CHROMOSOMAL ALTERATION AND THE DEVELOPMENT OF TUMORS. XVI.
KARYOLOGICAL STUDIES ON SENSITIVE AND RESISTANT SUBLINES
OF THE MOUSE LYMPHOCYTIC LEUKEMIA, L-1210,
TO SEVERAL ANTITUMOR AGENTS¹⁾

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The relationship between karyotype changes and tumor cell resistance to anticancer compounds has been studied by several investigators. Some of them found that karyotypes of the resistant sublines showed a remarkable change from the karyotypes of the sensitive parent line from which they were derived (Hauschka 1958, Bieseke *et al.* 1959, Vogt 1959, Stone and Kang 1962, Biedler *et al.* 1963, 1965, Schrecker *et al.* 1963, and Yosida 1966a), while others observed no change of karyotypes in resistant sublines (Hauschka 1958, Cailleau 1960, Hakala and Ishihara 1962, Harris and Ruddle 1960, 1961). In the mouse lymphocytic leukemia, P388, growing *in vitro*, Yosida (1966a), observed that the karyotypes of drug resistant sublines were markedly different from those of sensitive line. In an amethopterin resistant line of the lymphocytic leukemia L-1210 a different chromosome pattern from that of sensitive cells was observed by Bieseke *et al.* (1959), Biedler *et al.* (1963, 1965) and Schrecker (1963).

The present paper deals with the investigation of the karyotypes in sensitive and five drug resistant sublines of the ascitic form of the mouse lymphocytic leukemia, L-1210.

MATERIAL AND METHODS

All sensitive and drug resistant sublines in the ascites form of mouse leukemia L-1210 used in the present study were developed by one of us (L. W. Law). In this study, a sensitive L-1210/s line of a transplantable lymphocytic neoplasm and the following 5 drug resistant sublines were used: (1) a resistant subline, L-1210/AM, selected by continuous treatment with the antifolic compound, methotrexate (amethopterin). (2) a resistant subline, L-1210/6MP-FU, selected by two different drugs, 6-mercaptopurine and 5-fluorouracil. (3) a resistant subline, L-1210/6MP-FU-AM, selected by three different drugs, 6-mercaptopurine, 5-fluorouracil and methotrexate. (4) a resistant subline L-1210/38280 selected by the antileukemic compound NSC-38280 (terephthalanilide, 2-amino-4', 4''-di-2-imidazolin-2-yl-, dihydrochloride). (5) a resistant subline, L-1210/TG, selected by antipurine compound, thioguanine. The procedures used for establishment of the several

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resistant sublines have been described by Law (1951, 1953, 1962) and Law *et al.* (1956). The resistant L-1210 sublines were developed at different times during the transplantation of the parental line (Law 1962). The most closely related sublines were; L-1210/6MP, the doubly resistant L-1210/6MP-FU and the triply resistant L-1210/6MP-FU-AM. Those were developed sequentially. It should be noted here that all sublines, once developed, had stable heritable characters, and were carried in transplantation in the absence of the selecting anticancer drugs.

The ascites tumors were fixed with Carnoy solution 3 : 1 after pretreatment with 1 percent sodium citrate. The fixed material was placed on a wet slide, dried quickly, and stained with acetic orcein by the usual cytological technique.

RESULTS

1. Chromosome number distribution

Distribution of chromosome numbers in the sensitive L-1210/s subline and in 5 resistant sublines are shown in Table 1. As the table shows, in the sensitive line the modal chromosome number was 40, but secondary peak was at 39 chromosomes. Resistant sublines, L-1210/AM, L-1210/6MP-FU, L-1210/6MP-FU-AM and L-1210/38280, also had a chromosome mode at 40 similar to that in the sensitive L-1210/s subline. The secondary peak of chromosome number distribution was at 39 chromosomes in the resistant sublines L-1210/6MP-FU and L-1210/6MP-FU-AM, but at 41 chromosomes in the resistant sublines, L-1210/38280 and L-1210/AM. In only one resistant subline L-1210/TG, cells with 41 chromosomes were observed most frequently and the secondary mode was found at 40.

2. Karyotypes

a) *Sensitive L-1210/s line*: The karyotypes of 49 cells were analysed. All cells

Table 1. Distribution of chromosome numbers in the sensitive and various resistant sublines of mouse lymphocytic leukemia L-1210

Subline	L-1210/s	AM	6MP-FU	6MP-FU-AM	38280	TG
Chrom. no.						
34	6.1%					
35						
36	4.1	1.8	2.0			
37	4.1	5.3	2.0	1.8	7.8	
38	8.2	7.0	9.8	23.6	7.8	9.3
39	28.6	12.3	27.5	29.1	11.8	11.1
40	38.8	45.6	52.9	32.7	45.1	22.2
41	4.1	19.3	5.9	5.5	21.6	48.1
42		7.0			5.9	7.4
43		1.8		1.8		1.9
44	2.0			1.8		
45				3.6		
46	4.1					
Total no. of cells observed	49	57	51	55	51	54
%	100.1	100.1	100	99.9	100.1	100

were characterized by containing one large submetacentric chromosome in their karyotypes. It is described as an M-marker in the tables and figures (Fig. 1). Among 49 cells observed, 46 (95 %) had one large submetacentric marker chromosome, but the remaining 3 cells had a small minute (m-marker) together with the large submetacentric marker (Table 2). Arm ratio (long arm to short arm) in the large submetacentric chromosomes was at 1.2 to 2.0, the highest frequency being at 1.4 to 1.59.

Table 2. Frequency of marker chromosomes in the sensitive L-1210/s line

Marker Chrom. no.	1M, 1m	1M.	Total no. of cells observed
34		3	3
36		2	2
37		2	2
38		4	4
39	1	13	14
40	1	18	19
41	1	1	2
44		1	1
46		2	2
Total no. of cells observed	3	46	49

M = large submetacentric marker
m = minute

b) *Resistant subline L-1210/AM*: The large submetacentric marker which was always observed in the cell population of the sensitive L-1210/s line could not be found in this subline. However, the minute marker was frequently observed (Fig. 2). Among 59 cells analysed, 39 cells had the minutes. Minutes could be classified into two types, one very small and the other considerably larger. The former is a dot-like element designated as m_1 , and the latter as m_2 , in Table 3. Among 39 cells with the minutes, 18 cells had one m_1 marker, 8 cells had one m_2 marker, one cells has three m_1 markers, 7 cells had two m_1 markers, and remaining 5 cells had one m_1 and one m_2 markers. No marker chromosome was observed in 18 among the 59 cells analysed.

Table 3. Frequency of marker chromosomes in a resistant subline L-1210/AM

Marker Chrom. no.	3 m_1	2 m_1	1 m_1 , 1 m_2	1 m_1	1 m_2	None	Total no. of cells observed
36				1			1
37		1		1		1	3
38				1		3	4
39		1		1	2	4	8
40	1		1	12	3	9	26
41		3	3	2	2	1	11
42		2	1		1		4
Total no. of cells observed	1	7	5	18	8	18	57

m_1 = Very small minute. m_2 = Larger than m_1 .

c) *Resistant subline L-1210/6MP-FU*: The karyotype was analysed in 51 cells, and no marker chromosome was found in 47 cells (Fig. 3). Only 3 cells had a large submetacentric marker as observed in L-1210/s line (Fig. 4), and remaining one cell had two minute markers (Table 4).

d) *Resistant subline L-1210/6MP-FU-AM*: As in the former resistant subline, marker chromosomes were not observed in most cells (Fig. 5). Only one cell among 55 analysed had one large submetacentric marker (Table 5).

e) *Resistant subline L-1210/38280*: In this subline the large submetacentric marker disappeared completely in their karyotypes. 29 (57 %) among 51 cells analysed had no marker at all, and the remaining 22 cells had one small minute marker similar to the m₁ in L-1210/AM subline (Table 6).

Table 4. Frequency of marker chromosomes in a resistant subline L-1210/6MP-FU

Marker Chrom. no.	1M	2m	None	Total no. of cells observed
36			1	1
37			1	1
38			5	5
39	2	1	11	14
40	1		26	27
41			3	3
Total no. of cells observed	3	1	47	51

M = large submetacentric marker
m = minute

Table 5. Frequency of marker chromosomes in a resistant subline L-1210/6MP-FU-AM

Marker Chrom. no.	1M	None	Total no. of cells bsserved
37		1	1
38		13	13
39	1	15	16
40		18	18
41		3	3
42			
43		1	1
44		1	1
45		2	2
Total no. of cells observed	1	54	55

M = large submetacentric marker

Table 6. Frequency of marker chromosomes in a resistant subline L-1210/38280

Marker Chrom. no.	1 m ₁	None	Total no. of cells observed
37		4	4
38		4	4
39	2	4	6
40	6	17	23
41	11		11
42	3		3
Total no. of cells observed	22	29	51

m₁ = small minute marker

f) *Resistant subline L-1210/TG*: The thioguanine resistant subline in contrast to all others had 41 modal chromosomes. A large telocentric chromosome with remarkable secondary constriction near the centromere was found in many cells. The chromosome

Table 7. Distribution of marker chromosomes in a resistant subline L-1210/TG

Chrom. no.	Marker	1 m ₁	1 m ₃	2 m ₁	1 m ₁ , 1 m ₃	1 m ₁ , 1 T	1 m ₃ , 1 T	3 m ₁	2 m ₁ , 1 m ₃	2 m ₁ , 1 T	1 m ₁ , 1 m ₃ , 1 T	2 m ₃ , 1 T	Total no. of cells observed
38			1		2	1					1		5
39		1			1	1	2				1		6
40					4				1	4	3		12
41				3	7			1		5	9	1	26
42								1	1		1	1	4
43					1								1
Total no. of cells observed		1	1	3	15	2	2	2	2	9	15	2	54

m₁ = small telocentric minutem₃ = small metacentric minute

T = large telocentric chromosome with secondary constriction

was designated as the T marker (Table 7). Two kinds of minutes, one small telocentric and the other a small metacentric, were observed as other markers (Fig. 6). The former is similar to the m₁ marker in the L-1210/AM and L-1210/38280 sublines, but the latter is a new marker designated here as m₃. Several combinations of these markers occurred in these cells as shown in table 7. 15 among 54 cells analysed had one T, one m₁ and one m₃ markers, 15 cells had one m₁ and one m₃ markers, 9 cells included one T and two m₁ markers, and the remaining 15 cells were characterized by having several combinations of the above three markers.

DISCUSSION

A possible relationship between the change in karyotypes and the development of resistance to drugs has been discussed by several investigators. Change of karyotypes in resistant lines from those of the parental sensitive lines was observed in mouse lymphosarcoma P1798 and Ehrlich tumor cells (Hauschka 1958), HeLa cells (Vogt 1959, Stone and Kang 1962), mouse leukemia L-1210 (Bieseke *et al.* 1959, Biedler *et al.* 1963, 1965, Schrecker *et al.* 1963) and mouse lymphocytic leukemia P388 (Yosida 1966a), but no significant change of karyotypes was found in mouse plasma cell tumor 70429 (Hauschka 1958), mouse leukemia L-4946 (Cailleau 1960) mouse sarcoma S180 (Hakala and Ishihara 1962) and pig kidney cell lines (Harris and Ruddle 1960, 1961). Eventhough a change of karyotype was found in resistant lines, the mode of karyotype change was not always constant. But a similarity of karyotype change in those resistant lines developed by different concentrations of 8-azaguanine and methotrexate (amethopterin) was found in mouse lymphocytic leukemia P388 (Yosida 1966a). In this case an increase of chromosome number was found in several resistant sublines developed by treatment with rather low drug concentrations, and secondarily a decrease of chromosome numbers was observed in several resistant lines which had been established by treatment with more increased concentrations of the two drugs.

Figs. 1-6. Metaphase chromosomes in sensitive and drug resistant sublines in mouse leukemia L-1210. (All figures are drawn by camera-lucida). 1. Sensitive L-1210/s line. 40 chromosomes are counted. Large submetacentric chromosome is shown by an arrow. 2. Methotrexate (amethopterin) resistant subline, L-1210/AM. 40 chromosomes are counted. Minute (m_1) marker is observed (arrow). Large submetacentric marker was not observed. 3. 6-mercaptopurine and 5-fluorouracil resistant subline, L-1210/6MP-FU. 40 chromosomes are counted. Large submetacentric and minute markers were not observed. 4. Subline L-1210/6MP-FU. 39 chromosomes including one large submetacentric marker (arrow) are observed. 5. 6-mercaptopurine, 5-fluorouracil and methotrexate resistant subline, L-1210/6MP-FU-AM. 40 chromosomes are counted. No marker chromosome is observed. 6. Thioguanine resistant subline, L-1210/TG. 41 chromosomes are counted. Two minute markers (m_1 and m_3) are observed (arrow). M_3 marker is shown in the right side. Long telocentric marker does not appear in this figure.

Chromosome changes of sensitive and drug resistant sublines of mouse leukemia L-1210 were observed by several investigators as stated above. According to Bieseke *et al.* (1959) a sensitive L-1210 line was characterized by a bimodal distribution at 41 and 40 chromosomes, and most of cells contained two marker chromosomes; one long-element with submedian centromere and one minute. In an methotrexate-resistant line, the large subtelocentric element was not observed in its cell population, but in 6-mercaptopurine and 5-fluorouracil resistant lines a lack of the marker was not observed. It should be pointed out that the drug resistant lines reported by Bieseke were of a different origin and developed in a different laboratory from those reported in the present study. Schrecker *et al.* (1963) reported that absence of the subtelocentric marker was found in the three resistant sublines, established (i) by treatment with methotrexate, (ii) by two different drugs, azaserine and methotrexate, and (iii) by the three different drugs, methotrexate, 6-mercaptopurine and 5-fluorouracil. In the present study all 5 resistant sublines of L-1210 which were selected by treatment with (i) methotrexate alone, (ii) 6-mercaptopurine and 5-fluorouracil, (iii) 6-mercaptopurine, 5-fluorouracil and methotrexate, (iv) antileukemia compound NSC-38280 and (v) thioguanine alone showed the absence of the large submetacentric marker, which was however found in the sensitive parental line. On the other hand, a morphological change of the subtelocentric chromosome in methotrexate resistant L-1210 sublines was found by Biedler *et al.* (1965). In that case, cells of the sensitive line had a subtelocentric marker, but those of resistant sublines had submetacentric or metacentric markers instead of the subtelocentrics. The sublines studied by Schrecker *et al.* also have different origins from those reported in this laboratory and those reported by Biedler.

A striking difference in the shape of large marker element was found in sensitive L-1210/s lines used by us and those used by Bieseke *et al.* (1959), Schrecker *et al.* (1963) and Biedler *et al.* (1963, 1965). The arm ratio (long arm to short arm) in the marker chromosome in the present material was 1.2 to 2.0, the highest frequency being at 1.4 to 1.59, but that in the sensitive lines used by latter authors was about 3 to 4. The marker in the present material was clearly a submetacentric chromosome, while that in the latter studies was a subtelocentric element. From the above investigations, it is suggested that the karyotypes in sensitive L-1210 differentiated into two types; subtelocentric type observed in Biedler's material and submetacentric type in Law's material; the stemline cells with the subtelocentric marker in the former might be sensitive only to methotrexate, but those with submetacentric element were sensitive to various antileukemic drugs as observed in the present study. Thus, all 5 drug resistant sublines of L-1210 showed the lack of the submetacentric marker.

Besides the disappearance of the submetacentric marker in the drug resistant sublines studied in this laboratory new minute markers, m_1 , m_2 , and m_3 , and a T marker appeared in the present resistant sublines. The small m_1 minute marker was found in few cells of the sensitive L-1210/s line as seen in table 2, but the other minute and T markers were not observed in the sensitive line. They appeared therefore to develop in the course of selection of resistant sublines. On the other hand, the submetacentric marker which was usually observed in parental sensitive line was found rarely in some resistant sublines. Based on the above investigations, it can be concluded that when

the L-1210/s cells are treated with antitumor agents several kinds of chromosomal rearrangement may occur, among them unaltered cells with submetacentric markers may be selected from the cell population, and some altered cells without the submetacentric marker and with minute markers can be established as new stemline cells in resistant sublines. In the other words, it can be said that the resistance to antileukemic agents in the mouse leukemia L-1210 can develop by sequential events of mutation and selection as described already by Yosida (1966b) in other several tumors.

According to Biedler *et al.* (1963, 1965) and Schrecker *et al.* (1963) an increase of dihydrofolate reductase activity was associated with the development of resistance to methotrexate and they suggested a correlation between the loss of a large subtelocentric chromosome and increase of the enzyme activity. On the other hand, loss of nucleotide pyrophosphorylase was observed in 6-mercaptopurine and thioguanine resistant (TG) L-1210 by Brockman (1960). The latter author did not study the karyotypes of the resistant lines, but the thioguanine resistant subline, studied by Brockman was identical with the subline L-1210/TG, reported here. Although the present study we did not examine biochemical characteristics in the resistant lines, the relationship between change of biochemical characteristics and karyotypes in drug resistant lines remains as a further interesting problem.

SUMMARY

Chromosomes in a drug sensitive and 5 drug resistant sublines of mouse leukemia L-1210 were studied. The 5 sublines used in the present study were resistant to methotrexate (L-1210/AM), 6-mercaptopurine and fluorouracil (L-1210/6MP-FU), 6-mercaptopurine, fluorouracil and methotrexate (L-1210/6MP-FU-AM), antileukemic agent NSC-38280 (L-1210/38280) and thioguanine (L-1210/TG). The chromosome numbers in sensitive L-1210/s cells varied from 34 to 46 with the mode at 40. Resistant sublines L-1210/AM, L-1210/6MP-FU, L-1210/6MP-FU-AM and L-1210/38280 also had 40 chromosomes as the mode of variation, fluctuating from 36 to 45. The L-1210/TG subline had only 41 chromosomes as a mode. A large submetacentric marker chromosome was usually found to characterize the sensitive line. In none of resistant lines, however, this marker chromosome was observed, while a small minute telocentric or metacentric and long telocentric T-markers were often observed in them. The relationship of these changes in karyotypes and type to resistance was discussed.

LITERATURE CITED

- Biedler, J. L., A. W. Schrecker, and D. J. Hutchison, 1963 Selection of chromosomal variant in amethopterin-resistant sublines of leukemia L1210 with increased levels of dihydrofolate reductase. *J. Natl. Cancer Inst.* **31**: 575-601.
- Biedler, J. L., A. M. Albrecht, and D. J. Hutchison, 1965 Cytogenetics of mouse leukemia L1210, 1. Association of a specific chromosome with dihydrofolate reductase activity in amethopterin-treated sublines. *Cancer Res.* **25**: 246-257.
- Bieseke, J. J., J. L. Biedler, and D. J. Hutchison, 1959 The chromosomal status of drug resistant sublines of mouse leukemia L1210. In "Genetics and Cancer" (Univ. Texas Press, Austin, Texas), pp. 295-307.

- Brockman, R. W., 1960 A mechanism of resistance to 6-mercaptopurine: Metabolism of hypoxanthine and 6-mercaptopurine by sensitive and resistant neoplasms. *Cancer Res.* **20**: 643-653.
- Cailleau, R., 1960 Establishment of two mouse ascites tumors in tissue culture: Ehrlich's ascites carcinoma and L-4946. Abstracts American Tissue Culture Association, Chicago, pp. 26.
- Hakala, M. T., and T. Ishihara, 1962 Chromosomal constitution and amethopterin resistance in cultured mouse cells. *Cancer Res.* **22**: 987-992.
- Harris, M., and F. H. Ruddle, 1960 Growth and chromosome studies on drug resistant lines of cells in tissue culture. In "Cell Physiology of Neoplasia" (Univ. Texas Press, Austin, Texas), pp. 524-546.
- Harris, M., and F. H. Ruddle, 1961 Clone strain of pig kidney cells with drug resistance and chromosomal markers. *J. Natl. Cancer Inst.* **26**: 1405-1411.
- Hauschka, T. S., 1958 Correlation of chromosomal and physiologic changes in tumors. *J. Cellular Comp. Physiol.* **52** (Suppl. 1): 197-233.
- Law, L. W., 1951 Observations on properties of leukemic cells resistant to folic acid antagonists. *J. Natl. Cancer Inst.* **11**: 849-865.
- Law, L. W., 1953 Resistance in leukemic cells to an adenine antagonist, 6-mercaptopurine. *Proc. Soc. Exptl. Biol. Med.* **84**: 409-412.
- Law, L. W., 1962 Studies of inhibitory effects of terephthalanilide derivatives against several variants of leukemia L1210. *Cancer Chemotherapy Rep.* **19**: 13-18.
- Law, L. W., V. Taormina, and P. J. Boyle, 1956 Resistance to thioguanine in an acute lymphocytic leukemia, L-1210. *Proc. Am. Assoc. Cancer Res.* **2**: 129.
- Schrecker, A. W., J. M. Venditti, N. H. Greenberg, J. L. Biedler, D. L. Robinson, and D. J. Hutchison, 1963 Association of increased dihydrofolate reductase levels and chromosome alteration in amethopterin-resistant sublines of leukemia L1210. *J. Natl. Cancer Inst.* **31**: 557-574.
- Stone, D., and Y. S. Kang, 1962 Differences in chromosome stem-lines of a strain of HeLa cells inhibited in growth by certain steroids, and of steroid-resistant sub-lines selected from the sensitive strain. *Endocrinology* **71**: 238-243.
- Vogt, M., 1959 A study of the relationship between karyotype and phenotype in cloned lines of strain HeLa. *Genetics* **44**: 1257-1270.
- Yosida, T. H., 1966a Chromosomal alteration and the development of tumors XV. Change of chromosome pattern in 8-azaguanine and amethopterin-resistant sublines of the mouse lymphocytic neoplasm, P388, cultured *in vitro*. *Japan. J. Genetics* **41**: 59-74.
- Yosida, T. H., 1966b Relation between chromosomal alteration and development of tumors. *Japan. J. Genetics* **41**: 439-451.