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The acute-phase response of cultured rat hepatocytes

System characterization and the effect of human cytokines

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1. Hepatocytes were isolated from adult livers and cultured for periods of up to 5 days as monolayers at an initial density of 10^6 cells/10 cm^2 in Williams E medium containing insulin, dexamethasone and 5% foetal-calf serum. The daily production of 11 plasma proteins was measured by electroimmunoassay and compared with the concentrations of the same proteins in the plasma of normal rats and of those with experimental inflammation. 2. Hepatocytes from normal rats synthesized proteins in relative amounts which were similar to the relative proportions of the same proteins in the plasma of turpentine-injected animals. The pattern changed only slowly during 5 days in culture, but it did so profoundly either when the medium was devoid of dexamethasone or when human cytokines (from endotoxin-stimulated monocytes or unstimulated human squamous-carcinoma cell line COLO-16) were added. 3. The cytokines consistently increased the synthesis of α_2-macroglobulin and fibrinogen and depressed that of albumin; variable increases in the synthesis of α_1-acute-phase globulin, α_1-acid glycoprotein, haptoglobin and α_1-proteinase inhibitor, and variable decreases in transferrin synthesis, were seen, whereas the synthesis of antithrombin III, α_1-macroglobulin and prothrombin remained virtually unaffected. The cytokine effects on protein synthesis required the presence of dexamethasone. 4. The hepatocyte-stimulating activity derived from monocytes chromatographed on Sephadex G-100 corresponding to 30000 Da, as opposed to the lymphocyte-activating factor, which was eluted as a molecule of approx. 15000 Da. This suggests that both activities probably reside with distinct molecular species in the preparations of human cytokines.

AP proteins are liver-produced plasma proteins, the concentration of which changes in the blood during the early stages of inflammation (for reviews see Koj, 1974, 1983; Kampschmidt, 1981; Kushner, 1982). A low-M_r protein (or family of proteins), produced by peritoneal-exudate cells and stimulated blood monocytes, has been identified as the inducing factor and variously named 'leukocytic endogenous mediator' (Kampschmidt *et al.*, 1973, 1982; Wannemacher *et al.*, 1975; Gordon & Limaos, 1979), 'serum amyloid A inducer' (Sipe *et al.*, 1979; Sztein *et al.*, 1981), or 'hepatocyte stimulating factor' (Ritchie & Fuller, 1983). Since its active component reveals striking similarities to endogenous pyrogen and LAF (Sztein *et al.*, 1981; Dinarello *et al.*, 1982; Kampschmidt *et al.*, 1983), it has been proposed that the diverse biological responses to injury, infection and inflammation are elicited by one cytokine, interleukin 1, that shows a broad, hormone-like, activity (for a review see Oppenheim & Gery, 1982). Since human interleukin 1 is not yet available in a homogeneous form, the question of whether all these functions are expressed by a single factor or by a group of factors has yet to be determined.

Abbreviations used: AP proteins, acute-phase proteins; LAF, lymphocyte-activating factor.

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Studies *in vivo* of the cytokine effect on AP-protein synthesis are difficult to interpret because of the complexity of the system. Understandably, interest began to shift to working with primary hepatocyte cultures, and preliminary observations made with fibrinogen (Ritchie & Fuller, 1981, 1983), serum amyloid proteins (Selinger *et al.*, 1980; McAdam *et al.*, 1982; Tatsuta *et al.*, 1983) and α_1-acid glycoprotein, haptoglobin and serum amyloid A (Baumann *et al.*, 1983*b*, 1984) demonstrate the feasibility of such studies with both homologous and heterologous cytokines. However, the picture thus far available is incomplete. First, the optimal experimental conditions remain to be established; second, the spectrum of investigated proteins requires broadening; and third, a comparison of the synthetic response of stimulated hepatocytes with that of the liver *in situ* is needed. With 11 monospecific antisera to different rat proteins and two distinct human cytokines available, we undertook to close some of the gaps just outlined. The biological potency of the cytokines used was evaluated through their activity in an LAF assay.

Materials and methods

Materials

Williams E medium (Williams & Gunn, 1974), Dulbecco's minimal essential medium (Smith *et al.*, 1980), foetal-calf serum and a mixture of antibiotics (see below) were from GIBCO; Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], L-glutamine, insulin and collagenase (type IV) were from Sigma, dexamethasone phosphate and heparin from Organon, Sephadex G-100 and Ficoll/Hypaque from Pharmacia, and collagen and culture dishes (Linbro plates) from Flow Laboratories (McLean, VA, U.S.A.). Lipopolysaccharide W from *Escherichia coli* 055 : B5 was obtained from Difco and extracted with hot phenol. Agarose for immunoelectrophoresis was from FMC Corp., Marine Colloids Division (Rockland, ME, U.S.A), and Spectrapor dialysis tubing from Arthur Thomas Co. (Philadelphia, PA, U.S.A). All other reagents were A.R. grade, where available.

Purification of rat proteins and preparation of antisera

α_1-Acid glycoprotein, α_1-acute-phase globulin, albumin, antithrombin III, fibrinogen, haptoglobin, α_1- and α_2-macroglobulins and α_1-proteinase inhibitor were isolated as described previously (Koj *et al.*, 1982), prothrombin was purified by the method of Miletich *et al.* (1980), and transferrin as described elsewhere (Regoeczi *et al.*, 1974). Antiserum to rat transferrin was from Cappel Laboratories (West Chester, PA, U.S.A.), and antisera to the other proteins were raised in New Zealand rabbits by established procedures. All antisera were monospecific, except one that reacted both with α_1- and α_2-macroglobulins. However, these two antigens were easily distinguishable as separate peaks on rocket immunoelectrophoresis (see Fig. 3).

Hepatocyte cultures

Hepatocytes were isolated under sterile conditions from livers of adult male Wistar rats after perfusion with collagenase by the procedure of Sweeney *et al.* (1978). Cell viability (Trypan Blue exclusion) was in the range of 80–95%. The hepatocytes were suspended to a concentration of 2×10^6 cells/ml in Williams E medium containing 0.075% $NaHCO_3$, 10mM-Hepes, 2mM-L-glutamine, heparin (5 units/ml), 1 μM each of insulin and dexamethasone phosphate, 5% (v/v) foetal-calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and gentamycin (48 μg/ml). In some experiments dexamethasone was omitted or other hormones were added as indicated. Hepatocyte monolayers were obtained by pipetting 10^6 cells (0.5 ml of suspension) into 35 mm-diam. culture dishes that had been coated with collagen and contained 1 ml of the medium. After 2h incubation at 37°C in air/CO_2 (19 : 1), the unattached cells were aspirated and fresh medium (1 ml) was added, followed by 0.25 ml of a serially diluted cytokine preparation. Control cultures received 0.25 ml of phosphate-buffered saline (2.7mM-KCl, 16.7mM-Na_2HPO_4, 130mM-NaCl, pH 7.4). The cultures were incubated at 37°C in air/CO_2 (19 : 1). The medium was changed daily and the appropriate addition repeated. On the last day, the medium was collected and the viability of the monolayer assessed. To measure the cellular content with respect to plasma proteins, the layer was washed twice with phosphate-buffered saline (1 ml) before solubilization in 0.1% (v/v) Triton X-100 (1 ml) by freezing and thawing twice.

Quantification of plasma proteins

Concentrations of 11 plasma proteins in the medium and cell lysates were determined by electroimmunoassay (Laurell rocket electrophoresis) as described by Weeke (1973), by reference to the corresponding protein standards. To do so, cell lysates had to be concentrated 10-fold by freeze-drying to assay any protein and the media similarly concentrated to assay antithrombin III, α_1-macroglobulin and prothrombin. Concentration was also done whenever protein synthesis was sluggish, e.g. in the absence of dexamethasone. Fibrinogen and transferrin, both yielding broad rockets, were quantified by the area, and the other proteins by the height, of the rockets. The results were ex-

pressed as μg released by, or contained in, 10^6 cells per 24h. A heparinized plasma sample from the liver donor was suitably diluted and processed for comparison.

Source and preparation of cytokines

Cytokines were obtained from human squamous-carcinoma cells (line COLO-16, kindly provided by Dr. S.-K. Liao), which had previously been shown to produce LAF constitutively (Baumann *et al.*, 1984), and from human monocytes. The former cells were cultured in Dulbecco's minimal essential medium containing 5% foetal-calf serum. After 4 days at 37°C in air/CO_2 (19:1), the supernatant was collected, subdivided, and kept frozen at −20°C. Monocytes were isolated from fresh heparinized blood by gradient centrifugation in Ficoll/Hypaque (Böyum, 1976), washed, suspended in the above medium at 2×10^6 cells/ml and plated in Petri dishes. Bacterial lipopolysaccharide (5 μg/ml) was added and the cells were cultured for 24h at 37°C in air/CO_2 (19:1), after which the supernatant was collected, subdivided, and frozen at −20°C.

For further processing, samples of cytokine from either source were dialysed in Spectrapor tubing (phosphate-buffered saline, 24h) and concentrated approx. 10-fold by ultrafiltration with an Amicon YM 10 membrane. The concentrate (approx. 100mg of protein) was filtered through a column (2.2cm × 60cm) of Sephadex G-100 at 4°C with phosphate-buffered saline as the eluent, and the fractions were assayed for the A_{280} as well as for LAF activity (see below). Fractions having the highest LAF activities (and usually chromatographing in the 10000–40000Da range), were pooled and concentrated by ultrafiltration. This material, designated 'column-purified cytokine', was subdivided and kept at −20°C. Volumes (μl) of cytokine quoted in the Results section refer to these preparations. In separate experiments, aimed at the comparison of the M_r values of LAF and the hepatocyte-stimulating activity, individual fractions from the Sephadex column were tested directly on hepatocyte and thymocyte cultures.

Miscellaneous techniques

Bioassay of LAF activity was based on augmentation of the proliferation of phytohaemagglutinin-stimulated mouse thymocytes (Sauder *et al.*, 1982). Briefly, 1.5×10^6 thymocytes from C3H/HeJ mice were cultured in Costar flat-bottomed tissue-culture plates in Dulbecco's minimal essential medium (0.1ml) containing 5% foetal-calf serum, 1mM-sodium pyruvate, 2mM-L-glutamine, 10mM-Hepes, 50μM-2-mercaptoethanol, phytohaemagglutinin (1 μg/ml) and 100 μl of the test material. After culture for 72h, the cells were pulsed for 16h with [^{3}H]deoxythymidine (1 μCi), and then collected on filter paper with the aid of a Mash II harvester. Each sample was tested in triplicate.

Any additive to a cell culture (hepatocyte or thymocyte) was sterilized by filtration (Millipore; 45 μm pore size). For column-purified cytokine this meant treatment of the filter with albumin (bovine, 1%, in PBS) to minimize adsorptive losses. When added to hepatocytes, albumin at a final concentration of 2mg/ml had no detectable effect on plasma protein synthesis.

Results

Synthesis of plasma proteins by hepatocytes from normal donors when cultured for 3 to 5 days

Hepatocytes obtained from normal donors produced all the proteins under study, though not at constant rates (Fig. 1). Thus, by day 5, albumin output usually decreased by 10–30%, and that of α_2-macroglobulin and fibrinogen by 40–50%, as compared with the first 24h. Other proteins were less affected, and the synthesis of transferrin, α_1-acid glycoprotein and α_1-proteinase inhibitor even increased slightly, with a maximum between days 2 and 4.

A prerequisite for the acceptability of the protein content of the medium as an index of plasma protein synthesis by cultured hepatocytes is that newly synthesized molecules are not accumulated within the cell to any significant extent. Under all experimental conditions and for all proteins tested, the quantity of a protein in the cell lysate was less than 10% of the amount of the same protein in the medium obtained during the final 24h of culture.

In five experiments in the absence of dexamethasone, all proteins were produced in smaller amounts. This was particularly true for albumin (Fig. 1), α_1-acid glycoprotein (10–20% of control value) and, above all, for α_2-macroglobulin, which could not be detected at any time. Cells kept without the hormone for several days began to detach, thus resulting in further decreases in protein synthesis. The effect of dexamethasone deprivation on protein synthesis was fully reversible after 3 days (Fig. 1). Less hormone (0.05 μM) was equally effective in maintaining normal protein synthesis, but not the increased output in response to cytokines. Glucagon (0.1 μM) or adrenaline (10 μM), added with 1 μM-dexamethasone, did not noticeably alter the synthesis of any of the 11 proteins. No significant response to cytokines was observed in the absence of dexamethasone.

Preliminary studies showed that maximal depression of albumin synthesis and maximal stimulation of α_2-macroglobulin synthesis by either cyto-

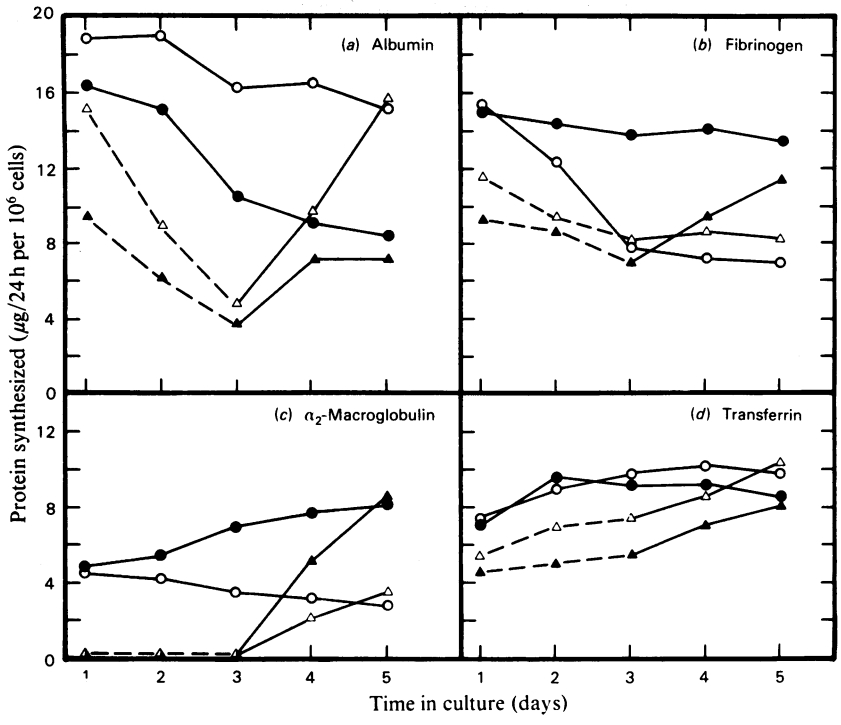

Fig. 1. *Synthesis of albumin (a), α_2-macroglobulin (b), fibrinogen (c) and transferrin (d) by hepatocytes from a normal rat* Cells were cultured for 5 days with daily changes of the medium in the absence (△, ▲) or presence (○, ●) of dexamethasone (1 μM). ▲, ●, Cells cultured in the presence of column-purified cytokine; △, ○, control cells cultured in the presence of phosphate-buffered saline. After 3 days, dexamethasone was given to all cultures and supplementation with cytokine continued wherever appropriate. Proteins were measured in the medium by rocket immunoelectrophoresis.

kine occurred on days 3–5 (Fig. 1). Day 3 was therefore adopted for subsequent studies with cytokines.

Plasma protein synthesis by hepatocytes obtained from donors with experimental inflammation

Synthesis of four plasma proteins by hepatocytes obtained from turpentine-stimulated donors is contrasted with that from control hepatocytes in Fig. 2. The stimulated cells, during the first 24h, produced less albumin and more acute-phase proteins than did the control cells, and the differences between control and test cells became smaller during the ensuing 48h. Also shown in Fig. 2 are the effects of a cytokine added to either type of cultured cells. The effect on normal cells is explained in the section below. When hepatocytes from turpentine-stimulated donors were used, the cytokine caused an additional suppression of albumin synthesis and a retardation in the 'normalization' of fibrinogen and α_2-macroglobulin synthesis; it had no effect, however, on α_1-acute-phase globulin synthesis. Indomethacin, at concentrations in the range 1.5–10 μg/ml, had no effect on the synthesis of α_2-macroglobulin and albumin regardless of whether cytokine was present or not (results not shown).

Of interest is the observation that the ratio of synthesis of most acute-phase proteins to synthesis of albumin in hepatocyte cultures considerably (and consistently) exceeded the corresponding ratio of the amounts of these same proteins in the plasma of the cell donor (Table 1).

Effect of cytokines

The presence of a cytokine (from monocytes or COLO-16 cells) in the hepatocyte culture affected the synthesis of plasma proteins, the largest and most consistent changes being observed with albumin, α_2-macroglobulin and fibrinogen. Figs. 3(*a*) and 4 show the dose-dependent responses of albumin and α_2-macroglobulin to different cytokine preparations from monocytes; in log–log plots, the relationship conformed to a straight line, though a tendency to a plateau became apparent on increasing cytokine concentration beyond a certain point; still higher concentrations of cytokine brought about an inhibition of the synthesis of all

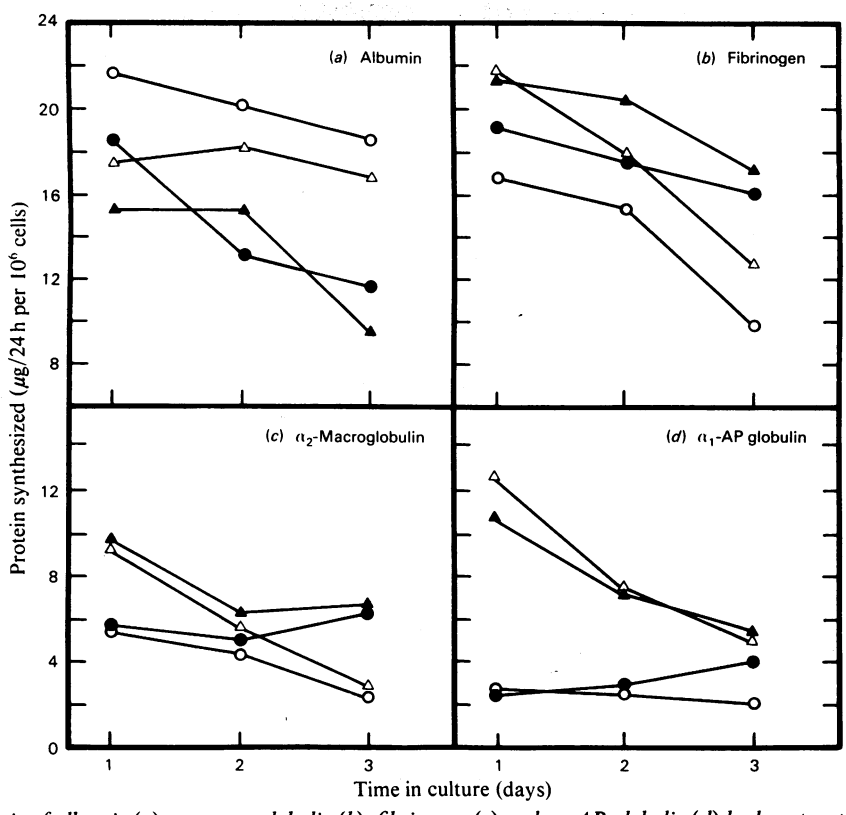

Fig. 2. *Synthesis of albumin (a), α_2-macroglobulin (b), fibrinogen (c) and α_1-AP globulin (d) by hepatocytes obtained from a control (○, ●) and a turpentine-injected (△, ▲) rat*

Turpentine (0.2ml/100g) was given subcutaneously 24h before removing the liver. Each culture contained 1 μM-dexamethasone. ●, ▲, Cultures were maintained in the presence of column-purified cytokine from COLO-16 cells; ○, △, cultures were maintained in the presence of phosphate-buffered saline. Proteins were measured in the medium by rocket immunoelectrophoresis.

Table 1. *Concentrations of four proteins in the plasma of liver donors and in the medium of hepatocytes cultured for 24h*

C, concentration, in mg/ml for plasma and in μg/1.25ml for the medium from 10^6 cells. R, ratio obtained by dividing the concentration of a protein by that of albumin. The concentration of α_2-macroglobulin in the plasma of the control rat could not be accurately measured with the present technique. Results are from a typical experiment.

		Protein concentration						
		Albumin	α_2-Macroglobulin		Fibrinogen		α_1-AP globulin	
Liver donor	Material	C	C	R	C	R	C	R
Control rat	Plasma	25.4	<0.01	<0.001	2.40	0.094	0.92	0.060
	Medium	21.7	5.6	0.258	16.8	0.774	2.65	0.122
Turpentine-injected rat	Plasma	20.2	0.25	0.012	5.95	0.294	2.83	0.140
	Medium	17.6	9.5	0.540	21.90	1.244	12.78	0.726

plasma proteins tested. Dialysis against phosphate-buffered saline of the cytokine-containing medium from monocytes did not impair its stimulating effect on α_2-macroglobulin synthesis (Fig. 4).

In view of the fact that increased synthesis of a positively reacting acute-phase protein was always accompanied by a negative effect on albumin synthesis, it seemed appropriate to express these two effects combined, i.e. as the ratio of protein con-

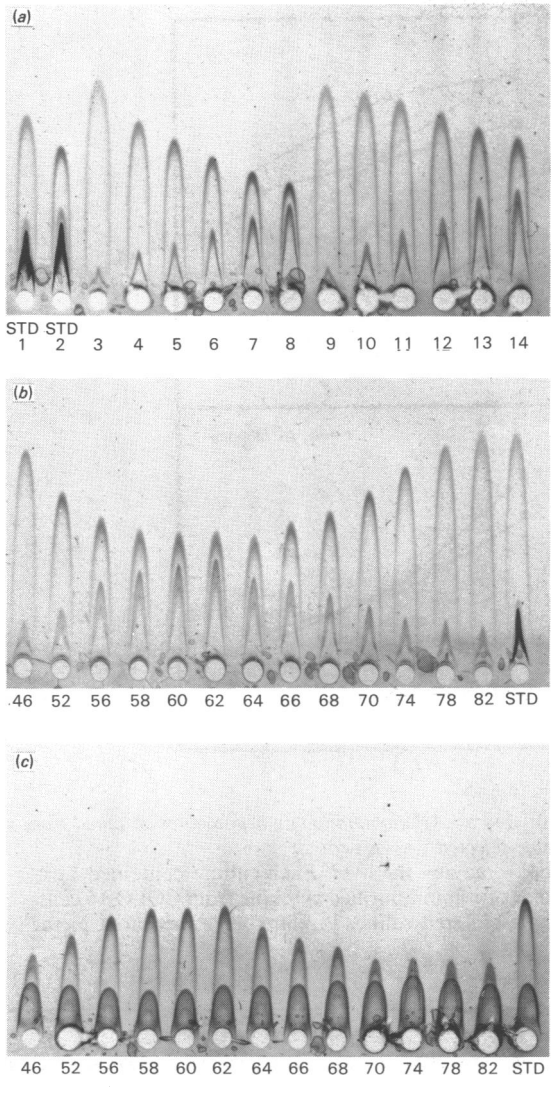

Fig. 3. *Quantification of plasma proteins in the medium of cultured hepatocytes by rocket immunoelectrophoresis*
(*a*) Effect of the supernatant from stimulated monocytes on plasma protein synthesis. The standards (STD 1 and 2) are: albumin (tall rockets), 84ng and 63ng; α_2-macroglobulin (middle-sized, faint rockets), 75ng and 100ng; α_1-macroglobulin (small, intense rockets), 360 and 480ng. Wells 3 and 9, media from control cultures; wells 4–8, media from cells cultured in the presence of 5, 10, 20, 40 and 80 μl of dialysed supernatant respectively; wells 10–14, the same as wells 4–8, except that the supernatant was not dialysed. The arc formed by α_1-macroglobulin is just above the rims of wells 3–14. For the dose–response curves drawn from this plate, see Fig. 4. (*b*) and (*c*), the effect of column-purified cytokine from monocytes on plasma protein synthesis. The standards (well 14 in each plate) are: for (*b*), albumin (105ng), α_1-macroglobulin (280ng) and α_2-macroglobulin (60ng); for (*c*) α_1-AP globulin (38ng, tall rocket) and transferrin (85ng, small rocket). In both plates, the media analysed were from cultures which had been incubated with 0.25ml portions of fractions from the chromatography depicted in Fig. 6.

centrations (e.g. the α_2-macroglobulin/albumin ratio). The advantage of these ratios, which can be obtained from single plates, is that they are largely insensitive to errors caused by variations in the number of plated cells and in the size of the samples placed into the agarose wells.

A tacit assumption underlying the use of protein-concentration ratios is that both the positive and negative effects of a cytokine are exerted by the same component. Support for this assumption is gained from the experiment illustrated in Fig. 5. Here, a concentrated supernatant from lipopolysaccharide-stimulated monocytes was chromatographed on Sephadex G-100, and the potencies of the fractions to alter the synthesis of seven plasma proteins were compared. Within the experimental error, maximal effectiveness with respect to any of the proteins was confined to the same region of the chromatogram. Therefore the agent is either a single component or several components having very similar M_r values.

Gel filtration and analysis of individual fractions not only freed monocyte-derived cytokine from the bulk of protein that lacked hepatocyte-stimulating activity, but it also achieved a partial resolution between this activity and LAF activity (Fig. 6). In four chromatograms of three batches of the supernatant from stimulated monocytes, the hepatocyte-stimulating activity was consistently

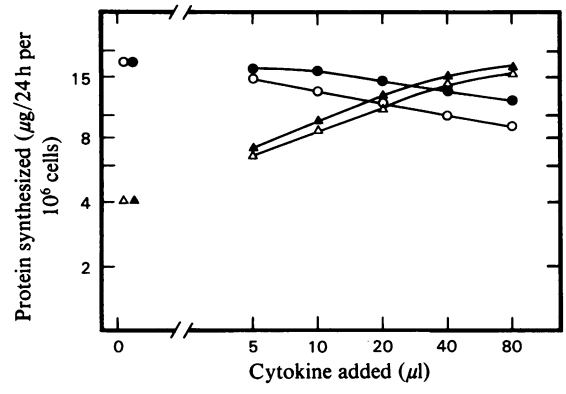

Fig. 4. *Response of albumin* (○, ●) *and* α_2*-macroglobulin* (△, ▲) *synthesis to different doses of cytokine*
Data were calculated from Fig. 3(*a*). The source of cytokine was the supernatant from stimulated monocytes and it was either dialysed (○, △) or not dialysed (●, ▲).

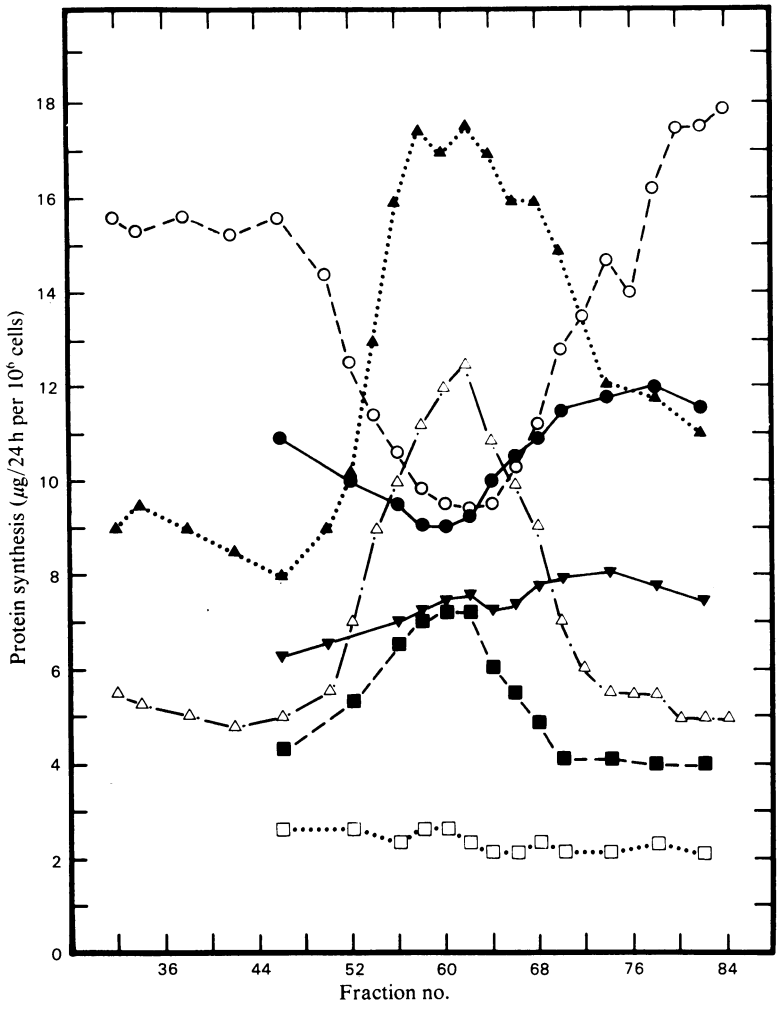

Fig. 5. *Comparison of cytokine fractions eluted from Sephadex G-100 for their potencies to affect the synthesis of different plasma proteins*

The column was loaded with concentrated supernatant from stimulated monocytes, and the fractions indicated on the abscissa were tested on individual hepatocyte cultures. The proteins assayed were albumin (○), α_1-AP globulin (■), α_1-macroglobulin (□), α_2-macroglobulin (△), fibrinogen (▲), α_1-acid glycoprotein (▼) and transferrin (●). For comparison also see Figs. 3(*b*) and 3(*c*).

eluted as a molecule of approx. 30000Da; in contrast, the LAF activity chromatographed corresponding to approx. 15000Da with an occasional peak, as in Fig. 6, also at 45000Da.

A summary of the diversified effects of cytokines on the synthesis of 11 plasma proteins by cultured hepatocytes is presented in Table 2. A value of <1.0 in Table 2 signifies depression of synthesis, 1.0 the lack of a clear response, and >1.0 an increased synthesis. The control values underline the point made above in connection with the data in Table 1, namely that the plasma proteins are synthesized by hepatocytes *in vitro* at relative rates which are quite different from the absolute amounts seen in the plasma of normal rats (Koj *et al.*, 1982).

Discussion

In agreement with previous observations by others (Bissell *et al.*, 1973; Jeejeebhoy *et al.*, 1975, 1980; Ritchie & Fuller, 1981; Marceau *et al.*, 1982; Baumann *et al.*, 1983*a*,*b*, 1984; Andus *et al.*, 1983; Guguen-Guillouzo & Guillouzo, 1983), the present study shows that hepatocytes cultured in the presence of insulin and dexamethasone continue to

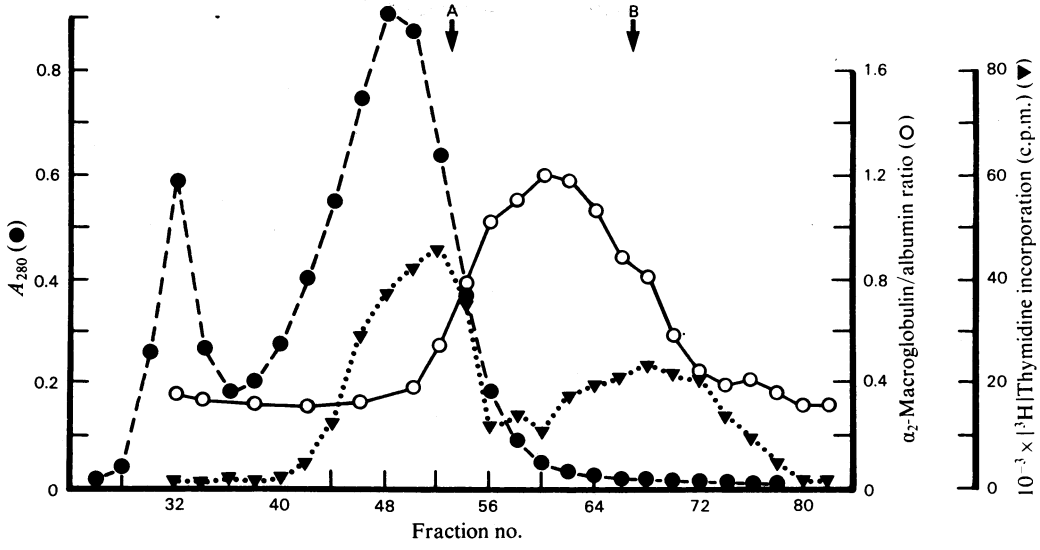

Fig. 6. *Chromatography of the concentrated supernatant from stimulated monocytes on Sephadex G-100*
Supernatant (10-fold concentrated; 2ml) was chromatographed as described in the Materials and methods section. Arrows denote the elution volumes of chicken ovalbumin (A, M_r 43000) and lactalbumin (B, M_r 15000). Fractions (2.7ml each) were tested for A_{280} (●), LAF activity (▼) and hepatocyte-stimulating activity (○). The last is expressed as α_2-macroglobulin/albumin ratios in the media of hepatocytes that had been cultured for 3 days in the presence of the test fractions.

Table 2. *Synthesis of plasma proteins by rat hepatocytes after 2 days in culture in the absence or presence of a cytokine*
The medium was collected on day 3. Values are means (±S.D.) from 6 to 22 experiments. Column-purified cytokines were obtained from monocytes or COLO-16 cells. Maximal responses are expressed as fractions of the synthesis rates in control cultures.

Protein	Control culture (μg of protein/24h per 10^6 cells)	Maximal response to cytokines
Albumin	19.66±3.21	0.4
Transferrin	10.41±2.95	0.7
Antithrombin III	1.38±0.28	~1.0
Prothrombin	0.58±0.19	~1.0
α_1-Macroglobulin	1.52±0.54	~1.0
α_1-Acid glycoprotein	4.86±1.33	1.4
Haptoglobin	10.95±4.06	1.4
α_1-Proteinase inhibitor	10.38±1.83	1.4
α_1-AP globulin	2.34±1.09	2.0
Fibrinogen	9.04±1.77	3.0
α_2-Macroglobulin	3.30±1.28	5.0

synthesize and secrete plasma proteins. However, experimental conditions used by different laboratories vary widely, thus rendering a direct comparison of the data in Table 2 with published values difficult.

From observations *in vivo* (for references see Koj, 1974) and with the perfused liver (John & Miller, 1969; Thompson *et al.*, 1976), the importance of glucocorticoids for the acute-phase response seems well established. As shown here, cultured rat hepatocytes (but not mouse hepatocytes: Baumann *et al.*, 1983*a*) require dexamethasone not only for the maintenance of plasma protein synthesis but also for the response to human cytokines. Since the changed protein synthesis seen after injury is preceded and accompanied by an increased transcription of specific mRNA (Princen *et al.*, 1981; Ricca *et al.*, 1981; Northemann *et al.*, 1983; Baumann *et al.*, 1984), the hormone may exert its effect at this level.

A striking observation made during the present studies was the fundamentally different composi-

tions of the culture media and of the liver donors' plasma with respect to plasma proteins (Table 1). Although we are comparing the ratios of rates of synthesis and absolute plasma concentrations, the differences were far too great to be explained by catabolism and transcapillary diffusion prevailing in the system *in vivo*. Furthermore, α_2-macroglobulin, which is only detectable in the plasma of injured rats and was presumed to be only minimally synthesized, was always present in the media from normal cultures. Thus it appears that hepatocytes isolated from normal rats synthesize plasma proteins *in vitro* at a rate that is more consistent with the plasma concentration changes seen *in vivo* during the acute-phase response. Whatever the explanation, it appears that cultured normal hepatocytes are already in a state of basal stimulation, presumably induced by the isolation procedures involving collagenase digestion. This helps us to understand why the changes effected by cytokines *in vitro* are less spectacular than those produced by turpentine *in vivo* (Koj *et al.*, 1982; Schreiber *et al.*, 1982). Time-dependent subsidence of this stimulated state may also explain our finding that hepatocytes were more responsive to cytokines after 2–3 days in culture. Hepatocytes cultured in suspension also secrete plasma proteins in proportions which are distinct from their proportions in plasma (Jeejeebhoy *et al.*, 1980).

Synthesis rates of plasma proteins by different preparations of normal hepatocytes exhibited considerable variations (Table 2). A part of the overall variability may be assumed to reflect biological scatter; another part, however, was certainly due to technical error, which can be decreased by using protein concentration ratios. Support for the latter is seen by a comparison of the coefficients of variation (CV): CV for α_2-macroglobulin between assays was 39%; CV for α_2-macroglobulin/albumin measurement between assays was 18% and within the same assay was 4%; CV for units of standard cytokine (volume needed to raise α_2-macroglobulin/albumin ratio from 0.3 to 0.4) between assays was 10%. Such precision would allow the assay to be used to detect low concentrations of cytokine.

On the basis of their maximal responses to cytokines, plasma proteins produced by hepatocytes *in vitro* may be divided into four groups (Koj, 1983). (1) Strongly positive acute-phase proteins (α_2-macroglobulin and fibrinogen); they responded regularly and with increases of approx. 3–5-fold. (2) Weakly positive reactants, notably α_1-AP globulin, α_1-acid glycoprotein, haptoglobulin and α_1-proteinase inhibitor. Of these, α_1-AP globulin gave the highest (less than 2-fold) and most consistent values. Only rarely did these proteins give dose–response curves for cytokines. (3) Neutral proteins (α_1-macroglobulin, antithrombin III and prothrombin), which were practically unaffected by cytokines. (4) Negative acute-phase proteins, i.e. albumin and transferrin. Depressed albumin synthesis was the most consistent response to active cytokines in all cultures tested. From immunochemical studies of the reciprocal modulation of secretion of fibrinogen and albumin by single cells (Bernau *et al.*, 1983), it may be inferred that this effect is intimately linked to the enhanced formation of positive acute-phase reactants. Unlike in the hepatocyte culture, haptoglobin, α_1-acid glycoprotein and α_1-AP globulin are classified *in vivo* as strong reactants. The reason for this difference in responsiveness is unknown. It is possible that additional regulatory factors are operational in the whole organism.

From the present experiments with human cytokines, a single component in the preparations may have been responsible for the changes in the synthesis rates of all positive and negative acute-phase reactants by stimulation of the hepatocyte, whereas differentiation of the response could have occurred at a cellular level. This differentiation could have been aided by additional components, for individual batches of cytokines possessed certain characteristic features, such as the ability or inability of stimulating haptoglobin or α_1-AP globulin synthesis, which were reproducible in several hepatocyte cultures.

The chromatographic behaviour of the hepatocyte-stimulating activity on Sephadex G-100 as found by us agrees well with the results of Ritchie & Fuller (1983), who reported that the protein from human monocytes, which stimulates fibrinogen synthesis by cultured rat hepatocytes, was eluted from Sephadex G-75 with an apparent M_r of 25000–30000. This position is distinct from that of the LAF activity, which chromatographed either as a broad single peak with a maximum at 15000 Da, or as two peaks (45000 and 15000 Da respectively), as in Fig. 6. The dual elution profile was also repeatedly observed by Dinarello and his colleagues, who suggest that the larger form, a trimer, may arise by aggregation from the smaller (Dinarello & Wolff, 1982; Dinarello *et al.*, 1983). Further heterogeneity of human LAF can be demonstrated by chromatofocusing between pH 7 and 4; in a preliminary study, we resolved column-purified cytokine from monocytes into three distinct peaks (pH 6.9, 5.5 and 5.1) with respect to LAF activity, whereas the hepatocyte-stimulating activity was eluted at pH 5.1 (A. Koj, J. Gauldie, D. N. Sauder, E. Regoeczi & G. D. Sweeney, unpublished work). These observations lend support to the view that the lymphocyte-activating factor and the hepatocyte-stimulating factor contained in human cytokines are probably different molecular entities. If this can be confirmed by the isolation of

the active components, the activity in question should be specified when using the term 'interleukin 1'.

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