summation of the inactivation indices of the separately transplanted cells), and when the ratio between the cells was 2 : 5 : 1 the inactivation index was only 44.4%, instead of the expected 100%. These results show that the composition of the subpopulation of T lymphocytes used includes cells protecting the stem cells against the inactivating action of allogeneic T lymphocytes. It will also be evident that the population of splenic T lymphocytes contained more cells abolishing the inactivating action of the syngeneic killer cells than the population of T lymphocytes from LN. This fact can evidently explain the lower inactivating power of the Spl than of the LN T lymphocytes (Table 1).

The nature of the protector cells found in these experiments is unknown. The possibility cannot be ruled out that they are a subpopulation of lymphocytes intended to protect stem cells against the harmful action of various factors. Another possibility is that the experimental conditions chosen facilitate the accumulation of large numbers of the cells known as suppressor cells, with an important role in the regulation of immunity reactions [4].

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SYNTHESIS OF α-FETOPROTEIN AND ALBUMIN BY HUMAN EMBRYONIC HEPATOCYTES

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Population aspects of the production of specific secreted proteins (serum albumin and α-fetoprotein) were studied in cultures of hepatocytes from human embryos at 6-12 weeks of development. A method based on local hemolysis in gel using sheep's erythrocytes conjugated with antibodies against the proteins for testing was used. The overwhelming majority of hepatocytes were shown to synthesize both proteins.

KEY WORDS: human embryo; hepatocyte cultures; fetoprotein; serum albumin; local hemolysis in gel.

In mammalian development a strictly regular change in the composition of the blood serum is observed. In the early stages of development α-fetoprotein (α-FP) is predominant, whereas albumin and transferrin are present in very low concentrations. In the course of development the α-FP level falls from 3-5 to 10^{-6} mg/ml, whereas the concentrations of albumin and transferrin rise sharply [1]. All three proteins are synthesized by the liver [1]. However, it was not previously known whether there exists in the liver a "mosaic" of hepatocytes, each synthesizing one particular protein, or whether they are produced by the same cells. In particular, it was not known whether different populations of cells synthesizing embryonic and "adult" proteins exist in the

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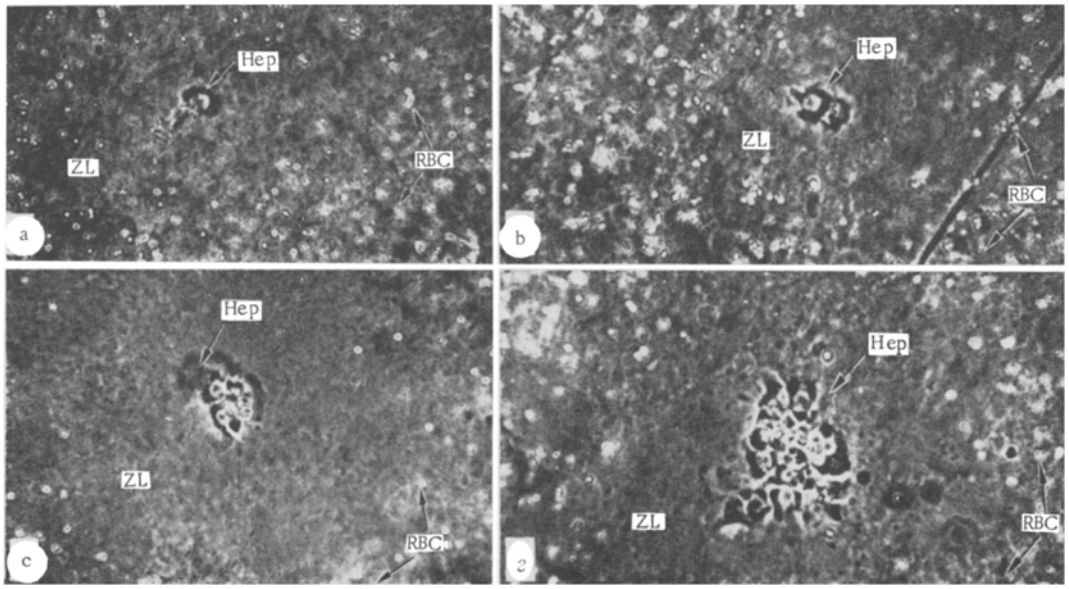

Fig. 1. Local hemolysis of RBC conjugated with antibodies against HSA or α-FP, after incubation with cultures of human hepatocytes; plaques around one (a), two (b), and three (c) cells and colonies of cells (d). Hep) Hepatocytes; RBC) sheep's erythrocytes; ZL) zone of lysis. Phase contrast, 140 $\times$.

embryonic liver or whether they are produced by the same cells, which alter their profile of synthesis in the course of differentiation.

In the investigation described below serum protein synthesis was studied by the method suggested by Molinaro et al. [7] as a modification of the method of local hemolysis in gel. The overwhelming majority of hepatocytes in the embryonic human liver were shown to produce serum albumin and α-FP simultaneously.

EXPERIMENTAL METHOD

Primary cultures of hepatocytes from human embryos at 6-12 weeks of development (obtained at therapeutic abortions) were used for analysis. The residue of hepatocytes obtained after primary treatment [5], washing, and centrifugation was poured into a series of petri dishes (Anumbra glass dishes 60 mm in diameter) containing culture medium (Eagle's medium with 20% bovine serum) until not more than one to three single cells or groups were present in a field of vision under a magnification of 10×7. To prevent the layer of gel with the erythrocytes from sliding, the bottom of the dish was smeared at the perimeter with 0.1% agarose before sterilization. The cells were cultured at 37°C in an atmosphere containing 7% CO_2. The time of performance of the experiment was chosen depending on how quickly the cells spread out in a monolayer: 18-42 h after seeding. Before the experiments the medium was drawn off and the dishes were washed twice with medium without serum.

Antibodies against human serum albumin (HSA) were isolated from commercial antiserum against human serum proteins (N. F. Gamaleya Institute of Epidemiology and Microbiology) by means of an immunosorbent prepared from a commercial preparation of HSA on a basis of Sephadex G-200 [3]. Antibodies against α-FP were isolated from commerical antiserum against α-FP (N. F. Gamaleya Institute), additionally exhausted by the addition of normal human serum (1 vol. of normal serum to 10 vol. of antiserum), with the aid of an immunosorbent prepared from human fetal serum on the basis of aminocellulose ester [2] or Sepharose 4B, activated by CNBr (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Antibodies were conjugated with sheep's erythrocytes (RBC) by the use of $CrCl_3$ [6, 7]. After each batch of RBC conjugated with antibodies against HSA or α-FP had been obtained, these erythrocytes were tested for reactivity, specificity, and sensitivity in the lysis test [7]. All possible combinations of antibodies, antigens, and antisera were tested, including antiserum against human γ-globulin (HGG). The lowest concentration of antigen causing lysis was 100 ng/ml for α-FP and 10 ng/ml for HSA. The method of local hemolysis suggested by Molinaro [7] for the detection of cells producing HSA and α-FP was modified as follows. A mixture (19 : 1) of 0.85% agarose, made up in Hanks's solution, and a 20% solution of thrice-washed erythrocytes conjugated with

TABLE 1. Synthesis of HSA and α-FP in Cultures of Hepatocytes from Human Embryos at 6-12 Weeks of Development (local hemolysis in gel method)

Expt. No.	Age of embryos, weeks	Incubation time, h	Fraction of cells and cell groups causing lysis of sheep's erythrocytes in gel (absolute number and percentages)					
			RBC anti-HSA			RBC anti-α-FP		
			1 cell	2—3 cells	3 cells	1 cell	2—3 cells	3 cells
1	8	3	22/22 100%		46/46 100%	19/24 79%		28/28 100%
			7/8 87%		12/12 100%	11/11 100%		7/7 100%
		6	8/10 80%		35/37 94%	7/7 100%		12/12 100%
2	8	3	1/4 25%		12/12 100%	5/10 50%		32/33 97%
		6	8/8 100%		7/7 100%	15/20 75%		32/35 91%
3	7—8	4	1/5 20%		4/12 33%	7/7 100%		10/10 100%
			3/8 37%		5/21 24%	5/6 83%		12/12 100%
4	7	4	4/6 67%	3/4 75%	16/16 100%	7/7 100%	11/12 92%	13/16 81%
			9/11 82%	21/29 72%	22/22 100%	2/11 18%	4/12 33%	23/29 79%
5	9	4	2/7 27%	13/16 81%	32/36 89%	0/1 0%	4/4 100%	34/36 95%
			2/2 100%	13/15 86%	49/53 93%	0/1 0%	4/5 80%	12/13 92%
6	11	3	11/21 52%	13/21 62%	27/33 82%	12/14 86%	12/13 92%	24/29 83%
						10/11 91%	8/11 72%	24/28 86%

Legend. Numerator – number of cells forming plaques, denominator – total number of cells.

antibodies, prepared immediately before use, was poured in volumes of 1 ml over the washed dishes with cultures. The cells were incubated with erythrocytes for 3-4 h (37°C, 7% CO_2), 1 ml of the developing antiserum was added to each, and incubation continued for 1 h. The antisera were drawn off, 1 ml of complement (freshly frozen guinea pig serum in a dilution of 1 : 10) was added to each dish, and incubation continued for a further 1 h. The dishes were examined under an inverted phase-contrast microscope and the percentage of cells giving hemolytic plaques was counted. In each variant of the experiment there were two to four dishes. In each dish the single cells, groups of two or three cells, and larger colonies were counted separately, there being usually 50 discrete groups to each dish.

EXPERIMENTAL RESULTS

The formation of hemolytic plaques was observed around hepatocytes secreting the test antigen (Fig. 1). The results of determination of the fraction of embryonic hepatocytes around which plaques were formed are given in Table 1 (several typical experiments). Clearly all or nearly all the hepatocytes could produce both HSA and α-FP. However, scatter of the data obtained in the different experiments, or even in the same experiment in parallel dishes, was observed and was evidently due to differences in the adaptability of the cells to in vitro conditions.

The specificity of the results was confirmed by a series of control experiments. When homologous antiserum used for development was replaced by antiserum of a different specificity (RBC anti-HSA by anti-α-FP or anti-HGG, RBC anti-α-FP by anti-HSA or anti-HGG) no plaques were formed. No plaques likewise were formed after replacement of erythrocytes conjugated with antibodies by normal erythrocytes or when cultures of embryonic fibroblasts were used instead of hepatocytes.

In some experiments the incubation time of the cells with the erythrocytes was changed from 0 to 6 h. In this case a tendency was observed for the number of plaques to increase with time and, in particular, for them to increase in size, showing that plaque formation depends on the synthesis of HSA and α-FP de novo by the cells.

The method revealed high sensitivity. The quantity of both antigens produced by one cell, even in 1 h, was often sufficient to cause lysis of erythrocytes over an area several times larger than the area of the cell.

The initial suspension of human embryonic liver cells was heterogeneous in composition, only 60% of the total number of cells being hepatocytes. This suspension could not therefore be used to study population aspects of protein synthesis by the cells by the local hemolysis in gel method. The modification of the method used, consisting of preliminary culture of the cells in petri dishes followed by superposition of a layer of erythrocyte suspension in agarose on the cell monolayer and microscopic assessment of the results, had three advantages: First, it enables hematopoietic cells to be eliminated during culture; second, it enables the morphology of each cell, whether giving or not giving a plaque, to be determined; and third, it enables living cells to be clearly differentiated from dead.

The results, namely 100% of cells producing both α-FP and HSA (the maximal result), mean that human embryonic hepatocytes, at the period of development studied (6-12 weeks) can in the overwhelming majority of cases synthesize α-FP, and that the synthesis of both HSA and α-FP takes place in the same cells. However, despite the high sensitivity of the method and the strong dependence of protein synthesis on the physiological state of the cells, the population structure cannot be estimated exactly by this method – for example, the ratio between cells producing and not producing α-FP cannot be so established, or a cell system more adapted to growth in vitro is required. Analysis based on the maximal result in parallel versions of the test will probably be correct in every case.

In some experiments the formation of hemolytic plaques was observed around dead hepatocytes which had separated from the glass and were in the body of the gel. This phenomenon could be due to several causes. Dead cells could have taken up bovine serum from the culture medium, and the proteins of that serum, diffusing into the agarose, could then have formed hemolytic plaques by crossed reactions with HSA or α-FP. However, neither RBC anti-HSA nor RBC anti-α-FP gave lysis in the bovine serum lysis test [7]. Another explanation, namely "suicide" of the cell as a result of what is called "bystander lysis," i.e., lysis of cells bordering on the antigen–antibody complex activating the C_5 component of complement, is possible.

The results of these experiments confirm those obtained for the human embryonic liver by methods of immunofluorescence and microelectrophoresis in polyacrylamide gel [4].

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