Chemotherapeutic Effects on Mammalian Tumor Cells.

II. Biologic and Chromosomal Instability of a Cyclophosphamide Treated Murine Leukemia

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SUMMARY

Murine leukemia L1210 has been treated with cyclophosphamide and 4 sublines have been developed from the tumor cells surviving drug therapy. Biologic studies have demonstrated a marked alteration of virulence compared to the parent tumor and striking cytogenetic modifications have been found to exist. Serial examinations over an interval of 22 weekly transplant generations have demonstrated a biologic and cytogenetic instability characterized by, generally, a tendency for return toward control values.

The observation that an alkylating agent altered the growth kinetics and karyotype of L1210 leukemia in mice has recently been reported (2). Leukemia sublines, developed from tumor cells surviving maximally sublethal treatment with cyclophosphamide, manifested a prolonged generation time, and cytogenetic studies demonstrated highly significant alterations. These findings have been confirmed (unpublished data) using a DBA-carried L1210 line in DBA mice, indicating that immunologic incompatibilities resulting from the employment of hybrid mice were not responsible for the original observations.

Four consecutively studied sublines, developed from recurrent tumors following cyclophosphamide treatment of L1210 leukemia, have now been examined serially over a period of 22 weekly passages. The instability of the induced modifications of virulence and karyotype are described in this paper.

MATERIALS AND METHODS

CDF, [(BALB/c X DBA/2)F] female mice were used and housed in a constant temperature facility. Water and laboratory chow were provided ad libitum. The L1210/M46G ascitic variant developed from the L1210 sensitive line (Goldin) was employed. The mice received 180–320 mg/kg body weight of cyclophosphamide i.p. in a single dose on day 3 following s.c. inoculation of 10⁶ cells. The drug was dissolved in saline in such concentration that the mice received 0.01 ml volume/gm body weight.

The subcutaneous tumors were individually homogenized in saline, and 1 mouse was inoculated i.p./homogenate. Individual cell suspensions obtained from the 8 mice 1 week later were bioassayed by inoculating 10 mice with 10⁶ cells for each suspension, 25 mice being inoculated with the parent line as a control.

RESULTS

The bioassay procedure summarized in Table 1 demonstrated that the virulence of untreated tumors undergoing ascitic to solid to ascitic conversions was essentially iden-
tical to that of the parent line ($P = 0.53$). The determin-
nations of median survival time for the sublines and control
(parent line) are shown in Chart 1. The deviation of each
subline from the control value was highly significant
($P \leq 0.001$) for the 1st transfer generation and remained so
for several generations. Eventually, all 4 sublines re-
covered a virulence approximating that of the parent line.
It is of interest that Subline B persisted with a slightly
prolonged median survival time at the 22nd transfer gen-
eration whereas Subline D killed more rapidly; both dif-
fferences are significant ($P \leq 0.01$). The prolonged
survival seen with mice inoculated with the sublines ap-
peared to be a consequence of a longer generation time as
estimated by direct cell counts (peritoneal washings) on
successive days following i.p. inoculation of $10^8$ cells. As
illustrated by Chart 2, the tumor doubling times are seen
to return toward the control time (15–16 hr) concomit-
tant with the decreasing median survival times of Subline B
during successive transplant (weekly) generations.

The cytogenetic findings are summarized in Tables 2
and 3. The parent line was evaluated at intervals during
the experiment and each subline was examined serially,
beginning with the 1st transfer generation, except for
Subline A which was initially karyotyped at Generation 10.
The parent L1210 tumor contains 3 predominant cell
lines, each with at least 1 easily recognizable marker
chromosome. These cell lines are characterized by (a)
a chromosome number of 40 with a minute chromosome,
(b) a chromosome number of 40 including both the minute
chromosome and a submetacentric chromosome, and (c)
a chromosome number of 41 including the minute chromo-
some. The stability of the parent tumor is indicated by a
similar chromosome count distribution and incidence of
predominant cell lines throughout the course of the study.
In addition, no modification in the parent L1210 tumor
resulted when it was converted from ascitic to solid to
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CHART 2.—Estimation of tumor generation times (Subline B) by direct ascitic cell counts. The markedly prolonged doubling time in the first transplant generation subsequently is seen to return toward the control rate. Each point represents the mean value for twenty cell counts for the control and ten cell counts for the subline determinations.

Marked instability of the cytogenetic modifications was observed in the serial studies, with a tendency for the chromosome count to return toward the normal (mouse) diploid number of 40. No consistent relationship between the cytogenetic findings and biologic changes was apparent, although the former would suggest that the observed modification of virulence in these cell lines is due to genetic alterations.

DISCUSSION

These studies were initiated to evaluate mammalian tumor cell characteristics when such cells had been exposed to chemotherapy under conditions where the dose levels would be maximal without being lethal. In this manner it was hoped that some insight could be gained into the kinetics of tumor cells receiving such maximally sublethal drug exposure. In theory, it was considered that this could best be accomplished by employing a system in which the fraction of cells surviving treatment would be minimal (hopefully a single cell). The expecta-
tion of this occurring would be greatest when some animals in a group (all having received a standard inoculum of cells and treated with an identical dose of drug) were “cured,” while others eventually developed recurrent leukemia.

As described in “Results,” it has been possible to demonstrate a substantial modification of growth kinetics, similar to that reported by other workers (1). The sublines exhibited a tendency to “recover” and in 1 instance, bioassay showed greater virulence in the subline than in the parent line. Over an interval of 16 months (encompassing this study), the parent L1210 line has not manifested even a slight instability of virulence. “Spontaneous” mutants with a decreased generation time would be expected to gain ascendance with a resulting increase in virulence. The failure to observe spontaneous instability of the parent tumor renders the increased virulence of Subline D particularly notable and the latter is considered indicative of an “induced” genetic change as the mechanism for the biologic and cytogenetic modifications.

Likewise, the cytogenetic alterations tended to be reversible with a progressive shifting of the chromosome count distributions toward the normal diploid number. This seemingly homeostatic tendency might be explained by an absence of sustained reproductive integrity of cells with hypo- or hyperdiploid chromosome numbers, by a continuous mutagenic process, or by selection with overgrowth of cells in the near-diploid range.

The rationale for studying tumor cell behavior under these conditions relates to the potential clinical situation in which chemotherapy succeeds in reducing the cell population to numbers not detectable and only 1 cell or very few cells might survive intensive drug exposure. Experimental studies indicate convincingly that the major action of most chemotherapeutic agents is to reduce fractionally (by cell kill) the tumor population (3). The prolonged lag phase and lengthened generation times observed are not in basic disagreement with this concept. Our studies have rather been directed toward the kinetics of the presumably few cells which, perhaps by random chance or by some inapparent process of selection, survive maximally sublethal drug exposure. It is worthy of comment that only under conditions where few cells are known to survive (as in a group of mice where a fraction are “cured” and the remainder develop recurrent leukemia) are alterations in the growth kinetics of the surviving cells likely to be detected. When a larger cell population survives treat-

### Table 2

<table>
<thead>
<tr>
<th>L1210 Line</th>
<th>No. of Cells</th>
<th>Chromosome Count Distribution (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent L1210 line</td>
<td>700</td>
<td>≤38</td>
</tr>
<tr>
<td>Subline A</td>
<td></td>
<td>89</td>
</tr>
<tr>
<td>Generation 10</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Generation 14</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>Generation 18</td>
<td>50</td>
<td>2</td>
</tr>
<tr>
<td>Generation 22</td>
<td>100</td>
<td>2</td>
</tr>
</tbody>
</table>

| Subline B | | 2 | 1 | 4 | 9 | 30 | 29 | 18 | 7 | 2 |
| Generation 1 | 100 | 3 | 4 | 42 | 27 | 16 | 6 | 1 |
| Generation 5 | 100 | 17 | 44 | 34 | 3 |
| Generation 10 | 100 | 2 | 3 | 16 | 63 | 9 | 1 | 5 |
| Generation 14 | 100 | 2 | 9 | 82 | 3 | 2 | 2 |
| Generation 18 | 100 | 2 | 94 | 3 | |
| Generation 22 | 100 | 2 | 9 | 2 | 1 |

| Subline C | | 3 | 5 | 10 | 28 | 29 | 16 | 6 | 3 |
| Generation 1 | 100 | 1 | 33 | 8 | 4 | 1 | 1 | 50 | 2 |
| Generation 5 | 100 | 1 | 89 | 4 | 1 | 1 |
| Generation 10 | 100 | 2 | 91 | 7 |
| Generation 14 | 100 | 2 | 94 | 4 |
| Generation 18 | 100 | 2 | 94 | 4 |
| Generation 22 | 100 | 0 | 97 | 1 | |

* Cells counted at the beginning of, during, and at completion of the study are summarized here.

³ X denotes 1 cell counted with given chromosome number.
TABLE 3

INCIDENCE OF CELL LINES, MARKERS, AND POLYPLOIDY

<table>
<thead>
<tr>
<th>L1210 LINE</th>
<th>NO. OF CELLS</th>
<th>INCIDENCE OF CELL LINES (%)</th>
<th>INCIDENCE OF MARKERS (%)</th>
<th>POLYPLOID (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>40+ minute</td>
<td>40+ minute + metacentric</td>
<td>41+ minute</td>
</tr>
<tr>
<td>L1210</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beginning of study</td>
<td>200</td>
<td>35</td>
<td>12.5</td>
<td>39</td>
</tr>
<tr>
<td>Early in study</td>
<td>200</td>
<td>47</td>
<td>9.5</td>
<td>43</td>
</tr>
<tr>
<td>Later in study</td>
<td>100</td>
<td>35</td>
<td>10</td>
<td>51</td>
</tr>
<tr>
<td>Completion of study</td>
<td>200</td>
<td>56</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>Ascitic/solid/ascitic</td>
<td>100</td>
<td>36</td>
<td>15</td>
<td>42</td>
</tr>
</tbody>
</table>

Subline A
- Generation 10: 100, 58, 5, 5 | 97, 13 | 2
- Generation 14: 100, 59, 2, 16 | 93, 5 | 2
- Generation 18: 50, 48, 0, 22 | 94, 4 | 3
- Generation 22: 100, 65, 0, 12 | 100, 0 | 2

Subline B
- Generation 1: 100, 0, 0, 0 | 95, 6 | 11
- Generation 5: 100, 0, 0, 0 | 96, 6 | 4
- Generation 10: 100, 2, 0, 2 | 96, 2 | 3
- Generation 14: 100, 3, 0, 15 | 100, 0 | 2
- Generation 18: 100, 1, 0, 7 | 98, 4 | 1
- Generation 22: 100, 0, 0, 0 | 100, 0 | 1

Subline C
- Generation 1: 100, 28, 0, 3 | 100, 0 | 4
- Generation 5: 100, 69, 0, 14 | 97, 0 | 6
- Generation 10: 100, 81, 0, 10 | 99, 0 | 3
- Generation 15: 100, 84, 0, 10 | 98, 0 | 2
- Generation 22: 100, 92, 0, 5 | 100, 0 | 0

Subline D
- Generation 1: 100, 0, 0, 0 | 82, 0 | 11
- Generation 5: 100, 0, 0, 0 | 71, 0 | 38
- Generation 10: 100, 3, 0, 2 | 7, 2 | 3
- Generation 14: 100, 3, 0, 3 | 7, 0 | 0
- Generation 18: 100, 1, 0, 2 | 3, 0 | 1
- Generation 22: 100, 2, 0, 0 | 3, 1 | 3

ACKNOWLEDGMENTS

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REFERENCES

Chemotherapeutic Effects on Mammalian Tumor Cells: II. Biologic and Chromosomal Instability of a Cyclophosphamide Treated Murine Leukemia

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