Presence of Antibody against Mouse Fetal Antigen in the Sera from C57BL/6 Mice Immunized with Rauscher Leukemia

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SUMMARY

Presence of cross-reacting antibodies against embryonic cells and Rauscher leukemia cells was demonstrated in the sera collected from the C57BL/6 mice immunized with Rauscher leukemia cells, with the use of the indirect immunofluorescent test. Such cross-reacting antigenicity was also found in the SV40-transformed BLSV cells and AKR lymphoma cells. The antibody was completely absorbed with embryonic cells but not with adult spleen cells. Absorption tests showed that this antigen was different from Gross and Friend-Moloney-Rauscher antigens. However, the cross-reacting antibodies were not detectable in the sera from 13-month-old mice in which the natural antibody against Gross antigen was clearly demonstrated.

INTRODUCTION

Various reports on fetal antigens associated with plasma membrane of tumor cells have appeared recently (6–9, 24). Baranska et al. (5) showed that the antiserum raised against unfertilized mouse eggs was cytotoxic to SV40-transformed mouse cells. Coggin et al. (10) also showed that embryonic cells induce antibodies and cytotoxic lymphocytes, which react with the SV40-transformed hamster cells.

Antigenic properties of leukemia in the mouse have been studied extensively. Old and Boyse (22) classified them into 5 distinct antigenic systems, recognized in vitro (G, FMR, TL, ML, and E).2 Hanna et al. (12) reported the suppressive effect of immunization with mouse fetal antigens on the growth of cells infected with Rauscher leukemia virus. This suggested the presence of fetal antigen, in addition to the 5 antigens, on the cell membrane of the Rauscher virus-induced leukemia cells.

This paper reports on the detection of antibody against mouse fetal antigens in the sera collected from C57BL/6 mice immunized with Rauscher leukemia cells.

MATERIALS AND METHODS

Virus. A Rauscher virus preparation, obtained through the courtesy of Dr. F. J. Rauscher, National Cancer Institute, Bethesda, Md., has been maintained by serial passage in BALB/c mice.

Mice. BALB/c, AKR, and C57BL/6 mice of both sexes were used. C57BL/6 and BALB/c mice were inbred in our colony from a stock supplied by the Department of Anatomy and Inbred Animal Center, School of Medicine, Kyoto University, Kyoto, Japan. AKR mice were obtained through the courtesy of Dr. B. Wahren, Karolinska Institute, Stockholm, Sweden.

Cells. SV40-transformed cells were kindly supplied by Dr. Ryoichi Morii, School of Medicine, Kyushu University, Kyushu, Japan. These cells, designated as BLSV cells, were established from C57BL/6 whole embryo cultures transformed in vitro with SV40. Origins of RD-12 cells (17, 20) and AKR-CI cells (16) are summarized in Table 2. R-17 cells are Rauscher virus-infected C57BL/6 spleen cells (16). These cell lines were maintained in vitro. Cell suspensions of embryos were prepared from 16-day-old C57BL/6 embryos with 0.25% trypsin. Age of the embryos was determined by keeping male and female mice in separate cages for more than 20 days, then placing them in the same cage for 48 hr after which they were separated again. Sixteen days after the 2nd separation, embryos from pregnant mice were used and were assumed to be 16-day-old embryos.

Antiser. Antisera against Rauscher leukemia cells were prepared by immunization of C57BL/6 mice with the homogenate of an enlarged spleen from a BALB/c mouse inoculated with Rauscher virus or with R-17 cells. Mice were immunized every 20 days at least 5 times. Detailed data are listed in Table 1.

Absorption. Equal amounts of test serum and cells were mixed and incubated for 1 hr at 37° and then for 3 hr at 4°, with intermittent agitations to absorb the antibody. After centrifugation, the supernatants were collected and tested.

Immunofluorescent Staining. Fetal, G, and FMR antigens were detected by the indirect immunofluorescent test according to the methods of Möller (21) and Klein and Klein (18). BLSV cells grown in TD40 culture flasks were dispersed by 0.25% trypsin. Lymphoma cells grow without attachment on the glass surface. The cells were washed thoroughly in PBS and suspended in a concentration of approximately 10⁷ cells/ml in PBS. About 10⁶ cells in 0.10-ml volume were added to 0.10 ml of serially 2-fold diluted antiserum and incubated for 30 min at 37°. The cells were then washed twice and incubated with 0.10 ml of 5-fold diluted FITC-conjugated anti-mouse γ-globulin rabbit serum (Cappel Laboratories, Inc., Downingtown, Pa.) for 30 min at 37°. After 2 additional washings with PBS, the cells were examined under the Tiyoda fluorescent microscope. In consideration of the results of the
experiment by Möller (21), cells with diffuse fluorescence were identified as nonviable cells. All cells showing dotted circular or semicircular and annular fluorescence patterns at the membrane were considered positive. However, results of the tests that showed the specific fluorescence on the cells with a ratio less than 0.5% were described as negative (Table 1). Data pertaining to the evaluation of the tests will be referred to further under “Discussion.”

### RESULTS

Rauscher leukemia cells (RD-12), 16-day-old mouse embryonic cells, AKR lymphoma cells (AKR-CI), and SV40-transformed cells (BLSV) were stained with various mouse sera, diluted 2-fold as shown in Table 1, and with FITC-labeled anti-mouse γ-globulin rabbit serum.

The presence of cross-reacting antibody against embryonic

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Designation</th>
<th>Collected from</th>
<th>Immunized with</th>
<th>Absorbed with</th>
<th>16-day-old embryonic cells (C57BL/6) BLSV (C57BL/6) AKR-CI (AKR) RD-12 (C57BL/6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N(P16)</td>
<td>Both sexes 35-day-old (8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N.D.&lt;sup&gt;b&lt;/sup&gt;</td>
<td>N.D. Embryonic cell</td>
<td>-&lt;sup&gt;c&lt;/sup&gt; - - -</td>
</tr>
<tr>
<td>2</td>
<td>R(P22)</td>
<td>Male, 5-mo.-old (10)</td>
<td>Rauscher leukemia (5)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>N.D. Embryonic cell</td>
<td>++ ++ (+++)&lt;sup&gt;e&lt;/sup&gt; +++</td>
</tr>
<tr>
<td>3</td>
<td>R(P26)</td>
<td>Male, 6-mo.-old (10)</td>
<td>Rauscher leukemia (6)</td>
<td>N.D. Embryonic cell</td>
<td>++ ++ (+++)&lt;sup&gt;e&lt;/sup&gt; +++</td>
</tr>
<tr>
<td>4</td>
<td>R(P27)</td>
<td>Female&lt;sup&gt;f&lt;/sup&gt; 6-mo.-old (6)</td>
<td>Rauscher leukemia (6)</td>
<td>N.D. Embryonic cell</td>
<td>++ ++ (+++)&lt;sup&gt;e&lt;/sup&gt; +++</td>
</tr>
<tr>
<td>5</td>
<td>R(P30)</td>
<td>Male, 8-mo.-old (10)</td>
<td>Rauscher leukemia (7)</td>
<td>N.D. Normal spleen cells from 35-day-old C57BL/6 mice</td>
<td>++ ++ (+++)&lt;sup&gt;e&lt;/sup&gt; +++</td>
</tr>
<tr>
<td>6</td>
<td>R(P18)</td>
<td>Male, 5-mo.-old (10)</td>
<td>Rauscher leukemia (5)</td>
<td>N.D. AKR-CI RD-12</td>
<td>+ + (++) +++</td>
</tr>
<tr>
<td>7</td>
<td>R(P17)</td>
<td>Male, 6-mo.-old (8)</td>
<td>R-17 cell (5)</td>
<td>N.D. Embryonic cell AKR-CI</td>
<td>+ + ++ +++</td>
</tr>
<tr>
<td>8</td>
<td>N(P34)</td>
<td>Male, 13-mo.-old (8)</td>
<td>N.D.</td>
<td>N.D. Embryonic cell AKR-CI</td>
<td>- - + +</td>
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<tr>
<td>9</td>
<td>N(P35)</td>
<td>Female&lt;sup&gt;f&lt;/sup&gt; 10-mo.-old (2)</td>
<td>N.D.</td>
<td>N.D.</td>
<td>- - ++</td>
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<tr>
<td>10</td>
<td>O(P41)</td>
<td>Male, 6-mo.-old (8)</td>
<td>Normal spleen cells from 2-month-old BALB/c mice (6)</td>
<td>N.D.</td>
<td>- - (++) ++</td>
</tr>
</tbody>
</table>

<sup>a</sup>Numbers in parentheses, number of mice pooled.
<sup>b</sup> Without immunization or absorption.
<sup>c</sup> Results of the tests on mice treated with 2-fold diluted serum. Grade of immunofluorescence: -, 0 to 0.5%; +, 0.5 to 5%; ++, 5 to 50%; +++; 50 to 100%.
<sup>d</sup> Numbers in parentheses, number of times of immunization.
<sup>e</sup> Symbols in parentheses include the results of reaction with common H-2 isoantigens between AKR and BALB/c mice, since Rauscher leukemic cells from BALB/c mice were used for immunizations.
<sup>f</sup> Female mice with a history of pregnancy were used for immunization.
<sup>g</sup> Collected from the mice with a history of 4 pregnancies; bled 30 days after the last delivery.
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cells and Rauscher leukemia cells was demonstrated in the sera collected from the C57BL/6 mice that had been immunized with crude homogenate of Rauscher virus-infected enlarged BALB/c spleens and R-17 cells (Table 1, Experiments 2 to 7). This cross-reacting antigenicity was also found in the BLSV cells and in the AKR lymphoma cells. The antibody was completely absorbed with 16-day-old embryonic cells from C57BL/6 mice (Experiments 2 to 4) but not with normal spleen cells from 35-day-old C57BL/6 mice (Experiment 5). Absorption tests with the embryonic cells and the AKR-CI cells showed that this antigen was different from G (Experiments 7 and 8) and FMR antigens (Experiment 7).

Presence of antibody against any one of the cell lines was not detected in the sera collected from 35-day-old C57BL/6 mice (Experiment 1). However, in the sera from 13-month-old male mice, presence of the antibody was demonstrated against AKR-CI and Rauscher leukemia RD-12 cells but not against embryonic cells and SV40-transformed BLSV cells.

Chart 1. Percentage of (A) embryonic cells, (B) BLSV cells, and (C) RD-12 cells with specific fluorescence treated in suspension with serially 2-fold diluted serum. O, unabsorbed serum; ●, absorbed serum with embryonic cells. Detailed data on sera and cells are listed in Tables 1 and 2.
Absorption tests for these sera with AKR-CI cells showed that this was the natural antibody against G antigen as already shown by Aoki et al. (2) (Experiment 8).

Sera collected from 10-month-old female mice, 30 days after the 4th delivery, failed to react with embryonic cells and BLSV cells (Experiment 9).

It was confirmed that a cross-reacting antibody against C57BL/6 embryonic cells and Rauscher leukemia cells was not demonstrated in the sera collected from C57BL/6 mice immunized with the crude homogenate of normal spleens from 2-month-old BALB/c mice (Experiment 10).

Antibody titers in the sera absorbed with embryonic cells are shown in Chart 1.

As shown in Fig. 1, most of the embryonic cells that showed specific fluorescence on the cell membrane were the large cells.

**DISCUSSION**

Since Möller detected the cell surface antigen on the living cells by the indirect immunofluorescent test (21), various cell surface antigens have been detected on the tumor cell membrane (14, 15, 18, 25). However, there are many technical difficulties in the test when applied to the living cells. First, it seems unsuitable to apply this technique to the target cells prepared in vivo except from embryonic cells. FITC-labeled anti-γ-globulin serum could stain directly γ-globulin-producing cells, which may be involved among the target cells. This often leads to errors in the evaluation of the results. Especially when the percentage of cells with specific fluorescence is very low, the evaluation of the results seems to be impossible. In our experiments, we used only the cells from embryo and in vitro cultures. The 2nd problem is nonspecific fluorescence found on the cell surface which looks like truly specific fluorescence. This type of nonspecific fluorescence was often seen when undiluted sera were used as the 1st serum for which the blocking test was not feasible. On the contrary, the 2nd serum did not show this type of nonspecific staining, because the labeled serum was always used after the adequate dilutions. We consider that this nonspecific fluorescence depends on the γ-globulin taken in by the phagocytosis or absorbed onto the cells for some unknown reasons. In this paper, we showed the results of treatment with 2-fold diluted serum (Table 1) and the results of treatment with serially diluted serum (Chart 1). Absorption tests were also applied to evaluate the results more precisely. The 3rd problem is concerned with the function of the fluorescent microscope. It is well known that the intensity of radiation from the mercury lamp decreases in proportion to the time used. We often measured the intensity by an actinometer and by the observation of the standard.
antigen was found neither on any normal C57BL/6 tissues nor TL antigens. However, the following facts could rule out the possibility that the antigen found in embryonic cells may be identified with any one of them. Aoki et al. (2) showed that E antigen demonstrated in this report was different from G and FMR antigens as classified by Old (22). However, there may be another cell surface-antigenic system related to murine leukemia viruses in addition to the G, FMR, and gs antigens.

Another interesting problem is whether or not the antigen detected by the immunofluorescent test is related to murine leukemia virus. Electron microscopic tests failed to demonstrate the presence of the type C virus in BLSV cells (R. Mori, personal communication). Detection of the type C virus in the embryonic cells from C57BL/6 mice has not yet been carried out. However, there is a possibility that the presence of the genome of the type C murine leukemia virus may be revealed in BLSV cells by 5'-bromodeoxyuridine and certain other antimetabolites (4, 19). Huebner et al. (13) reported that the gs antigens are present in embryo preparations but not in neonatal cell preparations. On the other hand, it is also well known that gs antigen of murine leukemia virus is hardly immunogenic to mice (23). Absorption tests showed that the antigen demonstrated in this report was different from G and FMR antigens as classified by Old (22). However, there may be another cell surface-antigenic system related to murine leukemia viruses in addition to the G, FMR, and gs antigens.

**ACKNOWLEDGMENTS**

We acknowledge the able technical assistance of Miss Yumiko Suzuki and Miss Toyoko Yoshida. We are also indebted to Dr. M. Maeda, Kyoto University, for supplying RD-12 cells and tissue culture medium, and Dr. R. Mori for supplying SV40-transformed cells and for his valuable suggestions.

**REFERENCES**


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### Table 2

Summary of cell surface antigenicity detectable by indirect immunofluorescent tests combined with absorption tests

<table>
<thead>
<tr>
<th>Designation</th>
<th>Cell Origin</th>
<th>Antigenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fetal antigen</td>
</tr>
<tr>
<td>Embryonic cells</td>
<td>16-day-old whole embryo from C57BL/6 mouse</td>
<td>+</td>
</tr>
<tr>
<td>BLSV</td>
<td>C57BL/6 whole embryo cells transformed in vitro with SV40</td>
<td>+</td>
</tr>
<tr>
<td>AKR-CI</td>
<td>Spontaneous AKR lymphoma</td>
<td>+</td>
</tr>
<tr>
<td>RD-12</td>
<td>Rauscher virus-induced C57BL/6 lymphoma</td>
<td>+</td>
</tr>
</tbody>
</table>
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