CELL SURFACE ANTIGENS OF A MOUSE TESTICULAR TERATOMA

IDENTIFICATION OF AN ANTIGEN PHYSICALLY ASSOCIATED WITH H-2 ANTIGENS ON TUMOR CELLS*

LINDA R. GOODING† AND MICHAEL EDIDIN

(From the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218)

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Testicular teratomas are thought to arise from the proliferation of primary germ cells (1). Though almost unknown in most mice, these tumors occur in 1% of newborn strain 129 males, apparently due to multiple genetic factors in this inbred strain (1). Strain 129 spontaneous teratomas usually contain a bizarre collection of differentiated tissues, and differentiated cells persist indefinitely in most transplantable teratomas (2). These differentiated cells appear to derive from undifferentiated pluripotent stem cells or “embryonal carcinoma” (3). Teratoma lines adapted to culture appear to remain undifferentiated. However, even after extended periods in culture, clones of teratoma were found to differentiate when reimplanted in mice (4-7). The range of teratoma differentiation in vivo is similar to that of normal embryos grafted to adults (8, 9). In particular both may produce “embryoid bodies,” structures which morphologically and histologically resemble early gestation embryos (10, 11). Teratoma cells then would seem an excellent starting point for an investigation of antigens common to tumors and normal embryos.

One of us reported previously that a rabbit antiserum to strain 129/J mouse teratoma 402AX reacts specifically with SV-40 transformed 3T3 cells and early mouse embryos (12). Only cells of the embryonic portion of the conceptus, those cells which would later express H-2 antigens, reacted with the antiserum, suggesting that perhaps the teratoma antigens are precursors to H-2 in normal development. Antigens common to early embryos and virus-transformed cells have been reported in hamsters (13, 14) and mouse antimouse embryo sera also define antigens common to a range of murine tumor cells (15, 16).

This report concerns our efforts to determine the antitumor reactivities of

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† Present address: Division of Immunology, Duke University Medical Center, Durham, N. C. 27710.

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rabbit anti-teratoma serum, and the relationship of these antigens to H-2 on
tumor cells. A second report will deal with teratoma antigens on developing
mouse embryos.\footnote{Gooding, L. R., Y.-C. Hsu, and M. Edidin. Teratoma cell-surface antigens expressed by
early mouse embryos. Manuscript in preparation.}

\textit{Materials and Methods}

\textit{Tissue Culture}—All tissue culture media were obtained from Grand Island Biological Co.,
Grand Island, New York. (Gibco). Media were routinely supplemented with Gibco 1%
antibiotic/antimycotic mixture.

\textit{Cells.—} Teratoma 402AX: Teratoma 402AX, originally an embryoid body-rich ascite tumor, was
obtained from Dr. Leroy Stevens (2) 8 yr ago and is passaged in our laboratory in male 129/J
mice. The tumor grows rapidly and must be transplanted every 12–14 days. Currently the
ascites consists almost exclusively of single cells although floating masses are occasionally
observed. True embryoid bodies are not seen. When cells are injected subcutaneously the
resultant tumor consists primarily of small undifferentiated stem cells, epithelial tissue re-
ssembling chorionic epithelium, and rhabdomyoblasts, precursors of striated muscle. Cartilage,
originally present in solid teratoma 402 AX (2), is not observed. When not in use for prolonged
periods, the teratoma cells were stored in ascites fluid containing 10\% dimethylsulfoxide
under liquid nitrogen. Thawed cells are more than 90\% viable (by dye exclusion), and the
growth of tumors from frozen cells occurs without a detectable lag.

A subline of teratoma 402AX was adapted to culture by Dr. Ed McGuire 3 yr ago and is
maintained in modified Eagle's medium supplemented with 5\% calf and 5\% fetal calf serum.
Like the ascites tumor, the cultured line consists almost entirely of single cells some of which
are epithelial in appearance. Aggregates of live cells are observed to lift off the plate in heavy
cultures, but these are not embryoid bodies. The cells were cloned twice without a feeder
layer before the onset of these studies. The cultured cells used in this work had been adapted
to growth in 20 \mu g/ml 8-azaguanine for another purpose. Two other lines of cultured teratomas,
adapted to culture independently within the past year and not grown in 8-azaguanine, react
with the antisera in a manner indistinguishable from the original subculture. “Teratoma A”
refers to the ascites tumor and “teratoma C” to the cultured subline.

\textit{Cultured cell lines:} BALB/c 3T3, SV-40 transformed BALB/c 3T3 (SV-3T3), BALB/c
3T12, and polyoma transformed BALB/c 3T3 cells were obtained from Dr. Stephen Roth
and are maintained in Dulbecco's medium with 10\% fetal calf serum. Flat revertants of SV-
C 3T3 (10A-7-4) were isolated by Dr. H. S. Smith (17) and given to us by Dr. Roth. These
cells are characterized by slow growth in factor-free medium and cease to divide at confluence,
but express T antigen and yield infectious SV-40 virus on fusion with permissive cells. The
flat revertants (FR-SV-3T3) are maintained in Dulbecco's medium with 10\% fetal calf serum.
LM-Tk\textsuperscript{+}-clone 1D (CI 1D), a thymidine kinase negative subline of the C3H “L” cell, was
isolated by Dubbs and Kitt (18), and given to us by Dr. H. G. Coon. CI 1D is maintained in
Eagle’s minimal essential medium with 5\% fetal calf serum.

\textit{Embryonic fibroblasts:} Primary fibroblasts were obtained by trypsinizing eviscerated 17-

\footnote{Abbreviations used in this paper: CI 1D, Clone 1D; FL-GaM, fluorescein-conjugated goat
antimouse IgG; FR-SV-3T3, flat revertants of SV-40 transformed BALB/c 3T3 cells; HH5, 0.01 M Hepes-buffered Hanks’ solution (pH 7.3) containing 5\% fetal calf serum; NRS, normal rabbit serum; Py-3T3, polyoma virus transformed BALB/c 3T3 cells; SV-3T3, SV-40 transformed BALB/c 3T3 cells; TMR-GaR, tetramethylrhodamine-conjugated goat anti-
rabbit IgG (F\text{c}).}
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20 day mouse embryos (19) and maintained in culture with Eagle's minimal essential medium supplemented with 20% fetal calf serum.

**Transplantable tumors:** Adenocarcinoma BW 10232 (C57BL/6J), melanoma B16 (C57BL/6J), and lymphatic leukemia BW 5147 (AKR/J) were obtained directly from Jackson Laboratories, Bar Harbor, Maine, and were not passaged by us. Hepatoma BW 7756 (C57L/J), also from Jackson Laboratories, was passaged in the laboratory of Dr. G. M. Williams and generously supplied to us by him. Mastocytoma P815, a DBA/2 ascites tumor, was obtained from Dr. Christopher Henney. Two methylcholanthrene-induced C57BI/6J fibrosarcomas were obtained from Dr. Leon Parks; they were at passage 6 and 7, respectively, at the time of assay.

**Mice.**—129/J, B10.Br/SgSn, B10.A/SgSn, C57BL/10SgSn, and C3H/HeJ mice were purchased from Jackson Laboratories. Pregnant CF1 females were given to us by Dr. Y.-C. Hsu.

**Antiserum.**—Anti-teratoma serum was produced by injection of $2 \times 10^6$ teratoma C cells suspended in $1.8 \times 10^8$ BCG (Research Foundation, Chicago, Ill.) into multiple intradermal sites on the flanks of New Zealand white rabbits. Rabbits were boosted with $2.5 \times 10^7$ live teratoma C intravenously and bled 7-9 days later.

Anti-teratoma serum was absorbed with 129/J male mesenteric lymph node, spleen, thymus, and kidney cells obtained by gentle teasing and pressing tissues through a 200-mesh wire screen. 0.7 ml of twice washed, packed cells were mixed with 2 ml of antiserum. After 3-5 h on ice, the cells were removed by centrifugation. After three such absorptions the antiserum did not react by immunofluorescence with fresh samples of the cells used for absorption.

Normal rabbit serum was obtained prior to immunization with teratoma. Antimouse cell reactivity was removed by absorption with 129/J cells as above.

Rabbit anti-teratoma serum was tested for reactivity against a variety of murine viruses by Microbiological Associates, Bethesda, Md. The serum did not react with reovirus type-3, sendai, ocmotemia, mouse adenovirus, mouse hepatitis virus, Theiler's mouse encephalomyelitis (GD VII), minute virus of mice, newborn mouse pneumonitis (K virus), pneumonia virus of mice, or lymphocytic choriomeningitis virus. It did, however, significantly inhibit poliovirus induced agglutination of guinea pig red blood cells, and our own tests confirm this finding (Table I).

We have further observed that extensive absorption of the anti-teratoma C serum with

| TABLE I |
|---|
| **Reaction of Anti-Teratoma Serum with Polyoma Virus and a Polyoma-Associated Antigen** |

| Anti-teratoma serum absorbed with: | HAI titer | % staining cells* |
|---|---|---|
| Teratoma A | 1/640 | 99  100  90 |
| Teratoma A + Py virus | <1/40 | —  86  85 |
| Teratoma A + gp RBC's | 1/640 | —  —  — |
| Teratoma C | 1/640 | 9   5   4 |
| NRS | <1/10 | 1   2   1 |

Hemagglutination inhibition (HAI) was performed by incubation of 8 HAU of purified polyoma virus (the kind gift of Dr. Carlo Croce) with serial dilutions of antiserum in a final volume of 0.1 ml. After 18 h at 4°C, 0.05 ml of guinea pig red blood cells at $10^8$ ml were added and samples incubated at 37°C for 15 min and then at 4°C for 2 h.

* Cell surface immunofluorescence was performed as described in Materials and Methods at a serum concentration of 1:10. NRS was obtained before immunization with teratoma.
teratoma A cells fails to remove all antibody reacting by immunofluorescence with live teratoma C (Table I), indicating the presence of an antigen expressed by the cultured subline but not in detectable amounts by the parent ascites tumor. Polyoma 3T3 cells also react with teratoma A absorbed serum (Table I), suggesting that perhaps the serum is reacting with a polyoma-specific cell surface antigen. The two activities, antipolyoma virus and anticell surface, are distinct from one another since appropriate absorption of the antiserum removes one without affecting the other (Table I).

It is uncertain why rabbits injected with live teratoma C produce antibody to polyoma virus. It is probable from the results in Table I that teratoma C is transformed by the virus, but the cells are not producing virus in detectable amounts. Attempts to rescue polyoma virus from teratoma C by Sendai virus fusion with both mouse and rabbit embryonic fibroblasts have been unsuccessful. Perhaps most surprising is the finding that the cultured cells express this polyoma-associated cell surface antigen, while the ascites tumor cells from which they were derived do not. Two separate lines of teratoma 402AX have subsequently been adapted to culture and both express the polyoma antigen.

None of the tumor and cultured cells reported in this study react with teratoma A absorbed antiserum by immunofluorescence. Tumor cells, eliminated from consideration because they did react with teratoma A absorbed serum were solid Sarcoma I, ascites Sarcoma I, and melanoma HP, all from Jackson Laboratories.

Anti-H-2\textsuperscript{a} allantiserum was prepared by intraperitoneal injection of C57BL/6 × DBA/2 F\textsubscript{1} mice with B10.Br lymphocytes. Serum was made H-2-specific by three serial absorptions with B10.D2 lymph node and spleen cells performed as described above.

Monospecific anti-H-2.11 and H-2.32 were obtained from the Transplantation and Immunology Branch, NIAID. Rabbit anti-T-cell antigen was kindly provided by Drs. Gerry Cole and Christopher Henney. This antiserum, prepared by injection of mouse brain and absorbed on mouse red cells, reacts specifically with thymus-derived lymphocytes (20).

**Fluorescent Reagents.**—Fluorescein-conjugated goat antimouse IgG (FL-GaM) and tetramethylrhodamine-conjugated goat antirabbit IgG (TMR-GaR) were prepared as described previously (21).

**Indirect Immunofluorescent Staining of Cells.**—Unless otherwise indicated, cultured cells were harvested with 2.5% heat-inactivated chicken serum, 0.2% trypsin, 0.002% purified collagenase (Worthington Biochemical Corp., Freehold, N. J.) in Moscona's solution (22). 5 × 10\textsuperscript{5} cells, washed twice in 0.01 M Hepes buffered Hank's solution (pH 7.3) containing 5% fetal calf serum (HH5), were incubated with 0.05 ml of antiserum on ice for 15 min. Antiserum was diluted in HH5 containing 2.5 mM dinitrophenol (DNP-HH5) to inhibit pinocytosis. The cells were washed twice with DNP-HH5 to remove excess serum and stained with 0.05 ml of the fluorescent conjugate for 15 min on ice. Excess conjugate and DNP were removed by three washes with HH5. All samples were encoded before observation and read as unknowns.

Fluorescence microscopy was performed as described previously (21). Counts were done on 100–200 cells to determine the percentage of stained cells. Pictures were taken using a Leica camera and GAF-500 color film. Film was processed with a GAF developing kit to an ASA of 1,000.

**Capping.**—Cl 1D cells were stained as described above except that DNP was omitted. After final washes, the cells were incubated at 37°C for 90 min for maximal capping of the fluorescent label. At the optimal serum concentration 60–90% of cells were capped by anti-H-2\textsuperscript{a} and approximately the same percentage with anti-teratoma.

**RESULTS**

**Reactivity of the Anti-Teratoma Serum with Normal Mouse Tissues**—The rabbit anti-teratoma serum displayed high titres of reactivity with the immu-
nizing cells (Fig. 1). Surprisingly little of this activity is directed against antigens common to normal mouse cells, since as shown in Fig. 1 the antiserum reacts poorly with 129/J lymph node cells. A single absorption with lymph node cells removes this activity while having no effect on the staining of teratoma itself. To ensure removal of this activity, antiserum absorbed on cells from a collection of normal 129/J tissues (see Materials and Methods) was used for all experiments described here.

The reactivity of the absorbed anti-teratoma serum with cells from a variety of adult mouse tissues is shown in Table II. With the exception of ovary, none of the normal cells, either teased apart or dissociated with trypsin, reacted with the antiserum. If any antigens which react with the anti-teratoma serum are present on these normal cells, they are expressed at levels too low to be detected by our assay system.

The fluorescent stain on dissociated ovary cells could not be removed by further absorption of the anti-teratoma serum with normal cells other than ovary itself. Staining of frozen sections of ovary with the anti-teratoma serum revealed bright cytoplasmic fluorescence on cells of the theca interna and luteinized stromal cells.

Teratoma Antigens Expressed by Cultured Mouse Fibroblast Cells.—As shown in Fig. 2 a, the anti-teratoma serum reacts strongly with living SV-40 transformed 3T3 cells and with 3T12 cells. It reacted weakly with 3T3 cells and SV-40 transformants which have reverted to normal growth regulation. However, if these “normal” cells are treated briefly with crude trypsin before
TABLE II

Staining of Cells from Normal 129/J Tissues by Anti-Teratoma Serum

| Cells             | NRS* | Anti-teratoma* |
|-------------------|------|----------------|
|                   | Teased | Trypsin       |
| Lymph node        | 4     | 0             | 0 |
| Spleen            | 7     | 1             | 1 |
| Thymus            | 3     | 0             | 0 |
| Kidney            | 4     | 6             | 10 |
| Intestine         | 14    | 12            | —  |
| Brain             | 3     | 6             | 0  |
| Liver             | 10    | 6             | 0  |
| Peritoneal exudate cells | 2 | 1 | 6 |
| Testis            | 4     | 7             | 1  |
| Ovary             | 4     | 73            | 61 |

129/J organs were removed and dissociated by gentle teasing. A portion of each was dissociated with 0.25% Difco trypsin in phosphate buffered saline at room temperature. Peritoneal exudate cells were washed from the peritoneal cavity with HH1 and a portion treated with trypsin. NRS controls were done on trypsinized cells.

* Sera at 1:10 dilution; results presented as percentage stained cells.

staining (Fig. 2 b), their reaction with antiteratoma serum is greatly increased. Trypsin treatment has a slight effect on staining of the transformed cells as well, but this may be due simply to rounding of the cells, thus increasing the apparent brightness of the fluorescent stain. Intact primary and early passage mouse embryonic fibroblasts do not react significantly with the anti-teratoma serum, even after treatment with trypsin (Table III).

Co-capping of the Teratoma Antigens and H-2 on CI 1D.—A subline of the C3H L-cell, CI 1D, also expresses teratoma antigens, most of which are available for fluorescent staining only after the cells are treated with trypsin (Fig. 3). Trypsinized CI 1D cells were stained for the teratoma antigens and then incubated at 37°C. Under these conditions, between 60 and 90% of the cells “cap,” that is, the fluorescent antigen-antibody complexes migrate to one pole of the cell (Fig. 4 a). These cells were then incubated with a mouse alloantiserum which reacts with several H-2$^k$ specificities and FL-GaM in ice so that no further capping could occur. The co-incidence of rhodamine and fluorescein stain on the capped cells (Fig. 4 a and b) indicates that both the H-2 antigens and the teratoma antigens had been capped by the antiteratoma serum. The same result is observed when the H-2 antigens are capped and the cells stained in the cold with anti-teratoma (not shown).

H-2D and H-2K molecules cap independently of one another in lymphocyte membranes (23), and the same is true of H-2 antigens on CI 1D (M. Edidin and A. Weiss, unpublished). Therefore, monospecific anti-H-2 sera were used to determine which of the alloantigens was responsible for co-capping with teratoma antigens. When H-2D molecules are capped and the cells then stained
with anti-teratoma, again capping of the teratoma antigens is observed (Fig. 4 c and d). Capping of the H-2K molecules (with anti-H-2.32) also caps the teratoma antigens (not shown). In both cases capping of the teratoma antigen was incomplete.

Fig. 2. Reaction of anti-teratoma serum with transformed and normal fibroblast cell lines. (a) Cells, plated onto cover slips 48 h before assay, were stained in situ with serial dilutions of anti-teratoma serum and a constant amount of TMR-GaR. All samples were subconfluent. (b) Plated cells were treated with 0.25% Difco trypsin in phosphate-buffered saline 1 min on ice before staining. Treated cells remained attached to cover slips.

| Cells    | Passage | % stained cells |
|----------|---------|-----------------|
|          |         | NRS  | Anti-teratoma | NRS  | Anti-teratoma |
| CF1      | 0       | 8    | 5             |
| B10. Br  | 3       | 5    | 4             |
| C57Bl/10 | 4       | 1    | 12            |
| B10.A    | 5       | 2    | 17            |
| 3T3      |         | 4    | 87            |

NRS and anti-teratoma sera at 1:40 dilution.
TERATOMA ANTIGENS

Fig. 3. Reaction of anti-teratoma serum with Cl 1D cells. Cells were stained on cover slips as described for Fig. 2.

To be certain that the observed co-capping of H-2 and teratoma antigens was in no way inherent to the procedure employed, the same sera and fluorescent reagents were used to look at capping with another fibroblast cell marker. Fibroblasts express \( \theta \)-antigen (24), and a heterologous rabbit anti-T-cell serum reacts with Cl 1D cells. In Fig. 4 e and f, Cl 1D cells were capped with the polyspecific anti-H-2\( ^k \) and then stained with the rabbit anti-T cell. No co-capping was observed. The reciprocal experiment, capping of the T-cell antigens and then staining with anti-H-2\( ^k \) also failed to produce co-capping (not shown).

Controls, shown in Table IV, were performed to ensure that the co-capping of H-2 and the teratoma antigens on Cl 1D was not due to unexpected interactions among the reagents employed. The fluorescent reagents react only with the specified antiserum and not with the cells themselves or with each other. The anti-teratoma does not react with H-3\( ^k \) lymphocytes, nor does it bind anti-H-2\( ^k \) antibody. The anti-H-2\( ^k \) does not bind to teratoma cells and does not react with the anti-teratoma antibody.

Teratoma Antigens Expressed by Transplantable Murine Tumor Cells.—The anti-teratoma serum reacts with living cells from a variety of transplantable mouse tumors (Table V). Staining of these cells is little affected by trypsin. One tumor tested, a lymphatic leukemia, reacted poorly with the antiserum, and because of the limitations of the assay system, expression of antigens on these cells is questionable. Two tumors, both early passage methylcholanthrene-induced fibrosarcomas, clearly did not react with the antiserum even after treatment of the cells with trypsin.

It was of interest to determine whether the various cells which react with the antiserum are expressing the same antigens, particularly the antigens which co-cap with H-2 on Cl 1D cells. This question was approached by the absorption studies shown in Table VI. Absorption of the antiserum with any reactive cell tested completely removes antibody binding to Cl 1D, melanoma and SV-3T3. This indicates that of the cells tested, all those reacting with the antiserum...
Fig. 4. Capping of surface antigens on CI 1D. CI 1D cells were capped and restained on ice as described in Materials and Methods. (a) and (b) TMR and FL fluorescence, respectively, of cells capped with rabbit anti-teratoma (1:40) and TMR-GaR then restained on ice with mouse anti-H-2¹ (1:4) and FL-GaM. (c) and (d) FL and TMR fluorescence, respectively, of cells capped with mouse anti-H-2.11 (undiluted) and FL-GaM then restained on ice with rabbit antimouse T-cell antigen (1:40) and TMR-GaR.
express a common antigen or antigens, and furthermore these antigens are cross-reactive with those expressed on Cl 1D cells. Also, since absorption of the serum with Cl 1D removes all staining on melanoma and SV-3T3, these cells are expressing only teratoma-defined antigens found on Cl 1D.

**TABLE IV**

*Controls for Reagent Specificity*

| Cells           | Sera                     | % stained cells |
|-----------------|--------------------------|----------------|
|                 | First | Second | Third | Fourth |
| CI 1D           | Anti-$H_{2b}$ (abs.)*    | FL-GaM         | -     | -     | 2   |
|                 | Anti-teratoma (abs)†     | TMR-GaR        | -     | -     | 1   |
|                 | Anti-$H_{2b}$            | TMR-GaR        | -     | -     | 0   |
|                 | Anti-teratoma            | FL-GaM         | -     | TMR-GaR | 91 (FL) |
|                 | Anti-$H_{2b}$            | FL-GaM         | TMR-GaR | -     | 0 (TMR) |
|                 | Antiteratoma             | TMR-GaR        | FL-GaM | -     | 92 (TMR) |
|                 | Anti-teratoma (abs)†     | TMR-GaR        | -     | -     | 0   |
|                 | Anti-$H_{2b}$ (abs)*     | FL-GaM         | -     | -     | 7   |
|                 | Anti-teratoma            | TMR-GaR        | -     | -     | 0   |
|                 | Anti-$H_{2b}$            | FL-GaM         | Anti-teratoma | TMR-GaR | 82 (FL) |
|                 | Anti-teratoma (abs)†     | TMR-GaR        | -     | -     | 0   |
|                 | Anti-$H_{2b}$ (abs)*     | FL-GaM         | -     | -     | 7   |
|                 | Anti-teratoma            | TMR-GaR        | Anti-$H_{2b}$ | FL-GaM | 95 (TMR) |
|                 | Anti-teratoma (abs)†     | TMR-GaR        | -     | -     | 0   |
|                 | Anti-$H_{2b}$ (abs)*     | FL-GaM         | -     | -     | 7   |
|                 | Anti-teratoma            | TMR-GaR        | Anti-$H_{2b}$ | FL-GaM | 95 (TMR) |
|                 | Anti-teratoma (abs)†     | TMR-GaR        | -     | -     | 0   |
|                 | Anti-$H_{2b}$ (abs)*     | FL-GaM         | -     | -     | 7   |
|                 | Anti-teratoma            | TMR-GaR        | Anti-$H_{2b}$ | FL-GaM | 95 (TMR) |

C3H lymphocytes were prepared from mesenteric lymph node by pressing through a fine mesh wire screen. All reagents were at the same concentrations used in Fig. 4. Controls: (*), anti-$H_{2b}$ serum absorbed on B10-Br ($H_{2b}$) lymph node and spleen cells; (†), anti-teratoma serum absorbed on teratoma C.

**TABLE V**

*Reaction of Anti-Teratoma Serum with Cells of Transplantable Mouse Tumors*

| Cells                | % stain on trypsinized cells at 1:16 serum dil. | Fluorescence titer |
|----------------------|-----------------------------------------------|--------------------|
|                      | NRS   | Anti-teratoma | Tensed | Trypsin |
| Melanoma B16         | 14    | 90            | 1:40   | 1:80    |
| Adenocarcinoma BW10232 | 3    | 64            | 1:20   | 1:40    |
| Hepatoma BW7756      | 4     | 91            | 1:80   | 1:160   |
| Mastocytoma P815      | 0     | 72            | 1:40   | 1:40    |
| Lymphatic leukemia BW5147 | 2  | 22            | —      | —       |
| Fibrosarcoma MC1      | 3     | 3             | —      | —       |
| Fibrosarcoma MC3      | 0     | 2             | —      | —       |

A portion of each solid tumor mass was dissociated by gentle teasing in HHS. Another portion was dissociated with 0.25% Difco trypsin in phosphate buffered saline at room temperature. Mastocytoma cells were washed twice in HHS and a portion treated with trypsin as above. "Titer" is defined as the lowest concentration of antiteratoma where more than 50% of the cells appear stained.
TABLE VI

Absorption of Anti-Teratoma Serum with Tumor Cells

| Cells, stained with: | NRS | Anti-teratoma absorbed with: |
|---------------------|-----|------------------------------|
|                     |     | Teratoma A | C1 1D | Adeno-carcinoma | Melanoma | Hepatoma |
| CI 1D†              | 4*  | 82*       | 4     | 8               | 8        | 2        |
| Melanoma‡           | 4   | 94        | 3     | 4               | 6        | 1        |
| SV-40-3T3‡          | 2   | 91        | 3     | 3               | 1        | 3        |
| Hepatoma∥           | 0   | 82        | 6     | 90              | 81       | 77       |
| Teratoma A¶         | 0   | 83        | 1     | 84              | 82       | 64       |

1 vol of anti-teratoma serum (absorbed on normal tissues as described in Materials and Methods) at 1:5 was absorbed twice with 1 vol of trypsinized packed tumor cells for 3-5 h on ice. Test cells were incubated with the lowest serum concentration giving about 100% stained cells with unabsorbed antiteratoma serum. All test cells were treated with trypsin prior to staining.

* Results are expressed as percent stained cells. Serum dilutions: (†) 1:20; (‡) 1:40; (¶) 1:80.

Aside from this common antigenic activity, the absorption studies revealed other reactivities in the anti-teratoma serum. Absorption with CI 1D, adeno-carcinoma or melanoma, which removes the common antigens, has little effect on the staining of hepatoma and teratoma itself. Absorption with hepatoma however removes most antibody reacting with the teratoma, indicating that a second activity is shared by teratoma and hepatoma but not by the other tumor cells tested.

Because absorption of the antiserum which removes the common antigens does not affect staining of the teratoma, most of the very high titer against teratoma cells must be due to the antigens shared by teratoma and hepatoma. It is probable that the serum reacts with a third antigen, one specific for teratoma only, since in repeated experiments absorption with hepatoma did not remove all antibody which reacts with teratoma.

The antigen or antigens responsible for the very high titer of the anti-teratoma serum with teratoma itself appear to be secreted or shed by teratoma cells into their ascites fluid. Absorption of the antiserum with teratoma ascites fluid removes all reactivity when teratoma cells are stained at a serum dilution of 1:80, but extensive absorption with ascites fluid has no effect on staining at a dilution of 1:20 (Table VII). This result would indicate that absorption with ascites fluid removes activity with one antigen expressed by teratoma, probably the high-titered antigen shared by teratoma and hepatoma, but does not remove a second, lower titered, activity. Absorption with ascitic fluid has no effect on staining of CI 1D cells.

Teratoma Antigens of Mouse Ovarian Cells.—Absorption studies were also used to determine which of the antigens reacting with anti-teratoma serum was responsible for staining of ovarian cells (Table VIII). Absorption of the antiserum with ovary removes antibody reactive with both teratoma and CI 1D
TABLE VII
Teratoma Cell Antigens in Teratoma Ascites Fluid

| Cells, stained with: | Serum dill. | Anti-teratoma Ascites fluid:anti-teratoma serum* |
|---------------------|-------------|-----------------------------------------------|
|                     |             | 2:1  | 20:1 |
| Teratoma A          | 1:80        | 83‡  | 4    |
|                     | 1:20        | 96   | 91   |
| C1 D                | 1:20        | 85   | —    |

* Ascites fluid from 129/J teratoma carriers was spun at low speed to remove cells and then at 100,000 g for 60 min to remove cell fragments and debris. Antiteratoma serum at 1:2 was absorbed with an equal or 10-fold volume of clarified ascites fluid at room temperature 1-2 h. Ascites fluid alone does not stain either teratoma A or C1 D cells.

† Results are expressed as percent stained cells.

TABLE VIII
Teratoma Antigens in Normal Mouse Ovary

| Cells stained with: | NRS         | —       | Ovary  | C1 D  | Hepatoma |
|---------------------|-------------|---------|--------|-------|----------|
| Teratoma A*         | 1           | 85      | 13     | 84    | 38       |
| C1 D‡               | 2           | 62      | 9      | 2     | 8        |
| Ovary‡              | 0           | 66      | 21     | 65    | 11       |

Absorptions were performed as described in Table VI. Ovaries from C3H females were used as absorbing and test cells. Ovary cells were obtained by gentle teasing in the presence of 0.25% Difco trypsin in phosphate-buffered saline. Results are expressed as percent cells stained. Serum dilutions: (*) 1:80; (‡) 1:20.

suggesting that both common and teratoma/hepatoma antigens are expressed in the ovary. C1 D absorption, which removes reactivity with the common antigen only, has no effect on staining of ovarian cells while absorption with hepatoma, which removes both activities, removes essentially all antiovary reactivity. This indicates that, as was the case with the teratoma, most of the stain on ovarian cells is due to the teratoma/hepatoma antigens. Staining of ovarian sections with absorbed antisera (not shown) gave results identical to those obtained with membrane stain on live cells.

Cellular Distribution of the Common Teratoma Antigens.—The common teratoma antigens are not confined to the plasma membrane. Fixed C1 D cells stain in the cytoplasm, and this fluorescence is removed by absorption of the antiserum with live C1 D (Fig. 5 a and b). Cytoplasmic staining is also observed in melanoma, SV-40-3T3, and 3T3 cells. In each case absorption of the antiserum with live C1 D removes the stain.

At least a portion of the cytoplasmic antigens are soluble, not membrane bound, since absorption of the antiserum with a 100,000 g, 120 min, supernate of lysed C1 D cells abolish cytoplasmic staining (Table IX). This supernate also absorbs antibody which binds to live cells, indicating that the cytoplasmic and plasma membrane antigens are identical, by serological criteria at least.
Fig. 5. Anti-teratoma serum staining of fixed CI 1D. Cells, plated onto cover slips 48 h before staining, were air dried then fixed in acetone at −70°C. Fixed cells were incubated at room temperature with undiluted normal goat serum, rabbit antiserum, and TMR-GaR for 10 min each with rinses in HH5 between each step. All sera were at 1:20 dilution. (a) CI 1D plus anti-teratoma serum. (b) CI 1D with anti-teratoma serum absorbed on live CI 1D (see Table VI).

| Anti-teratoma serum absorbed with: | % stained cells |  |
|----------------------------------|-----------------|
|                                  | Live (surface)  | Fixed (cytoplasmic) |
| Live CI 1D                       | 85              | 95                |
| CI 1D supernate*                 | 2               | 5                 |

Fixed CI 1D cells were prepared and stained as described in Fig. 5. Anti-teratoma serum was absorbed with live CI 1D as described in Table VI. Sera were used at 1:20 dilution.

* CI 1D cells were lysed by gentle homogenization in a loose-fitting glass homogenizer and particulate debris removed by centrifugation at 100,000 g for 120 min. 1 vol of anti-teratoma serum at 1:2 was absorbed with 4 vol of this supernate overnight at 4°C.

**DISCUSSION**

Absorption studies of anti-teratoma serum reveal at least three antigens. One antigen is apparently common to most tumor and transformed cells investigated thus far. It is not a "universal" tumor antigen since two tumors, both early-passage chemically induced fibrosarcomas, failed to react with the antiserum. A second antigen is expressed only by the hepatoma and teratoma. The third antigen, inferred from the failure of hepatoma absorption to remove all antibody reacting with the teratoma, may be specific for teratoma.

Two BALB/c fibroblast cell lines, 3T3 and FR-SV-3T3, react well only after treatment with trypsin while SV-3T3 and 3T12 cells react strongly without trypsinization. The difference in expression of the common antigen on
these cells is unclear. However, the results indicate that similar concentrations of reactive antigens are present on both “normal” and transformed fibroblast lines though their availability to antibody varies. This situation strongly parallels that observed for binding of concanavalin A (Con A) to normal and transformed fibroblasts (25). At 0°C, the temperature at which our incubations are performed, polyoma-transformed 3T3 cells bind three times as much [3H]Con A as do 3T3 cells. Trypsin treatment of 3T3 cells results in an almost threefold increase in binding at 0°C.

While both transformed and normal mouse fibroblast cell lines express the common antigen on their surface, significant levels of the antigen were not detected on live primary and early-passage fibroblast cell cultures from several strains of mice. If these cells express the common antigen, it is at a level below the limit of sensitivity of our assay. Thus, expression of the antigen, in a quantitative sense at least, is a property of fibroblast cell lines, and not of cultured fibroblasts per se.

The common teratoma antigen was found to co-cap with both the $H-2D$ and $H-2K$ molecules on C1 1D cells. Although we will refer to this antigen in the singular, it is not known whether the same or two different antigens are involved in the co-capping. Capping behavior of membrane markers in other experiments has agreed well with biochemical association of molecules following their removal from the membrane. The two $H-2$ polypeptides, $H-2D$ and $H-2K$, are readily separable by specific immunoprecipitation following either detergent (26) or papain (27) solubilization and do not co-cap on lymphocytes (23). On the other hand, β-2 microglobulin co-purifies with the human histocompatibility antigens, HL-A (28, 29), and co-caps with the HL-A antigens of lymphocytes (30).

Recently Fujimoto and co-workers (31) reported that $H-2$ antigens and a tumor-associated antigen co-purify on immunoabsorption of serum from lymphoma bearing mice. This tumor antigen was also defined by a heterologous antiserum, but nothing further is known about the tumor specificity of the antigens involved.

Of the cells tested thus far, all those which express the common teratoma antigen in the plasma membrane stain for the antigen in the cytoplasm as well. At least a portion of this cytoplasmic antigen in C1 1D is not associated with particulate fractions. A similar cellular distribution has been observed for a tumor-associated embryonic antigen. Baldwin and his co-workers found that an antigen defined by multiparous rat serum was localized both in the plasma membrane and cell sap of many chemically-induced rat tumors (32). It is possible that the soluble, cytoplasmic antigen is not associated with $H-2$. While preliminary results indicate that most of the common antigen in C1 1D cells is soluble, $H-2$ in cells is always membrane-bound (33).

Of the normal tissues tested, only ovary contained the common tumor antigen. The identity of the ovarian cells expressing the common antigen is un-
known since the very bright staining due to the teratoma/hepatoma antigen on ovarian sections effectively masks the presence of the weaker common antigen. However, since the common antigen is expressed by unfertilized mouse ova,1 it is possible that immature ova and germ cells in the ovary are also expressing the antigen.

Of the three antigens thus far found to react with our rabbit anti-teratoma serum, the strongest is the teratoma/hepatoma antigen. This antigen appears to be a secretory product of teratoma cells, and it is also expressed on the surface of parietal yolk sac cells.1 The tissue distribution of this antigen suggests it might be α-fetoprotein, a fetal serum protein found associated with and presumably synthesized by parenchymal cells of fetal liver and by parietal yolk sac (34). It is also secreted by many teratoma and hepatoma cells including hepatoma BW 7756 and several 129 teratomas (35). Although the homologous antigen is not immunogenic, α-fetoproteins from several species proved strong heteroantigens (36). Thus, if 402AX secretes α-fetoprotein, a rabbit antisera to the cells would certainly react with this antigen.

In a recent paper, Artzt and her co-workers describe the reactivities of an anti-teratoma serum prepared by injection of irradiated cells into syngeneic mice (37). Their teratoma F9, a subline of OTT-6050 (9), is composed entirely of embryonal carcinoma cells which have lost the ability to differentiate in vivo. Anti-F9 serum reacts with other embryonal carcinoma lines, with early cleavage embryos, and with testicular cells. It did not react with any of a variety of mouse tumor cells, while our rabbit anti-teratoma defines an antigen common to most of our tumor test cells. Very similar though not identical cells were used in the two studies, i.e., Artzt's 3T3 and SV-3T3 cells were derived from Swiss embryonic fibroblasts while ours were from BALB/c embryonic fibroblasts. One possible explanation for this difference is that the common antigen is not immunogenic in mice. A second possibility is that F9 does not express the common antigen. Teratoma 402AX differs from teratoma F9 in at least one important regard; 402AX retains the ability to differentiate in vivo. Perhaps then the common antigen reflects a commitment before differentiation, but is not expressed on fully differentiated normal tissues. It is re-expressed by cultured cells concomitant with the requisite changes involved in establishment of a cell line and by tumor cells.

SUMMARY

Rabbit antisera to a mouse testicular teratoma, absorbed with normal mouse tissues, react by immunofluorescence with plasma membrane antigens of a variety of transplantable mouse tumor cells and transformed fibroblast cell lines including Clone 1D, SV-40-3T3, and 3T12. Trypsin treatment of cells of "normal" lines, 3T3 and FR-SV-3T3, uncovers reactivity on these as well. Early passage mouse embryo fibroblast cell cultures do not react even after trypsinization. By cross-absorption studies, the anti-teratoma serum appears
to react with an antigen common to most tumor cells investigated thus far. When this antigen on Clone 1D cells is "capped," H-2 antigens collect with the teratoma antigens in the cap indicating a physical association between the molecules. Molecules specified by both the H-2D and H-2K regions are bound to the teratoma antigens in the Clone 1D plasma membrane. This antigen is also found in soluble tumor cell fractions where it is believed to be free of H-2. A second cell surface antigen defined by anti-teratoma serum is expressed only by hepatoma and teratoma itself. This second antigen is apparently a secretory product of teratoma cells. A third surface antigen defined by anti-teratoma serum appears to be specific for the teratoma.

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