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Electron microscopic evidence for the presence of an asialoglycoprotein receptor on isolated foetal rat hepatocyte surface

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SUMMARY

The ontogeny of asialoglycoprotein receptor was investigated by electron microscopic cytochemistry in hepatocytes isolated from foetal and adult rat. The binding capacity for asialofetuin coupled to horseradish peroxidase was lacking before the 18th day of intrauterine life; it arises at this time and increases with developmental age.

The ligand-receptor complexes form small patches. The distribution pattern of positivity is very similar in pre and postnatal age, covering the entire cell surface.

These results indicate a rather late appearance of the galactose-binding capacity related to the asialoglycoprotein clearance function, which is typical of adult mammalian liver.

INTRODUCTION

Several carbohydrate recognition systems are present in mammalian liver involved in the specific binding and internalization of different glycoproteins. Among them, the asialoglycoprotein (ASG) receptor is the most widely studied, being located on parenchymal cells, specifically recognizing galactose residues and thus mediating the removal from the plasma of these modified glycoproteins (Ashwell & Harford, 1982).

This system, first described by Ashwell & Morell (1974), since then has been well characterized, the receptor molecule has been isolated and many biochemical and morphological data concerning the binding, internalization and degradative pathway of ASG have been obtained both in whole liver and in isolated hepatocytes (Tolleshaug, Berg, Frolich & Norum, 1979; Wall, Wilson & Hubbard, 1980; Weigel & Oka, 1982; Geuze *et al.* 1982). The experiments performed on isolated parenchymal cells have conclusively demonstrated that the ASG receptor remains present in isolated hepatocytes and is also preserved

Dedicated to the memory of Professor Enrico Urbani, who died on the 9th of June 1983.

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in cultures of adult rat hepatocytes (Deschuyteneer *et al.* 1982; Zeitlin & Hubbard, 1982).

All these data have been obtained from adult liver, the only study relating to foetal life, to our knoweldge, being that of Hickman & Ashwell (1974), who incidentally reported that foetal liver was essentially devoid of binding activity. Although several aspects of the ontogeny of protein receptors (mainly for hormones and growth factors) have been carefully investigated (Csaba, 1981), no information on the time of appearance in the embryonic development of liver ASG clearance function is available so far.

These considerations, together with the difficulties in administering *in vivo* any substances to the foetal liver, have prompted us to utilize dissociated foetal hepatocytes to investigate the ontogeny of ASG receptor. We have therefore investigated the presence and distribution of the ASG receptor on foetal rat hepatocyte surface using electron microscopic cytochemistry to study the localization of asialofetuin coupled to horseradish peroxidase (ASF-HRP).

MATERIALS AND METHODS

Hepatocytes preparation

Isolated adult hepatocytes were obtained from liver of Wistar rats perfused with collagenase (Boehringer), according to the method of Moldeus, Högberg & Orrenius (1978).

Foetal livers were obtained after rapid hysterectomy from pregnant females anaesthesized with Farmotal (Farmitalia) 20 mg/100 g.b.w.; embryonal age was calculated on the basis of the appearance of vaginal plug and checked by foetus weight and length. The hepatocytes were isolated by immersion of small liver fragments in a collagenase solution, according to the procedure set up in our laboratory and detailed elsewhere (Conti-Devirgiliis *et al.*, 1981). This procedure was sometimes applied to adult livers to test the uniformity of results obtainable by the two isolation methods.

Cell yeld was $40–60 \times 10^6$ hepatocytes/g of fresh liver.

Cell viability was evaluated by the Trypan blue exclusion test and LDH leakage (Dickson & Pogson, 1977) and further assessed by morphological examination under electron microscope. In some experiments hepatocytes were purified by centrifugation in a Percoll (Pharmacia) gradient as described by Conti-Devirgiliis *et al.* (1981).

Asialofetuin-horseradish peroxidase (ASF-HRP) preparation and characterization

Fetuin (type IV, Sigma) was desialated by treatment with insoluble neuraminidase (Sigma) at 37 °C for 48 h as described by Dunn, Labadie & Aronson (1979).

Orosomucoid (α_1-acid-glycoprotein, Sigma) used for controls, was desialated in the same way.

Removal of sialic acid was checked by a gas chromatographic assay (Varma & Varma, 1976).

The conjugate between ASF and HRP (Boehringer) was obtained according to the method described by Wall *et al.* (1980) for asialorosomucoid, with minor modification. The ^{3}H-ASF added to cold ASF (1:20) during conjugation was prepared by the reductive methylation procedure of Means and Feeney, as described by Weigel & Oka (1982).

The specific radioactivity obtained was 2×10^7 dpm/mg of protein. The complex ASF-HRP was separated from the monomeric forms of ASF and HRP by means of a Sephadex G 200 column (cm 3×68) equilibrated in PBS. 2·5 ml fractions were collected at flow rate of 3·85 ml/h/cm^2 and were monitored for absorbance at 280 and 403 nm, and for ^{3}H-radioactivity.

Two peaks were eluted from the column: the first between 150 and 180 ml, and the second between 185 and 205 ml. Both peaks contained material absorbing at 403 nm (indicating the presence of the peroxidase haem group) but the ^{3}H-ASF radioactivity was almost entirely detected (97 %) in the first peak.

Analysis of this peak by SDS gel electrophoresis, performed according to Weber & Osborne (1969) shows only one band, corresponding to a M_r 80 000, which was positive for HRP activity. HRP, ASF and fetuin simultaneously analysed show RF values clearly different from ASF-HRP complex.

Hepatocyte treatment with conjugate

Freshly isolated hepatocytes, filtered through a nylon gauze (holes 100 μm dia) and twice rinsed with Krebs-Henseleit solution to remove the collagenase, were resuspended 1×10^6/ml in Waymouth medium (Gibco) supplemented with 2 % BSA (Miles), 10 i.u./ml of heparin, 1000 i.u./ml of penicillin and 1 mg/ml of streptomycin, continuously bubbled with carbogen (95 % O_2 and 5 % CO_2) and maintained for at least 120 min at 37 °C in a rotating bath. This period was demonstrated to be the minimum essential to recover a good metabolic capacity after collagenase treatment (Weigel & Oka, 1982; Zeitlin & Hubbard, 1982).

After the restoring period, the conjugate (30 to 300 μg ASF-HRP for 5–30 min) was added to incubation medium. All the experiments were performed at 37 °C, with oxygenation and rotation, in presence of 25 mM-$CaCl_2$. After incubation, cells were washed with PBS, fixed with 2 % glutaraldehyde in 0·1 M-cacodylate buffer for 15 min at 4 °C, rinsed twice in the same buffer before detection of HRP activity. The hepatocytes were resuspended in 3 mg/ml DAB, 0·02 M-3 amino 2,4 triazole and 0·03M-H_2O_2 in 0·1 M-Tris HCl buffer, pH 7·5 and incubated in the dark for 120 min at 24 °C (Wall *et al.* 1980).

Finally the cells, after rinsing in Tris buffer, were postfixed for 1 h in 1 % OsO_4 in 0·1 M-cacodylate buffer, dehydrated and embedded in Epon 812 for

electron microscopy. Ultrathin sections unstained or stained by lead citrate and uranyl acetate were observed under a Philips 400 T electron microscope.

Controls

The hepatocytes were treated in the following ways. Before treatment with conjugate: 15 min incubation in Ca^{++}-free medium, containing 20 mM-EDTA, after several rinsing of the cells by Krebs-Henseleit Ca^{++}-free containing EDTA for complete removal of calcium; preincubation and/or incubation with unconjugated ASF 20–100 fold exceeding the ASF-HRP concentration.

Incubation in presence of 65 mM-N-acetyl galactosamine.

Incubation in presence of a 33-fold excess of asialorosomucoid (ASOR), as asialoglycoprotein competitor.

RESULTS

Isolated adult hepatocytes exposed to saturating concentration of ASF-HRP at 37 °C for 5–15 min, exhibit a quite uniform staining of the entire cell surface; sometimes the label is particularly concentrated between the microvilli and in the position of the pits. A few vesicles lying below the plasma membrane are also stained (Figs 1, 2).

By increasing the incubation time (until 30 min) the labelling pattern is practically unchanged. Among the cells of each preparation, all showing a good preserved morphology, some are not at all stained.

As far as foetal hepatocytes are concerned, the most relevant data deal with the absence of asialoprotein-binding capacity before the 18th day of intrauterine life; in fact hepatocytes isolated from foetal rats aging 15, 16 or 17 days, treated with ASF-HRP at the highest concentration employed, are devoid of cell surface reaction product (Figs 4, 5). The hepatocytes of these preparations show always 90 % of viability as evaluated by the Tripan blue exclusion test and LDH leakage.

On the contrary, starting from the 18th day of development the positivity arises and is maintained as the developmental age goes on (Figs 6, 7 and 8).

In spite of the different morphological appearance of foetal hepatocytes which exhibit a more regular surface, with few microvilli, the distribution pattern of positivity is quite similar to that observed in the adult hepatocytes, i.e. small patches cover the entire cell surface, even if the foetal cells are always less intensely stained (Figs 6, 7 and 8).

Neither specific areas of reaction product accumulation nor engulfed regions and/or internalization pattern are detectable with the experimental conditions employed, although some structures similar to coated pits and vesicles are present since the earliest prenatal stage observed (Figs 4, 5).

Therefore the cytochemical results suggest a rather late appearance of binding capacity for asialoglycoproteins by the hepatocytes during the ontogenesis.

In the control experiments carried out in presence of excess of ASF (Fig. 3)

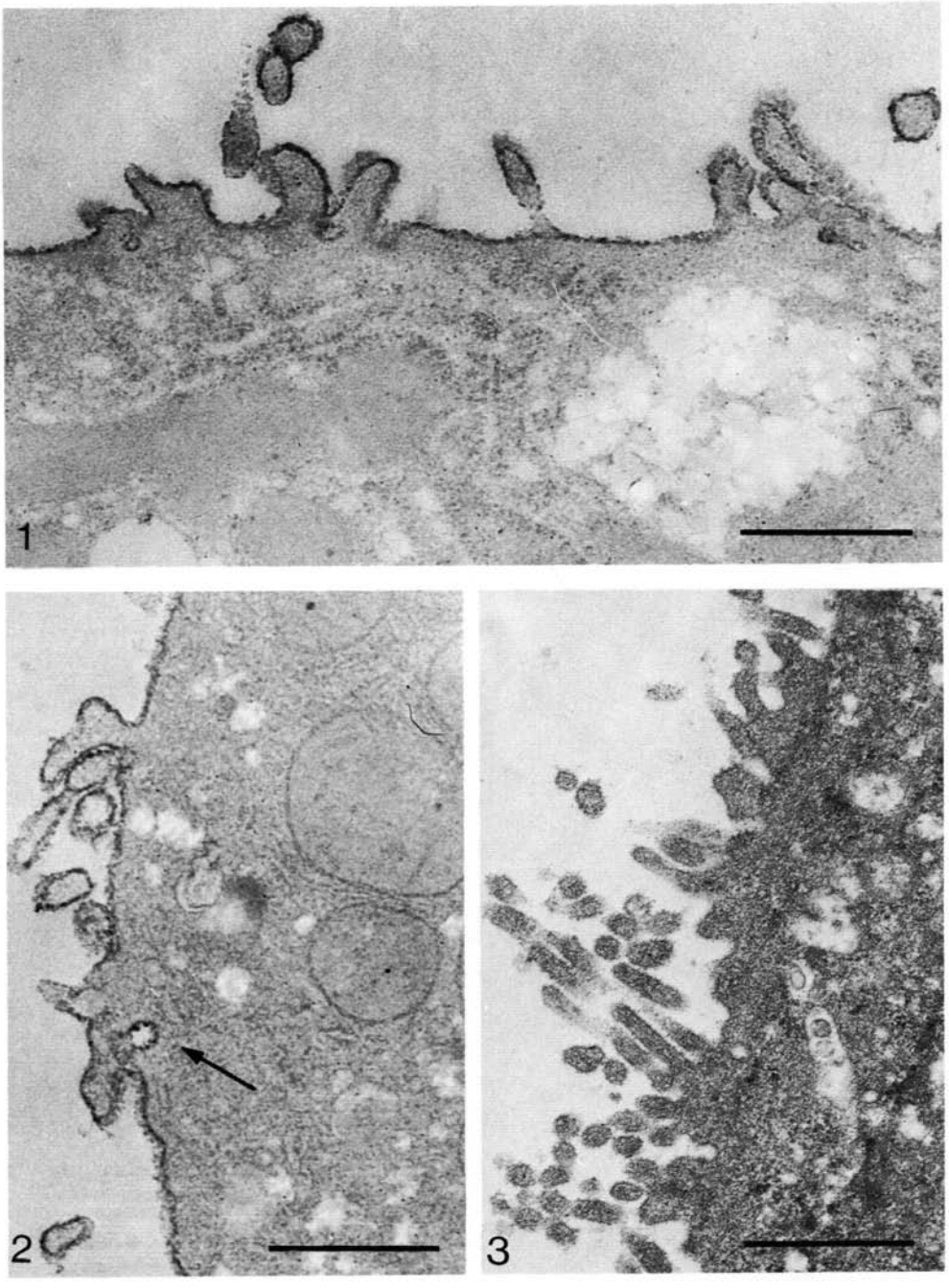
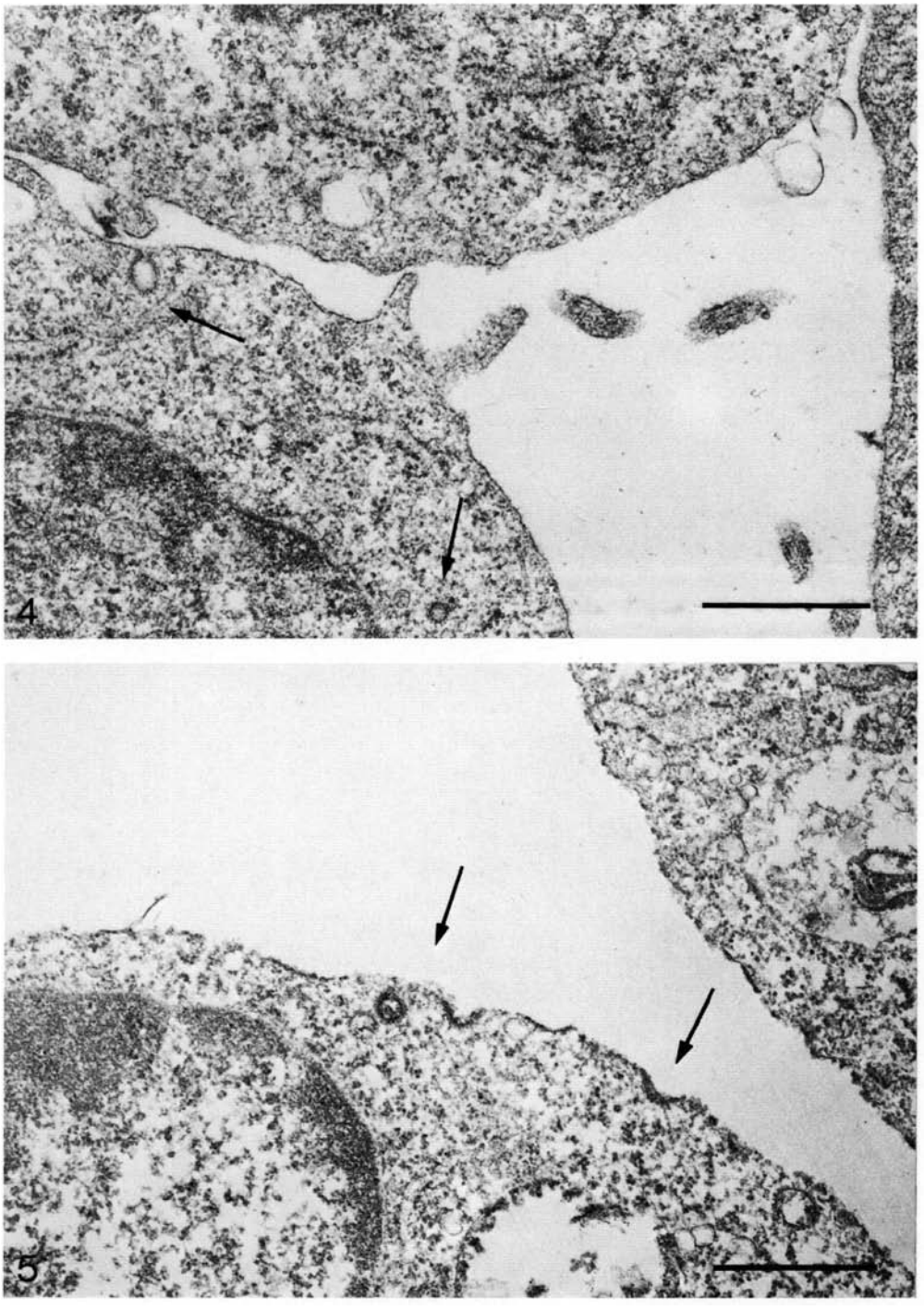


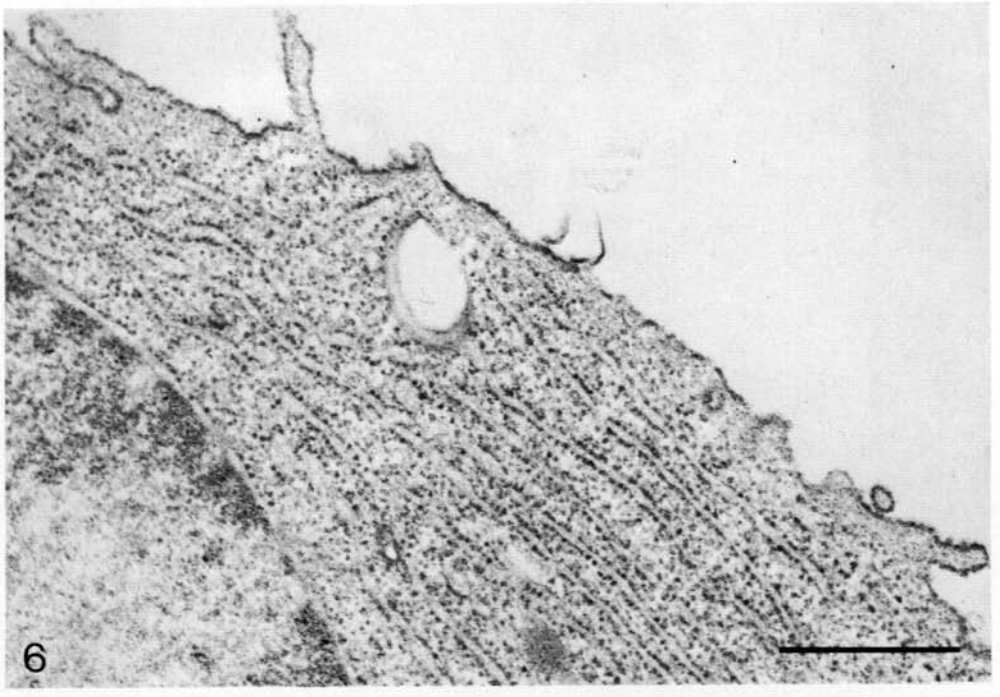
Fig. 1. Hepatocyte isolated from adult rat (viable cells 95 %) and treated for 5 min with ASF-HRP. The positivity is quite uniformly distributed along cell surface. Unstained. Bar = 0·5 μm.

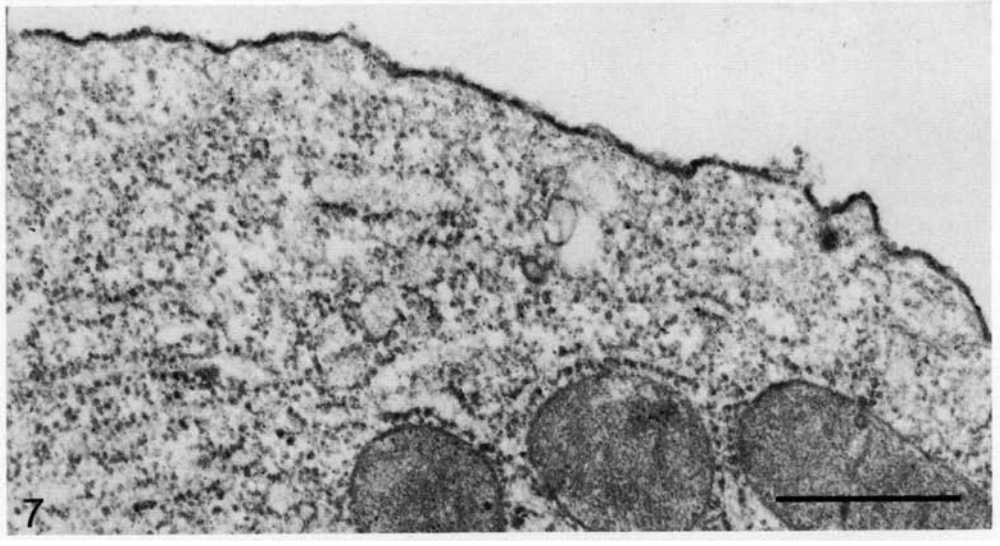
Fig. 2. Hepatocyte isolated from adult rat and treated for 30 min with ASF-HRP. Near the surface, an internalized vesicle is visible (arrow). Unstained. Bar = 0·5 μm.

Fig. 3. Hepatocyte isolated from adult rat. 3 mg/ml ASF as competitor were added to 30 μg/ml ASF-HRP: reaction product is absent from the cell surface. Bar = 0·5 μm.



Figs 4 & 5. Foetal hepatocytes isolated at 16th (4) and 17th (5) day of development (viable cells 90 %) and treated for 15 min (4) and 30 min (5) with ASF-HRP 300 μg/ml. Cell surfaces appear free of reaction product; coated pits and vesicles are present (arrows). Bar = 0·5 μm.





Figs 6 & 7. Hepatocytes isolated at 18th (6) and 19th (7) day of intrauterine life (viable cells 90 %) after 15 min of incubation with ASF-HRP. The positivity is well visible. Bar = 0·5 μm.

or ASOR as competitors, or after EDTA treatment, the reaction product is totally absent, indicating that the binding of ASF-HRP is specific and requires Ca^{++} (not shown).

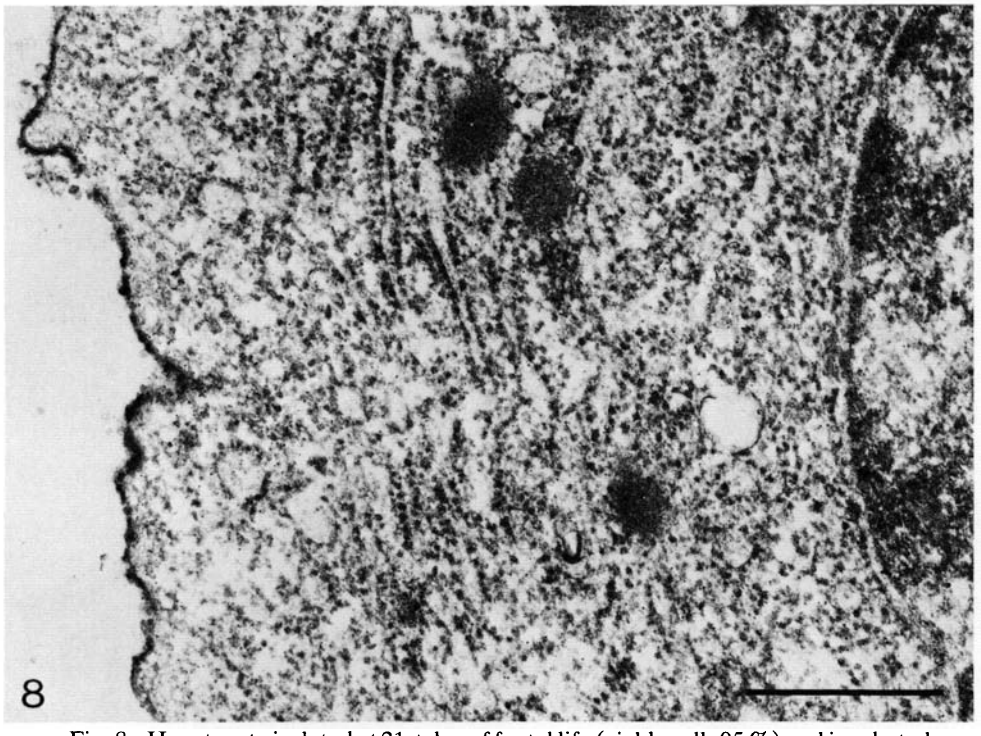


Fig. 8. Hepatocyte isolated at 21st day of foetal life (viable cells 95 %) and incubated for 30 min with ASF-HRP. Bar - 0·5 μm.

When the incubation is performed in presence of N-acetylgalactosamine, the labelling is not completely abolished and a faint positivity is still evident (not shown).

DISCUSSION

The data reported here provide another demonstration that isolated rat hepatocytes from adults and during prenatal life have properties comparable to those found in the intact liver, making them a suitable *in vitro* system to study metabolism.

Among the differences that must be considered the first is the loss of cell polarity; the sinusoidal and biliary domains are no longer segregated in isolated hepatocytes (Zeitlin & Hubbard, 1982; Matsuura, Nakada, Sawamura & Tashiro, 1982): therefore, in many instances receptor–ligand complexes, including ASG receptor complexes, show an uniform distribution on the cell surface, both in the adult and in the foetus, in contrast to the hepatocytes *in situ*. A loss of plasma membrane specialization has also been observed as regards the

cytochemical localization of several surface enzymes (Groothuis, Hulstaert, Kalicharan & Hardonk, 1981).

The heterogeneity of ASF-HRP positivity among hepatocytes of some preparations, observed both in adult and in foetuses, may be explained on the basis of variability in the number of exposed receptors. This could be related to a variable degree of loss of receptors during preparation or to a different recovery during incubation at 37 °C in Waymouth medium, due to the recycling mechanism operating on an internal receptor pool (Bridges, Harford, Ashwell & Klausner, 1982; Weigel & Oka, 1982). A pre-existing heterogeneity among hepatocytes cannot be ruled out, and may perhaps be linked to their position in the lobule (Hardonk & Scholtens, 1980).

As far as embryonic development is concerned, in the experiments presented here the presence of an ASG receptor could be demonstrated by electron microscopic cytochemistry of ASF-HRP on freshly isolated foetal rat hepatocytes after the 17th day of intrauterine life; cells isolated from younger embryos do not show in fact any surface reaction even after incubation in a medium containing the highest concentration of ASF-HRP and after 30 min of incubation.

These data seem to indicate a rather late appearance on the hepatocyte surface of the galactose-binding capacity to which the circulating ASG clearance function, typical of adult mammalian liver, may be related.

Some general aspects of receptor ontogenesis have been extensively reviewed in recent papers (Csaba, 1981), in which it has been recalled that receptor maturation seems to represent one of the key events in cell-membrane differentiation; the ontogenetic patterns of specific receptors may however be quite different in the same cell type. The glucagon-binding capacity of embryonic rat liver cells is only 1 % relative to adult liver at 15 days of prenatal life, and still not more than 23 % at the birth (Blasquez *et al.* 1976). Insulin-binding capacity of embryonic hepatocyte membrane, on the other hand, has been found to be less, similar to, or even greater than, that of adult cells (Blasquez *et al.* 1976; Neufeld, Scott & Kaplan, 1980; Autuori *et al.* 1981; Vinicor & Kiedrowski, 1982).

The absence of a clearance mechanism of circulating ASG in the liver of early foetuses, which is suggested by our results, may be related either to the absence in the early embryonic metabolism of extensive desialization processes of plasma glycoproteins, which become operative only after the 17th day of development, or to the fact that the removal of these modified proteins could be carried out by placental tissues.

A point which remains to be investigated is the route of internalization and the degradative pathway of ASG after the appearance of binding capacity, i.e. in the last days of intrauterine life, when the lysosomal system of hepatocytes has not yet reached the functional and morphological development characteristic of the adult cells (Ciofi-Luzzatto, 1981).

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