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Selective transport of cytokine-induced neutrophil chemoattractant from the lung to the blood facilitates pulmonary neutrophil recruitment

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Quinton, Lee J., Steve Nelson, Ping Zhang, Darren M. Boé, Kyle I. Happel, Weihong Pan, and Gregory J. Bagby. Selective transport of cytokine-induced neutrophil chemoattractant from the lung to the blood facilitates pulmonary neutrophil recruitment. Am J Physiol Lung Cell Mol Physiol 286: L465–L472, 2004. First published November 14, 2003; 10.1152/ajplung.00153.2003.—The CXC chemokines cytokine-induced neutrophil chemoattractant (CINC) and macrophage inflammatory protein-2 (MIP-2) are potent neutrophil chemoattractants in rats. We have previously shown that CINC, unlike MIP-2 and most other proinflammatory cytokines, is elevated in the systemic circulation in response to an intratracheal (IT) challenge. Therefore, we hypothesized that CINC generated within the lung selectively enters the vascular compartment to facilitate pulmonary neutrophil recruitment. Rats were administered IT LPS, and plasma CINC and MIP-2 levels were measured 90 min and 4 h after injection, along with mRNA expression in lung, spleen, liver, and kidney. Ninety minutes and 4 h after IT LPS, CINC and MIP-2 mRNA expression were largely confined to lung homogenate, but of the two chemokines, only CINC was present in plasma. In separate experiments, rats received IT injections of recombinant CINC and/or MIP-2. Here, plasma levels of CINC, but not MIP-2, were significantly increased throughout the 4-h observation period. This finding was verified by individually administering 125I-labeled forms of each chemokine. Instillation of recombinant MIP-2 or CINC into the lung increased the number of neutrophils recovered in bronchoalveolar lavage fluid at 4 h, and this effect was enhanced when both chemokines were administered together. In addition, intravenous (IV) CINC, but not IV MIP-2, increased pulmonary neutrophil recruitment in response to IT MIP-2. Our results show that CINC, in contrast to MIP-2, is selectively transported from the lung to the systemic circulation, where it promotes neutrophil migration into the lung in response to a chemotactic stimulus.

SUCCESSFUL CLEARANCE of bacterial pathogens from the lower respiratory tract is dependent on neutrophil (i.e., polymorphonuclear leukocyte or PMN) recruitment from the systemic circulation into the infected lung (40). The migration of neutrophils toward an area of infection is a complex process, involving multiple factors produced in response to an invading organism. Alveolar macrophages, which constitute the major phagocytic population of the lung, recognize pathogen-associated molecular patterns, such as lipopolysaccharide (LPS) associated with bacteria, and produce many proinflammatory mediators, including cytokines and chemokines (26, 51). Among these are the CXC chemokines, some of which are largely responsible for the recruitment and activation of neutrophils. Once within the alveolar space, recruited neutrophils augment the pulmonary host defense response by many mechanisms, including phagocytosis and the production of reactive oxygen species (31).

CXC chemokines are a family of cytokines characterized by the presence of one nonconserved amino acid between the conserved cysteine residues within the NH2-terminal region of the mature peptide. Of the CXC chemokines, those bearing a glu-leu-arg (ELR) amino acid motif upstream of the CXC sequence (such as human interleukin-8) are potent inducers of neutrophil chemotaxis (28). Two important ELR+ CXC chemokines in rats are cytokine-induced neutrophil chemoattractant (CINC) and macrophage inflammatory protein-2 (MIP-2) (43, 46). These two chemokines share the same receptor (CXCXR2) and bear structural and functional homology to members of the human interleukin-8 (IL-8/CXCL8) family of CXC chemokines, although CINC more closely represents the rat counterpart of human growth-related oncogene (GRO/CXCL1–3) than IL-8 itself (44, 45). Formal systematic CXCL designations have not yet been ascribed to rat CINC and MIP-2, although they are each loosely categorized as rat CXCL1, 2, or 3 (20a). In spite of their homology, CINC and MIP-2 differ somewhat in their biological potency. Not only does MIP-2 bind CXCR2 with 72-fold higher affinity than CINC, but it also desensitizes subsequent neutrophil responses to either chemokine. This desensitization does not occur on exposure to CINC (36).

In addition to their direct effects on neutrophil migration, CINC and MIP-2 enhance other aspects of neutrophil function such as β2-integron adhesion molecule expression, phagocytosis, and the respiratory burst (3, 11, 12). Both CINC and MIP-2 are produced in response to an intrapulmonary challenge, and neutralization of either chemokine significantly decreases neutrophil recruitment into an infected alveolus (12, 14, 35, 42). The same observation has been reported by others who have
shown that the absence (−/−) or neutralization of CXCR2 significantly decreases pulmonary neutrophilic inflammation (13, 41). Such defects in neutrophil recruitment reduce bacterial clearance in several rodent models of pneumonia, resulting in increased susceptibility to infection and increased mortality (6, 14).

We and others have shown that many proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, IL-6, and IL-8 remain compartmentalized within an infected locus (7, 8, 10, 30, 32). This phenomenon may serve to prevent the generation of a systemic inflammatory response during a local infection such as pneumonia. Recent data from our group have shown that rat CINC, in contrast to MIP-2, appears in the systemic circulation after an intratracheal (IT) challenge with LPS or Streptococcus pneumoniae (6, 19, 49). However, the source and functional significance of the systemic CINC response following IT LPS remain undefined. A similar finding has also been observed in a murine model of peritonitis, where keratinocyte-derived chemokine, the murine homolog of CINC, was detectable in the systemic circulation but not MIP-2 (9). The differential distribution of CINC vs. MIP-2 after IT LPS administration may result from a number of factors, including extrapulmonary gene expression and/or selective CINC translocation from the alveolar compartment into the circulation.

The present study was initiated to determine whether CINC, unlike MIP-2, moves from the pulmonary compartment into the vascular compartment, and to identify a physiological role for systemic CINC during an intrapulmonary inflammatory challenge. By implementing the use of multiorgan mRNA expression analysis and recombinant chemokine administration, we demonstrate that CINC is selectively transported from the intra-alveolar space. Our results also show that the presence of systemic CINC, but not MIP-2, enhances pulmonary neutrophil recruitment, where it functions to augment the pulmonary host defense response to invading pathogens.

MATERIALS AND METHODS

Animals. Male virus antibody-free Sprague Dawley International Gold Standard rats (Charles River Laboratories) weighing 175–200 g were housed in a controlled environment (12-h light:dark cycle) and had access to standard rodent chow and water for at least 1 wk before initiating experiments. All animal experimentation guidelines set forth by Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee were strictly followed during all animal experimentation.

IT and IV endotoxin. Depending on the experimental protocol, rats were administered Escherichia coli-derived LPS (Serotype 026:B6, Sigma-Aldrich) IT or intravenously (IV) under ether anesthesia. For IT injections, 0.5 ml of PBS containing 0.1 mg of LPS or PBS alone was instilled using a 28-gauge needle. IV injections of LPS (1.0 mg/kg body wt in 0.5 ml of PBS) or vehicle (PBS alone) were delivered via the penile vein. Rats were killed either 90 min or 4 h after IT administration to obtain heparinized blood from the abdominal aorta. Rats were killed either at 6 or 24 h after IV endotoxin.

Measurement of CINC and MIP-2. Plasma and BALF CINC levels were determined by a specific ELISA using paired antibodies (R&D Systems) as previously described (48). MIP-2 was determined in all samples using the Cytoscreen ELISA kit for MIP-2 (Biosource International) and the procedures supplied by the manufacturer.

Sample preparation for real-time quantitative RT-PCR assays. Total RNA extraction was performed on 50- to 100-μg tissue samples homogenized in TRIzol reagent using the isopropanol precipitation procedure suggested by the manufacturer (Invitrogen Life Technologies). 1-Bromo-3-chloropropane phase-separation reagent (100 μl; Molecular Research Center) was substituted for 200 μl of chloroform in the appropriate step of the protocol. Isolated RNA samples were quantified spectrophotometrically at 260 nm and stored at −80°C.

Real-time RT-PCR for quantitative measurement of CINC and MIP-2 mRNA. The primers and probes used for CINC and MIP-2 mRNA determination were the same as described previously (6). Real-time RT-PCR was carried out using the ABI PRISM Sequence
Detection System 7700 (PE Applied Biosystems). The RT-PCR assay used to detect CINC, MIP-2, and 18s rRNA was performed according to the Taqman One-Step RT-PCR Master Mix Reagents kit protocol supplied by the manufacturer (PE Applied Biosystems) as previously described (6). Actual copy numbers of CINC and MIP-2 were quantified by comparing the measured values of the unknown samples with a cRNA standard curve for each chemokine. The development of cRNA standards for CINC and MIP-2 has been previously described (33, 50). Both assays ranged from 10^3 copies to 10^10 copies, with an intra-assay coefficient of variation of 0.999. CINC and MIP-2 copy numbers were then normalized to the content of 18s control rRNA (PE Applied Biosystems), which was also calculated on the basis of its own standard curve (10^-7 to 10^1 ng/µl). All final values were expressed as copies of CINC or MIP-2 mRNA/nanogram 18s rRNA.

Statistics. Statistical analyses were performed using statistical software programs (SigmaStat; SPSS, San Rafael, CA and SAS version 8; SAS Institute, Cary, NC). Data are presented as means ± SE of the number of animals indicated in each figure. Comparisons were performed with one-way ANOVA, followed by a Tukey test unless otherwise stated. If data did not pass the tests for normality and equal variance, they were log_{10} transformed to fit ANOVA requirements for normality and homogeneity. Differences were considered statistically significant at P < 0.05.

RESULTS

Chemokine protein and mRNA expression in response to intrapulmonary or systemic endotoxin. Our laboratory has previously reported that large increases in both CINC and MIP-2 protein are detected in BALF after IT LPS administration, but of the two chemokines, only CINC increases in the systemic circulation (49). In the present study, CINC and MIP-2 mRNA, in addition to plasma protein concentrations, were determined after IT endotoxin to determine whether extrapulmonary gene expression might account for the differential protein distribution of the two chemokines. The effects of IV endotoxin were also measured to positively identify each tissue’s ability to produce CINC and MIP-2 in response to the inflammatory stimulus. Animals were killed 90 min or 4 h after the LPS challenge to measure CINC and MIP-2 protein levels in plasma, along with mRNA expression in lung, spleen, liver, and kidney samples.

IV LPS caused significant increases in plasma CINC and MIP-2 concentrations compared with control animals (Fig. 1). This effect was evident for both chemokines at 90 min and 4 h. Increases in plasma CINC and MIP-2 protein levels were associated with high mRNA expression in all organs analyzed, verifying their capacity to express these two chemokines in response to endotoxin (data not shown).

After IT LPS, plasma CINC levels were significantly increased compared with vehicle-treated control animals at 90 min and 4 h (Fig. 1). Unlike CINC, MIP-2 remained undetectable in the plasma at both time points after the IT challenge. Increases in plasma CINC were associated with large increases in lung CINC mRNA expression compared with control animals at 90 min and 4 h after IT LPS (Fig. 2). In contrast, only small increases in CINC mRNA were detectable in extrapulmonary tissues at both time points. Similarly, MIP-2 mRNA expression was highly increased in lung tissue compared with vehicle-treated controls at 90 min and 4 h after IT LPS (Fig. 2). Small increases in MIP-2 mRNA were also detected in liver and kidney at the 4-h time point, but these increases were not associated with detectable plasma levels of this chemokine.

Chemokine distribution in response to IT rCINC and/or rMIP-2. To determine whether CINC, as opposed to MIP-2, can be selectively transported from the alveolar space to the intravascular compartment, rCINC and/or rMIP-2 was instilled IT into rats. Blood samples were then taken over a 4-h time course for the measurement of plasma CINC and MIP-2.

As early as 5 min after a combined IT injection of rCINC and rMIP-2, plasma CINC levels were significantly higher than baseline values (Fig. 3). Plasma CINC remained elevated throughout the entire 4-h observation period. Similar results were seen when rCINC was injected by itself (Fig. 4A). No change in CINC mRNA expression was detected in lung, spleen, liver, or kidney samples of animals receiving IT rCINC (data not shown). Neither IT rMIP-2 alone nor PBS resulted in any change in plasma CINC concentration. In contrast to CINC, plasma MIP-2 levels did not increase after IT injection of rMIP-2 alone or in combination with rCINC (Figs. 3 and 4B).

BALF levels of CINC and MIP-2 were determined in all animals receiving IT injections of the two chemokines. Upon death at 4 h, MIP-2 levels were substantially higher than CINC in animals receiving combined injections of CINC and MIP-2, even though equal amounts of the two chemokines were initially injected (Fig. 5). Similar results were observed when the two chemokines were administered individually (data not shown).

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**Fig. 1.** Plasma cytokine-induced neutrophil chemoattractant (CINC) and macrophage inflammatory protein-2 (MIP-2) after intravenous (IV) or intratracheal (IT) administration of endotoxin or PBS. Measurements for both chemokines were made at 90 min (A) and 4 h (B) after the IV/IT challenge. ND, not detectable. Values are expressed as means ± SE (n = 5). *P < 0.05 compared with time-matched controls.
Distribution of radioactivity in response to IT 125I-labeled rCINC or rMIP-2. After IT administration of 125I-rCINC but not 125I-rMIP-2, 125I counts were elevated in plasma TCA precipitate at all time points (Fig. 6). Increases in counts corresponded to increased concentrations of plasma CINC protein (data not shown), such that 125I-CINC specific activity did not change during the 4-h observation period and was comparable to the specific activity found in BALF at the conclusion of the procedure.

Pulmonary neutrophil recruitment after IT CINC and/or MIP-2. CINC and MIP-2 are potent chemokines for the recruitment of neutrophils into the rat lung (35, 42). To monitor this important biological effect, neutrophil recruitment was assessed in BALF after IT chemokine instillation (Fig. 7). Four hours after IT rCINC or rMIP-2 alone, the number of neutrophils was significantly increased compared with animals receiving IT PBS. No statistical difference was observed in neutrophil recruitment between rats receiving IT rCINC vs. IT rMIP-2.

Animals receiving a combined injection of CINC and MIP-2 had a robust increase in the number of neutrophils recovered from BALF (Fig. 7). This increase was significantly greater (~6-fold) than that seen in rats receiving either chemokine individually.

Effect of systemic chemokines on pulmonary neutrophil recruitment in response to IT rMIP-2. To determine the effect of systemic CINC on pulmonary neutrophil recruitment, rCINC was administered IV 20 min after an IT challenge with rMIP-2. Neutrophil numbers were measured in BALF 4 h after IT rMIP-2. The effect of IV rCINC on pulmonary neutrophil migration was compared with the effects of IV rMIP-2 and IV PBS (Fig. 8A). After IT rMIP-2, IV rMIP-2 did not result in a statistical increase in the number of neutrophils recovered in BALF compared with vehicle-treated controls. In contrast, IV rCINC induced a considerable increase in pulmonary neutrophil recruitment 4 h after IT MIP-2. Thus the systemic presence of these two chemokines produced a differential effect on pulmonary neutrophil recruitment.

Total and differential white blood cell counts were also determined in animals treated with IT MIP-2, followed by IV CINC, MIP-2, or PBS (Fig. 8, B and C). No difference was identified in total white blood cell numbers in animals receiving IV rCINC, rMIP-2, or PBS. However, the percentage of blood neutrophils was significantly increased in animals treated with IV rCINC or rMIP-2 compared with rats treated with IV PBS.
DISCUSSION

The results of our study indicate that intrapulmonary CINC enters the vascular compartment to augment neutrophil recruitment. Furthermore, the data show that the translocation of CINC out of the lung is a selective process in that MIP-2 remains within the intra-alveolar space.

While lung, spleen, liver, and kidney were highly responsive to IV LPS with regard to MIP-2 and CINC mRNA synthesis, the gene expression of these two chemokines was largely restricted to the lung in response to an intrapulmonary LPS challenge. These data imply that the lung is the primary source of both chemokines after IT LPS, despite clear differences in distribution between the pulmonary and systemic space. However, this evidence alone cannot preclude the possibility of extrapulmonary CINC production after the local LPS challenge. For this reason, additional studies were performed to assess the hypothesis that intrapulmonary CINC, as opposed to MIP-2, can selectively access the vascular compartment.

Although many cell types within the lung are capable of expressing CINC and MIP-2, alveolar macrophages are the predominant cell source in response to an intrapulmonary challenge (14, 15, 20). If the source of plasma CINC is the alveolar macrophage, then CINC would have to cross several barriers, including epithelial and endothelial cell layers, to enter the intravascular space. To examine this possibility, we administered equal amounts of rCINC and rMIP-2 IT and found that only CINC was detected in the plasma. We propose that the CINC detected in the plasma is the same CINC that was delivered into the lung. Indeed, the higher quantity of MIP-2 recovered by bronchoalveolar lavage compared with CINC is consistent with this hypothesis. In the latter case, however, chemokine levels recovered by bronchoalveolar lavage could be influenced by other factors such as differential receptor binding, differences in binding affinity to extracellular matrix molecules such as sulfated sialomucins and glycosaminoglycans, and/or differences in protein degradation within the lung. Furthermore, because our assay for CINC does not discriminate between rCINC and endogenous CINC, it is possible that the CINC administered IT is not the same as that found in the plasma. In this regard, two possibilities exist. First, administered rCINC may stimulate endogenous CINC production. Second, the increase in plasma CINC could occur through the release of preformed CINC.

Several pieces of evidence obtained in our experiments argue against these two scenarios. First, CINC appeared in the...
plasma within 5 min of rCINC administration, an interval likely to be too short for the de novo synthesis of CINC. Second, IT CINC did not increase CINC mRNA expression in the lung or in any of the extrapulmonary tissues examined. Third, if rCINC was capable of inducing endogenous CINC production, one would expect rMIP-2, which also signals through CXCR2 (37), to elicit the same response. However, rMIP-2 caused no such effect in the current study. Collectively, these results strongly imply that IT rCINC did not increase plasma CINC by stimulating de novo production. Finally, we used $^{125}$I-rCINC and $^{125}$I-rMIP-2 to specifically demonstrate the selective decompartmentalization of CINC out of the intra-alveolar space. In this experiment, $^{125}$I appeared in plasma TCA precipitate after IT administration of $^{125}$I-rCINC but not $^{125}$I-rMIP-2. In addition, the specific activity of CINC in plasma throughout the 4-h observation did not differ from that found in the lung at the conclusion of the experiment. Thus we conclude that the CINC found in the plasma was the same as that administered into the lung and did not result from the release of preformed endogenous CINC.

The mechanism(s) underlying the selective transport of CINC, but not MIP-2, from the lung into the systemic circulation is/are yet to be defined. Although we demonstrate that intra-alveolar CINC can selectively enter the vascular compartment, it is also possible that differences in cell source for these chemokines could provide CINC with closer access to the vascular compartment after an intrapulmonary challenge. In fact, others have shown (using nonpneumonia models) that pulmonary microvascular endothelial cells are capable of producing both rat CINC and human IL-8 (4, 25). Although our current data do not preclude the possibility of pulmonary endothelial-/epithelial-derived CINC in the circulation, they do show that intra-alveolar CINC potentially contributes to the systemic increases of this chemokine observed during local lung infection. Similarities in size and amino acid structure make it unlikely that CINC, but not MIP-2, is capable of passive movement out of the alveolar space. Furthermore, it is unclear how the common receptor identified for these two chemokines, CXCR2, could permit selective active transcytosis of CINC but not MIP-2. A separate binding moiety possibly exists for CINC on the alveolar epithelial surface that induces active transcytosis of CINC out of the alveolar space. In this regard, it has been reported that an intact COOH-terminal heparin-binding domain is necessary for transcytosis of IL-8 across postcapillary venules in rabbits and that this action is integral to IL-8-mediated induction of a chemotactic response (27). Because both CINC and MIP-2 have a COOH-terminal heparin-binding domain (36, 47), small differences between these domains could account for the differential distribution of these two molecules.

MIP-2, which has a 72-fold greater affinity to the CXCR2 receptor than CINC (29), is a more potent chemotactic factor for PMNs both in vivo and in vitro (36, 52). The differences in potency between MIP-2 and CINC may be related to their different distributions and/or concentrations in response to an IT stimulus. In our study, however, IT rMIP-2 showed no greater effect on pulmonary neutrophil recruitment than IT rCINC. The robust increase in pulmonary PMN recruitment in the presence of both chemokines (~6-fold greater than either chemokine alone) corresponds to work from other laboratories demonstrating a synergistic effect between CINC and MIP-2 on in vivo neutrophil recruitment (11, 12, 42, 52). Therefore, although the presence of multiple neutrophil chemoattractants during infection is seemingly redundant, it is feasible that these proteins possess separate characteristics that potentiate the migration of blood neutrophils to extravascular sites of infection.

On the basis of this rationale, we sought to identify physiological significance for the decompartmentalization of CINC into the systemic circulation and found that IV rCINC, but not rMIP-2, significantly enhances pulmonary PMN recruitment in response to IT rMIP-2. The mechanism by which systemic CINC enhances neutrophil delivery to the challenged lung is unclear. One may propose that the effect of systemic CINC is largely due to neutrophil mobilization from the bone marrow and/or other tissues. Indeed, others have shown that related chemokines, such as human IL-8 and murine MIP-2, in addition to other chemoattractants, can induce the mobilization of neutrophils and progenitor cells into the bloodstream within minutes (21–23, 39). However, it seems unlikely that this process contributed to the selective effects of rCINC on lung PMN migration in the current study, since IV rMIP-2 induced similar changes in blood neutrophil numbers without a significant effect on local PMN recruitment. It is also unclear whether not the effects of IV CINC on PMN migration are attributable to chemotaxis itself as opposed to other processes such as PMN arrest. Much like their human counterpart IL-8, CINC and MIP-2 upregulate $\beta_2$-integrins on the surface of neutrophils (11, 12). Upon activation by systemic CINC, this process could then serve to position circulating PMNs within the pulmonary microvasculature. We hypothesize that systemic CINC activates circulating neutrophils, facilitating their response to subsequent, more potent chemotactic stimuli such as MIP-2 in the lung. Perhaps MIP-2 is not capable of such neutrophil “priming” due to its higher potency for receptor binding, calcium mobilization, chemotaxis, or other yet-to-be-defined functions. Thus exposure to MIP-2 may result in PMNs becoming refractory to further stimulation. In support of this speculation, investigators have shown that neutrophils activated with CINC can still respond to MIP-2, but the opposite is not true (2, 36, 37).

The same phenomenon is witnessed with GRO-α, the closest human homolog to rat CINC, in that IL-8 pretreatment abolishes IL-8-induced calcium mobilization in human neutrophils, whereas GRO-α pretreatment actually primes neutrophils to respond to IL-8 (16, 17). In the same study, CXCR2 stimulation upregulated CXC1 expression on human neutrophils, whereas CXCR1 binding actually decreased CXCR2 expression. Unlike GRO-α, which binds only to CXCR2, IL-8 binds to both CXCR2 and CXCR1, possibly contributing to the above-mentioned results. Because rat CINC and MIP-2 are believed to share a single receptor, CXCR2, comparisons between the human and rat chemokine interactions must be made carefully.

The generally accepted paradigm of chemokine function is based on leukocyte migration along a chemoattractant gradient, which seems to contradict our working hypothesis that systemic CINC enhances PMN chemotaxis into the alveolar space (5, 38). In fact, other investigators have shown that human IL-8 has no effect, or in some cases decreases, neutrophil delivery to sites of acute inflammation when present systemically (18, 24, 34). Here, we do not challenge the importance of solid-phase
chemokine gradients during the process of leukocyte migration. Instead, we hypothesize that soluble chemokines serve other purposes once presented and released from the luminal surface of the systemic vasculature.

Functional diversity exists among related chemokines, which may partially explain the preservation and redundancy of many chemokines across species. We conclude from our data that CINC is selectively released from the lung into the intravascular space, where it then augments neutrophil migration toward intrapulmonary chemotactic stimuli. Further studies in this area will improve our understanding of the mechanisms underlying the transport of certain cytokines and chemokines out of the intra-alveolar space and how these mechanisms may serve host defense.

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