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ATTACHMENT OF RAT HEPATOCYTES TO PLASTIC SUBSTRATA IN THE ABSENCE OF SERUM REQUIRES PROTEIN SYNTHESIS

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SUMMARY Rat hepatocytes attach within 1 hour to plastic petri dishes if foetal calf serum is added to the culture medium. In the absence of serum rat liver cells attach only after a lag phase of 4 hours. However hepatocyte monolayers are formed under either culture condition demonstrating that hepatocyte culture does not have an absolute requirement for foetal calf serum. Cycloheximide is without effect on cell attachment in serum containing medium which is compatible with the concept that attachment is mediated by a "cold insoluble globulin" present in foetal calf serum. In contrast cycloheximide markedly inhibits the attachment of hepatocytes cultured without serum suggesting that rat liver cells are capable of making their own "attachment" factor. This factor appears to be released into the medium and has a similar sensitivity to heat and acid treatment as the "attachment" protein present in foetal calf serum.

INTRODUCTION

In a systematic study of the basic requirements for hepatocyte attachment, Seglen and Fossa (1) concluded that hepatocytes do not attach readily to untreated polystyrene petri dishes in the absence of foetal calf serum (FCS).

These results could be misinterpreted suggesting that the culture of rat liver cells in media without FCS does not result in monolayer formation. This interpretation would be incompatible with findings of other laboratories (2), (3), as well as our own (4) which show that monolayers are consistently produced in the absence of serum.

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Abbreviations HBSS = Hank's balanced salt solution $-Ca^{2+}$ $-Mg^{2+}$
FCS = Foetal calf serum

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In order to clarify these apparently contradictory observations we have examined the mechanism of attachment of rat liver parenchymal cells cultured in serum free and serum containing media.

MATERIALS AND METHODS

Isolation and culture of hepatocytes Isolated hepatocytes were prepared from the livers of 150-200 g male rats of the Porton derived Wistar strain by perfusion with 0.05% v/v collagenase (Worthington Type II, supplied by Cambrian Chemicals, Croydon, Surrey, U.K.) in HBSS* containing 5 mM $CaCl_2$, essentially as described by Seglen (5). Viability was always 80-90% as determined by trypan blue exclusion. Cells were washed and resuspended in Williams medium E (Flow Laboratories, Irvine, Scotland, U.K.) at a density of 0.9-1 x 10^6 cells/ml and incubated in 60 mm culture dishes (Lux Scientific, Newbury Park, California, U.S.A.) under a humidified atmosphere of 5% CO_2 in air. When foetal calf serum (Seralab, Crawley Down, Sussex, U.K.) was added, the final concentration was 10% v/v.

Measurement of attachment Attachment was measured essentially as described by Ballard and Tomkins (6). After incubation the dishes were shaken on a platform (Minishaker, A. Kühner A.G., Basel, Switzerland) at an axial rotation (diameter = 1 cm) of 100 rpm for one minute. The turbidity of the medium containing non-attached cells was measured at 650 nm (Unicam S.P. 500, 1 cm light path). Absorbance at this wavelength was not affected by the pH indicator in the medium (phenol red) over the range of pH 1-12 and was a linear function of cell concentration up to an absorbance of 1.0. Attachment is expressed, according to Seglen and Fossa (1) as a percentage of the number of cells in the medium at zero time.

The method of measuring cell attachment by turbidity was correlated (r = 0.979) with attachment as determined by measuring the dry weight of unattached cells after filtering culture medium through Whatman GF/A glass fibre filters.

RESULTS AND DISCUSSION

Fig.1 shows that rat hepatocytes cultured in media containing 10% (v/v) foetal calf serum (FCS) attach to plastic petri dishes within 60 minutes. In agreement with Seglen and Fossa (1) the exact time for the attachment to be essentially complete varies between cell preparations but within 1 hour about 80% of all cells are attached. In contrast hepatocytes from the same preparation cultured without serum show very little attachment after one hour (Fig.1). However, after 4 hours incubation these cells attain the same level of attachment as cells cultured in medium containing serum. In spite of the delayed

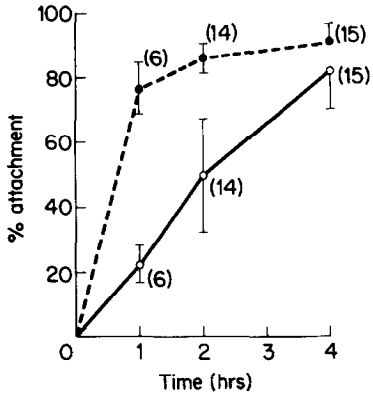

Fig.1. Time course of attachment of rat hepatocytes cultured without serum (O——O) or with 10% (v/v) FCS (●——●) to plastic petri dishes. Results are given as mean ± s.d. and n, shown in parenthesis, represents the number of experiments with hepatocytes prepared from different rat livers.

attachment, hepatocytes cultured without FCS form parenchymal cell monolayers which have a similar appearance as cells cultured in the presence of serum (Fig. 2A and B).

It is known that the attachment of cells to culture dishes is mediated by a protein present in FCS known as "cold insoluble globulin" or fibronectin (1,7). Therefore, a possible explanation for the slow rate of attachment of hepatocytes cultured without serum could be a requirement for these hepatocytes to synthesise a similar protein before attachment is achieved. Indeed, Table 1 shows that culture of cells in a medium containing 3 μM cycloheximide inhibits the attachment of hepatocytes cultured in a medium without FCS. Cycloheximide is without effect on the attachment of hepatocytes cultured in the presence of serum (Table 1). 3 μM Cycloheximide was found to inhibit protein synthesis in these cultures by 90% in the absence as well as in the presence of FCS.

However, it is interesting to note here, that hepatocytes cultured in a medium containing 10% serum plus 3 μM cycloheximide do not spread (i.e. flatten) to form a confluent monolayer after 24 hours culture. At this time the cells appear much like control hepatocytes

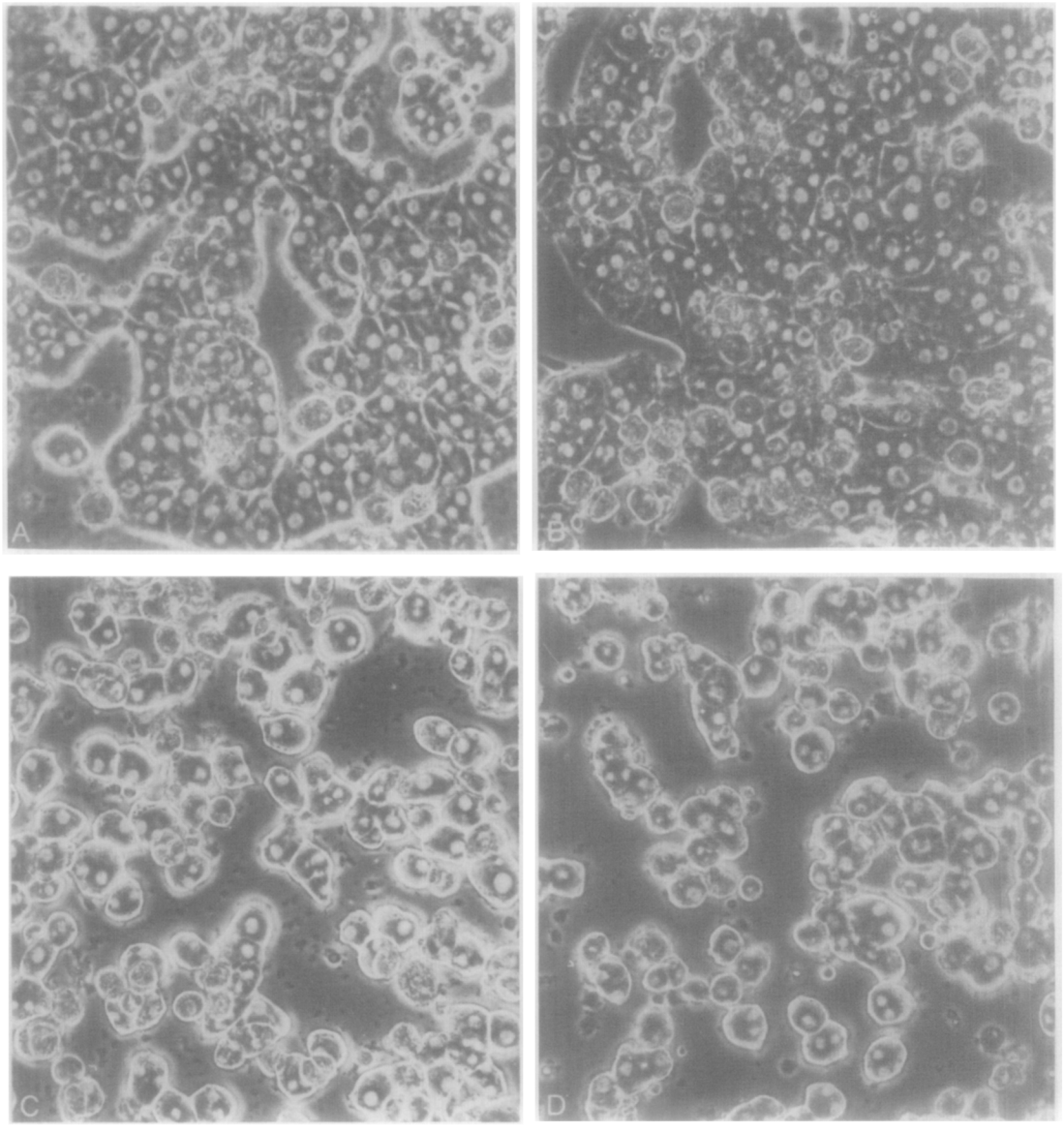

Fig.2. Appearance of rat hepatocyte cultures: effect of cycloheximide and FCS. Phase contrast
A: 24 hours, in medium without serum
B: 24 hours, in medium containing 10% (v/v) FCS
C: 24 hours, in medium containing FCS and 3μM cycloheximide
D: 4 hours, in medium without serum.

after 4 hours of culture (compare Fig. 2A with 2C and 2D). Apparently protein synthesis is required for spreading and monolayer formation, possibly involving an association of intracellular actin with cold insoluble globulin or fibronectin (8).

TABLE 1 Effect of cycloheximide and medium treatment on the attachment of rat hepatocytes

	- Treatment	+ Cycloheximide (3μM)	Acid treatment	Heat treatment
Medium - serum	35 ± 18 (6)	15 ± 8 (6)	47.7	44.5
Medium + FCS	83 ± 6* (6)	79 ± 9* (6)	55.8	41.3
"Conditioned medium"	75 ± 7* (4)	68 ± 8* (4)	52.8	41.1

Rat hepatocytes were isolated and cultured in Williams medium E ± FCS and attachment was measured after 2 hours as described in the methods section. "Conditioned medium" was prepared by culturing hepatocytes in Williams medium E - FCS for 24 hours. This medium was then centrifuged at 3000 g for 10 min before use. Medium was heat-treated at 70°C for 10 min. Acid-treated medium was prepared by adding $HClO_4$ to final concn. of 0.3 M. After 24 hours at 4°C this medium was neutralized with 3 M KOH and the buffer capacity was restored by addition of 0.9 M $NaHCO_3$ to a final concn. of 15 mM. Potassium perchlorate crystals in this medium were removed by centrifugation at 3000 g for 10 min.
Results are given as the mean ± s.d. and n, shown in parenthesis, represents the number of experiments with hepatocytes prepared from different rat livers. Results of acid and heat treatment are the average of separate experiments with cells prepared from 2 rat livers. Variation between these experiments did not exceed 10%.

* indicates significantly different from medium - serum ($P < 0.05$)

In addition Table 1 provides evidence for the "attachment" - protein to be released into the medium. This is shown by the ability of medium without FCS, but "conditioned" by incubation with cells for 24 hours, to promote attachment of freshly isolated hepatocytes to the same extent as medium containing FCS (Table 1). The attachment promoting activity of "conditioned" medium is not inhibited by cycloheximide, in the same way as medium containing serum (Table 1).

Although attachment occurs in "conditioned" medium here too, cycloheximide prevents spreading and monolayer formation and cells have the same appearance as those in Fig. 2C. In contrast, hepatocytes cultured in normal serum free medium containing cycloheximide never attach and appear as floating dead cells after 24 hours culture.

The attachment promoting activity of conditioned medium has the same characteristics as that present in serum with regard to the sensitivity to heat and acid treatment (Table 1).

These findings suggest that rat hepatocytes cultured in serum free medium attach to plastic substrata via a protein, which is synthesised and released into the medium by the cells themselves. Since protein synthesis is required for attachment, the higher level of attachment than that reported by Seglen and Fossa (1) in serum free medium is likely to be due to incubation in a medium containing amino acids rather than in a simple salt solution. Although epithelial cells (i.e. hepatocytes) are not generally accepted to synthesise fibronectin (9) the present work suggests that these cells can synthesise a similar protein with regard to its attachment promoting activity, as well as its sensitivity to heat and acid treatment.

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