CLONAL VARIATION IN PARAMECIUM

III. HETEROGENEITY WITHIN CLONES OF IDENTICAL SEROTYPES¹

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ABSTRACT

Clones of genetically uniform paramecia differ in the extent to which they retain the antigenic type of a common ancestor. Some are faithful and are considered stable. Others are unstable. Apparently there are two kinds of "unstable" clones. One is composed of cells all of which tend to produce subclones with some cells which have transformed to new serotypes. Other "unstable" clones apparently are really composed of two or more kinds of cells, each of which tends to yield subclones which are made up almost exclusively of cells of one serotype, although some of these subclones are not of the original serotype. Support for the existence of such heterogeneous unstable clones is presented, and several possible mechanisms to account for their existence are discussed.

WHEN two cells with identical genotypes possess different characteristics, one commonly accepted explanation invokes differential gene expression; the same genes are not active in these cells. In ciliated protozoa the phenomenon assumes higher levels of complexity due to the physical distribution of the genomes. In place of the single nucleus of most higher forms an individual cell may contain two nuclei, morphologically and functionally quite distinct. A large macronucleus serves as the "somatic" nucleus and is derived from the very much smaller "gametic" micronucleus. A second level complexity stems from the fact that—at least in *Paramecium aurelia*—the macronucleus is highly polyploid, with many subnuclei (SONNEBORN 1947). Thus, in an important sense, a single paramecium may be considered a population and a clone of paramecia simply a larger population consisting of subsets of populations physically separated from each other.

Despite this complexity there are numerous examples of uniformity of gene expression within single cells and clones. For example, individual paramecia are of only one mating type even when having the potentiality for expressing two types. Also, a cell with the capacity for making a dozen or so ciliary antigens usually will synthesize only one of these (BEALE 1954). Not only do these examples of mutual exclusion show that not all genes are expressed, but they sug-

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gest that if all subnuclei of a cell are physiologically active they are functioning identically or nearly so.

In another ciliate, *Tetrahymena*, NANNEY (1964) and ALLEN (1960) have dealt with differences in isozymes and antigens controlled by *alleles* at single loci. They interpret their data as best fitting a model of irreversible differentiation of subnuclei. Once a heterozygote expresses only one allele, this expression becomes fixed and the expression of the other allele is excluded from the vegetative descendants of that cell. This permanent alteration is contrasted with the labile system of transformations that occur between the various antigenic types specified by *different loci*. Lability suggests cytoplasmic intervention; stability, nuclear differentiation. In *Paramecium*, allelic exclusion of the sort described for *Tetrahymena* has not been observed.

With so many potential contributors to phenotypic variability, it is not surprising that relatively little progress has been made in determining the molecular basis for such phenomena as:

- 1) mutual exclusion: the expression of but one antigenic type by a given cell at any one time; and
- 2) transformation of serotype: the reversible induction of a new antigenic type and the complete disappearance of the old type.

A further difficulty stems from the nature of the character studied—antigenic type. To assign a serotype to a cell, the cell is generally killed by the action of specific immobilizing antiserum. Obviously, since only if sister cells of identical genotype and phenotype are available can an experiment be continued, it is important to know whether a clone is uniform.

This paper describes the application of several criteria for the recognition of clonal heterogeneity. Unfortunately, because the parameters used necessarily require operations which may take as long as a week, this knowledge is not on hand at the outset of an experiment. Aside from providing data of technical usefulness, these studies also have yielded information about mutual exclusion and the nature of unstable clones.

MATERIALS AND METHODS

Stocks and cultures: Stock 7, syngen 2, of *P. aurelia* was used. All clones originated as isolates of this stock and were maintained at 17°C in a Cerophyl medium (FINGER 1957).

Serotype classification: A cell was designated as of either a C, G or X serotype if after 1–2 hr at room temperature it was immobilized by one of the antisera prepared against each purified antigen (FINGER 1957). To classify a test tube culture, several hundred cells were exposed to each antiserum. With subclones grown in depression slides, generally 40–80 cells were tested against each of the three sera.

Recognition of stable and unstable clones: To classify a clone as stable or unstable a sample of cells from a test tube culture was mixed with an equal volume (2–5 drops) of fresh culture fluid. A second sample was added to an equal volume of cell-free medium from the original culture. After 18 hr at room temperature, sixty cells were isolated from each mixture into depression slides containing culture fluid. After a week's growth, during which time fresh culture fluid was added to each depression periodically, the contents of each of the 60 subclones (or those that had at least 100 cells at this time) were tested. (In about 75% of the clones examined, cells were exposed to only C and G antisera. Twenty-five percent of the clones were tested for all

three serotypes (C, G and X). Each subclone was scored as to which serotypes were present and whether the subclone was pure for any of the serotypes. If all subclones from a clone (both from the fresh culture fluid sample and from the "used" medium sample) consisted exclusively of cells of the same serotype as the parental clone, the parental clone was considered stable. An unstable clone, in contrast, was one characterized by: a) the appearance of cells with new serotypes in some subclones and often, b) a difference between the two samples in the frequencies of subclones with cells of each serotype (FINGER *et al.* 1972b).

Radioimmunoassay: Four-hour pulse labeling of antigen was achieved through feeding of *E. coli* labeled with ^{14}C -leucine as described elsewhere (FINGER *et al.* 1972a). The procedure for setting up and measuring quantitative precipitin mixtures was that followed in the same studies. Uniformly labeled ^{14}C -leucine was purchased from New England Nuclear Corp.

RESULTS

It might be thought that there can only be two kinds of cell populations that would comprise an unstable clone. Unstable clones should be composed of cells *all* of which or *some* of which tend to transform to a new serotype. The former situation would be anticipated with a homogeneous clone, the latter would be encountered with a heterogeneous clone. However, when the disposition of the genomes within an individual paramecium is taken into account, these simple models might not prove sufficient. A single macronucleus derived from a diploid fertilization micronucleus serves as the somatic or vegetative nucleus and is compound, consisting of many sets of genomes. There is also genetic and cytologic evidence that the genomes are arranged as subnuclei, with each subnucleus containing a complete genome (JURAND, BEALE and YOUNG 1962).

While stable clones, in general, can be viewed as uniform clones made up of only one kind of cell, unstable clones conceivably can be of several types. There could be either homogeneous clones, all of whose cells possess macronuclei with either identical subnuclei or a *mixture of subnuclei*, or heterogeneous clones composed of a *mixture of cells*. Thus, the following kinds of cell populations—*all of which appear identical by the surface immobilization reactions*—might be encountered in test tube cultures.

Stable populations: All cells are alike. All subnuclei of each macronucleus are indistinguishable or those subnuclei that are functioning have the same locus derepressed. For the criterion applied, i.e., stability of serotype, the clone is homogeneous.

Unstable populations:

Homogeneous clones:

- a) All cells are alike, with the macronucleus of each cell composed of identical subnuclei. Each subnucleus is in an unstable condition.
- b) All cells are alike. However, the macronucleus of each cell is heterogeneous, with the majority of the subnuclei generally having that locus functioning which determines the serotype then being expressed by an individual cell. The remaining subnuclei have this locus repressed and a second locus derepressed.

Heterogeneous clones:

Not all cells are alike. A heterogeneous population may be defined as one in

which the cells have different kinds of macronuclei. Paramecia that are phenotypically indistinguishable by immobilization tests may, according to this model, differ from each other with some cells having a macronucleus with only subnuclei determining one serotype, while other cells may possess subnuclei activated for another serotype. Or, alternatively, cells may have mixed subnuclei, with each macronucleus composed of different proportions of the various kinds of subnuclei. Such heterogeneity might be detectable through studies on the stability characteristics of the progeny of isolates such as a clone.

The critical parameter that should distinguish heterogeneous from homogeneous clones would be the *proportion* of the two kinds of subclones: subclones pure for a serotype and mixed subclones. The larger the number of subnuclei induced for the expressed serotype, the greater would be the proportion of subclones having cells of this serotype and *nothing but cells of this serotype*.

If one assumes that most unstable clones are homogeneous (regardless of whether *a* or *b* applies) one should be able to draw a straight line through a plot of percent subclones with *any* cells of the original serotype versus the percent

FIGURE 1.—A plot of subclones derived from individual clones that were initially C. Each point represents the result of testing about 50 subclones for serotypes 7–10 days after isolation. % C = % subclones with any C cells. % 100 % C = % of these subclones all of whose cells are C.

The solid line is the fitted line and the broken lines are the boundaries of 1 standard deviation from the regression line.

FIGURE 2.—A plot of subclones derived from individual clones that were initially G. Each point represents the result of testing about 50 subclones for serotypes 7–10 days after isolation. % G = % subclones with any G cells. % 100 % G = % of these subclones all of whose cells are G.

The solid line is the fitted line and the broken lines are the boundaries of 1 standard deviation from the regression line.

of these subclones that are 100% of this serotype. Figure 1 is such a plot for initially C clones and Figure 2 for initially G clones. Each point represents a separate clone using data obtained from testing 40–60 subclones originating from this clone. As the statistical analysis summarized in Table 1 indicates, the correlation coefficients support the suggestion that most clones are homogeneous.

As a test of the validity of the assumption that most clones are uniform, there should also be an inverse correlation between the percent subclones with cells of a new serotype and the proportion of subclones all of whose cells are of the initial serotype. (The shorthand that will be used henceforth for “the proportion of subclones all of whose cells are of one serotype” is “% 100%”). Figure 3 is a graph using these parameters for initially C clones. The correlation coefficient is very high, -0.68 (Table 1). This relationship might be thought to be trivial as it is simply another way of describing mutual exclusion—a cell is exclusively of one antigenic type. A clone that gives rise to subclones with cells of a new serotype must perforce no longer have subclones composed exclusively of the original type. On the other hand, were most clones not homogeneous no such relationship need exist. For example, some of the subclones with cells of the new serotype may have been derived from paramecia with macronuclei whose sub-

TABLE 1

Parameters for correlations obtained with data from clones initially of one serotype

	Number of clones from which subclones were isolated	Regression coefficient	Standard deviation	Correlation coefficient
CLONES INITIALLY C				
% 100% C <i>vs</i> % G*	86	-.75	14	-.68
% 100% C <i>vs</i> % C	85	.41	10	.55
% 100% G <i>vs</i> % G	86	.15	19	.23
% 100% G <i>vs</i> % C	84	-.15	11	-.37
CLONES INITIALLY G				
% 100% G <i>vs</i> % C	16	-.63	12	-.83
% 100% G <i>vs</i> % G	20	.23	11	.48
% 100% C <i>vs</i> % C	21	.14	19	.22
% 100% C <i>vs</i> % G	21	-.22	15	-.42

The equation for a fitted regression line is $y = a + bx$ (See BAILEY, N. T. J. (1959)).

* See body of text for meaning of these notations.

nuclei would have the expressed locus depressed. Other subclones may have arisen from cells with macronuclei composed of nearly equal numbers of at least two kinds of subnuclei. Thus, this relationship is evidence that most clones are homogeneous.

To demonstrate that the correlation between % *serotype* and % 100% is not an attribute of any serotype, but holds only for the originally expressed serotype, a plot of % G *vs.* 100% G for subclones derived from an initially C clone is presented in Figure 4. As indicated in Table 1, the correlation coefficient (r) is among the lowest of any of the bivariate comparisons (.23).

One can conclude, then, that for homogeneous clones there is a constant proportionality (i.e., the slope or regression coefficient) between the % of derived subclones with cells of the original serotype and the % of the subclones composed entirely of cells of this serotype. Also, it can be assumed that relatively few of the subclones with cells of a *new* serotype are 100% of this serotype (of 86 clones initially examined only 4 had even half of their derived subclones pure for the new serotype).

If these relationships typify a homogeneous clone, then a heterogeneous clone might be characterized both by a high proportion of subclones with cells of the initial serotype, many of which are pure for this serotype, and a high proportion of subclones that exclusively consist of *newly appearing* serotypes. The first category of subclones could originate from cells with macronuclei primarily consisting of subnuclei of the original serotype. Cells with macronuclei having a considerable portion of their nuclei already determined (or differentiated) for the new serotype could account for the second class of subclones.

Of the 13 initially C clones that fell outside one standard deviation of the % C *vs* 100% C plot (Figure 1)—i.e., those with higher than expected proportion of subclones consisting entirely of C cells—9 also had G-containing subclones most

FIGURE 3.—A plot of subclones derived from individual clones that are initially C. Each point represents the results of testing about 50 subclones for serotypes 7–10 days after isolation. % G = % subclones with any G cells. % 100% C = % of subclones with any C cells all of whose cells are C.

The solid line is the fitted line and the broken lines are the boundaries of 1 standard deviation from the regression line.

of which consisted entirely of G cells. Similarly, of the 9 clones deviating significantly from the regression line for the plot of % G *vs* 100% C (Figure 3), 6 had the majority of their subclones with G paramecia composed exclusively of G cells. These data strongly indicate that these clones represent heterogeneous clones, clones composed of two subpopulations—one primarily of C cells whose macronuclei had subnuclei chiefly determined for the C antigen; the other subpopulation consisted primarily of C cells whose macronuclei contained predominantly G determined subnuclei.

Data obtained from additional experiments are presented in Tables 2 and 3 in which representative clones are classified as either homogeneous unstable or heterogeneous unstable. For the purposes of comparison, a clone that possessed a ratio of

$$\frac{\% \text{ subclones of initial serotype}}{\% \text{ subclones with 100\% of cells of the initial serotype}}$$

of about 1.0 or less was considered a heterogeneous unstable clone. The clones were further grouped according to the degree of their apparent instability, i.e.,

FIGURE 4.—A plot of subclones derived from individual clones that were initially C. Each point represents the result of testing about 50 subclones for serotypes 7–10 days after isolation. % G = % subclones with any G cells. % 100 % C = % of subclones with any C cells all of whose cells were G.

The solid line is the fitted line and the broken lines are the boundaries of one standard deviation from the regression line.

whether more or less than 60% of their derived subclones had cells of the initial serotype.

Two characteristics of a heterogeneous unstable clone are made plain from this information. Homogeneous unstable clones give rise to more subclones with a new serotype than do heterogeneous unstable clones (Table 2). Moreover, when subclones with the original serotype have a disproportionate number of subclones pure for this serotype (indicated by a ratio of $\frac{\%}{\% 100\%}$ less than 1.0), subclones with cells of a new serotype also tend to be composed exclusively of this new serotype with a ratio <1.0 (Table 3). These correlations hold for both highly unstable and moderately unstable clones.

Antigen synthesis in heterogeneous clones: In all of the discussions of the data thus far, the question has been moot as to whether the subnuclei of a macronucleus have been “determined” (but not functioning) or differentiated (i.e., functioning) for a new serotype. Experiments in which paramecia were fed radioactively labeled bacteria, and the amino acid leucine was incorporated into immobilization antigen have led to this pertinent conclusion: a heterogeneous

TABLE 2

Distribution of serotypes among subclones derived from unstable clones

Clone	Percent subclones with G serotype New serotype		Percent subclones with 100% of G serotype New serotype	
INITIALLY G CLONES				
<i>Heterogeneous unstable</i>				
7-83	86	19	78	91
7-1	68	49	67	70
7-7	70	33	76	61
7-54	63	23	80	82
Average	71.8	31.0	75.3	76.0
<i>Homogeneous unstable</i>				
7-222a	79	68	24	6
7-7	73	30	41	17
7-122	83	47	45	28
7-222b	60	53	23	30
Average	73.8	49.5	33.3	20.3
<i>Heterogeneous unstable</i>				
7-202	16	53	47	60
7-14	48	27	60	90
7-6	40	42	100	88
Average	34.7	40.7	82.3	79.7
<i>Homogeneous unstable</i>				
7-4	39	91	9	25
7-211	40	81	32	69
7-91	52	79	33	50
Average	43.7	83.7	25	48.3
INITIALLY C CLONES				
<i>Heterogeneous unstable</i>				
7-47	66	23	97	92
7-220	71	32	81	48
7-26	67	35	95	50
7-107	66	34	77	75
Average	67.5	31.0	87.7	66.5
<i>Homogeneous unstable</i>				
7-26	60	88	47	64
7-8	70	57	62	48
7-27	72	51	65	40
7-10	79	48	56	25
Average	70.3	61.0	60	44.5
<i>Heterogeneous unstable</i>				
7-71	51	64	73	50
7-177	39	61	50	68
Average	45.0	63.0	61.5	59.5
<i>Homogeneous unstable</i>				
7-30	43	96	9	55
7-107	50	59	38	40
Average	46.5	77.8	23.5	47.5

TABLE 2—Continued

Clone	Percent subclones with		Percent subclones with 100% of	
	G serotype	New serotype	G serotype	New serotype
INITIALLY X CLONES				
<i>Heterogeneous unstable</i>				
7-36	78	34	67	54
7-1a	79	24	89	75
7-1b	78	20	88	27
7-7	91	14	98	100
7-1d	62	13	97	67
Average	77.6	21.0	87.8	64.6
<i>Homogeneous unstable</i>				
7-304	80	31	68	27
7-122	62	51	41	13
7-4	92	33	66	6
Average	78.0	38.3	58.3	15.3
<i>Heterogeneous stable</i>				
7-5	29	64	75	94
7-1c	48	35	67	33
7-178	46	52	78	92
Average	41.0	50.3	73.3	73.0

In these experiments there were no unstable X clones which produced fewer than 60% of their derived subclones with X cells.

TABLE 3

Distribution of pure subclones among heterogeneous and homogeneous unstable clones

	Initial serotype %	New serotype %
	% 100%	% 100%
CLONES INITIALLY G		
Heterogeneous unstable clones		
7-83	1.10	0.20
7-1	1.01	0.70
7-7	0.92	0.54
7-54	0.78	0.28
Mean	0.95	0.43
Homogeneous unstable clones		
7-222a	3.29	11.33
7-7	1.78	1.76
7-122	1.84	1.67
7-22b	2.60	1.77
Mean	2.38	4.13
Heterogeneous unstable clones		
7-202	0.34	0.88
7-14	0.80	0.30
7-6	0.40	0.48
Mean	0.51	0.55

TABLE 3—Continued

	Initial serotype	New serotype
	$\frac{\%}{\% \ 100\%}$	$\frac{\%}{\% \ 100\%}$
Homogeneous unstable clones		
7-4	4.33	3.64
7-211	1.25	1.17
7-91	1.57	1.58
Mean	2.38	2.13
CLONES INITIALLY C		
Heterogeneous unstable clones		
7-47	0.68	0.25
7-220	0.87	0.66
7-26	0.70	0.70
7-107	0.86	0.45
Mean	0.78	0.52
Homogeneous unstable clones		
7-26	1.27	1.37
7-8	1.12	1.18
7-27	1.10	1.28
7-10	1.21	1.92
Mean	1.18	1.44
Heterogeneous unstable clones		
7-71	0.70	1.28
7-177	0.78	0.89
Mean	0.74	1.09
Homogeneous unstable clones		
7-30	4.78	1.74
7-107	1.31	1.48
Mean	3.05	1.61
CLONES INITIALLY X		
Heterogeneous unstable clones		
7-36	1.16	0.63
7-1a	0.89	0.32
7-1b	0.89	0.74
7-7	0.93	0.14
7-1d	0.64	0.19
Mean	0.90	0.40
Homogeneous unstable clones		
7-304	1.17	1.14
7-122	1.52	3.92
7-4	1.39	5.50
Mean	1.36	3.52
Heterogeneous unstable clones		
7-5	0.39	0.69
7-1c	0.71	1.06
7-178	0.59	0.56
Mean	0.56	0.77

unstable clone, although possessing only one antigen recognizable by the immobilization test, is actively manufacturing at least two antigens which are indistinguishable from the same antigens made by a mixture of two stable clones (FINGER *et al.* 1972c).

One way in which two antigens can be compared is by their ability to be precipitated by antiserum made against either antigen. Each antigen will have a characteristic "profile" in terms of amount of precipitate when mixed separately with a number of different crossreacting antisera (FINGER *et al.* 1972a). G and X antigens strongly react with serum prepared against either antigen. When aliquots of immobilization antigens extracted from unstable pulse-labeled C clones were precipitated by anti-G and anti-X sera, counts were precipitated. From the data in Table 4 it can be seen that stable G and stable X antigens precipitate counts with an anti-G and anti-X serum in a ratio of about 1.0. G antigens from heterogeneous C clones also give a ratio near unity, while those from homogeneous unstable clones give ratios ranging from 0.04 to 4.8. Thus, secondary antigens from homogeneous unstable clones do not react like either G or X antigens alone, while those from heterogeneous unstable clones do.

It is possible, however, that unstable clones contain a mixture of X and G antigens and that in certain proportions a mixture of these antigens gives a

TABLE 4
Comparison of the incorporation of ^{14}C -leucine into antigens from homogeneous and heterogeneous stable C clones

		Counts per minute*			
Date		1150 (Anti-C)	1152 (Anti-G)	1154 (Anti-X)	1154/1152
Homogeneous unstable clone					
32	2-25-70	24	25	1	.04
9	5-6-70	1236	212	148	.70
98	6-15-70	126	25	120	4.80
44	6-15-70	92	30	80	2.67
16	6-15-70	88	23	48	2.09
1	6-15-70	50	11	36	3.27
Heterogeneous unstable clone					
32	3-11-70	166	52	52	1.00
9	4-9-70	231	20	38	1.90
41	4-9-70	450	15	16	1.07
41	5-6-70	540	72	84	1.17
Stable G clone					
48	3-30-70	—	190	240	1.26
42	5-6-70	60	612	600	0.98
48	5-6-70	66	564	640	1.13
42	6-8-70	50	248	257	1.04
Stable X clone					
28	3-11-70	47	205	188	0.92
28	3-30-70	—	490	480	0.98

— = not tested.

* cpm = less pre-immune counts.

TABLE 5

Precipitin counts of mixtures of G and X antigens

Serum	Conc.	Counts per minute		Antibody or antigen excess
		Observed	Predicted	
G + X (1:1) Mixture				
1153				
(Anti-G)	1/2	95	103	Ab.
	1/4	97	88	Ab.
1154				
(Anti-X)	1/2	86	80	Ab.
	1/4	71	19	Ag.
G + X (3:1) Mixture				
1153				
	1/2	123	141	Ab.
	1/4	113	138	Ab.
1154				
	1/2	119	103	Ab.
	1/4	78	20	Ag.
G + X (1:3) Mixture				
1153				
	1/2	75	69	Ab.
	1/4	54	57	Ab.
1154				
	1/2	54	52	Ab.
	1/4	53	18	Ag.

different ratio from either alone. Artificial mixtures of these antigens were reconstructed and the radioactivity of mixed antigens and antiserum was measured (Table 5). From the counts precipitated by each antigen separately (and from the complete precipitin curves) the counts expected from each mixture, in which the proportions of the two antigens were varied, could be calculated and compared with those observed. The results clearly indicate that mixtures of antigens precipitate as anticipated from the behavior of each antigen individually. The ratios are moderately constant regardless of the proportions, and the predicted and observed counts are in fairly good agreement as long as both antigens fall within antibody excess.

The data in Tables 4 and 5 also show that under the conditions employed even stable clones synthesize a small amount of an antigen other than the antigen on the surface, although not nearly to the extent that unstable clones do. The unusual ratios exhibited by the unstable clones could be due to the presence of antigens in configurations that are not the same as those of the native antigen or of incomplete antigens. There is some evidence for the existence of low molecular weight antigens in unstable clones (FINGER unpublished).

To summarize, homogeneous unstable clones can be distinguished from heterogeneous unstable clones by the following criteria:

1. Unstable clones yield subclones many of which are not pure for the original serotype, the proportion of pure subclones being correlated with the degree of instability; the more stable the original clone, the larger the number of subclones composed entirely of the initial serotype. Heterogeneous unstable

clones do not manifest this relationship; regardless of the apparent degree of instability, a relatively high proportion of derived subclones are entirely of the original serotype.

2. Heterogeneous unstable clones give rise to a proportionately large number of subclones pure for a new serotype; homogeneous unstable clones do not.
3. Homogeneous unstable clones produce a larger number of subclones of a new serotype than do heterogeneous unstable clones.
4. From experiments described elsewhere (FINGER *et al.* 1972b) it has also been shown that: a) While homogeneous unstable clones respond differently to being kept overnight in fresh culture fluid rather than in their own bacteria-exhausted medium, heterogeneous unstable clones generally react in the same way to these two conditions. b) Heterogeneous unstable clones are generally more resistant than are homogeneous unstable clones to the transforming action of antisera and antibiotics. In both of these respects, heterogeneous unstable clones are more like stable clones than homogeneous unstable clones.

DISCUSSION

The findings described here strongly suggest that cells with indistinguishable surface antigens can produce clones that differ markedly in their serotype composition. Unlike the conventional type of antigenic transformation, the new characteristics manifest themselves within a uniform external milieu. What occurs apparently is an unfolding of differentiations already determined or actively maintained at the time the isolates were initiated. Prior to isolation these cells of identical serotype must have differed in some way which set in motion events leading either to maintenance or loss of the original antigenic type.

As stated above, this description also applies to the phenomenon of unstable clones, which give rise to subclones with new serotypes, and stable clones from which only subclones of the original serotype are produced (FINGER, HELLER and LARKIN 1967). Stable and unstable clones also respond identically *en masse* to immobilization antibodies. What has been reported in this paper, however, may be quite different from the stability character when the two phenomena are considered in terms of underlying mechanisms. A homogeneous unstable clone could be thought of as a population of a single kind of cell, with each cell having a certain probability that the synthesis of its original antigenic type is "turned off" or, put positively, the synthesis of a new antigen is "turned on." A stable clone has a high probability of keeping its original type, and an unstable clone a low probability. A heterogeneous unstable clone, however, is probably composed of two kinds of cells. One kind has a very high probability for "transforming" to a particular serotype and the other kind has an equally high probability for retaining its original serotype.

The critical word in these statements is "probability" and what it corresponds to in physiological terms. If something akin to autocatalysis or feedback plays a major role in the maintenance of antigenic type, then "probability" may refer to the quantity of an antigen possessed by a cell. An unstable cell would have at least

two kinds of antigens, the surface antigen and another "surface antigen" that is not accessible to immobilization antibodies but is nonetheless present at the outset of an experiment. A stable cell would have only one "surface antigen," and that antigen is already on the surface. In contrast, a cell from a heterogeneous unstable clone might have *either* one "surface antigen" or two "surface antigens," one of which would replace the antigen initially present.

If probability is viewed within this context, then the phenomenon of mutual exclusion of antigenic type must be approached from a somewhat different perspective than that of differential gene activation. In unstable clones or heterogeneous stable clones several genes may be activated within single cells but the *product* of only one of them is allowed on the surface (or recognized as a surface antigen). Thus exclusion would not be initiated at the level of the gene but at a secondary level.

Probability could also be couched in terms that refer to the structure of the macronucleus. Unstable cells would have a macronucleus made up of diverse subnuclei, with some subnuclei "differentiated" (i.e., producing messenger) for one antigen, other subnuclei differentiated for a second antigen, etc. Stable cells would have only one kind of subnucleus. A cell from a heterogeneous unstable clone might also have only one kind of subnucleus, with different cells having different kinds of macronuclei. Similar descriptions could be devised involving active and inactive subnuclei within a macronucleus. In any event, it would seem that a cell from a heterogeneous unstable clone is probably more nearly like a cell from a stable clone than a "true" unstable cell.

Were "probability," however, simply a finite chance that a series of events would be triggered, the origin and existence of heterogeneous stable clones would be unexplained. Ultimately, however probability is defined, the question remaining is what is the initial stimulus which determines that a cell will transform.

The most intensively studied instances of subnuclei determinations are those investigated by NANNEY (1964) and ALLEN (1960) in *Tetrahymena*. The interpretation favored in these cases is that subnuclei become determined and their descendants, similarly determined, become sorted out to a single macronucleus. A cell with such a uniform macronucleus yields a clone with a single phenotype. For this model to hold for paramecium antigens, assuming a macronuclear basis for serotype (PREER, BRAY and KOISUMI 1965), one would have to say that determination occurs early with heterogeneous stable clones and spreads rapidly throughout a macronucleus. In unstable clones determination either would have to occur simultaneously among a number of subnuclei within a macronucleus and not spread to the other subnuclei, or occur late and then spread to all the subnuclei of a cell. Either mechanism could account for the observed distributions of serotypes within subclones (% *vs* % 100%) with transformed cells.

An "ideal" heterogeneous unstable clone might be expected to yield subclones almost entirely pure for the initial serotype and almost entirely pure for a new serotype. As is clear from the data in Table 2, such clones are rarely encountered. When they are found, apart from the fact that the cells of the clone react to only one antiserum, they should be more like a mixture of stable cells than of unstable

cells. In other words, they should resist transformation by reagents and the proportion of these "stable" cells should persist over many generations as true stable cells.

Evidence bearing on some of the points discussed here is presented in the preceding paper in this series. Together with the serotype distribution within subclones derived from stable and unstable clones noted here, these data raise numerous questions about the regulation of antigen synthesis, the origin of unstable clones, the functioning of subnuclei, and the true nature of the "serotype."

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