Growth Characteristics of Free C1498 (Granulocytic Leukemia) Tumor Cells in the Peritoneal Fluid and the Blood of C57 Mice*

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Subcutaneous implants of C1498 tumor grow as diffuse, soft tissue, with indefinite edges, closely adherent to the skin, spreading into muscle and invading peripheral blood and hematopoietic organs. The histologic type of tumor and the cytotologic characteristics of tumor cells in the blood were identified as granulocytic leukemia. The tumor was used for immunogenic (6) and immunological (7) studies. It seemed to us that various growth types of these cells—tumor tissue formation, blood invasion, infiltration of organs, growth in the pleural exudate (2)—indicate their exceptionally high ability to grow and to migrate as free cells in body fluids. We have studied, therefore, the growth potentialities of C1498 tumor cells, using our method of free tumor cell culture in peritoneal fluid (1). Moreover, in an attempt to assay the effect of a therapeutic agent on this tumor strain, we have treated animals bearing neoplastic cells with colloidal Au+98 and have recorded parallel changes in survival of the host and in tumor cell counts in the blood and peritoneal fluid, as outlined in our work on lymphatic leukemia in mice (3).

MATERIALS AND METHODS

Tumor and animals.—C1498 tumor (551st transfer generation) was obtained from the R. B. Jackson Memorial Laboratory, where it had spontaneously originated in 1941. C57BL/6 mice, were supplied by the same laboratory.

Technics.—We have previously (1–5) described our methods of suspending tumor tissue or spleen cells for inoculation, of counting and computing the number of cells in the inoculum, of withdrawing peritoneal fluid for repeated cell counts, of testing the cell viability in the inoculum, and of counting the total number of cells and the proportion of tumor cells in specimens of peritoneal fluid withdrawn from inoculated mice.

Serial transfers in peritoneal fluid.—Eight to 10 days after six mice were given an intraperitoneal inoculation of a suspension of ground tumor tissue, specimens of pooled peritoneal fluid from these mice (0.5 ml. of 1:5 dilution) were injected into five new mice. In this way more than twenty serial intra-peritoneal transfers were carried out and used as material for inoculation of tumor cells. Smaller inocula of these cells were prepared by serial dilution of peritoneal fluid.

Pattern of the experiment.—Groups of ten or twenty mice were inoculated intraperitoneally (i.p.) or subcutaneously (s.c.) with requisite numbers (computed by diluting spleen cell suspension or peritoneal fluid) of tumor cells. In i.p. inoculated mice, the data were recorded on (a) the total number of cells and on the percentage of tumor cells counted in specimens of peritoneal fluid and blood which were withdrawn at various intervals, (b) the infiltration of organs with tumor cells as found at autopsy, and (c) the mortality of mice after inoculation. The results of s.c. implantation were recorded on the same lines, except for examination of the peritoneal fluid. Each experiment was repeated at least 3 times, and the results were pooled and plotted in graphs. The pattern of each group of experiments varied as to route of inoculation (s.c. or i.p.), source of the inoculum (spleen or peritoneal fluid), and its size (15 × 10⁴ to 10⁶ cells).

Therapeutic assay of radioactive colloidal gold.—Twenty or 40 mice were inoculated i.p. with high doses (10⁴ or 10⁵) of tumor cells; 4 days later, one-half of mice in each group received 0.2–0.4 mc. of colloidal Au+98, while the same number of simultaneously inoculated mice remained untreated (controls). Specimens of peritoneal fluid and of blood from both groups were withdrawn at various intervals and counted; the pooled results and the data on mortality in both groups were plotted in graphs.

RESULTS

Growth of free C1498 tumor cells in the peritoneal fluid.—It is possible to withdraw from the peritoneal cavity of normal C57 mice about 0.1 ml. of fluid containing an average (of ten mice) 7,500 cells/cc (with the extremes of 4,300 to 115,200), mostly small lymphocytes (35–67 per cent throughout), large monocytes including macrophages (29–61 per cent), and a few polymorphonuclears (up to 5 per cent) and mast cells (0.8 to 3 per cent). After i.p. inoculation of 15 ×

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$10^6$ or $10^7$ cells from the spleen or from the peritoneal fluid, there was a rapid increase in the volume and the cellular content of peritoneal fluid (Chart 1, solid graphs) and, moreover, in the proportion of tumor cells in the cellular composition of the fluid (Chart 2, solid graphs). Numerous mitoses reaching their maximum (14–22 per cent of all tumor cells) on the sixth or the seventh day presented evidence of intense tumor cell proliferation in the peritoneal fluid. As a rule, the accumulation of cells in the fluid proceeded at a higher rate and reached a higher level in mice inoculated with higher numbers of tumor cells. However, this correlation was only approximate and subject to variations, since total cell counts reflected not only tumor cell content in the fluid, but also leukocytic reaction and diluting effect of accumulated serous exudate. On the contrary, the percentage of tumor cells in the fluid was consistently proportionate to the number of inoculated tumor cells (Charts 2 and 3). Accordingly, the concentration of tumor cells in the fluid was considered as a reliable indicator of their growth level.

Besides the size of the inoculum, its source, either from the spleen (S cells) or from the serial i.p. transfers in the fluid (F cells), was another factor determining the growth rate of inoculated cells, as it appears from the comparison of solid graphs S and F in Charts 1 and 2. The rate of growth for F cells was higher than for S cells, but the same maximum of 90–98 per cent of tumor cells was reached on the 8th day (Chart 2), independently from the source of growth.

In two groups each of twenty mice inoculated with $15 \times 10^6$ F cells (Chart 2), differential cell counts were carried out, beginning 80 minutes after inoculation (not after 2 days as in all other experiments), at intervals of 2–4 hours (night interval of 8 hours) until the 3d day. It was found that in all mice, without exception, the inoculated tumor cells almost disappeared from the peritoneal fluid (which was scanty in amount) at the end of the first 24 hours, but reappeared the next day at a high rate parallel to rapid accumulation of serous exudate. At the autopsy of ten animals sacrificed...
after 20–24 hours, i.e., at the lowest concentration of tumor cells in the fluid, many of these cells were found in the tissue of abdominal lymph nodes. Thus, the inoculated cells passed, before their multiplication, through a lag period or a stage of latency (adjustment to the new environment).

It was consistently recorded that the majority of the leukocytes found in the peritoneal fluid during proliferation of tumor cells from the inocula of $10^6$ cells or more were polymorphonuclears (about 90 per cent), while large monocytes were rare (less than 10 per cent), and the lymphocytes scanty or absent. With the decrease in the size of the inoculum, the proportion of large monocytes and lymphocytes rose, so that during tumor cell growth resulting from an inoculum of $10^6$ cells, the lymphocytes were often the predominant type of the leukocyte.

**Blood invasion by tumor cells from peritoneal fluid.**—As in the peritoneal fluid, the concentration of tumor cells in the blood was proportional to the number of i.p. inoculated cells (Charts 2 and 3). Accordingly, the maximum level of cell concentration in the blood, which was relatively low even for mice with large inocula (not more than 50 per cent for those inoculated with $15 \times 10^6$ cells—Chart 2, dotted graphs), was still lower (less than 20 per cent) for smaller inocula (Chart 3, dotted graphs). There was a constant relationship between the maximum level of tumor cell growth in the fluid and in the blood. However, seven out of eight dotted graphs of Charts 2 and 3 show the tendency of tumor cells to disappear from the blood stream, partly or entirely, (in mice with small inocula), temporarily or definitely.

In mice inoculated s.c. with graded numbers of tumor cells, the proportion of tumor cells in the blood was always higher than in analogous groups of i.p. inoculated animals, but their level showed the same trend to variations.

The changes in leukocyte formula of the blood of inoculated mice followed the pattern observed in peritoneal fluid.

**Relationship between the growth rate and growth site of tumor cells and the mortality rate of their host.**—Proliferation of tumor cells in the peritoneal fluid was terminated by death in all 200 mice inoculated i.p. with not less than $10^6$ cells, in 38 out of 40 mice inoculated with approximately $10^5$ cells, in 32 out of 40 with approximately ten cells, and in five out of twenty inoculated with the last dose 10 times diluted (i.e., presumably one cell). The comparison of Charts 2 and 3 with Chart 4 illustrates the close parallelism between tumor cell growth rate and mortality rate for each group (inoculated with the same number of cells). The lethal effect of tumor cell proliferation on the host was due to progressive invasion of the blood and the hematopoietic organs by high numbers of tumor cells. Autopsy findings showed that i.p. inoculated tumor cells had already begun to invade organs at the early stage of their proliferation in the peritoneal cavity, when the level of tumor cells in the blood was low. In s.c. inoculated animals, this invasion occurred at a much later stage, presumably only by blood route. These data may account for the relatively late death of these animals.

![Chart 4](image)

**DISCUSSION**

The term “tumor cells” implies tumor tissue cells as a rule. Tumor cells growing free in the peritoneal fluid were described recently as special types of tumors (ascites tumors [5], Yoshida tumor [9]), while invasion of the blood stream...
with tumor cells is considered as still another type of neoplasm—the leukemias. The data of our previous work (1, 2) suggested that free growth in the fluids of serous cavities—peritoneal and pleural—is a specific potentiality of any malignant cell and not of special tumor types. Moreover, it has been shown that proliferation of tumor cells in the peritoneal fluid can be associated with their passage in the blood stream (3). The data recorded above illustrate the effect of experimental growth factors—size and source of the inoculum and route of inoculation—on various growth manifestations of granulocytic tumor cells—free growth in peritoneal fluid, leukemia, tissue infiltration—and, moreover, on the relationship between tumor cell growth and host mortality. The rate of tumor cell proliferation in the peritoneal fluid was the essential factor of blood invasion and infiltration of hematopoietic organs. It was closely paralleled by the rate of host mortality.

It was possible to obtain abundant growth in the peritoneal fluid from the inocula of 1 ml. prepared by 1:30 \( \times 10^6 \) and 1:30 \( \times 10^6 \) dilution of peritoneal fluid containing 30 \( \times 10^6 \) tumor cells, i.e., by implantation of approximately ten and one cells. Previously, Kahn and Furth (4) succeeded in producing lymphatic leukemia in AK4 mice with a single cell, and Yoshida (9) reported free tumor growth from a single cell of Yoshida's tumor. This growth from very small inocula, as well as the ability to reach consistently the concentration of almost 100 per cent in the peritoneal fluid, gives evidence of exceptionally high growth potency of granulocytic tumor cells. Their use is therefore indicated for all studies requiring a nearly "pure culture" of free tumor cells, such as biochemical analysis (5) or virus cultures.

The higher growth potency of free tumor cells, as compared to those grown in the spleen, implies that the tissue tumor cell needs to adjust its nutritional requirements, surface activity, and other factors, in order to start rapid free growth in the fluid. Tumor cells in the blood showed a very low proportion of mitoses (1 to 3 per cent), apparently because during their transit from the peritoneal fluid or from a s.c. tumor to the organs they had not had sufficient time to adjust themselves to the blood plasma as a medium.

In the peritoneal fluid the percentage of leukocytes was inversely proportional to the number of inoculated tumor cells and, therefore, to their rate of multiplication. Since numerous leukocytes are found in the fluid of untreated mice or those injected with any proteinic material, their disappearance in proportion to tumor cell growth may be attributed to changes induced in the fluid by growing cells.

In our study of AK4 leukemic cells (8), we have shown that they have to reach a sufficiently high level of growth in the peritoneal fluid (as a rule, on the 5th day) in order to invade the blood in high numbers. An analogous phenomenon occurs in granulocytic leukemia but only with very high i.p. inocula (over 10^6, Charts 1 and 2). Smaller inocula induce blood invasion with tumor cells only intermittently or irregularly. We have presumed (2) that the passage of AK4 tumor cells from the site of their initial multiplication—the peritoneal cavity—to their definitive destination in the organs is regulated by natural barriers between peritoneal
fluid and the blood and between the blood and the organs. In following the same line of interpretation, it can be inferred that early appearance of tumor cells in the blood and the organs was due to early relaxation of both barriers, and that the intermittent or irregular course of leukemia initiated by smaller inocula was a reflection of the varying capacity of organs to accept cells from the blood.

Our therapeutic experiment showed that the number of tumor cells in the fluid and in the blood was reduced and the mortality of their hosts delayed by i.v. injection of colloidal Au198. It is obvious that this agent was able to destroy a certain number of tumor cells but was unable to prevent their penetration into vital organs. This result may emphasize the conclusion of our previous work (3) that the treatment of leukemias should aim not only at the destruction of leukemic cells but also at the maintenance of barriers between the site of tumor cell proliferation and the organs. Similar conclusions were reached recently by Whitby (8) in his clinical studies on leukemia.

SUMMARY AND CONCLUSIONS

1. Graded numbers (15 × 10⁶ to 10⁹) of C1498 tumor cells were inoculated i.p. or s.c. into C57BL/6 mice. From these animals peritoneal fluid and blood were withdrawn at various time intervals for total and differential cell counts. The results were plotted in graphs and compared to graphs of mortality in the same groups of mice and with their autopsy findings.

2. The i.p. inoculated tumor cells provoked an accumulation of serous exudate and grew abundantly (numerous mitoses) in that medium, rapidly invading blood and hematopoietic organs. Their maximum concentration in the peritoneal fluid was reached earlier and at a higher level (almost 100 per cent) by high inocula; later and at a lower level (80-90 per cent) by smaller inocula (less than 10⁶ tumor cells). Tumor cells adjusted to free growth by serial i.p. transfers grew faster than those grown in the spleen. The leukocytic pattern of peritoneal fluid during tumor cell proliferation was characterized by very early disappearance of lymphocytes, scarcity of monocytes, and prevalence of polymorphonuclears. This pattern was reversed by the use of smaller inocula.

3. Blood invasion by tumor cells paralleled, for large inocula, their growth in the fluid, but at much lower levels (not more than 50 per cent), and for smaller inocula it was intermittent and irregular. The changes in the leukocytic pattern of the blood followed those of the peritoneal fluid. Similar trends in the blood picture were found in s.c. inoculated animals.

4. The mortality rate of i.p. inoculated mice paralleled closely the growth rate of tumor cells and their invasion of hematopoietic organs. For s.c. implants these processes could not be correlated with the size of the tumor.

5. Treatment with colloidal Au198 reduced the number of tumor cells in the blood and the peritoneal fluid, but delayed only slightly the death of their hosts.

6. It is concluded that the ability of tumor cells to grow out of minimal inocula (single cell or few cells) in almost pure culture in the peritoneal fluid and to invade blood and hematopoietic organs should be attributed to their exceptionally high growth potency.

7. It is presumed therefore that leukemic cells are malignant cells of high growth potency and that variations in leukemic syndromes depend on the degree of preservation of natural barriers between body fluids and organs.

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