Culture of Neoplastic Mast Cells and Their Synthesis of 5-Hydroxytryptamine and Histamine in Vitro

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Among the experimental leukemias, mast-cell tumors are of especial interest because of their high content of histamine, heparin, and also, in certain species including the mouse, 5-hydroxytryptamine (serotonin; 5-HT). Two transplantable mouse mastoctymomas have been reported recently, and their mast-cell character has been established by histological, biochemical, and pharmacological methods (1, 8, 13). Culture of these cell lines offers a possibility of studying their reproduction and their synthetic functions under controlled conditions. This paper presents a system for continuous culture in vitro of the mast-cell neoplasm P-815 (1). The special nutritional needs of these cells, reflected in a growth medium which is significantly different from conventional tissue culture media, are described, and studies of the mast cells in culture with regard to production of their 5-hydroxytryptamine and histamine and to their neoplastic infectivity are reported.

MATERIALS AND METHODS

The P-815 mast-cell neoplasm was carried as an ascitic tumor in (AKR X DBA/2)F₁ hybrid mice. Since the mast cells did not multiply in those conventional tissue culture media which sustain the reproduction of HeLa and many other cell types, a different medium had to be devised; this is shown in Table 1.

Culture technique.—The neoplastic mast cells, obtained as an ascitic suspension from mice, were washed and resuspended in the new medium and incubated at 37° C. During the 1st day of culture the histiocyes which also were present in the ascitic fluid became attached to the glass surface of the culture flask. On the other hand, only a variable proportion of the mast cells became attached to the glass during the 1st day. Therefore, it was possible to eliminate the histiocyes by daily transfer of the cell suspension into a new flask during the first few days of culture. Subsequently, the mast cells, now free from histiocyes, continued to reproduce without attachment to the glass.

Since the mast cells multiply in suspension, relatively simple technics, such as those commonly used in bacteriology, were employed. Cell reproduction was measured by hemocytometer counts after gentle agitation of the culture. Trypsinization was not required. The medium was renewed daily to approximately 3 X 10⁵ cells per ml. by dilution with medium. Thus, the cultures were almost always in the logarithmic phase of growth.

To develop a genetically pure mast-cell line, single cells were isolated and subsequently allowed to multiply on feeder layers of chick embryo fibroblasts. For this procedure the technics adapted and described by Fischer(1) for another cell line growing in suspension, L5178, were used.

Nutritional experiments.—Experiments designed to measure the requirement of the mast cells for 5-hydroxytryptamine (5-HT; serotonin; 5-HT) and its 5-formyl-tetrahydro derivative, leucovorin or citrovorum factor (CF), were extended, for reasons of comparison, to other cell lines, namely L5178 and Sarcoma 180. In the studies on Sarcoma 180, 2 X 10⁵ cells, suspended in 2 ml. of Eagle's medium containing 10 per cent horse serum (8), were introduced into each culture flask (Earle's T-15) (6). After 96 hours this medium was replaced by Eagle's medium (omitting folic acid) containing 5 per cent dialyzed horse serum and various concentrations of folic acid or citrovorum factor. This medium was renewed daily. After 7 days cell growth was measured by determining the protein content of the cell layer by the method of Lowry and Passonneau (11).

Bioassay of 5-hydroxytryptamine and histamine.—The 5-HT-content of the mast cells was measured by bioassay on the heart of Venus mercenaria by a modification of the procedure described by Gaddum and Passonneau (9). The histamine content of the cells was determined by bioassay on the guinea pig ileum. For histamine determination a suspension of the cells was assayed on the guinea pig ileum suspended in a 5-ml. bath of Tyrode's solution containing atropine (0.1 mg/ml) at 37° C. The specificity of the action was checked by the use of Neostigmin (Mepyramine) (0.01 mg/ml). The histamine and 5-HT values given refer, in each case, to the base.

G. A. Fischer, manuscript in preparation.
Histological examination.—Fresh, thin smears of ascitic fluid from the mouse and of cells from culture, after fixation by drying in air, were stained with Azure A (0.1 per cent in 30 per cent alcohol), as described by Kramer and Windrum (10).

RESULTS

Nutritional requirements of the mast cells.—Eagle and others have shown that a large variety of mammalian cell lines have very similar nutritional requirements when grown in culture and reproduce indefinitely in a medium containing minerals, glucose, thirteen amino acids, nine vitamins, and, as the only chemically undefined component, dialyzed serum (5). The neoplastic mast cells, however, showed two major differences in their nutritional requirements.

First, dialyzed horse serum did not support the reproduction of these cells, while the corresponding undialyzed serum (in combination with the other compounds of the medium, as listed in Table 1) enabled the mast cells to multiply indefinitely. In a typical experiment, $3 \times 10^5$ cells/ml were incubated in a medium containing either 10 per cent dialyzed or 10 per cent undialyzed serum. After 24 hours in the medium containing the undialyzed serum, the cell count was doubled, whereas with dialyzed serum the cell density had decreased to $2.3 \times 10^5$/ml. The nature of the essential material or materials which are present in undialyzed serum and also in embryo extract, and which are either removed or inactivated by dialysis of the serum, has not yet been elucidated. The amount of the factor or factors supplied by the horse serum often appeared to limit growth (for instance, see Chart 3). Furthermore, the growth response of the mast cells depended on the sample and possibly on the age of the serum used; fresh serum seemed to give the best results.

The second nutritional peculiarity of the mast cells is an unusually high requirement for folic acid, a requirement which could be satisfied, as previously shown on other cell types (3, 7), by much lower concentrations of leucovorin.

The dependence of the growth rate of the mast cells on the concentration of PGA and CF in the medium is shown in Chart 1. In this experiment, aliquots of a cell suspension were incubated in a medium (as in Table 1, omitting CF) containing different levels of these two forms of the vitamin. After 3–5 days a constant submaximal rate of growth was obtained in the cultures with growth-limiting concentrations of either PGA or CF. Subsequently, the growth rates at both limiting and excessive levels of PGA or CF were measured for 4 additional days. The average growth rates during these 4 days of constant response are plotted on a logarithmic scale against the levels of PGA and CF in the medium. As can be seen in Chart 1, PGA was required at about 500 times the concentration at which CF served as a growth factor for the mast cells. The levels of PGA and CF required for 50 per cent of maximal growth are shown in Table 2, together with corresponding data for other cell lines. The experiments with L5178-Y cells were carried out in a manner similar to those with the mast cells, with a medium described elsewhere. The values for Sarcoma 180 cells were obtained as described, while the data for Earle’s L-strain and HeLa are taken from publications by Eagle (3, 4).

It can be seen that the two leukemic lines, namely, the mast cell P-815-T and L5178-Y, have a markedly higher requirement for folic acid than the three nonleukemic lines. Thus, the inability of conventional culture media to support the continuous multiplication of these leukemic lines is owing, at least in part, to the low levels of folic acid which they provide. On the other hand, the differences in the requirements for CF are less pronounced, and, since the media used for culture of

![Chart 1](image-url)
the different lines were not identical, these differences may be less significant. It should be noted, however, that Swan et al. (14) have reported a stimulatory effect of CF on marrow cell reproduction in vitro.

When the neoplastic mast cells, obtained as ascitic suspensions from mice, were incubated in the medium described, they began to multiply after a lag period of 1–2 days. Owing to this relatively short lag period, a mast cell population in culture was obtained which was highly representative of the original tumor cell population. After the initial lag period the mast cells (now free from histiocytes) continued to multiply with a constant doubling time of approximately 4 hours. The constant doubling time which was observed over a period of 30 consecutive cell generations indicates that all the nutrients required by the mast cells were supplied by the medium.

The characteristics of the neoplastic mast cells during progressive culture.—In order further to characterize the neoplastic mast cell population in culture, the 5-hydroxytryptamine (5-HT) and histamine content, as well as the neoplastic infectivity, of the cells in culture were determined at regular intervals. The infectivity was determined by intraperitoneal injection of 100 cells into ten (AKR X DBA/2) F1 hybrid mice, and the proportion of the mice which were killed by the leukemia was observed. Table 3 shows that the high intracellular levels of 5-HT and histamine and the neoplastic infectivity were fully maintained with progressive culture covering a cell increase of 1012-fold; in fact, the 5-HT and histamine content of the cells appeared to increase somewhat with time.

The cells in culture also retained their morphological characteristics and metachromatic staining properties and continued to multiply in suspension. Furthermore, autopsy of the mice which died 16–30 days following injection of 100 cells showed the typical signs of the original mast cell leukemia; ascites, enlarged spleen, invasion of the mesentery and often of the liver.

The concentrations of 5-HT and histamine were also determined in the serum used for the medium. The histamine level was too low to be detectable in the horse serum (less than 0.04 μg/ml), while the 5-HT-concentration was 0.06–0.1 μg/ml.

Since these levels of 5-HT and histamine in the serum were much too low to account for the observed increases in the absolute amounts of these amines which occurred with cell multiplication, it follows that the mast cells were actively forming 5-HT and histamine, presumably from tryptophan and histidine in the medium.

A more detailed experiment concerning the infectivity of the cells during culture is shown on Chart 2; this demonstrates the relationship between number of cells injected and survival time (time from injection of the mice with the tumor cells to the death of the animals caused by the leukemia) and shows that during 20 generations in culture this relationship is essentially unchanged. The difference between the two curves is within the experimental variation, although a small change in degree of malignancy owing to a moderate selection in culture cannot be excluded.

The characteristics of two sublines, as compared with the original P-815 line.—In one of the early culture attempts with the P-815 cells, a cell line with distinctly different characteristics, as compared with those of the original cell population, was obtained. The reason for the emergence of this different line, subsequently called the “T-line,” was possibly a selection due to a medium deficient in the growth factor supplied by the serum, an assumption supported by the finding that the original line had a higher requirement for horse serum.

<table>
<thead>
<tr>
<th>No. generations</th>
<th>Multiplication</th>
<th>5-HT (μg/10⁶ cells)</th>
<th>Histamine (μg/10⁶ cells)</th>
<th>Mice killed by 100 cells (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>0.04</td>
<td>0.12</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>10⁶</td>
<td>0.09</td>
<td>0.18</td>
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</tr>
<tr>
<td>20</td>
<td>10⁹</td>
<td>0.10</td>
<td>0.14</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>10¹⁰</td>
<td>0.19</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>10¹²</td>
<td>0.19</td>
<td>0.54</td>
<td>100</td>
</tr>
</tbody>
</table>

Chart 2.—The neoplastic infectivity of the mast cells before and after twenty generations in culture. Each dot represents the average survival time of ten mice.

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than the T-line. This is demonstrated by Chart 3, which describes the growth response of the two lines to undialyzed horse serum. In this experiment $3 \times 10^6$ mast cells per ml. of medium were incubated for 24 hours in a medium containing 5 per cent dialyzed serum (to supply the cells with the nondialyzable growth factors present in serum) and various amounts of whole horse serum. The requirement for undialyzed horse serum of the original line is shown to be 10-20 times higher than that of the T-line.

In addition to the different horse serum requirement, the two cell lines showed marked differences in their 5-HT and histamine production. Table 4 demonstrates the 5-HT and histamine content of the mast cells after 1 week in culture. As the P values indicate, the differences between the two lines were statistically significant. The T-line, because of its higher content of these amines, seems preferable as a tool for studies of 5-HT and histamine biosynthesis in vitro.

To combine these favorable characteristics of the T-line with the advantages of genetic homogeneity, a clone was developed from a single cell of the T-line by the technics described. The genetically pure mast cell line which was thus obtained had the same general properties as the parent line, and even higher levels of 5-HT and histamine than those observed with the presumably genetically heterogeneous T-line were found. The cells in culture had average 5-HT and histamine contents of 1.0 and 0.9 $\mu g.$, respectively, per $10^6$ cells. Studies of the characteristics of a genetically pure line obtained through two consecutive cloning procedures are in progress.

### DISCUSSION

The short lag period of 1-2 days before the onset of logarithmic multiplication of the mast cells in vitro provides a cell population which is highly representative of the original ascitic mast cell population. The data of Table 3 suggest, however, that in prolonged culture a very moderate selection pressure favors the emergence of a line with the properties of the T-line. Elucidation of the nature of the growth factor supplied by the undialyzed horse serum might permit the design of a medium in which no such selection would occur. On the other hand, the selected lines appear to be superior to the original population both because of their higher 5-HT and histamine production in culture and because the quality of the horse serum was less significant to their reproduction in vitro.

Several lines of evidence have been obtained which indicate that the culture method described results in a cell population consisting exclusively of neoplastic mast cells. The fact that the mast cells multiply in suspension provides a simple means of separating them from other cell types. Furthermore, the mast cells retain their morphological and staining characteristics and their capacity to produce, when reinjected into mice, a typical neoplastic state characterized particularly by leukemia, and to continue to synthesize 5-HT and histamine with progressive culture in vitro. Finally, single cells of the culture, when placed on a feeder layer, multiply to produce pure cell populations with the same general properties as those exhibited by the parent population.

The high requirement for PGA of the P-815-T mast cells, as well as the L5178-Y lymphoblasts, is in sharp contrast to the much lower requirement of the three nonleukemic cell lines in which this has been studied. These results suggest the possibility that a high PGA requirement might be a common feature of leukemic cells or perhaps of leukocytes. On the other hand, L5178-Y has a

### TABLE 4

<table>
<thead>
<tr>
<th></th>
<th>Original line</th>
<th>T-line</th>
<th>MEAN DIFFERENCE</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>5-hydroxy tryptamine</strong> (six experiments)</td>
<td>0.06</td>
<td>0.33</td>
<td>0.27</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Histamine</strong> (five experiments)</td>
<td>0.2</td>
<td>0.6</td>
<td>0.4</td>
<td>0.03</td>
</tr>
</tbody>
</table>
special requirement for peptone (but not for undialyzed serum) (7), while the mast cells under these conditions require undialyzed serum, but not peptone, for reproduction. In addition, it has not been possible as yet to grow another strain of malignant lymphoblasts, L1210, in culture either in the medium suitable for L5178-Y, or that described here-in, or in various modifications of these. These several findings indicate that different leukemic lines may have quite characteristic requirements for additional growth factors. Such additional nutrients may be necessary for the successful culture of other leukemias.

It is of considerable interest that the malignant mast cells in culture maintain their characteristic capacity to store and, as will be shown in more detail elsewhere, to manufacture both 5-HT and histamine without any sign of de-differentiation. This makes the described culture system a very useful tool for pharmacological and biochemical studies. The lack of de-differentiation with regard to infectivity, 5-HT and histamine production may be attributable to the fact that highly representative mast cell populations were obtained in culture and that the possibility of mixed populations of normal and tumor cells has been excluded.

SUMMARY

A system for the continuous culture in vitro of cells of the P-815 mast cell leukemia is described. For the reproduction of these cells an unusually high folic acid concentration (replaceable by relatively minute amounts of citrovorum factor) and the presence of a growth factor supplied by undialyzed (but not by dialyzed) horse serum were required. In the medium which has been developed the mast cells multiply, without becoming attached to the glass surface of the culture flask, with a constant generation time of approximately 4 hours. Also, they maintain fully their high intracellular levels of 5-hydroxytryptamine and histamine as well as their neoplastic infectivity. Evidence is presented that 5-hydroxytryptamine and histamine are actively synthesized by the mast cells in culture. The characteristics of two sublines, obtained through selection and through a cloning procedure, are described and compared with those of the original P-815 cell population.

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