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Acute-phase-response induction in rat hepatocytes co-cultured with rat liver epithelial cells

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The response of rat hepatocytes co-cultured with rat liver epithelial cells to conditioned medium (CM) from lipopolysaccharide (LPS)-activated monocytes was investigated by measuring the concentration of α_2-macroglobulin (α_2M), α_1-acid glycoprotein (AGP), albumin and transferrin, as well as the changes in glycosylation of α_1-acid glycoprotein. During an initial 8-day treatment with CM, concentrations of α_2M and AGP increased markedly over those of control culture, whereas concentrations of albumin and transferrin decreased. The glycosylation pattern of AGP indicated an important relative increase of the concanavalin A-strongly-reactive (SR) variant upon treatment. When CM addition to hepatocyte culture medium was stopped, the concentrations of the four proteins and the glycosylation pattern of AGP reverted to those of control cultures. Further addition (on day 15) to cultures of CM increased the concentration of α_2M and decreased albumin and transferrin concentrations. Although AGP concentrations did not increase above those of controls, the appearance of the SR variant was again stimulated by CM. These results show that, in co-culture, rat hepatocytes remain able to respond to repeated inflammatory stimuli.

INTRODUCTION

A major feature of the mammalian acute-phase response is the increase in hepatic synthesis of a set of plasma proteins termed 'acute-phase reactants'. This response is mediated by hepatocyte stimulating factors (HSFs) released into the circulation after tissue damage [1,2]. The action of HSFs has been extensively studied in pure cultures of rat hepatocytes [2], and it has been recently demonstrated that the major hepatocyte stimulating activity present in conditioned medium from lipopolysaccharide (LPS)-stimulated human monocytes was a cytokine identical with interferon β_2, β-lymphocyte stimulating factor, 26 kDa protein, interleukin HP-1 and HSF-1 [3–5] and which has been called 'IL-6' [6]. However, the response of pure hepatocyte culture to cytokines *in vitro* cannot be related to the liver acute-phase response *in vivo*, since hepatocytes in primary culture are in a stimulated state [7]. This is supported by the fact that even the highest doses of cytokines fail to reproduce the full extent of the response *in vivo*, and there are differences in the pattern of plasma proteins stimulated *in vitro* [2].

The reason for the failure of pure hepatocyte cultures to reproduce exactly the response *in vivo* is unknown, but may be due to the absence of additional regulatory factors in cytokine preparations or the instability of the hepatocyte phenotype in pure culture [8]. Rat hepatocytes in pure culture rapidly lose their functional differentiation and revert to a more foetal phenotype [9] and are therefore not typical of the rat liver cell *in vivo*. Recently a method of co-culturing hepatocytes with untransformed rat epithelial cells of biliary origin has been described [10], which allows long-term maintenance of hepatocyte-specific functions [11], including the synthesis of acute-phase reactants [7,12].

The aim of the present study was to investigate the action of conditioned medium (CM) from LPS-activated monocytes upon rat hepatocyte co-cultures. The response of the cells to the CM was monitored by measuring the concentrations of α_2-macroglobulin (α_2M), α_1-acid glycoprotein (AGP), albumin (Alb) and transferrin (Tf). The effects of this CM on the glycosylation pattern of AGP were also investigated by crossed immunoaffinity electrophoresis (CIAE) with concanavalin A (Con A), since it has been previously shown that the microheterogeneity of this glycoprotein changes significantly during the acute-phase response *in vivo* [13].

METHODS

Rat hepatocytes were obtained by the two-step collagenase-perfusion method of Guguen-Guillouzo & Guillouzo [14] and were co-cultured with rat liver epithelial cells as described in [10]. Hepatocytes (2×10^6) in 2 ml of Hams F-12 medium with insulin (10 μg/ml) and 5% (v/v) foetal-calf serum were seeded in 25 cm^2 flasks. After 3 h the medium was removed and 2×10^6 rat liver epithelial cells, from early passages, prior to spontaneous

Abbreviations used: CM, conditioned medium from lipopolysaccharide (LPS)-activated monocytes; AGP, α_1-acid glycoprotein; α_2M, α_2-macroglobulin; HSF, hepatocyte stimulating factor; LPS, bacterial lipopolysaccharide; CIAE, crossed immunoaffinity electrophoresis; Con A, concanavalin A; SR, Con A-strongly-reactive; NR, Con A-non-reactive; Alb, albumin; Tf, transferrin; PBS, phosphate-buffered saline; CRP, C-reactive protein.

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transformation, were added. Confluency was achieved within 24 h, and the above medium, supplemented with 3.5×10^{-6} M-cortisol hemisuccinate, was changed daily.

Human peripheral-blood monocytes were separated by buoyant-density centrifugation, purified by adherence and stimulated for 24 h with chromatographically purified LPS (10 μg/ml) from *Salmonella minnesota* (Sigma Chemical Co., St. Louis, MO, U.S.A.). The CM was dialysed against phosphate-buffered saline (PBS; 0.15 M-NaCl/5 mM-phosphate, pH 7.2) and sterilized by filtration through a 0.22 μm-pore-size membrane. This CM, at 25 and 10% (v/v), was added to the confluent co-cultures from day 1 to day 8, after which addition of CM was stopped until day 15 and then resumed until day 20. One set of controls was included, which consisted of cultures to which identical volumes of sterile PBS were added. All cultures were performed in triplicate, and medium was collected daily and stored at −30 °C.

Alb and Tf were measured in culture medium by using laser nephelometry. α_2M and AGP were determined by electroimmunoassay as described previously [7]. The microheterogeneity of AGP was checked by CIAE as described by Bog-Hansen *et al.* [15] and Nicollet *et al.* [16]. Briefly, Con A in 1 mM-$CaCl_2$/1 mM-$MnCl_2$/1 mM-$MgCl_2$ was added to the first-dimension gel to a final concentration of 1.5 mg/ml. Electrophoresis of each sample (15 μl of a 20-fold-concentrated cell culture supernatant) was performed in 1%-agarose gel for 1.5 h at 10 V/cm in a 50 mM-Tris/20 mM-barbital buffer, pH 8.6. The second-dimension electrophoresis was carried out at 2 V/cm for 18–20 h in 1%-agarose gels containing 100 μl of rabbit antiserum against rat AGP and 1-*O*-methyl D-glucopyranoside (40 mg/ml). After staining with Coomassie Blue R-250 the areas under the immunoprecipitate curves were determined by planimetry. By using this method, four microheterogeneity variants were characterized: variant 1, strongly reactive (SR), which is precipitated with Con A in the first dimension and eluted by 1-*O*-methyl D-glucopyranoside; variant 2, reactive with Con A; variant 3, weakly reactive; and variant 4, which is Con A-non-reactive (NR).

RESULTS

Morphological changes were observed in hepatocyte co-cultures treated with CM. In PBS-treated controls the characteristic hepatocyte co-culture morphology was observed with extensive bile canaliculi formation. Within 8 h of CM addition, bile canaliculi began to regress and were not evident after 2 days treatment. Furthermore the hepatocytes assumed a more elongated form reminiscent of hepatocytes in pure culture on plastic, although throughout treatment with CM contacts with epithelial cells were maintained. When CM addition was stopped, the hepatocytes had resumed their original co-culture morphology, with numerous bile canaliculi, within 2 days.

α_2M was detected in control cultures treated with 0.5 and 0.2 ml of PBS from days 6–8 and was present at a concentration not exceeding 10 μg/ml throughout the culture period. In cultures treated with CM, whether at 25 or at 10%, α_2M was detected on day 2, and concentrations increased to greater than 60 μg/ml until day 8, when addition of CM was stopped (Fig. 1). Thereafter levels of α_2M decreased and had returned to those of control cultures by day 13. Re-addition of CM on day 15 caused a rapid increase of α_2M, although, in contrast with the first stimulation, two peaks were observed. α_2M concentrations increased to greater than 80 μg/ml during the second stimulation with CM and decreased rapidly when addition was stopped on day 20.

Addition of CM to co-cultures decreased the concentrations of Alb (Fig. 2) and Tf (Fig. 3), whether added at day 1 or day 15. Compared with their respective PBS-treated controls, CM at 25 or 10% decreased Alb concentrations by 75% and those of Tf by 25–50%. Upon cessation of CM addition Alb and Tf concentrations increased and reached those of control cultures within 4 days. Alb and Tf concentrations declined within

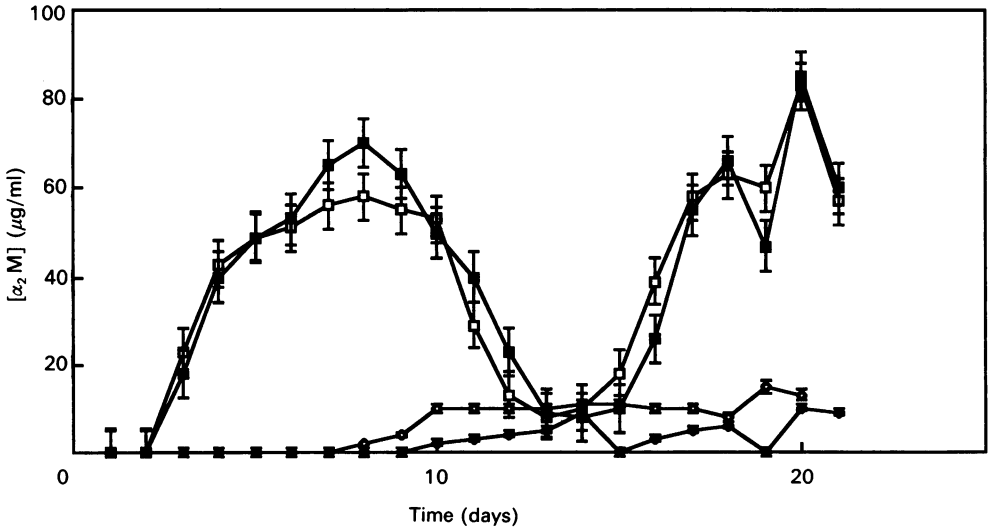

Fig. 1. Concentration of α_2M in medium obtained daily from hepatocyte co-culture

Each point is the mean result for triplicate cultures. Cultures were treated on days 1–8 and 15–20 with 200 μl (■) and 500 μl (□) of CM respectively or 200 μl (◆) and 500 μl (◇) of PBS respectively.

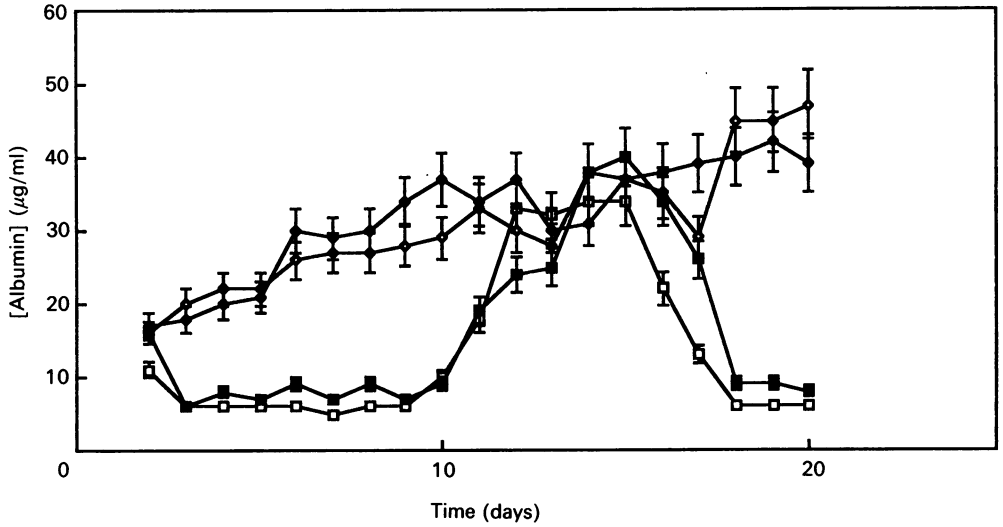

Fig. 2. Concentrations of albumin in co-cultured hepatocytes treated with CM or PBS

Each point is the mean result for triplicate cultures. Cultures were treated on days 1–8 and 15–20 with 200 μl (■) and 500 μl (□) of CM respectively or 200 μl (◆) and 500 μl (◆) of PBS respectively.

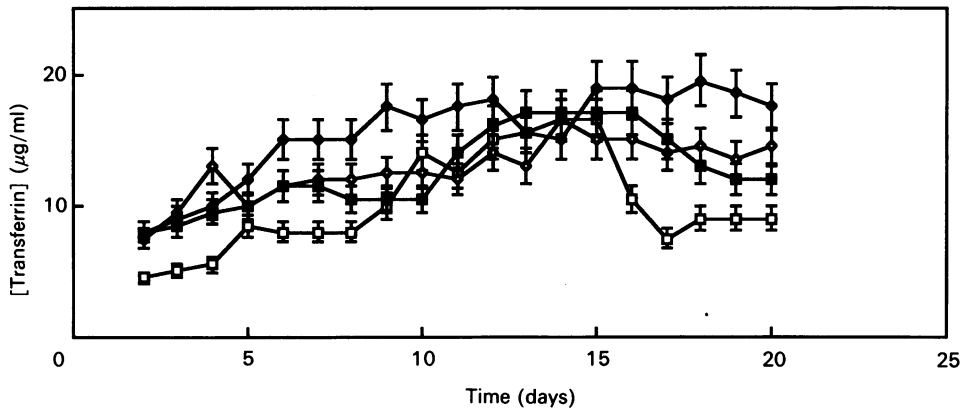

Fig. 3. Concentrations of Tf in co-cultured hepatocytes treated with CM or PBS

Each point is the mean result for triplicate cultures. Cultures were treated on days 1–8 and 15–20 with 200 μl (■) and 500 μl (□) of CM respectively or 200 μl (◆) and 500 μl (◆) of PBS respectively.

24 h after resumption of CM addition on day 15, and the magnitude of the decreases were similar to those obtained when the CM was added between days 1 and 8.

In control cultures four variants of AGP were detected by CIAE (Fig. 4), and the concentration increased throughout the culture period (Fig. 5). From day 2 until day 20 the concentration of AGP increased from 2 to 20 μg/ml. Addition of CM at 25 or 10% after 24 h of culture caused a 2-fold increase in AGP, and, although addition of CM was stopped on day 8, the concentration of AGP did not decrease until day 10. Thereafter the concentration of AGP was comparable with that of control cultures, despite the resumption of CM addition on day 15.

The four AGP variants identified in control culture medium arise because of different reactivities with Con A (Fig. 4*a*). Their number and relative distribution were identical with those observed for AGP in the non-inflamed rat [13]. In control cultures from day 4 to day 20 these four variants were observed, with the NR variant present in higher quantities than the SR variant (Figs. 4*a* and 5*a*). Addition of CM on days 1–8 resulted in a substantial increase in the SR variant and the disappearance of the NR variant (Figs. 4*b* and 5*b*). When CM was stopped on day 8, the relative amount of SR variant decreased, with a concomitant rise in the NR variant, although the control-culture levels were not achieved (Fig. 5*b*). A new addition of CM to culture medium on day 15 caused the disappearance of the NR variant and resurgence of the SR form of AGP. This last modification of the AGP glycosylation pattern was not related to an increase in AGP concentration, since the level in stimulated co-culture was similar to that of the controls during this period.

DISCUSSION

Our observations clearly demonstrate for the first time the ability of rat hepatocytes, when co-cultured with rat liver epithelial cells of biliary origin, to respond to CM

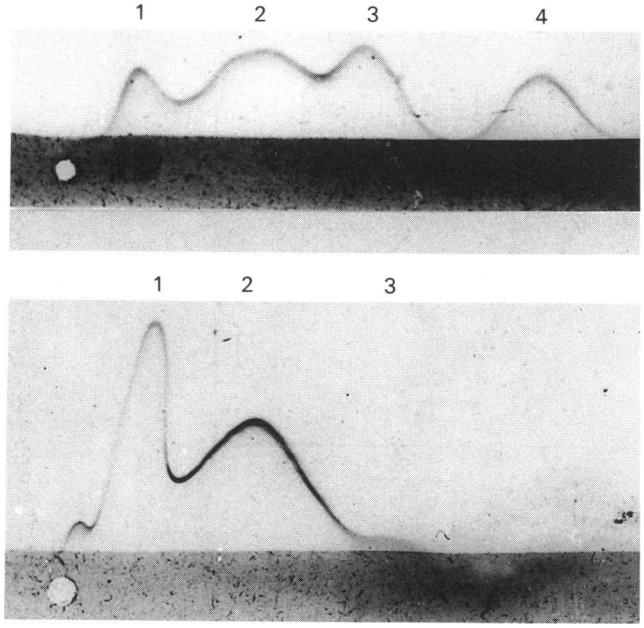

Fig. 4. CIAE patterns of AGP as present in media of monolayers of rat hepatocyte co-culture

1, 2 and 3, peak positions of AGP SR variants; 4, that of the NR variant. (*a*) CIAE pattern at day 12 of co-culture, 4 days after stopping the addition of CM. (*b*) CIAE pattern at day 20 of culture in the presence of CM.

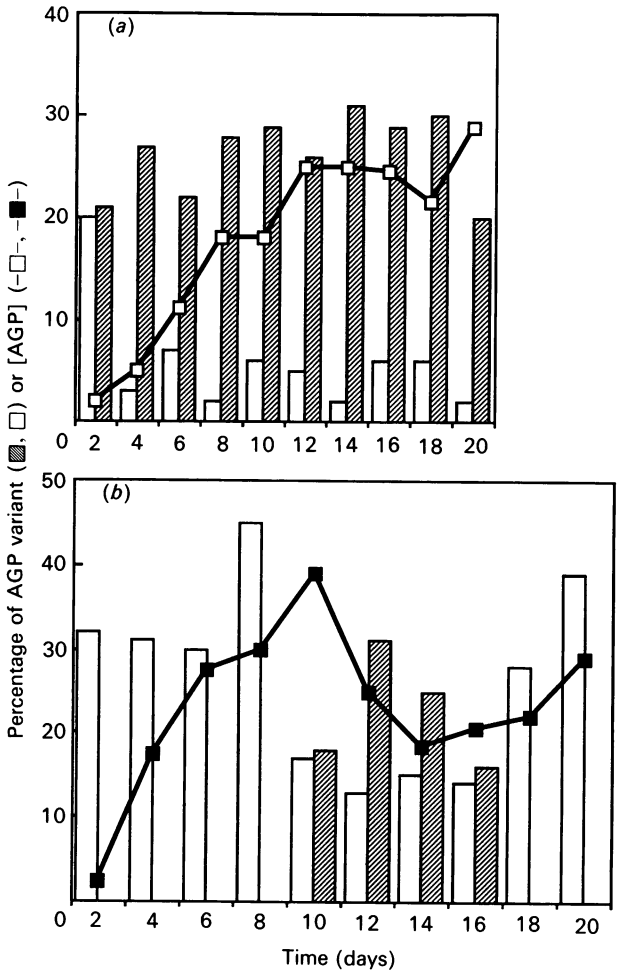

Fig. 5. Concentrations of AGP in medium obtained daily from hepatocyte co-culture

Each point is the mean result for triplicate cultures; cultures were treated on days 1–8 and 15–20 with 500 μl of PBS (*a*) or with 500 μl of CM (*b*). Histograms represent the percentages of the NR variant of AGP (▨) and the SR variant of AGP (□). The overall AGP concentration (μg/ml) is also shown (-□- in *a*; -■- in *b*).

from LPS-activated monocytes with increased synthesis of α_2M and a concomitant decrease in Alb and Tf production. This responsiveness was not lost when CM addition was stopped for 7 days and resumed on day 15 of culture. In parallel the glycosylation pattern of AGP was affected, resulting in an increase in the Con A SR variant and a decrease in the Con A NR variant. Such alterations are comparable with the inflammatory response *in vivo* [1,16].

During the initial 8 days of stimulation the concentrations of α_2M and AGP were increased 7–10-fold and 2-fold respectively. Alb synthesis was strongly depressed, with a less severe decrease in the concentration of Tf. When CM addition was stopped, the concentrations of the four proteins returned to those of the control cultures, as did the glycosylation pattern of AGP. Resumption of CM treatment produced changes only in α_2M, Alb and Tf. However, although the concentration of AGP failed to rise above control values, which increased throughout the culture period, the pattern of AGP glycosylation variants was modified as in the first stimulation. It has been previously shown that the epithelial cells do not participate in the production of plasma proteins in the co-culture system [7,10], and it can be assumed that it is the hepatocytes which are responding to the cytokines present in the conditioned medium.

In control cultures α_2M was present during the culture period despite the fact that this protein is virtually absent from normal rat serum [17]. The production of α_2M in control cultures has been previously observed [7] and can be attributed to the presence of cortisol in the culture medium inducing α_2M synthesis [18]. Cortisol has also been shown to stimulate AGP production [18], and it can be assumed that it is responsible for the increasing levels of AGP in control culture. The presence of cortisol *in vitro* is necessary for the hepatocytes to respond to cytokines [8].

The second stimulation of co-cultures by CM differed from the initial stimulation in two respects. When co-cultures were treated with conditioned medium on days 15–20, a double peak was observed for α_2M, and there was no detectable increase in AGP concentration. The appearance of two peaks for α_2M may possibly be due to a stabilization of the mRNA or to a recycling or induction of receptors for HSFs. However, in the case of recycling of receptors it has been shown by Macintyre *et al.* [19] that repeated inflammatory stimuli in the rabbit results in a diminishing C-reactive protein (CRP) response. Thus, after a series of seven biweekly turpentine injections, the CRP response progressively decreased such that after the fifth and seventh injections, occurring only 3 days after the previous injections, there was no CRP response. This effect was attributed to receptor down-regulation. Alternatively the two peaks of α_2M

could be explained by contaminating production of cytokines by the epithelial cells, although the rapid decrease in α_2M concentration upon cessation of CM addition argues against this hypothesis. The failure to detect an increase in AGP concentrations during the second period of CM addition may be due to the high levels of AGP in control cultures at this time, masking any cytokine-mediated increase.

During the first 2 days of culture the SR and NR variants were detected in equal amounts in control cultures, but by day 4 the relative proportion of the SR variant had decreased to a minimal value. This initially high proportion of the SR variant may reflect the adaptation of the hepatocytes to the culture environment. Treatment of the cultures with CM, whether started at day 1 or day 15, effectively decreased the NR variant to neglible levels and raised the proportion of the SR variant. When CM addition was stopped between days 9 and 14 the proportion of the SR variant decreased, with a concomitant increase in the NR variant. Despite the failure to detect an increase in AGP concentration during the second stimulation with CM, the glycosylation pattern changed to that of the acute-phase response, which suggests that different factors might control these responses. A lack of correlation between AGP concentrations and the glycosylation pattern has also been observed in humans in various disease states [20]. Further, by using the human hepatoma cell line Hep 3B it has been shown that, although CM altered the glycosylation pattern of caeruloplasmin and proteinase inhibitor, interleukin-1, tumour necrosis factor and HSF-1 were not responsible for these alterations [21]. The mechanism by which glycosylation is altered by acute-phase-response mediators is unknown, but it is possible that the effect is controlled via the glycosylating enzymes [21].

It has been shown both *in vivo* and *in vitro* that HSFs mediate the liver-cell response by increasing the amount of mRNA for the acute-phase proteins [2,8,22,23]. In the case of α_2M *in vivo* and AGP *in vitro* this increase in mRNA has been shown to arise from an enhanced nuclear transcription rate [8,24]. However, in the case of α_2M, although the transcription rate of the α_2M gene is increased during inflammation, the increase is insufficient to account for the rise in cytoplasmic α_2M RNA, and other factors, such as intranuclear processing of α_2M transcripts, must also be altered [25]. In pure culture the transcriptional activity of hepatocyte-specific genes remains very low, even in hormonally defined medium [26], and it is probable that this, plus the rapid loss of the hepatic phenotype in pure culture, is responsible for the differences between the response to cytokines *in vitro* and *in vivo* [8]. In co-culture it has been shown that transcriptional activity is well maintained [27], and, since the hepatocyte response to cytokines involves, at least in part, the activation of transcription of specific genes, co-culture of hepatocytes may provide an excellent system for the study of cytokine–hepatocyte interactions, particularly the cellular mechanisms responsible for the increase in acute-phase-protein synthesis. Certainly the 10-fold maximum increase in α_2M concentration and the 2-fold increase in AGP are higher than those reported previously for pure rat hepatocyte culture [4,28].

The results obtained in the present study have shown that rat hepatocytes maintained in co-culture respond to cytokines. This gives a further demonstration of the ability of co-culture in maintaining the differentiated functional state of hepatocytes. Pure cultures of rat hepatocytes have been used extensively in studying the action of cytokines, but suffer from the disadvantage that they can only be maintained in culture for a short period and that they are physiologically and phenotypically unstable. In co-culture the hepatocyte differentiation is maintained longer, as is the responsiveness to CM, and this, consequently, may be of use in further investigations of the action of HSFs on liver cells.

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REFERENCES

1. Kushner, I. (1982) Ann. N.Y. Acad. Sci. **389**, 39–48
2. Koj, A. (1985) in The Acute Phase Response to Injury and Infection (Gordon, A. H. & Koj, A., eds.), pp. 73–131, Elsevier Science Publishers B.V. (Biomedical Division), Amsterdam
3. Andus, T., Geiger, T., Hirano, T., Northoff, H., Ganter, U., Bauer, J., Kishimoto, T. & Heinrich, P. C. (1987) FEBS Lett. **221**, 18–22
4. Gauldie, J., Richards, C., Harnish, D., Lansdorp, P. & Baumann, H. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7251–7255
5. Poupart, P., Vandenabeele, P., Cayphas, S., Van Snick, J., Hafeman, G., Kruys, V., Fiers, W. & Content, J. (1987) EMBO J. **6**, 1219–1224
6. Vandamme, J., Cayphas, S., Van Snick, J., Conings, R., Put, W., Lednaerts, J. P., Simpson, R. J. & Billiau, A. (1987) Eur. J. Biochem. **168**, 543–550
7. Guillouzo, A., Delers, F., Clement, B., Bernard, N. & Engler, R. (1984) Biochem. Biophys. Res. Commun. **120**, 311–317
8. Baumann, H., Hill, R. E., Sauder, D. N. & Jahreis, G. P. (1986) J. Cell Biol. **102**, 370–383
9. Guguen-Guillouzo, C. & Guillouzo, A. (1983) Mol. Cell. Biochem. **53/54**, 35–56
10. Guguen-Guillouzo, C., Clement, B., Baffet, G., Beaumont, C., Morel-Chany, E., Glaise, D. & Guillouzo, A. (1983) Exp. Cell Res. **143**, 47–54
11. Guguen-Guillouzo, C. (1986) in Isolated and Cultured Hepatocytes (Guillouzo, A. & Guguen Guillouzo, C., eds.), pp. 259–283, John Libbey Eurotext, London/INSERM, Paris
12. Lebreton, J. P., Daveau, M., Hiron, M., Fontaine, M., Biou, D., Gilbert, D. & Guguen-Guillouzo, C. (1986) Biochem. J. **235**, 421–427
13. Dairra-Mehrpour, M., Bourguignon, J., Leroux-Nicollet, I., Marko-Vercaigne, D., Biou, D., Hiron, M. & Lebreton, J. P. (1985) Biochem. J. **225**, 681–687
14. Guguen-Guillouzo, C. & Guillouzo, A. (1986) in Isolated and Cultured Hepatocytes (Guillouzo, A. & Guguen-Guillouzo, C., eds.), pp. 1–12, John Libbey Eurotext, London/INSERM, Paris
15. Bog-Hansen, T. G., Prahl, P. & Lowenstein, M. (1978) J. Immunol. Methods **22**, 293–307
16. Nicollet, I., Lebreton, J. P., Fontaine, M. & Hiron, M. (1981) Biochim. Biophys. Acta **668**, 235–245
17. Koj, A. (1974) in Structure and Function of Plasma Protein (Allison, A. C., ed.), pp. 73–131, Plenum Press, London
18. Gross, V., Andus, T., Tran-Thi, T.-A., Bauer, J., Decker, K. & Heinrich, P. C. (1984) Exp. Cell Res. **151**, 46–54

19. Macintyre, S. S., Schultz, D. & Kushner, I. (1982) Ann. N.Y. Acad. Sci. **389**, 76–87
20. Mackiewicz, A., Marcinkowska-Pieta, R., Ballou, S., Mackiewicz, S. & Kushner, I. (1987) Arthritis Rheum. **30**, 513–518
21. Mackiewicz, A., Ganapathi, M. K., Schultz, D. & Kushner, I. (1987) J. Exp. Med. **166**, 253–259
22. Northemann, W., Andus, T., Gross, V., Nagashima, M., Schreiber, G. & Heinrich, P. C. (1983) FEBS Lett. **161**, 319–322
23. Baumann, H., Jahreis, G. P., Sauder, D. N. & Koj, A. (1984) J. Biol. Chem. **259**, 7331–7342
24. Northemann, W., Heisig, M., Kunz, D. & Heinrich, P. C. (1985) J. Biol. Chem. **260**, 6200–6205
25. Fey, G. H., Gehring, M. R., Shiels, B. R., De Bruijn, M. H. L., Chain, A. C., Kan, C. C., Noonan, D. J., Ueberberg, H. & Northemann, W. (1986) Protides Biol. Fluids Proc. Colloq. **34**, 255–258
26. Jefferson, D. M., Clayton, D. F., Darnell, J. E. & Reid, L. M. (1984) Mol. Cell. Biol. **4**, 1929–1934
27. Fraslin, J. M., Kneip, B., Vaulont, S., Glaise, D., Munnich, A. & Guguen-Guillouzo, C. (1985) EMBO J. **4**, 2487–2491
28. Koj, A., Gauldie, J., Regoeczi, E., Sauder, D. N. & Sweeney, A. D. (1984) Biochem. J. **224**, 505–514

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