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The human hepatoma Hep3B cell line as an experimental model in the study of the long-term regulation of acute-phase proteins by cytokines

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The regulation of the synthesis by the cytokines interleukin-1 (IL-1) and IL-6 of the positive acute-phase protein α_1-acid glycoprotein (AGP) and of the negative acute-phase protein α_2-HS glycoprotein (AHSG) has been studied in a long-term culture system of the human hepatoma cell line Hep3B. The culture system contained 30 nM-sodium selenite as the only supplement. This allowed maintenance of the synthesis of the proteins under study at a near steady state for over 3 months. An increase in AGP mRNA and a decrease in AHSG mRNA were observed when cells were treated for two successive 48 h-periods with monocyte-conditioned medium. A return to basal levels was obtained after cessation of the cytokine addition. Two further additions of cytokines led to alterations in mRNA levels similar to those observed following the first cytokine treatment. The amounts of AGP and AHSG secreted were altered in accordance with the mRNA modifications. These results suggest that new cytokine receptors were being constantly synthesized during cell culture. When cytokines were present in the culture medium for 10 days, maximum alterations in AGP and AHSG synthesis were obtained following 2 and 4 days of treatment respectively, but further alterations in protein levels could not be observed afterwards. Expression of IL-6 receptor mRNA was not up-regulated by cytokines, but only by 1 μM-dexamethasone. Our results show that, in this long-term culture system, cytokines induce a response in hepatoma cells similar to that observed *in vivo* during human inflammatory states. This model could be used to evaluate the effects of agonists or antagonists of cytokines responsible for the hepatic acute-phase protein response.

INTRODUCTION

The host response to inflammation is characterized by a dramatic change in the synthesis of a subset of proteins secreted by the liver, known as the acute-phase reactants. The plasma levels of proteins such as α_1-acid glycoprotein (AGP), fibrinogen and C-reactive protein increase in response to acute inflammation, and they are known as positive acute-phase proteins. On the other hand, plasma levels of proteins such as albumin, transthyretin and α_2-HS glycoprotein (AHSG) decrease, and these are known as negative acute-phase proteins (Koj, 1974; Kushner, 1982). The cytokine interleukin-6 (IL-6) has been identified as the major hepatocyte-stimulating factor accounting for the observed changes in acute-phase plasma proteins (Gauldie *et al.*, 1987; Andus *et al.*, 1987), although an optimal acute-phase response requires a combination of at least IL-1, IL-6 and glucocorticoids in hepatocytes and in hepatoma cell cultures (for a review, see Baumann & Gauldie, 1990). However, owing to the fact that hepatocytes maintained under standard tissue culture conditions are very short-lived and that the proliferative capacity of hepatoma cells is important, the hepatocyte-specific expression of genes coding for the plasma proteins during a prolonged exposure of hepatic cells to cytokines has not been studied. Thus, so far, most of the data concerning the regulating activities of purified cytokines and of cytokines contained in monocyte-conditioned media have been obtained following 12–72 h exposure of hepatic cells to cytokines (Koj *et al.*, 1984; Baumann *et al.*, 1987; Andus *et al.*, 1988; Castell *et al.*, 1988).

In the present study we have used a culture system that results in a decrease in the growth of the human hepatoma cell line Hep3B and which permits a steady state of protein synthesis to be maintained for over 3 months. We have used this system to evaluate the pattern of regulation of acute-phase protein synthesis in response to prolonged treatment of human hepatoma cells with cytokines.

MATERIALS AND METHODS

Dexamethasone and sodium selenite were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Fetal calf serum was obtained from Boehringer (Mannheim, Germany). The culture medium was RPMI-1640 purchased from Gibco/BRL (Cergy-Pontoise, France). Guanidine thiocyanate was obtained from Fluka AG (Basel, Switzerland). Human recombinant IL-6 (rhIL-6), with a specific activity of 10^7 units/mg, was purchased from Genzyme (Tebu, France). Human recombinant IL-1α (rhIL-1α), with a specific activity of 2.5×10^7 units/mg, was obtained from Hoffman–La Roche (Basel, Switzerland). Nylon N^+ membranes and [α^{32}P]dCTP (specific radioactivity 3000 Ci/mmol) were from Amersham Corp.

Cell culture and adaptation in serum-free medium

The human hepatoma cell line Hep3B (Knowles *et al.*, 1980) was adapted to growth *in vitro* and was a gift from Mrs. L. Michel (Institut Pasteur, Paris, France). Hep3B cells were plated at a density of (2–4) $\times 10^4$/cm^2 in RPMI-1640 supplemented with heat-inactivated 10% (v/v) fetal calf serum (FCS), 50 units of penicillin/ml, 50 μg of streptomycin/ml, 10 mM-Hepes and 2 mg of D-glucose/ml. Cells were cultured in plastic 25 cm^2 flasks (Falcon 3013) and incubated in a humid atmosphere at 37 °C in 5% CO_2/95% air. Cells which were fed every 48 h with 3 ml of

Abbreviations used: AGP, α_1-acid glycoprotein; AFP, α-fetoprotein; AHSG, α_2-HS glycoprotein; IL-6, interleukin-6; IL-1, interleukin-1; rhIL, recombinant human IL; LPS, lipopolysaccharide; Mo-CM, conditioned medium from LPS-activated human monocytes; FCS, fetal calf serum; PBS, phosphate-buffered saline (0.15 M-NaCl/5 mM-sodium phosphate, pH 7.2).

medium reached about 80% confluency at 5–6 days. Then cultures were washed with phosphate-buffered saline (PBS) and medium was replaced with RPMI containing 30 nM-sodium selenite. Media were harvested and changed every 48 h. Growth in selenium-supplemented medium was evaluated by measuring cellular protein in the attached monolayer. The cellular protein was dissolved in a solution of 4 M-guanidine thiocyanate, 25 mM-sodium acetate and 5 mM-EDTA disodium salt, pH 7.0, and the proteins were assayed using the Bio-Rad protein kit (Bio-Rad Laboratories, Richmond, CA, U.S.A).

Monocyte-conditioned media

Human peripheral blood monocytes were separated by buoyant-density centrifugation, purified by adherence for 1 h and stimulated for 24 h with chromatographically purified lipopolysaccharide (LPS) (10 μg/ml) from *Salmonella minnesota* (Sigma). The monocyte-conditioned media (Mo-CM) were dialysed against PBS and sterilized by filtration through a 0.22 μm-pore-size membrane (Millipore). To evaluate the activities of hepatocyte-stimulating factors contained in Mo-CM, the increase in α_1-antichymotrypsin secretion following addition of Mo-CM to HepG2 cells was measured as described (Baumann *et al.*, 1986; Daveau *et al.*, 1988). IL-6 in Mo-CM was assayed by a hybridoma growth stimulation assay using the mouse hybridoma 7TD1 line obtained through the courtesy of Dr. J. Van Snick (Ludwig Institute for Cancer Research, Brussels, Belgium) (Coulie *et al.*, 1987).

Cell stimulation

For the stimulation experiments, Hep3B cells in selenium-supplemented medium were incubated with control medium, 33% (v/v) Mo-CM, IL-1α (50 units/ml), IL-6 (100 units/ml), or a combination of IL-1α and IL-6. After 48 h, the medium was removed and replaced with either fresh medium containing an identical amount of the stimulants or fresh medium without stimulants. Media were analysed for protein production and the cell monolayers were obtained for extraction of total cellular RNA.

Protein quantification

Secretion of human plasma proteins by Hep3B cells was measured without concentration of the supernatant fluid using rocket immunoelectrophoresis as described (Daveau *et al.*, 1988). Rabbit anti-(human AHSG) and rabbit anti-(human AGP) were obtained in our laboratory as already described (Lebreton *et al.*, 1979; Nicollet *et al.*, 1981). Rabbit anti-(human albumin), rabbit anti-[human α-fetoprotein (AFP)] and plasma protein standards were obtained from Behringwerke (Marburg, Germany).

Cellular RNA preparation

At the end of the indicated periods in the stimulation experiments, the cell monolayers were rinsed with PBS and scraped into a denaturing solution containing 4 M-guanidinium thiocyanate, 25 mM-sodium citrate, pH 7.0, 0.5% sarcosyl and 0.1 M-2-mercaptoethanol. The total cellular RNA was obtained by a single-step method using an acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski & Sacchi, 1987; Daveau *et al.*, 1990). The total RNA was measured by spectrophotometry (A260/280), and isolation and standardization of undegraded RNA was monitored by Minigel electrophoresis in the presence of 0.5 μg of ethidium bromide/ml and identification of the 28 S and 18 S ribosomal bands.

cDNA probes

Human AGP cDNA was excised from the plasmid pUC19 with *Eco*RI and the ^{32}P-labelled insert was used as the hybridization probe. Human AHSG cDNA was obtained as previously described (Arnaud *et al.*, 1987). The 1.7 kb *Hin*dIII/*Xba*I fragment of the cDNA encoding human IL-6RpBSF2R-236 was provided by Dr. T. Kishimoto, Osaka, Japan (Yamasaki *et al.*, 1988). Probes were labelled with a random-primed labelling kit as indicated by the manufacturer (Boehringer). The IL-6 receptor cDNA probe was phenol/chloroform-extracted after the random primer reaction.

Northern blot hybridization analysis

Samples of total RNA (20 μg) were denatured for 15 min at 65 °C in 10 mM-sodium phosphate buffer, pH 7.0, containing 2.2 M-formaldehyde and 50% formamide, and then applied to 1.2% agarose gels containing 2.2 M-formaldehyde and separated at 25 V overnight. After staining of the gel with ethidium bromide, the gel was destained and transfer was performed for 4 h in NaOH (50 mM) on to Hybond N$^+$ membranes. The filters were prehybridized at 42 °C for 3 h and hybridized at 42 °C overnight, with the exception of the IL-6R cDNA probe, which was hybridized for 48 h. Nylon filters were exposed to Kodak XR-5 films at −70 °C for 24 h using intensifying screens. Exposure of IL-6R mRNA required 3–6 days and the unspecific labelling of the 28 S RNA could not be avoided.

RESULTS

Long-term plasma protein production in chemically defined medium

In our present study, it was possible to grow the human hepatoma Hep3B cells in serum-free medium in the presence of the trace element selenium (as sodium selenite). When the culture medium of subconfluent Hep3B cells supplemented with FCS was replaced with a selenium-supplemented medium (Nakabayashi *et al.*, 1982), no adaptation period was required for the growth of the Hep3B cells. Moreover, it was previously shown that cell attachment occurred with very low efficiency and cell growth decreased when the human hepatoma cell line HepG2 was grown in a serum-free medium supplemented with 30 nM-Na_2SeO_3 (Tecce & Terrana, 1988). In our culture conditions, Hep3B cells could be maintained in this medium for over 3

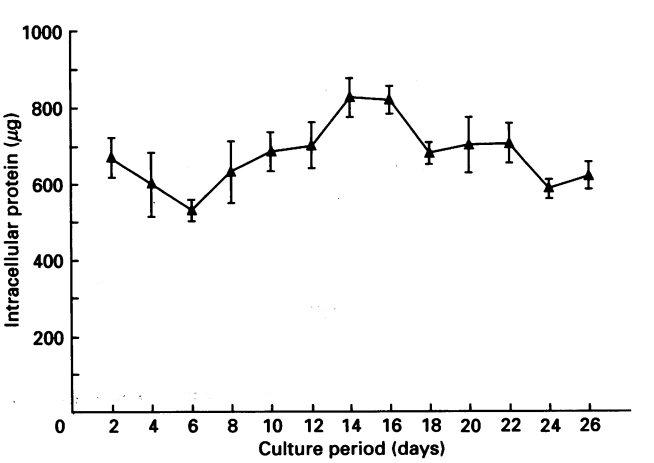

Fig. 1. Cellular protein in monolayers of Hep3B cells maintained in selenium-supplemented medium

Cells grown to reach about 80% confluency in RPMI-1640 with 10% FCS were deprived of FCS by replacing the initial medium with RPMI-1640 supplemented with 30 nM-sodium selenite. The medium was changed every 2 days. Growth was assessed by measuring cellular protein in the attached monolayer every 2 days. Values represent the means ± S.D. of three separate measurements.

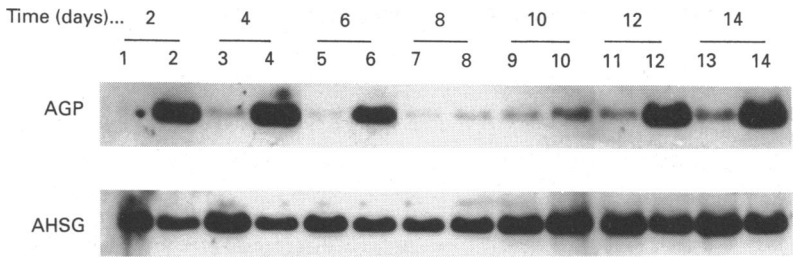

Fig. 2. Effect of Mo-CM on AGP and AHSG mRNA levels

Hep3B cells were cultured for 14 days in medium supplemented with 30 nM-selenium. Cells were stimulated with 33% Mo-CM containing 50 units of IL-6/ml for periods of 48 h on days 0, 2, 10 and 12, but not stimulated on days 4, 6 and 8. Total RNA was extracted on days 2, 4, 6, 8, 10, 12 and 14 as indicated. Lanes 1, 3, 5, 7, 9, 11 and 13, RNA extracted from Hep3B cells from flasks containing fresh culture medium alone; lanes 2, 4, 12, 14, RNA extracted from Hep3B cells from flasks supplemented with Mo-CM at days 0, 2, 10 and 12; lanes 6, 8, 10, RNA extracted from Hep3B cells from the latter set of flasks, in which Mo-CM was replaced by fresh culture medium on days 4, 6 and 8. For each experiment, total RNA was extracted from three flasks of cells as described in the Materials and methods section, and 20 μg portions were used for separation of 0.8% agarose/16.6% formaldehyde gels. RNA was transferred on to Nylon membranes and hybridized with a ^{32}P-labelled AGP cDNA or a ^{32}P-labelled AHSG cDNA.

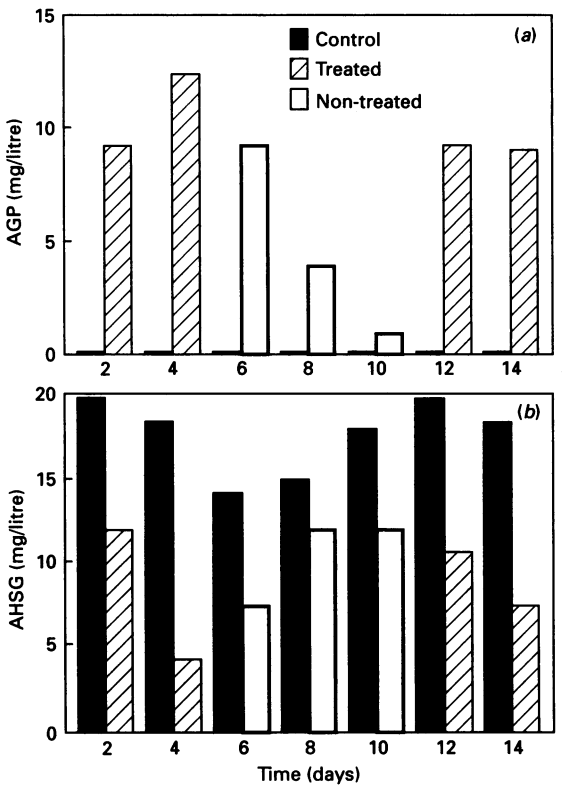

Fig. 3. Effect of Mo-CM on AGP (*a*) and AHSG (*b*) secretion in a long-term culture of Hep3B cells

Hep3B cells were cultured for 14 days in medium supplemented with 30 nM-selenium. Cells were stimulated with 33% Mo-CM for periods of 48 h on days 0, 2, 10 and 12, but not stimulated on days 4, 6 and 8. Media were harvested and changed every 48 h. Equal aliquots (30 μl) of the culture media were analysed by rocket immunoelectrophoresis. The mean value obtained from three flasks of the culture is shown.

months. Cellular protein in the attached monolayer showed a near steady state for a 26-day period, indicating that the amount of attached cells remained relatively constant during the period under study (Fig. 1). In five separate experiments, secretion of albumin was 1105 ± 335 ng/h per ml at the outset of the culture period in selenium-supplemented medium, and 1319 ± 243 ng/h per ml 16 days later. Under the same conditions, secretion of AHSG was 372 ± 248 ng/h per ml and 456 ± 125 ng/h per ml, and that of AFP was 143 ± 97 ng/h per ml and 159 ± 96 ng/h per ml respectively. AGP secretion was barely detectable both at the outset and at the end of the period investigated.

Effect of Mo-CM on gene expression and secretion of AGP and AHSG during the long-term culture of Hep3B cells

To investigate whether cytokines are able to regulate protein synthesis when added to Hep3B cells cultured for 14 days in selenium-supplemented medium, we studied the induction of the mRNA of AGP, a positive acute-phase protein, and of AHSG, a negative acute-phase protein. Northern blot analysis (Fig. 2) showed that Mo-CM treatment induced a sustained and drastic increase in AGP mRNA and a decrease in AHSG mRNA following two successive 48 h periods of stimulation. mRNA levels returned to a basal level when Mo-CM addition to Hep3B cells was stopped. Again, a marked up-regulation of AGP mRNA and a down-regulation of AHSG mRNA were obtained after a further addition of Mo-CM on days 10 and 12 of culture.

The quantities of the secreted proteins AGP and AHSG were also measured at various time points during the long-term culture. The amounts of secreted AGP and AHSG in the culture medium following treatment with cytokines were compared with those observed in control cultures (Figs. 3*a* and 3*b*). A marked and progressive increase in AGP secretion (Fig. 3*a*) was noticed following addition of Mo-CM at days 0 and 2 of the culture. In the absence of Mo-CM, AGP secretion decreased over time and returned to a basal level by day 10. A further addition of Mo-CM to the Hep3B cells at days 10 and 12 again increased AGP secretion. In contrast to the increase in AGP secretion following Mo-CM addition, a progressive decrease in AHSG secretion (Fig. 3*b*) was obtained, compared with that observed in control cells. AHSG secretion then increased until day 10 in the absence of Mo-CM, but without returning to a basal level. A further addition of Mo-CM at days 10 and 12 again decreased the AHSG secretion.

In another experiment (results not shown), Hep3B cells were cultured for 34 days in the selenium-supplemented medium and Mo-CM was added without interruption every 48 h for 10 days. Following cytokine addition, a maximal increase in AGP secretion was seen at 48 h and a maximal decrease in AHSG secretion was seen at 96 h. Interestingly, in spite of successive challenges with Mo-CM repeated every 48 h for 10 days, the increase in AGP secretion and decrease in AHSG secretion remained constant during this period.

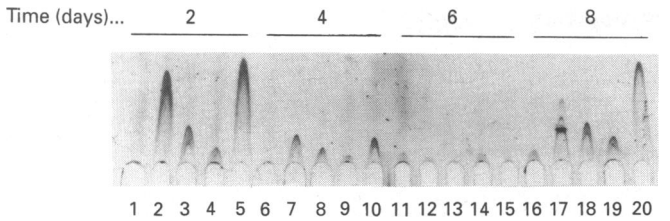

Fig. 4. Effect of cytokines on AGP secretion in a long-term culture of Hep3B cells

Hep3B cells were cultured for 10 days in a 30 nM-selenium-supplemented medium. Media were harvested and changed every 48 h, and analysed at days 2, 4, 6, 8 as indicated. Wells 1, 6, 11 and 16, media harvested from flasks of cells cultured in the presence of fresh culture medium alone; wells 2 and 17, media harvested from cells cultured in the presence of 33% Mo-CM at days 0 and 6; wells 7 and 12, media harvested from the latter cells in which Mo-CM was replaced by fresh culture medium at days 2 and 4; wells 3 and 18, media from cells cultured in the presence of IL-6 (100 units/ml) at days 0 and 6; wells 8 and 13, media from the latter cells in which IL-6 was replaced by fresh culture medium at days 2 and 4; wells 4 and 19, media from cells cultured in presence of IL-1 (50 units/ml) at days 0 and 6; wells 9 and 14, media from the latter cells in which IL-1 was replaced by fresh culture medium at days 2 and 4; wells 5 and 20, media from cells cultured in presence of IL-1 and IL-6 at days 0 and 6; wells 10 and 15, media from the latter cells in which IL-1 and IL-6 were replaced by fresh culture medium at days 2 and 4. Each set of cells was from three flasks. Equal volumes (30 μl) of three pooled flasks of the culture media were analysed by rocket immuno-electrophoresis using a rabbit anti-(human AGP) immuneserum.

Effect of IL-1 and IL-6 on the secretion of AGP during the long-term culture of Hep3B cells

The effects of recombinant cytokines IL-1α and IL-6, added either alone or together, were assayed on the synthesis of AGP in a long-term culture of Hep3B cells for 10 days. In this set of experiments, the effect of the above cytokines was compared with that obtained following the culture of another set of Hep3B cells in the presence of Mo-CM under the same conditions. Cytokines and Mo-CM were added for 48 h periods at day 0 and at day 6 of the culture, and the culture media were harvested every 48 h. For comparison, medium from cells cultured in the presence of fresh medium alone was analysed. It could be seen (Fig. 4) that an increase in AGP secretion occurred at days 2 and 8 of the culture, both with Mo-CM and with the combination of IL-1α and IL-6. In contrast, only a small increase in AGP secretion was observed in the presence of IL-1α or IL-6 alone. The time course experiment showed that the effects of Mo-CM and of IL-1 and IL-6 were no more apparent at day 6 following the addition of cytokines to the cell culture.

Expression of IL-6 receptor mRNA

To find out whether the variations in AGP and AHSG gene regulation are associated with variations in the expression of the IL-6 receptor during the period of cytokine addition, we evaluated IL-6 receptor mRNA levels. Hep3B cells were cultured in the selenium-supplemented medium in the presence or in the absence of 1 μM-dexamethasone (Bauer *et al.*, 1989; Snyers *et al.*, 1990; Rose-John *et al.*, 1990). Preliminary studies (results not shown) indicated that the presence of dexamethasone increased the production of AGP following Mo-CM addition compared with that obtained without dexamethasone. In contrast, the decreases in albumin and AHSG following addition of Mo-CM were not significantly modified by the presence of dexamethasone.

Hep3B cells were incubated in the presence of Mo-CM containing 50 units of IL-6/ml either with or without dexamethasone for 48 h. Total RNA was extracted following the period of Mo-CM treatment. Fresh medium without Mo-CM was then added every 48 h, and RNA was extracted on day 8. Northern blot analysis with an IL-6 receptor cDNA probe detected IL-6 receptor mRNA in cells cultured in the presence of dexamethasone (Fig. 5*a*), but not, in our study, in cells cultured in the absence of dexamethasone (Fig. 5*b*). Further, Mo-CM had no effect on the expression of IL-6 receptor mRNA in cells cultured in the presence of dexamethasone (Fig. 5*a*, lane 2) compared with that found in control cells (Fig. 5*a*, lane 1), whereas IL-6 receptor mRNA was not detectable in cells cultured in the presence of Mo-CM without dexamethasone (Fig. 5*b*, lane 6). At day 8 of the culture, i.e. 6 days after stopping the addition of Mo-CM, the patterns of IL-6 receptor mRNA expression, in the presence (Fig. 5*a*, lanes 3 and 4) or in the absence (Fig. 5*b*, lanes 5 and 8) of dexamethasone were unchanged.

DISCUSSION

We have previously reported that rat hepatocytes co-cultured with untransformed rat epithelial cells of biliary origin show long-term synthesis of acute-phase proteins and are able to respond to cytokines (Guillouzo *et al.*, 1984; Lebreton *et al.*, 1986; Conner *et al.*, 1990). In fact, the transcriptional activity of hepatocytes in co-cultures is well maintained (Fraslin *et al.*, 1985), in contrast to pure cultures of rat or human hepatocytes, which suffer from the disadvantage that they can only be

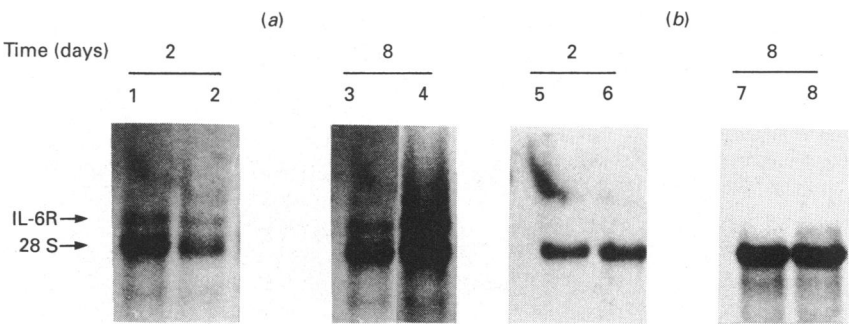

Fig. 5. Expression of IL-6 receptor mRNA

Hep3B cells, cultured in a selenium-supplemented medium, were incubated for 48 h with 33% Mo-CM containing 50 units of IL-6/ml and 1 μM-dexamethasone (*a*) or without dexamethasone (*b*). Total RNA was obtained at 48 h from control cells (lanes 1 and 5) and from stimulated cells (lanes 2 and 6). Thereafter, fresh medium without Mo-CM was added every 48 h and total RNA was obtained at day 8 from control cells (lanes 3 and 7) and non-stimulated cells (lanes 4 and 8). Total RNA was extracted as in Fig. 1 and hybridized with a ^{32}P-labelled IL-6 receptor cDNA. The probe used detects a non-specific hybridization on the 28 S ribosomal RNA.

maintained in culture for a short period and are physiologically and phenotypically unstable. However, the co-culture system requires the presence of FCS and glucocorticoids. Corticosteroids appear to be essential for the long-term survival of active albumin-secreting hepatocytes co-cultured with epithelial cells. These effects are dose-dependent and, in the absence of the corticosteroids, rat hepatocytes do not survive for more than 2 weeks (Baffet *et al.*, 1982).

We report here, for the first time, that the use of a medium containing selenium as the only supplement (Nakabayashi *et al.*, 1982; Tecce & Terrana, 1988) maintains the synthesis of plasma proteins by human Hep3B hepatoma cells at a near steady state for over 3 months. In this model, the long-term behaviour of hepatic cells in response to repeated or prolonged exposure to cytokines could be studied without the presence of FCS and glucocorticoids. Accordingly, the role of cytokines in the synthesis of acute-phase proteins by hepatic cells could be analysed in the absence of co-factors that are possibly present in FCS.

The results clearly indicated that the cells cultured in the selenium-supplemented medium could respond to cytokines by a co-ordinate rise in AGP acute-phase protein mRNA and a decrease in AHSG mRNA. When Mo-CM addition was stopped this responsiveness was not lost, since cells were always able to modify the gene expression of the acute-phase proteins under study on resumption of the cytokine addition. Moreover, analysis of the changes in protein production and of the return to a basal level of protein secretion following addition of cytokines indicated that increase in AGP production and its return to basal level were more rapid than the changes observed for the negative acute-phase protein AHSG. Also, when cytokines were present in the culture medium for 10 days, a maximum increase was obtained 2 days following cytokine addition for AGP synthesis and a maximum decrease was obtained 4 days following cytokine addition for AHSG synthesis. No further changes were observed. This permanence of responsiveness to stimulation by cytokines also suggested that, during cytokine treatment, either cytokine receptors were constantly recycled, or these receptors were consumed and new receptors were synthesized. Finally, our data suggested that the transcription factors acting in hepatocyte-specific gene expression were effective during the long-term culture of Hep3B cells.

In contrast to the *in vitro* system of hepatocytes cultured with rat liver epithelial cells, Hep3B cells cultured in a medium containing selenium do not require the presence of glucocorticoids. These hormones increase the synthesis of AGP in primary cultures of rat hepatocytes (Baumann *et al.*, 1983) and during co-culture (Conner *et al.*, 1990). In this latter work, an increase in AGP synthesis was observed following an initial 8-day period of cytokine addition, whereas no further increase was observed during a second period of cytokine addition. In our present work, in contrast, an increase in AGP synthesis by Hep3B cells could always be obtained following a second period of cytokine addition (Figs. 2 and 3).

IL-6 hepatic receptor expression was found not to be regulated by IL-6 in HepG2 cells (Rose-John *et al.*, 1990). In the present study we have found that expression of IL-6 receptor in Hep3B cells in a long-term culture system was up-regulated by dexamethasone, but not by cytokines present in Mo-CM (including IL-6). This result is in agreement with that obtained by Rose-John *et al.* (1990) using HepG2 cells in a short-term culture in the presence of dexamethasone. However, in our study, exposure of Hep3B cells to cytokines in the absence of glucocorticoids resulted in a marked modification of AGP and AHSG synthesis. In conclusion, our work shows that, in this *in vitro* culture system, cytokines induce a response similar to that observed in humans *in vivo* during inflammatory states. Such a model could thus be used to examine the effects of cytokine agonists and antagonists which induce modifications in the hepatic acute-phase protein response.

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