Effects of CXC chemokines on neutrophil activation and sequestration in hepatic vasculature

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Bajt, Mary Lynn, Anwar Farhood, and Hartmut Jaeschke. Effects of CXC chemokines on neutrophil activation and sequestration in hepatic vasculature. Am J Physiol Gastrointest Liver Physiol 281: G1188–G1195, 2001.—The initiating step of neutrophil-induced cytotoxicity in the liver is the recruitment of these phagocytes into sinusoids. The aim of our study was to compare the efficacy of systemic exposure with individual inflammatory mediators on neutrophil activation and sequestration in the hepatic vasculature of C3Heb/FeJ mice as assessed by flow cytometry and histology, respectively. The CXC chemokine macrophage inflammatory protein-2 (MIP-2; 20 μg/kg) induced a time-dependent upregulation of Mac-1 (318% at 4 h) and shedding of L-selectin (41% at 4 h). MIP-2 treatment caused a temporary increase of sinusoidal neutrophil accumulation at 0.5 h (97 ± 6 polymorphonuclear leukocytes (PMN)/50 high-power fields (HPF)), which declined to baseline (8 ± 2) at 4 h. The CXC chemokine KC was largely ineffective in activating neutrophils or recruiting them into the liver. Cytokines (tumor necrosis factor-α and interleukin-1α) and cobra venom factor substantially increased Mac-1 expression and L-selectin shedding on neutrophils and caused stable sinusoidal neutrophil accumulation (170–220 PMN/50 HPF). Only cytokines induced venular neutrophil margination. Thus CXC chemokines in circulation are less effective than cytokines or complement in activation of neutrophils and their recruitment into the hepatic vasculature in vivo.

complement activation; cytokines; adhesion molecules; L-selectin; Mac-1; CD11b/CD18

POLYMORPHONUCLEAR LEUKOCYTES (neutrophils) contribute to liver injury in a number of disease states including hepatic ischemia-reperfusion (21, 23), endotoxemia (24), sepsis (36), alcoholic hepatitis (1), remote organ injury (17), hemorrhagic shock (41), and certain drug-induced liver injuries (7). The basic mechanism of a neutrophil-mediated pathophysiology consists of three steps: accumulation of activated neutrophils in the hepatic vasculature, transmigration, and adherence-dependent cytotoxicity against target cells (26). Pre-requisite for toxicity is the accumulation of these phagocytes in sinusoids and in postsinusoidal or portal venules (4, 43). Previous investigations demonstrated that systemic exposure to inflammatory mediators such as complement factors (20, 44), tumor necrosis factor-α (TNF-α) (10, 14), interleukin-1 (10), platelet-activating factor (3), or leukotriene C₄ (3) can cause neutrophil sequestration in the hepatic vasculature.

In recent years, CXC chemokines, which are potent chemoattractive mediators for neutrophils, have been described (33). Antibodies against members of the CXC chemokine family were shown (6, 18, 32) to be protective against hepatic ischemia-reperfusion injury. Corresponding to the reduced injury, fewer neutrophils were found in the postischemic liver. Furthermore, substantial neutrophil accumulation in the liver was observed (40) in transgenic mice with a systemic overproduction of the CXC chemokine interleukin-8 (IL-8). In addition, transfection of the rat cytokine-induced neutrophil chemotaxicant-1 (CINC-1) gene into hepatocytes resulted in hepatic neutrophil sequestration (34). These results suggested that CXC chemokines can be responsible for neutrophil recruitment into the liver (6, 18, 32, 34, 40). However, in a complex pathophysiology, neutrophils are exposed to local CXC chemokine gradients in the tissue and, at the same time, are also systemically exposed to a number of potent inflammatory mediators including CXC chemokines. Thus to determine which mediator(s) is potentially responsible for hepatic neutrophil recruitment, it is necessary to evaluate the systemic effects of chemokines and other inflammatory mediators separately from local tissue gradients.

Most compounds that recruit neutrophils into the liver are able to upregulate Mac-1 (CD11b/CD18) on neutrophils (19). However, whether CXC chemokines are able to activate neutrophils, as indicated by the increased expression of Mac-1, is still unclear. Previous in vitro experiments showed that human IL-8 (8), rat CINC-1 (48), and mouse KC (2) can increase Mac-1 expression on neutrophils. However, anti-CINC-1 antibodies did not affect endotoxin-induced upregulation of Mac-1 in vivo (48). This could mean that a temporary activation was missed or that CXC chemokines are not relevant for neutrophil activation in the presence of 

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other, more potent inflammatory mediators. To clarify these issues, we compared the effects of individual cytokines, CXC chemokines, and complement on the systemic activation of neutrophils in vivo by flow cytometry and their effect on hepatic neutrophil sequestration in sinusoids and postsinusoidal venules by histochecmstry. Our data show that CXC chemokines alone were only weak activators of neutrophils and therefore were only moderately effective in inducing neutrophil accumulation in the hepatic vasculature.

 MATERIALS AND METHODS

 Animals. Male C3Heb/FeJ (20–25 g body wt) were obtained from Jackson Laboratories (Bar Harbor, ME). The animals had free access to food (certified rodent diet no. 5002C, PMI Feeds, Richmond, IN) and water. The experimental protocols followed the criteria of the University of Arkansas for Medical Sciences and the Guide for the Care and Use of Laboratory Animals [DHHS Publication No. (NIH) 85-23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892]. Animals were treated with 8 or 20 μg/kg murine recombinant macrophage inflammatory protein-2 (MIP-2), KC, TNF-α, IL-1α (all from Endogen, Woburn, MA), or cobra venom factor (CVF; 120 μg/kg; Sigma, St. Louis, MO). Groups of animals were killed at various time points after injection of an inflammatory mediator (0.5, 2, and 4 h). Blood was collected from the right ventricle into a heparinized syringe. In some experiments, blood was obtained and allowed to clot. Serum levels of MIP-2 and KC were measured with the respective ELISA kits (R & D Systems, Minneapolis, MN). Sections of the liver were frozen in liquid nitrogen or fixed in phosphate-buffered formalin for histological analysis.

 Histology. Formalin-fixed portions of the liver were paraffin embedded, and 5-μm sections were cut. Neutrophils were stained using the AS-D chloroacetate esterase technique as described in detail previously (22). Neutrophils were identified by positive staining and morphology and were counted in 400 high-power fields using a Nikon Labophot microscope. Only neutrophils present within sinusoids or extravasated into the tissue were counted; the number of neutrophils margined within large vessels, e.g., hepatic venules, were evaluated separately (counted in 10 vessels of equal diameter). Cell damage was evaluated in parallel sections stained with hematoxylin and eosin. The percentage of necrosis was estimated by evaluating the number of microscopical fields with necrosis compared with the entire histological section. The pathologist (A. Farhood) performing the histological evaluation was blinded as to the treatment of animals.

 Flow cytometric analysis of neutrophils. Peripheral blood neutrophils were incubated with FITC-conjugated RB6-8C5 (anti-Gr-1) and phycoerythrin (PE)-conjugated MI/70 (anti-CD11b) or PE-conjugated Mel-14 (anti-L-selectin) antibodies (PharMingen, San Diego, CA) and stained using a whole blood lysis kit (Coulter Immunology, Hialeah, FL) as previously described in detail (9, 29). Two-color analysis of antibody binding to cells was analyzed by flow cytometry using a FACSscan flow cytometer (Becton Dickinson, San Diego, CA). Peripheral blood neutrophils were gated by the forward and light angle scatter and Gr-1 FITC fluorescence. Nonspecific fluorescence was determined on cells incubated with isotype- and fluorochrome-matched control antibodies.

 Nuclear extracts and electrophoretic mobility shift assay. Nuclear extracts were prepared from frozen liver sections as described in detail previously (11). Gel shift reagents were used according to the manufacturer’s suggested protocol (Promega, Madison, WI). Double-stranded nuclear factor-κB (NF-κB) consensus oligonucleotide probe (5'-AGTTGAGGG-GACTTCCCAGGC-3') was end-labeled with [γ-32P]ATP (10 μCi at 222 TBq/mmol; Amersham, Arlington Heights, IL). Binding reactions, containing 35 fmol (~1 × 10⁶ dpm) of oligonucleotide and 5 μg of nuclear protein were conducted at room temperature for 20 min in a total volume of 10 μl in binding buffer [10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol (vol/vol), and 0.5 μg poly(dI-dC)]. For competition reactions, unlabeled oligonucleotide was added 5 min before addition of radiolabeled probe. After the binding reactions, 1 μl of 10× gel loading buffer was added, and the reaction was subjected to nondenaturing 4% PAGE in low-ionic-strength buffer (45 mM Tris-borate and 1 mM EDTA) at 100 V/20 mA for 2 h (13). Gels were vacuum dried and exposed to X-ray film (Hyperfilm MP; Amersham) at −70°C.

 Northern blot analysis. Total cellular RNA was isolated from liver tissue as previously described in detail (9). RNA was hybridized overnight with 4 μg of RNA samples were electrophoresed on denaturing agarose-formaldehyde gels and transferred to Gene Screen Plus hybridization membranes (NEN Research Products, Boston, MA) (10, 11). RNA was cross-linked by baking the membranes at 80°C for 2 h under vacuum. Mouse intercellular adhesion molecule-1 (ICAM-1) hybridization probe was prepared by PCR using a mouse ICAM cDNA (16) and the following oligonucleotides: 5'-TGAAACTGCACGTGCTGTTAT-3' and 5'-ACCATTCTGTTCAAACGCAG-3' encompassing nucleotides 500–900. The probe for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was prepared with a PCR-Amplimer kit (Clontech, Palo Alto, CA). Purified fragments were radiolabeled with [α-32P]dCTP using a random hexanucleotide primer kit (Stratagene, La Jolla, CA). Transferred membranes were prehybridized with Rapid-Hyb buffer (Amersham) at 65°C for 2 h and then hybridized with labeled probes overnight at 65°C. Membranes were washed in 1× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) containing 0.1% SDS for 15 min at room temperature. Membranes were washed twice at 55°C in 0.2× SSC containing 0.1% SDS for 30 min. The washed blots were exposed to Hyperfilm MP X-ray film (Amersham) at −80°C.

 RNase protection assay. All protocols followed the instructions of the RiboQuant multiprobe RNase protection assay system (PharMingen, San Diego, CA). With the use of an in vitro transcription kit and a customized template set (containing mouse MIP-2, mouse KC, and L32), a radiolabeled probe set was synthesized using [α-32P]UTP (30). These probes were hybridized with total RNA isolated from liver tissue for 16 h. After digestion of nonhybridized RNA with RNase, the protected probes were separated on a denaturing acrylamide gel. The gel was dried and then exposed to X-ray film (Kodak X-OMat, Fisher Scientific, Pittsburgh, PA) for 12 h at −80°C. The developed X-ray films were scanned using a calibrated imaging densitometer (GS-710, Bio-Rad Laboratories, Hercules, CA) and Quantity-One software (Bio-Rad).

 Peritonitis experiments To test the chemotactic effects of CXC chemokines, KC or MIP-2 (8 μg/kg) was injected intraperitoneally (5). Controls received 10 ml/kg PBS. After 2 or 4 h, the animals were killed and the peritoneal cavities lavaged twice with 2 ml of PBS. The lavage fluids were centrifuged (1,000 g) for 10 min to sediment the neutrophils. The pellets were resuspended in Tris-buffered 0.75% NH₄Cl for 10 min to lyse erythrocytes. After centrifugation, the pellets were resuspended in detergent buffer (50 mM Phos-
phate buffer containing 0.5% cetyltrimethylammonium bromide), briefly sonicated, and freeze-thawed twice. Myeloperoxidase (MPO) activity as an index for neutrophil accumulation was determined spectrophotometrically in 50 mM phosphate buffer (pH 6.0) containing 0.165 mg/ml o-dianisidine hydrochloride and 0.15 mM hydrogen peroxide. The change in absorbance was determined at 460 nm.

Statistics. All data are given as means ± SE. Comparisons between multiple groups were performed with one-way ANOVA followed by a Bonferroni t-test. P < 0.05 was considered significant.

RESULTS

CXC chemokines and neutrophil activation. Flow cytometric analysis of peripheral blood neutrophils indicated that intravenous administration of a high dose of MIP-2 caused a progressive increase in Mac-1 expression on circulating neutrophils. Expression of Mac-1 reached values of 318% of baseline levels at 4 h (Fig. 1A). At the same time, L-selectin was shed, resulting in a 41% loss of the adhesion molecule at 4 h (Fig. 1B). MIP-2 administration induced a transient accumulation of neutrophils in sinusoids with a maximum at 0.5 h (Fig. 2). All neutrophils were located within sinusoids; no extravasation was detected. In contrast to MIP-2, KC caused only a very moderate and transient increase in Mac-1 expression and L-selectin shedding at 0.5 h after administration of the CXC chemokine (Fig. 1). Similarly, only a very minor increase in the number of sinusoidal neutrophils was observed at 0.5 h (Fig. 2). No margination of neutrophils was found in postsinusoidal venules after KC or MIP-2 injection during the 4-h time course of the experiment (data not shown).

Chemotaxis of CXC chemokines in vivo. Because of the substantial difference between KC and MIP-2 in activating neutrophils and causing hepatic neutrophil sequestration, we evaluated the chemotactic potential of both chemokines in vivo. Intraperitoneal injection of 8 μg/kg of KC or MIP-2 resulted in peritoneal neutrophil accumulation as indicated by the increased MPO activity of lavaged cells (Fig. 3). However, there was no significant difference in neutrophil recruitment between KC and MIP-2 (Fig. 3).

Neutrophil activation by cytokines and complement. To evaluate the capacity of other proinflammatory mediators to activate neutrophils and cause hepatic neutrophil recruitment, murine TNF-α or IL-1α (20 μg/kg) was injected intravenously or complement was maximally activated in vivo by injecting 120 μg/kg CVF. All three mediators increased Mac-1 expression on circulating neutrophils by 500–700% above baseline values (Fig. 4A). However, there was a substantial difference

Fig. 1. Effect of CXC chemokines on Mac-1 (CD11b/CD18) and L-selectin expression (CD62L) on circulating neutrophils. Upregulation of Mac-1 expression (A) and shedding of L-selectin (B) were determined by flow cytometry. Animals received 20 μg/kg iv of recombinant murine macrophage inflammatory protein-2 (MIP-2) or KC. Blood samples were obtained from controls and at 0.5, 2, and 4 h after chemokine injection. Values are given as means ± SE (expressed as %mean fluorescence of control neutrophils) of n = 4 animals per group and time point. *P < 0.05 vs. time 0.

Fig. 2. Effect of CXC chemokines on sinusoidal neutrophil sequestration. Neutrophils were stained and quantitated in histological sections. Animals received 20 μg/kg iv of recombinant murine MIP-2 or KC. Liver samples were obtained from controls and at 0.5, 2, and 4 h after chemokine injection. Values are given as means ± SE of n = 4 animals per group and time point. PMN, polymorphonuclear leukocytes; HPF, high-power fields. *P < 0.05 vs. time 0.

Fig. 3. CXC chemokine-induced peritoneal neutrophil infiltration. Animals received 8 μg/kg ip of recombinant murine MIP-2 or KC. Myeloperoxidase (MPO) activity was determined in lavaged cells obtained from controls or after chemokine injection (at 2 or 4 h). Values are given as means ± SE of n = 4 animals per group and time point. *P < 0.05 vs. time 0.
in the time course. Animals treated with CVF or TNF-α showed a rapid onset of the response and a consistent increase over the entire observation period. In contrast, IL-1α injection resulted in a delayed increase at 2 h and a reduction of the activated Mac-1 levels at 4 h (Fig. 4A). Although L-selectin shedding after treatment with these mediators supported the concept that circulating neutrophils are activated, there are differences from the response of Mac-1 expression (Fig. 4B).

TNF-α and IL-1α were equally potent in inducing L-selectin shedding. Only a minor delay was observed with IL-α. However, activation of complement was significantly less potent in inducing L-selectin shedding despite the fact that it was the most potent activator of Mac-1 expression (Fig. 4B). All three mediators induced sinusoidal neutrophil sequestration at 2 and 4 h but had no effect at 0.5 h (Fig. 5A). TNF-α and IL-1α were most potent with a 27- to 29-fold increase over baseline. In contrast, complement activation induced only a 10-fold increase at 2 h. The response after TNF-α injection was more stable than after IL-1α or CVF (Fig. 5A). Neutrophil margination in postsinusoidal venules was absent in controls and at 0.5 h after injection of cytokines (Fig. 5B). However, at 2 and 4 h a substantial number of neutrophils adhered in these vessels. With the exception of a minor increase at 2 h, CVF did not cause venular neutrophil margination.

Administration of TNF-α and IL-1α induced formation of MIP-2 and KC. Serum levels of MIP-2 increased 2,700- and 6,500-fold 1 h after TNF-α and IL-1α administration, respectively (Fig. 6). Compared with MIP-2, the absolute increase of KC serum levels was much higher after cytokine injection. However, due to the higher baseline, there was only a 236- and 525-fold increase with TNF-α and IL-1α, respectively (Fig. 6).
CVF did not increase serum levels of KC or MIP-2 (data not shown). To verify that at least part of the chemokines measured in plasma were generated in the liver, mRNA levels of KC and MIP-2 were evaluated by RNase protection assay in controls and 1 h after TNF-α injection (Fig. 7A). In controls, mRNA levels of both CXC chemokines were almost undetectable. However, TNF-α induced a substantial increase in MIP-2 and in particular in KC mRNA. Densitometric analysis indicated that the KC-to-L32 (housekeeping gene) ratio increased by 280-fold and the MIP-to-L32 ratio increased by 16-fold (Fig. 7B). Similar results were obtained when the CXC chemokine mRNA levels were compared with GAPDH (data not shown).

**Fig. 7.** RNase protection assay for detection of chemokine mRNA levels in the liver. (A) RNA isolated from the livers of individual animals was hybridized with probes for KC (286 nt), MIP-2 (205 nt), and the control genes L32 (112 nt) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 97 nt). RNA samples from untreated controls (lanes 1–4) and animals treated with 20 μg/kg of recombinant murine TNF-α (lanes 5–8) for 1 h were compared. Densitometric analysis of the gel was performed, and the CXC chemokine-to-L32 ratio was calculated (B). Values are means ± SE of n = 4 animals per group. *P < 0.05 vs. controls.

TNF-α but not complement activates NF-κB and ICAM-1 mRNA formation. Because CVF caused margination of neutrophils in the sinusoids but not in venules, we tested whether CVF could activate the transcription factor NF-κB and induce ICAM-1 mRNA formation. In livers of control animals, only minor amounts of NF-κB are present in the nucleus and only traces of ICAM-1 mRNA were detectable (Fig. 8). CVF induced neither the translocation of NF-κB into the nucleus nor ICAM-1 transcription at 1 h. In contrast, administration of TNF-α caused a dramatic activation of NF-κB and induction of ICAM-1 mRNA formation (Fig. 8).

**Fig. 8.** Effect of TNF-α and CVF on nuclear translocation of nuclear factor-κB (NF-κB; A) and intercellular adhesion molecule-1 (ICAM-1) mRNA expression (B) in the liver. Animals received 120 μg/kg iv CVF or 20 μg/kg of recombinant murine TNF-α. Liver samples were obtained from controls and 1 h after mediator injection. A: NF-κB activation was determined in nuclear extracts by electrophoretic mobility shift assay. An NF-κB consensus oligonucleotide probe was used as described in MATERIALS AND METHODS. The position of the specific NF-κB oligonucleotide/protein complex is marked. Each lane represents the nuclear extract from an individual animal (n = 3–4 animals per group). Lane 11 represents a sample of a TNF-treated animal with the addition of a 50-fold excess of unlabeled NF-κB oligonucleotide (Comp). B: levels of mRNA for ICAM-1 and the housekeeping gene were assessed by Northern blot hybridization as described in MATERIALS AND METHODS. Each lane represents a sample from 1 animal.

**DISCUSSION**

The objective of this study was to evaluate the capacity of high systemic levels of CXC chemokines to activate neutrophils as indicated by Mac-1 upregulation and L-selectin shedding and to induce neutrophil sequestration in sinusoids and venules as assessed by histochemistry. Our results showed that a high dose of the CXC chemokine KC caused only a minor, temporary activation of circulating neutrophils in vivo. This temporary activation correlated with a minor, transient accumulation of neutrophils in sinusoids. On the other hand, the CXC chemokine MIP-2 induced a sustained activation of circulating neutrophils but also only a temporary accumulation of neutrophils in sinusoids. These data suggest that sinusoidal neutrophil sequestration requires some degree of neutrophil activation. However, systemic neutrophil activation does not necessarily lead to a sustained accumulation of these phagocytes in sinusoids. Moreover, the magnitude of neutrophil activation as determined by Mac-1 expression and L-selectin shedding does not correlate with sinusoidal neutrophil sequestration. This effect was not only observed with CXC chemokines but also with activated complement. CVF induced the largest increase in Mac-1 expression but triggered only a moderate response of neutrophil accumulation in sinusoids. Thus systemic neutrophil activation may trigger...
neutrophil accumulation in the liver. However, it is neither qualitatively nor quantitatively a reliable assessment of hepatic neutrophil accumulation. This finding is of particular importance for human pathophysiologies in which, in most cases, the activation status of circulating neutrophil can be evaluated at different time points but liver tissue is only rarely available.

The comparison between the effects of CXC chemokines and other mediators known to systemically activate neutrophils and cause accumulation in the liver suggests that TNF, IL-1, and activated complement factors are substantially more potent in causing Mac-1 upregulation and L-selectin shedding on circulating neutrophils. The response to IL-1 was slightly delayed, which may explain why in previous studies (10) IL-1 was thought to be unable to increase Mac-1 expression. TNF, IL-1, and to some degree activated complement induced a pronounced and a sustained accumulation of neutrophils in sinusoids. Interestingly, the sequestration of neutrophils in sinusoids after cytokine administration and complement activation was delayed compared with the effect of CXC chemokines despite the fact that all mediators rapidly upregulated Mac-1. In general, it is assumed that neutrophil accumulation in sinusoids is independent of adhesion molecules (19). There is no rolling in sinusoids (43, 45), and consequently, P (12, 45)-, L (29)- and E-selectin (14, 29) did not affect neutrophil accumulation in sinusoids. Furthermore, antibodies against β2-integrins (22), ICAM-1 (10, 22), or vascular cell adhesion molecule-1 (9) had no effect on the initial sinusoidal neutrophil sequestration induced by cytokines. After injection or generation of these inflammatory mediators, neutrophils may be trapped because of reduced deformability (46), sinusoidal cell swelling (35), and active vasoconstriction (47) rather than the interaction of adhesion molecules. The different behavior of neutrophils after CXC chemokine injection compared with cytokines and complement suggests potentially different mechanisms of accumulation. However, this mechanism requires further study to be understood.

In contrast to sinusoids, the adherence of neutrophils in postsinusoidal venules requires adhesion molecules. Selectins are involved in rolling, and ICAM-1/ integrin interactions mediate firm adhesion (12, 14, 29, 39, 42) similar to the general mechanism of neutrophil adhesion in postcapillary venules (15). Our results showed that CXC chemokines, in contrast to TNF and IL-1, did not induce any margination of neutrophils in postsinusoidal venules. P-selectin is not expressed in the hepatic vasculature of control animals (12). Adhesion in these vascular beds requires either recruitment of P-selectin from Weibel-Palade bodies (39) or transcriptional upregulation of P-selectin as an initiating step (12). The induction of P-selectin in postsinusoidal venules is mediated by cytokines and dependent on NF-κB activation (12). Only TNF or IL-1 can activate NF-κB and upregulate adhesion molecules and therefore only cytokines induced venular adherence. Because neither CXC chemokines (27, 37) nor activated complement (Fig. 8) can trigger these effects, these mediators were unable to cause relevant neutrophil adherence in postsinusoidal venules. CXC chemokine formation is regulated by the transcription factor NF-κB (28, 38). Thus TNF and IL-1 induced the synthesis of KC and MIP-2 in vivo (Fig. 6). At least part of the chemokines was generated in the liver as indicated by the increase in mRNA levels (Fig. 7). However, these chemokines had only a moderate, transient effect on neutrophil accumulation in sinusoids and did not trigger venular adherence. These data suggest that the effects of TNF and IL-1 on neutrophil activation and their sequestration in different vascular beds of the liver were not secondary to CXC chemokine formation.

An important issue regarding our study is the relevance of the effect of systemic administration of inflammatory mediators to their mechanism of action in vivo. In general, neutrophil accumulation in liver sinusoids requires either systemic activation by inflammatory mediators and/or a local gradient of a chemotactic factor. During endotoxemia, high systemic levels and local gradients of CXC chemokines are present at the same time. In addition, high levels of cytokines are generated, and depending on the dose of endotoxin, complement can be activated. To investigate the role of CXC chemokines in this pathophysiology, we address one of several aspects in this study: the relative importance of systemic levels of CXC chemokines compared with cytokines and complement in activating neutrophils and inducing their accumulation in sinusoids. Our results suggest that CXC chemokines are of limited relevance for neutrophil recruitment into the liver through this mechanism. This conclusion is also supported by preliminary data that show no effect of antibodies against CXC chemokines on hepatic neutrophil accumulation during endotoxemia (25). However, in the absence of elevated systemic cytokine levels and activated complement, local gradients of chemotactic factors such as CXC chemokines may become important for neutrophil recruitment into the liver. Alternatively, local gradients of CXC chemokines may be important for directing movement of already sequestered neutrophils in the liver. Because transmigration is necessary for neutrophil cytotoxicity in the liver (4), CXC chemokines may be more relevant for extravasation.

Another important aspect of our study was the finding that there are substantial differences in the degree of neutrophil activation and hepatic neutrophil sequestration between individual CXC chemokines. MIP-2 and KC are murine CXC chemokines, which are related to the GRO family of human CXC chemokines (33). Both chemokines act on the only CXC chemokine receptor in mice, CXCR2 (31), and trigger neutrophil chemotaxis and intracellular Ca²⁺ fluxes (31). However, MIP-2 appears to be ~10-fold more potent than KC in triggering these effects in isolated murine neutrophils (31). However, the 10-fold higher generation of KC compared with MIP-2 in response to cytokines in vivo may compensate for the lower efficacy of KC at the receptor. Our in vivo data also indicate that MIP-2 was
more potent than KC in causing systemic neutrophil activation, but we observed a similar chemotactic response in the peritonitis experiments. However, the difference in neutrophil chemotaxis between MIP-2 and KC was only observed at very low concentrations (0.5 nM) (31). The dose used in our in vivo experiment resulted in considerably higher concentrations, which may explain the similar response to both chemokines. On the other hand, our data on neutrophil activation suggest that even with these high doses of CXC chemokines, which should have resulted in initial serum levels of one to two orders of magnitude beyond peak levels after cytokine administration (Fig 6), there was still a difference in the neutrophil response to KC and MIP-2. This suggests that for realistic in vivo levels of these chemokines, only MIP-2 may be of importance for systemic neutrophil activation and hepatic neutrophil recruitment. Furthermore, low concentrations of CXC chemokines effectively trigger a chemotactic response (31) but do not upregulate Mac-1. This indicates that CXC chemokines may be more important for directing neutrophil movement than for activation.

In summary, our investigation demonstrated that the CXC chemokine MIP-2 has the capacity to activate neutrophils at high concentrations in vivo and induce a transient neutrophil accumulation in sinusoids but not in venules. The CXC chemokine KC is much less effective in triggering these effects. In contrast, the cytokines TNF and IL-1 and activated complement factor C3b are potent mediators for upregulation of Mac-1 and shedding of L-selectin. These mediators also induce a sustained accumulation of neutrophils in sinusoids. In addition, cytokines cause adherence in post sinusoidal venules due to their capacity to induce upregulation of adhesion molecules. Thus CXC chemokines are less effective than cytokines or complement in activation of neutrophils and their recruitment into the hepatic vasculature in vivo. This suggests that local gradients of CXC chemokines may direct neutrophil movement within the liver. Systemic CXC chemokines may only be relevant to hepatic neutrophil sequestration if more potent mediators are absent.

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