Quantitative Studies on Circulating Cancer Cells in the Mouse

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SUMMARY

Varying numbers of cells of two isologous tumors were injected intravenously into groups of mice, and a nearly complete disappearance of the cells was noted after 3 minutes. Only when over 500,000 cells were injected could cells be regularly found in the blood after 3 minutes.

Blood obtained at 1, 3, and 10 minutes following the intravenous injection of a large number of tumor cells showed that most of the cells were removed in 1 minute and virtually all were removed in 10 minutes.

The disappearance rate with a homologous tumor was essentially similar to that with the isologous tumors, and the addition of heparin to these cells did not alter their rate of disappearance. No relationship could be shown between the removal of the tumor cells from the blood and the metastatic potential of the tumor.

The possible significance of such cells in animals bearing primary tumors has been discussed, and certain parallels have been drawn with clinical studies.

A commonly used procedure for the study of metastasis in experimental animals has been the intravascular injection of tumor cells (1, 3, 8). The numbers of cells required to produce a metastasis and the organs in which such metastases occur have been studied in several tumor-host systems. However, little is known about the length of time these cells continue to circulate or, conversely, how rapidly they are removed from the circulation. Also, no relationship has been established between the number of cells injected and the rate of their removal.

It would seem desirable in a study concerned with these questions also to have information on the viability of the injected cells, since the rate at which the cancer cells are removed from the circulation may be related to the integrity and viability of the cells. Another conceivable influence could be genetic. That is, the behavior of the host toward circulating cancer cells may be different in a homologous system from what it is in an isologous or even an autogenous one. The following studies were undertaken in an attempt to answer some of these questions with a system used which resembled as closely as possible that which exists in spontaneous tumor metastases.

MATERIALS AND METHODS

Single cell suspensions were injected intravenously into mice using various concentrations of cells. At precisely timed intervals, blood was obtained from the intact animals and examined for tumor cells by a quantitative technic. Tumors of both isologous and homologous types were used. In one case the possible effect of heparin was tested.

In selecting tumors for this study we have used the following criteria: (a) the cells must be large and easily identified; (b) they are not normally found in the circulating blood; and (c) single-cell preparations can be obtained. Two isologous tumors of recent origin in an inbred mouse strain and one homologous tumor were used. One of the isologous tumors, MPC-3, is a plasma-cell tumor which arose in BALB/c mice following intraperitoneal implantation of millipore filter chambers

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containing Sarcoma 37. This was used from its 46th through 53rd transplant generation. It is a widely metastasizing tumor when inoculated by the intravenous route but not by the subcutaneous route, and has been carried in both the solid and ascites form. The other isologous tumor, MS-2, is a methylcholanthrene-induced sarcoma in BALB/c mice and was used from its 21st through 24th transplant generation. It does not metastasize from subcutaneous solid tumors but has been found to give rise to lung metastases following intravenous injection. It has no ascites form. BALB/c mice were used with these tumors as both donor and recipient animals. The third tumor, a homologous tumor, is the ascites form of Ehrlich sarcoma. Swiss mice were employed with this tumor as donor and recipient animals. The cells of each of the three tumors are large enough to be easily seen when scanned against a background of leukocytes.

Heparinized ascitic fluid obtained 8–11 days after transplantation was the source of MPC-3 cells, and nonheparinized ascitic fluid obtained 14 days after transplantation, the source of the Ehrlich cells. Viable MS-2 cells were obtained in single-cell suspension from solid tumors by an enzymatic method described elsewhere (6). All dilutions were made in a modification of Earle's salt solution.2 Inoculations were made into the tail vein in volumes between 0.1 and 0.25 ml., and cell counts were made in a standard hemocytometer before and after each series of injections. The pre- and postinoculation counts were considered necessary not only to increase the accuracy of counting but also to insure that the cell concentration had not changed during the time of inoculations.

We have employed the exclusion of trypan blue from the cell as the criterion for viability. Although viability in the sense that each cell which excludes dye is potentially able to form a metastasis is certainly not proved, there is ample evidence by our microscopic observations that stainable cells are physically altered. These alterations vary from loss of cytoplasmic detail and membrane clarity to fragmentation and disintegration. The data of Kaltenbach (4) would also suggest that stainable cells have ceased to respire. The cell viability index was determined in every case by trypan blue. This was used in the calculation of the cell count so that the counts were considered to represent the number of viable cells present.

Since cell counts were made on wet preparations, the distinction between tumor cells and other normal cellular elements had to be made. This was especially true for the ascites preparations. In most cases the difference in cell size facilitated this distinction. By comparing stained millipore filter preparations, the morphologic characteristics of the tumor cells and normal cellular elements could be clearly established.

Most experiments involved the use of MPC-3 and MS-2 cells. One minute following tumor cell inoculation the mice were etherized and blood was collected from the right heart chamber into a heparinized syringe. The total elapsed time from inoculation to obtaining blood was usually between 3 and 3½ minutes, but in no case exceeded 5 minutes. Four to seven mice were used in each experiment. The weights of the animals ranged between 21 and 30 gm. In one experiment using the MPC-3 tumor, blood was obtained at 1 minute and at 10 minutes, respectively, following inoculation. In the 1-minute series the animals were killed by neck fracture. An additional experiment involved the injection of Ehrlich ascitic cells into two groups of Swiss mice. In one group the cells were heparinized (100 units to 20 million cells), and in the other they were not. The mice were sacrificed 3 minutes after tumor cell injection.

The volumes of blood obtained were between 0.4 and 0.6 ml. To this blood 45 ml. of 1 per cent acetic acid was added rapidly with shaking. Following centrifugation at 200 X g for 10 minutes the pellet was broken up with a syringe and 3-inch #15-gauge needle and washed once in a similar volume of 0.9 per cent saline. The final pellet was resuspended in 5 ml. saline, and 5 ml. of 10 per cent formalin was added. This final suspension was then collected on a type SM millipore filter at 10 mm Hg. pressure. All filters were stained by the Papanicolaou method, mounted, and scanned at 10 mm Hg. pressure. All filters were stained by the Papanicolaou method, mounted, and scanned for tumor cells. Cells identified by one of us (REM,) as suspicious or positive were examined by the other (RAM) for final interpretation.

In a control experiment to establish the quantitative recovery of tumor cells, varying numbers of MPC-3 cells were added to 0.5 ml. mouse blood, which was then processed as above.

RESULTS

Control recovery of cells.—The recovery of known numbers of MPC-3 cells added to mouse blood in vitro is shown in Table 1. It is seen that the method employed permits nearly quantitative recovery at several concentrations of cells. Careful handling of the specimen in transfer and mixing and the use of clean glassware were observed. With these precautions the use of the millipore filter proved not only technically simple and rapid, but reliably quantitative. Quantitative cell re-
covery, with the use of the millipore filter in a method for human blood specimens, has been previously reported (7).

Cell recovery following injection of varying numbers of cells.—Several groups of mice were given injections of increasing numbers of single tumor cells and sacrificed in 3–5 minutes. This was done with the two isologous tumors, and the results are shown in Table 2. Blood of control animals in which no tumor cells were injected failed to show circulating abnormal cells. Figures 1 and 2 show the appearance of MS-2 and MS-3 tumor cells, respectively, on the Papanicolaou-stained milli-

TABLE 1

<table>
<thead>
<tr>
<th>Cells added</th>
<th>Cells recovered</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>902 904</td>
<td>903</td>
</tr>
<tr>
<td>500</td>
<td>454</td>
<td>454</td>
</tr>
<tr>
<td>100</td>
<td>92 92 94</td>
<td>92</td>
</tr>
<tr>
<td>50</td>
<td>46 45 52</td>
<td>48</td>
</tr>
</tbody>
</table>

Virtually all the cells were removed from the circulation, even at the massive and often lethal dose of $5 \times 10^8$ cells. In these latter animals, a cardiac decompensation appeared to be taking place. Although only surviving animals were recorded, it was noted that, in several of these and in those that died, the right side of the heart was dilated. Pulmonary blockade by tumor cells had apparently caused cor pulmonale.

Cell recovery following varying time intervals.—Detection of isologous tumor cells in the blood at 1 minute and 10 minutes after intravenous injection is shown in Table 3. Five million cells were injected not only because this appears to be the maximal possible dose, but also because it was the only level at which substantial numbers of cells were present in the blood after 3 minutes. Again a wide range of cell recovery was noted. A rapid decrease in the number of circulating cancer cells was seen between 1 and 3 minutes. A much slower rate of removal was noted by comparing the 3-minute and the 10-minute series, and by the latter time the blood of two of five mice was negative.

Cell recovery with homologous cells with and without heparin.—In a final experiment a high dose, five million cells, of the homologous Ehrlich ascites tumor was injected into a series of four Swiss mice. These cells were collected without the use of heparin. An average of 23 cells was recovered in

| 0.4–0.6 ml. blood obtained per mouse.

TABLE 2

TUMOR CELLS FOUND IN BALB/c MOUSE BLOOD* 3–5 MINUTES FOLLOWING GRADED INTRAVENOUS INOCULATION

<table>
<thead>
<tr>
<th>Cells inoculated</th>
<th>MPC-3</th>
<th>MS-2</th>
<th>Average</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>50,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>100,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>500,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,000,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5,000,000</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| 0.4–0.6 ml. blood obtained per mouse.

TABLE 3

TUMOR CELLS FOUND IN BALB/c MOUSE BLOOD* 1, 3, AND 10 MINUTES FOLLOWING INTRAVENOUS INOCULATION OF 5,000,000 MPC-3 TUMOR CELLS

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Tumor cells found</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80 80 59 10 90</td>
<td>68 59 100</td>
</tr>
<tr>
<td>3</td>
<td>82 84 43 8 6</td>
<td>64 64 43 8 6</td>
</tr>
<tr>
<td>10</td>
<td>82 82 6 4 6</td>
<td>82 82 6 4 6</td>
</tr>
</tbody>
</table>

| 0.4–0.6 ml. blood obtained per mouse.

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0.4–0.6 ml. of blood after 3 minutes. A comparison of the range of cell recovery at the same dose and postinjection interval with that of the isologous tumors indicates that the behavior is essentially similar. Since a possible heparin effect was sought, a second series of mice was similarly given injections in which the cells were pretreated with heparin. The average recovery after 3 minutes was nineteen cells. It was concluded that no significant alteration in cell recovery had occurred.

DISCUSSION

The results in all three mouse tumors studied indicate an extremely rapid rate of removal of circulating tumor cells. Although the mechanism of removal is not known, it is quantitatively similar for three dissimilar tumors. There is apparently a very wide range over which this removal mechanism operates, and it is only when the cell dose becomes massive, almost lethal, that tumor cells are repeatedly seen in the blood.

A wide variation in the absolute number of cells recovered was noted between mice of the same series. Although the mice in a given series were of the same age and sex, and although the experiments were carried out as identically as possible, the rapid early decline in the number of circulating cells suggests that differences of a few seconds in the time from tumor injection to bleeding may be responsible for this variation. In terms of the number of cells injected this variation in the number of cells removed is small.

Should an animal bearing a primary tumor display a constant malignant cytemia, then the rate of cancer cell release into the circulation would have to equal or exceed the cell removal rate. If, for example, $5 \times 10^6$ MPC-3 cells are released into the blood stream we could expect only one to three cells in one-third of the total blood volume after 3 minutes (see Table 2). However, the packed cell volume of this tumor has been determined to be $5 \times 10^6$ cells/cu mm. Thus, the tumor volume removed, if the removal rate were constant at this high early level, would be 20 cu mm/hour or nearly 0.5 cu mm/day. This surely exceeds the growth rate of the primary tumor itself and therefore obviously does not occur. One can assume that maximal removal occurs, if ever, only during momentary showers of cells, and yet such massive release of cells appears to be necessary to permit recovery of circulating cancer cells, since injection of less than 500,000 cells did not result in an average recovery of any tumor cells. Perhaps after repeated showers of cells the mechanism for removal becomes blocked and allows circulating cancer cells to persist, but this is a matter for further study. In addition, if cells are to be found in animals bearing these tumors, not only must showering of tumor cells probably occur, but the time when the blood samples are removed must be fortuitous. It is apparent from Table 3 that, 10 minutes after the inoculation of 2.5 million cells, only a few can be demonstrated.

On the other hand, Moore et al. (8), using different technics and different tumors, have recently demonstrated the presence of malignant cells in mouse blood up to 72 hours after inoculation. Such cells, of course, could have been in continuous circulation, but they may also have been arrested and re-released. Since the present study, which depends upon the morphological demonstration of cancer cells, was not extended beyond 10 minutes after tumor cell inoculation, discussion of this question is only speculation.

Zeidman (12) and Wood (10) have demonstrated by direct cinematography the capillary arrest of single cells of transplantable tumors in the rabbit. According to Zeidman both the size of the cell and the tumor type are important factors. Although most of the cells he observed passed the mesenteric capillaries, perhaps with continuous recirculation these cells also would have rapidly disappeared from the blood.

It was felt initially that metastasizability might be related to the removal rate of circulating cells. Two of the tumors studied, MPC-3 and MS-2, have been shown to metastasize widely following intravenous inoculation. It has been our experience and that of others that Ehrlich sarcoma does not tend to produce metastasis following intravenous injection. Yet all three tumors displayed a similar rate of removal from the blood stream. It would appear in the case of the mouse tumors studied that cells will be rapidly removed from the circulation regardless of metastatic potential and genetic background.

Heparin has been shown to affect metastasizability (11), probably by altering the thrombus formation at the point of intravascular arrest of the malignant cells. If the cells failed to stick to the vessel wall, they might continue to circulate. One might, therefore, expect to find more tumor cells in the blood of an animal receiving heparin. As shown above, no effect of heparin on the number of tumor cells found in the blood was demonstrable.

Numerous clinical reports on circulating cancer cells have appeared since Engel's monograph (2) was published. There are inherent difficulties in
doing controlled studies on human material which can be overcome to some extent in animal experimentation. It is, however, difficult and often risky to generalize excessively from the behavior of animal tumors to the clinical behavior of human tumors. In the present studies two of the tumors used, MPC-3 and MS-2, were of recent origin and isologous to the inbred strain in which they were used. Thus, there was at least a genetic similarity to the clinical situation of the spontaneous cancer.

Some parallelism to the present animal work can be seen in two clinical reports. In the first (5) signs and symptoms of pulmonary embolization have been associated with large numbers of circulating cancer cells. This had been observed in our animals in which a very large concentration of cells was used. In the other report momentary showering of malignant cells into the blood was associated with uterine curettage in patients with uterine malignancy (9). This can probably be correlated with the present findings of a rapid disappearance of cancer cells from the blood of experimental animals.

REFERENCES


FIG. 1.—An MS-2 tumor cell is shown among lymphocytes and polymorphonuclear leukocytes. Note the size difference between the tumor cell and the white cells. The prominent nucleoli and chromatin clumping are also evident. Papanicolaou stain, mag. X725.

FIG. 2.—An MPC-3 tumor cell is shown among lymphocytes and polymorphonuclear leukocytes. Note the prominent nucleoli and chromatin clumping of the tumor cell. Papanicolaou stain, mag. X800.
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