ALPHA-FETOPROTEIN SYNTHESIS BY CLONOGENIC CELLS OF RAT HEPATOMA McA-RH 7777

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As a rule tumors preserve the features of differentiation of the tissue from which they arose. These features are usually unequally expressed in different cells of the same tumor. This inequality may be the results of the fact that a stem line capable of producing a progeny going on to terminal differentiation exists in tumors. The stem line of a tumor does not carry features of differentiation, but it is determined for a particular direction of development, which is realized in some of its progeny. The ratio of stem cells to differentiated cells determines the level of differentiation of the tumor and its heterogeneity with respect to expression of features which can be studied. Such a tumor structure has in fact been demonstrated for embryonic teratocarcinomas [11, 12], mouse virus erythroblastosis [15], and myeloid leukemia [13], in which stem lines and terminally differentiated cells segregating from them have been discovered.

The origin of many tumors from determined but not differentiated precursor cells of a definite tissue is in complete harmony with the scheme described previously [7]. At the same time the possibility cannot be ruled out that in malignant cell transformation ability for self-support, which is a feature under normal conditions of only stem or semistem cells, is acquired by differentiated cells, which preserve the level of their differentiation in the tumor.

This problem has not been resolved for the overwhelming majority of tumors, for experimental approaches to the determination of the level of differentiation of the stem cell in a tumor are almost completely absent. The exceptions are leukemias, which have differential membrane antigens, antibodies against which can suppress the corresponding stem cells [6].

Hepatomas of animals and man preserve many features of normal hepatocytes, including ability to synthesize serum proteins [1]. It is quite unclear whether they are produced by the stem cells of the tumor or by their differentiated progeny. The latter would seem to be the most likely explanation [2], and for several years the writers have endeavored to find a way of testing this hypothesis experimentally [2, 4].

The object of this investigation was to study the ability of clonogenic (stem) cells of a hepatoma to synthesize the embryonic serum protein alphafetoprotein (AFP). AFP-producing cells have been demonstrated by the method of local hemolysis in gel [8, 10], with certain modifications introduced by the writers. The clonogenic ability of AFP-producing cells was determined by colony formation by them during subsequent culture.

EXPERIMENTAL METHOD

Rat hepatoma McA-RH 7777, cultured in vitro, was generously provided by Dr. Becker and Professor Van Potter under the aegis of the Soviet-American Collaboration on the Immunology of Tumors. This hepatoma actively synthesizes AFP and a number of serum proteins of adult animals [3].

Rabbit antibodies (AB) against rat AFP, purified on an immunosorbent for use in the work were generously provided by D. A. Él'gort. Activity of the AB was completely suppressed by a purified preparation of AFP, in equivalent proportions. The AB were dialyzed against the culture medium and kept at −20°C. Sheep's red blood cells (SRBC) were conjugated with protein A (from Pharmacia, Sweden) with the aid of $CrCl_3$ by the method in [9], suggested for conjugation of SRBC with AB: 0.1 ml of a 0.05% solution of protein A was conjugated with 0.5 ml of a 20% suspension of SRBC with the aid of 0.6 ml of 0.01% $CrCl_3$. The material was cultured in plastic Petri dishes 35 mm in diameter (Falcon Plastics, 3001), treated with a 0.1% solution of poly-

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TABLE 1. Characteristics of Clonogenic Cells and Clones of Hepatoma McA-RH 7777

Type of clonogenic cells	Number of cells	Type of clone	Number of clones
+AFP	106	+AFP	60
		−AFP	31
		Mixed	15
−AFP	66	+AFP	2
		−AFP	61
		Mixed	3

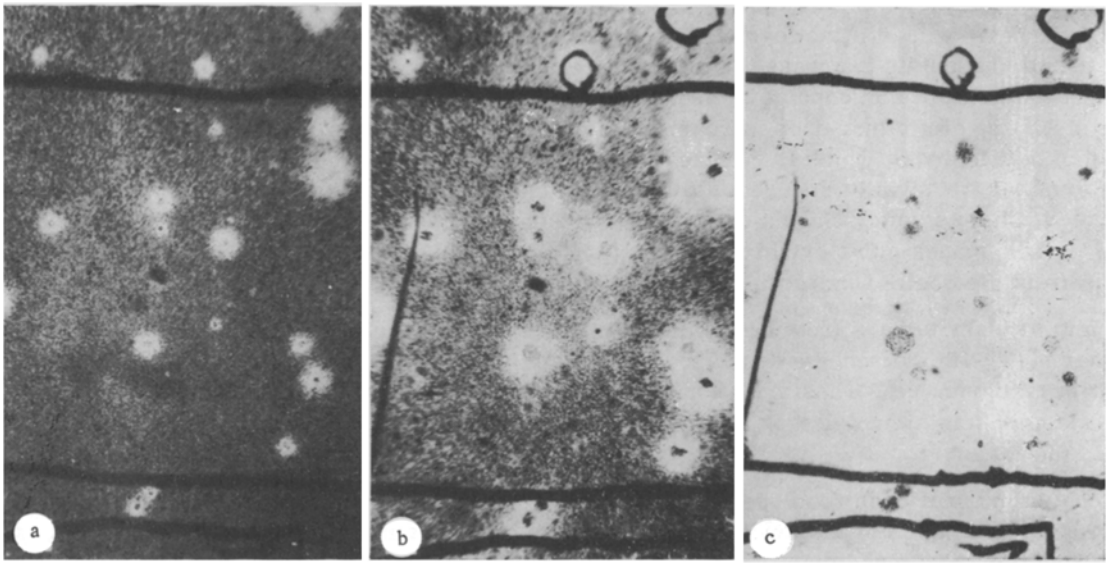

Fig. 1. Photographs of the same area of a Petri dish on the 2nd (a), 5th (b), and 8th day (c) of culture. a) Magnification 4 (Diavert microscope), field of vision was outlined immediately after testing with a needle on the outer side of the floor of the dish; b) lysis of the erythrocytes had not yet taken place on the 5th day of culture, the plaques were enlarged; c) clone formation can be distinguished on the 8th day. Clones growing from +AFP and −AFP cells are visible.

lysine (from Serva, West Germany). The trypsinized suspension of hepatoma cells was seeded into dishes, washed to remove free polylysine (2000 cells per dish) in Hanks' solution without serum. After attachment of the cells to the dish 0.01 ml of a 20% suspension of SRBC, conjugated with protein A (SRBC-A), was added. The SRBC-A was uniformly distributed over the dish and adhered firmly to its surface covered with polylysine, to form a continuous monolayer. The monolayer of cells and SRBC-A was then covered with 0.5 ml of 0.3% agarose solution (from Calbiochem, USA) in DMEM medium with 20% embryonic calf serum (ECS) containing 10 μg of AB. After solidifying the agarose formed a layer about 0.5 mm thick. The dishes were incubated at 37°C in an atmosphere containing 5% CO_2 for 20-24 h. During this time the cells still had not begun to divide. The AFP secreted by the cells was adsorbed on SRBC-A, which had previously bound AB of the IgG class. Free diffusion of AFP in the gel was blocked by AB, and the excess of AB in the medium enabled the formation of an antigen−antibody complex to red blood cells, binding complement (C'), to take place. After incubation, guinea pig serum (C'), diluted 1/30-1/40 with culture medium, was layered above the layer of agarose. The concentrations of AB and C' were chosen beforehand. The dishes were examined under a "Diavert" inverted microscope (from Leitz, West Germany). After only 30 min clear zones of hemolysis (plaques) were visible around single cells. After 60 min the number of plaques did not increase any more, neither did their size.

Single plaque-forming cells and also cells without plaques were outlined with a pointed needle from the under side of the dish, under microscopic control. The cells thus outlined were photographed.

Next, 0.5 ml of culture medium with 20% ECS was added to each dish, after which the dishes were incubated for 7-10 days and the same fields of vision were periodically photographed. On the 2nd day most of the adherent cells had begun to divide, and for a period of several days they formed colonies (clones). Under these

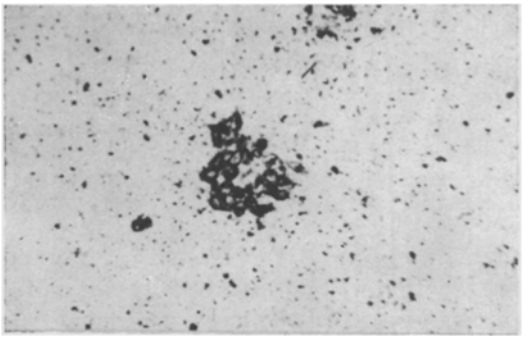

Fig. 2. +AFP clone growing from plaque-forming cell. Here and in Fig. 3 cells were stained by the immunoperoxidase method with antibodies against rat AFP. Diavert microscope (Leitz, West Germany). Magnification 70.

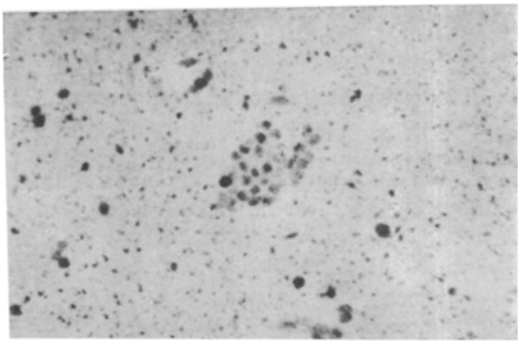

Fig. 3. AFP clone growing from a cell not forming a plaque.

circumstances the SRBC and plaques were sometimes preserved until the end of the experiment.

The cells were fixed in 4% formalin in buffered physiological saline and stained for AFP by the immunoperoxidase method [5, 14].

Cells in the same dishes which did not form plaques, and absence of plaques in the dish before addition of C' served as the internal control.

EXPERIMENTAL RESULTS

Plaques formed by single hepatoma cells producing AFP, just like the cells of that hepatoma which did not form plaques, are illustrated in Fig. 1a. The use of an SRBC-A monolayer in the version described above enabled very distinct plaques to be obtained, located in the same plane, and the daily incubation of cells, comparable with the cell cycle, before addition of C' enabled all the AFP-producing cells evidently to be discovered. Two days after seeding the cells, most cells, whether or not they formed plaques, could be seen to be dividing. By the 8th day in culture colonies of about 30 cells were formed (Fig. 1b, c). Consequently, neither the polylysine covering of the base of the dish nor the presence of erythrocytes, activated C', or lysis products of erythrocytes prevented colony formation. Attempts were therefore made to remove C' and erythrocyte lysis products as quickly and completely as possible, and this was facilitated by the thin layer of gel used in this variant of the method.

The reproducibility of the method is very high. Quantitative results of one experiment are given below.

The cloning efficiency in this experiment was close to 70% – 250 colonies from 359 single cells. It was the same both for AFP-producing (+AFP) cells and for cells not producing AFP (–AFP): 69% (163 of 236) and 71% (87 of 123) respectively.

Some colonies were studied before the stage of staining (Figs. 2 and 3; Table 1). It will be clear from Table 1 that the colonies in most cases preserved the type of synthesis of the ancestral cell. "Switching" of the type of synthesis was observed more often from +AFP to −AFP than vice versa. This result perhaps does not reflect the true relationship, for sensitivity of local hemolysis in AFP determination may be much higher than its detection by the immunoperoxidase method in cells. The study of "switching" in clonal lines with maximal AFP production may give the precise answer to this question. In just the same way the nature of "mixed" clones requires further study.

The results thus show that hepatoma McA-RH 7777 consists of +AFP and −AFP clones which, in most cases, do not possess intraclonal heterogeneity for this character. Most cells in both types of clones are self-maintaining (clonogenic). Ability to synthesize AFP does not correlate with the clonogenic potential of the hepatoma cells. Consequently, this character is expressed in the stem line of the tumor and is not connected with the formation of terminally differentiated cells, as the present writers postulated previously [2]. The answer to the question, to what extent is the synthesis of adult serum proteins produced by this hepatoma expressed in its stem line, will be obtained by similar experiments.

The approach suggested in this paper enables the presence of characters of differentiation inherent to this tumor to be detected in tumor stem cells. The method developed for this purpose, in the writers' view, has a more general value and can undoubtedly be used for selecting rare clones with assigned antigenic properties for the quantitative analysis of the clonal structure of cell populations and stem lines of various tumors producing soluble antigens.

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