Asparagine-requiring tumor cell lines and their non-requiring variants: cytogenetics, biochemistry and population dynamics

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ABSTRACT

Asparagine-requiring Jensen and Walker rat tumor cells and their asparagine-independent variants have been analyzed. The following results were obtained: (1) Both cell lines have very low levels of asparagine synthetase, and non-requiring revertants isolated from these lines have elevated levels of the enzyme. (2) No differences in chromosome number were detected between the parent Jensen line and five Jensen non-requiring revertants isolated from it. (3) Both Jensen and Walker cells undergo asparaginless death when deprived of this amino acid, although the Jensen cells do so at a more rapid rate. (4) Jensen requiring lines are at a selective advantage when grown in competition with non-requiring variants in complete medium, and their growth rate is more rapid when grown separately. The selective coefficients for the variant with respect to the asparagine-requiring parent ASN- line were 0.94 for the competition experiments and 0.83 for growth rate estimates. (5) A somatic cell hybrid between Chinese hamster cells (which require asparagine at low densities, and possess measurable synthetase activity) and the Walker line was found to be asparagine-independent, and it possessed enzyme levels equivalent to the hamster parent. The results of these investigations suggest a parallel with microbial auxotrophic mutants and can be understood in terms of alterations within nuclear structural genes.

The requirement for L-asparagine (ASN)* by certain malignant cells is one biochemical property that distinguishes them from their normal counterparts as well as from other malignant cells. For this reason, the system is of interest both from a theoretical standpoint regarding an elucidation of the ASN requirement, and also from an applied standpoint in the chemotherapeutic treatment of neoplasia.

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3 Present address: Department of Anatomy, Tufts University School of Medicine, Boston, Massachusetts 02111.
4 Abbreviations: ASN = L-asparagine, ASNase = asparaginase, amidohydrolase E.C. 3.5.1.1; ASN SYN = L-asparagine synthetase, E.C. 6.3.1.1; CHO/Pro- = Proline-requiring Chinese hamster ovary cell line; LMTK- = BUdR-resistant L cell variant.

ASN-requiring malignant cells of the rat, mouse, dog and human have been extensively studied. Acute leukemias, especially tumors which involve the lymphocytic series, are the predominant types of ASN-requiring tumors among humans. The requirement for ASN in such tumor cells of all species is due to the absence or low level of ASN synthetase (ASN-SYN) in these cells (Patterson and Orr 1967; Horowitz et al. 1968).

Guinea pig serum has been found to cause regression of certain rat carcinomas and mouse lymphomas due to its relatively high content of L-asparaginase (ASN-ase) (Kido 1958; Broome 1961). ASN-ase has also been identified in E. coli cell extracts and has been shown to possess tumor inhibitory properties (Mashburn and Wriston 1964; Roberts, Prager and Bachynsky 1966). The discovery of significant levels of ASN-ase in E. coli made it possible to obtain a purified enzyme for use and evaluation of antitumor activity in man. From these studies, it was found that certain neoplastic cells have an absolute requirement for exogenous sources of ASN and that this requirement is not present in the majority of normal cells. Although requiring cells probably need ASN to meet their needs for protein synthesis, there may exist other pathways of ASN utilization which have not yet been elucidated.

Studies have shown that the antitumor effects of ASN-ase are related to a specific depletion of L-ASN which results in the death of those cells which are deficient in ASN synthetase. For this reason, L-ASN-ase has considerable potential in amino acid depletion therapy. One hindrance to this method of chemotherapy is that nutritional variants arise from the parent population of ASN-requiring cells which no longer require exogenous asparagine for growth (Haskell and Canellos 1970).

The present studies were undertaken to elucidate the genetic nature of ASN-requiring cells and their non-requiring variants, and also to test the hypothesis that ASN-requiring cells as well as their non-requiring variants arise through mutational events occurring in nuclear genes. Jensen sarcoma and Walker 256 carcinosarcoma cells (both originating in the rat) were used in these studies since both cell lines have an absolute in vitro requirement for ASN, are sensitive to L-ASN-ase and give rise to ASN-non-requiring variants. The results of the present experiments suggest a parallel with auxotrophic microbial systems.

MATERIALS AND METHODS

Cell lines: Jensen cells were grown in the autoclavable modification of Eagle's Minimal Essential Medium (Flow Laboratories) supplemented with 5% calf serum (Colorado Serum Company), non-essential amino acids, antibiotics, and glutamine. W256 cells were grown in Leibovitz medium to which the above supplements were added. Both cell lines possessed rather low plating efficiencies, ranging between 20% and 30%. CHO/Pro- cells are a proline-requiring Chinese hamster ovary cell line originally isolated by Kao and Puck (1967) and given to us through the courtesy of Dr. M. W. Taylor. LMTK- cells are a BUdR-resistant derivative originally isolated from the L cell line by Krr et al. (1963). Other procedures for cell cultivation have been previously described (Morrow 1971; Morrow et al. 1973).

Reversion frequency: Jensen sarcoma cells ($10^6$) were plated in 30 ml Falcon tissue culture flasks each containing medium lacking ASN. After a seven to fifteen days period, visible clones
of ASN-independent cells appeared. The flasks were briefly heated in a drying oven to attach the cells tightly and then stained with 1% crystal violet (Vernick and Morrow 1973).

Rescue experiments: Jensen ASN-dependent and WAG (W256, 8-azaguanine-resistant; Morrow, Colettiore and Rintoul 1973) cells were tested for their ability to survive in medium lacking ASN for periods of one to six days. Initially cells were plated at cloning densities (10^3 cells per 30 ml flask) in medium devoid of ASN. At various times thereafter fresh medium containing ASN was added to the flasks and the cells were allowed to grow until visible clones formed, which were stained with crystal violet and counted. The ability of each cell line to survive in medium minus ASN was measured as a fraction of a control which had not been exposed to asparagine-free medium.

Cell competition experiments: In these experiments artificial mixtures of Jensen ASN-dependent and ASN+-independent cells were prepared consisting of 2 x 10^6 cells from each cell line. The cells were plated as a mixture in flasks containing medium plus ASN. At weekly intervals of growth under these conditions, the cells were harvested with Viokase, counted and replated. Samples were also taken at this point at cloning densities (10^3 cells per flask) in two groups of 30-ml Falcon flasks. One group of flasks contained medium plus ASN while another group contained medium lacking ASN. This entire process was repeated at weekly intervals for twelve weeks. The change in ratio of the two cell types was measured by comparing the number of clones in asparagine-deficient medium with the number in complete medium. Thus

\[
\frac{\text{Clones in ASN- medium}}{\text{Clones in complete medium}} \times 100 = \text{Fraction of population which is asparagine-independent.}
\]

Chromosome studies: Chromosome counts of all cell lines, variants and somatic cell hybrids were performed. The chromosome preparations were carried out by the method of Sumner, Evans and Burkland (1971). In all cases, a minimum number of 40 cells was examined to determine the modal number of chromosomes. Chromosomal analysis was also performed to verify the hybrid nature of fusion products of CHO/Pro- and WAG cell lines.

Somatic cell hybrids: CHO/Pro- WAG cell lines were hybridized in flasks containing medium lacking proline and asparagine but which contained HAT (hypoxanthine, aminopterin, and thymidine; Littlefield 1964). Sendai virus was added to facilitate the fusion of these parent cell lines (Harr is et al. 1966). Appropriate controls were also performed to measure the reversion frequency of the parent cell line.

Asparagine synthetase assays: ASN synthetase levels in all cells and hybrids were measured by previously reported methods (Patterson and Orr 1967, 1968).

Growth rate studies: The relative growth rate of Jensen ASN-dependent and -independent variants was measured. Cells were plated at densities of 10^3 or 10^4 in plastic petri dishes (Falcon) and incubated in 5% CO₂ in air. At the end of 7 days and at the end of 14 days replicates of 4 plates per cell line were harvested and counted. Growth rates of the ASN variants were measured by growing the cells in plates containing medium plus ASN and in another group of plates containing medium devoid of ASN.

RESULTS

We have previously shown (Morrow 1971; Patterson, Maxwell and Conway 1969) that ASN-nonrequiring mutants preexist in a population of ASN-requiring W256 and Jensen cells from the rat, and that the mutation rate is of the order of 1.4 x 10⁻⁶ to 3.5 x 10⁻⁶ per cell per generation. In the work reported here frequency data closely agreed with that previously reported (Table 1). It should be noted that the distribution of colonies per flask fit the Poisson distribution, indicating that the distribution of colonies is random.

In order to measure the differential survival of both cell lines after a period of ASN deprivation, cells of the Jensen sarcoma and Walker 265 carcinosarcoma were tested (Figure 1). The cloning efficiency, expressed as percent control of
TABLE 1

Frequency of asparagine non-requiring clones among the Jensen sarcoma cell line

<table>
<thead>
<tr>
<th>Number of colonies</th>
<th>Number of flasks</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
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</table>

Mean colonies per flask = 0.94
Variance = 1.10; standard deviation = 1.0495
\[ \chi^2 = 7.87 \quad P > 0.05 \]

Frequency of mutant colonies = \(9.4 \times 10^{-7}\)

Results represent data pooled from three experiments performed on different dates. Each flask contained \(10^6\) cells. Chi square and probability are calculated on the assumption of a Poisson distribution.

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FIGURE 1.—Effect of asparagineless medium on the cloning efficiency of Wag and Jensen ASN- cells after varying periods of deprivation. Cells were plated in asparagineless medium and at the indicated times thereafter complete medium was added to the cultures.
the Jensen sarcoma cells, markedly decreased until after four days no clones were detected. By contrast, after six days the Walker 256 cells could still be rescued by the addition of ASN to the medium.

In order to investigate the population dynamics of this system, selection was studied in artificial mixtures of Jensen ASN-requiring and non-requiring cells (Figure 2). As a control, two non-requiring clones were grown in complete medium and plated out in the two selective media in an equivalent manner. The control served the additional purpose of testing the possibility that ASN+ clones would re-revert to asparagine requirement when placed in non-selective (complete) medium. As can be seen in the graph in both cases, the two asparagine-independent clones declined in plating efficiency in asparaginless medium, but after several weeks appeared to stabilize and regained a high plating efficiency in both media. The artificial mixture, on the other hand, showed a rapid and

![Graph showing population dynamics of Jensen ASN- and ASN+ cells in artificial mixtures.](Figure 2)
continuous loss of plating efficiency in asparaginless medium, indicating that
the asparagine-independent component of the mixture was being selected against.

The results of the artificial mixture experiments were corroborated by the
growth rate experiments in which the asparagine-requiring parent and two non-
requiring variants were tested for their growth rate by simply growing the cells
for several days, harvesting, and counting the increase in cell number. As can
be seen from Table 2, the difference in growth rates is consistent with the differ-

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Medium</th>
<th>Generation time (days)</th>
</tr>
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<tbody>
<tr>
<td>Jen ASN+1</td>
<td>—ASN</td>
<td>1.135 ± 0.073</td>
</tr>
<tr>
<td>Jen ASN+4</td>
<td>+ASN</td>
<td>1.208 ± 0.086</td>
</tr>
<tr>
<td>Jen ASN+1</td>
<td>+ASN</td>
<td>1.208 ± 0.086</td>
</tr>
<tr>
<td>Jen ASN+4</td>
<td>+ASN</td>
<td>1.004 ± 0.041</td>
</tr>
<tr>
<td>Jensen ASN-</td>
<td>+ASN</td>
<td>1.004 ± 0.041</td>
</tr>
</tbody>
</table>

Cells were grown in 60 mm diameter petri dishes and treated as described in the METHODS. A selective coefficient or s value of 0.744 to 0.931 of the mutant with respect to the wild type is obtained.

ence obtained in the selection experiments. Thus in both cases asparagine-
requiring cells appear to possess a distinct advantage over their non-requiring
derivatives when both types are grown in complete medium.

Chromosome counts were performed on five independently arising Jen ASN+
clones. The results (Figure 3) show that there is no significant difference in
modal chromosomal number of these variant clones. Three of the non-requiring
variant clones had a modal number of 59, one variant had a modal number of 58,
and one variant had a modal number of 60. The modal chromosomal number of
ASN-dependent Jensen cells was also determined and found to be 59. Thus there
is no gross chromosomal change associated with ASN prototrophy.

The asparagine synthetase levels of the Jensen parent and its non-requiring
variants were measured (Table 3). All five variants had measurable activity,
although the levels obtained were lower than those seen in the LMTK-, which is
reproduced for comparison. However the values were of the same order of magni-
tude as the CHO/Pro- line (see below). The level of enzyme did not appear to be
correlated with modal chromosome number or origin of the cell lines (Table 3
and Figure 3).

Somatic cell hybrids were isolated from the CHO/Pro- and WAG lines through
the use of HAT medium lacking asparagine and proline. One such clone was iso-
lated and characterized; its hybrid nature was established on the basis of its
modal chromosome number and also on the basis of the fact that clones arose
only in mixed cultures of CHO and WAG. The hybrids grow vigorously in
asparaginless medium and possess synthetase levels equivalent to the CHO
parent (Table 3).
Figure 3.—Histogram of chromosome number distribution of Jensen line and its asparagine-independent derivatives.
TABLE 3

Asparagine synthetase mean values in various cell lines

<table>
<thead>
<tr>
<th>Asparagine synthetase values (nmol/mg protein/hour)</th>
<th>Jensen (ASN⁻)</th>
<th>Walker 256 (ASN⁻)*</th>
<th>LMTK⁻ (ASN⁺)*</th>
<th>CHO/Pro⁻</th>
<th>W256 x LMTK⁻*</th>
<th>WAG x CHO/Pro⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.089 ± 0.027 (6)</td>
<td>0.15 ± 0.06 (6)</td>
<td>33.3 ± 10.5 (6)</td>
<td>5.38; 4.94-5.83 (2)</td>
<td>3.05 ± 0.86 (9)</td>
<td>5.6 (1)</td>
</tr>
</tbody>
</table>

Jensen non-requiring variants

| Jensen ASN+1 | 1.15; 1.05-1.24 (2) |
| Jensen ASN+2 | 1.43; 0.39-3.38 (3) |
| Jensen ASN+3 | 2.83; 2.66-3.00 (2) |
| Jensen ASN+4 | 1.28; 0.31-1.95 (5) |
| Jensen ASN+5 | 0.82; 0.23-1.82 (4) |

Numbers in parentheses refer to number of assays; standard errors are given where more than five assays were performed; otherwise range is given. All clones were derived by UV irradiation, with the exception of Jen ASN⁺3, which was of spontaneous origin.

* Data from Morrow et al. (1973).

DISCUSSION

The studies reported here were designed to bring further evidence to bear on the nature of the asparagine requirement and its relationship to the malignancy of the cell lines in question. Although it is not possible at this time to make a final statement on this problem, we feel that our evidence supports the contention that the variation which we are observing has a mutational, genetic basis due to alterations in nuclear genes. We have been drawn to this point of view by the following body of evidence.

The previous data that we (Morrow 1971; Patterson, Maxwell and Conway 1969) and others (Handschumacher, personal communication) have accumulated from fluctuation tests clearly demonstrates that rare ASN⁺ variants are preexisting in the population and not induced by the selecting agent. Additional data which we have produced on mutation rates in the Jensen line and which will be reported subsequently confirm this belief. Furthermore the ASN⁺ clones are extremely stable, even in medium supplemented with asparagine, as shown by the control experiments reported in Figure 2. We have cultivated some ASN⁺ clones for over a year in asparagine-deficient medium and some for over four months in complete medium without observing any alteration in their asparagine-independent phenotype. Thus enzyme induction or other modifications of gene expression can be conclusively ruled out as possible explanations. It should, however, be noted that ASN⁺ cell lines are inducible with respect to ASN SYN (Patterson, unpublished).

Accepting that the alterations are hereditary modifications, we have attempted to ascertain whether gross chromosomal alterations are responsible for the ASN phenotype. Based on chromosomal counts, this does not appear to be the case, al-
though modification involving parts of chromosomes would not be detected using our procedures. Since the Jensen and Walker lines show a good deal of karyotypic heterogeneity, this does not appear to be a question technically feasible to investigate. Other studies (Hsu and Manna 1959; J. G. Kidd, personal communications) have indicated high frequencies of chromatid aberrations or other alterations in asparagine-independent derivatives of requiring lines, but we did not observe such an increase in our work. Although a number of investigators have looked for correlations between karyotype and phenotype in cultured cells (Morrow 1970; Harris and Ruddle 1960; Szybalski and Szybalska 1962), most such efforts have produced negative results. The one exception is the work of De Carli and his coworkers (1964) in which a consistent correlation was observed between the level of alkaline phosphatase and the number of chromosomes in a specific group in cultured human heteroploid cells. Thus the vast majority of genetic variations in cultured cells must find their origins in mechanisms beneath the level of resolution of the light microscope.

It is not possible at this time to answer the question of whether the asparagine requirement results from a mutational lesion in a structural or a regulatory gene. Since no detailed information is yet available on regulatory systems in eukaryotic cells, extensive speculation may be unwarranted. However, it should be stated that the asparagine requirement behaves as a genetic recessive in the CHO/Pro− × Jen ASN− hybrids as was previously seen to be true in the WAG × LMTK− hybrids (Morrow et al. 1973). Several presumptive regulatory systems have been identified in somatic cell hybrids (Klebe, Chen and Ruddle 1970; Weiss, Bertolotti and Peterson 1972); all behave as dominants, suppressing enzyme activity in hybrids. This information indicates that regulatory signals are not species-specific, but are highly gene-specific in their action. Since the asparagine requirement is recessive in hybrids, this suggests that it is not a regulatory alteration of the type so far described. The levels of asparagine synthetase observed in the hybrid CHO × Jensen were about the same as those found in the CHO parent, whereas in the previously reported WAG × LMTK− hybrids the levels in the hybrids were about 10% of the high enzyme parent. This discrepancy between the two systems may result from extensive loss of rat chromosomes in rat-hamster hybrids, yielding a cell which is quite comparable to the original CHO parent.

A further point of interest concerning the hybrids relates to the fact that the CHO parent possesses a marginal requirement for asparagine and thus the two characters are mutually complemented in the hybrid. We suspect that the requirement of the CHO line at low densities for asparagine is due to a lowered ability of the cells to transport or concentrate asparagine; thus complementation of two different mechanisms of asparagine requirement could account for those results.

There have been suggestions (Mezger-Freed 1972; Harris 1967) that certain phenotypic alterations in cultured mammalian cells are difficult to explain in terms of classical mutational hypotheses, and may be the result of cytoplasmic or epigenic modifications. This does not appear to be true in the case of the as-
paragine requirement, since the data presented here and positive results of mutagenesis experiments (VERNICK and Morrow 1973) are explicable in terms of alterations in nuclear genes. Furthermore, the data reported here show that both spontaneous and UV-induced ASN+ clones have recovered asparagine synthetase activity.

The cell rescue experiment demonstrates that both cell lines die when deprived of asparagine, although the demise of the Jensen cell is much more dramatic, which may be due to the fact that its basal level of ASN SYN is much lower than that of the W256 line. In any case these results are noteworthy in that they parallel recent observations by KAO, CHASIN and PUCK (1969) on the behavior of glycine-requiring cell lines and are in contrast to the observations on the behavior of proline-requiring mutants which lose viability only very slowly when deprived of this requirement (KAO and PUCK 1967).

The results of the cell competition experiments demonstrate that ASN- cells have a selective advantage over ASN+ revertants in artificial mixtures. We believe that this is due to properties that are intimately associated with the asparagine requirement, and that these observations can be used to explain the existence of asparagine-requiring tumors. If we assume that some unique property of the genes responsible for the synthesis of asparagine render unto cells which possess them in a functional state a lower growth rate, then in a newly proliferating tumor population any mutation to asparagine requirement would be selected for, and one might expect at least some tumors to be asparagine-requiring and respond to asparaginase. Thus revertants to asparagine independence would not take over such cell populations, even though they are constantly being produced by mutation. This situation would be analogous to the azaguanine-resistance system where it has been demonstrated (Morrow 1972) that newly arising azaguanine-resistant mutants are selected against in artificial mixtures and, in the case of alkaline phosphatase, where alkaline-phosphatase-negative variants are selected against in artificial mixtures with the AP-positive parent line (Morrow and De CARLI 1967). However, the situation of ASN requirement would be somewhat unique, in that the mutant, rather than the wild type, would possess the selective advantage.

The selective advantage of the ASN- lines with respect to the ASN+ types was estimated quantitatively both in the competitive experiments (Figure 2) and in the case in which the growth rates of the Jensen line and its variants were measured (Table 2). From this data an $s$ value can be calculated to yield a selective coefficient for the mutant with respect to the parent line. Thus for the growth rate experiments

$$s = \frac{\text{Generation time of wild type}}{\text{Generation time of mutant}}$$

while for the mixture experiments

$$s = \text{Change in frequency of mutant per generation} = \frac{N/N_0}{N_{total}} = e^{rT}; \; T = \text{Time in days}; \; N_0 = \frac{\text{ASN+ cells}}{\text{total}} \text{ at time 0}; \; N = \frac{\text{ASN+ cells}}{\text{total}} \text{ at time T}.$$
For the growth rate data the $s$ value of the Jen ASN$^+$4 and Jen ASN$^+$1 lines was 0.83 with a range from 0.744 to 0.931, taking into account the statistical error of the estimates for the cell lines. This is somewhat lower than the figure of 0.94 (95% confidence limits 0.91 and 0.97) which was obtained from the competition experiments, assuming a generation time of 24 hours (Table 2). Since the limits overlap, this difference may reflect experimental error inherent in the system or a crossfeeding rescue between the two types when grown together in mixed culture. Thus our data support the contention that the original asparagine requirement endowed the tumor line with a selective advantage, and that the lack of large scale reversion of the ASN$^-$ populations can be accounted for on this basis. If this is true then a logical conclusion would be that there is no causal relationship between the malignancy of the cell lines and their asparagine requirement.

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LITERATURE CITED


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