INTRACLONAL VARIATION IN HEPATOMA CELL LINE McA-RH 7777 REVEALED BY ANALYTICAL CLONING

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Cell heterogeneity in a tumor population is well known. It is based on the accumulation and selection of mutations ensuring survival of the population under the given conditions. Besides mutational variation, however, heterogeneity which cannot be reduced to the accumulation of classical mutations also is found in tumors. It is characterized by two features: the rapid appearance of an intracellular clone and the establishment of equilibrium in the population between the original feature and its variant. Heterogeneity of this type has been observed with respect to ability to metastasize and to morphological and growth features [3-5].

In a study of cell heterogeneity of rat hepatoma McA-RH 7777 with respect to alpha-fetoprotein (AFP) expression the authors showed previously that AFP^{+}- or AFP^{-}-cells can give rise to clones of alternative type and that the frequency of these switches is much higher than the frequency of possible mutations [2].

In this paper a scheme of "analytical cloning" is suggested (two consecutive clonings carried out after a short time interval), whereby intraclonal variation in this cell line can be demonstrated and characterized quantitatively.

EXPERIMENTAL METHOD

McA-RH 7777 is a cell line obtained from Morris rat hepatoma, generously provided by Dr. Becker in 1977. The line is maintained on a mixture of L15 and Eagle's media (1:1) with 10% fetal calf serum. Fresh seedings were made once a week and the cells removed from the support with trypsin.

Cloning was carried out by the method of low-density seeding on a mixture of CMRL and L15 media (1:1) with 20% fetal calf serum and sodium pyruvate. With a cloning efficiency of 40-70%, 3000-4000 cells were seeded on a Petri dish 30 mm in diameter. Dishes with adherent cells (3-4 h after seeding) were examined in an inverted microscope and single cells were encircled underneath on the outer side with a dissecting needle. After 2 days the medium was replaced by a mixture of 0.25% low-temperature agarose and growth medium. To prepare the mixture, 2% agarose in Hanks' solution, sterilized in a water bath, was diluted 1:4 with growth medium for the clones with the addition of fetal serum up to 20%. To solidify the agarose the dishes were kept in a refigerator for 15 min.

After 7 days of culture samples were taken from the marked clones for further subculture and they were photographed in phase contrast. By means of an inverted microscope clones were withdrawn from beneath the agarose with siliconized capillary tubes in the order in which they were photographed, and they were transferred into wells of a 24-well plate, with pipeting to obtain a unicellular suspension. Adherent single cells were marked and the resulting clones were cultured in the same way as the original ones. On the 7th day they were fixed with 4% formaldehyde in Hanks' solution and AFP in the cells was stained by the standard immunoperoxidase method, after pretreatment with 70% alcohol. Rabbit antibodies to rat AFP were obtained and generously provided by D. A. Él'gort. (Fab)'-fragments of donkey antibodies to rabbit IgG, conjugated with peroxidase, were obtained and generously provided by V. S. Poltoranina.

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TABLE 1. Distribution of Primary Clones (obtained from mass culture) by Phenotype

Phenotype of clone	Number of clones	
	absolute	%
AFP^+	121	51
$AFP^{+/-}$	83	35
AFP^-	34	14
Total number analyzed	238	100

Legend. AFP^+) All cells of clone are AFP-positive, AFP^-) all cells of clone are AFP-negative, $AFP^{+/-}$) clone is mixed.

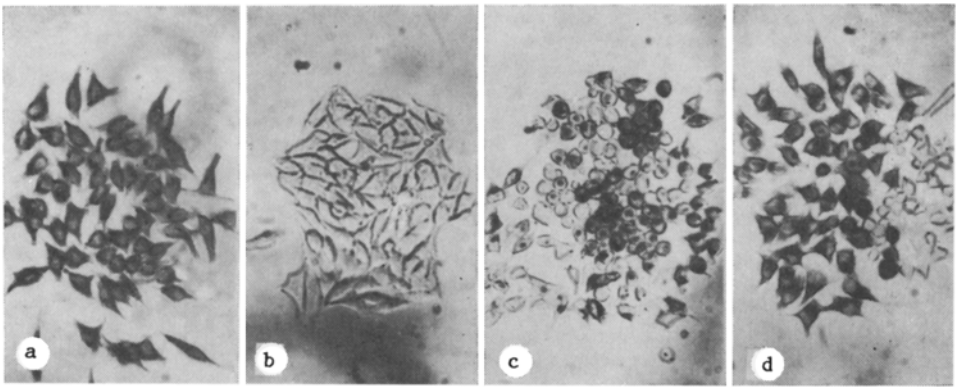

Fig. 1. Clones of cells taken from mass culture ("primary"). a) AFP^+-clone; b) AFP^--clone; c,d) $AFP^{+/-}$-clones. Indirect immunoperoxidase reaction for AFP. Here and in Fig. 2, magnification 140.

EXPERIMENTAL RESULTS

The efficiency of primary cloning varied from 50 to 75%. The size of the clones on the 7th day of culture varied from 30-60 to 100-200 cells and the mean size of the clone was 120 cells. The distribution of primary clones by phenotype in eight experiments is summarized in Table 1. Examples of clones of different types are given in Fig. 1. Clones growing beneath a layer of agarose did not differ morphologically, and clones positive and negative for AFP did not differ in size.

Efficiency was significantly lower for the subclones. If the number of growing subclones was expressed as a ratio of the number of cells of the original clone, it did not exceed 25%. Some cells were lost during transfer or they divided poorly after pipeting. If there were fewer than 10 clones in a well, it was not analyzed. Thus clones with between 10 and 60 progeny were analyzed. The subclones were smaller than the original clones cultured for the same length of time, evidently due to stress caused by mechanical dispersal. The size of the subclone population varied from well to well, but mainly it was 30-60 cells, although in some wells the number of cells in the subclones reached 120-140. Altogether the progeny of 46 clones were analyzed. Phase-contrast photographs of the original clones and their immunohistochemically stained subclones are given in Fig. 2.

During analysis the number of AFP^+-, $AFP^{+/-}$-, and AFP^--subclones was counted in each well. Depending on their relative proportions the clones were divided into groups (Table 2). The two extreme groups (Nos. 1 and 9) were clones giving 100% of AFP^+- and 100% of AFP^--subclones on subcloning. Intermediate groups were formed in order of increasing numbers of progeny of mixed and negative subclones (groups Nos. 2-8).

The results of analytical cloning were as follows. Phenotypic variations of AFP were inherited in several cell generations, which follows from at least five clones (of groups Nos. 1 and 9), all the subclones of which had the identical phenotype.

Changes in the AFP-phenotype of the cells took place as a rule quite quickly. In 17 of the 46 clones (groups Nos. 4-6) these changes took place actually in the original clone, i.e., in the course of 1-7 generations, for both homogeneous phenotypic variants were rep-

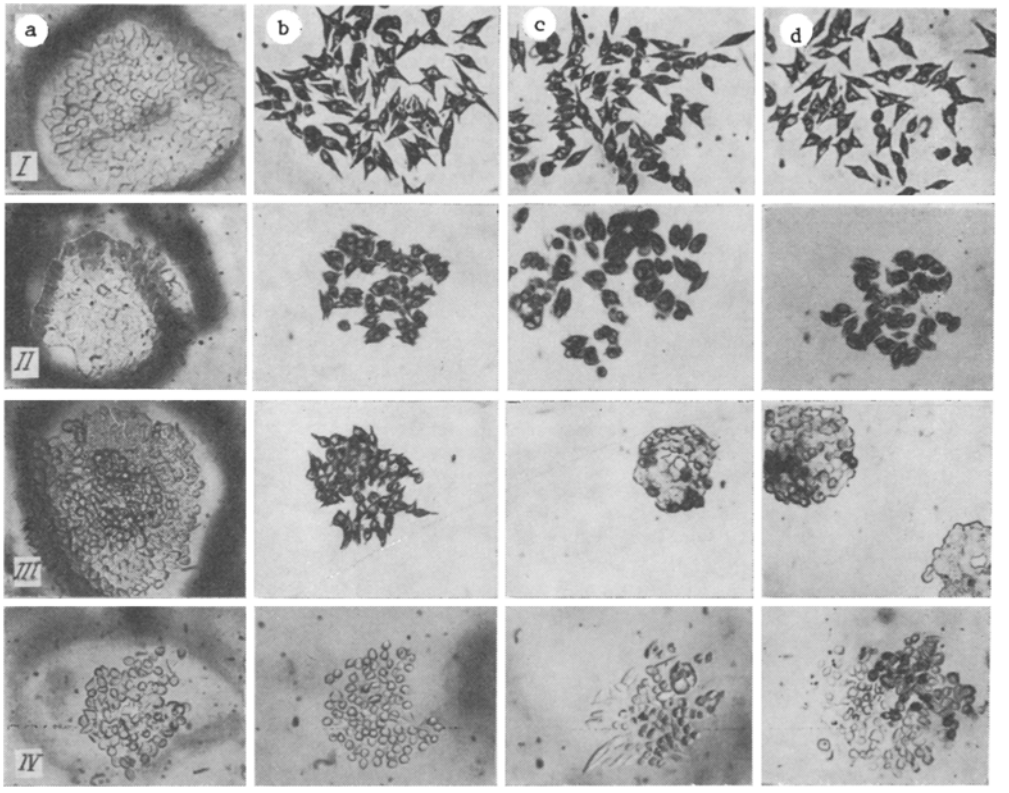

Fig. 2. Examples of intraclonal variation. a) Primary clone (phase contrast); b, c, d) subclones (immunoperoxidase reaction for AFP). I) Clone from group 1:18 of AFP^+-subclones (b, c, d); II) clone from group 2:18 of AFP^+-subclones (b, c) and two $AFP^{+/-}$-subclones (d); III) clone from group 6:1 of AFP^+-subclone (b), 14 $AFP^{+/-}$-subclones (c, d), and seven AFP^--clones (d); IV) clone from group 8:14 of AFP^--clones (b, c) and four $AFP^{+/-}$-clones (d).

TABLE 2. Intraclonal Heterogeneity of Hepatoma McA-RH 7777

No. of group	No. of primary clones	No. of subclones in well (group average)	Distribution of subclones by phenotype, % (average for group)		
			AFP^+	$AFP^{+/-}$	AFP^-
1	4	23	100	—	—
2	16	27	83	17	—
3	2	15	23	78	—
4	8	50	68	28	4
5	2	13	65	—	35
6	7	24	9	77	14
7	3	17	—	76	24
8	3	16	—	26	74
9	1	10	—	—	100

resented among the subclones. Thus the frequency of changes from AFP^+ to AFP^- and vice versa was several orders of magnitude higher than the frequency of mutation.

AFP-positive and AFP-negative cells forming the original population differed from one another not only in their AFP-phenotype, but also in the stability of this pheotype in several successive cell generations. The greatest stability observed in this investigation was about 7 + 7 = 14 cell generations for the four AFP^+-clones (group No. 1) and it was rather less for one AFP^--clone in group No. 9 (the preparation of subclones of this clone was fixed on the 5th day of culture and it had 10 clones each of about 30 cells). The least stability could be judged in the case of clones included in group No. 6 (Table 2; Fig. 2, III). In these clones all three phenotypic types of subclones were present, but mixed predominated. Hence it follows that the changes took place actually in the

original clone and that in general these cell lines exhibit high variability. Least stability could also be postulated on the basis of preparations of primary clones with the mixed $AFP^{+/-}$ phenotype. Mixed positive and negative cells (Fig. 1c) were most likely to be the result of several $AFP^{+} \rightleftarrows AFP^{-}$ changes during the life of the clone; the sectoral arrangement of the cells of the alternative phenotype (Fig. 1d) is evidence of the great probability of one change. The size of the sector was determined by the generation at which this change took place. Thus an equal number of positive and negative cells may indicate that the change of phenotype took place during division of the original cell.

Two basic features of development of the cell line investigated can thus be noted: the AFP-phenotype oscillates at different rates in a series of cell generations and this variability is of nonmutational origin.

The greatest variability, found in the present investigation, extends over a period of under seven generations, the least, which we observed previously, extends over about 20 passages or 140 generations [1].

An important question which arises from this investigation is: what is the relationship between the cause of this phenomenon of unstable inheritance of the AFP phenotype to regulation of the AFP gene in the liver. At present this can be answered only in the most general form. It is clear that the changes observed are changes in the regulatory element and not in the structural gene of AFP itself. The less frequent occurrence of AFP-negative cells and the faster transition from AFP^{-} to AFP^{+} is evidence that in this particular hepatoma the AFP^{-}-state of the hepatocyte is more differentiated than the AFP^{+}- (constitutive) state. The transition to the next stage of differentiation for the AFP^{+}-hepatocyte probably means an increase in the complexity of regulation, for example, activation of additional syntheses. The existence of a regulator with negative action, a repressor, may be postulated, which must be activated in order that the AFP-positive cell become negative. The presence of cell lines with different degrees of stability within populations may perhaps reflect the property of this repressor of being multiplied to a varied degree.

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