A STUDY OF THE RELATIONSHIP BETWEEN KARYOTYPE AND PHENOTYPE IN CLONED LINES OF STRAIN HeLa[1]

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IT has been shown repeatedly by various workers (HAUSCHKA and LEVAN 1958; HAUSCHKA 1958; CHU and GILES 1958; CHU *et al.* 1958; BAYREUTHER and KLEIN 1958) that cloned lines of tumor cells with distinct physiological or morphological properties frequently differ from one another in observable characteristics of their karyotypes. The obvious question arises: are the distinct physiological or morphological properties of the various cell lines caused by the differences in the karyotypes, or, are the latter only an additional expression of the great variability of tumor cells? Most workers in this field consider the present evidence insufficient to decide the question in either way. The purpose of this paper is to present further data bearing on the same question. As will be seen, the data favor strongly the assumption of a causal relationship between karyotype and phenotype in a tissue culture line of human neoplastic cells, the strain HeLa (GEY *et al.* 1952).

Several factors may be held responsible for the difficulties encountered in a study of the relationship between karyotype and phenotype in tumor cell populations. One of the factors is the great frequency with which the karyotype changes in populations carrying aneuploid chromosome constitutions. In strain HeLa, for example, one out of a hundred mitoses on the average is abnormal (CHU and GILES 1958). Since most of these abnormal mitoses lead to an unequal distribution of the chromosomes to the daughter cells, new karyotypes are formed at a high frequency. Cloned cell populations contain therefore after a short period of growth a number of variant karyotypes. It was found possible to overcome this difficulty by studying the karyotypes of cloned populations within one to two months after the isolation from the single cell.

Another difficulty in this study lies in the great variety of karyotypes that arise as a consequence of the large number of chromosomes and their more or less random reassortment during abnormal mitoses. Due to the great variety of possible chromosome combinations, many different karyotypes may be expected to lead to phenotypes indistinguishable in respect to the mutant character studied. This situation excludes, therefore, *a priori*, the possibility to find a one-to-one relationship between karyotype and phenotype, which would be the most direct evidence of a causal relationship between karyotype and phenotype.

It is, however, possible to look for a partial solution of the problem by studying the relationship between karyotype and phenotype in one direction only, that is,

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by studying the karyotypes which correspond to given phenotypes. The possible results can be examined in the light of the following two hypotheses:

Hypothesis 1. The phenotype is not determined by the karyotype; any karyotype can correspond to a given phenotype.

Hypothesis 2. The phenotype is determined by the karyotype. Different phenotypes have, in general, different karyotypes.

Under hypothesis 1, each cell has, independent of its karyotype, the same probability to mutate to a new phenotype. The karyotype of most (if not all) variants should therefore fall into the same karyotype classes most frequently represented in the parental population. Under hypothesis 2, on the other hand, where each change in phenotype is caused by a karyotype change, the karyotypes of the variants should differ from those of the parental line. Hypothesis 2 predicts furthermore that karyotypes which have been selected for and which are maintained under strong selection pressure should vary less than karyotypes grown under nonselective conditions. In fact, the selection pressure would select against most of the new karyotypes arising from abnormal mitoses since most of these would not give rise to adaptive phenotypes.

The data to be presented give support to both predictions of hypothesis 2.

MATERIAL AND METHODS

Cell line: The two clones, *St1* and *F8*, of strain HeLa used throughout this work were isolated from the clonal line S3 (PUCK and MARCUS 1956), kindly made available to us by DR. PUCK. The method of isolation and the properties of the two clones have been described previously (VOGT 1958).

Culturing conditions: Unless otherwise stated, all cell cultures were grown in 60 mm petri dishes in EAGLE's medium (1955), containing four times as much amino acids and vitamins and 4×10^{-5}M inositol (EAGLE *et al.* 1956), supplemented with 15 percent human and 15 percent horse serum. The cultures were incubated at 37°C in an incubator containing a well humidified five percent CO_2-air mixture. Single-cell platings for the counting of colony formers or for the isolation of clones were done according to the method described by PUCK *et al.* (1956). The aminopterin (4-amino-pteroylglutamic acid) used in this work was kindly supplied by Lederle Laboratories.

Staining and counting of chromosomes: For chromosomal studies, cells grown on coverslips were treated for three to eighteen hours with a 10^{-7}M colchicine solution, exposed 2–10 minutes to a hypotonic solution, air-dried, fixed with methanol, stained with Feulgen and embedded in euparal. Each cell selected for counting was photographed with a 97× phase-contrast oil immersion objective and a 10× eyepiece. The position of each cell on the slide was recorded by using the calibration of the microscope stage. To minimize counting errors, special care was taken in choosing only cells with sharp outlines and widely spread chromosomes. The chromosomes were counted from seven fold enlargements of the negatives; each enlargement was then checked for details with the original preparation. Cases in which unresolvable ambiguities were encountered were

discarded. Cells with higher degrees of polyploidization were omitted from the counts, since they comprised only a small percent of the total population.

RESULTS

A number of variants were obtained from two recently cloned lines of strain HeLa, S3, and the karyotypes of the parental and of the variant lines were compared. Two different methods for obtaining the variants were used. One method consisted in the use of selective agents, such as drugs or suboptimal growth media; variants capable of good growth in the presence of the drug or the suboptimal growth medium were selected for. The second method consisted in the isolation of spontaneous morphological variants which had accumulated in the cloned lines after a cultivation period of 15–19 months.

In the classification of a karyotype, two parameters have been used: the total number of chromosomes counted per cell, and the number of chromosomes corresponding to the three largest (L) metacentric chromosome pairs of the human idiogram. Although this is a limited characterization of the karyotype, it proved to be adequate, since it revealed karyotype differences in all cases in which the phenotype had changed.

Karyotype distributions of the parental lines: The origin of the two parental lines, *St1*, and *F8*, has been described previously (VOGT 1958). At the time of isolation the cells of clone *St1* were polygonal in shape and formed tightly packed colonies in single-cell platings. The cell line was characterized by a high sensitivity to poliovirus. The distribution of chromosome numbers counted over a culturing period of four to 14 months since the isolation of the line is shown in Figure 1. The two most frequent karyotypes present in the line after four to five months of cultivation were a karyotype with 78 (12 large) chromosomes, and a karyotype with 77 (11 large) chromosomes. After a culturing period of nine months, a new karyotype of 76 (12 large) chromosomes was found in 23 percent of the cells. The proportion of cells carrying the new karyotype increased with further cultivation. Parallel with the shift in the chromosome mode of the population, a change in the phenotypic appearance of the cell line was noticed. An increasing proportion of cells showed a phenotype intermediate (I) in its morphology between a polygonal (P) and a fusiform (F) cell type.

The second parental line, designated as *F8*, was isolated from the survivors of a S3 population that had been repeatedly exposed to poliovirus. Its cells were, at the time of isolation, fusiform in shape. Due to their tendency to migrate, they formed colonies of loose texture in single-cell platings. Cells of the *F8* line were 10–15 times more resistant to poliovirus than the cells of the *St1* line (VOGT 1958). A karyotype with 70 (nine large) chromosomes remained the most frequent karyotype over a culture period of six to 13 months (Figure 2).

Influence of suboptimal growth conditions on the distribution of karyotypes: Parallel to the passages in standard medium, which contains 15 percent human and 15 percent horse serum, parallel cultures of both parental lines were grown for seven to eight months in media containing as sole source of serum either ten

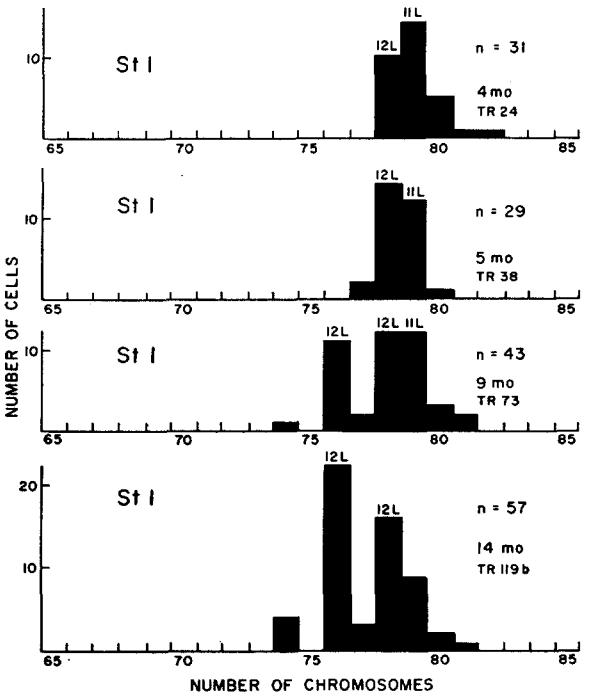

FIGURE 1.—Distribution of chromosome numbers in the parental *St1* clone after four to 14 months of growth in standard medium. The numbers above the columns indicate the numbers of large chromosomes present in each karyotype. The number of months refers to the time since the isolation of the single cell.

percent horse serum (*F8* line), or ten percent calf serum (*St1* line). In both media, a certain proportion of the cells died at the early passages, indicating that the cell populations were not well adapted to these media. At later passages, no cell death was observed, and the adapted lines had a growth rate similar to that of the control cultures grown in standard medium. Chromosome counts of the *F8* line after its growth in ten percent horse serum medium are given in Figure 2. A new karyotype with 72 (nine large) chromosomes, which was not present in the parental population, was found in approximately half of the cells after four months (tr 115), and in 28 out of 29 cells after eight months (tr 148) of growth in horse serum medium. The substitution of the parental karyotypes by a single new karyotype suggests a high selective advantage for the cells carrying this karyotype. The stability of the new karyotype over a culturing period of several months in the selective medium—a stability usually not encountered if cells are grown in standard medium—agrees well with the prediction of hypothesis 2. Single-cell platings of the horse-serum-adapted line in standard medium showed that the fusiform (F) phenotype of the original *F8* line had been replaced by an intermediate (I) phenotype. In addition, the resistance of the new line to poliomyelitis virus had decreased to one fifth of that of the original *F8* line.

The chromosome numbers obtained after seven months cultivation of a *St1* culture in ten percent calf serum are shown in Figure 3 (tr 40). Only two karyotypes were observed: a karyotype with 77 (ten large) chromosomes and a karyotype with 78 (ten large) chromosomes, respectively. Neither of the two karyotypes corresponded to any of the three most frequent classes found in the control parental population (Figure 1).

The analysis of cloned populations derived from the calf serum-adapted line showed the presence, in roughly equal proportions, of two cell types of markedly different colonial morphology. The cells of one type adhered strongly to the glass and formed colonies which were less compact than those of the original *St1* line. The cells of the second type had a small affinity to the glass and tended to pile up

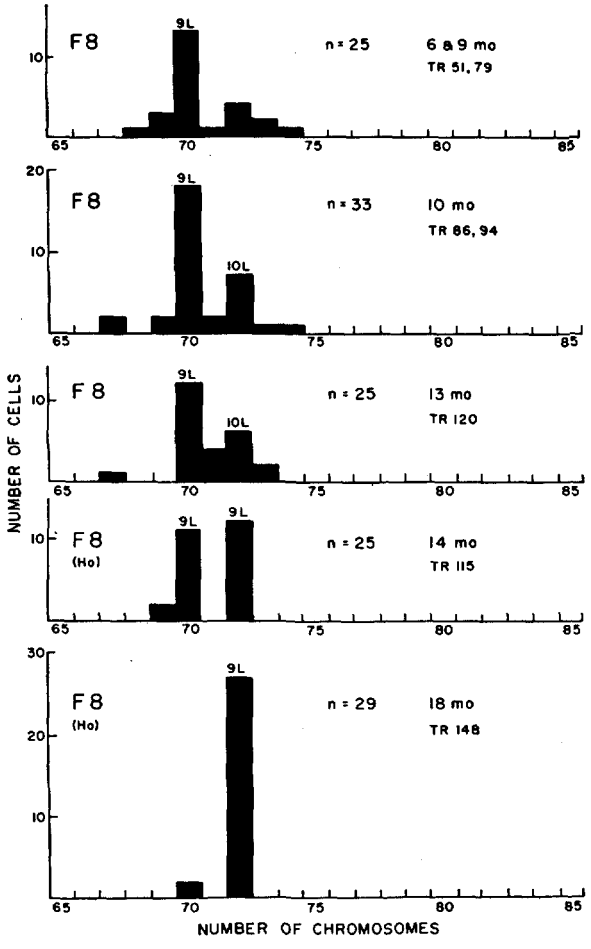

FIGURE 2.—Distribution of chromosome numbers in the parental *F8* clone. The upper three figures show the distribution after six to 13 months of growth in standard medium, the lower two figures after ten months in standard medium and four months (tr 115) or eight months (tr 148) of growth in horse serum medium.

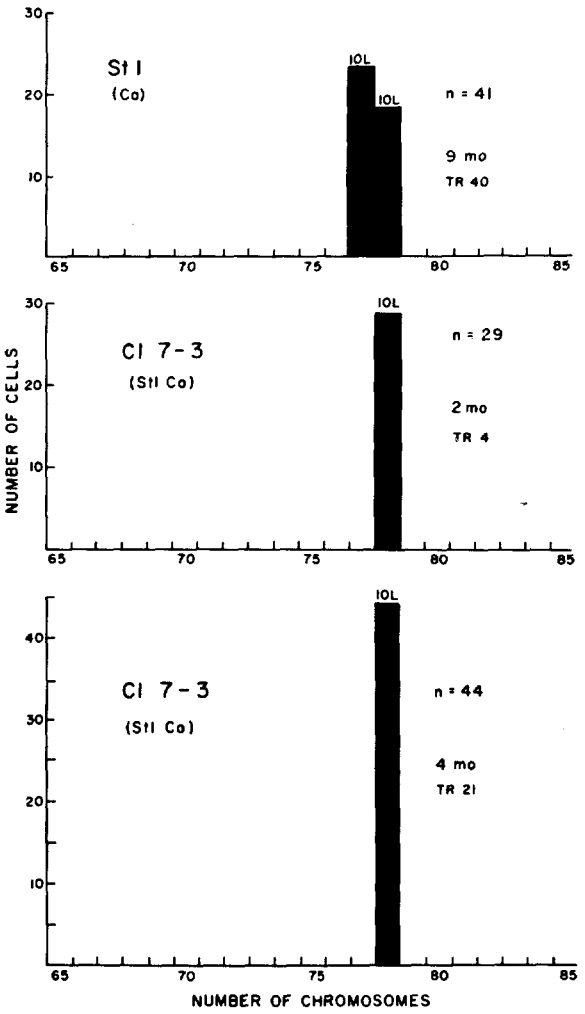

FIGURE 3.—Upper figure: chromosome numbers in the *St1* (Ca) line after two months of growth in standard medium and seven months in calf serum medium. Lower figures: chromosome numbers in clone *C1 7–3* after two and four months of growth in calf serum medium.

inside each colony. A clone of the second type was isolated and has now been grown for four months in the selective calf serum medium. In two samples of cells from this clone, examined two and four months after the isolation of the clone, a single karyotype was found, which corresponded to one of the two karyotypes present in the uncloned (adapted) population (tr 40, Figure 3). The stability of the karyotype over a culturing period of four months in selective medium is again best explained by assuming a strong selective advantage of this karyotype. Experiments have recently been started in which independent cultures of the calf serum-adapted clone are grown in parallel in standard, nonselective

medium, and in the selective calf serum medium. The comparison of the rates at which new karyotypes accumulate under both conditions will provide a more direct test of the correctness of the interpretation.

Karyotype distributions in three cell populations with increased resistance to aminopterin: The sensitivity to aminopterin (Am) was measured by plating aliquots of a given cell suspension into medium containing various concentrations of aminopterin, and counting the number of cell colonies after ten to 14 days of incubation. Fresh drug-containing medium was added to the cultures on the fourth, seventh and tenth day after the plating. The results of such experiments are given in Table 1. As may be seen from the table, a concentration of 0.0064 micrograms of aminopterin per milliliter suppresses completely colony formation in the parental *St1* line. A concentration of 0.004 μg/ml is only slightly inhibitory, since the number of colonies counted corresponded to 70 percent of the number of colonies found in the absence of the drug. In repeated platings of 2×10^4 *St1* cells per plate over a culturing period of 14 months, no colony formation was ever obtained at a concentration of 0.0064 μg Am/ml. The results in Table 2 show that

TABLE 1

Survival, as colony formers, of St1 *cells and of cells from three different aminopterin-resistant cell populations*

Cell line	Aminopterin concentration in μg/ml	No. of cells plated per plate	No. of colonies counted per plate	Relative cell survival
St1	....	520	213,316	1.0*
tr 60	0.0040	520	179,198	0.71
	0.0046	520	156,121	0.52
	0.0054	520	48,37	0.16
	0.0064	26,000	0,0	<0.00008
	0.0064	5,200	0,0	...
	0.0080	26,000	0,0	<0.00008
	0.0080	5,200	0,0	...
Am 16–6	....	480	297,286	1.0*
(aminopterin	0.0046	480	127,229	0.62
independent)	0.0054	480	112,215	0.56
	0.0064	480	16,11	0.05
	0.0080	480	7,10	0.03
Am 36	...	670	212,256	1.0*
(aminopterin	0.032	670	238,189	0.91
independent)	0.064	670	112,144	0.55
	0.128	2,010	121,135	0.18
Am 32	...	510	5,7	0.03
(aminopterin	0.016	510	190,246	1.0†
dependent)	0.032	510	92,114	0.47
	0.064	510	64,73	0.32

* The survival is referred to the number of colonies counted in the absence of the drug.
† Since this cell line is aminopterin dependent, the survival is referred to the number of colonies counted at an aminopterin concentration of 0.016 μg/ml.

TABLE 2

Plating efficiency of Am-resistant cells (line Am 36*) in presence of Am-sensitive cells (line* St1*)*

Aminopterin concentration in μg/ml	No. of cells plated per plate		No. of colonies counted per plate	Efficiency of plating
	Am-sensitive cells	Am-resistant cells		
0.0064	0	500	220, 243	0.46
0.0064	5×10^4	500	171, 189	0.36
0.0064	5×10^4	0	0,0, 0,0	0
0.032	0	500	153	0.31
0.032	5×10^4	500	207	0.41

the efficiency of plating of Am-resistant cells is not significantly affected by the presence of 5×10^4 Am-sensitive cells on the plate; the presence of Am-resistant cells in the parental population should therefore have been detected. It can thus be concluded that the proportion of Am-resistant cells in the parental *St1* population remained always below a value of 10^{-4}.

Cell populations with an increased resistance to aminopterin could be obtained from the parental *St1* line by exposing populations of 2–4 million *St1* cells to the drug and by varying either the length of exposure time or the concentration of the drug. Cell clones resistant to a concentration up to 0.512 μg/ml were obtained in successive selection steps. A selection for resistance to higher levels of the drug has not been attempted.

The karyotype distributions have been studied for three cell populations with different degrees of resistance to aminopterin. They are given in Figure 4. All three Am lines were started from parallel *St1* cultures two to three months after the isolation of the *St1* clone. The two main karyotypes found in the parental population at this time were a karyotype of 78 (12 large) and a karyotype of 79 (11 large) chromosomes (Figure 1 and VOGT 1958).

Line *Am 16–6* was exposed for 1–3 days to high doses of aminopterin, varying from 0.5 to 10 μg/ml. The exposures to the drug were spaced at 2–3 week intervals, the average time required until the survivors had grown out to form a confluent sheet. After four months, the culture was continuously grown in the presence of 0.008 μg Am/ml. The chromosome numbers and the aminopterin resistance of the line were determined five months after the selection had been started. As shown in Table 1, 56 percent of the colony formers of the line *Am 16–6*, as compared to 16 percent of the parental line, survived an aminopterin concentration of 0.0054 μg/ml. A small proportion of the cells were in addition able to form colonies in the presence of 0.0064 and 0.0080 μg Am/ml. The chromosome counts revealed two new classes of karyotypes (Figure 4) not encountered among the karyotypes of the parental *St1* population (Figure 1). It may be of interest to mention that the karyotype of 75 chromosomes carried a translocation in one of the largest chromosomes. In certain mitotic figures, this chromosome had the

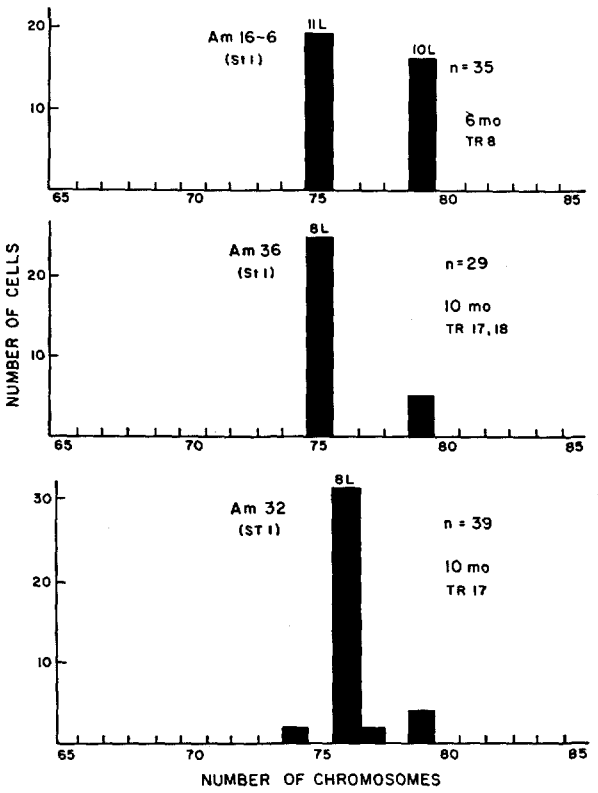

FIGURE 4.—Chromosome numbers in three populations with an increased resistance to the drug aminopterin.

appearance of a dicentric chromosome similar to that described by CHU and GILES (1958) for the clone S3-RA1.

Line *Am 36* was—within a three month period—exposed twice for one and three days, respectively, to 2.5 μg Am/ml. Subsequently the line was grown for three months in the continuous presence of aminopterin in concentrations increasing from 0.004, 0.008, 0.012, 0.016 to 0.032 μg/ml. During the seventh month, the aminopterin was omitted from the medium and the culture was allowed to grow for approximately 30 cell generations without drug. The intercalation of a growth period without drug was thought to eliminate any aminopterin-dependent cells from the population, should such cells have been present. The aminopterin resistance and the karyotype distribution of the population were determined after one and two passages in the presence of a concentration of 0.032 μg Am/ml immediately following the drug-free growth period. Table 2 shows that the survival of the population as colony formers was practically unaffected by a concentration of 0.032 μg Am/ml, the highest drug concentration at which the cells had been grown previously. The survival of the colony formers was reduced to 18 percent at a drug concentration of 0.128 μg/ml. Two karyo-

types were found in a sample of 29 cells (Figure 4). The most frequent karyotype with 75 (eight large) chromosomes was again a karyotype that had not been observed in the parental *St1* population and which differed also from the karyotype present in the *Am 16–6* population.

The selection procedure used to obtain line *Am 32* differed from that described for line *Am 36* only in two respects. The drug was not omitted during the seventh month of selection and the highest concentration of the drug used during the last month before the culture was analyzed was only 0.016 μg/ml. As shown in Table 1, the highest cell survival for line *Am 32* was obtained at a concentration of 0.016 μg Am/ml. It decreased to one third at 0.064 μg Am/ml. In contrast, only one percent of the plated cells were capable of giving rise to colonies in the absence of the drug, which indicates that the majority of the population was aminopterin dependent. The most frequent karyotype found in this cell line (Figure 4) was a karyotype with 76 (eight large) chromosomes. It differed from the karyotypes of the parental *St1* population and of the two other *Am*-lines.

The results show therefore that cultivation of the parental *St1* population in the presence of various concentrations of aminopterin led in three cases to populations with an increased resistance to aminopterin. The karyotype of the three selected populations differed from one another and from those of the parental *St1* population.

Karyotypes of clones of different morphology: Under hypothesis 2, discussed in the introduction, cells of different phenotype should have different karyotypes. To test the hypothesis, clones of different cell or colonial morphology were isolated from the two parental populations and their karyotypes determined within the first two months of cultivation. Figure 5 shows the chromosome counts obtained for five morphologically different clones isolated from a single-cell plating of the parental *St1* line at its 130th passage in standard medium.

Clone *St1–3I* had a phenotype intermediate between the polygonal and fusiform type and corresponded to the most frequent phenotype of the parental *St1* population at this late passage. Its main karyotype of 76 chromosomes (and 12 large chromosomes) is the same karyotype that was most frequently present at late passages of the parental *St1* line (Figure 1).

Clone *St1–8P* was characterized by polygonal cells and a less compact colonial morphology than that from the original *St1* clone. Clones *St1–1F*, *St1–4F*, and *St1–5F* all had fusiform cells. They differed in their growth characteristics from one another: the cells of clone *St1–1F* grew mostly in suspension, whereas the cells of clone *St1–4F* adhered to the glass and had a tendency to migrate away from one another, which in turn differentiated them from the more tightly packed growth of clone *St1–5F*. The sensitivity to poliovirus, as measured by the plaque-forming titer obtained for a standard virus suspension, was the same as that of the original *St1* line for clones *St1–3I*, *St1–5F*, and *St1–8P*. It was reduced to one third for clones *St1–1F* and *St1–4F*. As seen in Figure 5, each of the five morphologically different clones obtained from the parental *St1* line had a different karyotype.

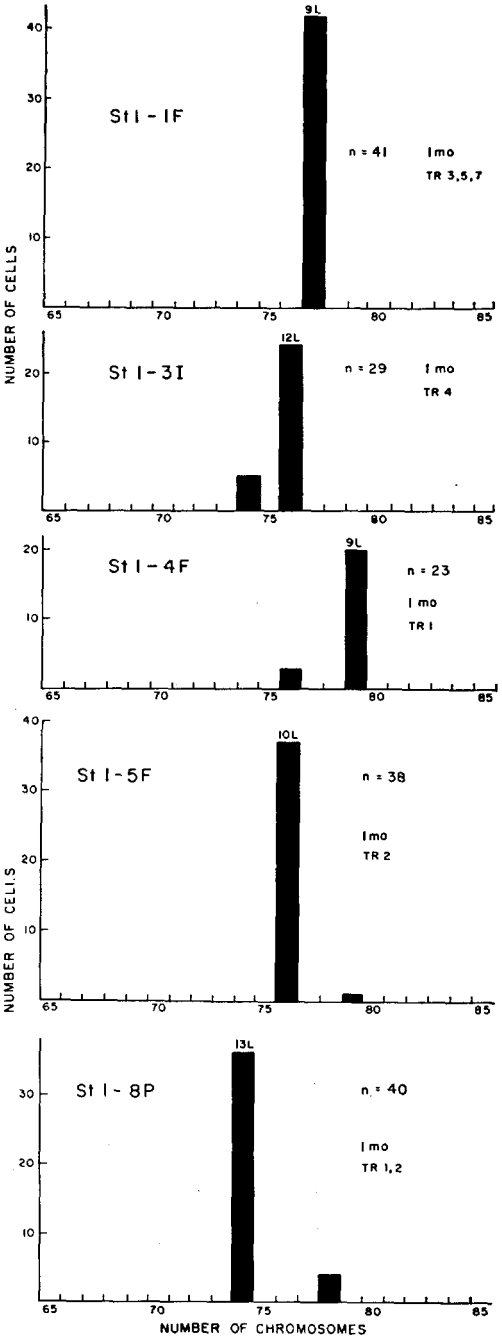

FIGURE 5.—Chromosome numbers in five different morphological clones selected from the parental *St1* line after 15 months of growth in standard medium.

Figure 6 shows the same for the karyotypes of two morphologically different clones isolated from the *F8(Ho)* line after two months of growth in standard medium. Clone *F8–4I* had the same intermediate phenotype as the prevalent type in the *F8(Ho)* population. Its karyotype was the same as that most frequently found in the *F8(Ho)* population (tr 148, Figure 2). Clone *F8–3F* had fusiform cells. Its colonial morphology was more compact than that of the original *F8* clone. The karyotype of clone *F8–3F* contained 69 chromosomes and differed thus from that of clone *F8–4I*.

DISCUSSION

As discussed in the introduction, the hypothesis that the karyotype determines the phenotype in aneuploid cells would be supported by two findings: 1) that clones selected for different phenotypes have also different karyotypes, and 2) that cell lines selected and maintained under strong selection pressure maintain a relatively stable karyotype.

In the present study of two lines of HeLa cells, both findings were obtained in all cases. The first finding is not in disagreement with HAUSCHKA's (1958) report that in only three out of seven amethopterin resistant cell lines could a karyotype change be observed, since in the remaining four cases the changes could have involved a shift in the proportion of indistinguishable chromosome classes.

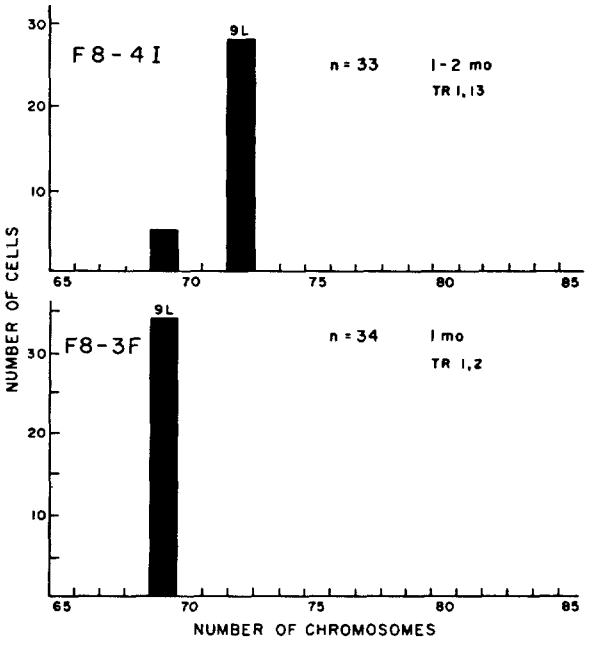

FIGURE 6.—Chromosome numbers of two morphologically different clones isolated from the *F8* (Ho) line at its 160th passage. (After eight months of growth in horse serum medium, the line had been grown for the last two months in standard medium.)

The second finding is especially significant, since it shows that the selective value of a cell depends on its karyotype. This is best explained if the karyotype directly determines the phenotype.

Furthermore it was found that whenever clones with the most frequent parental phenotype were isolated, their karyotype corresponded to that prevalent in the parental population (clones *St1–3I*, *F8–4I*, and *C1 7–3*). This finding argues against the possibility that the karyotype differences between morphologically different clones were artifacts, resulting either from the exceptional conditions or from the manipulations involved in the isolation of the clones.

The results described lend strong support to the view that the abundant variation observed in aneuploid cell populations, as for instance in many tumors, is mainly a consequence of irregular chromosome segregation and of the resulting shift in gene balance.

SUMMARY

A comparative study of the karyotypes of two cloned lines of strain HeLa, S3, and their variant sublines has been made. A given karyotype was classified by the total number of chromosomes and by the number of large chromosomes, corresponding to the three largest chromosome pairs of the human idiogram. All variant sublines—two sublines adapted to growth in horse or calf serum as sole source of serum and three sublines showing an increased resistance to the drug aminopterin—were found to have karyotypes which had not been observed in the parental lines. Similarly, clones selected for their different morphology were found to differ in their karyotypes from the parental type and from one another. Two of the selected sublines maintained a stable karyotype while grown under strong selection pressure. All these findings support the hypothesis that the phenotypic variability of aneuploid cell populations is mainly caused by changes in the chromosomal constitutions of the cells.

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