In Vitro Studies on the Anemia of Tumor-Bearing Hamsters

By JOSEPH D. SHERMAN, CARMEN RICKARD, ROBERT S. CHRISTIAN AND GILBERT H. FRIEDELL

THE POSSIBILITY that the anemia in some cancer patients was due to the hemolytic effect of toxins produced by the tumor was first analyzed by Weil in 1908. He demonstrated that extracts of normal human and animal organs had a lytic effect in vitro on red blood cells although there was considerable variability in the hemolytic potency of these extracts. Moreover, he reported that both necrotic and non-necrotic tumors had some hemolytic effect, with more hemolysis produced by the necrotic tumors. Weil concluded from these studies that the anemias associated with malignant tumors were probably due in part to the direct hemolytic action of products of necrosis in the tumor.

Gross reported that hemolysis of the donor's red blood cells could be produced in vitro by a saline-extracted, filtrable, thermolabile substance derived under aseptic conditions from mouse and human neoplasms. He extracted his lytic material from tumors which were primarily composed of viable-appearing tumor tissue. There was no separation of the extracts obtained from necrotic and from healthy tumor tissue. Other investigators have reported hemolysins to be present in extracts derived from tumor tissue. Ponder and Nesmith and Brüchmann and Westheimer suggested that these hemolysins were derived from the necrotic foci within growing tumors.

In studies reported by Sherman concerning the development of anemia in nontumor-bearing hamsters it was found that repeated injections of an extract of necrotic tumor tissue could produce an anemia in hamsters comparable to that found in tumor-bearing animals. It was also shown that repeated injections of an extract of apparently viable tumor tissue did not produce this effect. It was felt that the anemia was probably related to an immunologic response to the altered or "foreign" proteins found in the necrotic homologous tumor tissue. However, the possibility of direct destruction of red blood cells by lytic agents found in the necrotic portions of growing tumors could not be ruled out. It was, therefore, decided to investigate the hemolytic effect in vitro of extracts of whole tumor and also of extracts of both the necrotic and the viable portions of these same tumors when they were separately extracted. The hemolytic effect in vitro of extracts of normal organs from nontumor bearing hamsters was also studied.
ANEMIA OF TUMOR-BEARING HAMSTERS

MATERIALS AND METHODS

All extracts were prepared from a 100 per cent transplantable, methylcholanthrene-induced sarcoma of the hamster which has been serially propagated in our laboratory by transplants both in the cheek pouch and in the subcutaneous tissue of the flank. Flank tumors were used to prepare the extracts. The tumors had central areas of necrosis, but had not ulcerated the overlying skin of the flank.

Prior to removal of the tumor from the anesthetized donor hamster 0.5 to 1.0 ml. of blood was obtained by cardiac puncture. This blood was kept refrigerated in lightly heparinized tubes until used a few hours later. Red blood cells from these animals are referred to later as donor red blood cells (MCP Donor). The tumor was then removed, weighed, cut in half, and each half weighed separately. One half, containing both viable and necrotic portions, was used to prepare the extract of whole tumor (WT). The remaining half was dissected carefully into viable and necrotic portions. On gross examination the necrotic and viable portions are fairly readily distinguished from one another. However, in order to keep this distinction as sharp as possible, the tissue in the border zone between necrotic and viable tumor was dissected away from the remaining tumor, weighed and added to the half of the tumor used to make whole tumor extract.

The dissected necrotic tissue was then weighed and used in the preparation of the necrotic tumor (NT) extract. Similarly, the viable portion of the tumor was weighed and an extract (VT) was prepared.

Most of the extracts were prepared by adding 2 ml. of sterile saline to each g. of tissue used. The material was then placed in a Waring blender, and, after blending, the mixtures were centrifuged. The supernatant fluids were filtered through a Büchner funnel and 9 thicknesses of no. 42 Whatman filter paper. The filtrates were then passed through Seitz filters. The end products were sterile, cell-free, extracts of whole tumor (WT), necrotic tumor (NT), or viable tumor (VT). The extracts were kept in ice baths during the filtration and extraction procedures and after preparation were kept refrigerated. The time for preparing each of the tissue extracts in this fashion averaged 2½ hours.

Additional extracts using 4 ml. and 30 ml. of sterile saline per g. of tissue were also prepared in a similar fashion and some preliminary determinations of their hemolytic effect were made. The results, however, revealed that these extracts were not hemolytic, and further studies were not carried out.

In addition to erythrocytes obtained from the animal donating the tumor, red blood cells were obtained by cardiac puncture from other hamsters bearing the methylcholanthrene-induced sarcoma (MCP other), from hamsters bearing heterologous transplants of human epidermoid carcinoma (Deac 1)* and malignant melanoma (ME-1)* from hamsters bearing a malignant hamster melanoma (MM-2)* and from normal, nontumor-bearing hamsters. The blood from each animal was kept refrigerated in lightly heparinized tubes until its use within a few hours after it was drawn. Immediately before use the red blood cells were washed 3 times with saline and a 1 per cent saline suspension was made.

Duplicate 0.5 ml. samples of each of the viable, necrotic and whole tumor extracts made with 2 ml. of saline per gram of tissue were combined in small test tubes with 1.0 ml. of the red blood cell suspension prepared from animals in the above noted tumor-bearing and non-tumor bearing groups. After incubation at 37 C. for 2½ to 3 hours the tubes were centrifuged for 5 minutes at 3000 r.p.m. and then examined for hemolysis. Since some of the extracts were noted to be pink or red prior to combination with the erythrocyte suspension the degree of hemolysis if present was determined by the size of the white to pink halo around the button of packed erythrocytes at the bottom of the test tube.

In an effort to assess the potency of the hemolytic action of the extracts, 1:2, 1:4,

*Tumors were originally obtained from the laboratory of Dr. W. B. Patterson, Boston City Hospital.
1:8, and 1:16 dilutions of all three extracts (WT, NT, VT) were prepared. Duplicate samples of each extract were incubated with the various 1 per cent suspensions of hamster red blood cells and then read after centrifugation as described above.

The sterile, cell-free extracts of normal hamster organs were made in the same manner as the extracts described above. Two types of extracts were prepared. The first (extract I) was prepared from the livers of 3 normal, nontumor-bearing hamsters after the gall bladders were dissected away. The second extract (extract II) was prepared from the combined kidneys, adrenals, hearts, lungs, and spleens of the same 3 animals. The organs used for each extract were weighed, 2 ml. of sterile saline were added per gram of tissue, and tissue and saline were placed in a Waring blender. The resulting mixtures were centrifuged and filtered as described above, and then incubated with 1 per cent suspensions of erythrocytes freshly drawn from tumor-bearing and nontumor-bearing hamsters. The degree of hemolysis in each tube was determined by the same method as that followed when extracts of tumor tissue were used.

RESULTS

The proportion of viable to necrotic tumor varied in the tumors used (table 1). The relative amounts of each of these components is obviously of particular importance when the hemolytic effect of whole tumor extract is evaluated and when an attempt is made to interpret the contribution of each factor to the hemolytic reaction.

As seen in tables 2 and 3, the extracts from the viable portion of the flank tumors were hemolytic, whereas those from the necrotic portion of the tumors were not. The whole tumor extracts proved to be hemolytic in some cases, while in other cases they did not. An explanation for this apparent contradiction is found in the fact (table 1) that some tumors were composed largely of necrotic material, the fraction shown to have almost no hemolytic effect in vitro. Only slight hemolysis occurred at a 1:2 dilution with the active whole tumor extract.

With viable tumor extract the greatest hemolytic effect was noted on red blood cells from hamsters carrying the MCP tumor. Lysis occurred with red cells obtained both from donor animals from which tumors were taken for preparation of the extracts, and from nondonor animals that were also carrying the MCP tumor. Slight hemolytic effect by viable tumor extract was also noted on red cells from hamsters bearing hamster malignant melanoma, human malignant melanoma and human epidermoid carcinoma. This extract, however, had little effect on the red cells from normal, nontumor bearing hamsters. The hemolytic effect of the viable tumor extract decreased markedly when it was diluted. Only slight hemolysis occurred in 1:2 dilutions of the extract, and no hemolysis was noted in 1:4 dilutions.

The extracts of necrotic material prepared with either 2 ml., 4 ml., or 30 ml. of saline per Gm. of necrotic material, produced no hemolysis when incubated with the whole range of red cells. Dilutions of the most concentrated necrotic extract likewise failed to produce hemolysis.

The hemolytic effect in vitro of the sterile, cell-free extracts of organs from normal, nontumor-bearing hamsters is shown in table 4. The liver extract (extract I) produced marked hemolysis of a fairly uniform type on all hamster red blood cell suspensions. Extract II produced hemolysis of all of the test red blood cell suspensions, but to a lesser degree than the liver extract and to a lesser degree than the viable tumor extracts.
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Table 1.—Weights of Necrotic and Viable Portions in Half of the Selected Tumors

<table>
<thead>
<tr>
<th>50 Day Flank Transplant</th>
<th>Half-Tumor</th>
<th>Necrotic Portion</th>
<th>Viable Portion</th>
<th>Borderline* Portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor A</td>
<td>22.3 Gm.</td>
<td>6.4 Gm.</td>
<td>14.9 Gm.</td>
<td>1.6 Gm.</td>
</tr>
<tr>
<td>Tumor B</td>
<td>29.6</td>
<td>19.6</td>
<td>9.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*This tumor tissue lying between necrotic and viable portions was used in preparation of whole-tumor extract.

Table 2.—Degree of Hemolysis Produced by Viable, Necrotic and Whole Tumor Extracts from Tumor A on a Battery of Test Red Blood Cells

<table>
<thead>
<tr>
<th>Extracts</th>
<th>MCP (Donor)*</th>
<th>MCP (Other)*</th>
<th>MMC†</th>
<th>ME††</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Tumor</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Viable Tumor</td>
<td>2</td>
<td>±</td>
<td>0</td>
<td>±</td>
<td>0</td>
</tr>
<tr>
<td>Necrotic Tumor</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Hamster methylcholanthrene-induced sarcoma. Donor RBC's represents animal bearing tumor from which extract was made. Other is hamster with same tumor but not used to make extract.
†Hamster malignant melanoma.
††Human malignant melanoma.

Discussion

These studies in vitro demonstrating the presence of a saline extractable, hemolytic factor in this transplantable hamster sarcoma, confirm the findings of others that extracts of tumor possess hemolytic capabilities. Our data, however, are not in agreement with the results of Ponder and Nesmith who found maximal hemolytic activity in necrotic material from the center of the tumors used. Another point of disagreement is the finding by these authors that tumor homogenates of C3H mouse tumor usually contained a greater concentration of lysin than did those prepared from normal tissues. In our studies, the extracts of normal liver tissue produced as much hemolysis as did the extract of viable tumor tissue. The rather marked hemolytic effect of the liver extract is probably due to the bile salt content of this organ.

The hemolytic effect of the combined heart, lung, kidney, spleen, adrenal extract is less easily explained.

As noted briefly before, the apparent discrepancy between the effects of whole tumor extract from one tumor and that from another tumor is rapidly resolved if one refers to table 1. The relative amounts of necrotic material, the component which appears to have the least hemolytic potency, and of viable tumor, the component with the greatest effect, would seem to determine the hemolytic potency in vitro of the whole tumor extract. Thus, where the tumor is composed in large part of viable tissue, the hemolytic effect will be at its maximum.

The presence of hemolysins in the viable portion but not in the necrotic portion of the tumor used in this study may also hold for other tumors and other species. The differing results of other investigators studying the hemolytic effect of extracts of whole tumor may perhaps also be explained on
TABLE 3.—Degree of Hemolysis Produced by Viable, Necrotic and Whole Tumor Extracts from Tumor B on a Battery of Test Red Blood Cells

<table>
<thead>
<tr>
<th>Extracts</th>
<th>MCP (Donor)*</th>
<th>MCP (Other)*</th>
<th>Red Blood Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Tumor</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Viable Tissue</td>
<td>3</td>
<td>3</td>
<td>2, 3</td>
</tr>
<tr>
<td>Necrotic Material</td>
<td>0 ±</td>
<td>0 ±</td>
<td>± ± ±</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Hamster methylcholanthrene-induced sarcoma. Donor RBC's represents animal bearing tumor from which extract was made. Other is hamster with same tumor but not used to make extract.

†Hamster malignant melanoma.
‡Human malignant melanoma.
§Human epidermoid carcinoma.

TABLE 4.—Degree of Hemolysis Produced by Extracts of Normal Hamster Organs on a Battery of Test Red Blood Cells

<table>
<thead>
<tr>
<th>Extract</th>
<th>MCP (Other)*</th>
<th>Red Blood Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (Liver)</td>
<td>3</td>
<td>3, 3</td>
</tr>
<tr>
<td>II†</td>
<td>1</td>
<td>1, 1</td>
</tr>
</tbody>
</table>

*Hamster methylcholanthrene-induced sarcoma. Donor RBC's represents animal bearing tumor from which extract was made. Other is hamster with same tumor but not used to make extract.

†Hamster malignant melanoma.
‡Human epidermoid carcinoma.
§Combined kidneys, adrenals, hearts, lungs and spleens.

the basis of varying proportions of viable to necrotic tissue in the tumors used.

The results obtained by Greenfield, Sterling and Price⁶ using radioactive iron (Fe⁵⁹) may possibly be explained by the presence of hemolysins in viable tumor tissue. They found a rather high concentration of radioactive iron in tumors which showed very little necrosis, a finding which may well have resulted from hemolysis of red blood cells within the tumor by hemolysins produced by the growing viable tumor. The failure to find increased amounts of radioactive iron in the spleen, liver and other components of the reticuloendothelial system was interpreted by these investigators as evidence against a tumor-produced hemolysin acting systemically within the vascular system or with the assistance of the reticuloendothelial system. Their interpretation is supported by the findings of this study that the optimal hemolytic effect is obtained with the highest concentration of viable tumor extract, and that all hemolytic activity is lost by the effective extracts when dilutions of 1:4 or higher are prepared. Thus, it would seem highly unlikely that the hemolytic factor found in tumor extracts could be effective in low dilutions within the blood stream, and most likely that the hemolysin produced by viable tumor functions within the tumor.

According to studies in vivo reported elsewhere,¹⁸ however, necrotic tumor tissue is a most important factor in producing the anemia found in tumor-bearing hamsters. This effect, unlike the one discussed above, is a
systemic effect involving the reticuloendothelial system, and apparently is based on an antigen-antibody response. A sterile, cell-free filtrate of necrotic tissue obtained from the hamster sarcoma used in the above in vitro experiments was shown to produce a significant degree of anemia when injected into non-tumor bearing hamsters. Histologic examination of the enlarged spleens from the injected group of animals revealed marked reticuloendothelial hyperplasia, proliferation of plasma cells and their precursors and extramedullary hematopoiesis of all blood cell elements. The livers also showed reticuloendothelial hyperplasia although to a lesser degree, and some amount of extramedullary hematopoiesis. These changes were comparable to those seen in anemic, tumor-bearing hamsters, although not quite as pronounced.

Anemia of the same degree was not produced by the repeated injection of a sterile, cell-free extract of viable tumor tissue, although there was a slight drop in average hemoglobin values. Similarly, although there was some histologic evidence of reticuloendothelial hyperplasia and extramedullary hematopoiesis in these animals, it was seen much less frequently and to a lesser degree than in those receiving injections of necrotic tumor extract.

It would, therefore, appear that there may be at least two mechanisms operable in producing the anemia in tumor bearing hamsters—a direct hemolytic effect occurring in or near the tumor itself, and a more gradual effect mediated through the reticuloendothelial system, plasma cells, and some antigen-antibody reaction affecting the red blood cells. Radioactive iron \((\text{Fe}^{59})\) studies suggest that in animals with advanced tumors of this type, containing large areas of necrosis, the second mechanism is a more important one than the first in the production of anemia.

**Summary**

1. Sterile, cell-free extracts of the viable portion of a methylcholanthrene-induced sarcoma of the hamster were capable of hemolyzing in vitro hamster red blood cells from the donor animals, and from animals with homologous and heterologous tumors.
2. Sterile, cell-free extracts of the necrotic material from this same tumor had little in vitro hemolytic action.
3. Whole tumor extracts varied in their in vitro hemolytic activity depending upon the proportion of viable to necrotic tissue present, with the maximum hemolysis observed when the whole tumor contained more viable than necrotic tissue.
4. Sterile, cell-free extracts of normal hamster liver had a strong hemolytic action on a whole range of red blood cells.
5. Hemolysins elaborated by the viable tissue in transplanted hamster tumors may be one factor contributing to the anemia in hamsters bearing transplantable sarcomas.

**Summario in Interlingua**

1. Sterile extractos sin contento cellular, obtenite ab le portion viabile de sarcomas inducite in hamster per methylcholanthrena, se provava capace a hemolysar in vitro erythrocytos de hamster ab le donatores mesme e ab animales con tumores homologe o heteroloe.
2. Sterile extractos sin contento cellular, obtenite ab le portion necrotic del mesmo tumores, mostrava pauc activitate hemolytic in vitro.

3. Extractos ab tumores total variava in lor activitate hemolytic in vitro secundo le proportion inter histo viabile e histo necrotic in illos. Grados maximal de hemolyse eseva observate quando le tumor contineva plus histo viabile que histo necrotic.

4. Sterile extractos sin contento cellular, obtenite ab normal hepates de hamster, habeva un forte action hemolytic super un grande varietate de erythrocytos.

5. Hemolysinas elaborate per le histos viabile in transplantate tumores de hamster es possibilemente un del factores contribuente al causation del anemia in hamsters que forma sarcomas de typo transplantabile.

REFERENCES
1. Adelsberger, L.: Differences in immunologic reactions of red blood cells of a tumor-susceptible (C3H) and a tumor-resistant (C57) mouse strain. Cancer Res. 11:653-657, 1951.

2. —: Differences in the hemolytic behavior of red blood cells of a tumor-susceptible (C3H) and a tumor-resistant (C57) mouse strain. Cancer Res. 11:658-662, 1951.


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