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Killed *Escherichia coli* Stimulates Macrophage-Mediated Alterations in Hepatocellular Function During In Vitro Coculture: a Mechanism of Altered Liver Function in Sepsis

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Hepatic dysfunction is a poorly understood and highly lethal component of multiple-system organ failure. Both in vivo and in vitro studies of "liver" function have generally neglected hepatocyte-Kupffer cell interactions. In the following experiments, isolated hepatocytes were cocultivated with unstimulated peritoneal cells, predominately macrophages, which served as a readily available Kupffer cell analog. Coculture of hepatocytes with peritoneal cells resulted in little or no change in [^{3}H]leucine incorporation into hepatocyte protein. When gentamicin-killed *Escherichia coli* cells (GKEC) were added to coculture, there was a marked decrease in hepatocyte [^{3}H]leucine incorporation. In contrast, GKEC added to hepatocytes alone had no effect. Kinetic data revealed an 8-h delay before any significant decrease in leucine incorporation into hepatocyte protein after the addition of GKEC to the coculture. The maximal decrease in hepatocyte [^{3}H]leucine incorporation occurred 24 h after GKEC were added. The decrease observed 24 h after GKEC were added disappeared almost completely after 48 h of coculture. Similar alterations in cocultured hepatocyte protein synthesis were observed after the addition of phorbol myristate acetate, lipopolysaccharide, or muramyl dipeptide, a component of bacterial peptidoglycan. Hepatocyte viability by trypan blue exclusion was unchanged, and gross morphology by light or electron microscopy was unaffected. We propose that during sepsis, macrophages (Kupffer cells) respond to circulating microbial products and mediate alterations in hepatocyte function. These experiments underscore the important role of Kupffer cell function in attempts to understand hepatic malfunction in multiple-system organ failure.

Hepatic dysfunction is frequently associated with uncontrolled sepsis (6, 7, 31, 42). We hypothesized that alterations in hepatocellular function might arise from interactions between hepatocytes and contiguous phagocytic Kupffer cells. Although evidence of intrahepatic Kupffer cell division has been reported (3), it is generally thought that Kupffer cells are derived from bone marrow (17) precursors and repleted by circulating monocyte-macrophages (36). Previous investigations have shown that macrophages can mediate alterations in adipocyte (33), adrenal cortical cell (27), and muscle cell (8) metabolism. Kupffer cells have likewise been shown to modulate hepatocyte carbohydrate metabolism (15) and fibrinogen biosynthesis (37). In addition, interleukin-1 (14) a product of activated macrophages, is well known to increase hepatocyte acute-phase protein synthesis (24, 43).

Because cells of monocyte-macrophage lineage have known cytotoxic potential (32), we hypothesized that alterations in hepatocellular function in sepsis might possibly arise from interaction between hepatocytes and the phagocytic Kupffer cells that lie in direct contiguity with hepatocytes. To test whether the macrophages could mediate alterations in hepatocyte function, we examined leucine incorporation into protein of cultured hepatocytes in vitro. We compared leucine incorporation into hepatocyte protein of hepatocytes cultured alone or cocultured with a Kupffer cell analog (peritoneal macrophages).

The present studies were designed to clarify several issues raised in our previous report of a macrophage-mediated decrease in hepatocyte protein synthesis during in vitro cocultivation (22). Those studies utilized unstimulated peritoneal cells (70% macrophages) cocultured with freshly isolated hepatocytes. Thus, our results could have been a reflection of aggravated damage to hepatocytes rather than induced changes in function. In the experiments performed here we used hepatocytes in stable cultures (19) containing serum (18), insulin (41), and dexamethasone (24). Further, we stimulated the macrophages with a nonviable bacterial inoculum to simulate a septic stimulus.

MATERIALS AND METHODS

Hepatocyte isolation. Adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 200 to 300 g were utilized for hepatocyte isolation. Unfasted rats were anesthetized with intraperitoneal pentobarbital (Abbott Laboratories, North Chicago, Ill.), and then the skin was washed with betadine solution. The abdomen was opened widely, and hepatocyte isolation was performed by a modification of the Seglen perfusion technique (38). Briefly, the portal vein was cannulated, and the liver was perfused at 37°C in situ with a calcium-free solution containing 0.145 M NaCl, 6.7 mM KCl, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4; GIBCO Laboratories, Grand Island, N.Y.), 2.4 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid, and 1% bovine albumin (Sigma Chemical Co., St. Louis, Mo.). This perfusion was continued for 7 min, then the perfusate was switched to 0.05% collagenase (Sigma; C-0130 type I, 200 U/mg), 67 mM NaCl, 6.7 mM KCl, 100 mM HEPES buffer (pH 7.6), and 1% albumin. While the perfusion

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continued, the hepatic attachements were divided, and the liver was transferred to a sterile Büchner funnel. The collagenase perfusion continued ex vivo for a total perfusion time of 10 min. After this the liver capsule was incised, and the liver was gently combed to produce a hepatocyte suspension. The hepatocytes were washed three times in minimal essential medium (GIBCO) with centrifugation at 60 × *g* between washes. The cells were enumerated, and their viability was determined by trypan blue exclusion. This procedure usually yielded 2.0×10^8 to 3.0×10^8 hepatocytes (80 to 90% viability). After enumeration the suspension was diluted in culture medium and plated.

Peritoneal cell isolation. Peritoneal cells were obtained from adult male Sprague-Dawley rats weighing 200 to 300 g. A peritoneal cell harvest solution was prepared containing minimal essential medium (pH 7.4), 7 mg of low-endotoxin fetal calf serum (FCS; Hyclone) per dl, 10,000 U of penicillin per dl, 10 mg of streptomycin per dl, and 500 U of heparin per dl. A sample (20 to 25 ml) of this solution was drawn into a sterile syringe and injected percutaneously into the peritoneal cavities of the rats, which had been previously sacrificed by CO_2 asphyxiation. The abdominal cavity was agitated for 1 to 2 min and then opened aseptically through a generous midline incision, and the lavage fluid was removed with a sterile pipette. The cells were transferred to a 50-ml conical plastic centrifuge tube (Becton Dickson and Co., Paramus, N.J.) and centrifuged at 250 × *g* for 5 min. Hypotonic lysis of contaminating erythrocytes was performed by suspension in 0.2% NaCl, followed immediately by 1.6% NaCl to restore isotonicity. The cells were centrifuged at 250 × *g*, and the resulting pellet was suspended in 5% FCS–Williams E culture medium. The cells were enumerated and viability determined. This procedure yielded approximately 1.0×10^7 to 2.0×10^7 peritoneal cells per rat with a viability of >95%. The differential count of the peritoneal cells was 70% macrophages, 8% lymphocytes, and 25% granulocytes (predominately mast cells and eosinophils).

Cell culture techniques. The enumerated hepatocytes were suspended in Williams medium E (GIBCO) containing 6.5 ml of HEPES buffer per dl, 1.0 μM insulin (U-100 Iletin; Eli Lilly Co., Indianapolis, Ind.), 1.0 μM dexamethasone (Sigma), 30 mg of L-glutamine (Sigma) per dl, 10,000 U of penicillin G per dl, 10 mg of streptomycin per dl, and 10 ml of low-endotoxin FCS per dl (22). The final cell concentration, 5×10^4 hepatocytes, was added in 1.0 ml to 15-mm Linbro (Flow Laboratories, Inc., Rockville, Md.) tissue culture wells. The hepatocytes were incubated at 37°C in 5% CO_2 and air. After 2 to 3 h of incubation the medium was changed. The hepatocytes were allowed to recover function for at least 24 h before the biochemical studies or the addition of peritoneal cells.

After the initial 24-h culture period in 10% FCS–Williams E containing 1.0 μM dexamethasone, the medium was changed to 5% FCS–Williams E with 0.1 μM dexamethasone. Peritoneal cells were added at appropriate dilutions in Williams medium E containing 0.1 μM dexamethasone and 5% FCS. In general coculture was established 24 h before the addition of gentamicin-killed *Escherichia coli* cells (GKEC) or other stimuli. Peritoneal cells had been cultured for 48 h in vitro before the [^{3}H]leucine pulse.

Tritiated leucine incorporation into protein. At intervals dictated by the experimental design, protein synthesis was assessed by measuring tritiated leucine incorporation into protein. Protein labeling was performed by exchanging culture medium for 1.0 ml of leucine-free Eagle minimal essential medium to which 1 to 2 μCi of L-[4,5-^{3}H]leucine (5.0 mCi/mol; New England Nuclear Corp., Boston, Mass.) had been added. Incorporation of radioactive leucine into protein was allowed to continue for 1 to 4 h as dictated by the experiments. At the end of the [^{3}H]leucine pulse, the cells were lysed by the addition of 1 ml of 0.1% Triton X-100 (Rohm and Haas, Philadelphia, Pa.). After the addition of the detergent samples of the cell lysate were transferred to glass centrifuge tubes and precipitated with ice-cold 10% trichloroacetic acid. The protein precipitates were washed three or four times in 3 ml of 10% trichloroacetic acid with centrifugation at 750 × *g* between washes. The resulting pellet was solubilized in 0.5 M Protosol (New England Nuclear) and 7 ml of scintillation cocktail (made by adding 8.4 g of 2,5-diphenyloxazole from RPI to 1 gallon [ca. 3.79 liters] of toluene) was added. Radioactivity was enumerated with a Beckman LS 2800 liquid scintillation counter. Separate [^{3}H]leucine incorporation into protein was measured in hepatocytes alone, hepatocytes plus peritoneal cells in coculture, and peritoneal cells alone. The portion of [^{3}H]leucine incorporation by hepatocytes in coculture was determined by subtracting the peritoneal cell counts per minute from coculture counts per minute. Appropriate controls were performed with specific hepatotoxins (galactosamine) to inhibit hepatic protein synthesis. Peritoneal cell protein synthesis was shown not to be affected by the presence of hepatocytes. As a control for nonspecific trapping of unincorporated [^{3}H]leucine in the protein precipitate, a blank well without cells was carried through identical culture and assay techniques. This blank consistently yielded values <2% of [^{3}H]leucine incorporation into hepatocyte protein.

Bacterial killing. The *E. coli* strain used in these experiments was a clinical isolate which is maintained in our laboratory. The virulence of this organism remained consistent in in vivo studies. Gentamicin killing was performed (11) by incubating washed, enumerated, stationary-phase susceptible bacteria in 20 ml of brain heart infusion containing 40 μg of gentamicin sulfate per ml on a rotor mixer at 37°C for 2 h. The treated bacteria were sedimented by centrifugation at 1,600 × *g* for 10 min and washed twice by suspension in sterile normal saline. The nonviability of gentamicin-killed organism was confirmed by 24-h culture of a 0.1-ml sample of suspended bacteria.

Methods of in vitro peritoneal cell stimulation. (i) Killed bacteria. Killed bacteria were prepared as described above and added to cocultures at intervals dictated by individual experiments as a suspension in 50 μl of culture medium (5% low-endotoxin FCS–Williams E).

(ii) Lipopolysaccharide. Appropriate dilutions were made in culture medium, and lipopolysaccharide from *E. coli*

TABLE 1. Effect of duration of in vitro culture on hepatocyte protein synthesis

Culture time (h)[a]	[^{3}H]Leu incorporation into protein	
	cpm ± SEM	% ± SEM[b]
24	18,233 ± 253	100.0 ± 1.9
48	18,618 ± 673	102.1 ± 3.7
72	17,935 ± 603	98.4 ± 3.3
96	15,436 ± 591	84.7 ± 3.2

[a] Replicates of 12 wells were analyzed for each time point. The culture medium was changed daily.

[b] Percentage of 24 h hepatocyte counts per minute.

O11B4 (Difco Laboratories, Detroit, Mich.) was added in a 50-μl volume to yield final concentrations of 5 or 20 μg/ml.

(iii) Muramyl dipeptide. 9-Acetyl-L-alanyl-D-isoglutamine (Calibiochem-Behring, La Jolla, Calif.) was reconstituted in 2 ml of sterile normal saline to achieve a stock concentration of 500 μg/ml. This stock solution was diluted in culture medium immediately before use so that a 50-μl volume yielded a final concentration of 1 μg/ml.

(iv) Phorbol myristate acetate. Phorbol myristate acetate (Sigma; P-8139) was stored at −70°C in 1 mg of dimethyl sulfoxide per ml. Samples were diluted in normal saline to 0.1 mg/50 μl immediately before use and added to cultured cells in this volume.

Electron microscopy. Cultured cells grown on 15-mm Thermanox plastic cover slips (LUX Scientific Corp.) were quickly rinsed in isotonic phosphate-buffered saline (pH 7.4) and then immediately fixed with 2.0% glutaraldehyde in phosphate-buffered saline for 1 h at 20°C. The cells were washed in phosphate-buffered saline and postfixed in 1.0% osmium tetroxide for 1 h at 20°C. The cells were washed again. After graded ethanol dehydration they were critical point dried with a Dupont-Sorvall apparatus. The specimens were then coated with gold-palladium alloy and examined in a Hitachi 5-450 scanning electron microscope.

Statistical analysis. At least three separate wells constituted an experimental group. The mean, standard deviation, and standard error were calculated for each group. Leucine incorporation by hepatocytes in coculture was calculated by subtracting the counts per minute (cpm) of a parallel group of Kupffer cells from the counts per minute of hepatocytes plus Kupffer cells [calculated hepatocyte cpm = (cpm of hepatocytes and Kupffer cells) − (cpm of Kupffer cells alone)]. The pooled variance and standard error were determined for cocultured hepatocyte counts per minute. When pooled data representing several experiments were analyzed, the means of the groups were compared, and the variance was expressed by standard error. The Student *t*-test was used for statistical analysis. Linear regressions were performed with the least-squares method, and the coefficient of determination (R^2) was calculated.

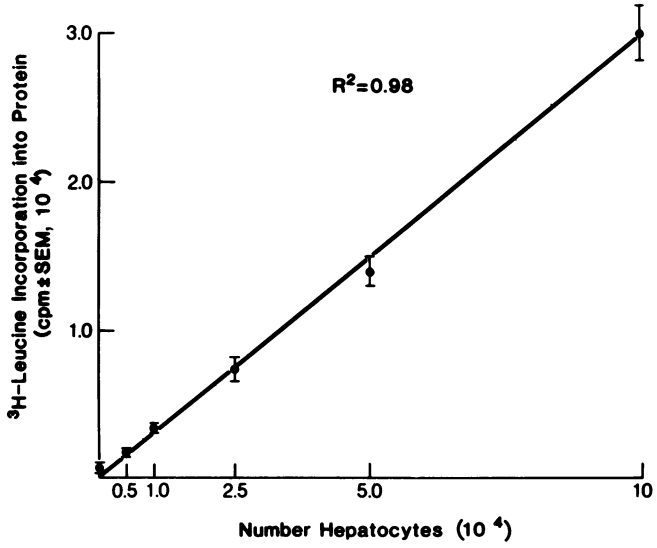

FIG. 1. Hepatocytes (5×10^3 to 1×10^5) were plated in 1.0 ml of Williams medium E containing 10% FCS and 10 μM dexamethasone. The cells were allowed to recover function for 24 h, after which the medium was changed to Williams E with 5% FCS and 0.1 μM dexamethasone. After an additional 24-h culture period a 4-h pulse of [^{3}H]leucine (7 μCi) in leucine-free minimal essential medium was added. [^{3}H]leucine incorporation into protein was measured after 10% trichloroacetic acid precipitation. The regression line and coefficient of determination (R^2) were calculated by using the mean counts per minute for each group.

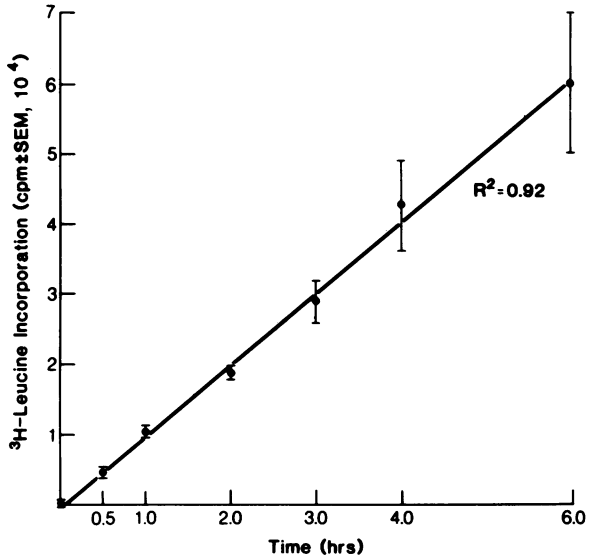

FIG. 2. Time course of [^{3}H]leucine incorporation into hepatocyte protein. Hepatocytes (5×10^4) were obtained and plated in 10% FCS–Williams E containing 10 μM dexamethasone for 24 h to allow recovery of function. The medium was changed after 24 h to Williams E containing 5% FCS and 0.1 μM dexamethasone. After an additional 48-h culture period a 7-μCi pulse of [^{3}H]leucine was added for 0, 0.5, 1.0, 2.0, 3.0, 4.0, or 6.0 h, and incorporation into protein was measured after cell lysis and protein precipitation in 10% trichloroacetic acid. The regression line and coefficient of determination (R^2) were calculated by using the mean counts per minute for each time interval.

RESULTS

The use of these modified culture and medium conditions allowed us to maintain hepatocyte function (leucine incorporation into hepatocyte protein) at >85% of base line for at least 96 h in culture (Table 1). Trypan blue exclusion was likewise unaffected throughout this extended culture period. Figure 1 shows that [^{3}H]leucine incorporation into hepatocyte protein was a linear function of the number of hepatocytes in culture. Furthermore, over the range of interest, cell crowding had no significant effect on incorporation of [^{3}H]leucine into protein. We chose to do subsequent studies with 5×10^4 hepatocytes. The time course of [^{3}H]leucine incorporation into hepatocyte protein is shown in Fig. 2. This shows that after a 5 to 10 min lag, incorporation of [^{3}H]leucine into protein was linearly related to the duration of labeling for up to 6 h. Subsequent experiments, described below, generally utilized a 2 to 4 h labeling pulse.

Using these modified culture conditions, we cocultured the hepatocytes with unstimulated resident peritoneal cells (Table 2). In contrast to our previous results with freshly isolated hepatocytes, which showed a decrease in protein synthesis with coculture (26), our modified culture conditions now showed a mild dose-dependent increase in hepatocyte protein synthesis after coculture with unstimulated peritoneal cells. The hepatocytes in these experiments

TABLE 2. Incorporation of [^{3}H]leucine into protein by hepatocytes alone or cocultivated with peritoneal cells

No. of peritoneal cells	Effector cell/hepatocyte ratio	[^{3}H]Leu incorporation (% ± SEM)[a]			
		Hepatocytes alone	Peritoneal cells alone	Hepatocytes cocultured with peritoneal cells	Calculated hepatocytes in coculture
0	0:1	100.0 ± 7.5			
0.05×10^6	1:1		3.6 ± 0.2	104.8 ± 7.6	101.2 ± 7.6[b]
0.25×10^6	5:1		11.1 ± 0.2	120.9 ± 5.1	109.8 ± 5.1[b]
0.5×10^6	10:1		20.3 ± 0.3	151.5 ± 5.6	131.2 ± 5.6[c]
1.0×10^6	20:1		27.9 ± 1.0	153.0 ± 7.0	125.2 ± 7.1[c]

[a] Results are expressed as the percentage of counts per minute incorporated by 5×10^4 hepatocytes (100% is 20,330 ± 1,531 cpm).
[b] P is not significant compared with hepatocytes alone.
[c] $P < 0.05$ compared with hepatocytes alone.

had been cultured in vitro for 72 h, and coculture had been established for 48 h at the time of the [^{3}H]leucine pulse.

Because our hypothesis involved possible activation of Kupffer cells by bacterial products leading to altered hepatocellular function, we next investigated how such stimuli affected our in vitro hepatocytes. In our coculture system peritoneal macrophages were intended to serve as a readily available, unstimulated, Kupffer cell analog. We examined the effect of the addition of GKEC to cocultures. Killed bacteria were utilized to avoid potential contamination problems. We utilized high effector cell/hepatocyte ratios (20:1) in these experiments to exaggerate any possible effects. Table 3 shows that addition of GKEC to cocultured hepatocytes resulted in a marked decrease in hepatocyte protein synthesis after 24 h. In contrast, the same inoculum of GKEC added to hepatocytes alone had no significant effect. Protein synthesis by peritoneal cells alone was actually lower 24 h after stimulation with GKEC. This experiment utilized a fixed dose of GKEC such that the ratio of bacteria/peritoneal cell was 5:1. This inoculum is one that we have previously found to be rapidly and completely phagocytosed (11).

To determine whether the observed changes after GKEC stimulation were due to particulate phagocytosis, we next examined the effect of a soluble stimulus, *E. coli* (O11B4) lipolysaccharide. We found identical changes when 5 or 20 μg of lipopolysaccharide was added to cocultured hepatocytes. Again, the addition of lipopolysaccharide to hepatocytes in the absence of peritoneal cells had no significant effect. Similar changes were seen with muramyl dipeptide, a component of bacterial peptidoglycan. This suggests that the effects seen after the addition of GKEC or lipopolysaccharide were not due solely to endotoxin or to particulate phagocytosis. Dead bacteria, lipopolysaccharide, and muramyl dipeptide are stimuli that are all known to activate macrophages and that could potentially circulate during clinical sepsis. Phorbol myristate acetate is a different type of soluble stimulus that will also activate macrophages. The addition of phorbol myristate acetate to cocultures of hepatocytes and macrophages resulted in a significant, but less marked, decrease in protein synthesis after 24 h, but did not alter the protein synthesis of hepatocytes cultured alone. Phorbol myristate acetate, however, did not depress leucine incorporation in the macrophage alone. These experiments suggested that diverse stimuli, known to activate macrophage function in several different ways, all resulted in decreased hepatocyte leucine incorporation when cocultured, but had no direct effect on hepatocytes cultured alone.

To further characterize this phenomenon, we concentrated on GKEC as the stimulus most readily applicable to the clinical situation. Admittedly, nonviable bacteria represent a complex stimulus that may simultaneously affect macrophages via several mechanisms. The addition of GKEC to cocultures of macrophages and hepatocytes appeared to have an effect at all effector/target cell ratios (Fig.

TABLE 3. Effect of the addition of GKEC, lipopolysaccharide, or muramyl dipeptide on incorporation of [^{3}H]leucine into protein by hepatocytes and peritoneal cells alone or in coculture

Additive[a]	[^{3}H]Leu incorporation (% ± SEM)[b]			
	Peritoneal cells alone (10^6)	Hepatocytes plus peritoneal cells in coculture[c]	Hepatocytes alone (5×10^4)	Calculated hepatocytes in coculture
Medium (control)	42.9 ± 5.4	131.4 ± 9.7	100.0 ± 5.4	88.5 ± 11.1[d]
GKEC (5×10^6)	17.1 ± 2.7	37.0 ± 6.7	94.4 ± 8.2[d]	19.9 ± 7.2[e]
LPS (5 μg/ml)	21.1 ± 1.7	42.3 ± 2.5	97.6 ± 1.7[d]	22.8 ± 3.0[e]
LPS (20 μg/ml)	20.9 ± 2.4	52.6 ± 6.0	105.5 ± 3.0[d]	31.7 ± 6.7[e]
MDP (1 μg/ml)	23.3 + 4.2	46.1 + 7.8	109.8 + 2.7[d]	21.2 + 8.9[e]
PMA (0.1 μg/ml)	60.0 ± 6.6	125.0 ± 12.3	91.4 ± 3.4[d]	64.6 ± 14.0[f]

[a] Abbreviations: LPS, lipopolysaccharide; MDP, muramyl dipeptide; PMA, phorbol myristate acetate.
[b] Results are expressed as the percentage of counts per minute incorporated by control hepatocytes (100% is 8,729 ± 474 cpm).
[c] Cocultures contained 5×10^4 hepatocytes and 1×10^6 peritoneal cells.
[d] P is not significant compared with hepatocytes alone.
[e] $P < 0.01$ compared with hepatocytes plus respective additives.
[f] $P < 0.05$ compared with hepatocytes plus phorbol myristate acetate.

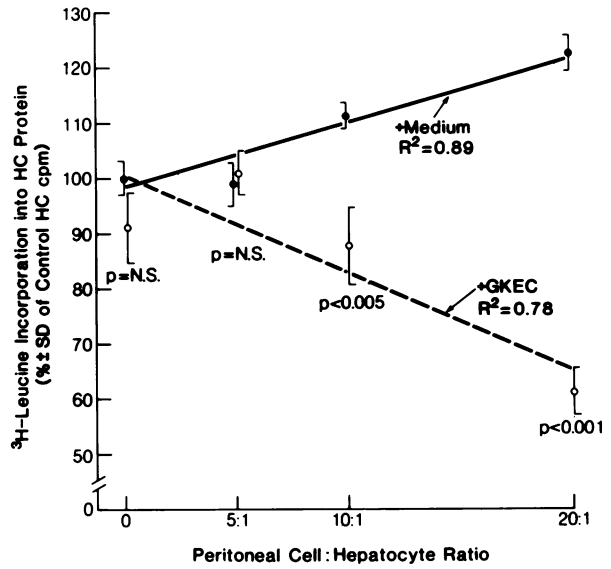

FIG. 3. Dose response of increasing peritoneal cell/hepatocyte ratio on hepatocyte protein synthesis after the addition of medium or GKEC. Hepatocyte coculture was established as described in Table 3. A constant ratio of 5 GKEC per peritoneal cell or medium was added in a 50-μl volume at each peritoneal cell/hepatocyte ratio. After 24 h the cells were pulsed for 4 h with 7 μCi of [^{3}H]leucine, and incorporation into protein was measured after precipitation in 10% trichloroacetic acid. The results shown represent pooled data from four experiments. The mean and standard deviation were determined for each. The counts per minute for each experiment are converted to percentages of counts per minute from 5 × 10^4 hepatocytes.

3). Under these culture conditions, at least, no plateau in response was seen. In these experiments the inoculum of GKEC was adjusted to maintain a constant ratio of 5 bacteria per plated peritoneal cell. No gross morphological abnormalities were apparent despite the marked alterations demonstrated in cocultured hepatocyte protein synthesis. Hepatocyte viability as assessed by trypan blue exclusion was not changed throughout the experimental culture period and could not account for the observed results.

Figure 4 shows the time course of the altered protein synthesis of hepatocytes cocultured with macrophages versus hepatocytes or macrophages cultured alone. This experiment clearly demonstrated that there was an 8-h lag before any demonstrable change in cocultured hepatocyte protein synthesis. A maximal decrease in hepatocyte protein synthesis was seen after 24 h of coculture.

In a subsequent experiment (Table 4), we demonstrated that the decrease seen 24 h after the addition of GKEC was almost completely reversed by 48 h after the addition of the stimulus. In contrast, hepatocytes alone or in coculture without GKEC or hepatocytes alone with GKEC showed a slight increase in protein synthesis after an additional 24 h culture period. This is strong evidence that the observed alterations are reversible. To ensure that macrophages were indeed responsible for the alterations seen with coculture, we enriched the proportion of macrophages by utilizing their properties of adherence. In this experiment (Fig. 5), hepatocytes were plated and allowed to recover function for 24 h. Coculture was then established with resident peritoneal cells at an effector/hepatocyte ratio of 20:1. Coculture was established, and macrophages were allowed to adhere for 24 h, after which they were vigorously washed to remove the nonadherent cells. This left a residual population that was enriched in macrophages. Although washing was performed in situ, we have found that cultured hepatocytes are very adherent, and the washing did not alter hepatocyte leucine incorporation. We compared the ability of peritoneal cells or macrophages in coculture to alter leucine incorporation into hepatocyte protein after the addition of medium or GKEC, respectively. The addition of GKEC resulted in identical decreases in hepatocyte protein synthesis whether hepatocytes were cocultured with peritoneal cells or adherent peritoneal macrophages. Furthermore, reconstitution of the peritoneal cell population by the addition of the nonadherent cells to the adherent macrophages did not significantly alter the response. In contrast, coculture of the nonadherent cells and hepatocytes followed by GKEC triggering was not different from the response after the addition of culture medium to hepatocytes and nonadherent cells. Qualitatively, the response seen after the addition of GKEC to hepatocytes cocultured with nonadherent cells was identical to that observed when GKEC were added to hepatocytes cultured alone.

DISCUSSION

The etiology of the hepatic dysfunction seen in sepsis is poorly understood (6, 29). The data presented here are consistent with our hypothesis that macrophages or Kupffer

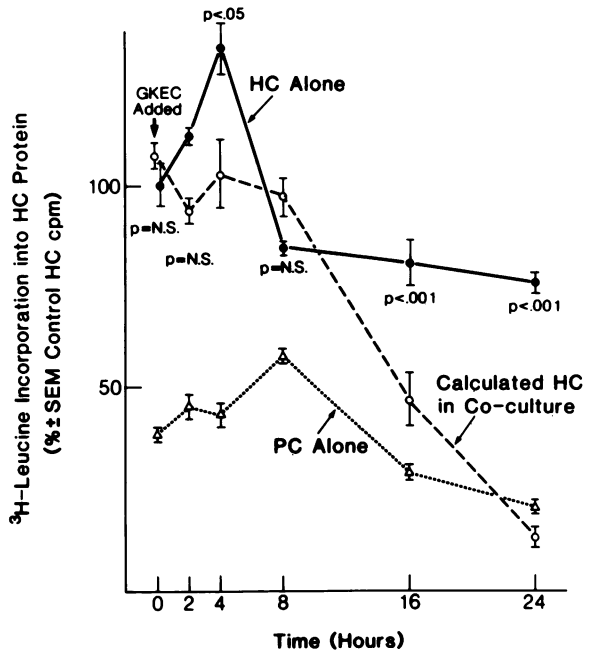

FIG. 4. Time course of protein synthesis by hepatocytes alone or cocultured with peritoneal cells after the addition of GKEC. Coculture was established as described in Table 3 at a ratio of 20 peritoneal cells per hepatocyte. During the final 24-h period GKEC were diluted in medium and added in a 50-μl volume (5 bacteria per peritoneal cell) at specified times before labeling. All parallel groups were pulsed with 10 μCi of [^{3}H]leucine per well in leucine-free minimal essential medium for 2 h, 22 h after GKEC had been added to the first coculture group. Therefore the total duration of in vitro culture or coculture was identical for all groups when protein synthesis was measured. The results are normalized to [^{3}H]leucine incorporation by 5 × 10^4 hepatocytes (control hepatocyte value is 13,054 ± 783 cpm).

TABLE 4. Effect of time after the addition of GKEC on hepatocyte incorporation of [^{3}H]leucine into protein

Time (h) after additive[a]	Additive	[^{3}H]leu incorporation into protein (% ± SEM)[b]			
		Peritoneal cells alone (10^6)	Hepatocytes plus peritoneal cells in coculture	Hepatocytes alone	Calculated hepatocytes in coculture
24	Medium	59.8 ± 5.4	147.4 ± 5.9	100.0 + 3.7	88.9 + 8.0[c]
	GKEC	43.7 ± 1.9	85.0 ± 3.0	91.8 + 4.1[c]	42.4 + 3.6[d]
48	Medium	81.5 ± 2.9	203.0 ± 5.0	127.4 + 2.2	124.2 + 5.8[e]
	GKEC	104.7 ± 1.0	199.7 ± 6.2	128.7 + 4.2[e]	97.8 + 6.3[f]

[a] Parallel wells were incubated for 24 and 48 h after the addition of GKEC or medium.
[b] Results are expressed as the percentage of counts per minute incorporated by control hepatocytes at 24 h (100% is 9,402 ± 349 cpm).
[c] P is not significant compared with 24-h hepatocytes alone.
[d] $P < 0.01$ compared with 24-h hepatocytes plus GKEC.
[e] P is not significant compared with 48-h hepatocytes alone.
[f] $P < 0.01$ compared with 48-h hepatocytes plus GKEC.

cells may be capable of altering, modulating, or regulating at least one hepatocellular function during sepsis. In these experiments, resident peritoneal cells were used as Kupffer cell analogs. The use of peritoneal cells allowed us to avoid potential problems associated with the severe enzymatic digestion procedures used to obtain Kupffer cells. We have clearly demonstrated that peritoneal cells, properly stimulated, mediate alterations in cocultured hepatocyte leucine incorporation into protein. In these experiments leucine incorporation into total cellular protein was measured. This parameter was chosen because it should be a highly sensitive indicator of integrated hepatocellular function. Most previous investigations of liver function in shock or sepsis have examined secretory proteins (26, 28, 29). In the culture system described herein, supernatant secretory protein represented 10 to 20% of the total.

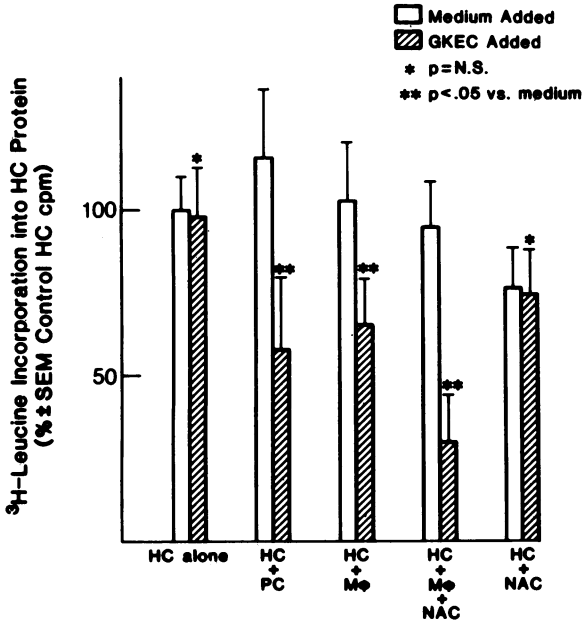

FIG. 5. Comparison of hepatocytes (HC) cultured alone or cocultured with various fractions of peritoneal cells (PC) after the addition of GKEC. Hepatocyte culture was as described in Table 3. One group representing peritoneal cells was left undisturbed. Another group had the nonadherent cells (NAC) removed by vigorous washing, leaving a population enriched in peritoneal macrophages (Mo). The nonadherent were removed, pooled, washed, enumerated, and added to hepatocytes alone or hepatocytes in coculture with macrophage enriched peritoneal cells at a ratio of 6 nonadherent cells per hepatocyte. After 2 h all groups were stimulated by the addition of GKEC (5×10^6 per well) or medium in a 50-μl volume. After a 24-h culture period the cells were pulse-labeled with 7 μCi of [^{3}H]leucine per well, and incorporation into 10% trichloroacetic acid precipitable protein measured. The results are the percentages of values for 5×10^4 hepatocytes (control hepatocyte value is 11,267 ± 665 cpm).

Decreased [^{3}H]leucine incorporation into hepatocyte protein will be referred to below as decreased hepatocyte protein synthesis. It is not clear whether the observed alterations in hepatocyte function represent modulation of normal cell function or actual injury of the hepatocyte. From the data presented we infer that these hepatocytes were not injured in a conventional sense. Nonetheless, the effects of these alterations could be deleterious to the host. Microscopic morphology and trypan blue exclusion were unchanged after the addition of GKEC or other stimuli that result in marked decreases in hepatocyte protein synthesis. Furthermore, the fact that this perturbation in hepatocellular function appeared to be partially reversible with additional culture argues against a cytolytic mechanism.

In these studies hepatocyte protein synthesis in coculture was calculated by subtracting the protein synthesized by a parallel group of peritoneal cells cultured alone from the total protein synthesis in coculture. We have previously shown that the presence of hepatocytes does not alter leucine incorporation into peritoneal cell protein (22). Indeed, if coculture caused increased peritoneal cell protein synthesis, the subtraction of a larger peritoneal cell contribution to coculture [^{3}H]leucine counts per minute would exaggerate the observed decrease in hepatocyte protein synthesis in coculture. Cell crowding effects did not appear to account for the results, since, in the absence of activating stimuli, coculture resulted in a small dose-dependent increase in hepatocyte protein synthesis. Coculture with nonadherent peritoneal cells, with or without GKEC, likewise had no effect on hepatocellular function. We have previously shown that coculture with spleen cells (22) or neutrophils (manuscript submitted for publication) have no effect upon hepatocyte protein synthesis.

Several different stimuli including GKEC, lipopolysaccharide, muramyl dipeptide, and, to a lesser extent, phorbol myristate acetate triggered cell-mediated alterations in cocultured hepatocyte protein synthesis without affecting protein synthesis of hepatocytes cultured alone. These stimuli represent diverse classes of soluble and particulate trig-

gering agents. Most recently we have observed that heat-killed *Bacteroides fragilis* and *Staphylococcus epidermidis* mediate similar results (data not shown). It is possible that minute quantities of contaminating endotoxin were present in the diluent of these additives, but this is unlikely because similar endotoxin contamination would occur in the untriggered peritoneal cells, which did not alter hepatocyte function.

The findings presented differ from the previously reported results of peritoneal cell coculture with freshly isolated hepatocytes, which resulted in decreased protein synthesis without additional triggering agents (22). We speculate that the resident peritoneal cells in our earlier reports were activated either in vivo by a subclinical infection, during the isolation and purification procedure, by hepatocyte membrane damage, or by particulate debris. In the present experiments hepatocytes were allowed to recover from their isolation procedure before the addition of peritoneal cells. Alternatively, the presence of dexamethasone (0.10 μM) during coculture in these experiments may have suppressed the peritoneal cells, although the addition of a sufficient stimulus (GKEC) could override this suppression.

One property shared by these diverse stimuli is the ability to cause macrophage activation (32). Activation of macrophages is not an all-or-none phenomenon, but rather a spectrum of changes in baseline activities above those of unstimulated cells. Cohn (9) has proposed that activation occurs in stages dependent on the activating stimulus. Stimuli that will activate macrophages, monocytes, and Kupffer cells (12, 21, 32) include bacteria, endotoxin, chemotactic peptides, muramyl dipeptide, phorbol myristate acetate, zymosan, complement components, immune complexes, hypoxia, and specific cellular targets. Macrophage activation has been noted to be predominantly a local event (10, 40). The potential for activated macrophages to injure other cells or to mediate altered functions has been correlated with their ability to release several substances including active oxygen intermediates, lysosomal enzymes, activated complement components (5), plasminogen-activating factor, arachidonic acid metabolites, prostaglandins (30), and cytokines such as interleukin-1 (14). We have measured interleukin-1 release by our peritoneal cells by using the LAF assay as an indicator of their activation state. Resident peritoneal cells released significant amount of interleukin-1 with only a slight additional increase after GKEC triggering (submitted for publication).

The mechanism by which peritoneal macrophages mediate the alterations in hepatocyte protein synthesis after triggering is under investigation. The significant time delay between GKEC addition and decreased hepatocyte protein synthesis and the lack of apparent cytotoxicity do not support oxygen radicals as mediating the observed alterations. We have previously shown that the addition of hydrogen peroxide causes an immediate, rather than delayed, decrease in hepatocyte protein synthesis (G. A. Keller, R. Barke, J. T. Harty, E. Humphrey, and R. L. Simmons, Arch. Surg. in press). Furthermore, in experiments with exogenous hydrogen peroxide altered protein synthesis was accompanied by marked morphological changes and decreased viability. Preliminary experiments in which dimethyl sulfoxide, catalase, or superoxide dismutase was added to GKEC-triggered coculture showed no protective effect. We favor the concept that one or more macrophage-derived factors are secreted after appropriate stimulation and mediate the alterations in hepatocyte function. To date, inconclusive results have followed attempts to reproduce the observed coculture alterations with transfer of 24-h GKEC coculture supernatants to hepatocytes alone. This inability to reproduce the alterations in hepatocyte function by supernatant transfer does not disprove that soluble macrophage-derived factors are involved. It is possible that such a factor may be labile or rapidly degraded. High local concentrations achievable only when the cells are in close proximity may be required. Another possible explanation is that the microenvironment of macrophages cocultured with hepatocytes is hypoxic. Knighton et al. (23) have shown that hypoxia can be a powerful stimulus to induce macrophage activation. However, to date we cannot rule out the possibility that cell-cell contact is required.

Decreases in hepatocyte total protein synthesis after macrophage coculture as shown in these experiments have not been described. Plasma concentrations of albumin, transferrin, and alpha-2 HS glycoprotein have been reported to fall during the acute phase response (26), although it is unclear whether this reflects decreased hepatic synthesis, increased peripheral catabolism, or both. In vitro work with hepatocytes shows a decrease in albumin synthesis concomitant with increased acute-phase protein synthesis (37). There is growing evidence that the systemic manifestations of sepsis, fever (2), muscle wasting (1, 43), and synthesis of the hepatic acute phase proteins (14) may be mediated by monokines released during reticuloendothelial system activation. Many other, less well characterized, macrophage-derived soluble mediators undoubtedly are also released during sepsis.

These studies have shown that macrophages exposed to diverse stimuli can induce reversible alterations in hepatocellular function. These stimuli may well circulate during sepsis. Kupffer cells share with macrophages most of the major alterations seen after activation (30, 32, 34). Evidence supporting a role for Kupffer cell-mediated hepatic injury in systemic sepsis has been presented by several authors (4, 13, 39). Indirect evidence suggest that Kupffer cells form a cooperative system with hepatocytes (16, 20, 35, 40). Such observations support the concept that the Kupffer cell acts to receive stimuli and transmit messages to the hepatocyte, modulating its response, and that the hepatocyte feeds back information in turn. We are presently refining the model to utilize Kupffer cells rather than peritoneal macrophages in coculture. The data presented herein emphasize that macrophage and Kupffer cell function is critical in attempts to understand alterations in liver function during sepsis.

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