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CINC-1 is identified as an acute-phase protein induced by focal brain injury causing leukocyte mobilization and liver injury

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ABSTRACT

Following injury or infection, the liver releases acute-phase proteins (APP). After a severe focal injury, this systemic response can be excessive and may lead to multiorgan dysfunction (MODS). CINC-1 is a neutrophil chemoattractant, and we have now established that it also functions as an early APP after injury to the brain or to peripheral tissues. After induction of a focal inflammatory lesion in the brain, there is rapid hepatic and serum CINC-1 induction, which is associated with increases in neutrophil numbers within the liver and within the circulation. CINC-1-mediated recruitment of neutrophils to organs distant from the primary injury site may contribute to MODS. Indeed, we found that enzyme markers of liver tissue injury are increased in the serum following generation of a focal inflammatory lesion in the brain. Neutralization of CINC-1 in the periphery reversed brain-injury-induced neutrophil mobilization and inhibited recruitment of neutrophils to the brain and to the liver. Thus, a significant component of the hepatic acute-phase response is the release of chemokines by the liver, which act to amplify the inflammatory response and modulate the subsequent leukocytosis and secondary tissue damage. Hepatic CINC-1 synthesis following injury presents a novel focus for treatment of inflammation.

Key words: MODS • IL-1β • cytokine • chemokine • neutrophil

An acute injury to the brain such as a traumatic or ischemic insult results in an acute inflammatory response (1), and components of this inflammatory response, including the recruitment of leukocytes and the local release of inflammatory mediators, may exacerbate the brain lesion. Cytokines such as interleukin (IL)-1β may induce degeneration in neurons compromised by the initial injury (2, 3), and recruited neutrophils also play a significant role in lesion exacerbation (4). We have shown previously that when IL-1β is microinjected into the brain parenchyma, CXC chemokines are induced (5, 6). Cytokine-induced neutrophil

chemoattractant-1 (CINC-1) is the major chemokine involved in neutrophil recruitment to the brain and spinal cord following an inflammatory challenge in the rat, although the other members of the family, CINC-2α and CINC-3, are also elevated (6). Elevated expression of the CINC proteins also has been reported in the rodent central nervous system (CNS) following stress (7), stroke (8), or peripheral endotoxin injection (9), and in the periphery in response to acute and chronic injury (10, 11). Moreover, adenovirally mediated expression of CINC in the rodent liver is associated with neutrophil recruitment and hepatic damage (12). The CINC chemokines are the rat equivalent of IL-8 and growth-regulated oncogene (GRO) proteins in humans, and elevated expression is associated with a spectrum of human inflammatory diseases(13).

Local inflammation may be accompanied by a systemic acute-phase response (APR), which is characterized by leukocyte mobilization, fever, and changes in serum levels of glucocorticoids, cytokines, and hepatically derived acute-phase proteins (APPs) (14). Inflammation in brain is no exception. It has been shown that following acute brain injury, a hepatic APR is elicited by as-yet ill-defined routes (15, 16). The role of the APR in influencing the outcome of the brain lesion is unknown at present. However, it is of interest that following brain injury there is a leukocytosis, and in some instances, recovery may be impeded by multiorgan dysfunction syndrome (MODS) (17). Although many features of MODS may be driven by low-grade systemic infection commonly associated with acute brain injury (18), it is possible that injury to the brain *per se* and the associated APR is involved.

In some instances, inflammatory lesions in the periphery can give rise to local production of cytokines, which are sufficient to mobilize leukocytes into the circulation. For example, in the inflamed asthmatic lung, there is evidence that the secreted cytokine IL-5 and the chemokine eotaxin are required for the release of eosinophils from the bone marrow and the subsequent eosinophilia (19, 20). The levels of chemokines generated in the brain parenchyma after an acute injury (5, 6) appear insufficient to account for the systemic leukocytosis that accompanies brain injury (17). We have therefore sought a mechanism that might amplify the inflammatory response after local brain injury in the rat. In this study, we evaluate whether CXC chemokines synthesized in the liver function as APR proteins, how hepatic synthesis of CXC chemokines may contribute to the pathogenesis of experimental brain injury, and what are the consequences of hepatic chemokine synthesis for the maintenance of liver integrity after injury.

MATERIALS AND METHODS

Reagents

Rat recombinant (rr) cytokines (rrIL-1β and rrIL-6) and ELISAs for rat IL-1β, IL-6, and tumor necrosis factor α (TNF-α) were obtained from the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, UK). The IL-1 is fully active in a murine cell bioassay and also in receptor binding and *in vivo* models of IL-1 activity (21). By LAL test, the IL-1 contains 100 IU endotoxin/mg IL-1 (corresponding to 10 ppm by weight), which, in view of previous studies (22), was considered negligible in the context of these experiments. The IL-6 is fully active in the B9 bioassay, and on a weight basis is two- to threefold more active than the international standard for human IL-6 (23). By the LAL test, the IL-6 contains 0.25–2.5 IU

endotoxin/mg IL-6 (corresponding to 2.5–25 ppm by weight). The cytokines were dissolved in endotoxin-free saline (vehicle). All other reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Animal and stereotaxic surgery

Twelve-week-old (>200 g) male Wistar rats were used throughout (Charles River, UK). In each experiment, at least three animals were used per group. All procedures were performed with local ethical committee approval and under UK legislation, Animals (Scientific Procedures) Act, 1986.

All surgical procedures were performed under an operating microscope (Wild M650, Leitz). The animals were anesthetized using isoflurane (Rhodia Organique Fine Ltd, Bristol, UK) for induction and maintenance at 2.5–3% in entonox (50% N_2O, 50% O_2). Stereotaxic surgery was performed. In brief, anesthetized rats were held in a stereotaxic frame. A small hole was drilled in the skull, and 1 ng or 1 μg of rrIL-1β or vehicle, in a volume of 1 μl, was microinjected into the striatum with a glass capillary needle (tip <50 μm) (coordinates from bregma: anterior +1.2 mm, lateral +3.0 mm, depth of 4.5 mm). Injections were made over a period of 5 min.

Neutralization of circulating CINC-1

Adult male Wistar rats were anesthetized under isoflurane. An affinity-purified goat polyclonal anti-CINC-1 antibody (R&D Systems, Minneapolis, MN) (50 μg per animal) or an affinity-purified goat polyclonal anti-human IgG (control IgG) was diluted in endotoxin-free saline (200 μl) and was administered i.v. through the tail vein 10 min before surgery. rrIL-1β (1 μg) was then microinjected into the striatum, and the animals were killed after 6 h.

Intraperitoneal injections

rrIL-1β (4 μg/kg), rrIL-6 (4 μg/kg), or lipopolysaccharide (LPS) from *Escherichia coli* (0111:B4) (500 μg/kg) were diluted in vehicle and administered intraperitoneally (i.p). Volumes administered i.p. were 200 μl. The doses of cytokine and LPS where chosen on the basis of previous reports to reflect physiologically relevant final concentrations (24–28).

Spinal cord injury

Following a partial laminectomy at thoracic level (T8), a finely drawn capillary tube was inserted through the dura into the gray matter of the spinal cord and 1 μl of saline was microinjected. The animals were killed after 2 h.

Tissue and serum collection

After 2-, 4-, or 6-h survival times, rats were deeply anesthetized with sodium pentobarbitone. Blood was collected and allowed to clot for 2 h at room temperature, and then serum was collected by centrifugation. Trans-cardiac perfusions were performed using heparinized saline. Tissue was removed, immersed in Tissue Tek (Miles, Elkhart, IN), and frozen in isopentane (histology) or in liquid nitrogen (mRNA/protein).

Analysis of numbers of circulating neutrophils

Whole blood was collected in the presence of 2 mM EDTA (pH 8.0) and leukocyte numbers assessed in a Cell-Dyn 1600 hematology analyzer (Unipath, CA).

Identification of neutrophils

Frozen, 1-μm-thick serial coronal sections were cut from tissue blocks. Neutrophils were identified using the anti-neutrophil serum HB199(5). The numbers of neutrophils present in the brain were counted in the superficial layers of the cortex and in the injected striatum. The distribution of neutrophils was examined through all liver lobes and found to be homogenous. For each tissue section, two representative fields were chosen and the average number of neutrophils was calculated and is expressed as the number of neutrophils/mm^2.

mRNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA extraction and Taqman RT-PCR were performed essentially as described previously using CINC-1/3-specific probes (6). mRNA is expressed as copies per ng RNA corrected to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Protein extraction and assay

Liver was homogenized on ice in phosphate buffer (0.5 M NaCl; 2.5 mM NaH_2PO_4; 7.5 mM Na_2HP0_4; 0.1% polyoxyethylene-sorbitan monolaurate [pH 7.3]) containing protease inhibitors (100 mM amino-n-caproic acid, 10 mM Na_2EDTA, 5 mM benzamidine, 90 mM AEBSF). Homogenates were centrifuged for 1 h at 100,000*g* in a Beckman TL-100 ultracentrifuge at 4°C, and the supernatants were retained. Proteins were assayed using the Bio-Rad (Herts, UK) D_c protein assay against bovine serum albumin standards.

Measurement of cytokine production and liver enzymes

ELISAs for the rat cytokines IL-1β, IL-6, TNF-α, CINC-1, and CINC-3 were performed as described previously (6). ELISA data are expressed as pg per ml of serum or pg per mg total protein for liver. Levels of liver enzymes (units/l at 37°C), alanine transaminase, aspartate transaminase, and alkaline phosphatase in sera obtained 6 h after the intrastriatal injection of IL-1β were determined using a Bayer Advia 1650.

Statistical analysis

The data are presented as mean ± SE of the mean for a minimum of three animals at each time point. Although all the data are expected to be normally distributed, given the small sample sizes (required by UK legislation), where statistical analysis has been used, we have used nonparametric Mann-Whitney U-tests (Statview v5, SAS) to compare groups.

RESULTS

CINC-1 is elevated in the liver and serum by prototypical APR inducers

Working with a well-established rat model, we injected the classical inducers of the APR, endotoxin (LPS) (Fig. 1a–c), IL-1β (Fig. 1d–f), or IL-6 (Fig. 1g, h) i.p. and analyzed the hepatic and serum levels of CINC-1 and CINC-3 by Taqman RT-PCR and ELISA. The i.p. injection of saline (vehicle) produced small increases in CINC-1 and CINC-3 mRNA, but these were trivial compared with the profound elevations of hepatic CINC-1 mRNA 2 h after the i.p. injection of LPS (Fig. 1a), IL-1β (Fig. 1d), or IL-6 (Fig. 1g). Hepatic CINC-3 mRNA was also induced after 2 h by the i.p. injection of LPS, IL-1β, or IL-6, but in each case, the level of induction was lower than that for CINC-1. The i.p. injection of LPS (Fig. 1b), IL-1β (Fig. 1e), or IL-6 (Fig. 1h) also resulted in the rapid appearance of the CINC-1 protein in serum at well above control levels. CINC-3 was present in the serum at 2 h after the injection of LPS (Fig. 1c) or IL-1β (Fig. 1f), though at a lower level than CINC-1. However, we detected no CINC-3 protein in the serum at 2 or at 6 h following the i.p injection of IL-6 or at 6 h after the i.p. injection of IL-1β.

Levels of hepatic and serum CINC are proportional to the severity of tissue injury

The acute and transient increases in expression levels of hepatic and serum CINC-1 and CINC-3 in response to proinflammatory challenges are consistent with the characteristics of an acute-phase protein (APP). The magnitude of expression of hepatically synthesized APPs is known to be proportional to the degree of injury (14). Thus we examined hepatic CINC expression (surgery alone: Fig. 1i) and serum CINC levels (surgery alone: Fig. 1j) following surgical injuries of increasing severity. We injected saline i.p., or into the brain parenchyma (striatum), or into the spinal cord parenchyma. Our previous observations have revealed that the spinal cord is more permissive than the brain to proinflammatory stimuli, which may be related to a differential APR (29). During spinal cord injections, prolonged anesthesia is required as a partial laminectomy is performed, which is accompanied by significant trauma and tissue damage. We found that CINC-1 mRNA was up-regulated in the liver in proportion to the degree of tissue damage (Fig. 1i). Similarly, in the serum, there were significant elevations in CINC-1 levels following an injection of saline into the spinal cord, but not after an i.p. or intracerebral injection. Although we detected CINC-3 mRNA in the liver following the differential injuries, CINC-3 protein was not detected in the serum of any of the experimental groups when investigating the injury response. Thus, CINC-1, but not CINC-3, satisfies the criteria of an APP (30).

CINC is elevated in the liver following injury to the brain

Previous studies from our laboratory have demonstrated delayed induction of APPs in the striatum and in the liver following a focal proinflammatory challenge to the mouse brain (16). To further investigate the putative role of CINCs as APPs, we injected 1 ng or 1 μg IL-1β into the rat striatum and measured the hepatic CINC mRNA (Fig. 2a, b) and serum CINC levels (Fig. 2c). We examined hepatic CINC-1 and CINC-3 mRNA expression at 2, 4, 6, 8, and 12 h. Both CINC-1 and CINC-3 mRNA were significantly increased 2 h after the injection of either 1 ng or 1 μg IL-1β into the striatum. We also observed significant elevation of CINC-1 protein in serum 2 h after the microinjection of 1 ng IL-1β into the striatum. Levels returned to basal values after

6 h. Increasing the dose of IL-1β to 1 μg resulted in further elevations in serum CINC-1 protein at 2 h, which remained significantly elevated after 6 h. Despite elevations in hepatic CINC-3 mRNA following injection into the striatum of IL-1β, we observed only low levels of CINC-3 protein in the serum 2 h after the injection of the 1 μg dose, and CINC-3 protein was not detected at any other time point or after 1 ng (results not shown). Note that the injection of IL-1β into the striatum induces significant local production of CINC-3 in the brain (6), but, as we now show, this protein does not enter the circulation.

The liver produces CINC-1, but not CINC-3, following focal brain injury

To determine whether the time course of elevated serum CINC levels was coincident with elevated hepatic levels, and thus identify the liver as a source of the serum CINC, we assayed CINC protein levels in the liver by ELISA (Fig. 2d). We could not detect CINC-1 protein in the liver of naïve rats, or in the liver 2 or 6 h after injection of saline into the striatum, but we observed elevated hepatic CINC-1 protein levels after 2 or 6 h in a dose-dependent manner in rats injected with either 1 ng IL-1β or 1 μg IL-1β into the striatum. CINC-3 was not detected in the liver following the focal injection of IL-1β at either dose or at any time point.

IL-1β injection in the brain mobilizes neutrophils into the circulation

To investigate the role of circulating CINC-1, we examined the number of neutrophils in the blood 2, 4, and 6 h after 1 ng or 1 μg IL-1β injections into the striatum (Fig. 3b). The injection of 1 ng IL-1β resulted in a significant increase in the number of neutrophils in the circulation at 6 h. When 1 μg IL-1β was injected into the striatum, neutrophil mobilization occurred earlier, at 2 h, and was more marked.

Neutrophils are recruited to the liver following focal brain injury and are associated with increased levels of serum transaminases

We examined liver sections, after immunohistochemistry, to determine whether the presence of CINC-1 protein was associated with neutrophil recruitment (Fig. 3a). Small numbers of neutrophils were present in the livers of naïve rats that were not altered by the microinjection of vehicle into the striatum. In contrast, the number of neutrophils present throughout all liver lobes was significantly increased in a dose-dependent manner from 2 to 6 h after the injection of 1 ng IL-1β or 1 μg IL-1β into the striatum. Recruitment of neutrophils to the liver in models of endotoxic shock is associated with increased levels of the serum transaminases (31). Thus, we examined whether the recruitment of neutrophils to the liver following the intrastriatal injection of IL-1β could increase the levels of serum transaminases. The level of aspartate transaminase (AST) and alanine transaminase (ALT) in the serum was significantly elevated following the intrastriatal injection of IL-1β, but the level of alkaline phosphatase (ALP) was unchanged (Fig. 4).

Neutrophil recruitment to the brain

In our previous studies, we found that following the injection of IL-1β into the striatum, neutrophils are recruited to the brain parenchyma only after a delay (32). Once again, we found

that 1 ng failed to recruit neutrophils to the brain parenchyma by 4 h, despite marked, though unilateral, recruitment to the meninges (results not shown) and to the liver. We increased the dose of IL-1β to 1 μg to determine whether the intrinsic resistance of the brain could be overcome. The 1-μg dose also failed to elicit neutrophil recruitment to the brain parenchyma after 4 h, despite the presence of bilateral meningitis and increased neutrophil recruitment to the liver. Six hours after the injection of 1 μg IL-1β, neutrophil recruitment was more marked in the meninges and neutrophils were also present in the superficial layers of the cortex and in the striatum around the injection site.

Communication from the brain to the liver

We analyzed serum levels of the proinflammatory cytokines IL-1β, TNF-α, and IL-6 by ELISA following the injection of 1 ng or 1 μg IL-1β into the striatum to investigate whether the communication from the brain to the liver is by cytokines in the blood. However, none of these proinflammatory cytokines was detectable in serum by ELISA at any time point.

CINC-1 neutralization

We blocked the functional activity of CINC-1 with an i.v. injection of purified anti-CINC-1-neutralizing IgGs. Six hours after the i.v. administration of the anti-CINC-1 and microinjection of 1 μg IL-1β into the striatum, the number of neutrophils recruited to the liver was significantly reduced compared with the number recruited following an equivalent injection of IL-1β and nonimmune goat anti-human IgG (Fig. 5a). In the brain, the anti-CINC-1 blocked neutrophil recruitment to the meninges, the superficial layers of the cortex (Fig. 5b), and in the striatum (Fig. 5c). The CINC-1 antibody was also effective in reducing the number of neutrophils present in circulating blood after the injection of 1 μg IL-1β into the striatum, compared with the level observed following the administration of the control IgG (Fig. 4d).

DISCUSSION

We show for the first time that, in addition to its established role as a neutrophil chemoattractant, CINC-1 also functions as an early APP. Injury increases hepatic CINC-1 synthesis and secretion that leads to raised circulating CINC-1 levels and neutrophil mobilization to the blood, the magnitude of which is proportional to the degree of initial injury. Thus, a significant component of the hepatic APR following CNS trauma is the release of chemokines by the liver, which act to amplify the inflammatory response and modulate the subsequent leukocytosis and secondary tissue damage. Hepatic chemokine synthesis following acute brain injury, or indeed injury of any organ, presents a novel focus for the treatment of inflammation. Thus, injury to the brain elicits a peripheral response that is likely to represent a useful target for novel antiinflammatory agents, especially those that do not cross the blood-brain barrier.

CINC-1 is an APP

Acute brain injury results in the rapid induction of IL-1β synthesis within the brain. The microinjection of IL-1β allows precise control of the intensity of the inflammatory challenge and the spatial location. Acute brain injury has also been shown to give rise to a hepatic APR (15,

16). The pattern of induction of CINC-1 synthesis observed in our study suggests that this chemokine is behaving as an APP. Peripheral challenge with APR inducers (14) resulted in rapid transient elevation (>>25%) of hepatic and serum CINC-1. The potential of the liver to produce the CINC chemokines in response to IL-1 and IL-6 has been previously demonstrated by primary liver cultures *in vitro* (33). Moreover, CINC-1 protein is known to be released by the liver in response to high doses of LPS in *in vivo* models of endotoxaemia (34) or following direct liver injury (10, 35), but nonchemoattractant roles for CINC-1 were not considered. Thus, the pattern of CINC-1 expression and its effects on leukocyte mobilization fulfill the criteria for an early APP (30). Indeed, CINC-1 is produced by the liver well before most of the other classical APPs, such as α_1-acid glycoprotein; α_1-proteinase inhibitor; and the major rat APP, α_2-macroglobulin, which is up-regulated 4–8 h following injury (30). APP induction in the liver following intracerebroventricular (i.c.v.) or parenchymal administration of IL-1β in mice has been reported previously (15, 16), but, again, the response was delayed compared with the induction of CINC-1 by IL-1β.

Enhanced blood levels of CINC-3 were observed following peripheral proinflammatory challenge but were not provoked by severity-dependent CNS injury. The elevation of CINC-3 mRNA in the liver without translation to protein indicates a degree of posttranscriptional control, as has been described previously for this (6) and for other chemokines (36).

Induction of hepatic CINC synthesis

The signaling pathways leading to the rapid induction of CINC-1 in the liver following a focal inflammatory challenge to the brain parenchyma remain unclear. Interstitial fluid may drain through perivascular spaces to the vasculature on the base of the brain and pass through the cribiform plate, where macromolecules are taken up at the vascular bed or drain into the lymphatic system (37, 38). Following i.c.v. injection, IL-1 escapes from the CSF into the peripheral circulation (39, 40), but the fate of cytokine after injection into the brain parenchyma is less clear. Measurements of serum IL-6, after IL-1β injection i.c.v., suggest that peak levels of this cytokine occur at 2h (39), but we were unable to detect increased levels of IL-1β, IL-6, or TNF-α in the blood following the focal microinjection of IL-1β into the brain. Thus, if there is release of cytokine into the circulation, it is insufficient to induce detectable serum levels of the principal proinflammatory cytokines. IL-6 or IL-1β knockout mice can still mount a normal systemic APR, suggesting that there is redundancy within the APR-inducing molecules (41–43). A saturable and inhibitable bidirectional transport system for IL-1β has been identified in brain endothelium, but the rate of transport is thought to be modest (44). Neural mechanisms may be operating via vagal efferents to control peripheral cytokine and, indirectly, chemokine synthesis. Indeed, parasympathetic pathways have been described showing that the vagal release of acetylcholine can modulate the release of cytokines in the liver (45). In the current study, we show that the amount of CINC-1 mRNA measured following the focal microinjection of IL-1β (1 μg/250 g rat) into the brain parenchyma is 10 times that measured following the injection of an equivalent quantity into the periphery (1 μg/250 g rat), suggesting that central IL-1β induces and amplifies the hepatic CINC signal. Interestingly, the induction of serum IL-6 by centrally administered IL-1β is greater than the induction of serum IL-6 by the injection of an equivalent amount of IL-1β given in the periphery. This phenomenon has been observed in hypophysectomised or adrenalectomised rats, which indicates that activation of the

hypothalamus-pituitary-adrenal axis is not essential for this effect of central IL-1β on serum IL-6 production (46). However, the amplification of peripheral serum IL-6 production by centrally administered IL-1β has been shown to be dependent on central opiate receptors (39). Similar neural amplification pathways may account for the amplification of the hepatic CINC-1 response following centrally administered IL-1β compared with the equivalent peripheral injection.

Leukocytosis and recruitment

A role for chemokines in the release of cells from bone marrow into the circulation is not without precedent. Circulating IL-5 and eotaxin together have been shown to mobilize bone marrow eosinophils, which are critical for their recruitment to local tissue injury sites (47). Subsequently, other chemokines such as monocyte chemoattractant protein-1 and IL-8 homologues have been implicated in the mobilization of leukocytes (48, 49), but this activity was not linked to hepatic chemokine synthesis or the APR. We show that hepatic CINC-1 appears to be operating as a systemic amplifier of the local inflammatory response, with the levels of neutrophil mobilization and subsequent neutrophil recruitment to the brain and liver being proportional to the hepatic and circulating CINC-1 levels. Dampening of the CINC-1 response, using neutralzing sera, was sufficient to reduce neutrophil mobilization and neutrophil recruitment to tissues distant from the initial injury site. In patients with acute brain injury, levels of cytokines and neutrophils are elevated in the blood and are thought to play a role in the pathology of injury that often occurs in several organs distant from the brain and may be a factor determining clinical outcome (17). The levels of both AST and ALT were significantly elevated and indicate that there is hepatocellular injury associated with the appearance of neutrophils in the liver following the intrastriatal injection of IL-1β. ALP was increased but not significantly. ALP may be raised significantly in primary cholestatic injury but is not raised acutely in the context of hepatocellular injury. Peripheral injections of LPS (31) or the adenoviral-mediated expression of CINC in the liver (12) is associated with increases in the numbers of neutrophils in the liver and in elevations of the serum transaminases, but it was surprising to discover that a focal lesion within the brain could also result in neutrophil recruitment to the liver, giving rise to hepatocellular injury.

Anti-CINC antibodies have been successfully used following other tissue injuries (50–52). Previously, we have shown that the central injection of an anti-CINC antibody can inhibit neutrophil recruitment to the brain parenchyma by >50%, presumably acting at the local level (5). The present study has shown that systemically delivered anti-CINC-1 antibodies inhibit local neutrophil recruitment by >50%. Thus, manipulating the hepatic chemokine response may be beneficial for reducing both the neurological and the peripheral sequelae of acute brain injury. However, it is clear that the protective effects of neutralizing anti-chemokine antibodies need to gain access to multiple sites to be fully effective.

The current hypothesis of neutrophil recruitment describes local production of chemokine at the site of injury, establishing a gradient that attracts circulating leukocytes. When we now consider that the circulating neutrophils are exposed to CINC in the blood before local chemokine gradients are established, that hypothesis is seen to be a somewhat simplified scenario. CINC-1 expression peaks within the first 2 h following injury before falling dramatically to basal levels. We suggest that the acute production of chemokine is necessary to allow neutrophil mobilization. However, the levels must fall quickly to allow circulating neutrophils to detect increases in local

chemokine concentrations. This concept is supported by transgenic studies showing that prolonged exposure of leukocytes to circulating chemokine results in desensitisation of the chemokine receptors (53). The local chemokine gradient may also be supported by the sequestration of circulating CINC-1. It has been shown that i.v. administered CINC-1 enters the CNS compartment, but it is not clear precisely where the CINC-1 becomes bound (54). Exactly how local and systemic chemokines interact to control leukocyte recruitment remains to be elucidated. In addition, CINC proteins may have different functions; for example, CINC-1 may be involved in mobilization of neutrophils, whereas CINC-3 may provide a local chemokine gradient. Indeed, compared with CINC-1, the leukocyte recruitment profile is delayed following the microinjection of CINC-3 into the CNS (5). CINC-1 facilitates mechanical nociception in the CNS (55), and other atypical nonchemoattrattant roles may also exist for CINC-2 and CINC-3 in the CNS.

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Fig. 1

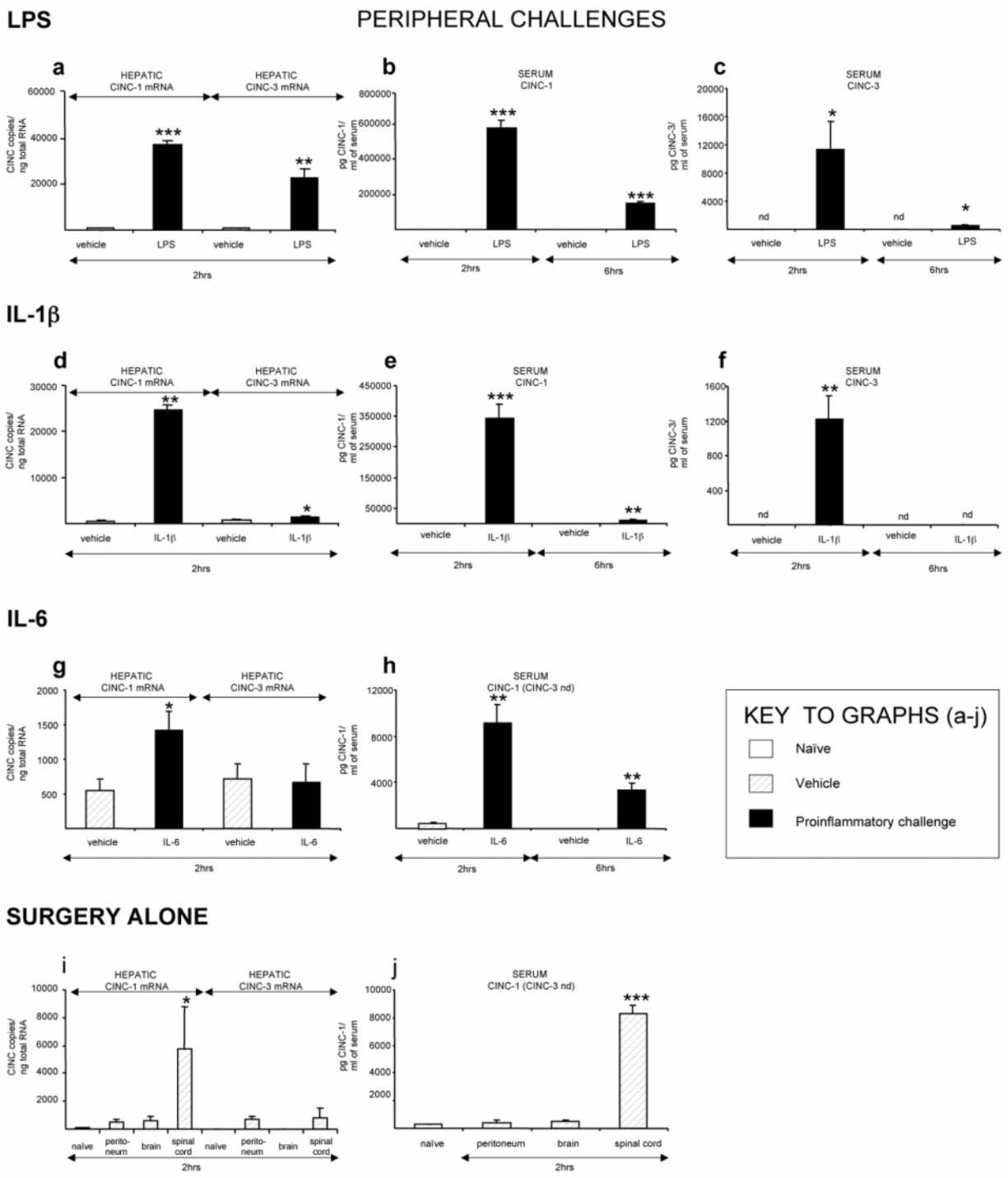

Figure 1. CINCs are elevated in the liver and serum following proinflammatory challenges or surgical injury in the periphery. Proinflammatory mediators: Hepatic CINC mRNA and serum levels were assessed by Taqman RT-PCR and ELISA 2 h after the injection of vehicle, lipopolysaccharide (LPS, 500 µg/kg), IL-1β (4 µg/kg), or IL-6 (4 µg/kg) into the peritoneum. Hepatic CINC-1 and CINC-3 mRNA 2 h after LPS (**a**), IL-1β (**d**), or IL-6 (**g**). ELISA-determined serum CINC-1 protein following LPS (**b**), IL-1β (**e**), or IL-6 (**h**) and serum CINC-3 following LPS (***c***) or IL-1β (**f**). Surgery alone: Hepatic CINC mRNA (**i**) and associated serum levels (**j**) 2 h after a saline injection into the peritoneum, brain, or spinal cord parenchyma. Key color coding of bars is shown. Results are expressed as number of copies of CINC mRNA per ng of total RNA as determined by measures of GAPDH ± SE of mean. ELISA results are expressed as pg CINC-1 or CINC-3 per ml of serum ± SE of mean. **P*<0.05, ***P*<0.01, and ****P*<0.001 when compared with naïve (**i-j**) or vehicle injection (**a-h**). nd, not detectable.

Fig. 2

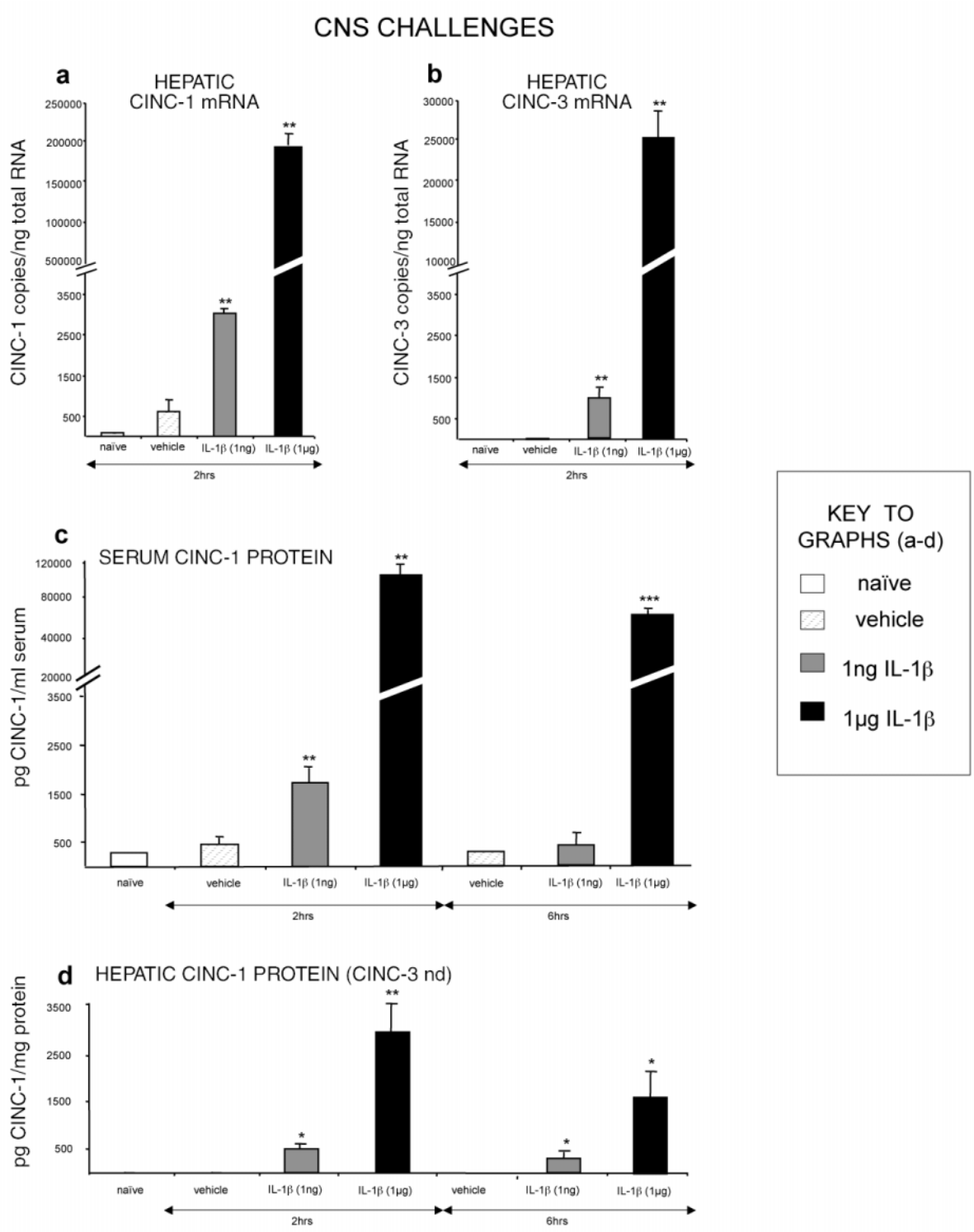

Figure 2. CINC mRNA is induced in the liver in response to proinflammatory challenge in the brain. Hepatic CINC-1 (**a**) and CINC-3 mRNA (**b**) 2 h after the microinjection of vehicle, 1 ng IL-1β, or 1 μg IL-1β into the striatum and associated serum CINC-1 protein (**c**) and hepatic CINC-1 protein (**d**) after 2 and 6 h as determined by Taqman RT-PCR and ELISA. Note the axis breaks to allow the direct comparison of CINC mRNA and protein expression at the different IL-1β doses. Key color coding of bars is shown. Results are expressed as number of copies of CINC mRNA per ng of total RNA as determined by measures of GAPDH ± SE of mean. ELISA results are expressed as pg CINC-1 per mg of protein or per ml of serum ± SE of mean. nd, not detectable. **P*<0.05, ***P*<0.01, and ****P*<0.001 compared with vehicle.

Fig. 3

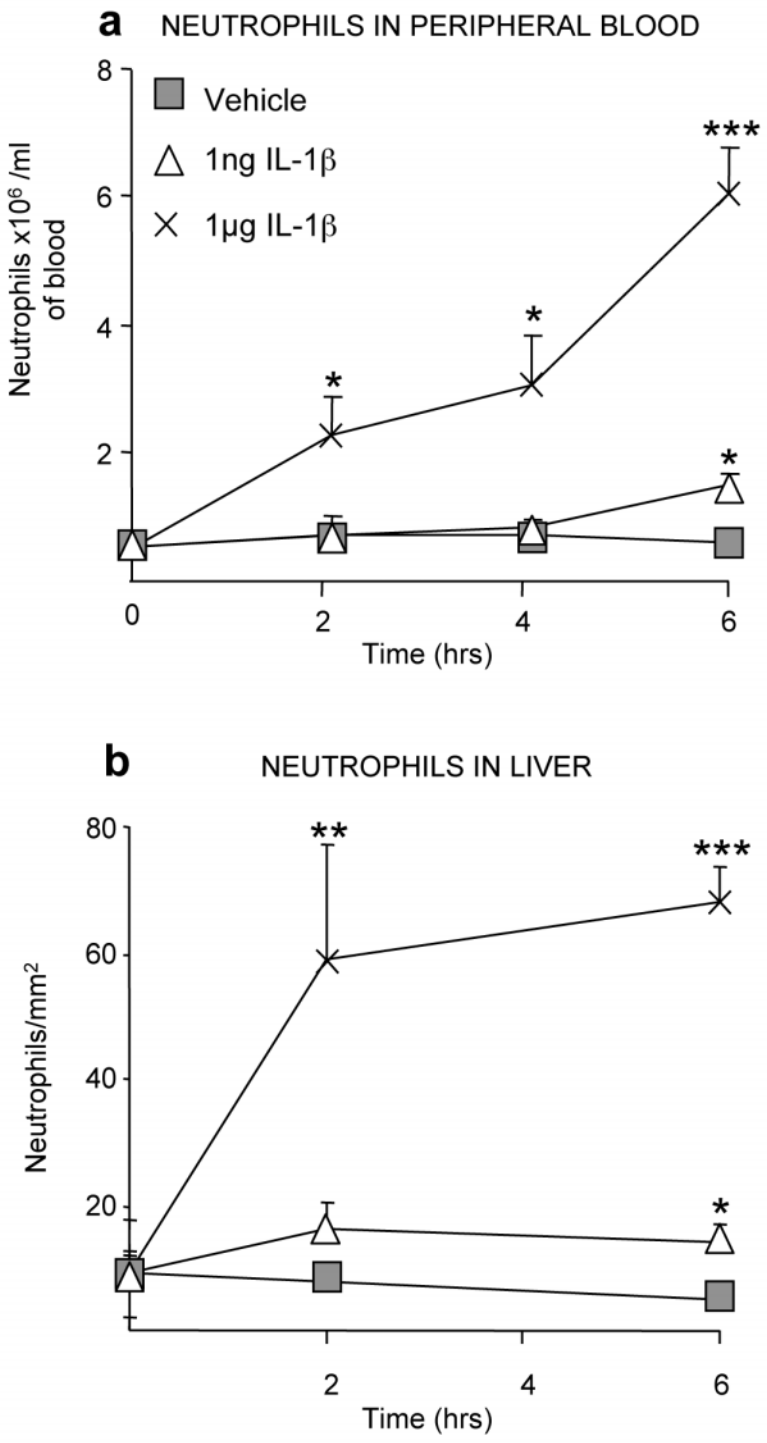

Figure 3. Neutrophils are recruited to the liver and blood following IL-1β challenge in the brain parenchyma. The number of neutrophils in circulating blood (**a**) at 2, 4, or 6 h following the microinjection of vehicle (squares), 1 ng IL-1β (triangles), or 1 μg IL-1β (crosses) into the striatum. Neutrophil numbers in circulating blood are expressed per ml of whole blood $\times 10^6 \pm$ SE of mean. Neutrophils, identified by immunohistochemistry, present in the liver 2 and 6 h after the microinjection of 1 ng IL-1β (triangles), 1 μg IL-1β (crosses), or vehicle (squares) into the striatum (**b**). Neutrophil numbers in liver are expressed per $mm^2 \pm$ SE of mean. **P*<0.05, ***P*<0.01, and ****P*<0.001 compared with vehicle.

Fig. 4

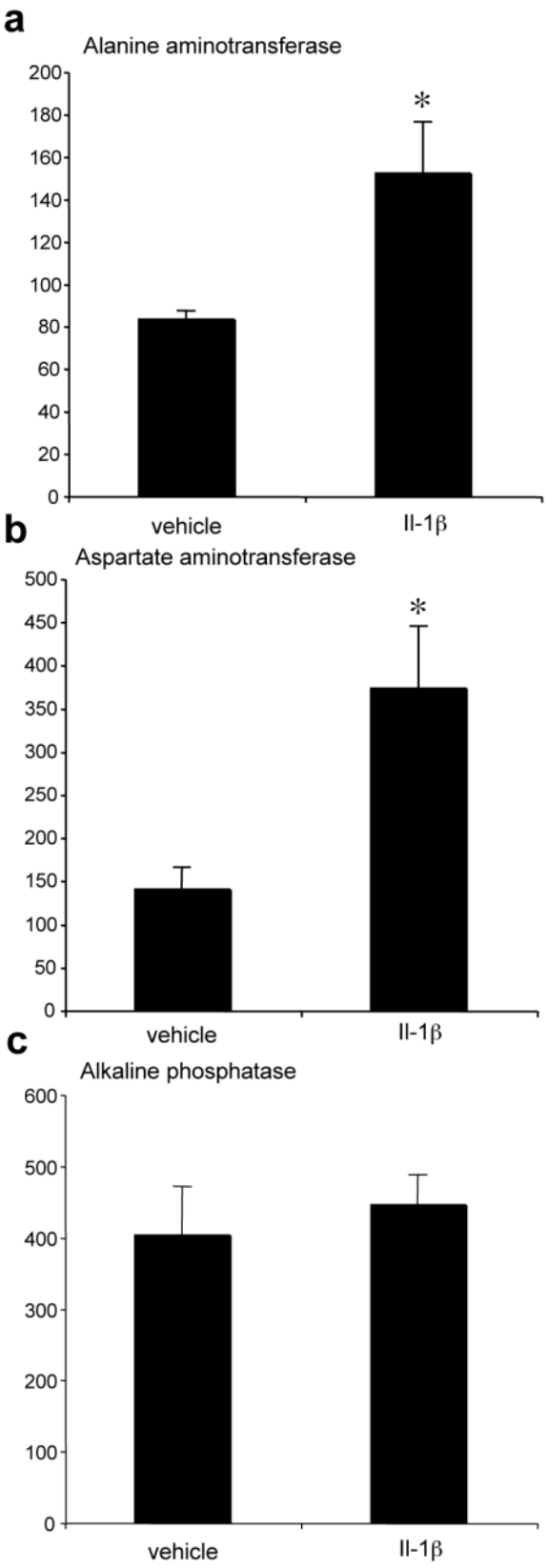

Figure 4. Liver enzyme levels. Alanine transaminase (**a**), Asparate transaminase (**b**), and alkaline phosphatase (**c**) levels in the serum 6 h after the injection of either IL-1β or vehicle into the striatum. **P*<0.05, ***P*<0.01, and ****P*<0.001 compared with vehicle.

Fig. 5

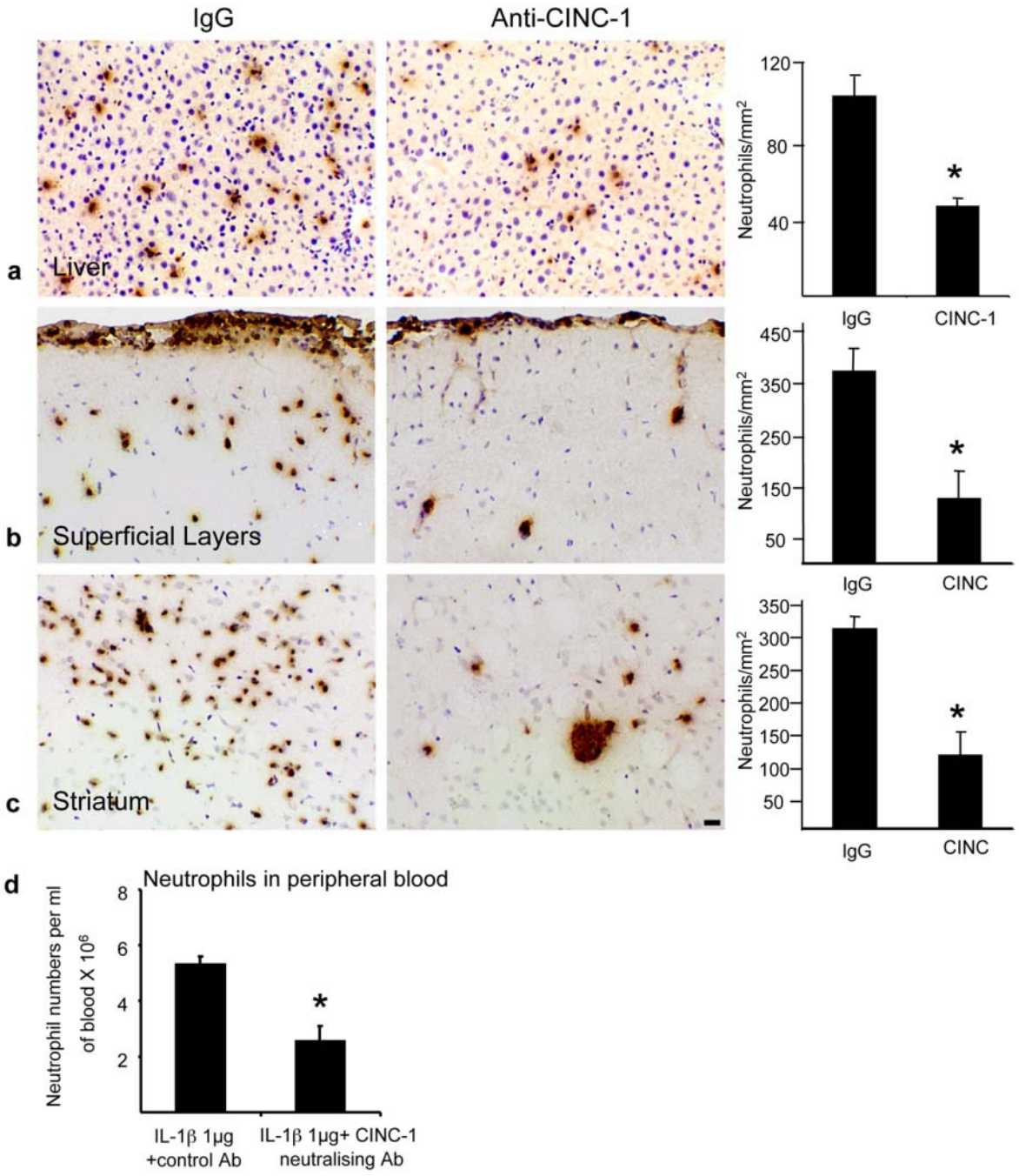

Figure 5. Leukocyte mobilization and recruitment are inhibited by neutralizing anti-CINC antibodies. Six hours after the injection of IL-1β into the striatum, the effect of the administration of anti-CINC-1 neutralizing antibody or purified IgG on neutrophil recruitment to the liver (**a**), to superficial layers of the cortex (**b**), and to the striatum (**c**) was assessed by quantitative immunocytochemistry. Representative photomicrographs of neutrophils on HB199-stained sections from the sites are shown after each treatment. Scale bar represents 20 μm. Note: Anti-CINC-1 antibody significantly reduces neutrophil recruitment to the liver and brain compared with the IgG control. Neutrophil numbers in liver and brain are expressed per $mm^2 \pm$ SE of mean. Hematological analysis of neutrophil numbers (**d**) following the microinjection of IL-1β into the striatum in the presence of either the anti-CINC-1 antibody or equivalent concentration of IgG. Note the reduction of neutrophils in the blood in the presence of the anti-CINC-1 antibody. Neutrophil numbers in circulating blood are expressed per ml of whole blood $\times 10^6 \pm$ SE of mean. **P*<0.05, ***P*<0.01, and ****P*<0.001 when compared with the matched IgG controls.