The Inactivation in Vivo of Mouse Lymphosarcoma 6C3HED by Antibodies Produced in a Foreign Host Species*

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In the past, several workers have reported that neoplastic tissues possess antigenic differences from normal tissues and that these differences can be demonstrated by immunological methods. General reviews of this field have been published (6, 9, 12). One technic that has been used has been the in vitro neutralization test similar to that employed for the immunological study of viruses. Using this general approach, Dulaney and Arnesen (2) have shown that antisera to components of both normal and leukemic cell exhibit in vitro cytotoxic effects for mouse leukemic cells, but that the leukemic antisera were much more effective than normal tissue antisera. More recently, Werder, Kirschbaum, MacDowell, and Syvertson (10, 11) have shown that granulocytic and lymphocytic mouse leukemic cells are inactivated in vitro more readily by antibodies produced in rabbits against the leukemic cells than by antinormal tissue sera. Green (5) found that rabbit antiserum prepared against mouse mammary tissue containing the milk factor possesses cytotoxic properties for mammary carcinoma cells when mixed with them in vitro. However, Law and Malmgren (8) have recently reported no consistent difference in the protective action of rabbit tumor antisera and those against normal tissues free of the tumor agent. Kidd (7) has shown that there is an antigenically distinct, sedimentable substance present in the Brown-Pearce rabbit carcinoma and that this substance is not detectable in a variety of normal rabbit tissues or in three other types of rabbit tumors. Also, from the results of experiments carried out by Burmester (1) with the avian lymphoid tumor, it appears that there is a factor, or factors, present in tumor tissue which is antigenically different from constituents of normal lymphoid tissue.

It is the purpose of this paper to record the results of experimental studies demonstrating that specific antibodies can be produced in a foreign host species against a fraction of mouse lymphosarcoma 6C3HED, separated by high-speed centrifugation, and that these antisera can be used to inactivate lethal inocula of tumor cells in vivo.

MATERIALS AND METHODS

Lymphosarcoma 6C3HED was employed in these studies. This tumor was originally induced in a C3H mouse by the administration of the estrogen, a-estradiol dipropionate, by Gardner, Kirschbaum, and Strong (3). The tumor line used in this work was obtained from Dr. John Kidd, Cornell University Medical School, and has been maintained in this laboratory by serial transfer in C3H mice of both sexes through many generations. Mice of the C3H strain for these experiments were obtained from the Jackson Laboratories and are designated Jax-C3H (Heston). Additional mice were obtained from Carworth Farms and are designated CF-C3H. Mice from these two sources were used interchangeably in the experiments reported here.

For controls, various mouse tissues have been used, including liver and splenic tissues from normal Swiss mice and liver tissue from normal C3H mice. Also, Flexner-Jobling rat carcinoma tissue was used as a source of antigen for the production of antisera which were subsequently tested for their in vivo effect on the growth of the mouse lymphosarcoma. This was to serve as an additional check on the species specificity of the in vivo inactivation of lymphosarcoma cells by immune sera.

Preparation of tissue fraction antigens used for immunization of rabbits.—Lymphosarcoma tissue 6C3HED was harvested using aseptic technic from C3H mice bearing 7- to 10-day-old tumor growths. These tumors were routinely produced in susceptible mice by the subcutaneous inoculation of 0.2 ml. of a 1.5 tumor homogenate in saline (0.85 per cent sodium chloride solution). The excised tumor tissue was minced with scissors and homogenized with saline using a Potter-Elvehjem type homogenizer. The tumor was homogenized in a concentra-
tion of 1 part of minced tissue to 4 parts of saline. The resulting 1:5 tumor homogenate was centrifuged in 10-ml aliquots at 5,000 r.p.m. for 40 minutes in a Servall SS-Type I centrifuge. Each 10-ml aliquot of tumor homogenate contained 2.5 gm. of homogenized tissue. Centrifugation was carried out at 5° C. The resulting supernatant fluid was withdrawn with a pipette and recentrifuged at 15,000 r.p.m. for 90 minutes at 5° C. The residue obtained as a result of the second centrifugation represents the immunizing antigen and is referred to as the pellet fraction. To prepare the pellet fraction for use as an immunizing antigen, the final supernatant was decanted off and the pellet material resuspended in saline so that 1.0 ml. of solution contained the material derived from approximately 0.5 gm. of tumor tissue. The pellet was resuspended by agitation and rehomogenized in a Potter-Elvejhem apparatus. The pellet fraction was then filtered by a double layer of nylon of 20xx mesh to remove the larger particles.

As control antigens, pellet fractions were isolated in an identical manner from liver and spleen tissue of normal Swiss mice, from liver tissue of normal C3H mice, and from Flexner-Jobling rat carcinoma tissue.

**Production of antiserum.**—Pellet fraction antigens were prepared from (a) lymphosarcoma mouse tumor tissue, (b) normal C3H mouse liver tissue, (c) normal Swiss mouse liver tissue, (d) normal Swiss mouse spleen tissue, and (e) Flexner-Jobling rat carcinoma tissue. All rabbits used for the production of antiserum were bled by cardiac puncture several days prior to immunization to obtain normal control sera. The immunization schedule was as follows: Each rabbit received a series of six inoculations of the pellet antigen, over a period of 3 weeks. Two to 4.0 ml. of resuspended pellet material was injected at each inoculation. The amount of antigen contained in each ml. of solution represents that amount of pellet fraction derived from approximately 0.5 gm. of tissue. All inoculations were made intravenously except the rat carcinoma pellet antigen. The latter was inoculated intraperitoneally to avoid the fatal anaphylactoid reactions produced in rabbits when this antigen was given intravenously. From 5 to 7 days after the last injection, the rabbits were bled by cardiac puncture; the serum was removed by centrifugation and stored at -40° C.

**Absorption of antiserum with mouse red blood cells.**—Immune serum inactivated at 56° C. for 30 minutes at 37° C. for 1 to 2 hours, and allowed to stand in the cold (4° C.) for 24 hours. The cells were centrifuged, and the erythrocyte agglutinating titer was determined. The absorption was repeated as many as 4 to 5 times until the agglutinating titer dropped to 1:8 or 1:16. One such absorption test was done with erythrocytes from C3H mice and several other tests done with red blood cells from the more available Swiss mice. No difference was noted in the results.

**Technic of the in vivo inactivation of lymphosarcoma 6C3HED**.—Each C3H mouse was inoculated subcutaneously with lymphosarcoma 6C3HED tumor homogenate at two sites, namely, in the left and right inguinal regions. Two-tenths ml. of a 1:100 homogenate of lymphosarcoma tissue in saline was used as the test inoculum at each site. The antisera were tested for their in vivo tumor-inactivating effect by intraperitoneal inoculation. The antisera were administered routinely in doses of 0.25 ml. of undiluted serum at 0, 48, 96, and 144 hours subsequent to the implantation of the tumor. In some of the earlier experiments, antiserum treatments were continued at 48-hour intervals up to the 14th day after tumor implantation, but such prolonged administration of antiserum was found to be unnecessary. In most of the experiments reported here, a total of 1.0 ml. of undiluted antiserum was administered to each mouse over a period of 6 days. The animals were examined daily for the presence of palpable tumors. For control purposes, tumor-injected mice were injected in an identical manner with normal rabbit sera. To provide a check on the viability of the 1:100 lymphosarcoma homogenate in the several experiments, animals injected with the tumor suspended in saline were observed for tumor development. In this investigation no evidence was found of a change in the in vivo protective capacity of heat-inactivated or noninactivated normal or immune rabbit sera. After this finding, sera were not inactivated. The results presented are with such sera unless otherwise stated.

**RESULTS**

From the data presented in Table 1, it will be noted that there is a marked difference in the in vivo action of the antitumor pellet sera and the several types of normal or antisera used on controls. Rabbit antiserum against mouse lymphosarcoma pellet fraction routinely agglutinated mouse red blood cells in vitro at a final serum dilution of from 1:64 to 1:512. The presence of a mouse erythrocyte agglutinating antibody was apparent even when antigenic material used for immunization
appeared on gross examination to be devoid of red
blood cells. The possibility of common antigens
from tumor cells and erythrocytes must be borne
in mind, as well as the probable contamination of
the tumor pellet by red cell stroma, in explaining
the erythrocyte-agglutinating properties of anti-
tumor sera. It was of interest to determine whether
or not the mouse red blood cell agglutinin was re-
sponsible for the in vivo inactivation of tumor im-
plants through some mechanism such as the occlu-
sion of the small capillaries in the area of tumor
proliferation. In addition, it was found that treat-
ment of mice with antisera showing mouse red
blood cell agglutinating titers of from 1:256 to
1:512 resulted in the death of the animal. Deaths
usually occurred 24-48 hours after the administra-
tion of antiserum and was characterized by marked
hemoglobinuria. As a result of this observation,

In further studies on the in vivo inactivation of
mouse lymphosarcoma by various antitumor sera,
it seemed desirable to determine the period of time
that the initiation of antiserum injection could be
delayed and still produce the inhibitory effect on
the growth of the tumor. In these experiments,
injection of tumor-bearing C3H mice with antitumor
pellet sera was started at 0, 48, 96, or 144 hours
after tumor implantation. The total amount of
antiserum given to each animal was 1.0 ml. This

**TABLE 1**

<table>
<thead>
<tr>
<th>Antiserum used for treatment</th>
<th>Tumor incidence</th>
<th>Tumor incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimouse lymphosarcoma pellet serum</td>
<td>0/6*</td>
<td>0/6</td>
</tr>
<tr>
<td>Antinormal CSH mouse liver pellet serum</td>
<td>1/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Antinormal Swiss mouse liver pellet serum</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Antinormal Swiss mouse spleen pellet serum</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Antilymphosarcoma pellet serum #2362</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Antilymphosarcoma pellet serum #2352</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Antilymphosarcoma pellet serum #2372</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Antilymphosarcoma pellet serum #2374</td>
<td>0/6</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Denominator signifies the total number of sites inoculated with 0.2 ml.
1:100 lymphosarcoma 6C3HED homogenate; the numerator, the total number of sites at which palpable tumors developed.

In these antisera, inactivated at 56o for 30 minutes,
were routinely absorbed with mouse red blood
cells to remove the mouse erythrocyte agglutinin
prior to their use in vivo.

Table 2 gives the results of an experiment which
indicate that the mouse red blood cell agglutinin
present in the antitumor sera was not associated
with the antibody responsible for the in vivo in-
activation of lymphosarcoma 6C3HED. Aliquot
portions of four antitumor mouse pellet rabbit
sera, with mouse erythrocyte agglutinating titers of
from 1:64 to 1:128, were absorbed with mouse
red blood cells from Swiss mice or C3H mice until
free of the agglutinin. The absorbed antisera were
then tested for their ability to inactivate the tu-
mor in vivo. The results show that there was no
appreciable change in the ability of these antisera
to inactivate the tumor in vivo when the mouse
red blood cell agglutinin was removed by absorp-
tion. Therefore, it appears that the protective
power of the antitumor sera was associated with
an antibody directed against some component of
the tumor cells rather than antibody against the
mouse erythrocytes.

**TABLE 2**

<table>
<thead>
<tr>
<th>Antiserum used for treatment</th>
<th>Tumor incidence with unabsorbed antisera</th>
<th>Tumor incidence with mouse red blood cell absorbed antisera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antilymphosarcoma pellet serum #2362</td>
<td>0/6*</td>
<td>0/6</td>
</tr>
<tr>
<td>Antilymphosarcoma pellet serum #2352</td>
<td>1/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Antilymphosarcoma pellet serum #2372</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Antilymphosarcoma pellet serum #2374</td>
<td>2/6</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* Denominator signifies the total number of sites inoculated with 0.2 ml. 1:100 lymphosarcoma homogenate; the numerator, the total number of sites at which palpable tumors developed.

**TABLE 3**

<table>
<thead>
<tr>
<th>Antiserum treatment started</th>
<th>Antiserum used</th>
<th>Antiserum treatment started</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiserum used for treatment</td>
<td>Antiserum treatment started (hours after tumor implantation)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antilymphosarcoma pellet serum #2362</td>
<td>0/6*</td>
</tr>
<tr>
<td></td>
<td>Antilymphosarcoma pellet serum #2352</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Antilymphosarcoma pellet serum #2372</td>
<td>0/6</td>
</tr>
<tr>
<td>Control: Normal rabbit serum</td>
<td>Antilymphosarcoma pellet serum #2372</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Denominator signifies the total number of sites inoculated with 0.2 ml. 1:100 lymphosarcoma homogenate; the numerator, the total number of sites at which palpable tumors developed.

was administered intraperitoneally in doses of 0.25
ml at 48-hour intervals after the initial injection.

The results of the delayed initiation of anti-
tumor serum injections on the growth of the tumor
are given in Table 3. The data show that the anti-
sera were just as effective in inactivating the tu-
mor when injections were started 48 hours after
tumor implantation as when initiated at 0 hours.
When antitumor serum injections were started 96
hours after tumor implantation, there was partial
in vivo inactivation of the tumors, as shown by the
development of tumors in nine of the twelve tumor
sites inoculated. From these results it appears that
the antitumor sera can inactivate the tumors in vivo.
during the initial stages of growth, but that the antisera, at least in the dosages tested, cannot cause the tumor to regress once it has established itself.

The effect of dilution of antitumor pellet sera on the in vivo inactivation of mouse lymphosarcoma is shown in Table 4. In this experiment, CSH mice given tumor implants were injected with antitumor serum doubly diluted 1:2 through 1:64 with saline. Antiserum injections were initiated at 0 hours after subcutaneous tumor implantation, and each mouse received a total of 0.75 ml. of the appropriate antiserum dilution at 48-hour inter-

<table>
<thead>
<tr>
<th>Dilution of antiserum used for treatment</th>
<th>Undiluted</th>
<th>1:2</th>
<th>1:4</th>
<th>1:8</th>
<th>1:16</th>
<th>1:32</th>
<th>1:64</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence</td>
<td>0/6*</td>
<td>1/6</td>
<td>1/6</td>
<td>1/6</td>
<td>1/6</td>
<td>1/6</td>
<td>1/6</td>
</tr>
</tbody>
</table>
| The denominator signifies the total number of sites inoculated with 0.3 ml. 1:100 lymphosarcoma homogenate; the numerator, the total number of sites at which palpable tumors developed.

The results indicate that there was a marked reduction in the number of tumor implants inactivated in vivo when the injected antitumor serum was diluted 1:16.

**DISCUSSION**

In any study of the immunology of tumors in experimental animals, the genetic constitution of the host must be considered. For immunological tests it is usually desirable to employ inbred strains of mice and tumors which have originated in these pure lines. For control purposes, normal tissues from the same inbred strain of mice should be used. In many of the experiments reported here, spleen and liver tissues from normal Swiss mice were used for the preparation of some of the normal tissue pellet antisera because of a limited supply of CSH mice. When these antinormal tissue sera were tested for their ability to inactivate the mouse tumor in vivo, there was little protection of the mice against subsequent tumor development. To determine whether this failure to protect was due to differences in the genetic make-up of the test animal and the tissues of the donor mouse, antisera were prepared in rabbits against pellet fractions isolated from liver tissue of normal CSH mice. The experimental results show that rabbit antisera against normal liver pellet fractions of CSH mice were no more effective than the antisera against normal Swiss mouse tissues in inactivating the tumor in vivo.

The observation that the antitumor sera contained an antibody against mouse red blood cells suggested that possibly the in vivo inactivation of mouse lymphosarcoma implants was due to a nonspecific interference of the blood supply in the new tumor implants resulting in the failure of the tumors to grow. Youngner and Algire (13) have shown that interference or occlusion of capillaries in areas of tumor growth can cause a regression of the neoplasm. However, it was found that there was no appreciable change in the ability of the antitumor sera to protect against subsequent tumor development when the mouse red blood cell agglutinins present in the antitumor sera were removed by absorption. This is in agreement with work by Gorer (4) who found that absorption of mouse erythrocyte agglutinins from antisera against leukemic cells of A strain mice did not alter the neutralizing power of these antisera. Thus, it appears that the in vivo inactivation of the tumor by antitumor pellet sera is due to antibodies directed against tissue elements other than those of the mouse red blood cell.

**SUMMARY**

From the results of these experiments, evidence is provided that antibodies can be produced in a foreign host species against a tissue fraction isolated from mouse lymphosarcoma 6C3HED. When this antiserum was used for the in vivo inactivation of the neoplasm, tumors developed in only 18.0 per cent of the sites inoculated with tumor. The injection of tumor-implanted mice with normal rabbit serum resulted in tumor takes in 96.9 per cent of the sites. Attempts to inactivate the tumor with antisera against various mouse tissue pellet fractions resulted in considerably less protection than when the specific antitumor serum was used. Rabbit antisera against a tumor pellet fraction isolated from rat carcinoma tissue were also ineffective (90.4 per cent tumor takes) in protecting mice.
against tumor development. When the administration of antitumor serum was delayed until 96 hours after tumor implantation or when the antitumor serum was diluted 1:16, no protection was noted.

REFERENCES
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