Studies on the Asparaginase Requirement of the Jensen Sarcoma and the Derivation of Its Nutritional Variant

M. K. Patterson, Jr., M. D. Maxwell, and E. Conway

Biomedical Division, The Samuel Roberts Noble Foundation, Inc., Ardmore, Oklahoma 73401

SUMMARY

When Jensen sarcoma cells were cultured in medium devoid of free L-asparagine, the cell population decreased exponentially for 10 days. The surviving cells then began an exponential proliferation phase. The resulting population did not require asparagine to maintain proliferation. Population growth curves and cloning studies showed that, per 10^6 original cells, approximately 10^2 variant cells were present that did not have a nutritional requirement for the amino acid. Cells derived from single cell isolates of the original cell population were tested for variant production. Some failed to give rise to variants initially but did so after several subcultures in medium containing asparagine. Others gave rise to variants after 10—40 days in the absence of the amino acid. These data suggested that the selected cell variant originated as the result of events not directly associated with asparagine deprivation.

INTRODUCTION

The nutritional requirement for L-asparagine by certain animal malignant cells is well documented (2, 3, 5, 8, 15—17) and is the basis for current clinical trials of L-asparaginase as a therapy for tumors of humans (9, 18). Whereas those tumors sensitive to the therapy regressed initially, relapse occurred in a number of instances, and the resultant tumors were found resistant to further treatment by the enzyme. Resistant cells have also been obtained after injections of guinea pig serum (10) or E. coli asparaginase (4, 6) into mice bearing C3HED lymphomas. Incubation of cells such as the Jensen sarcoma (13), Walker 256 carcinoma (20), and C3HED lymphoma (3) in asparagine-free medium or by the addition of L-asparaginase (19) to the complete incubation medium have given rise in vitro to variants that no longer require the amino acid.

The experiments described in the present report were designed to study the conditions necessary for the development of the variants (designated JA tumors) from the Jensen sarcoma and to determine whether these variants result from cell selection or from a metabolic alteration in the parent cells as a direct result of L-asparagine deprivation.

MATERIALS AND METHODS

Jensen sarcoma cells were obtained from freshly excised tumors grown for 7 days in the rectus femoris muscle of female Holtzman rats. Monolayer cultures were established from cell suspensions in T-15 flasks at an initial inoculum of 4 X 10^5 cells per flask in McCoy's complete medium 5a (14). This medium, which contains L-asparagine, was supplemented with 5% bovine serum that was twice dialyzed against cold Earle's solution or treated with Sephadex G-50 (22). After 48 hr, the medium was replaced with medium 5a minus asparagine and changed every 24 hr thereafter. After a medium change to remove loose cells, the cells were released from the glass surface by chilling at 4°C for several hours and counted with a Coulter counter. Cell counts were plotted as the log_2 so that each ascending unit represented a doubling of the cell population.

The standard assay used to establish asparagine requirement was an initial inoculum of 4 X 10^5 cells per flask, grown in triplicate, in complete medium 5a or medium 5a minus asparagine for 5 days and then counted.

To determine the incidence of mitosis and DNA synthesis, 60 x 15 mm Petri dishes containing 25 x 40 mm cover slips were inoculated with 400,000 cells in 5 ml of complete medium 5a and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. After 48 hr, the cultures were given medium 5a minus asparagine. Subsequent medium changes were made every 24 hr. Cells were counted by scraping the cover slips into 3 ml of fresh medium and enumerating in a hemocytometer. Four hours prior to termination, the medium was replaced in triplicate in Petri dishes with medium containing 0.05% colcemid or thymidine-3H (1 μc/culture). The colcemid- or thymidine-treated cultures were terminated with Carnoy's fixative by the procedure of Fogh and Fogh (7). Cover slips for mitotic counts were stained with acetic-orcein stain; only metaphasic figures were counted. The incidence of DNA synthesis was determined by the autoradiographic technics described by Baserga (1). After dipping in the film emulsion, cover slips were exposed for 12 days.

The parent cell population was cloned by diluting a suspension of cells, in which the viable cell count had been determined (trypan blue), so that 0.5 ml of medium would contain one cell. This volume was then introduced into 10-ml glass ampules, and the ampules were sealed. After 10 days of incubation at 37°C, the ampules were examined, and only those containing a single colony were subcultured to T-15 flasks and grown in complete medium 5a until a sufficient population was obtained for further subculturing. At this stage, approximately 23 cell doublings had occurred. One tenth of the population was subcultured in complete medium 5a. The remaining...
population was subcultured in replicate flasks in medium 5a minus asparagine and observed for variant production. These flasks were maintained for 60 days before being scored negative for variant production.

RESULTS

Serum Effects. To determine whether serum-adsorbed or -incorporated asparagine contributed to the derivation of the variant cells, the serum concentration was varied (Chart 1). Increasing the concentration of dialyzed or Sephadex-treated serum in the medium appeared to increase slightly the number of cells surviving at 10 days. In media containing 2%, 10%, and 20% serum, the cells surviving at 10 days represented 1.2%, 2.4%, and 4.0%, respectively, of the cell inoculum. In separate experiments with 40% serum, not shown in Chart 1, the nutritional variants also developed. In the proliferation phase of the curve, the cell doubling time was calculated to be 26—30 hr; and in the period of population decrease, the population halved every 26—40 hr. The cells surviving at 10 days showed marked morphologic changes from those observed in the general population of the parent cells. They were thin and transparent and lost their fibroblastic characteristic. The first visible evidence of the variants was the appearance of colonies which on proliferation contained cells morphologically resembling the original population.

Asparagine Concentration Effect. Supplementation of the medium with 0.01 mM asparagine maintained the cell population (Chart 2). Above this concentration the cells proliferated at a rate dependent upon the asparagine concentration, whereas below the 0.01 mM level the populations decreased and variants ultimately developed.

Population Dependency. Varying the initial cell inoculum from $5 \times 10^5$ to $4 \times 10^6$ cells did not alter the pattern of development of the variant (Chart 3). The population halving time, however, was 32.9, 43.6, 58.8, and 69.3 hr, respectively, for $5 \times 10^5$, $1 \times 10^6$, $2 \times 10^6$, and $4 \times 10^6$ initial cell inoculum. The percentage of the initial number of cells surviving at 10 days ranged from 1.5% to 6.3%.

Initial cell inocula, varying in number, were incubated in 60 x 15 mm petri dishes in medium 5a minus asparagine for 10 days in a 5% CO$_2$ atmosphere. The colonies were fixed with Bouin’s solution, stained with Giemsa stain, and counted. The number of colonies formed was found to be dependent upon the number of cells in the inoculum (Table 1). Thus, an average of 66 colonies were formed per $10^6$ cell inoculum over a wide range of initial inocula. Each colony contained an average of 27 cells. Corrected for a 24-hr lag growth period, this figure would correspond to a doubling time of 38.4 hr.

Mitotic Figure and Autoradiographic Studies. Chart 4 shows the number of metaphase figures per culture observed after 3, 6, 9, and 12 days of exposure of the cells to medium 5a minus asparagine. The number of metaphase figures increased during the period of population decrease. Chromosome counts at each time period showed a modal number of 56, which is the same as that observed for the parent Jensen.
The number of cells incorporating thymidine-\(^3\)H increased during the period of population decrease and continued to increase through 15 days (Chart 4). Cell labeling ranged from heavy to light throughout the culture; whereas in some cells, even after 24-hr exposure to the isotope, no labeling was observed. Exposure of the cells to the thymidine-\(^3\)H for 24 hr gave labeling indexes of 3\%, 6\%, 15\%, 32\%, and 78\% at 3, 6, 9, 12, and 15 days, respectively.

**Cloned Population Studies.** The response to L-asparagine deprivation of cell populations derived from single cell isolates of the Jensen sarcoma is shown in Table 2. In Experiment 1, of 25 clones, each grown in 5 replicate flasks, 20 failed to give rise to variants even after 60 days; 2 of 5 flasks of 2 clones gave rise to variants; 1 clone produced variants in 4 of 5 flasks; and all 5 replicate flasks gave rise to the variants in 2 clones.

### Table 1

<table>
<thead>
<tr>
<th>Initial inoculum (\times 10^6)</th>
<th>Average no. of colonies per Petri dish</th>
<th>Colonies formed per 10(^6) cells</th>
<th>No. of cells per colony</th>
<th>(N^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>52.9 ± 4.7(^b)</td>
<td>66 ± 6</td>
<td>29.2 ± 2.5</td>
<td>171</td>
</tr>
<tr>
<td>4.0</td>
<td>29.8 ± 3.4</td>
<td>75 ± 8</td>
<td>32.9 ± 3.3</td>
<td>161</td>
</tr>
<tr>
<td>2.0</td>
<td>15.6 ± 1.9</td>
<td>78 ± 9</td>
<td>27.0 ± 4.6</td>
<td>123</td>
</tr>
<tr>
<td>1.0</td>
<td>5.9 ± 0.8</td>
<td>59 ± 8</td>
<td>19.6 ± 4.7</td>
<td>43</td>
</tr>
<tr>
<td>0.5</td>
<td>2.7 ± 1.1</td>
<td>54 ± 22</td>
<td>24.8 ± 8.9</td>
<td>19</td>
</tr>
<tr>
<td>0.25</td>
<td>[6](^e)</td>
<td></td>
<td>(d)</td>
<td>6</td>
</tr>
<tr>
<td>0.10</td>
<td>[1](^e)</td>
<td></td>
<td>(e)</td>
<td>1</td>
</tr>
</tbody>
</table>

| Colony formation by Jensen sarcoma cultured in media minus L-asparagine. Values are the average of two experiments using 6 Petri dishes per inoculum level in each. |
|---|---|
| \(\text{No. of colonies observed to obtain cells per colony.}\) | \(\text{Standard error.}\) |
| \(\text{Total no. of colonies in 12 Petri dishes.}\) | \(\text{Thirty, 53, and 144 cells in 3 colonies; >500 cells in 3 colonies.}\) |
| \(\text{e>500 cells.}\) | |

In a second experiment, of 16 cloned cell populations, all failed to give rise to variants. Three of the original clones after 8 or 9 subcultures, or approximately 26—30 doublings in the presence of L-asparagine, were again tested for the presence of variants. One cloned population gave rise to the variant in all 5 replicate flasks, whereas in the other 2 populations, 2 of the 5 flasks showed variants.

In a third experiment, 20 cloned populations were established in triplicate flasks in medium 5a minus asparagine. Two of the clones failed to show variants; 4 showed variants in all 3 replicate flasks. The remaining 14 clones gave rise to the variants in either 1 or 2 of the triplicate flasks. The variation in time for the variants to appear as visible colonies is shown in Table 3. Although colonies are visible in the parent population 10 days after asparagine deprivation, the cloned population ranged from 10 to 40 days. The original cell population of the 2 clones that failed to give rise to the variants were again tested for variant production after 10 doublings in the presence of asparagine. With 1 clone, all 3 replicate flasks gave rise to variants, whereas in the second clone, 1 of 3 flasks was positive.

### Table 2

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>No. of clones</th>
<th>No. of flasks showing variants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>2(^e)</td>
</tr>
</tbody>
</table>

| Variants derived from cloned cell populations of the Jensen sarcoma. Values are clone numbers. |
|---|---|
| \(\text{Two after 8 subcultures.}\) | \(\text{One after 9 subcultures.}\) |
| \(\text{Original cloned population retested after 3 subcultures. Both gave rise to variants.}\) |

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**Chart 3.** Effect of initial inoculum on percent of surviving cells at various time intervals; 5 \(\times 10^5\) cell inoculum, \(--\); 1 \(\times 10^6\) cells, \(--\); 2 \(\times 10^6\) cells, \(--\); 4 \(\times 10^6\) cells, \(--\).**

**Chart 4.** Metaphase figures and thymidine-\(^3\)H uptake observed during growth of Jensen sarcoma cells in asparagine-deficient medium. Growth pattern, \(--\); metaphase figures, \(--\); thymidine-\(^3\)H-labeled cells per cover slip incubated for 4 hr in presence of isotope, \(--\).
DISCUSSION

Conditions under which the nutritional variant for L-asparagine of the Jensen sarcoma developed did not appear confined to narrow limits. This variant developed in culture medium devoid of asparagine but containing up to 40% serum, or in medium containing less than 0.01 mM supplemental asparagine, or in cultures initiated with 5 X 10^5 to 4 X 10^6 cells per T-15 flask.

The common observation in the parent cultures grown in the absence of asparagine was that a population decrease occurred for the first 8–10 days followed by a proliferation phase. Such a growth curve suggested two cell populations—one dying and one proliferating—i.e., cell selection. On the assumption that the proliferation phase represented the growth curve of the selected population, an extrapolation of this curve revealed that, in an initial cell population of 10^6 cells, 100 cells were present that survived and proliferated (Chart 5). Further, the data obtained in the present studies would support cell selection in that (a) the number of cells surviving at 10 days was directly proportional to the initial cell inoculum; (b) the number of cells forming colonies in the parent population was directly proportional to the initial inoculum and was of the same magnitude as that obtained by extrapolation of the proliferating phase curve; (c) during the period of decreasing cell population, the number of cells in mitosis and DNA synthesis was increasing; and (d) it was possible to clone cells from the parent cell population which failed initially to give rise to the variant. Although this does not constitute direct proof that an autonomous cell not requiring asparagine exists in the parent cell population, it would appear to exclude the probability that all cells are capable of giving rise to the variant as the direct result of cellular enzyme adaptation in the absence of asparagine.

Since these data would support a cell selection hypothesis, the question of the origin of the selected population was of interest. The studies on cloned cell populations, derived from the parent cells, demonstrated that a population failing to give rise to variants initially would do so upon further culture, even in the presence of L-asparagine. The variation in the time for the appearance of the variants in the cloned population would suggest an uncontrolled event. Such variations with cloned populations have been interpreted as indicative of spontaneous mutation by Law (11) in the case of folic acid-antagonist resistance of mouse leukemia L1210 and for the puromycin-resistant sublines of the mouse AMK2 studied by Lieberman and Ove (12). Definitive proof of such a phenomenon, however, must await further studies.

Asparagine synthetase activity (21) has been shown to be elevated in the nutritional variants (4, 20). Preliminary results show that the specific activity of the enzyme increases exponentially concomitant with the decrease in cell population during the first 10 days of asparagine deprivation. This observation would be compatible with cell selection. Recently, however, Prager and Bachynsky (23) reported that the asparagine-sensitive 6C3HED lymphosarcoma exhibited a transitory rise in asparagine synthetase activity, and the resistant tumor showed a large increase following L-asparaginase treatment. These authors suggest that depression accounts for the major part of the enzyme increase. These differences are under investigation.

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REFERENCES

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