Prostaglandin E_2 Production During Hepatic Regeneration Downregulates Kupffer Cell IL-6 Production

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The liver possesses the remarkable ability to regenerate to its original size after a 70% partial hepatectomy. There has been little effort to characterize the Kupffer cells' role in this unique mammalian reparative physiologic phenomenon. The capacity of rat Kupffer cells (KC) isolated at specific intervals after partial hepatectomy to produce interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) in response to endotoxin was evaluated in standard RPMI-1640 (1200 μM L-arginine) and arginine-depleted RPMI-1640 (10 μM L-arginine) media. Regenerating liver KC 48 to 120 hours after partial hepatectomy responded to endotoxin stimulation with a significantly greater ($p < 0.05$) production of IL-6 in standard RPMI-1640. Because Kupffer cells function in an environment where high arginase activity results in negligible L-arginine levels, the 10 μM L-arginine RPMI-1640 was used to simulate the true hepatic microenvironment. Production of IL-6 by regenerating liver KC was further increased ($p < 0.05$) by placing these same KC in 10 μM L-arginine RPMI-1640 tissue culture media. During the same period, regenerating liver KC produced significantly ($p < 0.01$) more PGE_2 than sham-operated KC in both standard and low-arginine media. When the cyclo-oxygenase inhibitor indomethacin (1×10^{-5} M) was added to cultures, the PGE_2 production was inhibited, and IL-6 production was upregulated ($p < 0.05$) in arginine-depleted cultures. The authors conclude that during hepatic regeneration KC IL-6 production is elevated but controlled in an autoregulatory fashion by KC PGE_2 production.

MAMMALIAN LIVER POSSESSES a remarkable regenerative capacity after surgical partial hepatectomy or hepatic injury.[1] Recognition of this unique mammalian process has led to extensive research attempting to identify substances that initiate and terminate hepatic regeneration.[2,3] These investigative efforts have identified several humoral factors,[4–6] portal blood factors,[2,7] and liver-derived growth factors[3,8] that stimulate liver regeneration in *in vivo* experimental models, but little effort has been made to characterize the alterations that occur in the liver's resident macrophage, the Kupffer cell (KC), during this unique phenomenon. Several investigators have suggested that the reticuloendothelial system plays an important role in regeneration of the rat liver because reticuloendothelial system blockade depressed hepatocellular proliferation after partial hepatectomy.[9,10]

Kupffer cells constitute over 90% of the host reticuloendothelial system,[11] and are juxtaposed anatomically with the hepatocytes, which are the predominant regenerating parenchymal mass.[12] West et al.[13,14] reported that the stimulated Kupffer cell may inhibit the types and quantities of individual proteins being synthesized by hepatocytes, and may play a role in the development of hepatic failure during sepsis, endotoxemia, or after extended liver resection. Therefore, prevention of the negative impact of the KC on hepatocyte function may promote hepatic regeneration and improve the survival of the host during these periods of physiologic stress. Kupffer cells produce the cytokine interleukin-6 (IL-6), which is known to affect the hepatocytes' acute phase response to injury[15] and plays a role in hepatocyte proliferation.[16] Prior studies on hepatic regeneration and KC–hepatocyte interactions have not determined the influence of this cytokine nor the L-arginine concentration of the tissue culture medium being used. Callery et al.[17] have recently reported that the production of prostaglandin E_2 (PGE_2)

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by the KC during the initial stage of hepatic regeneration was elevated and that the KC production of tumor necrosis factor-α was markedly decreased.[18] Because KC production of cytokines and eicosanoids may affect the systemic immune response, as well as the hepatocyte responses, we compared the capacity of KCs with other macrophage populations to produce IL-6 and determined whether this production was regulated by PGE_2 in both standard RPMI-1640 (1200 μM L-arginine) and low arginine (10 μM L-arginine) RPMI-1640 tissue culture media during hepatic regeneration after a 70% partial hepatectomy in the rat.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 225 to 250 g were used at 10 to 12 weeks of age, and were cared for according to specific National Institutes of Health guidelines. Animals were provided a nutritionally balanced rodent diet (5001, Purina Mills, St. Louis, MO) and water *ad libitum* and were not fasted before use.

Operative Procedures

Under ether anesthesia, 70% partial hepatectomies (PHx) were performed through a 3.0-cm. midline incision by resecting the median and left lobes of the rat liver, as outlined by Higgins and Anderson.[1] Sham operations consisted of a midline laparotomy with gentle manipulation and exteriorization of the median and left liver lobes. All operations were performed between 8:00 and 10:00 A.M. to avoid the effects of diurnal variations on the regenerative response.[19]

Kupffer Cell Isolation and Purification

Kupffer cells were harvested from animals for *in vitro* analysis at 48, 120, 168, and 336 hours after partial hepatectomy or sham operations. While under anesthesia, the animal was exsanguinated and the liver was perfused *in situ* through the portal vein with Hank's balanced salt solution (HBSS). The livers were dissected free and passed through 60-μm brass screens into HBSS containing 1% HEPES buffer and 1% penicillin/streptomycin. After centrifugation at 500*g* for 10 minutes (4 C), the pellet was resuspended in 30 mL antibiotic-supplemented HBSS containing 0.01% collagenase (type I, 240 U/mg, Worthington Biochemicals, Freehold, NJ), 0.001% deoxyribonuclease (type 1, 60 U/mg, Sigma Chemicals, St. Louis, MO), and 1 mM $CaCl_2$, and incubated for 45 minutes in a 37 C agitating water bath. After centrifugation at 50*g* for 2 minutes to sediment hepatocytes, an enriched nonparenchymal cell pellet was obtained by centrifugation of the supernatant at 500*g* for 10 minutes. Erythrocytes were lysed by incubation for 3 to 5 minutes with ammonium chloride (0.83%). The cells were washed twice with HBSS, and resuspended in either standard RPMI-1640 (1200 μM L-arginine) or low-arginine (10 μM) RPMI-1640 tissue culture media without arginine containing antibiotics and 10% fetal calf serum (low-endotoxin, heat-inactivated, Gibco, Grand Island, NY). After 4 hours of incubation at 37 C in 100-mm plastic petri dishes (Costar, Cambridge, MA), nonadherent cells were removed by three successive washes with warm HBSS. Adherent cells, phagocytosing 0.8 μm latex beads (Sigma), are designated as KCs. After 10 minutes' incubation at 37 C in Trypsin-ethylenediaminetetra-acetic acid (0.05%/0.02%), KC were liberated from the plastic dishes by vigorous pipetting, and washed and counted. Kupffer cell purity was >95% by latex bead ingestion, and viability was > 90%, as indicated by trypan blue exclusion (0.4% trypan blue stain in 0.85% saline, Gibco Laboratories).

Splenic, Peritoneal, and Alveolar Macrophage Isolation

Resident splenic, peritoneal, and alveolar macrophages were isolated from the same rats providing KC. Peritoneal macrophages were isolated before the harvest of liver by repeatedly lavaging the intact abdominal cavity with 37 C HBSS. Alveolar macrophages were recovered after KC harvest by directly cannulating the trachea and repeatedly lavaging the tracheobronchial tree with 37 C HBSS. Splenic macrophages were harvested by passage of the spleen through a 60-μm brass screen, differential centrifugation, and plastic adherence, as described for the KC isolation technique. Ninety-five per cent of the splenic, alveolar, and peritoneal macrophages excluded trypan blue and phagocytized latex beads.

Culture Media

Kupffer cells (5×10^5) were suspended in one of two different preparations of culture media of RPMI-1640 (Gibco Laboratories, Grand Island, NY) containing 1% HEPES buffer, 1% nonessential amino acids, 1% L-glutamine, 1% penicillin/streptomycin, and 10% fetal calf serum (low endotoxin, Hyclone Labs Inc., Logan, UT). Arginine concentration in the culture media was the only variable in these experiments. Standard RPMI-1640 (+Arg) contained 1200 μM L-arginine, whereas arginine-depleted RPMI-1640 (−Arg) contained only approximately 10 μM L-arginine (verified by high-performance liquid chromatography performed by the Gibco Corp., Grand Island, NY), which was derived from the addition of 10% fetal calf serum.

Culture Protocol

After purification, 5×10^5 sham or PHx KC, splenic macrophages, peritoneal macrophages, and alveolar mac-

rophages in 1 mL complete KC media were plated in 24-well culture dishes (Costar). After overnight culture, KC were repleted with fresh media and stimulated with 2.5 μg/mL bacterial lipopolysaccharide (LPS, *Escherichia coli* type 0111:B4, Difco Laboratories, Detroit, MI). Supernatants were collected at timed intervals, sterile filtered (0.2-μm Acrodisc, Gelman Sciences, Ann Arbor, MI), and stored at −80 C until use.

Prostaglandin E_2 Radioimmunoassay

Partial hepatectomy and sham KC supernatants were assayed for PGE_2 by competitive binding radioimmunoassay. Samples were combined with ^3H-labeled PGE_2 (New England Nuclear, Boston, MA) and a specific rabbit antisera to PGE_2 (provided by Dr. Aubrey Morrison, Department of Pharmacology, Washington University). After a minimum 12-hour incubation at 4 C, the excess ^3H-PGE_2 was removed with a charcoal–dextran mixture and the bound portion counted by liquid scintillation spectroscopy. Triplicate values were averaged and compared with a standard curve performed with each assay. The lack of cross-reactivity of the antisera with competing eicosanoids has been previously verified.[20]

Interleukin-6 Bioassay

Partial hepatectomy and sham KC supernatants were collected at timed intervals and IL-6 activity measured by proliferation of the IL-6–responsive B9.9 cell line (provided by Dr. Lucian Aarden, Netherlands, through Dr. Richard Nordan, National Institutes of Health). Serially diluted supernatant samples were incubated at 37 C in 5% CO_2 for 44 hours with 2×10^3 B9.9 cells. ^3H-thymidine (2 μCi/well) was added for the final 4 hours of culture. The cultures were harvested at 48 hours after culture initiation by using an automatic cell harvester onto glass fiber filters. Radioactivity was determined by liquid scintillation spectrometry using an LKB (1272 Clinigamma, LKB, Turku, Finland) liquid scintillation counter. Unknown supernatant IL-6 levels were determined by comparison with a standard curve of B9.9 proliferation to known quantities of recombinant human IL-6 (Genzyme, Boston, MA).

Statistical Analysis

Data are representative of three independent experiments, each performed in triplicate, and analyzed for significance by analysis of variance.

Results

Effects of 70% Partial Hepatectomy on KC IL-6 Production

Hepatic regeneration is greatest 48 to 120 hours after 70% PHx. Regenerating KC demonstrated a significantly ($p < 0.05$) greater capacity to produce IL-6 at all culture

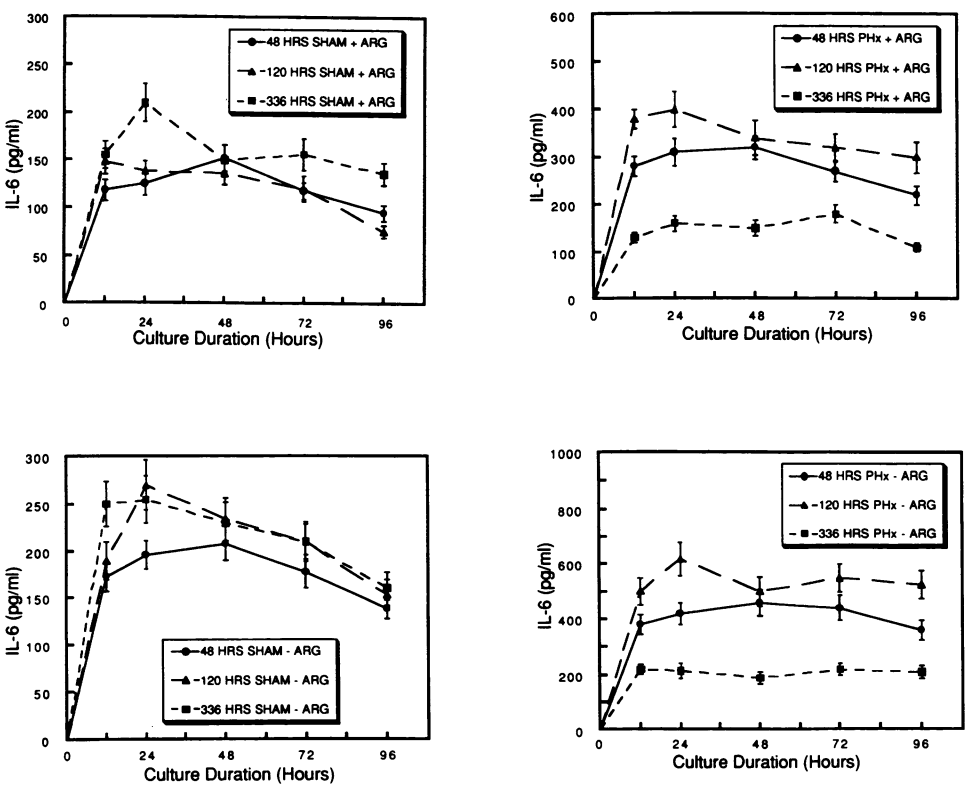

FIG. 1. Kupffer cell IL-6 production by 5×10^5 Kupffer cells after (left) sham-operation or (right) 70% partial hepatectomy in (top) standard RPMI-1640 (1200 μM L-arg) or (bottom) arginine-depleted RPMI-1640 (10 μM L-arg) 48, 120, and 336 hours after operation.

time points in response to LPS stimulation than did equal numbers of Kupffer cells from time-matched sham-operated control animals when cultured in standard (1200 μM L-arginine) RPMI-1640 (Fig. 1). This enhancement of regenerating Kupffer cell production was maximal between 48 and 120 hours after partial hepatectomy and persisted throughout the culture duration (48, 120, and 168 hours) after 70% partial hepatectomy. The depletion of arginine (to 10 μM) from *in vitro* KC cultures, however, resulted in a significant increase ($p < 0.05$ *versus* standard RPMI-1640) in KC IL-6 production, most notably during the first 48 hours of culture (Fig. 1).

To determine whether the observed increase in regenerating liver KC IL-6 production persisted once regeneration of hepatic mass neared completion, the kinetics of IL-6 production by the KC was determined 14 days (336 hours) after both 70% partial hepatectomy and sham operation (Fig. 1). Regenerating KCs no longer produced larger quantities of IL-6 in response to LPS than did equal numbers of sham-operated KCs in either standard or arginine-depleted RPMI-1640 tissue culture media.

Effects of 70% Partial Hepatectomy on KC PGE_2 Production

The production of PGE_2 by 5×10^5 KCs after stimulation with 2.5 μg/mL bacterial LPS was significantly ($p < 0.01$) higher than PGE_2 production by an equal number of time-matched sham-operated KCs, 48, 120, and 168 hours after partial hepatectomy in standard RPMI-1640 tissue culture media (Fig. 2). Because of the significant changes in KC IL-6 production related to arginine concentration, we next measured the affects of arginine availability on KC PGE_2 production in response to LPS. Regenerating Kupffer cells cultured in media depleted of arginine (10 μM) produced an earlier and sustained increase in PGE_2 than did sham KC, with levels reaching 30 ng/mL (Fig. 2). This marked difference in PGE_2 production was no longer present at 336 hours (14 days) after partial hepatectomy, when regenerating KC PGE_2 production had returned to levels comparable to those produced by sham-operated KCs 336 hours after operation.

Effects of PGE_2 on KC IL-6 Production

Prostaglandin E_2 is known to be immunosuppressive and to downregulate the KC's production of tumor necrosis factor-α.[21,22] To test the possibility of an autoregulatory effect by KC PGE_2 production on KC IL-6 production, the cyclo-oxygenase inhibitor indomethacin (1×10^{-5} M) was added to parallel standard RPMI-1640 and arginine-depleted RPMI-1640 cultures. The addition of indomethacin inhibited KC PGE_2 production by >95%.[21] Consequently, with elimination of the inhibitory influences of elevated levels of PGE_2, IL-6 production by both sham-operated and regenerating KCs was signifi-

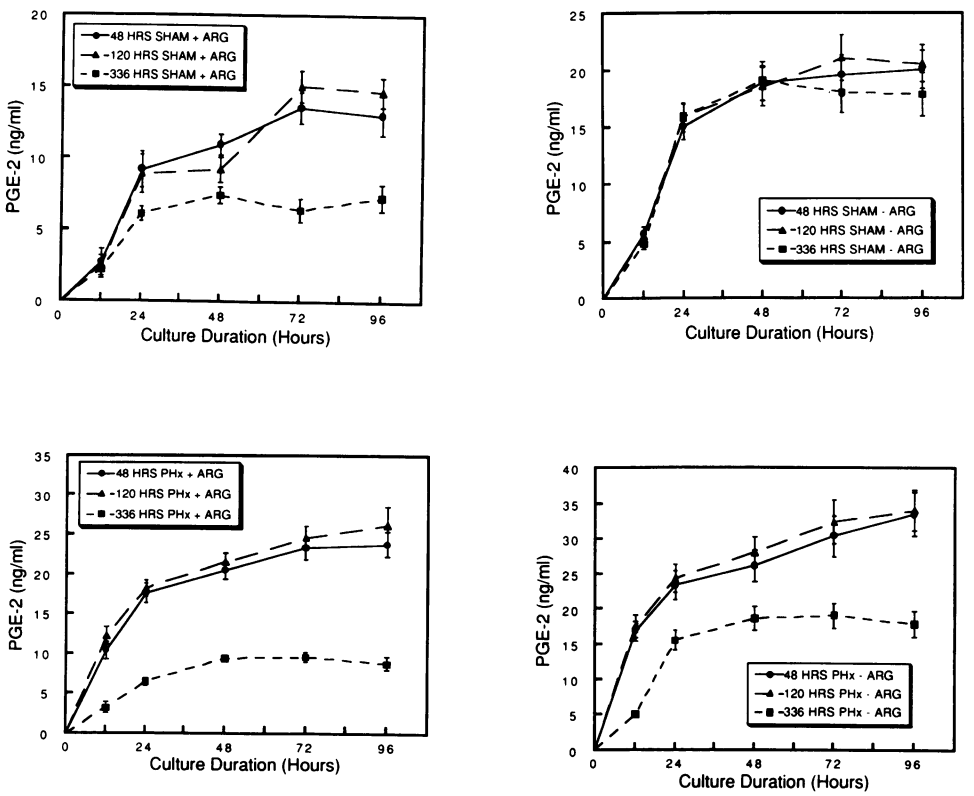

FIG. 2. PGE_2 production by 5×10^5 Kupffer cells after (top) sham operation or (bottom) 70% partial hepatectomy in (left) standard RPMI-1640 (1200 μM L-arg) or (right) arginine-depleted RPMI-1640 (10 μM L-arg) 48, 120, and 336 hours after operation.

cantly ($p < 0.05$) increased in arginine-depleted RPMI-1640 media, but not in standard RPMI-1640 media (representative sham and PHx KC data are presented in Fig. 3).

Effects of PGE_2 on Splenic, Peritoneal, and Alveolar Macrophages

To determine whether the regulatory effects of PGE_2 on IL-6 was unique to the KC, we measured the IL-6 responses of three macrophage populations in response to LPS stimulation, with and without PGE_2 blockade, in both standard RPMI-1640 and arginine-depleted RPMI-1640. Unlike KCs, nonelicited peritoneal macrophages, splenic macrophages, and alveolar macrophages do not normally encounter LPS and function in a microenvironment that is not depleted of L-arginine. When activated by LPS in a 1200-μM arginine culture, sham-operated, peritoneal, splenic, and alveolar macrophages produced low levels of IL-6 and PGE_2. After a 70% partial hepatectomy, however, the peritoneal, splenic, and alveolar macrophages all responded to LPS stimulation with elevated production of IL-6 and PGE_2 at 48, 120, and 168 hours. In the high-arginine tissue culture environment, inhibition of PGE_2 production by the addition of indomethacin did not alter the production of IL-6 by these cell populations. To determine whether these arginine-specific alterations in LPS response were unique to the KC, the peritoneal, splenic, and alveolar macrophages also were cultured in an arginine-depleted environment. With decreased arginine levels, both IL-6 and PGE_2 production by these cell populations were all increased. In contrast to the response by the KC, however, the peritoneal, splenic, and alveolar macrophage populations in an arginine-depleted environment did not further increase IL-6 production after blocking the production of PGE_2 with indomethacin (representative peritoneal macrophage IL-6 data are presented in Fig. 4. There was no difference in responsiveness between peritoneal, splenic, and alveolar macrophages).

Discussion

Hepatic regeneration in the rat is characterized by an initial 8-hour quiescent period, which is followed by an enhanced hepatocyte DNA synthesis, which is maximal 24 hours after hepatectomy. Rapid cellular proliferation follows this period of increased DNA synthesis and results in almost complete restoration of the liver's original mass within 10 to 14 days of PHx.[1] During hepatic regeneration, new hepatocytes are generated from existing hepatocytes by mitosis, which therefore appears to be a locally controlled process.[1,19] In this study, we demonstrate that the KC, which functions in a unique microenvironment with high arginase and negligible L-arginine levels,[23] and is anatomically juxtaposed with the hepatocyte, and produces

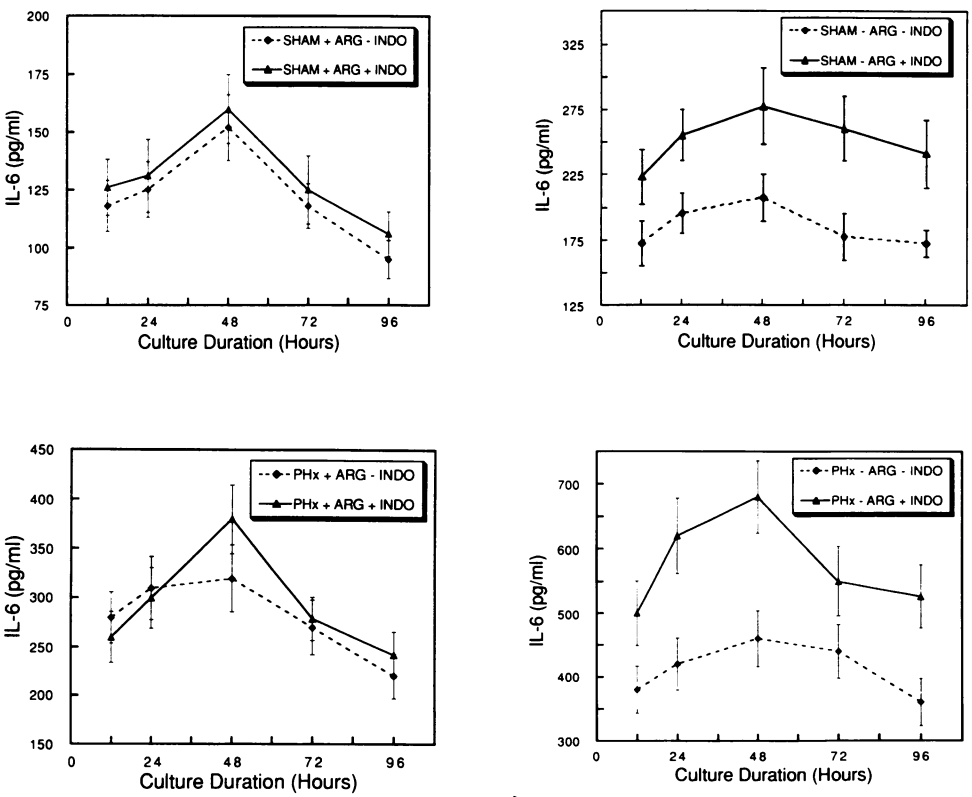

FIG. 3. Effects of 10 μM indomethacin on IL-6 production by 5×10^5 Kupffer cells stimulated with 2.5 μg/mL lipopolysaccharide in (top) sham-operated and (bottom) partially hepatectomized rats, cultured in (left) standard RPMI-1640 (1200 μM L-arg) and (right) low-arginine RPMI-1640 (10 μM L-arg).

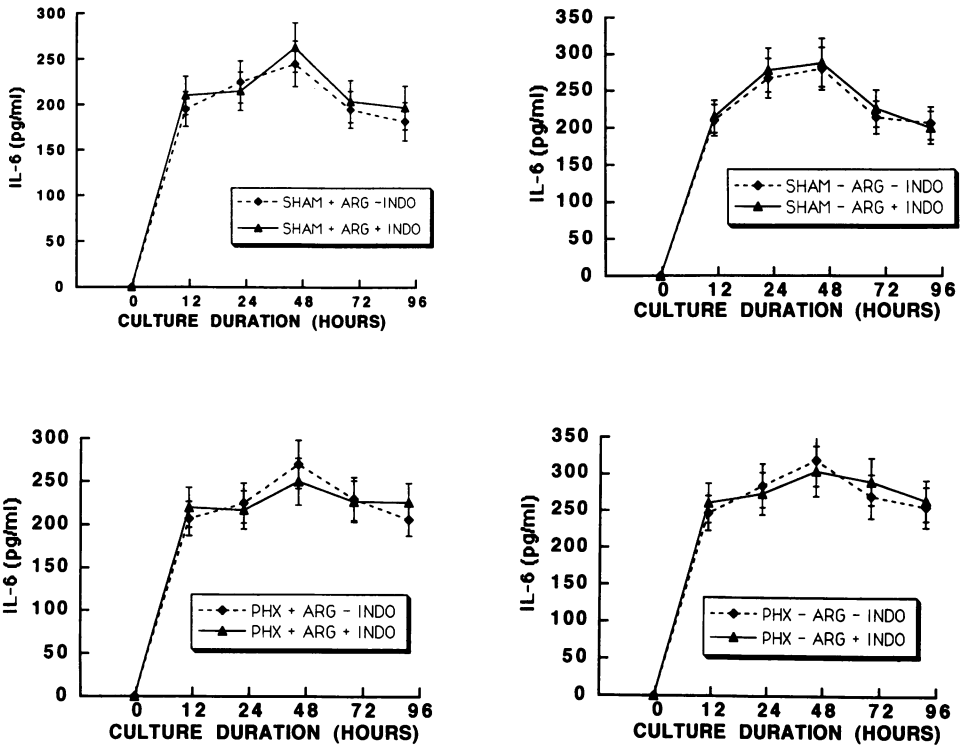

FIG. 4. Effects of cyclo-oxygenase blockade on IL-6 production by 5 $\times 10^5$ peritoneal macrophages stimulated with 2.5 μg/mL lipopolysaccharide in (top) sham-operated and (bottom) partially hepatectomized rats, cultured in (left) standard and (right) low-arginine RPMI-1640.

increased levels of IL-6 and PGE_2 during hepatic regeneration. Because it has previously been shown that KCs and hepatocytes are interdependent during health and illness, this finding leads us to conclude that the KC plays a more central role in hepatic regeneration than previously recognized.[13,14] Cornell[24] has demonstrated that endogenous bacterial LPS, which continuously bathes KCs, accelerates the regenerative process, and that liver regeneration is abrogated when gut-derived LPS is neutralized by polymyxin B or eliminated by chronic lavage with neomycin and cefazolin.

In addition to inducing the acute phase response,[15] IL-6 also activates the maturation of hematopoietic progenitor cells,[25] stimulates B-cell immunoglobulin synthesis,[25] and augments the differentiation of cytotoxic T lymphocytes.[26] During mammalian hepatic regeneration, however, the most important contribution of IL-6 produced by the KC is probably hepatocyte growth,[16] the essence of hepatic regeneration. This demonstration of elevated production of IL-6 by regenerating KCs during the early stages of hepatic regeneration provides additional support to our hypothesis that the cytokines produced locally by the KC play an important role in hepatic regeneration. Prostaglandin E_2 autoregulation of KC IL-6 production would be beneficial to the host because elevated IL-6 production is needed for a short period to induce hepatocyte growth, but if uncontrolled would lead to a state of continuous acute phase response and catabolism detrimental to the health of the host.

The increased amount of PGE_2 produced by the regenerating KC is also a documented mitogenic stimulus for hepatocytes.[27,28] Evidence that this potent paracrine growth factor could stimulate enhanced DNA synthesis and proliferation of neighboring hepatocytes within the regenerating liver is further supported by the time course of PGE_2 production during hepatic regeneration (Fig. 2). Because KC PGE_2 production is elevated during the induction and maximal regenerative response of the remaining hepatocytes, locally produced PGE_2 would qualify as a theoretical hepatic regeneration initiation substance. That this amplified KC PGE_2 response subsequently subsides as the hepatic mass returns to its original size and regeneration ceases provide additional support for the hypothesis that regenerative KCs produce PGE_2, which stimulates DNA synthesis and the proliferation of neighboring hepatocytes. More importantly, our finding that the KC uniquely controls its own IL-6 production through PGE_2, unlike other macrophage populations, may reflect an evolutionary adaptation of KCs to their position in the portal venous circulation to directly minimize the local and systemic adverse effects of gut-derived endotoxin. As specialized macrophages in an arginine-depleted microenvironment, the KCs are unique because they normally and continuously sequester the small quantities of LPS, which penetrate the intact gastrointestinal mucosal barrier and drain directly into the portal circulation.[11] Therefore, the kinetics of IL-6 and PGE_2 production by the regenerating KC with bacterial

LPS stimulation[24] and the known importance of IL-6 and PGE_2 on *in vitro* hepatocyte proliferation indicate the central importance of the Kupffer cell in this unique mammalian response.

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Discussion

DR. ROBERT ZEPPA (Miami, Florida): Dr. Bland, Dr. Jones, colleagues, I am indebted to Dr. Flye for his invitation to discuss this paper under the assumption that I know something about cytokines. That is in error. I think Wayne asked me to comment on this because 20 years ago Joe Levi and I were studying the regenerative problem of rodent liver. And, of course, in paired *in situ* perfused animals, we discovered that at 24 hours, as Dr. Flye has pointed out, there came into the circulation media some substance that we were never able to characterize, which had an effect on normal liver if they were cross-circulated, in that DNA synthesis went up at that 24-hour period in both livers. That is by way of some historical background. But what I would like to ask Dr. Flye about these experiments is, if in fact the animals are pretreated with indomethacin, what happens then to your pellet of Kupffer cells? This is fascinating and reminds me a little of the presentation that Dick Simmons gives in which he calls it "pillow talk in the liver," that is, the communications between the Kupffer cells and the hepatocytes, which appear now more and more, thanks to the work of Dr. Flye and his group, to be one of the most important factors that we have in terms of how the liver responds to a variety of stimuli, not merely regeneration. Thank you.

DR. COURTNEY M. TOWNSEND, JR. (Galveston, Texas): Dr. Bland, Dr. Jones, Fellows and Guests, Dr. Flye and his colleagues have long been interested in the problem of hepatic regeneration and the interaction of immunologically competent cells with hepatocytes. This study, I think, provides more information into the mechanisms by which Kupffer cell functions are regulated. It appears that there is a tight autocrine control, at least of prostaglandin, on interleukin-6 (IL-6) production.

What are the signals that are at play here? That is, what turns the Kupffer cells on to begin increasing their responsiveness to make IL-6 in response to lipopolysaccharide? Also, does a reciprocal relationship exist? That is, if you were to decrease IL-6, would you increase prostaglandin production? And is there any evidence that exogenously added prostaglandin would further affect the level of IL-6 produced? And, finally, do you know whether the mechanism of action of prostaglandin on IL-6 production is direct or indirect? And if it is indirect, what possible second messengers are involved? Thank you very much.

DR. GEORGE PARKER (Richmond, Virginia): Dr. Bland, Dr. Jones. Like Dr. Zeppa, I am not sure why I was asked to discuss this paper. I think the only reason is that 19 years ago Wayne Flye was my first senior