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RAT CORTICOSTEROID-BINDING GLOBULIN (CBG) BIOSYNTHESIS BY FETAL HEPATOCYTES IN CULTURE

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Summary—The ability of fetal liver to synthesize and secrete corticosteroid-binding globulin (CBG) was investigated using primary cultures of hepatocytes transplanted from 15- and 18-day-old rat fetuses. Analysis of culture medium, after labelling with [^{14}C]leucine, by crossed immunoelectrophoresis, clearly demonstrated CBG, albumin and alpha-fetoprotein (AFP) secretion. The relative rate of CBG secretion was more important at the younger stage. Indirect immunofluorescence permitted localization of CBG in fetal hepatocytes. The results suggest that the high level of CBG found in fetal serum is mainly produced by the liver of the fetus itself.

INTRODUCTION

The binding capacity of corticosterone in the serum is higher in the 19-day-old rat fetus serum than in the adult and pregnant rat [1]. The high binding capacity of the fetus begins to decline before birth. It drops to a minimum a few days after the delivery. It increases again and progressively reaches the adult value 3 weeks after birth [2, 3]. It has been demonstrated that these binding capacity variations of glucocorticoids are due to variations of concentration in CBG itself [4, 5].

The high level of fetal serum CBG and its drop at birth raise the question of its origin in the fetus [2]. Is it due to a specific transfer of CBG from the mother to the fetus as for other proteins [6, 7] or are the fetal hepatocytes able to synthesize and secrete CBG?

To answer this question, 15- and 18-day-old fetal rat hepatocytes were cultivated in the presence of [^{14}C]leucine or [^{14}C]glucosamine. We have investigated synthesis and secretion of CBG into the medium by these cultures using crossed immunoelectrophoresis followed by autoradiography.

EXPERIMENTAL

Radioactive products

[^{14}C]Leucine (> 300 mCi/mmol) and [^{14}C]glucosamine (> 300 mCi/mmol) were purchased from Centre de l'Energie Atomique [CEA] (Saclay, France). [^{14}C]corticosterone (52 mCi/mmol) was obtained from Amersham, England.

Antigens and antibodies

Anti-adult rat serum was purchased from DAKO, Sweden. Purified albumin and AFP were used to obtain rabbit antisera whose specificities were tested by crossed immuno-electrophoresis.

In the same way, an antibody against rat CBG was raised in the rabbit with CBG purified by single semi-preparative agarose electrophoresis (Barbital buffer pH 8.6, ionic strength μ: 0.02 containing 2 mM calcium lactate [8] under 10 V/cm, 2 h at 4°C) performed on a 19-day-old embryo rat serum. In these conditions, CBG is the protein with the highest anodic mobility.

Culture conditions

The hepatocyte cultures were established from 15- and 18-day-old rat fetal livers as described [9]. A cellular suspension was obtained after trypsinisation. The cells were re-suspended and plated in collagen coated culture dishes in a culture medium (NCTC 109 from GIBCO containing 10% of fetal calf serum (FCS) from SORGA, Paris). After 6 h incubation at 37°C under 5% CO_2, the hepatocytes adhere to collagen coated dishes, while the hematopoietic cells do not. The medium is replaced with 2 ml of fresh medium containing 2 μCi/ml of [^{14}C]leucine or [^{14}C]glucosamine. The culture was carried out during 2 days, i.e. $15d + 2$ and $18d + 2$. The medium of each Petri dish is concentrated 10 times by lyophilisation before being analyzed.

Crossed immuno-electrophoresis(CIE) and autoradiography

The conditions of electrophoresis in agarose gel were the same as those used to purify the CBG: 10 μl of concentrated medium was run in the first dimension at 10 v/cm. The electrophoretic second dimension was carried out at 2 v/cm overnight (14°C) in an agarose gel (1.2 mm thick) containing 10% of anti-adult rat serum supplemented with 5% of anti-AFP; an intermediate gel (1 cm wide and 1.3 mm thick) was used containing 10% (v/v) of specific antibody. The plates were washed, dried and stained with Coomassie blue and exposed to Ultrofilm LKB from 2 to 7 days.

In order to investigate the possibility of non specific binding of [^{14}C]leucine to the proteins con-

tained in the culture medium, 10 μl of unlabelled supernatant culture medium (SCM) was incubated during 3 h with 1,000,000 cpm of [^{14}C]leucine and processed as previously described. In the same conditions [^{14}C]corticosterone was used to assess the specific binding property of the CBG. After being washed and dried, the plates were exposed to the Ultrofilm LKB before being stained.

Localization of CBG by immunofluorescence procedure

Hepatocytes, grown for 2 days on collagen-coated glass cover slips, were washed 3 times with 0.1 M phosphate buffer saline pH 7.4 (PBS) and fixed with 95% ethanol–5% acetic acid at −20°C for 10 min. The cells were washed again with PBS before being incubated for 45 min. at room temperature with anti-CBG (diluted 1:100). After another washing, the cover slips were incubated with goat-anti rabbit IgG coupled with FITC (Fluorescein isothiocyanate) for 45 min. The cover slips were incubated with goat-anti rabbit IgG coupled with FITC (Fluorescein isothiocyanate) for 45 min. The cover slips were mounted and after thorough washing, observed through a Leitz/Ortoplan fluorescent microscope. Non-immune rabbit serum or the conjugate alone were used as controls.

RESULTS

Monospecificity of anti-CBG

The crossed immuno-electrophoresis (CIE) realized with an embryo rat serum shows, after staining, only one precipitin peak in anti-CBG containing intermediate gel (Fig. 1A). Moreover, the autoradiography of the electrophoretic run of the serum incubated with [^{14}C]corticosterone revealed a binding to this peak only (Fig. 1B). These results demonstrate clearly the specificity of this anti-CBG.

Choice of the labelling conditions

The CIE (Fig. 2A) of the supernatant culture medium of 18-day-old embryo hepatocytes revealed several proteins. The unspent culture medium containing 10% fetal calf serum (control) revealed that at least 2 proteins from the fetal calf serum (the major one is probably albumin) are precipitated against the anti-adult rat serum (Fig. 2B). Among murine proteins, albumin and α_1-fetoprotein can be easily identified by the use of monospecific antisera. The study of the CIE autoradiographies done with the supernatant culture medium obtained in presence of [^{14}C]leucine (Fig. 2C) reveals that all the precipitin lines were labelled except those which correspond to fetal calf serum proteins. Incubations of an unlabelled supernatant culture medium with [^{14}C]leucine does not show any labelling of the immunoprecipitates indicating that the protein labelling observed with [^{14}C]leucine is due to the biosynthetic incorporation of the labelled amino acid into the proteins.

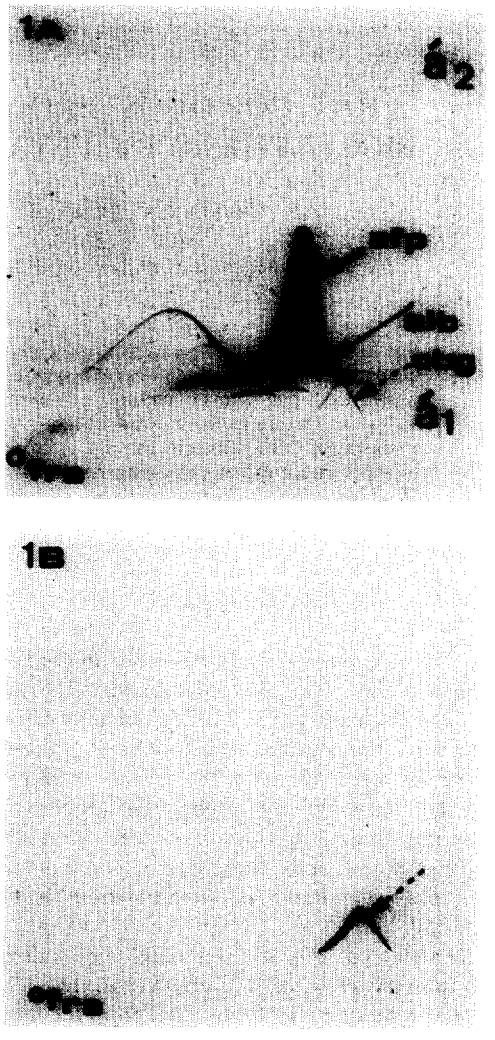

Fig. 1. (A) Crossed immunoelectrophoresis of 10 μl of a 19-day-old fetal rat serum incubated with [^{14}C]corticosterone against 10% anti-CBG (a 1) and 10% anti-adult rat serum supplemented with 5% anti-α-fetoprotein (a 2). AFP (thick arrow) indicates α-fetoprotein, ALB (thin arrow) albumin and CBG (dotted arrow) corticosteroid-binding globulin. (B) Corresponding autoradiography showing the [^{14}C]corticosterone binding of the precipitate in the intermediate gel.

On the contrary, when [^{14}C]glucosamine (Fig. 2D) is used to follow the biosynthesis of glycoproteins all the immuno-precipitates, including those of calf serum proteins, were labelled with various intensities. The more heavily labelled peaks seem to correspond to the true glycoprotein synthesis. A slight labelling was also observed on the FCS cross-reacting proteins.

Biosynthesis of CBG by the hepatocytes of 15- and 18-day-old rat fetuses

The CIE analysis of supernatant culture medium elaborated by the 18 day ($18d + 2$, Fig. 2A) and 15 day ($15d + 2$, Fig. 3A) fetal rat hepatocytes shows that a precipitate appears against the anti-CBG antibody. The [^{14}C]leucine labelling of these specific pre-

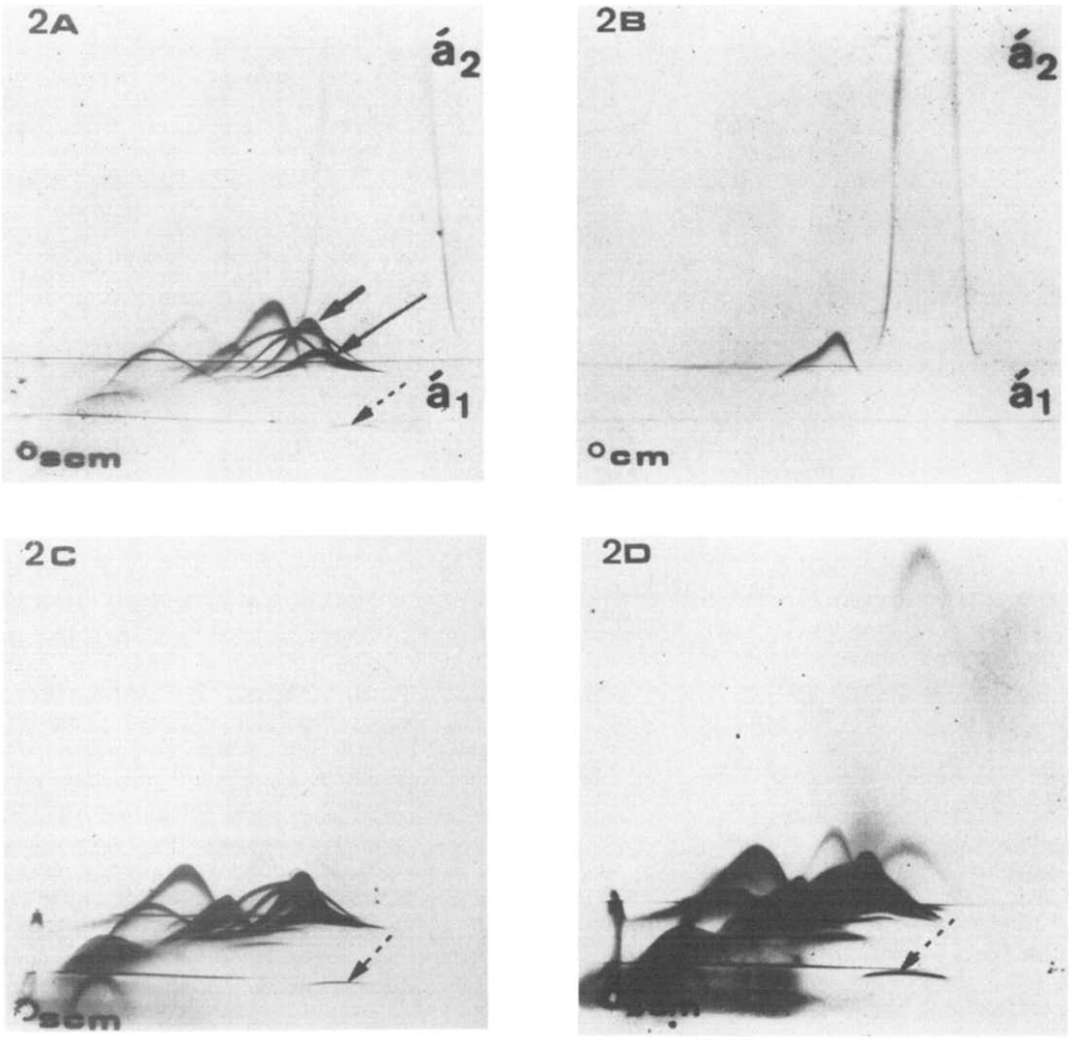

Fig. 2. (A) Crossed immunoelectrophoresis of 10 μl of concentrated supernatant culture medium (SCM) of 18d + 2 fetal hepatocytes showing a small corticosteroid-binding-globulin precipitate in contrast to large amount of the other precipitated rat proteins. (B) Crossed immunoelectrophoresis of unspent culture medium containing 10% FCS (CM) showing the cross reacting antigens from Fetal Calf Serum with anti-rat serum. (C, D) Autoradiographs of [^{14}C]leucine (C) and [^{14}C]glucosamine (D) labelled supernatant culture medium.

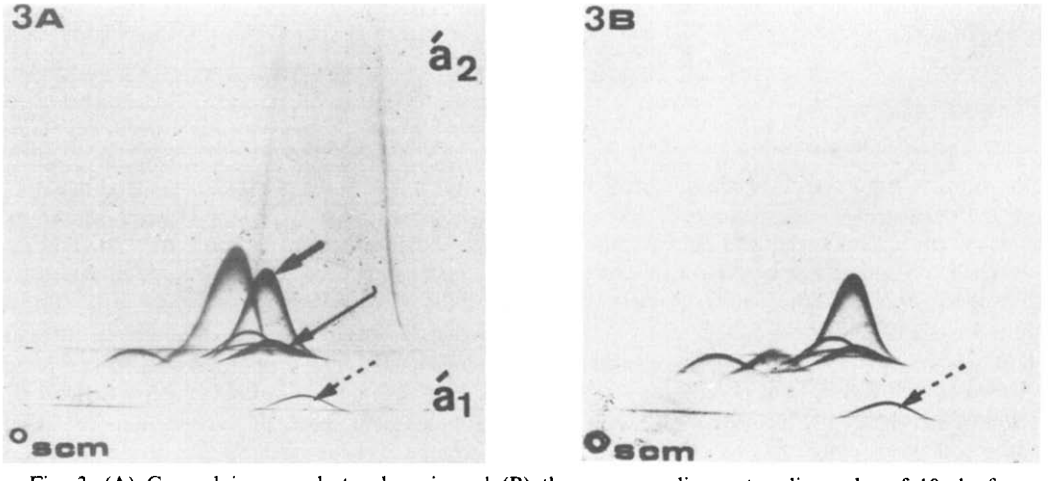

Fig. 3. (A) Crossed immunoelectrophoresis and (B) the corresponding autoradiography of 10 μl of supernatant culture medium form 15d + 2 fetal rat hepatocytes cultivated in presence of [^{14}C]leucine.

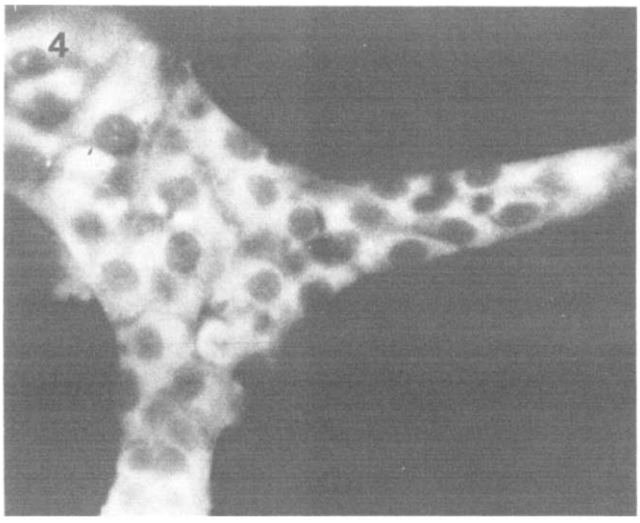

Fig. 4. Immunofluorescence localization of corticosteroid binding globulin in fetal rat hepatocytes.

cipitates clearly demonstrate the synthesis of CBG by the embryonic hepatocytes (Fig. 3B).

The functional activity of the CBG secreted into the supernatant culture medium is evidenced by the labelling of the CBG peak only, after CIE autoradiography of an unlabelled supernatant culture medium, previously incubated with [^{14}C]corticosterone (data not shown).

Comparison of the $15d + 2$ and $18d + 2$ supernatant culture medium patterns make obvious that the area of the CBG loop is smaller at $18d + 2$ (Fig. 2A) than at $15d + 2$ (Fig. 3A) whereas the number of protein peaks increases from $15d + 2$ to $18d + 2$, as well as the surfaces of some of them, as revealed by analysis with anti-adult rat serum.

Immunolocalization of the CBG

An indirect immunofluorescence procedure used for the localization of CBG revealed specific labelling of the hepatocytes (Fig. 4). Similar group of cells were also positive for albumin and α_1-fetoprotein (data not presented) indicating that these cells are hepatocytes. The minor contaminant fibroblast-like cells were totally negative.

DISCUSSION

Many authors report that primary culture is a suitable method to study the biosynthesis and secretion of serum proteins into the medium by adult [10] and fetal [11–13] hepatocytes. This allows investigations of the physiological and age-related secretions by the hepatocytes.

In this experiment the cells remaining attached to the collagen-coated dishes were mainly hepatocytes. The number of contaminant cells (fibroblasts and endothelial cells) accounted for less than 10% and was always very low for the 18-day-old hepatocytes culture [9]. Thus, most of the proteins found in the supernatant medium, especially those investigated, were expected to be secreted by the hepatocytes.

In order to prove the true synthesis of different proteins by fetal hepatocytes and their secretion into the medium, labelled supernatant culture media were analysed by CIE, which mono and polyspecific antibodies followed by autoradiography. This method has already been used to study the synthesis of proteins by cultured chicken fetal hepatocytes [14]. The technique as employed in our studies, has provided more information than several quantitative methods used by other authors [10–13], since the concomitant analysis by an anti-CBG containing intermediate gel with a polyspecific upper-gel allowed a semi-quantitative estimation of specific protein (CBG) as well as a comparative study of other proteins produced by the hepatocytes.

From the analysis of the stained plates, it was clear that some proteins from the FCS were recognized by the anti-adult rat serum (Fig. 2B). Interestingly the autoradiographies evidenced a labelling of these proteins in addition to those observed on the rat glycoproteins, when [^{14}C]glucosamine was used (Fig. 2D). Thus unwanted labelling was probably due to enzymatic or nonenzymatic secondary glycosylation. By contrast, only rat proteins truly synthesized and secreted into the medium were positively labelled when [^{14}C]leucine was used (Fig. 2C).

Thus, these results prove that rat fetal hepatocytes in culture are able to synthesize and secrete many proteins. Among them, albumin, AFP and CBG were identified. AFP production by the fetal hepatocytes has allowed us to confirm that the cells were fetal hepatocytes which did not differentiate into adult ones. The different results presented in this publication: (1) immunolocalization of CBG in fetal hepatocytes, (2) obtention of a precipitin line when a supernatant culture medium is tested against an anti CBG (3) labelling by covalent incorporation of [^{14}C]leucine into the precipitates, (4) functional activ-

ity of CBG produced by cultured fetal hepatocytes, as evidenced by [^{14}C]corticosterone binding studies, demonstrate without doubts that this protein is synthesized by the rat fetal hepatocytes.

The demonstration of CBG synthesis in liver, however, cannot exclude that part of the CBG found in the fetus can also come from other sources such as its embryonic membranes or maternal blood. For instance, a maternal transfer has been observed during early pregnancy for retinol binding protein [6] and α_2-macroglobulin [7]. It has earlier been observed that fetal rat serum CBG levels (expressed per mg serum protein) were several fold higher than those of the mother but similar in both compartments when expressed as CBG/ml serum [5]. CBG Levels in the fetus and newborns also showed a pronounced fall immediately after birth [2, 5]. These observations may lead to the conclusion that CBG in the fetus is arising due to specific transfer from the mother. In the present studies, we show that the proteins secreted by the fetal hepatocytes, especially CBG profiles, ($15d + 2$ SCM, Fig. 3A) reflect the serum protein pattern at the corresponding developmental stage. Moreover, there is an age related decrease in the ratio of CBG secretion by the hepatocytes with respect to other proteins (compare $15d + 2$ (Fig. 3A) and $18d + 2$ (Fig. 2A) and their respective autoradiographies Figs 3B and 2C). A suggestive conclusion could be drawn from the comparison of the hepatocytes secretion pattern and the serum pattern for albumin, AFP and CBG which are both reasonably similar and from the decreasing rate of synthesis of CBG by the hepatocytes. But, since serum levels depend to a great extent on clearance as well as synthesis, other sources of CBG besides fetal hepatocytes cannot be excluded. Nevertheless, it can be postulated that CBG in the fetus serum is mainly coming from fetal liver and the age related decrease in serum CBG level is due to a fall in its synthesis and secretion in the fetal hepatocytes.

From a biological point of view the implications of these CBG variations could be highly meaningful during the fetal postnatal period [15]. Thus the ratio of the free to the bound glucocorticoids could be significantly modulated by the variations of this protein [16]. In this way CBG could play an essential role in the onset of the activity of some crucial glucocorticoid regulated enzymes [17–18] involved in the post-natal cellular multiplication and differentiation of different organs, like liver, lung and lymphopoietic system.

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