ENUMERATION OF ALPHAFETOPROTEIN SECRETING CELLS USING A REVERSE HEMOLYTIC PLAQUE ASSAY DURING THE GROWTH OF MORRIS HEPATOMA 7777 IN CULTURE

Béatrice de Néchaud, Sylvie Fromont
and Josette Bergès

Institut de Recherches Scientifiques sur le Cancer
B.P. 8, 94800 Villejuif, FRANCE.

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SUMMARY: Enumeration of alphafetoprotein (AFP) secreting cells in subcultures of rat hepatoma McA-RH7777 has been achieved by a reverse hemolytic plaque assay. The proportion of the cell population engaged in AFP production varies during the growth. Its maximum is reached during the late exponential phase and precedes the maximum of daily AFP accumulation in the culture medium. Then the proportion of AFP secreting cells decreases whereas AFP accumulation remains high, suggesting a change in AFP secretion rate.

Some tumors and some normal immature organs produce alphafetoprotein (AFP). What proportion of the cells are involved in such a production remains to be established. There are several approaches to this question. In order to locate the AFP containing cells, intracellular AFP could be labeled by specific interaction with antibodies (1) or hormones (2). But AFP is mainly exported from the producing cells and seldom stored inside. Therefore a more rational approach would be to probe for AFP secretion rather than for its intracellular presence. Methods based on plaque forming cell assay are now available for the detection of antigen secreting cells (3, 4). They involve a complement mediated lysis of erythrocytes coated with antibodies and surrounded by antigen secreting cells. They have been successfully applied to enumeration of albumin secreting hepatocytes in dissociated mouse and rat liver (4, 5). We report here results

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TABLE I : COMPOSITION OF THE MIXTURE FOR PFC ASSAY

REAGENT	VOLUME (µl)	FINAL DILUTION
medium	110	
"developper"	10	1/1000
packed coated erythrocytes	20	1/50
viable hepatoma cells	50	from $5X10^2$ to $5X10^3$ cells per assay
complement	10	1/20

concerning AFP secreting cells in McA-RH 7777, a cell line derived from Morris hepatoma 7777 (6).

MATERIALS AND METHODS

The cell line is subcultured in $25cm^2$ plastic flasks (Falcon) at 37° without agitation in an atmosphere of 95% air 5% CO_2. The medium consists of Swim's S77 medium (Gibco) supplemented with 4mM L-Glutamine (Sigma), 5% fetal calf and 20% horse sera (Eurobio). Unless mentioned, it is changed every day. AFP content in culture media was estimated by electroimmunodiffusion (7) using the monospecific antisera described below.

Rabbits were hyperimmunized against a purified preparation of rat AFP. The antisera were made monospecific by adsorption on glutaraldehyde insolubilized normal rat serum (8). Pure antibodies were isolated from the IgG fraction of these monospecific antisera on glutaraldehyde insolubilized rat amniotic fluid (8), concentrated to 3mg/ml on microporous filters (Diaflo) and stored at -20°C. Coating of sheep erythrocytes with the pure antibodies was achieved by p-benzoquinone method recently described (9). Briefly 600 µg of antibodies were treated with p-benzoquinone (3mg/ml final) and filtrated through a Sephadex G200column. The activated antibodies were then coupled to 0,2 ml packed erythrocytes. The efficiency of coating was assessed by hemagglutination and the batches of coated erythrocytes stored at +4°C until use.

Monocellular suspensions of McA-RH7777 were obtained by trypsinization of the cell layer, washing by centrifugation and pushing the resuspended cells through a 0.4mm diameter needle. Viability was estimated by trypan blue exclusion.

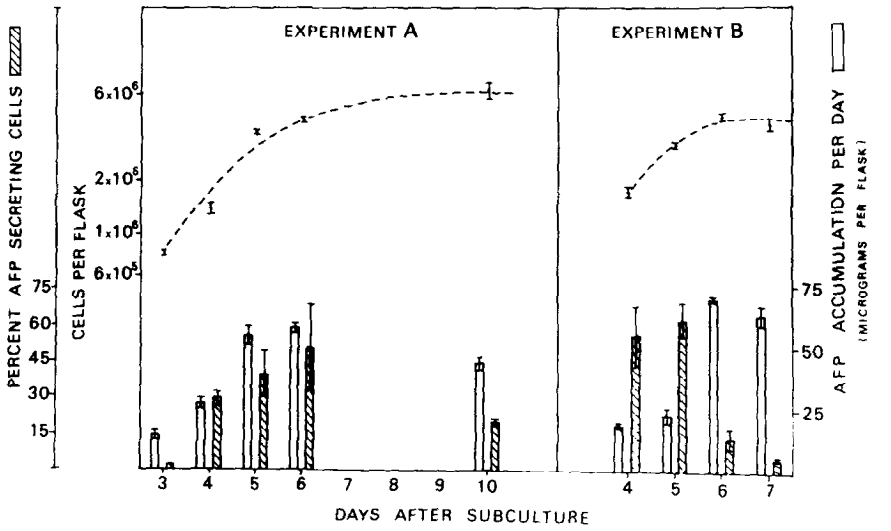

FIGURE 1 AFP SECRETION DURING THE GROWTH OF RAT HEPATOMA McA-RH7777 IN CULTURE.

At each time 3 culture flasks were harvested, the culture medium removed and its AFP content assessed. The cell layer was then trypsinized and the cell suspension collected, washed and counted in order to calculate the cell number per flasks, the proportion of viable cells and the dilution for PFC assay. PFC assays were performed immediatly and plaques scored five to seven hours later.

The plaque forming cell (PFC) assay was performed as described by Cunningham and Szenberg (3) adapted as indicated in Table 1. All the dilutions were done in air CO_2 gased S77 medium supplemented with 4mM L-Glutamine and 6% decomplemented horse serum. The volume and dilution of the reagents are given in Table 1. The "developper" (for terminology cf. 4) was the pure anti AFP antibody preparation; reproductible results were obtained for a final concentration range from 6 to 1.5 µg/ml. The complement was fresh guinea pig serum adsorbed by sheep erythrocytes. Each assay was done in triplicates and internal control obtained by replacing the "developper" by the same volume of medium. The assay chambers were sealed and incubated at 37°. The hemolytic plaques began to appear after two hours of incubation and were counted after five to seven hours in order to allow to develop completly.

RESULTS AND DISCUSSION

Two typical experiments are given in FIG. 1. It can be seen that the AFP accumulation in the medium increases from the early exponential phase to the beginning of the stationary phase. In experiment B the maximum amount of AFP perday (70.7 ± 1.3µg AFP/flask) was reached for a cell

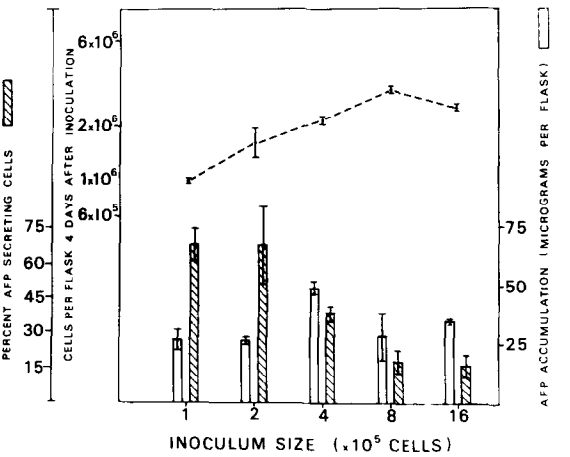

FIGURE 2 AFP SECRETION BY McA-RH7777 SUBCULTURES STARTED WITH VARIOUS INOCULUM SIZES.

At day 0 15 flasks were inoculated with five different cell densities. The medium was changed the second and the third day and the cells harvested the fourth day. The cell number per flasks, the AFP accumulation between the third and the fourth day and the percentage of AFP secreting cells were calculated as for FIG. 1.

density of 4.55 $\pm$ 0.20 X 10^6 cells/flask. During the stationnary phase there is a slight decrease in the daily AFP accumulation.

The PCF assays at different days were performed with the same batch of coated erythrocytes. One batch was used for experiment A (McA-RH7777 subculture number 106) and another for experiment B(subculture ## 116). Heterogeneity in the hemolytic plaque sizes has been noticed in every PFC assay suggesting variations in the amount of AFP released per cell.

The percentage of AFP secreting cells follows the same general picture than the AFP accumulation (Fig. 1). However its miximum is reached sooner during the growth and its decrease is more abrupt. In order to check whether the ageing of the erythrocyte batches could interfere with the PFC results; we assayed one batch at the same time with cultures started with various inoculum sizes (subculture ## 119) and having therefore reached different cell densities (Fig. 2). As in experiment B

TABLE 2 : PERCENTAGE OF AFP SECRETING CELLS IN SUBCULTURES OF DIFFERENT AGES

DAYS OF SUBCULTURE	NUMBER OF CELLS PER FLASK (X 10^6)	AFP SECRETING CELLS FOR 10^6 CELLS ASSAYED (mean ± S.D.)	PERCENT OF AFP SECRETING CELLS (mean ± S.D.)
2	1.26	675 ± 25	82 ± 8
	1.00	828 ± 39	
	1.26	952 ± 19	
7	2.16	630 ± 26	47 ± 14
	1.92	586 ± 75	
	0.92	196 ± 16	

Two subcultures of McA-RH7777 inoculated at different days were harvested at the same time and assayed for PFC simultaneously. They differ significantly ($P < 0.01$) for the percentage of secreting cells (fourth column) but not for the number of cells per flask (second column) although the two days subculture was in an actively growing phase. There is a significant difference among some flasks of the same subculture for the number of AFP secreting cells (third column) even though this number doesn't vary much among triplicate PFC assays done for each flask (third column, standart deviation = SD)

(Fig. 1) the maximum of AFP accumulation is dissociated from the maximal percentage of AFP secreting cells. Decrease of both occurs when the cultures arrive at their optimal cell density.

From the results reported in Fig. 1 and 2 it appears that enumeration of AFP secreting cells at anyone time during the growth of McA-RH7777 could be achieved by PFC assay in spite of the changes imposed to the cells when the assay is performed. Although the estimation of the number of secreting cells varies from one flask to the other, it remains homogenous among the triplicate assays done for any given flask (Table 2). Furthemore these results show that during the growth of McA-RH7777 there is at first an increase in both the percentage of AFP secreting cells and

the amount of AFP accumulated in the medium. Thereafter, there is a decrease in the number of secreting cells whereas the AFP accumulation doesn't drop as abruptly. This suggests a change in the AFP secretion rate per cell and could fit in with the concept of an AFP synthesis restricted to the pre-replicative part (G1) of the cell cycle (10, 11 and 12 for a tentative model). Thus, during the exponential phase of growth there are more and more cells enlisted in active cycling and passing through G1 and consequently the number of AFP secreting cells and the total amount of AFP released in the medium increase. Then, when the cell maximal density is reached, the number of cells engaged in the proliferative process drops and therefore the total number of AFP producing cells also diminishres. However since under these conditions the G1 phase of the cell cycle lasts longer, the amount of AFP synthesized and exported per cell increases resulting in the maintenance of a high AFP accumulation in the medium.

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