Human Keratinocytes and Monocytes Release Factors Which Regulate the Synthesis of Major Acute Phase Plasma Proteins in Hepatic Cells from Man, Rat, and Mouse*

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Most mammals, when undergoing an acute inflammation, show within hours a profound change in the hepatic synthesis and secretion of a subset of plasma proteins, the acute phase reactants (1). Although several plasma proteins, such as α1-AGP, haptoglobin, and hemopexin, are found to be elevated in many species (1), there are also species-specific acute phase reactants. C-reactive protein is prominent in humans and rabbits (2-4), SAA in humans and mice (5, 6), α1-ACh in humans (7, 8), and α1-AP and α2-MG in rats (1, 9, 10). In the past few years a major effort has been undertaken to determine the source and biochemical nature of the factors modulating the production of the acute phase plasma proteins. Identification of the responsible factors was complicated by the fact that during an acute inflammation the circulating concentration of many hormones was altered (11). Although some of these hormones, such as growth hormone, triiodothyronine, insulin, glucagon, and glucocorticoids were found to affect the production of acute phase plasma proteins (12-17), they could not, however, bring about the entire spectrum of changes as occurring during an inflammation. Based on the observation that mononuclear leukocytes play an important role during the initial stage of inflammatory processes (18-20), it was found that factors released by these cells had not only pyrogenic and thymocyte-stimulating (leukocytic pyrogen, lymphocyte-activating factor, interleukin-1) activity but were also able to modulate a variety of acute phase plasma proteins in liver cells (21-25). Recently it was also demonstrated that epidermal cells produce factors with activities similar to those derived from activated monocytes (26-29). Still open questions are whether the factor(s) promoting the thymocyte growth is also directly responsible for modulation of most, if not all, acute phase plasma proteins in liver cells and whether the liver-regulating activity is limited to a species-specific set of acute phase proteins. In this study we present evidence that human keratinocytes and monocytes produce factors which modulate the synthesis of different sets of major acute phase proteins in human and murine liver cells. With the exception of rat hepatocytes, this regulation does not require the presence of glucocorticoids. Furthermore, it appears that there is not a strict correlation between thymocyte-stimulating and liver cell-stimulating activity, suggesting the presence of separate factors.

EXPERIMENTAL PROCEDURES

Cells—Hepatocytes were prepared by in situ perfusion of livers of 2-4-month-old male and female Buffalo rats and C57BL/6J mice according to the method of Seglen (30). The parenchymal cells were enriched by differential centrifugation and placed in monolayer cultures using collagen-coated culture plates and DMEM containing 10% fetal calf serum as outlined elsewhere (17). Only cell preparations with viabilities greater than 95% were used. A subclone of the human hepatoma cells line HepG2 (31) (generously provided by Dr. B. ACh, α1-antichymotrypsin; α1-AP, α1-acute phase protein; α2-MG, α2-acute phase macroglobulin; DMEM, Dulbecco's modified Eagle's medium; CM, conditioned medium; SAA, serum amyloid A.

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Knowles, Wistar Institute, Philadelphia, PA) was maintained in monolayer culture using above culture medium. Normal human epidermal cells and the human skin carcinoma cell line COLO-16 (32) were cultured in DMEM containing 5% fetal calf serum without any additional stimulants. Monocytes were cultured in RPMI 1640 medium containing 10% fetal calf serum and a 1:2,000 dilution of cell-free conditioned medium of Staphylococcus aureus, which has been shown to be a potent stimulant for interleukin-1 production. Medium with S. aureus factors or bacterial lipopolysaccharides, but without monocytes, has no effect on the regulation of acute phase plasma proteins in cultured hepatic cells (data not presented, see also Fig. 6C).

**Conditioned Media**—Medium, designated here as CM, was collected after 5-days culture of subconfluent monolayers of COLO-16 cells, or 24-hour culture of epidermal cells or monocytes in DMEM containing 2-10% fetal calf serum. In order to measure thymocyte-stimulating activities, the CM had to be dialyzed first for 24 h against phosphate-buffered saline in order to remove inhibitory activities (26). Thymocyte-stimulating activity was determined and expressed in units/ml as described (29). In the following we use the term "thymocyte-stimulating activity," for the activity attributed to lymphocyte-activating factor or interleukin-1 of monocytes (33, 34) or to the epidermal cell-derived thymocyte-activating factor of epidermal cells (29) or COLO-16 cells (35).

In this paper we have employed two different CM of COLO-16 cells (1600 and 100 units/ml), two different CM of monocytes (1000 and 2100 units/ml), one CM of epidermal cells (2460 units/ml), and one G-100 fraction of CM from COLO-16 cells (2100 units/ml), one CM of epidermal cells (75 units/ml). To obtain CM with higher specific activities, the CM were concentrated between 5- and 28-fold by ultrafiltration using an Amicon ultrafiltration unit equipped with a YM-10 filter. A specific enrichment of thymocyte-stimulating activity was also achieved by gel filtration of 10-fold concentrated CM on Sephadex G-100 column using phosphate-buffered saline as eluant. The fractions eluted from the column in the region of M, = 10,000 and 30,000 epidermal cells, 75 units. To obtain CM with higher specific activities, the CM were concentrated between 5 and 28-fold by ultrafiltration using an Amicon ultrafiltration unit equipped with a YM-10 filter. A specific enrichment of thymocyte-stimulating activity was also achieved by gel filtration of 10-fold concentrated CM on Sephadex G-100 column using phosphate-buffered saline as eluant. The fractions eluted from the column in the region of M, = 10,000 and 30,000 units/ml.

In preparation.

**Cell Culture Condition and Radioactive Labeling**—For analyses of synthesis and secretion of proteins, cells were cultured in 6-well cluster plates (10 cm2 culture area). HepG2 cells were allowed to reach about 80% confluency. The treatments of primary cultures of mouse and rat hepatocytes were started 1 h after plating (17). The treatments were the same as outlined above; for each monolayer 1.0 ml of test medium containing either no further additions (=control), 1 μM dexamethasone, or various dilutions of CM. All CM were at least diluted 1:1 with fresh culture medium before adding to the culture medium (for details, see the legends to the figures and tables). Twenty-four h later the medium was replaced by fresh. After 48 h of treatment, the cell monolayers were washed three times with 2 ml of serum-free DMEM and then incubated with 1 ml of serum-free DMEM containing 10% of the normal concentration of methionine and 0.5-100 μCi of [35S]methionine (100 Ci/mmol, New England Nuclear). Six h later the radioactive media were collected and the few present cells were removed by centrifugation. To screen for activities in column fractions we used cells maintained in 24-well cluster plates (2 cm2 culture area).

The treatments were the same as above but proportionally less medium was used and no [35S]methionine was added to the medium of the final 6-h period. Aliquots of the media were subjected to two-dimensional gel electrophoresis or one-dimensional gel immunoelectrophoresis without further treatment, or, when used for crossed immunoelectrophoresis, were 10-30-fold concentrated by dialysis for 24 h against 50 mM NH4HCO3/0.1 mM phenylmethylsulfonyl fluoride followed by freeze-drying.

To obtain RNA for in vitro translation and for blot analysis, cells were maintained in 7.5-cm2 culture flasks. The treatments were the same as outlined above; for each monolayer 12 ml of test media was used.

**RNA Analysis**—Total DNA was extracted by the guanidine HCl procedure (36, 37) and translated in a reconstituted cell-free system (15, 38). Northern blot analysis was carried out by separation of 25 μg of RNA on agarose gels containing formaldehyde (39).
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TABLE I
Effect of CM of COLO-16 cells and activated monocytes on synthesis of secretory proteins in HepG2 cells

HepG2 cells in triplicate culture wells were treated for 48 h with: culture medium alone (control); CM of COLO-16 cells containing 50 units/ml of thymocyte-stimulating activity (CM-COLO); same, plus 1 µM dexamethasone (CM-COLO + Dex); G-100 fraction of CM of COLO-16 cells containing 60 units/ml of thymocyte-stimulating activity (G-100 fraction of CM-COLO); and CM of monocytes containing 170 units/ml of thymocyte-stimulating activity (CM-monocytes). After labeling of the cells with [35S]methionine (100 pCi/ml) for 6 h, the radioactivity incorporated into total cell and medium protein was measured. Twenty-five µl of labeled culture medium from each well was separated by two-dimensional gel electrophoresis (see Fig. 1), and the relative radioactivity present in the selected plasma proteins was determined. The values represent means and s.d. of the triplicate cultures.

| Treatment of cells | Incorporation | Relative incorporation (×10⁶) |
|--------------------|---------------|-----------------------------|
|                    | cpm/µg cell protein | Albumin | α₁-Fetoprotein | Transferrin | α₁-Antitrypsin | α₁-ACh |
| Control            | 32,100 ± 1,000 | 17,050 ± 470 | 1,122 ± 190 | 509 ± 10 | 550 ± 127 | 1,206 ± 246 |
| CM-COLO            | 34,350 ± 920  | 17,740 ± 1,540 | 611 ± 125 | 361 ± 55 | 307 ± 64 | 1,058 ± 185 |
| CM-COLO + Dex      | 34,770 ± 980  | 17,670 ± 1,100 | 675 ± 13 | 471 ± 61 | 269 ± 36 | 1,307 ± 89 |
| G-100 fraction     | 35,180 ± 3,310 | 17,790 ± 1,290 | 629 ± 86 | 306 ± 68 | 317 ± 74 | 1,007 ± 260 |
| CM-monocytes       | 34,270 ± 1,470 | 18,210 ± 1,660 | 522 ± 69 | 232 ± 6 | 252 ± 52 | 1,088 ± 177 |

FIG. 2. Combined effect of dexamethasone and CM of COLO-16 cells on the synthesis of secretory proteins of rat hepatocytes. Male rat hepatocytes were cultured for 48 h in normal culture medium (A), medium containing CM of COLO-16 cells with 50 units/ml of thymocyte-stimulating activity (B), medium containing 1 µM dexamethasone (C), and in medium containing both CM of COLO-16 cells and dexamethasone (D). The cells were labeled for 6 h with [35S]methionine (75 µCi/ml), and 50 µl of labeled medium were separated by two-dimensional gel electrophoresis. For the first dimension an ampholine composition was chosen which generated a narrower pH gradient than that in Figs. 1 and 4. This was necessary in order to achieve a better separation of the more acidic proteins. The fluorograms were exposed for 24 h. The spot indicated by numbers represent the following proteins: albumin (1), transferrin (2), α₁-antitrypsin (3), α₁-ACh (4), α₁-AGP (5), β-laptoglobin (6), haptoglobin (7), α₁-AP (8), α₂-MG (9), α₂-globulins (10), and protein increased after treatment with CM (14). BPB, bromphenol blue.
proteins were separated by two-dimensional polyacrylamide gel electrophoresis (42) and visualized by fluorography (43). Identification of product with differently glycosylated rat hepatocyte-derived α-ACh provided by Drs. R. E. Hill and N. D. Hastie, Medical Research Council CAPCI, Western General Hospital, Edinburgh, Britain.

**Analysis of Labeled Proteins**—Cellular and cell-free synthesized proteins were separated by two-dimensional polyacrylamide gel electrophoresis (42) and visualized by fluorography (43). Identification of the gel-separated spots was performed as follows (see also Ref. 17): by immunoprecipitation with monospecific antibodies: human, rat, and mouse albumin and transferrin; human α₂-fetoprotein, α₁-ACh; rat α₁-AP, α₁-AGP, α₂-MG; mouse SAA, haptoglobin, α₁-antitrypsin; by purification: human, rat, and mouse haptoglobin; rat α₂-antitrypsin and hemopexin; mouse hemopexin, α₁-AGP, and α₁-ACh; by cell-free translation of mRNA selected with specific cDNA containing insert sequences coding for: rat α₂-globulin and α₁-AGP (17); mouse major urinary proteins (44, 45), α₁-antitrypsin and α₁-ACh (46).3 All other cell-free synthesized products for which no specific cDNA probe were available, were identified by proteolytic mapping and comparison with the cellular synthesized counterparts (47).

Mouse α₁-ACh, which was purified by the method for human α₁-ACh (48) exhibited structural properties very similar to the human protein. Electrophoretic and functional properties, as well as the relative abundance in the circulation, suggest that the mouse α₁-ACh might be identical to the second trypsin-inhibitor "contrapsin" described by Takahara and Sinohara (49). Identification of rat α₁-ACh was based solely on mRNA selection using the cDNA probe for mouse α₁-ACh and proteolytic comparison of the cell-free synthesized product with differently glycosylated rat hepatocyte-derived α₁-ACh. The identification of this protein is, therefore, still considered to be tentative until confirmation by an independent technique.

In the gel pattern shown in the figures we have marked spots representing proteins which correspond in all three hepatic systems with the same number. The radioactivities present in the separated protein spots were determined as described (17). In order to compare the effects of various treatments, the measured values are expressed as fraction of the total protein-bound radioactivity analyzed.

Immunologic analyses of human and rat plasma proteins were carried out either by crossed or by rocket immunoelectrophoresis (50). Monospecific antibodies against the following proteins were used: human albumin, transferrin, and α₁-ACh (Miles Laboratories Inc); rat albumin (N. L. Cappel laboratories Inc.), α₁-AGP, α₁-AP, and α₂-MG. The amount of antigen present in the separated samples was determined by comparing the precipitin area to that of similarly separated standard protein. The values represent the means of the duplicate cultures. ND, not detectable.

**TABLE II**

| Treatment of hepatocytes | Incorporation | Relative incorporation (×10⁴) | Secretion rate |
|-------------------------|---------------|-------------------------------|---------------|
|                         | cpm/µg cell protein | α₂-MG | α₁-ACh | α₁-Anti-trypsin | Prohaptoglobin | ALbumin | α₂-MG | α₁-AGP | α₁-AP |
| Experiment I            |               |       |       |               |               |         |       |       |       |
| Control                 | 17,670        | 6,350 | ND    | 98           | 425           | 87       | 866   | ND    | 13     | 18     |
| Dex                     | 21,200        | 8,380 | 35    | 738          | 401           | 71       | 1,051 | 19    | 56     | 25     |
| CM-COLO                 | 20,430        | 7,900 | ND    | 184          | 293           | 67       | 820   | ND    | 67     | 38     |
| CM-COLO + Dex           | 27,150        | 11,500| 325   | 397          | 332           | 87       | 676   | 159   | 129    | 78     |
| CM-monoctyes            | 21,950        | 7,820 | ND    | 257          | 310           | 60       | 491   | ND    | 41     | 30     |
| CM-monoctyes + Dex      | 26,120        | 12,810| 296   | 365          | 403           | 70       | 641   | 110   | 75     | 54     |
| Experiment II           |               |       |       |               |               |         |       |       |       |
| Dex                     | 29,420        | 10,200| 20    | 235          | 431           | 56       | 512   | 45    | 38     | 35     |
| CM-COLO + Dex           | 36,450        | 15,160| 645   | 515          | 310           | 58       | 370   | 183   | 102    | 70     |
| CM-monoctyes            | 37,100        | 15,000| 515   | 670          | 403           | 73       | 353   | 121   | 86     | 60     |
| G-100 fraction of CM-monoctyes + Dex | 34,620 | 13,640| 521   | 499          | 356           | 136      | 388   | 77    | 71     | 57     |

**RESULTS**

**Effects of Conditioned Medium from COLO-16 Cells and Human Monocytes on the Production of Plasma Proteins in HepG2 Cells and Hepatocytes from the Rat and the Mouse**—HepG2 cells respond to a 48-h treatment with CM of COLO-16 cells, by a change in the synthesis of several plasma proteins (Fig. 1, A and B). Most notable difference is an increased amount of α₁-ACh. At the same time the production of albumin and transferrin appears to be reduced. There is no apparent change in the synthesis of haptoglobin, which in these hepatoma cells represents a relatively minor component. We were not able to detect any significant synthesis of other major acute phase proteins, such as C-reactive protein, SAA or α₂-MG, either in control or in CM-treated cells.

Since factors produced by activated monocytes were implicated in mediating the hepatic acute phase response (17, 21-23, 31), we determined the effect of CM derived from peripheral human monocytes on HepG2 cells (Fig. 1C). The monocyte conditioned medium showed similar activity as the
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Legend to Fig. 1: 1. a2-MG; 2. albumin; 3. aI-AP; 4. aI-ACh; 5. aI-haptoglobin; 6. haptoglobin; 7. transferrin; 8. aI-cysteine proteinase inhibitor. The second and third dimension gels (Table II) are shown.

COLO-16 medium: prominent reduction in the production of albumin, transferrin, and even a1-fetoprotein. However, the magnitude of increased a1-ACh production appears to be lower, even when we used CM with relative high thymocyte-stimulating activity (B), and medium containing both CM of monocytes and 1 mM dexamethasone (C). After labeling of the cells as described in the legend to Fig. 2, 500 ml of labeled medium were concentrated to 30 ml and then subjected to crossed immunoelectrophoresis. The second dimension gel contained a mixture of monospecific rabbit antibodies against the rat plasma proteins: albumin (1), a1-AGP (5), a1-AP (8), and a2-MG (9). Autoradiograms after 24-h exposure are shown.

Fig. 3. Effect of CM of monocytes on the secretion of the major rat acute phase reactants. Male rat hepatocytes were treated for 48 h with normal culture medium (A), medium containing CM of monocytes with 170 units/ml of a2-MG and then subjected to crossed immunoelectrophoresis. The second dimension gel contained a mixture of monospecific rabbit antibodies against the rat plasma proteins: albumin (1), a1-AGP (5), a1-AP (8), and a2-MG (9). Autoradiograms after 24-h exposure are shown.

Quantitative measurements (Table I) indicate that CM of COLO-16 cells and monocytes have no significant influence on the production of total cell and medium protein. At the concentration of CM used (the effect of different concentrations is described below), albumin and transferrin are almost reduced by half and a1-fetoprotein slightly less. The synthesis of a1-ACh is enhanced between 3- and 4-fold with CM of COLO-16 cells, but only 2-fold with CM of monocytes. The second major antiproteinase, a1-antitrypsin, is not affected by the same treatment although in vivo in man a1-antitrypsin is increased in inflammatory diseases (1). Surprisingly the presence of 1 mM dexamethasone in control cultures (data not shown) or in cultures treated with CM has no detectable influence on the synthesis of any plasma proteins. Treatment of HepG2 cells with a G-100 fraction of CM derived from COLO-16 cells and containing about the same thymocyte-stimulating activity as nonfractionated CM, has a much reduced capability to stimulate a1-ACh, although the effect on albumin, a1-fetoprotein, and transferrin is comparable. It appears that gel filtration might have resulted in the removal of some hepatic stimulating activity (see also Fig. 6).

The observation that keratinocytes and monocytes produce a new factor(s), which have the potential to modulate acute phase proteins in hepatic cells leads to two major questions. 1) Since HepG2 cells represent transformed liver cells and might, therefore, possess an altered response pattern, would normal adult hepatocytes react to the CM with a change of a wider spectrum of acute phase proteins, such as haptoglobin or a1-AP? 2) Is the hepatic stimulating activity from COLO-16 cells or human monocytes species specific? Answers to both questions were found in experiments using primary cultures of rat and mouse hepatocytes.

Rat liver cells are unique in that they respond to inflammation by a drastic increase in synthesis of specific acute phase proteins, a1-AP, a a1-cysteine proteinase inhibitor (52), and a1-MG (1). Furthermore, treatment of rat hepatocytes, but not HepG2 cells (Table I) or mouse hepatocytes (17), with glucocorticoids results in an elevated synthesis of several major acute phase plasma proteins, including a1-AGP and a1-MG (41). Treatment of primary cultures of adult rat hepatocytes with CM of COLO-16 cells elicited a complex pattern of changes in the production of plasma proteins (Fig. 2). a1-AGP and a1-ACh are increased by CM (Fig. 2B) and by dexamethasone (Fig. 2C), and this effect appears to be additive (Fig. 2D). A not yet identified protein with M, = 120,000 (spot 14) shows a similar behavior. A different mode of modulation can be seen for a2-MG. This protein is not synthesized in detectable amounts by untreated liver cells (Fig. 2A). Addition of CM to the hepatocyte culture has no inducing effect. In dexamethasone-treated cells a low level of a2-MG is produced. The combination of dexamethasone and CM has, however, potentiating activity. Surprisingly, there is no significant change in the production of haptoglobin (both pro- and a2-haptoglobin (spot 6). This protein represents a major acute phase reactant (10, 41).

In order to compare quantitatively the effects of treatments we determined the relative incorporation of [35S]methionine into the major plasma proteins clearly separated on two-dimensional gels (Table II). We noted in all experiments involving rat hepatocytes, that the combination of dexamethasone and CM of COLO-16 or monocytes have stimulatory activity on the uptake of the isotope (data not shown). Consequently, the specific activities of the cell and medium proteins are always higher in those cells. The measurements presented in Table II confirm the additive effect of dexamethasone and CM of COLO-16 cells on the synthesis a1-ACh and the potentiating effect on a2-MG. Again, there are no significant changes in the relative production of haptoglobin or a1-antitrypsin. Addition of CM of activated human monocytes provokes alterations in the plasma protein production by rat
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FIG. 4. Effect of CM of COLO-16 cells on the synthesis of secretory proteins of mouse hepatocytes. Female mouse hepatocytes were treated with the identical culture media as the rat hepatocytes in Fig. 3: A, control; B, CM of COLO-16 cells; C, dexamethasone; and D, CM of COLO-16 cells plus dexamethasone. The labeled secretory proteins were separated by two-dimensional gel electrophoresis. Fluorograms were exposed for 18 h. Spots indicated by numbers represent the following proteins: albumin (1), transferrin (2), α1-antitrypsin (3), α1-ACh (4), α1-AGP (5), β-haptoglobin (6), α-haptoglobin (6α), hemopexin (7), SAA (11), apolipoprotein A-1 (12), a protein increased after treatment with CM (14). BPB, bromophenol blue.

Effect of CM of COLO-16 cells and monocytes on mouse hepatocytes

Two independent preparations of male mouse hepatocytes (Experiments I and II) were treated either with media containing 1 μM dexamethasone (control) or dexamethasone-containing medium to which were added the same preparations of CM of COLO-16 cells and monocytes as used on the rat hepatocytes in Table II. The G-100 fraction of CM of COLO-16 cells contained 80 units/ml of thymocyte-stimulating activity. The cells were labeled with [35S]methionine (50 μCi/ml) for 6 h. The specific incorporation into total medium proteins was measured. Fifty μl of labeled medium were separated by two-dimensional gel electrophoresis (see Fig. 4) and the relative incorporation into the indicated proteins was determined. No correction for the overlap of hemopexin and albumin was done. The values represent means of duplicate cultures.

| Treatment of hepatocytes | Total medium protein | Relative incorporation (x10⁴) |
|-------------------------|----------------------|----------------------------|
|                         | Albumin | Transferrin | α1-Antitrypsin | α1-ACh | β-Haptoglobin | Hemopexin | α1-AGP | SAA |
| **Experiment I**        |         |             |               |        |              |          |        |     |
| Control                 | 3150    | 1542        | 170           | 406    | 328          | 104      | 194    | 22  |
| CM-COLO                 | 3980    | 1100        | 157           | 230    | 179          | 422      | 484    | 43  |
| G-100 fraction of CM-COLO | 2950   | 890         | 224           | 310    | 236          | 596      | 436    | 128 |
| CM-monocytes            | 3530    | 986         | 201           | 277    | 250          | 132      | 203    | 27  |
| **Experiment II**       |         |             |               |        |              |          |        |     |
| Control                 | 5140    | 1027        | 173           | 395    | 325          | 198      | 197    | 53  |
| CM-COLO                 | 6443    | 734         | 179           | 348    | 179          | 433      | 453    | 152 |
| CM-monocytes            | 6684    | 521         | 161           | 377    | 222          | 308      | 352    | 87  |

hepatocytes which are very comparable to those observed with CM of COLO-16 cells (Table II). The influence of lower concentration of monocyte CM is described below (Fig. 5). A G-100 fraction of monocyte medium, enriched for thymocyte-stimulating activity, still exhibit about the same spectrum of modulating activities as unfractionated CM containing a comparable concentration of thymocyte-stimulating activity.

Because two-dimensional gel electrophoresis does not yield a clear separation of α1-AP, α1-AGP, and albumin from other proteins, immunoelctrophoresis was employed to determine quantitatively their synthesis (Fig. 3). Immunoelctrophoresis has the advantage that absolute rather than relative amounts of plasma proteins can be measured (in Table II expressed as secretion rate). Treatment of hepatocytes with CM of COLO-16 cells or monocytes results in an up to 50% reduction of albumin synthesis, which is independent of the presence of dexamethasone. However, production of α1-AGP is influenced almost equally by dexamethasone and CM, whose activities are, as in the case of α1-ACh, additive. α1-AP, however, is not significantly affected by dexamethasone. Only in combination with CM an enhancing action is noted.

Recently we have shown that primary mouse hepatocytes represent a reproducible tissue culture system to study the regulation of acute phase plasma proteins (17). It is, therefore,
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A

FIG. 5. Correlation of thymocyte- and hepatocyte-stimulating activities. Serial dilutions of two different preparations each of CM of COLO-16 cells (closed circles) and CM of monocytes (open circles) were added to two independent preparations of male rat hepatocytes (A) and to HepG2 cells (B). All culture media contained in addition 1 μM dexamethasone. After 48 h of treatment, the cells were labeled for 6 h with [35S]methionine (5 μCi/ml). The labeled culture media were concentrated and aliquots were used for measurements of the indicated plasma proteins by rocket immunoelectrophoresis. The value of each culture is related to the concentration of thymocyte-stimulating activity.

of interest to see, whether the factors derived from COLO-16 cells or human monocytes can modulate the same spectrum of proteins as mouse acute phase plasma or CM of activated mouse monocytes. In addition we can compare the response pattern of mouse liver cells with that of rat hepatocytes and HepG2 cells, since these differ from the latter two in the production of high concentration of SAA (53). We treated primary cultures of adult mouse hepatocytes with the same CM of COLO-16 cells and monocytes as the rat hepatocytes. The gel electrophoretic analyses of the secretory proteins (Fig. 4; only the experiment with CM of COLO-16 is reproduced) and the quantitation of the relative incorporation (Table III) revealed that dexamethasone has no modulating activity on the action of the CM used. As described (17) the importance of dexamethasone lies in a better preservation of plasma protein production by the hepatocytes and in preventing the appearance of extraneous proteins (Fig. 4, A and C). The treatment of mouse hepatocytes with CM of COLO-16 cells leads to a 2-4-fold increase in the synthesis of haptoglobin, hemopexin, and α1-AGP. The magnitudes of these changes are comparable with those observed during an in vivo inflammation (17). SAA, however, is only minimally stimulated by COLO-16 cell medium. Transferrin and α1-antitrypsin are not significantly altered, while albumin and α1-ACh appear to be slightly reduced. Most of these modulating activities were also detected in G-100 fraction of CM from COLO-16 cells.

Mouse hepatocytes from the same preparation treated with CM of COLO-16 cells and with CM of monocytes showed that the latter was relatively ineffective. The stimulation of all major acute phase plasma proteins, especially haptoglobin, was much reduced as compared to that of CM of COLO-16 cells.

Comparison of the Thymocyte- and Hepatic-stimulating Activities Produced by Epidermal Cells and Monocytes—A correlation between thymocyte-stimulating activity (interleukin-1/lymphocyte-activating factor/epidermal cell-derived thymocyte-activating factor) and stimulation of SAA synthesis has been suggested by experiments of several laboratories (23, 28, 29). Since CM of COLO-16 cells and of monocytes contain thymocyte- as well as hepatocyte-stimulating activities, both testable in tissue culture cells, we have, therefore, a good opportunity to demonstrate a possible correlation of the activities. Analyses of G-100 fractions of CM from COLO-16 cells from monocytes, which contain the majority of thymocyte-stimulating activity, have already indicated that most but not all of the hepatocyte-stimulating activities are also present in these fractions (Tables I-III). To compare thymocyte- and hepatocyte-stimulating activities present in the same CM preparation we performed two separate determinations: 1) analyzing the response of HepG2 cells and primary cultures of rat hepatocytes to treatments with various dilutions of CM of COLO-16 cells and monocytes, and 2) separating the activities by gel filtration on Sephadex G-100.

The effects on the main regulated plasma proteins were determined and related to the concentration of thymocyte-
stimulating activity present in the test cultures (Fig. 5). It is apparent that not all hepatocyte-stimulating activities coincide with thymocyte-stimulating activity, for CM of COLO-16 cells has roughly 10-fold higher stimulatory activity than CM of monocytes on α2-MG and α1-AP in rat hepatocytes (Fig. 5A) and on α1-ACh in HepG2 cells (Fig. 5B). There are, however, no significant differences in modulation of albumin and α1-AGP in rat hepatocytes and of albumin and transferrin in HepG2 cells. Higher stimulation of the cells with CM of monocytes could not be achieved because concentrations of CM with thymocyte-stimulating activity above 300 units/ml were found to be inhibitory (see reduction in the secretion rates).

As noted above, G-100 fractions from CM of COLO-16 cells or monocytes do not contain the full spectrum of hepatocyte-stimulating activities as found in nonfractionated CM (Tables I–IV). This apparent loss of activities might be due to removal of components with molecular weights outside of the selected range of 10,000–30,000. To test this possibility, we chromatographed CM of COLO-16 cells and monocytes on Sephadex G-100 and measured the elute for both hepatocyte- and thymocyte-stimulating activities (Fig. 6). The majority of thymocyte-stimulating activity in CM of monocytes and COLO-16 cells migrated with an apparent Mr = 15,000–20,000. Activities regulating α2-MG in rat hepatocytes showed the same chromatographic profile as the activities regulating α1-ACh and albumin in HepG2 cells, but differed significantly in their elution position from the thymocyte-stimulating activity (Fig. 6A, A and B). The major peak of liver-regulating activities of monocytes has an apparent Mr = 25,000–30,000 (Fig. 6A). Surprisingly, CM of COLO-16 cells contained an additional peak of hepatocyte-stimulating activity in the Mr = 50,000–60,000 range (Fig. 6B). This higher molecular weight component co-migrated with the major activity present in CM of mouse peritoneal monocytes which stimulate the production of α2-MG in rat hepatocytes (Fig. 6C). A similar modulation of α1-ACh in HepG2 cells could not be demonstrated because the human cells did not show any significant response to the mouse-derived factors.

The question arises as to whether the production of relative high concentration of hepatocyte-stimulating activities by COLO-16 cells is specific to this tumor cell line or whether other epidermis-derived cells have the same properties. Therefore, we tested the activities present in the CM of normal epidermal cells, which have been found to release thymocyte-activating factor (epidermal cell-derived thymocyte-activating factor, 2B). Rat hepatocytes and HepG2 cells were exposed to the same test media containing CM of epidermal cells or of COLO-16 cells and then analyzed for the change in the synthesis of the major acute phase plasma proteins (Table IV). Surprisingly, CM of epidermal cells has an even higher stimulatory effect on the production of α1-ACh in HepG2 cells than CM of COLO-16 cells. In rat hepatocytes, however, CM of epidermal cells is less active than CM of COLO-16 cells, as notable in the reduced stimulation of α2-MG and α1-AP. An exception is α1-ACh which is equally well modulated by both CM. When CM of epidermal cells is fractionated by gel filtration, the fraction enriched for thymocyte-stimulating activity also contains hepatocyte-stimulating activities as was already observed for G-100 fractions of CM of COLO-16 cells (Table I).

Effect of Conditioned Medium of Epidermal Cells and Monocytes on mRNA Levels in Rat Hepatocytes and HepG2 Cells—CM of COLO-16 cells and monocytes are both able to provoke a relative large increase in the production of several plasma proteins in rat hepatocytes and HepG2 cells. The magnitude of change appears to be sufficient to be used for determining whether the increased cellular synthesis is correlated with an elevated level of functional mRNA for these proteins. The use of rat hepatocytes for this analysis is advantageous since we have already characterized the in vivo acute phase response

![Fig. 6. Separation of hepatocyte- and thymocyte-stimulating activities by gel filtration.](image)
of rat liver on the level of translatable RNA (41).

To assess changes in mRNA population, primary cultures of adult rat hepatocytes were treated for 48 h with or without factors from COLO-16 cells or monocytes and in the presence or absence of dexamethasone. RNA were extracted and translated in a cell-free system, and the products were separated by two-dimensional polyacrylamide gel electrophoresis. Regardless of the sex of the animals from which the hepatocytes derived, essentially identical results were obtained (an example for male hepatocytes is reproduced in Fig. 7): CM of COLO-16 cells and of monocytes cause, in the presence or absence of dexamethasone, a reduction in mRNA for albumin. Translatable mRNA for α1-AGP is increased independently by dexamethasone and by CM, and the effect appears to be additive (data not shown, for hybridization see below). Whether α1-ACh follows the same mode of regulation is not clearly apparent because the cell-free synthesized protein overlap to some degree with actin on two-dimensional gels. A clear difference between the two is that the cell-free product of the latter species is achieved by immunoprecipitation from the translation mixture of the liver RNA (41). Under any treatments of tissue culture cells, the mRNAs for haptoglobin and α1-AP are not nearly as much increased as they are in liver cells during acute inflammation. Then, mRNA for these proteins represent, next to that for α1-AGP, the major induced species in liver cells (see Fig. 7D and Ref. 41).

Since we have available a cDNA probe to mRNA for rat α1-AGP (41) and human α1-ACh (3) we followed the change of hybridizable RNA by Northern blot analysis (Fig. 8). As far as visual comparison of the hybridization intensities allows, an additive action of dexamethasone and CM of COLO-16 cells on rat α1-AGP can be observed (Fig. 8A). Under optimal conditions, treatment of rat hepatocytes with CM of monocytes yields a level of α1-AGP mRNA which is comparable to

### Table IV

| Treatment of cells | Total medium protein (cpm/μg cell protein) | Relative incorporation (×10⁴) | Secretion rate (ng/h × 10⁶ cells) |
|--------------------|------------------------------------------|-----------------------------|----------------------------------|
| Rat hepatocytes    |                                          |                             |                                   |
| Control            | 1240                                     | α2-MG: 29 α1-ACh: 561 α1-AGP: 390 | α1-AP: 12 α1-AP: 44 α1-AP: 29 |
| CM-epidermal cells | 2480                                     | 640 α1-AP: 980 α1-AP: 460      | 82 α1-AP: 57 α1-AP: 80           |
| G-100 fraction     | 1170                                     | 360 α1-AP: 1164 α1-AP: 200     | 49 α1-AP: 49 α1-AP: 45           |
| CM-COLO            | 2180                                     | 1019 α1-AP: 1003 α1-AP: 340    | 125 α1-AP: 71 α1-AP: 120         |
| HepG2              |                                          |                             |                                   |
| Control            | 930                                      | 1100 α1-ACh: 80               |                                   |
| CM-epidermal cells | 1000                                     | 750 α1-ACh: 305               |                                   |
| CM-epidermal cells | 1040                                     | 900 α1-ACh: 238               |                                   |
| (1:10 diluted)     | 980                                      | 700 α1-ACh: 220               |                                   |
| CM-COLO            | 1160                                     | 650 α1-ACh: 140               |                                   |

**Fig. 7.** Effect of CM of COLO-16 cells on translatable mRNA in rat hepatocytes. Male rat hepatocytes were treated for 48 h with normal culture medium (A) and with medium containing 1 μM dexamethasone plus CM of COLO-16 cells with 50 units/ml of thymocyte-stimulating activity (B). The RNA were extracted and translated in a cell-free system. The products were separated by two-dimensional gel electrophoresis. For comparison, the in vitro translation products from RNA of a liver 48 h after an in vivo inflammation were similarly separated (C). Identification of the cell-free products of α1-AP is achieved by immunoprecipitation from the translation mixture of the liver RNA (D). Spots indicated by numbers represent the following proteins: albumin (1), α1-antitrypsin (3), α1-ACh (4), α1-AGP (5), haptoglobin (6), hemopexin (7), α1-AP (8), α2-MG globulin (10), and actin (A).
Fig. 8. Modulated concentration of α1-AGP mRNA in rat hepatocytes and α1-ACh in HepG2 cells. A, total RNA, which have been extracted from cultured rat hepatocytes, were separated on agarose gel, transferrred to nitrocellulose, and probed with nick-translated p10-14 DNA, containing complementary sequence to rat α1-AGP mRNA. The fluorographic image was exposed for 5 days. The lanes were rearranged and represent the following RNA samples: 1–5, female rat hepatocytes immediately after perfusion (1); or treated for 48 h with normal medium (2), medium containing CM of COLO-16 cells with 50 units/ml of thymocyte-stimulating activity (3), medium with 1 μM dexamethasone (4), and medium containing both CM of COLO-16 cells and dexamethasone (5). Lane 6 contains RNA from rat hepatocytes treated for 48 h with medium containing 1 μM dexamethasone plus CM of monocytes with 170 units/ml of thymocyte-stimulating activity. B, total RNA from control HepG2 cells (1) and cells treated for 48 h with CM or epidermal cells with 100 units/ml of thymocyte-stimulating activity (2) were analyzed by Northern blot analysis using as probe nick-translated pHAC1 DNA containing complementary sequence to human α1-ACh mRNA. The same blot was exposed for 24 h (a) and for 5 days (b). The numbers to the left indicate the positions of the size markers (in kilobases): 28 S (5.0) and 18 S (1.9) ribosomal RNA, and rat α1-AGP mRNA (0.9).

that in cells exposed to CM of COLO-16 cells. Since nontreated hepatocytes have a very low amount of α1-AGP mRNA, a quantitative correlation between the change in mRNA level and cellular synthesis of the protein could not be performed. Such a comparison was, however, possible in the case of HepG2 cells. Treatment of these cells with CM of epidermal cells resulted in an elevated concentration of α1-ACh mRNA (Fig. 8B). Spectrophotometric measurements of major mRNA band with an apparent size of 1800 nucleotides indicated a 7-fold increase in hybridization, that correlated well with an 8-fold increased rate of α1-ACh synthesis of the same cells.

Taken together these results suggest that factors produced by human epidermal cells and activated monocytes are capable of stimulating cultured liver cells to express genes for acute phase plasma proteins. This stimulation results in an accumulation of functional mRNA in the cells, which may account entirely for increased synthesis and secretion of the acute phase plasma proteins.

Discussion

The most interesting result of our studies is that human monocytes and epidermal cells produce factors which specifically modulate the production of several major acute phase proteins in hepatic cells from man, rat, and mouse. The factors from epidermal cells and monocytes appear, however, not to possess identical spectra of liver-regulating activities. Furthermore, our results suggest that the hepatocyte-stimulating activities are structurally distinct from those stimulating proliferation of thymocytes.

None of the CM tested was able to initiate a full spectrum of changes in plasma protein synthesis as found in liver cells during an acute phase reaction. The restricted response pattern of HepG2 cells can probably be attributed to the transformed nature of the hepatoma cells. Indeed, the analysis of the plasma proteins from these cells indicated that at least one important acute phase reactant, α1-AGP, is not significantly expressed (Ref. 31 describes the plasma protein production of the original cell line). It appears that the HepG2 hepatoma cells have lost the ability to synthesize and to regulate this protein. However, this may be a more general phenomenon since Ko et al. (54) recently reported the inability of rat Morris Hepatoma 7777 to increase synthesis of several acute phase proteins after in vivo inflammation.

A restricted potential of CM to modulate the acute phase proteins was, however, also observed when we employed nontransformed hepatocytes. For instance, rat hepatocytes show no change in haptoglobin and a minor increase in α1-AP (Table II), while mouse hepatocytes responded with a rather insignificant increase in synthesis of SAA (Table III). All these proteins are synthesized at greatly increased rates by liver cells of inflamed animals (17). This incomplete response of tissue culture cells might have two reasons: 1) the CM tested do not contain all activities required for full stimulation of all acute phase proteins, or 2) the factors in CM would be sufficient, but the tissue culture conditions are not optimal. We and others have recently described that culture conditions have indeed a remarkable influence on the response pattern of mouse hepatocytes (17, 51, 55). Most notable is that the adult phenotype of the cells is not maintained in tissue culture, as apparent in the loss of hormone responsiveness and of the production of specific plasma proteins, and in the appearance of new forms of secretory proteins. From these observations, it must be concluded that the tissue culture environment most likely does not provide the entire spectrum of factors, which would be required to elicit a fully concerted change in expression of acute phase proteins.

Although our tissue culture systems do not strictly correspond to in vivo conditions, they nevertheless represent a suitable model to identify specific regulations associated with hepatic acute phase response. These regulatory mechanisms would be quite difficult to assess in vivo. The usefulness of hepatocytes as a test system is best illustrated in the case of rat hepatocytes. The results (Figs. 2 and 3, Tables II and IV) revealed that there are remarkable differences in the spectrum of factors, which would be required to elicit the hormonal requirements for the regulation of the major acute
phases. Previous studies with rats have indicated that α1-AGP can be stimulated by either dexamethasone or a stress-associated factor that is different from adrenal gland hormones (41). At that time it was not possible to answer the question whether the two active components act additively.

The present studies have now shown that the rat hepatocytes are indeed capable of responding to a factor of noncorticos-teroid nature by increase in α1-AGP and that the response is additive with that of dexamethasone (Table II, Fig. 8A). Moreover, since our studies are not limited to an individual plasma protein, we could also recognize a similar behavior of α1-ACh, but also a fundamentally different one for α2-MG.

In our studies we have analyzed two parameters: the effect of different CM and the responses of different hepatic cells. In essence we found that the influence of CM of epidermal cells, COLO-16 cells, or monocytes on the hepatic cells was not identical. HepG2 cells showed the highest stimulation of α1-ACh when treated with CM of epidermal cells (Table IV), but rat hepatocytes were more sensitive to CM of COLO-16 cells. Moreover, CM of monocytes, unlike CM of COLO-16 cells, lacked stimulating activity for mouse haptoglobin (Table III). At present, we do not know yet the basis for the differences between the results of the test cells and between the stimulating activities of the various types of CM. Possibilities are that either some activities are lost during culturing of the cells or during medium preparations, or the cells, which were used as a source of CM, produce a different composition of factors, each with a specific modulating activity on liver cells. Furthermore, we cannot rule out that there are some species-specific differences in the response of the hepatic cells to the human factors.

Our results demonstrate that we have in COLO-16 cells an important source of a factor, or factors, which modulate activity of liver cells. It is therefore imperative to characterize biochemically these activities and to compare their properties with those of the activities from activated monocytes. Such analyses will also indicate whether the activities are structurally and/or functionally related to any of the described lymphocyte-modulating activities (58), especially to already identified thymocyte-stimulating activities such as interleukin-1. We are currently in process to determine to what extent the hepatocyte-stimulating factor(s) produced by epidermal cells are recognized by antibodies against human endogenous pyr-o-gen of leukocytes (56). A further goal will be to assess whether the epidermal cell-derived factor(s), which exhibit hepatocyte-stimulating activities in tissue culture, do also play any role in regulating liver cells in vivo and whether these participate in mediation of the hepatic acute phase response.

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