A Study of the Leukemoid Response to Transplantable A-280 Tumor in Mice*

E. J. LAPPAT AND MADISON CAWEIN
(Department of Medicine, University of Kentucky Medical Center, Lexington, Kentucky)

SUMMARY

A preliminary study of the characteristics of the leukemoid response to transplantable A-280 tumor in BALB/c and B6D2 F1 mice is presented. A direct correlation between the magnitude of leukemoid response and the duration of tumor implant was noted. Liver, kidney, spleen, and adrenals of leukemoid tumor-bearing animals showed an increase in weight which was correlated with myelogenous cellular infiltration. The excision of tumor resulted in a disappearance of the leukemoid response and return of spleen weight to normal. In investigating the mechanism of the leukemoid response various tumors and tissue homogenates were injected subcutaneously in an attempt to provoke a similar type of response. A leukemoid response similar to that in tumor-bearing animals was found only with the injection of fresh tumor homogenate.

Electrophoresis of serum from leukemoid tumor-bearing animals revealed abnormal components in the a1, a2, and a3 fractions, which increased with duration of tumor growth and leukocyte response. A concept of a factor present in, or elaborated by, tumor cells that induces myelopoiesis directly or through enhancement of physiological mechanisms is discussed.

In 1953 a transplantable, rapidly growing and metastasizing tumor of BALB/c mice was found to have developed from treating a previously benign papilloma B3651 with injections of methylcholanthrene dissolved in tricaprylin. This tumor was designated A-280. Histopathological studies showed it to be a squamous-cell carcinoma (Fig. 1). Subsequently, it was observed that the animals carrying this tumor developed a leukemoid response with white blood cell counts ranging from 100,000 to 700,000 per cu. mm. and splenomegaly.

Although there has been frequent experimental use of the cellular elements from leukemoid blood produced in this manner, (3, 15, 16) no comprehensive cytological study of the peripheral blood and hematopoietic system or investigation of the mechanism of this response has been reported. It was the purpose of this investigation to study the splenomegaly and to correlate the histopathological findings in this organ with those of peripheral blood, bone marrow, liver, and kidney during tumor growth. Further, an investigation of factors possibly responsible for the leukemoid response was undertaken by evaluating the leukocytic response to the injection of various tumor and normal tissue fractions.

MATERIALS AND METHODS

Mice of BALB/c and B6D2 F1 strains were used. Initial stock of BALB/c tumor-bearing animals was supplied by Dr. W. R. Bryan of the National Cancer Institute. Initial stock of B6D2 F1 tumor animals was supplied by Dr. L. H. Smith of the Biology Division, Oak Ridge National Laboratories, where the tumor had been adapted to these animals from BALB/c A-280 tumor-bearing animals. All animals were maintained on Purina Mouse Breeder Chow and water ad libitum. Animals under study were marked and housed separately in groups of six. Stock animals were housed in groups of 25. Tumors were maintained by transplants made by the standard trocar technic. Approximately 50 mg. of finely chopped tumor tissue was implanted subcutaneously into the dorso-lateral aspect of the right flank.

Blood for peripheral blood counts was obtained by lancing tail veins. To obviate any leukocytic response that might be induced by bleeding, counts were made no oftener than once a week. Special calibrated pipettes for small

* This investigation was supported by Grant No. CA06900-01 from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

† H. B. Andervont's laboratory records, National Cancer Institute, 1943.

‡ W. Ray Bryan, personal communication.

§ Thelma Dunn, personal communication.

Received for publication August 19, 1963.
animals were used for red and white cell counts. Peripheral blood smears, bone marrow preparations, and spleen imprint preparations were stained with Wright's stain. Tissue obtained at autopsy was fixed in 10 per cent neutral formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Organ weights were obtained with a precision torque balance (±0.5 mg.). Cell-free fresh tumor homogenate was prepared by homogenizing freshly excised tumor tissue 20 minutes at ±6°C. in a Virtis "45" homogenizer, then rapidly freezing and thawing 3 times in a dry ice and ethanol mixture (—65°C.) alternately with a warm water bath (37°C.).

Cell-free, bacteria-free filtrates from tumor homogenate were made by homogenizing freshly excised tumor, adding normal saline as a diluent. A ratio of approximately 5 ml. of saline to 1 gm. of tissue was used. The homogenate was centrifuged in a refrigerated centrifuge at 2500 r.p.m. for 15 minutes. The supernatant was then filtered through a Seitz bacteriological filter with an asbestos pulp filter disc having a pore range of ±100 μm. Further handling was carried out by aseptic technic.

Nonsterilized necrotic material was obtained by aspiration from cyst cavities or by direct removal by spatula from open cavities. This material was then homogenized. The absence of intact tumor cells was insured by making Wright's stained smears of the preparation. Sterilized necrotic material was obtained by the manner above and then subjected to autoclaving at 250°F., 15 pounds' pressure for 20 minutes. This material was homogenized prior to injection. The homogenate from normal isologous animal tissues was made with the thigh muscles, heart, thyroid, and salivary glands from one animal.

In each instance 0.2 ml. of material was injected subcutaneously in the dorso-lateral flank. Injections were made daily for a period of 3 months. White cell counts were taken prior to injection and at 10- to 14-day intervals thereafter.

Paper electrophoresis was carried out on a Spinco Model R electrophoretic cell on Schleicher and Schuell 2043-A Mgl. filter paper strips with a Veronal B-2 buffer at pH 8.6.

Animals were sacrificed selectively as experiment controls or tumor replants dictated. However, occasionally massive hemorrhage into tumor cyst cavity or cannibalism required autopsy earlier. BALB/c animals were frequently sacrificed early because of impending death.

RESULTS

Tumor Growth

Transplanted tumors became palpable 0.5- to 1-cm. nodules in 2–3 weeks' time. Following an initially similar growth pattern, there was marked difference between growth in the two mouse strains. In the B6D2 F1 animals the tumor after 4 weeks' growth became soft and cystic. This was followed by ulceration and spillage of cyst contents. Following necrosis there was central scarring with a surrounding ridge of proliferating tumor tissue. Tumor regrowth locally, with secondary mushrooming and repeat cyst formation, followed. Rarely, there was metastasis to regional lymph nodes or to lung. These animals seemed minimally affected by the tumor, maintaining a sleek, healthy-appearing coat and continuing to gain weight. Death was generally by sacrifice.

In BALB/c animals, the cystic degeneration of tumor did not occur before 6–8 weeks, prior to which there was obvious regional metastasis to the axillary and/or inguinal nodes. By 2½ months these animals appeared ill and markedly dehydrated, with shaggy coats and obvious weight loss. Pulmonary metastasis as evidenced by marked respiratory distress and post mortem findings and local invasion by the retroperitoneal route into pelvic and peritoneal cavities was the rule.

Correlation of Tumor Age with Leukemoid Response

Serial blood studies showed a progressive rise in the white blood cell count which was directly related to the duration of tumor implant. During tumor growth the magnitude of leukemoid response was comparable in both mouse strains, as illustrated in Chart 1. However, the final cell counts in BALB/c animals did not reach the magnitude of those in B6D2 F1 owing to earlier death of the BALB/c animals.

Peripheral blood of control animals routinely showed 70–80 per cent lymphocytes. These were small mature lymphocytes with dark-staining pachychromatic nuclei and a scant rim of pale blue cytoplasm. Mature monocytes, larger cells (10–20 μ) with pale, blue cytoplasm and spherical nuclei with a fine chromatin pattern, varied in occurrence from 3 to 8 per cent. A variance of 12–20 per cent mature neutrophils with irregularly segmented nuclei was found. One to 3 per cent 'ring form' neutrophils and eosinophils were observed.

In mice with tumors of 2–3 weeks' duration no elevation in count was observed, but the peripheral smears showed a change in the differential counts from predominant lymphocytosis to that of a granulocytosis varying from 50 to 70 per cent. As the leukocyte count rose there was an increasing shift to the left in the granulocytic series with 20–30 per cent young ring forms, 5–8 per cent metamyeloocytes, 2–4 per cent myelocytes, and 1–2 per cent blast forms observed (Fig. 2).

Chart 1.—Correlation of tumor age with magnitude of leukemoid response. Each point represents a separate tumor-bearing animal.

O—BALB/c strain animals
●—B6D2 F1 animals
Pathological Findings

Only the pathology pertinent to the hematopoietic organ system is noted here. A complete detailed study of all autopsied tissues will be made in a separate report.

Bone marrow.—Bone marrow flushed from the femur was grossly pale or a light salmon color as compared with the deep red marrows in the normal animals of comparable age. Marrow smears showed intense hypercellularity. There was an increase in the number of myeloid elements as shown in Figure 4. This was reflected by an increase in myelocytes and metamyelocytes. There was a predominance of young ring forms and mature segmented forms. The most significant finding was the proportional decrease in cells of lymphocytic and erythroid series from that observed in the marrow of control animals. No change was observed in the number of megakaryocytes.

Spleen.—The spleens of all tumor animals were grossly enlarged (Fig. 3). There was a change from the normally deep burgundy color to one of pale red gray with fine white mottling. The consistency was firmer than normal, and the cut surface revealed a decrease in the normal soft pulpiness.

Microscopic section and imprints revealed a complete disruption of normal splenic architecture owing to proliferating myeloid elements. There was replacement of lymphoid tissue and red pulp by myeloid cells in all stages of development. Germinal centers were present but reduced in number and size. Primitive blast cells, myelocytes, and early metamyelocytes were more abundant than in the bone marrow. The predominant cell types were myelocytes, metamyelocytes, and young ring forms. Megakaryocytes were strikingly increased in number. Megakaryocytes were present but few in number. These changes are illustrated in Figure 5.

Liver.—The liver was a pale gray-brown. In three BALB/c animals white spots varying in size from 0.5 to 3 mm. were noted on subcapsular surface. No other gross changes were observed.

Sections of the liver showed extramedullary myelopoiesis as illustrated in Figure 6. This occurred in multiple small foci scattered throughout the section. However, the most extensive areas were those around the larger veins, radiating in a characteristic pattern from the subcapsular area. "Horse shoe" metamyelocytes and ring forms were present. The predominant cells were large, primitive mononuclear cells. Many mitotic figures were seen. Megakaryocytes were prevalent. Occasional nests of lymphocytes were observed. Plasma cells were scattered randomly throughout the area of cellular infiltration.

Kidney.—No gross changes were apparent in the kidneys. Microscopic sections revealed intense periarterial cellular infiltration (Fig. 7). This was observed mainly in the cortex and could be seen grossly in the stained section. The predominant cells were medium- to large-sized mononuclear cells and plasma cells. Lymphocytes, scattered singly and in nests, were seen throughout the area of infiltration.

Comparison of Organ Weights

The correlation between organ weights and leukocyte count in tumor and normal control animals is shown in Charts 2 and 3. Animals with a leukemoid response showed a significant increase in the average weights of liver, spleen, kidneys, and adrenals as compared with the averaged weights of organs from control animals.

Leukocyte Response to Injection of Various Types of Tissue Homogenate and Plasma

Table 1 illustrates the leukocyte response of BALB/c and B6D2 F1 mice given injections of various types of homogenates and plasma every day over a 3-month period. The most significant elevation occurred in those animals given injections of fresh, lysed-tumor-cell homogenate. The leukemoid response in these animals resembled that described in tumor bearing animals.

Surgical Removal of Tumor Tissues

Tumor tissue was removed surgically from six mice with leukemoid responses. Prior to tumor excision two control white cell counts were taken, and laparotomy was performed. At laparotomy the spleens in all mice were found to be greatly enlarged. The usual deep red color was replaced by fine white mottling. No discernible enlarge-
ment or change was observed in any other organ except that in one BALB/c animal the adrenals appeared enlarged. Following surgical removal of tumor, a fall in white count was observed over a 10-day period, with a return to the normal range between 6 and 8 weeks in all animals except one BALB/c. Repeated laparotomy in 2 months following the excision of tumor revealed normal spleen size and appearance. One BALB/c animal whose leukocyte count remained elevated died 6 weeks following surgical removal of the tumor from extensive pulmonary metastasis. These findings are summarized in Table 2.

**TABLE 1**

Leukocytic Response to Injection of Various Types of Tissue Homogenates and Plasma

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Injection material</th>
<th>Mean pre-injection WBC</th>
<th>Maximum leukocyte response</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fresh, lysed tumor homogenate</td>
<td>6,600</td>
<td>105,000</td>
</tr>
<tr>
<td>II</td>
<td>Cell-free filtrate of #1</td>
<td>15,200</td>
<td>15,500</td>
</tr>
<tr>
<td>III</td>
<td>Nonsterile necrotic tumor homogenate</td>
<td>15,400</td>
<td>25,150</td>
</tr>
<tr>
<td>IV</td>
<td>Sterile necrotic tumor</td>
<td>12,250</td>
<td>10,650</td>
</tr>
<tr>
<td>V</td>
<td>Normal tissue homogenate (a)</td>
<td>11,200</td>
<td>10,700</td>
</tr>
<tr>
<td>VI</td>
<td>(a) Normal plasma</td>
<td>12,325</td>
<td>22,450</td>
</tr>
<tr>
<td></td>
<td>(b) Tumor animal plasma</td>
<td>13,000</td>
<td>29,800</td>
</tr>
</tbody>
</table>

**ELECTROPHORESIS**

Electrophoresis of sera from tumor-bearing mice showed the appearance of abnormal components in the α₁ and α₂ globulin zones which were absent in the sera of normal control animals. This component increased with duration of tumor growth and increase of white blood cell count. In addition there was widening of the γ-globulin band as compared with that observed in sera of control animals. The further increase in γ-globulin fraction paralleled the rise in white blood cell count with tumor age (Fig. 8).

**DISCUSSION**

The difference in tumor growth patterns in the two strains of mice is probably a reflection of difference in host immunity or resistance to tumor growth. The A-280 tumor which originated in the BALB/c strain is considerably more malignant for this animal than for other strains and inevitably causes its death. However, this host resistance or susceptibility to tumor appears to have no direct effect on the magnitude of the leukemoid response. In both strains there is a direct correlation between the duration of tumor growth and rise in WBC. Although the maximum leukocyte response in BALB/c animals was generally lower than that of the B6D2 F₁ animals, this was due to earlier death of those animals.

The early change in peripheral blood picture from one of lymphocytosis to granulocytosis prior to direct elevation of total white count seems to indicate a preferential change in

**TABLE 2**

Effect of Tumor Excision on Leukemoid Response

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>AV. WBC/cu mm before tumor inoculation</th>
<th>Splenectomy #1</th>
<th>Pre-excision WBC</th>
<th>Tumor Age (days)</th>
<th>Post-tumor excision</th>
<th>Splenectomy at laparotomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>10,500</td>
<td>4+ enlarged</td>
<td>150,000</td>
<td>89</td>
<td>108,000</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28,000</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15,500</td>
<td>64</td>
</tr>
<tr>
<td>2*</td>
<td>17,000</td>
<td>2-3X enlarged</td>
<td>96,000</td>
<td>89</td>
<td>62,500</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>42,000</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12,500</td>
<td>64</td>
</tr>
<tr>
<td>3†</td>
<td>10,200</td>
<td>2-3X enlarged</td>
<td>82,000</td>
<td>89</td>
<td>53,500</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>37,000</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>45,000</td>
<td>47</td>
</tr>
<tr>
<td>4†</td>
<td>8,200</td>
<td>4X enlarged</td>
<td>260,000</td>
<td>112</td>
<td>175,000</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>44,000</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17,400</td>
<td>56</td>
</tr>
<tr>
<td>5†</td>
<td>12,500</td>
<td>3X enlarged</td>
<td>192,000</td>
<td>112</td>
<td>98,500</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>25,300</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14,600</td>
<td>56</td>
</tr>
<tr>
<td>6†</td>
<td>16,400</td>
<td>2X enlarged</td>
<td>84,000</td>
<td>112</td>
<td>38,200</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>15,800</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>17,200</td>
<td>56</td>
</tr>
</tbody>
</table>

* BALB/c.
† Died of pulmonary metastasis.
‡ B6D2 F₁.
hematopoiesis. Direct suppression of lymphopoiesis cannot be established. However, the histological appearance of bone marrow, spleen, and the liver show a marked stimulation of relative and real myelopoietic activity which would be extremely difficult to differentiate from chronic granulocytic leukemia on the basis of histology alone. This is most striking in liver sections, where there appears to be proliferation of vascular endothelium with an almost imperceptible merging of these cells into the surrounding myelopoietic cells; and with the marked splenomegaly, which is due entirely to proliferation of myelopoietic elements. The difficulties presented and a method of approach in differentiating this extramedullary myelopoiesis from malignant myelopoiesis have been pointed out by Barnes and Sisman (1).

The disappearance of the leukemoid reaction and splenomegaly after surgical removal of tumor tissue further correlates the myelopoietic activity with presence and growth of tumor. Although the accumulation of necrotic material in tumor cyst cavities has previously been thought to correlate with the leukemoid response, this study shows that continued injection of this material failed to produce a leukemoid response in similar host animals under similar conditions. Of the various fractions injected only the homogenate of fresh tumor produced a leukemoid response similar to that during actual tumor growth. This would seem to indicate the existence of a factor or substance that is a by-product or metabolic constituent of the tumor cell itself which is capable of stimulating granulopoiesis. The action of this substance may be direct or through an intermediate "effector" organ. The response is not that of mature granulocyte release, since single injections do not produce temporary increases in the granulocyte count. It would appear rather to be a constant increasing differentiation and proliferation of tissue with hematopoietic potential.

The appearance of proteins migrating in the \(a_2\) and \(a_4\) regions in the serum of tumor animals and the increase in \(\gamma\) globulin may represent nonspecific serum globulin changes associated with host reactivity to the tumor. However, there may be also a direct correlation with this and a tumor-produced granulopoietic factor. Further purification of these proteins and bio-assay are necessary.

There are many tumors either induced or of spontaneous origin in various strains of mice; yet a leukemoid blood picture has been reported in only a small percentage of them. Those noted to produce a leukemoid response have been a spindle-cell sarcoma, reported by Parsons (14), as associated with myeloid leukemia, and the fibrosarcomas and myoepitheliomas reported by Bateman (2). Extramedullary myelopoiesis in the liver and spleen of mice following inoculations of a transplantable tumor has been reported by Jaffe (7). Hueper (6) also found extramedullary myelopoiesis developed in mice with spontaneous breast tumors. Lewis (10, 11) working with transplantation of chemically induced (1, 2, 5, 6-dibenzanthracene) tumors in mice, reported a pronounced myeloid hyperplasia and splenomegaly in these animals.

The findings of this study are in agreement with those previously reported. Of particular importance is the correlation of leukemoid response with tumor duration, its subsidence with excision of tumor, and the inability to elicit a similar leukemoid response by injections of tumor necrotic material. In Bateman's (2) study, an attempt was made to obviate the possibility of a leukocytic response due to bacterial contamination of necrotic material by intense antibiotic treatment. This failed to influence the leukemoid response.

Hematopoietic effects of tumors in other animals and man have been reported, (5, 8, 12, 17), and leukemoid responses have been observed in association with various tumors (4, 9, 13). There appears to be no common de-
Fig. 8.—Paper electrophoresis of sera from tumor-bearing and normal control BALB/c mice, 16 hours at 3.0 ma., B-2 buffer veronal, pH 8.6.
TUMOR ANIMAL #5
WBC - 267,000
TUMOR GROWTH - 63 DAYS

TUMOR ANIMAL #4
WBC - 28,300
TUMOR GROWTH - 17 DAYS

CONTROL ANIMAL #2
nominate as to cell type. It would seem probable, therefore, that this response is not specific for any one cell type but rather is related to some metabolic activity of tumor cells. More specific characterization of the factor or factors responsible for the leukemoid response and study of the role of any tissues or organs mediating the response require further investigation.

ACKNOWLEDGMENTS

The authors wish to thank Dr. W. Ray Bryan of the National Cancer Institute and Dr. L. H. Smith of the Biology Division of Oak Ridge National Laboratory for their cooperation in supplying tumor-bearing mice. We are additionally grateful for the courtesy extended by Dr. W. Ray Bryan in making available laboratory records. We wish to thank Dr. Thelma Dunn of National Cancer Institute for her advice and for making available pathology slides of the original A-280 tumor-bearing animal. We are also grateful to Miss Patricia Kemper for technical assistance.

REFERENCES

A Study of the Leukemoid Response to Transplantable A-280 Tumor in Mice

E. J. Lappat and Madison Cawein

Cancer Res 1964;24:302-311.

Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/24/2_Part_1/302

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.