Humoral Antibodies in the Host Directed against Tumor Cells after Suppression of Ascites Tumor by Heteroimmune Sera

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SUMMARY

The biological activity of rabbit anti-Yoshida ascites sarcoma (YAS) sera was studied. Immune antisera exhibited cytotoxic effects at a 1:100 dilution on YAS cells in vitro, causing vacuolation and disruption of the cytoplasm accompanied by karyolysis. Rabbit antisera, given i.p. in gradually increasing daily doses over a period of 8 days, completely inhibited the growth of YAS in isogenic Wistar rats (A/IISc) and, after absorption with liver and spleen of the A/IISc Wistar rats, the animals still showed tumor suppression. Anti-rat liver and anti-rat spleen sera from rabbits could prolong the survival period of rats with YAS but were unable to inhibit tumor growth. These findings suggest the existence of tumor-specific antigens as well as the presence of normal tissue antigens in the tumor.

After tumor inhibition, the rats exhibited lasting immunity as evidenced by rejection of lethal doses after subsequent tumor transplantation. Electrophoresis of the sera of these rats showed considerable increase in the γ-globulin fraction. Agglutinating antibodies in the sera were detected by a passive hemagglutination test. With trypan blue dye exclusion techniques, the presence of heat-stable (for 30 min at 56°) and complement-dependent cytotoxic antibodies to YAS cells could be shown in immune rat sera. The cytotoxic effect was observed, but with a reduced titer, even after thorough absorption of the immune sera with rat spleen and lymphoid cells. The neutralizing effect of immune rat sera on YAS cells was shown by the inability of lethal doses of YAS cells (10 million) to induce tumors in rats after incubation with immune rat sera. At twice this dose of YAS cells (20 million), only 40% of the injected rats developed tumors; they died after a prolonged survival period of 20 days while controls survived for 10 days.

The heterologous antisera, although not specific, can still be a useful therapeutic measure for some tumors, and the lasting immunity conferred is likely to play a significant role in control of growth and metastatic dissemination of the tumor cells.

INTRODUCTION

The significant role of the immune mechanism and the development of immunity in the host after suppression of tumors by chemotherapy (17) or surgical removal (15) have been demonstrated by the rejection of tumor grafts. Alexander (2) and others (3, 10, 12) have studied the influence of vaccines and serotherapy separately or as adjuncts to chemotherapy in the treatment of Moloney sarcoma virus-induced tumors and in other malignant tumors. While the participation of cellular immunity in tumor rejection is well established (1, 3, 13), the presence and characterization of humoral antibodies and their role in tumor suppression is still a subject of investigation.

In our earlier studies on the antigenic analysis of YAS1 (20), we reported the presence of humoral antibodies to YAS cells in rabbit anti-YAS serum. Precipitin antibodies to 3 distinct antigenic components of the tumor cells were detected in rabbit immune sera by standard immunological techniques. The specificity of this reaction was shown by the presence of these 3 precipitin lines even after complete absorption of the immune sera with the various tissues, i.e., spleen, liver, kidney, erythrocytes, and plasma of the A/IISc Wistar rats. With suitable absorption and cross-reaction studies and a passive hemagglutination technique, the presence of some tissue antigens common to the liver and spleen could be detected in the YAS cells.

This communication discusses the tumor-inhibiting activity of the rabbit anti-YAS sera on YAS in A/IISc Wistar rats and their cytotoxicity on tumor cells in vitro. The immunological response in rats after tumor suppression has been studied by challenging them with lethal doses of the tumor cells, by performing cytotoxic and neutralization tests, and by examining their sera for the presence of circulating antibodies.

MATERIALS AND METHODS

Tumor. YAS (Indian Cancer Institute, Bombay, India), maintained in isogenic Wistar rats (A/IISc) by serial i.p. transfer of $2 \times 10^7$ tumor cells, is a rapidly developing, chemically induced (22) tumor, causing 100% mortality with an average survival period of 8 days (21). All experiments were on isogenic Wistar rats (A/IISc) weighing 120 to 140 g.

Tumor Antigen. On the 5th day after transplantation, the ascites fluid containing YAS cells was aspirated into cold HBSS, and the tumor cells were separated from ascites fluid by washing 3 times in HBSS after centrifugation at 2000 rpm for 5 min each time. The contaminating red cells were lysed by suspension in distilled water. Immediately after centrifugation,

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1 The abbreviations used are: YAS, Yoshida ascites sarcoma; HBSS, Hanks' balanced salt solution.

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Table 1

The effect of rabbit anti-YAS serum on YAS in rats

<table>
<thead>
<tr>
<th>Type of material</th>
<th>Total volume of sera used (ml)</th>
<th>Survivors/treated</th>
<th>Survival period of tumor-bearing rats (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9% NaCl controls</td>
<td>2.0</td>
<td>0/15</td>
<td>7.5 ± 0.50</td>
</tr>
<tr>
<td>Normal rabbit sera</td>
<td>2.0</td>
<td>0/15</td>
<td>10.0 ± 0.43</td>
</tr>
<tr>
<td>Rabbit antisera to rat liver</td>
<td>2.0</td>
<td>0/15</td>
<td>15.6 ± 0.53</td>
</tr>
<tr>
<td>Rabbit antisera to rat spleen</td>
<td>2.0</td>
<td>0/15</td>
<td>13.8 ± 0.32</td>
</tr>
<tr>
<td>Rabbit immune sera to YAS</td>
<td>2.0</td>
<td>15/15</td>
<td></td>
</tr>
<tr>
<td>Rabbit immune sera to YAS (absorbed)</td>
<td>2.0</td>
<td>15/15</td>
<td></td>
</tr>
</tbody>
</table>

a Administered by i.p. route in single daily doses, starting 24 hr after transplantation with 2 × 10⁷ cells.

b Mean ± S.E.

the tumor cells were suspended in cold 0.9% NaCl and stored. All operations were performed at 0–4°C.

Cell suspensions in cold 0.9% NaCl were subjected to sonic oscillation for 30 min at 4°C in a 10-kc Raytheon sonic oscillator. Cell-free extracts obtained after centrifuging at 5000 x g for 30 min at 0–4°C in a Servall centrifuge were dialyzed against distilled water at 4°C for 24 hr with 3 changes. The dialysates were concentrated in a vacuum and stored in a Deepfreeze.

Organ Antigens. Healthy rat liver tissue was cut into small bits in cold 0.9% NaCl and was homogenized and centrifuged. The supernatant was dialyzed against distilled water at 4°C for 24 hr and lyophiilized. Spleen tissue antigen was prepared similarly.

Determination of Protein. The protein content of the samples, viz., YAS, liver, and spleen antigens, was determined by the Folin phenol method of Lowry et al. (16).

Antisera. After preimmunization bleeding, the healthy adult rabbits were immunized by injecting s.c. 0.8 ml of Freund's adjuvant (containing 12 mg of tumor antigen) on their backs in 4 sites sufficiently apart from each other; the rabbits received the injection once every 15 days. Fifteen days after the 3rd injection, when antibodies at high titer were detected, the immunized rabbits were bled completely, and the separated serum was stored in a Deepfreeze at −20°C.

Rabbit anti-rat liver and anti-rat spleen sera were prepared separately in a similar manner.

Immunotherapy. A day after transplantation of 2 × 10⁷ tumor cells, treatment with immune sera was started with a single dose of 0.1 ml and continued with gradually increasing single daily doses (total dose, 2.0 ml) until the death of all untreated tumor-bearing rats. Tumor-transplanted rats treated with 0.9% NaCl, normal rabbit serum, liver antisera, spleen antisera, and tumor antisera (absorbed with rat liver and spleen tissue antigens) served as controls.

Rats showing tumor suppression were challenged 6 months later with 2 × 10⁷ cells, and the same animals were rechallenged twice with 3 × 10⁷ cells in the 2 successive months. The surviving rats are hereafter referred to as immune rats.
Electrophoresis. The γ-globulin concentration of immune and normal rat sera was determined by agar gel electrophoresis (9, 20).

Passive Hemagglutination. YAS antigen-coated tanned sheep erythrocytes were used to determine the hemagglutination titer of immune rat serum by a passive hemagglutination method (4, 6).

Cytotoxicity of Rabbit Anti-YAS Serum. The in vitro cytotoxicity of the immune sera on tumor cells was tested at different concentrations and time intervals. The incubating mixture (total volume, 4 ml) contained $3 \times 10^6$ cells in Eagle's basal medium supplemented with 15% inactivated calf serum and a known concentration of anti-YAS serum. Flasks with or without normal rabbit serum served as controls.

Spleen and Lymphoid Cells. Spleens or lymph nodes from A/HeJc Wistar rats were cut separately into small fragments, pressed with a glass rod, and shaken thoroughly in HBSS. The cell suspension was separated, and the cells were washed 3 times in HBSS, after centrifugation at 2000 rpm for 5 min each time. Both spleen and lymphoid cells were used in absorption studies.

Cytotoxic Studies of Immune Rat Sera. The test procedure was a modification of that of Boyse et al. (5). Equal volumes (0.2 ml) of immune rat sera, guinea pig sera as a source of complement, and tumor cell suspension were incubated in test tubes for 2 hr in a water bath at 37°C. The final concentration of tumor cells in each incubating tube was 0.3 million. After incubation the tubes were centrifuged, and the supernatant fluids were removed. The cells were suspended in 0.2 ml of HBSS, an equal volume of 0.1% trypan blue in 0.9% NaCl was added, and the resulting suspension was incubated at 37°C for 10 min. The cells were then examined for dye uptake (19). Three separate counts of 100 cells each were made, and the results are reported as the mean percentage of cells showing dye uptake.

Neutralization Test. Tumor cells ($3 \times 10^7$) were incubated with 1 ml immune rat serum for 1 hr at 37°C. Nonimmune rat serum served as a control. A known number of cells in suspension were then injected i.p. into susceptible rats which were observed for tumor growth.

RESULTS

Body weight and survival period (Chart 1 and Table 1) clearly reveal the tumor-inhibiting efficacy of anti-YAS rabbit sera. The antisera, both before and after absorption with rat liver and spleen tissues, showed complete suppression of tumor growth.

<table>
<thead>
<tr>
<th>Animals used</th>
<th>No. of rats</th>
<th>1st challenge&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2nd challenge&lt;sup&gt;b&lt;/sup&gt;</th>
<th>3rd challenge&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rats</td>
<td>20</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
<tr>
<td>Rats treated with rabbit immune sera (absorbed)</td>
<td>15</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Rats treated with rabbit immune sera (unabsorbed)</td>
<td>15</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Twenty million YAS cells administered i.p.

<sup>b</sup> Thirty million YAS cells administered i.p.
growth in all the treated animals. In comparison, the rat liver and rat spleen rabbit antisera merely prolonged the survival time of tumor-bearing rats but failed to prevent tumor development. These results indicate that, while the antisera against the common antigens of YAS, liver, and spleen tissues do exhibit slight inhibitory action, the complete suppression is mainly due to specific antibodies to soluble antigens of YAS cells in the rabbit immune sera.

**Cytotoxicity of Rabbit Anti-YAS Sera on Tumor Cells in Vitro**

Cytotoxicity of the immune sera at different concentrations on tumor cells is shown in Chart 2. The immune serum at 1:100 dilution was toxic to tumor cells; at higher dilutions, a slight reduction in cell count was noticed only in the initial stages (3 and 6 hr).

Vacuolation and disruption of the cytoplasm, leaving only the nuclei, was a prominent feature seen in all cells treated with the antisera (1:100 dilution) (Figs. 1 and 2). Faint staining of the nuclei and karyolysis in nondividing cells were noticed in smears prepared at 3 and 6 hr from cells treated with the same dilution of the antisera (Fig. 3).

After tumor suppression, the rats were found to be resistant to subsequent challenges with lethal doses of tumor cells, even 6 months after treatment (Table 2). Thus, it appears that serotherapy not only inhibits tumor growth but also brings into play immune mechanisms enabling the host to resist subsequent fatal doses of tumor cells.

No tumor cells could be seen in the peritoneal washings of immune rats at the time of sacrifice. The sera of these rats agglutinated the tumor antigen-coated erythrocytes up to a titer of 1:1024 (Table 3).

Fig. 4 shows the electrophoretic pattern of normal and immune rat sera. A rise in immunoglobulin concentration is seen in immune rat sera by an increase of the $\gamma$-globulin component by 25% over normal.

**Cytotoxicity of the Immune Rat Sera.** The presence of cytotoxic antibodies in immune rat sera and their specificity to YAS cells after absorption with spleen and lymph node cells is shown in Table 4. At 1:48 dilution, about 40% of the cells were affected. Higher concentrations exhibited a greater cytotoxic effect. These antibodies were heat stable (for 30 min at 56°) and complement dependent. A slight reduction in cytotoxic titer was noticed after thorough absorption of immune rat sera with spleen and lymphoid cells, while complete neutralization of cytotoxic effect was observed after absorption with tumor cells. These findings suggest that tumor cells, apart from possessing antigens common to spleen and lymphoid cells, have specific antigens.

**Neutralization Test.** The neutralization effect of immune rat sera on YAS cells is shown in Table 5. None of the animals given injections of 10 million treated cells showed tumor growth.

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### Table 3

<table>
<thead>
<tr>
<th>Test material</th>
<th>1:2–1:8</th>
<th>1:16–1:256</th>
<th>1:512–1:1024</th>
<th>1:2048</th>
<th>1:4096</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal rat sera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune rat sera</td>
<td>3+</td>
<td>2+</td>
<td>+</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

* The symbols used are: -, non-agglutination; +, degree of agglutination.

* Sera from rats resistant to challenging doses of YAS cells after treatment with rabbit immune sera.

### Table 4

<table>
<thead>
<tr>
<th>Test material</th>
<th>Serum dilution</th>
<th>Normal sera (1:3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:3</td>
<td>1:6</td>
</tr>
<tr>
<td>Immune sera</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune sera heated at 56° for 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune sera absorbed with rat spleen cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune sera absorbed with rat lymphoid cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immune sera absorbed with YAS cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
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<td>92</td>
<td>87</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Percentage of YAS cells stained.

* The abbreviations used are: -, fewer than 10% YAS cells stained; nt, not tested.

* One ml of immune rat sera absorbed twice with 3 x 10^7 cells for 1 hr at 37° with fresh cells each time.
cells; Group 2, 20 X 10^6 cells.

Normal rat sera

suppression of tumor growth by both unabsorbed and 1 hr. The cells were washed free from the sera. The cell suspension in the medium was injected i.p. into groups of 15 rats. Group 1, 10 X 10^6 cells; Group 2, 20 X 10^6 cells.

Some antigenic components common to tumor cells, spleen, certain tumors, has been postulated (7).

The existence of lasting immunity in rats after immune suppression may be multiple. It is well established that syngeneic tumors can give rise to a state of immunity in pure-line animals by (a) excision of a transplanted tumor after it has reached a certain size; (b) injection of tumor cells rendered incapable of indefinite growth, although metabolically active, by exposure to X-rays or chemical substances; and (c) injection into the animal, of a dose of malignant cells which is too low to give rise to a tumor. By these procedures, the production of immunity can be demonstrated by the resistance of the animals to subsequent challenges with tumor cells which induce tumors in nonimmunized animals (3, 14).

It is very likely that the immune state, as observed in our experimental rats after treatment with immune rabbit serum, is due to a reduction in the number of malignant cells to a sublethal concentration. Since viability tests of YAS cells after treatment with immune rat sera were based on a dye exclusion technique, the possibility that these cells were physiologically active and capable of acting as antigens but not capable of continuous division also must be considered. Since immune rat sera and ascitic fluid in the peritoneum are shown to contain potent cytotoxic antibodies (M. Sirsi, unpublished data), their effect on the tumor cells might also be sufficient.

Studies on the effect of rat immune sera on YAS cells in culture in vitro and on the ability of YAS cells after treatment with rat immune sera to remain physiologically active and act as antigen may help clarify the mechanism of induction of lasting immunity after tumor suppression.

ACKNOWLEDGMENTS

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REFERENCES


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YAS-specific Antibodies after Immune Suppression

<table>
<thead>
<tr>
<th>Test material</th>
<th>Survivors&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Normal rat sera</td>
<td>0</td>
</tr>
<tr>
<td>Immune rat sera</td>
<td>100</td>
</tr>
</tbody>
</table>

<sup>a</sup> Animals were observed for 8 weeks or until death occurred.

<sup>b</sup> YAS cells, 3 x 10^7/ml of test material, were incubated at 37° for 1 hr. The cells were washed free from the sera. The cell suspension in the medium was injected i.p. into groups of 15 rats. Group 1, 10 x 10^6 cells; Group 2, 20 x 10^6 cells.

Figs. 1–3. YAS cells in vitro after incubation with rabbit anti-YAS serum at 1:100 dilution. The hours refer to the time after incubation started.
Fig. 1. At 3 hr. Note the agglutination of cells with broken cell walls. Giemsa stain, X 360.
Fig. 2. At 6 hr. Note the broken cell walls, vacuolation, and disruption of cytoplasm. Giemsa stain, X 1620.
Fig. 3. At 3 hr. Note the disintegrating nuclear bodies with condensed nuclei. Feulgen stain, X 1400.
Fig. 4. Electrophoretogram of normal and immune sera of A/IISc Wistar rats by agar gel electrophoresis. Note the increased γ-globulin fraction of immune rat serum. IRS, immune rat serum; NRS, normal rat serum; Alb, albumin; α-, β-, and γ-gbl, globulin fraction.
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