Derivation of Stable Polyploid Sublines from a Hyper-diploid Ehrlich Ascites Carcinoma*

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Induction of polyploidy through various experimental procedures has been reported by many authors for plant and animal cells since the classical experiment of Boveri (5). Among the principal methods were exposure to the action of cold, CO₂, narcotics, or other types of poisoning during mitosis (29). The frequent appearance of heteroploid cells in tumor populations (2, 11, 20, 21, 24, 26); the greater resistance of such cells to irradiation (9); the frequent appearance of heteroploid cell suspensions to various unfavorable environments.

MATERIALS AND METHODS

The observations are based on the Ehrlich ascites carcinoma received in the spring of 1958 from Dr. H. Lettré in Heidelberg and serially kept in this laboratory through intra-peritoneal transplantation in adult female mice of the Swiss/Ha ICR strain. For the routine transfer 0.2 cc. of undiluted ascites, containing approximately 20—50 million tumor cells, were transplanted once a week. This ascites has a predominantly hyper-diploid chromosome constitution (2) and, on the basis of chromosome morphology as well as number, is distinguishable from the unrelated hypotetraploid Ehrlich ascites (11, 20).

To determine the ploidy of the various tumor cell populations here described, 100—400 metaphase plates were checked in each case under oil immersion on aceto-orcein smears. In the Ehrlich-Lettre ascites, over 90 per cent of the cells were hyper-diploid in all samples examined. Among the 71 sublines, those that contained more than 50 per cent of hyper-tetraploid and higher-numbered plates were classified as polyploid lines.

For the counting of chromosomes, aceto-orcein smears on siliconed slides were best. The colchicine-technic was helpful, since it greatly increased the number of countable plates without changing the normal numerical distribution of chromosomes (2, 19). Within 20 hours after intraperitoneal injection of 5 μg of colchicine/10 gm mouse body weight, the arrested metaphase plates were easily spread and many were countable. It should be stressed that colchicine was here used only to facilitate chromosome counting, not to induce doubling, which was favored either by low cell dosage or exposure of the cells to unfavorable environments prior to inoculation.

Small cell dosage.—The ascites, tapped usually 5—7 days after transplantation, was diluted 1:2,000 to 200,000 times in mammalian Ringer. The inoculum/mouse was 0.1 cc. of this nearly pure tumor cell suspension containing from 50 to 5,000 hyper-diploid neoplastic cells, i.e., much below the normal transfer dosage of 20,000,000 cells. In one experiment 500,000 cells were injected.

Starved cell inoculation.—Ascites diluted 20 times in Hauschka’s “starvation medium” (1,000 cc. redistilled water, 8.1 gm. NaCl, 0.2 gm. KCl, 0.2 gm. CaCl₂, 0.05 gm. dry heparin, 1 million units sodium salt of penicillin, buffered at pH 7.1 with sodium phosphate buffer) was agitated slowly with a magnetic stirrer at 20° C. for 14—48 hours. After stirring for 24 hours, some mitotic figures could still be observed, but many of the cells showed swelling. For inoculation into mice the starved ascites was diluted further, so that 0.2 cc. contained approximately 50,000 cells.

Refrigerated cells.—Twenty-fold dilutions of ascites were kept in a cold box at 4° C. for 7—14 days. Fourteen days of cold-storage reduced viability of the cells considerably, but after 7 days’ storage about half of the inoculations were successful.

All tumors which appeared after inoculation of material treated in the several ways shown above were studied for their chromosome constitutions. In most cases there were enough good mitotic figures for critical distinction between near-diploid and near-tetraploid tumors.

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RESULTS

CYTOLOGICAL OBSERVATIONS

The chromosomes could be counted exactly on smears fixed with aceto-orcein 18-20 hours after intraperitoneal administration of colchicine in the amounts recommended by Levan (19). Figure 1 shows a typical high dry field after colchicine treatment. Care was exercised not to include broken or overlapping plates in the counts. Two hundred of these random counts are presented in histogram form in Chart 1. The mode of the chromosome number of the original Heidelberg Ehrlich ascites which occurs regularly in a small proportion of the hyper-diploid population. This phenomenon is recognizable by temporarily four-stranded metaphase chromosomes, such as shown in Figure 8; these separate into two-stranded units, thus doubling the total chromosome complement of the cell. The doubled cells cannot overgrow the modal diploid component under the conditions of routine transplantation of large cell dosages exceeding twenty millions per mouse.

In addition to strictly numerical observations on chromosomes, certain morphologically distinct elements in the ideogram were always recognizable. Secondarily constricted chromosomes as well as metacentric ones, the so-called "A" and "B" chromosomes described by Bayreuther (2, 28) for the same tumor and several minute elements shown in Figure 4 were seen not only in the hyper-diploid Ehrlich, but these same chromosomes were duplicated in the hyper-tetraploid derivatives. That most of the hyper-tetraploid cells have two "A," one or two "B," and four to six minute chromosomes, while the cells in the original hyper-diploid tumor have usually one "A," and one "B," and three minute units, is further proof that rather exact doubling of the chromosome set has occurred.
On morphological grounds, it is impossible to confuse this very characteristic ideogram with that of the other hypo-tetraploid “Ehrlich” ascites tumors (20) and their clonal derivatives (9), which are usually more bloody and kill the host about twice as rapidly.1

**Derivation of Polyploid Sublines**

The sequence of events preceding recovery of polyploid tumors from the mice inoculated intraperitoneally with very dilute or pretreated hyper-diploid cell suspensions is outlined in Chart 2.

About one-half of the 275 injected animals developed no tumors at all. In a small proportion of cases, normal progressive accumulation of ascites occurred in typical fashion, and mice died in 20 ± 1.4 days; but these primary ascites were always hyper-diploid. In most of the positive animals, a subcutaneous solid tumor appeared at the inoculation site during the 1st month. During the 2nd month, these growths often infiltrated into the abdominal cavity and produced secondary ascites. When the latter sublines were checked for ploidy, about 40 per cent of them showed extensive chromosome doubling (life span of mice, 60 ± 4.0 days). Some of the secondary ascites were unsuitable for analysis because of infrequent mitosis. Such tumors were sub-transplanted into further hosts for later re-checking.

The results for the entire experimental series of 275 mice (125 tumor takes, exclusive of control tumors) are summarized in Table 1. Altogether, 28 polyploid sublines were found among the 71 lines which were analyzed cytologically.

The ratio of near-diploid to polyploid cells in 28 polyploid subline derivatives is shown in Chart 3; the frequency of hyper-tetraploid and near-octoploid cells was 60 to 95 per cent, compared with less than 10 per cent in the original tumor, which is shown on the extreme right of Chart 3.

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**INJECTION OF HYPER-DIPLOID CELLS**

1 Small dosage of cells
2 or starved tumor cells (Day 0)
3 or cold-storaged cells

Most tumor cells disappear from the abdominal fluid. (1–2 weeks)

Subcutaneous tumor appears at the inoculation site. (3–4 weeks)

Sc. tumor infiltrates i.p. forming secondary ascites. (5–14 weeks)

**RECOVERY OF MANY SECONDARY ASCITES WITH DOUBLED HYPER-TETRAPLOID CHROMOSOME CONSTITUTIONS**

**Chart 2.—Sequence of events in the doubling of chromosome number**

**Table 1**

**Derivation of Hyper-tetraploid Sublines from a Hyper-diploid Ehrlich Ascites Tumor**

<table>
<thead>
<tr>
<th>Method</th>
<th>Takes/total mice</th>
<th>No. tumor lines tested for ploidy</th>
<th>No. hyper-diploid</th>
<th>No. hyper-tetraploid</th>
<th>Per cent hyper-tetraploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-cell dosage</td>
<td>83/164</td>
<td>90</td>
<td>11</td>
<td>9</td>
<td>45.0</td>
</tr>
<tr>
<td>(50–500,000)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell starvation*</td>
<td>53/79</td>
<td>44</td>
<td>28</td>
<td>16</td>
<td>56.4</td>
</tr>
<tr>
<td>Cold storage†</td>
<td>9/32</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>42.8</td>
</tr>
<tr>
<td>Control</td>
<td>330/330</td>
<td>53</td>
<td>53</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>(20,000,000 cells)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Slowly stirred in vitro for 14–48 hours at 30°C. in heparinized buffered mammalian Ringer solution. From 20,000 to 50,000 cells of the stirred suspension were inoculated per mouse, but many cells may not have been viable.

† Seven to 14 days at 4°C. Inoculation dose: 500,000–4,000,000 cells per mouse, including damaged cells.
STABILITY OF THE POLYPLOID SUBLINES

The derived polyploid sublines were maintained by serial transplantation in Swiss female adults. The growth pattern of these sublines was grossly similar to that of the original hyper-diploid Ehrlich; typical abdominal distension, rather blood-free ascites, only slight invasiveness into the viscera, the appearance of fibrous clots of cellular debris, i.e., the so-called "Schwimmkulturen" (22) at the end-stages of growth. There also was close similarity of the life span after standard inocula of about twenty million cells:

\[
\begin{array}{ll}
\text{Ehrlich-Lettré (hyper-diploid)}: & 16.6 \pm 0.13 \\
\text{EL 88 (hyper-tetraploid)}: & 17.2 \pm 0.19
\end{array}
\]

(119 mice) (98 mice)

During serial transplantation of the polyploid sublines the relative proportion of hyper-diploid and polyploid cells was checked from generation to generation. Although in some sublines the frequency of polyploid cells showed a gradual or abrupt tendency toward decrease, others remained fairly stable during over 20 weeks of observation with a consistently high percentage of polyploid cells (Chart 4).

EXPERIMENTAL STABILIZATION OF POLYPLOIDY THROUGH "IMMUNO-SELECTION"

When the most stable of the lines EL 88 showed a slight trend toward return to hyper-diploid after fourteen passages, an attempt was made to test "immunological selection" (7–9, 18) as a possible stabilizing influence on the level of tetraploidy of this tumor.

The subline EL 88 was transplanted into mice which had been previously immunized with hyper-diploid Ehrlich by caudal inoculation (1). By this procedure, the frequency of polyploid cells in-

\[
\begin{array}{ll}
\text{COLD STORAGE OF CELLS} \\
\text{HYPER-DIPLOID} & \text{HYPER-TETRAPLOID} \\
\text{EL 88} & \text{NEL 888 (Chart 4)}
\end{array}
\]

CREASED and stabilized firmly at the high level of 92 per cent, which has remained constant up to the present, for a period of 15 weeks.

Similar results were obtained by the subcutaneous inoculation of a relatively small dosage of EL 88 cells which gives enough time for an immune response to develop. Fifty days later a secondary ascites, established as the stable subline EL 888 (Chart 4), was recovered. This line has 95 per cent hyper-tetraploid cells.

The question now arose whether or not the hyper-diploid population could be changed directly into a tetraploid one by passage through im-
mune hosts. Twenty-seven Swiss female adults were given subcutaneous injections near the tail tip with hyper-diploid Ehrlich ascites, and the tails were amputated near the base 3 weeks later. The immunized mice were then challenged intraperitoneally with 0.2 cc. of 1:10 diluted hyper-diploid Ehrlich (approximately five million cells). Normally, such an inoculum kills nonimmunized mice within 3 weeks and always produces hyper-diploid ascites. Fifteen of the 27 immunized animals were still tumor-free after 2 months. Development of tumors in twelve positive cases was followed in the same way as described before. Among the twelve ascites analyzed, five were hyper-tetraploid, four had 15–45 per cent tetraploid cells, and only three were hyper-diploid in the same way as the original tumor.

The probable iso-immunological implications of this particular experimental approach should be evident from Table 2 (see "Discussion"). The second line of Table 2 shows that, even after inoculation of 5 million cells from the hyper-diploid tumor into hosts previously immunized by Andervont's tail inoculation-amputation method (1), a high percentage of hyper-tetraploid sublines was recovered. This was never the case with similar large inocula in nonimmunized mice.

On the other hand, it was not possible to derive polyploid sublines when optically counted very small dosages of 49–165 cells were inoculated with glass capillary into newborn, hence immunologically inactive, mice. Under these conditions, 22 per cent of takes were obtained in 76 infants, and all the 17 takes examined for chromosome number were hyper-diploid. Inoculation of a single hyper-diploid cell into infant mice was unsuccessful in 47 cases, while we were successful in repeating Hauschka's result of about 15 per cent takes (9, 10) with the hypo-tetraploid Ehrlich lines (nine takes out of 75 infants).

![Chart 4](chart.png)

**DISCUSSION**

The "mosaic concept" of the tumor cell population has been suggested and discussed by Hauschka in the light of various facts (7, 8), among which...
he cites "the attainment of so-called autonomy, stepwise losses of histocompatibility genes, the spontaneous transitions from carcinoma through mixed tumor to sarcoma, and the replacement of differentiation by anaplasticity," as well as "the mutation-selection mechanisms" posing the chemotherapeutic dilemma of resistant lines (8). Of special interest here is the cytology of neoplastic cell populations: the chromosome number of transplantable tumors shows wider distribution (11, 20, 21, 24) than in normal adult or embryonic materials (21, 27); the clonal derivatives established by single cell transplantation (10) have different modes of chromosome number1 (9), and many cells fail to take. In agreement with chromosomal variability, values of DNA per nucleus are widely scattered in human neoplasms (18).

Transplantable tumors with modal chromosome numbers in the diploid region always have small components of polyploid cells, which normally remain very much in the minority. A spontaneous shift from diploid to near-tetraploid has been observed in the chromosome number of an ascites tumor "TA3" in A/Ha mice during serial transplantation. This was associated with loss of the formerly rigid host specificity (9), and was in keeping with the significant relationship between chromosome constitution and histocompatibility established for a variety of transplantable tumors (6, 12).

It was, therefore, of theoretical interest to develop a repeatable experimental procedure resulting in stable duplication of chromosome numbers. This was accomplished by intraperitoneal injection of a small cell dosage or starved cells or refrigerated cells of the hyper-diploid Ehrlich ascites into adult Swiss mice. The secondary ascites infiltrating from the subcutaneous solid tumor-nodules contained a high percentage of doubled chromosome constitutions. From the author's abstract of preliminary results (14), one might gain the impression that unfavorable external environments were selective in doing less damage to the small polyploid component in the hyper-diploid tumor before the treated ascites was inoculated into the mice. This may not have been the case; but the number of viable cells may have been generally reduced; because all the primary ascites tumors which occasionally developed after the injection of starved or chilled cell suspensions were hyper-diploid, while the polyploid sublines were found only among the secondary ascites which appeared after a long latent period in the same way as after transplantation of small cell dosages. The selective factor involved in these phenomena may be neither starvation nor cold-storage in vitro, but a difference in immune response between diploid and polyploid cells which shows up after inoculation of small immunizing dosages of viable cells. Small cell dosage—in contrast with "overwhelming" cell dosage—permits the host animal to build up at least temporarily effective defenses, so that cell types with the least immune response could be selected out. That "immunoselection" (7, 8, 18) may indeed be involved in the present observations is suggested by the data in Table 2.

Even when the cell inocula were relatively large, selection of polyploid cells occurred in mice immunized by the tail inoculation-amputation method. On the other hand, very small diploid inocula, which nearly always gave polyploid sublines in nonimmunized adult mice, were followed by near-diploid takes when they were put into infants, because infants are immunologically inactive (10, 25) and cannot respond differentially to the few tetraploid cells present in every inoculum. The major selective agent is probably a differential host response to minor differences of iso-antigenic efficiency between near-diploid and near-tetraploid cells (13).

Fig. 1.—A high dry field of a hyper-tetraploid subline of the Ehrlich ascites 18 hours after colchicine treatment. Note four well-spread metaphases, indicative of the high frequency of countable plates in such preparations.

Fig. 2.—A typical metaphase plate from nontreated hyper-diploid Ehrlich ascites with 46 chromosomes.

Fig. 3.—A metaphase plate from colchicine-treated hyper-diploid ascites with 45 chromosomes.

Fig. 4.—Higher magnifications of several structurally distinct chromosomes.

a) Secondarily constricted "A" chromosome.
b) Metacentric "B" chromosome.
c) Chromosome having small "head." The head should be distinguished from minute chromosome "d."
d) Minute chromosome.
e) Ordinary telocentric chromosome. Most of the mouse chromosomes are like "e."

"A" and "B" and minute chromosomes are characteristic of both hyper-diploid and hyper-tetraploid Ehrlich nuclei.
Fig. 5.—A typical hyper-tetraploid plate (92 chromosomes) from untreated EL 88, a polyploid subline derived from Ehrlich-Lettré. The micron scale in the upper left corner of Figure 5 also applies to Figures 6 and 7.

Fig. 6.—A hyper-tetraploid plate (88 chromosomes) from colchicine-treated EL 88. Note c-mitotic effect on chromosomes as compared with Figure 5.

Fig. 7.—A near-octoploid plate (163 chromosomes) from EL 88.

Fig. 8.—Endo-reduplication in a cell of the hyper-tetraploid EL 88, showing 89 four-stranded chromosomes.
It may be relevant here that approximately 15 per cent of the takes were obtained in the single cell transplantation of the hypo-tetraploid Ehrlich ascites (9), confirmed by the author, whereas we were unable to obtain any takes with single cells of the hyper-diploid Ehrlich. Recently, Lettré and Querner obtained only 1.2 per cent takes in 168 infant mice with the latter tumor (17). Thus, on the single cell level, the hyper-diploid tumor is nearly incompatible, while near-tetraploid cells of the same general type give a relatively high take percentage.

The new polyploid sublines of the Heidelberg Ehrlich ascites are now being put to wide experimental use as tools in studies of growth rate, chemotherapeutic sensitivity, and some biochemical differences (4) between precisely comparable diploid and tetraploid cells of common origin.

SUMMARY

Repeatable experimental production of polyploid sublines from the hyper-diploid Ehrlich ascites carcinoma has been achieved. After intraperitoneal injection of low cell dosage or starved cells or refrigerated cells into Swiss female adults, primary ascites often failed to develop. The secondary ascites infiltrating from the subcutaneous solid tumor nodules at the inoculation site contained from 60 to 95 per cent of polyploid cells. From these ascites, stable hyper-tetraploid sublines were established and maintained in serial transfer for over 20 weeks. The modal chromosome number of the derivative sublines is at 90–92, exactly twice that of 45–46, characterizing the hyper-diploid Ehrlich. The mechanisms involved in these shifts of ploidy are discussed from the point of view of “immunoselection.”

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