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Sensitivity of Populations of Clonal Lines of HeLa Cells to Polioviruses.* (25150)

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Fluctuations in growth potential and susceptibility to invasion by a given virus in HeLa cell "farm" populations have been a common experience. In addition to these changes, we have also experienced emergence of cells which had a rapid growth potential and which failed to take up neutral red adequately for the detection of plaques. Our own experience with these periodic changes suggests that they occur without any known alterations in environment which might select certain types; the degree of change at times necessitated interruption of our studies. In an attempt to obtain HeLa cell lines which exhibit greater stability of growth potential and virus susceptibility, populations were propagated from single clones isolated by the method of Puck (1). Six presumably clonal lines and a number of subclonal lines were isolated from a "farm" population of HeLa cells and examined for their sensitivity to types I and II polioviruses. The cell lines were found to differ in their sensitivity; two of the most sensitive have been maintained for two years and the above fluctuations have not recurred to an appreciable degree. Variation in susceptibility to poliovirus among clonal strains of HeLa cells has been reported and the variation appears to be a stable character(2).

Materials and methods. "Farm" population. The "farm" HeLa cell population, originally obtained from Dr. Scherer, has been carried with weekly passage in a variety of media. The medium used for the present study is that modified by Puck, *et al.*(3). It was sterilized by Seitz filtration. *Isolation of clonal lines.* The technic of isolation of single clones was that described by Puck, *et al.* 24-hour old subcultures of the cells were trypsinized for 10 or more minutes (until populations were comprised mostly of single cells) and, after appropriate dilution in the growth medium, were plated in 60 x 15 mm Petri dishes. The plate preparations, placed in containers that could be made air-tight, were gassed for a few minutes with 5 per cent CO_2 in air and then incubated at 37°C for 9 to 11 days. In estimating the percent of single cells in trypsinized suspensions, clumps of no more than 2 cells were considered to be incompletely divided cells and were grouped as single cells. On this basis, 90 per cent or more of the populations plated were single cells. Some of the trypsinized parental clonal populations used for the isolation of subclonal lines were virtually 100 per cent true single cells. The plating efficiency of all but one of the parental clonal lines at the time of subclonal isolation was between 30 and 65 per cent. Plating efficiency *per se* was not explored for the different clonal populations. Colonies of cells were selected for isolation on the basis of gross microscopic morphology and adequate separation from adjacent colonies. Selected clones were ringed by cylinders as described by Puck *et al.*, trypsinized, and then transferred to 16 x 150 mm test tubes containing 1 ml of growth medium. After adequate growth on the glass, the cells were transferred to 2-oz bottles in 3 ml of medium and subsequently to 5-oz bottles in 10 ml of medium. In general, single clone populations were first tested for

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their sensitivity to the polioviruses within 1 month of their primary isolation. *Test for sensitivity to polioviruses.* Clonal lines were tested for their sensitivity to types 1 and 2 polioviruses by the plaque assay technic. Approximately 1,000,000 cells of trypsinized week-old cultures of each cell line were used to seed 60 x 15 mm Petri dishes containing 4 ml of growth medium. The suspensions were then gassed for a few minutes with 5 per cent CO_2 in air and incubated at 37°C for 3 days in an airtight container. After incubation, the cell monolayers were washed twice with a Tris-buffered balanced salt solution (pH approximately 7.2) and inoculated with 0.1 ml of a dilution of the virus. After one half hour at 37°C, 4 ml of an agar overlay(4) was added and the plates were incubated for 4 days. 4 ml of a second agar overlay(4) containing neutral red was then added, and the number of plaques recorded after 4 to 5-hours incubation at 37°C. *Polioviruses used to test sensitivity.* Type 1 (Mahoney) and type 2 (MEF-1) polioviruses, originally obtained from the Connaught Labs (Toronto) were grown on "farm" populations of HeLa cells and diluted in Tris-buffered balanced salt solution before plating on monolayers. For most experiments, aliquots of a single preparation of each type of virus were used.

Results. Selection and sensitivity to polioviruses of parental clonal lines. Six single colonies of cells were selected from the "farm" population on the basis of morphology. The predominant type consisted of cells that were spread out so that a compact colony was not formed; 4 of this general type (clones 1, 2, 3 and 6) were isolated. Clone 4 was distinguished by its unusually transparent appearance. It gave rise to populations which in early passages were more transparent than those of the other cell lines, and which tended to grow in groups arrayed in a parallel fashion. With further passages, these characteristics were not constant; the cells assumed the gross morphology of the predominant clonal types. Clone 5 was clearly distinguishable from the others since it produced a much denser colony and was composed of cells that appeared small and were closely adherent; on subculture the cells tended to grow in circumscribed ovals or circles. Patches of this type of growth were noted in the "farm" population. Clone 6 on subculture appeared to contain a high proportion of spindle-shaped cells in young cultures.

TABLE I. Sensitivity of Populations of Clonal Lines of HeLa Cells to Types 1 and 2 Polioviruses.

Clonal line	Avg No. of plaques formed/monolayer			
	Type 1 virus		Type 2 virus	
	Exp. 1	Exp. 2*	Exp. 1	Exp. 2*
1	26	21	116	77
2	11	13	16	41
3	12	13	63	68
4	2	12	1	1
5	92	76	app. 280	app. 300 †
6		39		112

Each experiment (Exp.) represents simultaneous testing of all cell lines.

Aliquots of same virus preparation and same dilution used for each exp.

* Performed 2 wk after Exp. 1.

† app. = approximate.

These clonal lines were tested for their sensitivity to types 1 and 2 polioviruses approximately 1 month after their primary isolation. Monolayers of each clonal line were simultaneously seeded in triplicate with 0.1 ml of an appropriate dilution of each type of virus and treated as described under "Materials and methods." Two weeks later the experiment was repeated. Table I lists the average number of plaques formed per monolayer with each cell line and virus. The distribution of the number of plaques formed in each triplicate series was within the experimental error of the method.

The data indicate that at least 2 widely different clonal lines were isolated from the "farm" population; clone 4 appeared to be more resistant, and clone 5 more sensitive, to the viruses. In experiment 2, however, line 4 had lost its increased resistance to type 1 virus.

To establish that the differences in sensitivity noted in Table I were related to the clonal lines and not an extraneous factor, and to be more assured that the lines were of single cell origin, subclones were isolated from each of the 6 parental clonal lines. Approximately 2 months after the first clones were isolated, 4 to 6 subclones were selected from each clonal line except clone 2, from which only 2 subclones were isolated. Table II re-

TABLE II. Sensitivity of Populations of Parental Clones and Their Subclones to Types 1 and 2 Polio viruses.

Clonal line		Avg No. of plaques formed/monolayer			
		Type 1 virus		Type 2 virus	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
1	P*		37		59
	S_1†	28	36		53
	S_2	31	43		64
	S_3	20	24		39
	S_4	11	41		40
2	P	46		65	
	S_1	44		57	
3	P		22		43
	S_1	5	37	6	30
	S_2	12	44	12	41
	S_3	19	58	18	59
	S_4	15	47	15	40
4	P		41		21
	S_1	3	28	0	26
	S_2	2	32	0	26
	S_3	21	35	9	23
	S_4	2	29	0	25
5	P	41	85	223	110
	S_1	33	58	226	81
	S_2	33		223	
	S_3	46	63	232	87
	S_4	51	57	255	67
	S_5	56	75	239	67
	S_6	29‡		199‡	
6	P	46	39	41	61
	S_1	27	5	48	5
	S_2	68	55	111	70
	S_3	64	53		88
	S_4	58	58	91	69

Aliquots of same virus preparation used for each experiment; dilution used constant except for Type 2 virus, exp. 1. Parental and subclonal lines tested simultaneously.

* P = Parental clone.

† S = Subclone. Subscript refers to subclone number.

‡ Plaques smaller in size.

cords the sensitivity of the parental and subclonal lines as measured by the average number of plaques formed per monolayer of a triplicate series. Any particular parental line and its subclones were tested simultaneously with both types of virus; approximately 3 weeks intervened between the 2 experiments listed in Table II.

It is apparent that although an unexplained variability may occur on repetitive examination of a particular cell line, in general, any parental line and its subclones do not differ significantly in their sensitivity. The exception is subclone 6 of line 5 which on repeat examination was less sensitive than the parental or its sister subclonal lines, and produced a smaller plaque. It should be noted that this variant had no selective advantage in the population, since parental line 5 was maintained for more than a year with no evidence of decrease in sensitivity nor change in plaque morphology.

Line 4 and its subclones deserves comment. Three of 4 subclones were relatively resistant to the virus when first tested, but 3 weeks later this resistance was not apparent. This same sensitivity pattern had been noted with the parental line. Since no more than 3 cell passages were made in the time interval between experiments, it is unlikely that the increased sensitivity in line 4 is due to the emergence and overgrowth of a variant that is more sensitive to the viruses. Moreover, parental line 4 had already expressed its increased sensitivity at the time subclones were isolated. No detailed study of this change in sensitivity was attempted; the differences noted might have been correlated with the changes in growth characteristics which were mentioned earlier.

To minimize variables that are inherent in the test system used, all 6 parental lines and their subclones showing the highest and lowest sensitivity were tested simultaneously for their sensitivity to type 1 virus. Two different environments were used for the monolayers inoculated with the virus. One series was incubated with an agar overlay that was buffered with Tris (pH 7.2) and was not exposed to 5 per cent CO_2 in air; the other series contained an agar overlay buffered with bicarbonate which requires CO_2 to maintain pH. The results are shown in Table III. Although the average number of plaques formed per monolayer for each cell line is independent of the two environments, the plaques formed were clearer and more easily identified when a CO_2 environment was used to maintain pH. Line 5 (parental and subclone 3) is, as expected, more sensitive to the virus than any of the other lines, and its subclone 6 on repeat testing is, again, less sensitive than the parental and other subclonal lines. Line 4 has maintained its increased sensitivity.

Stability and sensitivity of lines 5 and 6 after continuous passage for 15 months. Par-

TABLE III. Simultaneous Comparison of Sensitivity of Parental and Subclonal Populations to Type 1 Poliovirus. Two different environments used for cultivation of monolayers after virus inoculation.

Clonal line		Avg No. of plaques formed/monolayer: CO_2 environment	Tris-buffered medium; no CO_2
1	P	38	29
	S_2	41	32
	S_3	33	21
2	P	33	18
	S_1	18	16
	S_2	28	24
3	P	50	43
	S_1	39	30
	S_3	60	48
4	P	37	28
	S_1	20	13
	S_3	32	21
5	P	91	102
	S_8	89	83
	S_6	31*	27*
6	P	29	29
	S_1	21	14
	S_2	45	35
Farm population		23	17

* Plaques smaller in size.

P = Parental clone; S = Subclone.

Virus preparation and dilution same as that used in Table II.

ental lines 5 and 6 were retained as the routine cell lines for the laboratory, the former because of its greater sensitivity and the latter because of its more dispersed type of growth. Line 5 produces a uniform, dark red background for plaques when exposed to neutral red, probably because the cells grow in a closely packed formation. The plaques formed are, however, rather small and have irregular edges. Plaques formed on populations of line 6 are larger and have a smooth margin, but the background color produced by cell uptake of neutral red is less intense than with line 5.

For at least a 15-month period, virus titers remained unusually constant and both cell lines maintained predictable growth rates. Despite this constancy, however, it became evident that line 5, within a year of its primary isolation, contained a small proportion of cells that appeared to grow in a looser, or more dispersed manner than the majority of cells. Subclonal lines were therefore again selected from both lines 5 and 6 and tested for their sensitivity to types 1 and 2 viruses. Two types of colonies were noted in line 5. One (subclone 7) was the closely packed type characteristic of the line; the other (subclone 8) was composed of cells that were spread out and not closely packed together. One clone of each type was isolated and subsequent passage established the difference in type of cell growth. Colonies from line 6 were uniform and characteristic; two were isolated. Populations derived from these isolates were examined for virus sensitivity. In Table IV, the average number of plaques formed on 3 to 6 monolayers per cell line and virus are listed for the parental and subclonal lines. Each experiment represents the simultaneous testing of all cell lines. Although subclone 8 (loose cell growth) of line 5 may be less sensitive than the characteristic cell type, the evidence is suggestive only since the counts in experiment 1 were too high for reliable results. Subclone 5 of line 6 appears to be less sensitive to type 2 virus than the parental line. In general, line 5 maintained its greater sensitivity to the polioviruses for more than a year despite the emergence of cells with different growth characteristics.

It is evident that variability in sensitivity exists from experiment to experiment. Within a single experiment, such factors as pH, individual serum effects, and virus dilution are

TABLE IV. Sensitivity of Populations of Parental Clonal Lines 5 and 6 and Their Subclones to Types 1 and 2 Polioviruses One Year after Primary Isolation of Parental Lines.

Clonal line		Avg* No. of plaques formed/monolayer: Type 1 virus, Exp. 1	Type 1 virus, Exp. 2	Type 1 virus, Exp. 3	Type 2 virus, Exp. 1	Type 2 virus, Exp. 2	Type 2 virus, Exp. 3
5	P	450†	137	80	58	87	92
	S_7	450†	160	86	83	112	97
	S_8	227	139	55	42	120	95
6	P	172	92	38	42	95	68
	S_5	129	59	34	26	32	34
	S_6		79	39		61	40

* Avg of 3-6 replicate platings.

† Approximate figure.

P = Parental clone. S = Subclone; subscript refers to subclone number.

All clonal lines tested simultaneously for each experiment.

Virus preparations used for Exp. 1 differed from that used for Exp. 2 and 3.

TABLE V. Influence of Size of Cell Population Seeded for Monolayers on the Sensitivity of Clonal Lines to Type 1 Poliovirus.

Clonal line		Avg No. and distribution of plaques formed in monolayers of varying seeding population sizes		
		2×10^6 cells*	1×10^6 cells*	0.5×10^6 cells*
5	P†	100 (89–104–107)	89 (106–78–84)	75 (74–76)
	S_7‡	87 (74–84–103)	101 (97–100–105)	71 (71–76–67)
	S_8	83 (93–88–74)	72 (74–62–80)	70 (75–61–74)
6	P	59 (64–59–54)	41 (36–46–42)	39 (45–35–37)

* Approximate figure. † P = Parental clonal line. ‡ S = Subclonal line.

kept relatively constant. One factor which might influence variability within a single experiment is the concentration of cells in a monolayer at the time of virus inoculation. The number of cells required to form a continuous monolayer would be expected to vary with cell size and growth pattern. To determine the importance of cell concentration, parental line 5 and its two subclones (7 and 8) and parental line 6 were tested simultaneously for their sensitivity to type 1 virus when the number of cells plated on monolayers was approximately 2 x 10^6, 1 x 10^6 and 0.5 x 10^6 cells. (One million cells was the standard concentration used.) The data are given in Table V; the average number of plaques formed per monolayer and plaque distribution in a triplicate series are shown for each cell line and concentration.

Twice the standard cell concentration does not appear to increase significantly the number of plaques formed. The slight reduction in plaque number when one-half the standard cell concentration was used was anticipated, since this concentration did not yield continuous cell monolayers. The two subclones of line 5 which differ in growth characteristics do not appear to differ significantly in their sensitivity to type 1 virus. Any difference in sensitivity between subclones 7 and 8 of line 5, if it exists, is only suggestive in this experiment, when the plating population for cell monolayers approaches the standard plating population. The greater sensitivity of line 5 does not, therefore, appear to be wholly related to its unique pattern of growth.

Summary. Propagation of HeLa cell populations from single clones isolated by the Puck technic provides cells which are more stable in growth potential and virus sensitivity than "farm" populations from which they were selected. Populations from 6 clones have been studied; 4 were of predominant cell type by microscopic examination (clones 1, 2, 3 and 6) and 2 differed from the predominant type (clones 4 and 5). Clone 5 was the most sensitive and clone 4 the most resistant to types 1 and 2 polioviruses. Although clone 5, as a generalization, maintained its characteristic morphology and sensitivity to poliovirus for 2 years, it was not possible to correlate morphology with sensitivity to the polioviruses. Selection and propagation of this most susceptible of the 6 clonal populations isolated provided a stable tool for studies of polioviruses. Since line 5 maintained its greater sensitivity for at least 2 years, a heritable factor (or factors) is probably responsible for the greater sensitivity.

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