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Banding Karyotype Analysis of Murine Leukemia L1210 Cell Lines with Special Regard to Changes Associated with a Shift from *in Vivo* to *in Vitro* Growth*

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Abstract—*An attempt was made to establish permanent* in vitro *cultures of L1210 leukemia cells under close chromosome control. The investigations involved three independent adaptations. The karyotype was analyzed by the G-banding method. Chromosome analysis of the* in vitro *cell populations was carried out as soon as they could be analyzed, i.e. immediately after they obtained stable growth in the culture. By comparing the karyotype of the* in vivo *parental cell line with that of the derivative sublines* in vitro, *it was found that only some karyotypic variants present in the heterogenous original population* in vivo *were able to produce cell lineages. In some cases rapid overgrowth of the cell populations by newly formed karyotypic variants was observed. In all independently obtained sublines changes in the karyotype involved the same chromosomes, thus suggesting that these changes were non-random for the process of cell line establishment in the system investigated. The observed changes in the number of copies of chromosomes 9 and 15 in the cells from the culture seem to indicate that the ratio of the genes localized in these chromosomes may be important for the growth in perpetuation of the cells.*

INTRODUCTION

THE PHENOMENON of establishment of cells *in vitro* has been studied by different systems and methods [1-6]. One debatable problem is whether the established cells were already present in the original *in vivo* population or whether the cells transferred *in vitro* went through changes which made possible unlimited proliferation in culture conditions. It seems that this is important problem could be solved by comparing the karyotype of the parental cell line growing *in vivo* with that of the derived sublines *in vitro* if at least two conditions are met: first, that techniques are used for chromosome banding permitting identification of individual chromosomes and their rearrangements, and second, that the analysis of the karyotype is carried out early enough, i.e. soon after the cell growth becomes stabilized. In this way it is possible to differentiate between the primary changes involved in the establishment of the *in vitro* cell line and those resulting from the evolution in the cell population during the long-term *in vitro* growth. Earlier, such trials were undertaken in the period when the techniques of chromosome banding had not yet been developed [7-10], were not yet used [11-13] or when the second of the above conditions had not been met [14-15]. In the present study the problem was approached by analyzing the karyotypic changes occurring during the establishment of mouse lymphoblastic leukemia L1210 cell lines.

This 35-yr-old [16] transplantable mouse ascites tumor was chosen because strains of the leukemia L1210 have long been employed as extremely useful material in various experimental biological systems and are still utilized in many laboratories for the evaluation of potential anticancer agents. Many of these studies used *in vitro* adapted cell lines, although the possibility that the tumor cells surviving in culture may not be entirely representative of the general tumor cell

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population cannot be excluded. For these reasons such cell lines must be examined carefully in order to determine whether and how the cell properties differ in *in vivo* and *in vitro* environments.

Three consecutive adaptations were studied, which resulted in three derivative sublines *in vitro*: VAI, VAII and VAIII. The karyotype was analyzed by the technique of G-banding patterns. A detailed analysis of the karyotype involved cell populations after 1 month of *in vitro* culture, that is, as soon as they could be analyzed. The results are referred to the karyotype of the *in vivo* parental cell line.

MATERIALS AND METHODS

Parental cell line L1210(V)

The origin and G-banding patterns of this cell line have been described in detail in our previous paper [32]. To our knowledge, this particular cell line has never been adapted to *in vitro* growth before. The line was maintained in serial passages in ascites form in female DBA/2w mice. The median survival time was 25 days (range 20-30 days) for mice inoculated i.p. with 10^5 viable cells.

Cultures were grown in suspension in MEM medium supplemented with 10% of calf serum, 100 u/ml penicillin, 5 μg/ml streptomycin and 5 μg/ml tylosine. Once established, the cultures were maintained by diluting the concentration to around 1-2 × 10^5 cells/ml every 2 days. Trypan blue exclusion test was used to assess cell viability.

Conventional air-dried chromosome preparations were banded according to a modified trypsin-Giemsa technique described by Wang and Fedoroff [33] (5 ml stock Giemsa solution and 75 ml phosphate buffer, pH 6.8). The slides were rinsed twice in distilled water and blotted dry. Metaphase plates were photographed and karyotypes were arranged according to the standard mouse karyotype described by Nesbitt and Francke [17]. The photographed cells were selected for karyotyping solely on the basis of their having fewer overlaps and better bands.

RESULTS

Initiation of in vitro *sublines and their growth characteristics*

The attempts to grow L1210 cells *in vitro* had to be repeated several times before achieving success. The time of cell collection and choice of medium were found to be important: all attempts with cells collected from mice 3 and 5 days after i.p. injection, as well as those with Parker 199, medium, failed.

Sublines *in vitro* were obtained in a similar way: the cells were derived from the ascites fluid of a mouse injected i.p. with L1210(V) cells 8 days before cell collection. Initial cell density in the culture ranged from 4 to 6 × 10^5/ml. During the first few days the culture was diluted every day by addition of fresh portions of medium to maintain a constant culture volume. For the VAII subline, between days 9 and 26 no observations were made and the medium was not replenished. The growth pattern of VAII cells during the initial 35 days of culture is shown in Fig. 1. Two phases of establishment can be distinguished. In fact in the first 26-day-long phase the number of viable cells decreased rapidly, only a small fraction of them increased, which indicated that some cell divisions were present. During the second phase, a typical growth pattern of the established culture was observed. The growth curves for VAI and VAIII sublines were of similar character. The population doubling times calculated for the period between days 30 and 90 of culture for the sublines VAI, VAII and VAIII were: 12.0 ± 4.5, 11.3 ± 4.2 and 12.0 ± 3.9 hr respectively.

In the experiment for determining the *in vitro* growth ability of VAII cells after successive passages *in vivo*, it was found that such an ability could be used as a stable phenotypic marker in the sense that after a given period (10 months in our case) of continuous passaging in mice the culture could be readily restituted.

Cytogenetic investigations

Chromosome numbers of the parental *in vivo* line in 50 cells examined varied from 33 to 40, with a mode of 38. The number of chromosomes in each of the *in vitro* sublines was close to that found in the *in vivo* parental L1210(V) cells, but their distribution was much less dispersed. Each subline ranged from 31 to 40 (mode 39) for VAI, from 34 to 42 (Mode 41) for VAII and from 34 to 44 (mode 38) for VAIII. In the case of the VAII

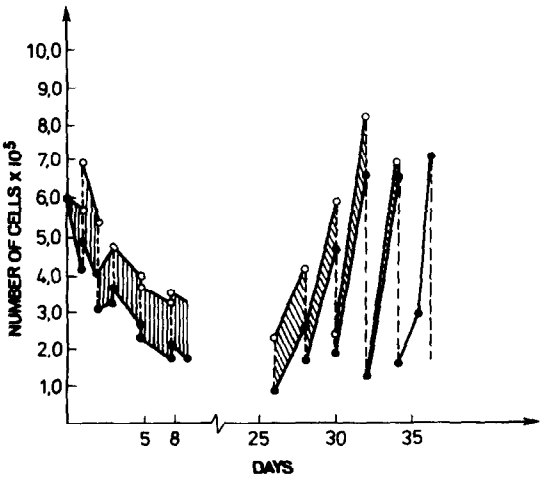

Fig. 1. Daily growth curve of VAII cells from 0 to 35 days of culture. O——O, total cells; ●——●, total viable cells.

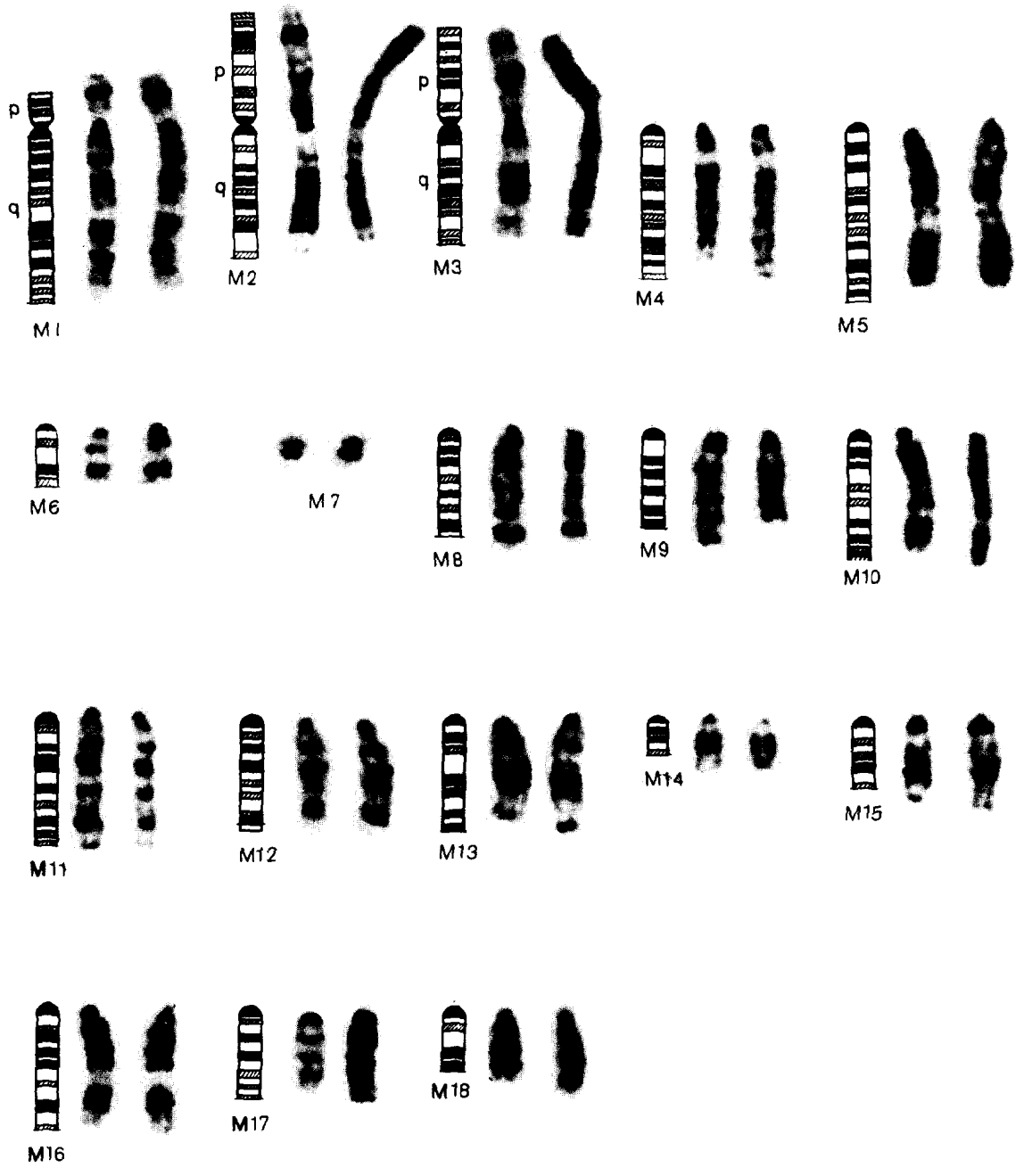

Fig. 2. Markers of L1210(V) cell line. For each of these markers there are given one diagrammatic drawing of the G-band pattern and two photographs.

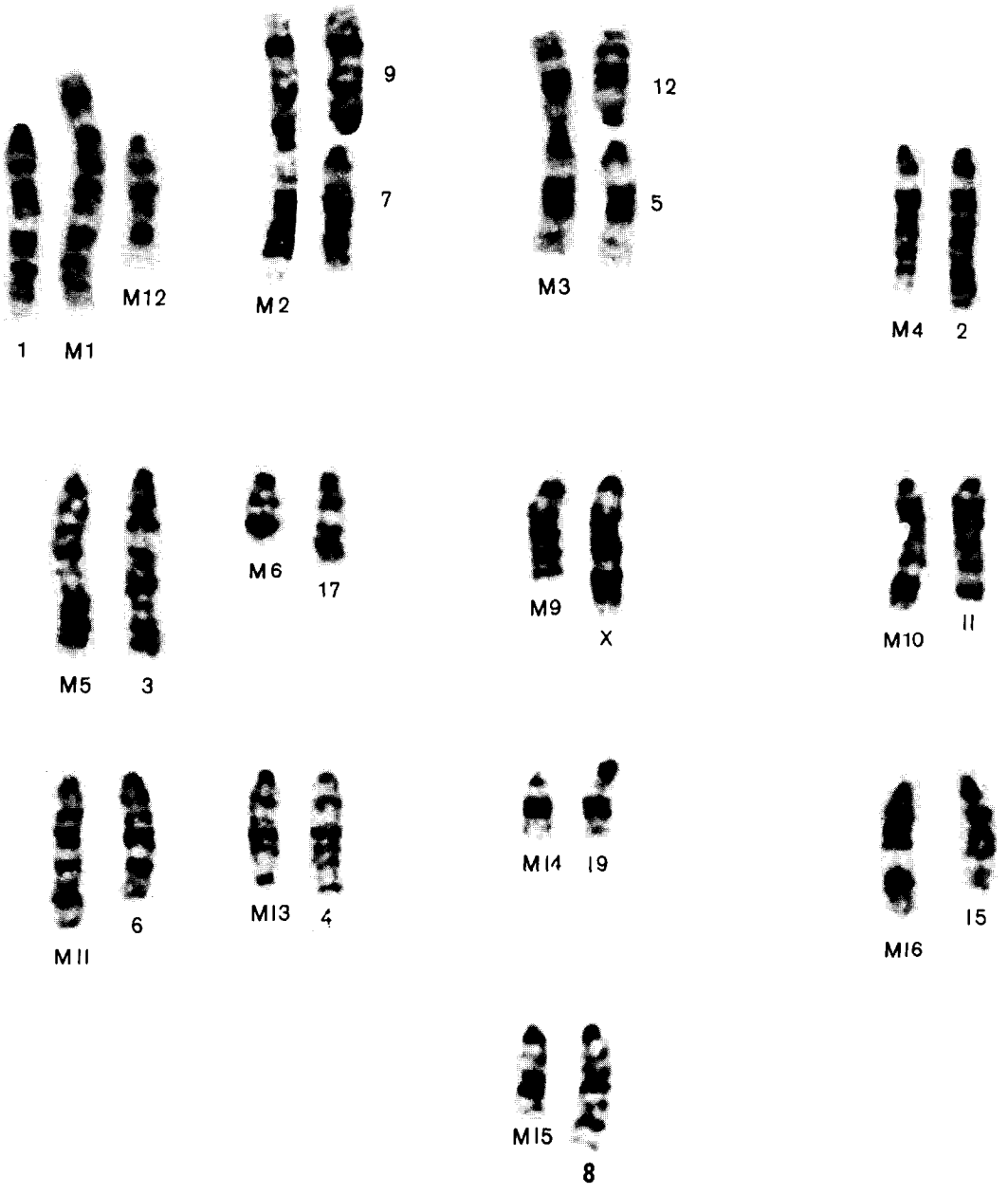

Fig. 3. Possible derivation of 14/18 marker chromosomes found in the L1210 leukemia cell lines under study. Each marker chromosome is placed next to a corresponding normal chromosome from the same tumor cell. Using an international system for human genetic nomenclature [18] they may be described as follows: M1, rob t(?; 1) (:? → cen:: 1 cen → 1 ter); M2, rob t(9; 7) (9 ter → cen:: 7 cen → 7 E3::?); M3, rob t(5; 12) (12 ter → cen:: 5 cen → 5 ter); M4, t(2; ?) (2 cen → 2 F1::?); M5, t(3; ?) (3 cen → 3 F3::?); M6, del (17) (cen → B::D → E4:); M9, t(X; ?) (X cen → X D::?); M10, t(11;?) (11 cen → 11 ter::?); M11, t(6;?) (6 cen → 6 A2::6 B1 → 6 F1::?); M12, del(1) (cen → F:); M13, del(4) (cen → C5::D1 → ter); M14, del(19) (cen::A → ter); M15, del(8) (cen → C3:); M16, t(15; ?) (15 cen → 15 E::?).

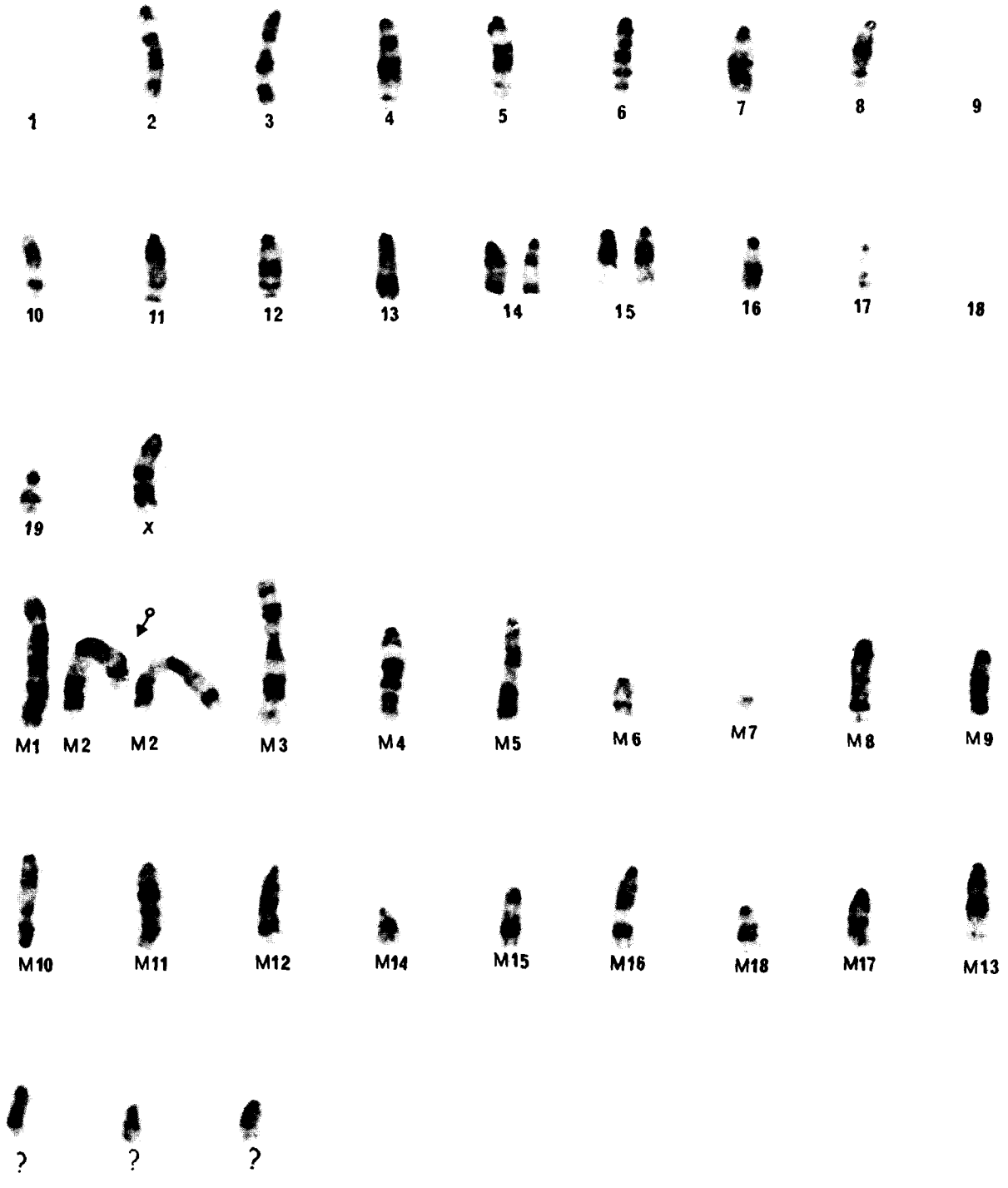

Fig. 4. Example of G-banded karyotype of a VAII cell belonging to the '2M2' stemline. Arrow indicates two copies of marker M2. M, marker chromosomes. Lowest row, unidentified chromosomes.

subline two distinct stemline populations, a larger one with 41 and a smaller one with 38 chromosomes, were present in the ratio of 5:3.

A total of 86 cells were karyotyped: 26 for the *in vivo* parental line and 20 for each subline *in vitro*. The data on karyotypic findings in the parental *in vivo* line are summarized in Table 1. Nearly all normal chromosomes were present in a single copy. In 17 cells trisomy 15 was determined, the remaining cells containing two copies of this chromosome. Marker chromosomes accounted for nearly one-half of the total number. Eighteen markers were distinguished, most of which occurred with a high frequency. Their G-band patterns and two photographs of each are shown in Fig. 2. The marker chromosomes included one subtelocentric (M1), two metacentric (M2, M3) and one minute chromosome (M7). Figure 3 shows the possible origin of 14 markers together with the description of their formation. As can be seen in this figure, when nullisomy or monosomy occurs for an intact chromosome there is usually a marker chromosome that can be shown by the banding technique to carry all or large segements of the missing chromosome, as in Nos 1 (M1), 9 (M2), 5 and 12 (M3), 2 (M4), 6 (M11) and 15 (M16). There were only some chromosomes whose frequencies of occurrence or numbers of copies varied, thus determining the karyotype heterogeneity of the original *in vivo* population. This group included autosome Nos 6, 9 and 15 markers M2, M11, M15, M16, M17 and M18. Modifications of the karyotype in the three derivative sublines *in vitro* exhibited changes in the number of copies or frequency of occurrence of the above-listed chromosomes, as shown in Table 2. While in the *in vivo* parental line the content of these chromosomes differed from cell to cell, in the

Table 1. *Distribution of the number of copies per cell of each normal and marker chromosome in parental* in vivo *line L1210(V)*

No. of copies/cell	Chromosome No.																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X
0	25						1	1	7					2						1
1	1	26	26	26	26	17	25	25	19	25	26	25	24	24		24	26	20	25	25
2						9				1		1	2		9	2		6	1	
3															17					
	Marker chromosome No.																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18		
0		1	1		1		1	2	2		10	4		1	11	17	12	21		
1	26	25	25	26	25	26	25	24	24	26	16	22	26	25	15	9	14	5		

Table 2. *Major differences in the number of copies and frequency of occurrence of some normal and marker chromosomes in the parental L1210(V) cell line and its three* in vitro *adapted derivatives*

Cell line	No. of copies/cell	Chromosome No.									
		9	9	14	15	M2	M11	M15	M16	M17	M18
L1210(V)	0		7	2		1	10	11	17	12	21
	1	17	19	24		25	16	15	9	14	5
	2	9			9						
	3				17						
VAI	0							1		2	
	1	20	20	1		M2q	20	19	20	18	20
	2			19	20						
VAII '1M2'	0		8								8
	1	8		8		8	8	8	8	8	
	2				8						
VAII '2M2'	0		12								
	1	12					12	12	12	12	12
	2			12	12	12					
VAIII	0		20	1			1				20
	1	19		19		20	19	20	20	20	
	2	1			20						

three *in vitro* sublines nullisomy, monosomy or disomy of a given chromosome were found in all, or nearly all, the karyotyped cells. All cells of subline VAI examined contained only one metacentric chromosome—M3. In these cells the arm q of the marker M2 appeared in the form of a separate chromosome (denoted as M2q), most probably as a result of dissociation of this marker. Only one copy of chromosome 9 was present, in contrast to the cells of the *in vivo* parental line, most of which had two copies, one of them appearing as arm p of marker M2. In the cells of sublines VAII and VAIII No. 9 appeared in the composition of marker M2 exclusively. Another characteristic feature of the *in vitro* sublines was the common presence of marker M16. This marker (Fig. 3) was formed by the loss of the distal part of No. 15 (break point at 15E) and translocation of a fragment of unidentified origin to its site and was found only in cells with disomy 15. Every cell of the three *in vitro* sublines contained the M16 marker, in contrast to its presence in only 34% of the *in vivo* cells. Thus the inclusion of the marker derived from No. 15 produced partial trisomy in all *in vitro* sublines.

In the case of the VAII subline, in the population after 1 month, two distinct stemlines designated '1M2' and '2M2' were present, according to the number of M2 markers in the cells. Of 20 cells karyotyped from this population 12 belonged to the '2M2' and eight belonged to the '1M2' stemline (Table 2). This finding indicates a tendency of '2M2' cells to predominate this culture. '2M2' cells were characterized by a karyotype consisting of 41 chromosomes with two copies of the marker M2, disomy 14 and M18 marker included. Figure 4 shows the karyotype of the modal cell of this stemline. The '1M2' stemline was made up of cells containing 38 chromosomes with one copy of M2, monosomy 14 and nullisomy M18. This stemline had cells which were of almost identical karyotype to those of the VAIII subline, the only difference being in the number of copies of chromosome 18.

The chromosomes listed in Table 2 made up 21 various combinations of sets in the sample of 26 karyotyped cells belonging to the parental *in vivo* line as illustrated in Table 3. In contrast, all the *in vitro* sublines exhibited a high degree of karyotype homogeneity (in the case of the VAII subline such homogeneity involved the cells within '1M2' and '2M2' stemlines). Of these 21 different cell types, two (metaphase Nos 15 and 26) had sets of chromosomes identical to those of the stemline '1M2' and VAIII. They could have been, therefore, parental cells of these stemlines. The cells constituting stemlines VAI and '2M2' were unlikely to be present in the original *in vivo* population. Both variants, easy to identify on the basis of the presence of one (VAI) or three (2M2) large metacentric chromosomes, were not found either within the karyotyped cells or among the several thousand metaphases on the preparations which provided 26 samples for the analysis. It seems that these variants were formed *in vitro*, and being particularly well adapted to the culture system they rapidly overgrew the cell population. In both cases the change, although different in character (VAI, dissociation; '2M2', duplication), involved the same chromosome, the M2 marker. Moreover, the cells of both stemlines contained the M18 marker, which was found in only five cells of the *in vivo* parental line and had disomy 14.

DISCUSSION

As a result of the adaptations of L1210(V) cells to growth *in vitro*, three sublines were obtained with a near-diploid number of chromosomes and similar growth characteristics.

All adaptations investigated evolved without the stage of polyploidization, a phenomenon denoted by Hsu [1] as heteroploid transformation and frequently observed in the course of the

Table 3. The karyotypic variants which were found in the original population

	Metaphase No.																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Chromosome					a	b	c						b		d	a	b			c						d
6	2	2	1	2	2	1	1	1	1	2	2	1	1	2	1	2	1	1	1	1	1	1	1	2	1	1
9	1	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	0	0	1	0	1	1	1	0	0
14	1	1	1	1	1	1	1	1	0	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M2	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
M11	1	0	1	0	0	1	1	1	1	0	0	1	1	0	1	0	1	1	1	1	0	1	0	0	1	1
M15	1	1	1	1	1	0	1	0	0	0	1	1	0	0	1	1	0	1	1	1	0	0	1	0	0	1
M16	0	1	0	0	0	0	0	1	1	0	0	1	0	0	1	0	0	1	1	0	0	0	1	0	0	1
M17	1	0	1	1	1	0	0	1	0	1	0	0	0	0	1	1	0	1	0	0	1	1	0	1	1	1
M18	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0

The karyotypic variants which could have been *in vitro* sublines of parental cells are put in frames. The same karyotypic variants are denoted by a, b, c, d.

establishment of various cell lines [1, 10, 13, 18-21], including leukemia L1210 [7, 8]. It has been postulated [1, 4] that polyploidization and the successive appearance of pseudo- and quasi-diploid cells play an important role in the evolution leading to the establishment of cell lines *in vitro* as a source of genotypic variability. As indicated by results presented above, in the line investigated such a genotypic variability has already been achieved and the spectrum of this variability is sufficiently broad to include among the genotypic variants of the *in vivo* population variants whose genotype reaction norm allows growth under *in vitro* conditions. In other words, the phenomenon of cell line establishment in the system investigated is likely to be the result of selection within a cell population. Todaro and Green [5] and Rosenfeld *et al.* [3] excluded the possibility that in the initial *in vivo* population parental cells for the *in vitro* lines were present. However, in both cases primary culture systems using cells taken directly from animals and patients were studied, i.e. cell populations with only a slight genotypic variability.

The transfer of tumor or transformed cells to a new environment significantly different from their original habitat *in vivo* always involves disturbances in their division leading to the formation of new karyotypic variants. The loss, acquisition or duplication of one of the chromosomes may lead to an increased adaptive value, and as an effect, to the dominance of the given variant over the whole population. Such a situation was found in the case of sublines VAI and VAII, whose dominant karyotypic variants most probably arose under the *in vitro* conditions. The rapid overgrowth of a heterogenous cell population by a single chromosomal marked variant originated *in vitro* was also observed by Navholz *et al.* [23] in the course of establishing murine cytolytic T cell lines.

In all the independently obtained sublines the changes in the karyotype, although different in character, involved the same chromosomes. Repeated changes of the karyotype may be interpreted as non-random for the process of cell line establishment in the given system. Such non-random changes include the loss of one copy of No. 9, partial trisomy 15 caused by the inclusion of the proximal two-thirds of chromosome 15 in the M16 marker, and the regular presence of markers M11, M15 and M17. Preferential loss or duplication of some chromosomes during establishment of cell lines *in vitro* was also described by Yang and Rosanoff [24], Puck *et al.* [2] and Steel *et al.* [25] for animal and human cell lines.

The most consistent change observed in the cells of the *in vitro* sublines under study was the loss of one copy of No. 9 and partial trisomy 15. Stanbridge *et al.* [26] observed that the hybrids resulting from fusion of diploid cells, in contrast to the 2n × 4n ones, died out after a limited number of population doublings. The authors suggest that a gene dosage effect may be operating in such a system, similar to the 'balance of chromosome' theory [27]. It seems that a similar interpretation may be offered for the observed changes in the number of chromosomes 9 and 15 in the cells of the L1210 leukemia lines. Recently several laboratories [28, 29] have located the *c.myc* oncogene in the distal part of chromosome 15 which, as discussed by Klein [30], plays an important role in the control of lymphoid cell differentiation and growth. This important fragment was missing as a result of M16 marker formation. It is interesting that some *in vitro* sublines of Friend virus-induced mouse erythroleukemia studied by Miller *et al.* [31] showed monosomy 9 together with partial trisomy 15 owing to the presence of a marker closely resembling the M16 chromosome.

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