heat inactivation (56° for 30 min) lowered this activity to acceptable levels (less than 20% F9 cells lysed at all serum dilutions). All sera used were therefore heat-inactivated before dilution with the test medium. Test conditions were the following: equal volumes (0.05 ml) of diluted antiserum, cell suspension, and diluted C' were incubated for 45 min at 37° with occasional vigorous shaking. All dilutions given refer to dilutions before use in the cytotoxic test. The percentage of dead cells in each tube was determined after addition of 0.02 ml of a 0.5% stock solution of trypan blue in 0.85% NaCl.

Testicular Cells were prepared by removing the tunica albuginea, teasing apart, rinsing the seminiferous tubules free of interstitial cells, and mincing the tubules. The tubules were vigorously pipetted; the cells released were washed three times in Hanks' solution and suspended in test medium.

FIG. 1. 129 male antiserum against F9 cells titrated on F9 cells. Antiserum against F9 after five immunizations (○); preimmunization serum of the same mouse (▲). Results of three independent experiments.

FIG. 2. Cytotoxic activity of antiserum against F9 on F9 before absorption (○), after absorption with PCD2 myoblasts (▲), PTE endoderm (□), 129 testicular cells (■), F9 (●), and PCC4 (△).

Absorptions were performed at 4° for 30 min with 1 volume of C':2 volumes of cells to diluted serum. The dilution of antisera selected for absorption was about two doubling dilutions below the titer.

Preimplantation Embryos. (a) *For peroxidase test.* Virgin female mice, 6-7 weeks old, were injected with 4 IU of pregnant mare serum (Endocrine-Paris) and 44 hr later with 5 IU of human chorionic gonadotropin (Ayerst Laboratory, New York). They were then mated with males of the same strain. Preimplantation embryos were flushed from the fallopian tubes at various intervals after detection of a copulation plug. They were washed and the zona pellucida was removed with Pronase (7). Embryos were incubated for 2 hr at 37° under 5% CO₂. They were washed three times in Eagle's medium and treated with glutaraldehyde [5% solution in 0.2 M cacodylate buffer (pH 7.4)]. After fixation, the embryos were washed twice in phosphate-buffered saline (pH 7.2) and once in Eagle's medium.

(b) *For cytotoxicity test.* Fertilized eggs and cleavage-stage embryos were washed into Whitten's medium (8) out of the oviducts of mice that had ovulated normally, treated with Pronase, washed three times, and incubated for 3-6 hr in Whitten's medium at 37° in 5% CO₂ in air before use in cytotoxicity tests. Antiserum and C' were diluted with Whitten's medium just before use, and mixed (0.1 ml of each) in depression slides at the beginning of the test; embryos were added and incubated for 45 min in a humidified incubator at 37° in 5% CO₂ in air. Lysis was assessed by inspection with a dissecting microscope at a magnification of $\times 60$.

Indirect Test with Antibodies Against Mouse Immunoglobulins Prepared in Sheep, Coupled with Peroxidase (Peroxidase Test). Sera were diluted in Eagle's medium containing 4% IPT. F9 cells or preimplantation embryos, previously fixed with glutaraldehyde, were incubated in 0.01 ml of diluted anti-F9 or in preimmunization serum for 20 hr at 4°. They were washed three times in Eagle's medium and transferred into 0.01 ml of Eagle's medium containing 4% IPT and 125 μ g/ml of purified antibodies against mouse immunoglobulins prepared in sheep coupled with peroxidase (9). After 3 hr at 4°, the cells were washed three times in Eagle's medium and once in distilled water. They were transferred into 0.005 ml of 0.05 M acetate buffer (pH 5.0). Then 0.005

ml of a reaction mixture [2 mg of 3-amino-9-ethyl carbazole in 10 ml of acetate buffer (pH 5.0) (10) and a drop of a 3% solution of hydrogen peroxide] was added. The reaction was stopped after 30 min by replacing the medium with distilled water.

RESULTS

Activity of Syngeneic 129 Male Antiserum Against PTC (F9) cells. After five immunizations, immune sera were extremely active in the cytotoxicity test on F9, with a prozone below 1:20, 95% killed, and titers in the range of 1:3000 (Fig. 1). These results were confirmed by the indirect peroxidase test. At serum dilutions between 1:100 and 1:3200, F9 cells were stained when treated with the immune serum but not with preimmunization serum. At serum dilutions lower than 1:100, nonspecific staining can be observed with the control serum.

Activity of Antiserum Against F9 Cells Against Other Teratoma Cells. By cytotoxicity test, antisera against F9 reacted with PTC but not with DTC cell lines, even though in some cases both were isolated from the same tumor. In addition to F9, three other independently isolated PTC lines were tested. T17 (2), which currently does not differentiate *in vivo* (J. Jami, personal communication), was susceptible to cytotoxic lysis to the same degree as F9. PCC3 and PCC4, two PTC lines isolated from OTT6050 in this laboratory by Dr. H. Jakob, are homogeneous in culture when maintained in exponential growth, the conditions under which they are tested. Unlike F9 and T17, these two lines give rise to derivatives of all three germ layers when injected into mice. Even at low dilutions, antiserum against F9 did not kill more than 40 and 60% of PCC3 and PCC4 cells, respectively. PCC4 cells, however, removed *all* activity against F9 by absorption (Fig. 2).

The activity of antiserum against F9 was also assayed by cytotoxicity tests against a series of differentiated cells: PCD1, PCD2, PTE, and neural cells from PCC3, all derived from the same original tumor (OTT6050). In addition, neural cells cultured from LS 4799.508 were also negative (see Table 1). In no case was any reaction detected, either by cytotoxicity or by comparative absorption tests (Fig. 2).

Activity of Antisera Against F9 Cells Against Nonteratoma Cells. Antiserum against F9 was assayed by cytotoxicity tests against several cells of various origins, some of which carry defined viral cell-surface antigens (Table 2). No reaction was detected except for male germ cells and early-embryo cells.

Male germ cells. In a direct cytotoxicity test (11) with absorbed rabbit C' (1:10) on normal BT BRTE/Nev sperm, antiserum against F9 lysed 35% more cells than controls with activity up to a serum dilution of 1:512, while control sera were negative. Furthermore, 129 testicular cells absorb out some 50% of activity on F9 (Fig. 2) and spermatozoa remove all activity. Since all antisera against F9 were prepared in male mice, H-Y antigen could not be involved.

Early-embryo cells. The peroxidase test showed a clear-cut reaction of antisera against F9 with cleavage-stage embryos of strain 129. Preimmunization control sera gave nonspecific weak staining below a dilution of 1:100. With antisera against F9, fertilized one-cell eggs do not stain significantly more than the control of a dilution of 1:25. Two-cell embryos stain

FIG. 3. Peroxidase test on 129 morulae (magnification: $\times 800$). (a) Preimmunization serum 1:800; (b) antiserum against F9, 1:800.

specifically up to a dilution of 1:200; 4- to 8-cell morulae stain up to a dilution of 1:1600 (Fig. 3). Similar results, but sometimes with a higher nonspecific background, were found with embryos of strains A, SJL/J, C57BL6, and random-bred NCS.

Preliminary results with cytotoxicity tests on cleavage-stage embryos of the ICR strain gave similar results: 8-cell morulae were more sensitive than 2-cell eggs; fertilized eggs were relatively insensitive. The test system, however, appears to be exquisitely sensitive to minor fluctuations in C' levels, with respect to both activity and toxicity. Furthermore, cleavage embryos of the same developmental age do not seem to be a homogeneous population with respect to susceptibility to lysis. So far these factors have prevented us from obtaining, in the cytotoxicity test, quantitative data of a sufficiently reproducible nature to permit direct comparison of embryos with F9 cells.

DISCUSSION

It is likely that specific cell-surface determinants are involved in developmental processes, even at early stages (12). Direct immunological approach with early-embryo cells for immunization is hindered by the scarcity of material, hence the interest of the mouse teratocarcinoma system from which various primitive and differentiated cell lines can be isolated and provided in quantity.

There has already been one report concerning immunological crossreaction between teratoma and embryo cells (13). These results, however, are difficult to interpret because (1) embryoid bodies—i.e., a mixture of cell types—were used as the immunogen and (2) sera prepared in rabbits can contain, even after absorption, antibodies against mice that are not specific for teratomas and eggs.

The studies described here show that PTC (F9) cells from culture elicit, in syngeneic male mice, formation of specific antibodies that react with the surface of all other PTC cell lines tested but not of differentiated cells that originate from the same tumor. This absence of crossreaction with cells of comparable origin underlines the specificity of the reaction. All these PTC cell lines have, therefore, in common some specific surface antigen(s). This hypothesis has been confirmed by the production in syngeneic mice of a serum against another PTC line (PCC4), a serum that lyses 95% of F9 cells but only 50% of PCC3 and PCC4. It is not possible to say whether this is an effect on antigen masking nor whether this effect is related to the capacity of these latter lines to differentiate.

The number of antigens observed here is unknown. PTC cells are highly tumorigenic, and some tumor antigens might be present on their cell surfaces. No crossreaction, however, has been observed with various viral tumor antigens. Antibodies against PTC do not react with any differentiated cell type derived from the same teratoma or from other teratomas or from adult mice, except male germ cells from different mouse strains. The latter reaction suggests the presence of at least two types of antibodies in antisera against F9 because (1) after absorption with testis cells, sera retain some anti-F9 activity and (2) the slope of the titration curve is much less steep than usually observed when one antigen is involved.

The most significant result reported here is the reaction of antisera against PTC with mouse morula cells. The reaction appears to increase from very little, if any, with 1-cell eggs to a maximum at the 8-cell stage. This result suggests the progressive expression of a new surface antigen(s) during the earliest stages of development. For such a component(s) to become immunogenic in a syngeneic organism, it has to disappear early from the embryo. This is precisely what can be expected of a surface antigen playing a role at an early stage of development.

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