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Level of Neutrophil Chemotactic Factor CINC/gro, a Member of the Interleukin-8 Family, Associated with Lipopolysaccharide-Induced Inflammation in Rats

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Rat cytokine-induced neutrophil chemoattractant (CINC), which is a counterpart of human gro and belongs to the interleukin-8 family, has been quantified by a new sandwich enzyme-linked immunosorbent assay. Administration of lipopolysaccharide (LPS) into an air pouch preformed by subcutaneous injection of air caused inflammation and severe neutrophil infiltration. After the LPS injection, changes in the concentration of CINC/gro, chemotactic activity, and the number of neutrophils in the air pouch exudate were determined. The chemotactic activity of neutrophils was augmented before practical neutrophil infiltration. More than half of the chemotactic activity was neutralized by the antisera. The time kinetics of the level of CINC/gro coincided with the changes in chemotactic activity. The maximal level of rat CINC/gro was 85 ng/ml, which is sufficient to cause neutrophil migration in vitro and in vivo as described previously. These data suggest that rat CINC/gro is a functional chemoattractant for neutrophils in LPS-induced inflammation in rats.

Human interleukin-8 (IL-8) is a chemotactic cytokine for neutrophils and T lymphocytes which is produced in several cell species upon stimulation with IL-1, tumor necrosis factor, and lipopolysaccharide (LPS) (2, 4, 5, 13, 21). IL-8 is a member of a cytokine family which includes the gro gene product, platelet basic protein, and platelet factor 4. The gro gene product was first identified as a growth factor (20), melanoma growth-stimulating activity (MGSA) (9). Recent studies, however, revealed that human gro/MGSA was highly chemotactic for human neutrophils and shared a receptor on neutrophils with IL-8 (1, 10). Although there is no significant difference in the chemotactic properties of these two cytokines, the production of IL-8 predominates in most in vivo and in vitro experiments compared with that of gro/MGSA (10, 11).

On the other hand, when treated with IL-1, tumor necrosis factor, and LPS, cultured rat cells such as the primary culture of rat glomerular epithelial cells and the normal rat kidney epithelial cell line NRK-52E predominantly produced an IL-8-like neutrophil chemoattractant, cytokine-induced neutrophil chemoattractant (CINC) (14, 17–19). The primary structure of CINC revealed that it belongs to the family of human IL-8, but it is closer in sequence homology to gro/MGSA (69.4%) than to IL-8 (47.2%). Therefore, CINC is a rat equivalent of human gro/MGSA and hereafter will be referred to as CINC/gro. A rat equivalent of human IL-8 has not yet been identified, despite efforts to do so.

In parallel with the above in vitro studies, we have performed in vivo experiments with rat chemotactic factors existing in the exudate of inflammatory tissues. When a solution of LPS is infused into an air pouch preformed on the backs of rats, a strong chemotactic activity for neutrophils appears in the air pouch fluid. In order to examine whether CINC/gro is responsible for the chemotactic activity, we have raised anti-CINC/gro rabbit antisera and developed an enzyme-linked immunosorbent assay (ELISA) to measure CINC/gro levels in the air pouch fluid. The present paper demonstrates that CINC/gro is in part responsible for neutrophil recruitment in LPS-induced inflammation in rats.

MATERIALS AND METHODS

Polyclonal antibody to rat CINC/gro. Rabbits were immunized subcutaneously and intramuscularly with chemically synthesized CINC/gro (Peptide Institute, Osaka, Japan), which was conjugated to keyhole limpet hemocyanin. Freund's complete adjuvant was used to ensure immunization. Subcutaneous and intramuscular booster injections were repeated every 2 weeks. A total of 1 (first through the third challenge) or 0.5 (fourth through the sixth challenge) mg of CINC/gro was injected into several sites on a rabbit. After the sixth immunization, blood was collected and the antisera were isolated. Anti-CINC/gro antisera were purified by ammonium sulfate precipitation, and the immunoglobulin G (IgG) fraction was obtained by ion-exchange chromatography with DEAE-cellulose (DE-52, Whatman). Horseradish peroxidase-conjugated anti-CINC/gro rabbit IgG was prepared by the periodic acid method (6) and purified by gel filtration chromatography (Sephadex G-200).

Double radial diffusion test. Immunological double diffusion tests were carried out with anti-CINC/gro serum and CINC/gro or zymosan-activated serum (ZAS) by the method of Ouchterlony (8) with 1% agarose gel in Veronal buffer. Reaction mixtures were allowed to diffuse for 2 days at 4°C.

ELISA. Samples of pouch fluid were assayed for CINC/gro by ELISA. Microplates were coated with 100 μl of purified anti-CINC/gro IgG at 10 μg/ml in 50 mM bicarbonate buffer (pH 9.0) and incubated for 4 h at room temperature. Wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween) and then blocked overnight with 200 μl of 0.05% bovine serum albumin (BSA) in PBS-Tween at 4°C. These were then incubated for 2 h at room temperature with 100 μl of diluted

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pouch fluid. Wells were washed as before and incubated for 2 h at room temperature in 100 μl of 10 μg of horseradish peroxidase-conjugated anti-CINC/gro rabbit IgG per ml in 0.05% BSA–PBS-Tween and then incubated with 100 μl of enzyme substrate solution (0.05% *o*-phenylenediamine in 50 mM Na_2HPO_4–24 mM citric acid buffer (pH 5.0) containing 0.0034% H_2O_2) for 5 min. The reaction was stopped with 50 μl of 6 N H_2SO_4, and the optical density at 492 nm was recorded.

LPS-induced inflammatory exudate. Male Wistar rats, specific pathogen free and weighing 250 g, were used. While the rats were under light ethyl ether anesthesia, 14 ml of air was injected subcutaneously on the dorsum to make an ellipsoid or oval-shaped air pouch. One day after the air injection, 7 ml of sterilized 1% (wt/vol) sodium carboxymethyl cellulose (CMC) (Cellogen F-3H; Dai-ichi Kogyo Seiyaku Co., Niigata, Japan) in saline containing 1 μg of LPS per ml (*E. coli* 0111:B4; Difco, Detroit, Mich.) was injected into the air pouch. The CMC suspension helps to retain the inflammatory exudate in the pouch for several hours. When sample solutions were injected into the pouch without the CMC solution, they were instantly absorbed and eliminated from the inner space of the pouch (3, 12). At various times after the injection, the rats were sacrificed by cutting the carotid artery and an opening (0.5 to 1.0 cm in length) was cut into the skin at the top of the pouch. Aliquots of pouch fluid were centrifuged at 750 × *g* for 10 min, and the pellets were resuspended in RPMI 1640 medium (Nissui, Tokyo, Japan) for cell counting in a hemocytometer. Leukocytes were differentially counted after Wright-Giemsa staining. A portion of the pouch fluid was ultracentrifuged at 105,000 × *g* for 30 min at 4°C and the supernatant was frozen at −40°C until chemotactic activity, CINC/gro levels, and adsorption of chemotactic activity by polyclonal rabbit anti-CINC/gro antiserum could be measured.

Chemotactic activity. Chemotactic activity was assayed as described previously (15). Elicited by intraperitoneal injection of 1% casein solution, rat neutrophils were washed three times with RPMI 1640 medium and adjusted to a concentration of 10^7 cells per ml. The upper chambers were filled with 150 μl of cell suspension, and the lower chambers were filled with 200 μl of the appropriate samples. After incubation for 80 min at 37°C 5% CO_2-95% air, the fluid in the upper chambers was decanted and completely washed with water. The medium in the lower chambers was collected, and the numbers of migrated neutrophils in the fluid were counted with a Coulter Counter.

One unit was defined as the chemotactic activity equivalent to 1 ng of CINC/gro per ml.

Neutralization by rabbit anti-CINC/gro antiserum. Neutralization of inflammatory exudate chemotactic activity by polyclonal rabbit anti-CINC/gro antiserum was performed as described below. The inhibitory action of polyclonal rabbit anti-CINC/gro antiserum on the chemotactic activity of CINC/gro and LPS-induced inflammatory exudate was tested. Synthesized CINC/gro was dissolved in RPMI 1640 medium containing 0.1% human serum albumin (LPS free) (Fujirebio, Tokyo, Japan), or 15-fold diluted inflammatory exudates were incubated with an equivalent volume of 50-fold diluted anti-CINC/gro antiserum for 1 h at 37°C. Normal rabbit serum was used to determine control values.

RESULTS

Antiserum to rat CINC/gro and the ELISA. Anti-rat CINC/gro antiserum, which effectively bound the antigen after a 50,000-fold dilution, was obtained by repeated immunization (data not shown). In order to determine whether the antiserum bound to the other chemotactic peptides, such as C5a, double radial diffusion with CINC/gro and ZAS containing C5a was performed with an agarose gel. Visible precipitates were formed with the antiserum and CINC/gro but not with the ZAS (Fig. 1).

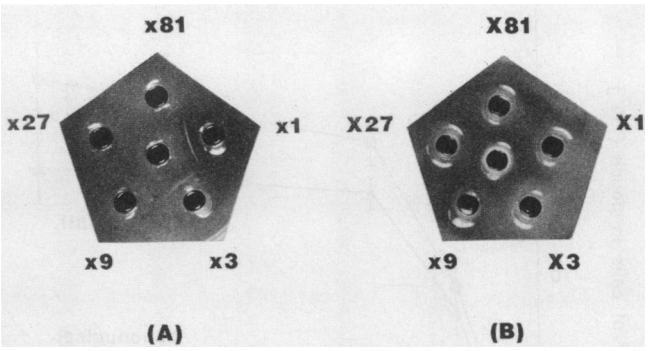

FIG. 1. Double radial diffusion in agarose gel. Central wells contained antiserum, and peripheral wells contained rat CINC/gro (A) or ZAS (B). CINC/gro (100 μg/ml) or ZAS was serially diluted with Veronal buffer (1-, 3-, 9-, 27-, and 81-fold dilution).

Polyclonal anti-CINC/gro IgG partially purified from the antiserum was used for ELISA. As shown in Fig. 2, the ELISA was sensitive enough to detect 250 pg to 10 ng of CINC/gro per ml.

Infiltrating cells in LPS-stimulated inflammatory exudate. Administration of CMC suspension containing 1 μg of LPS per ml into the air pouch preformed by subcutaneous injection of air caused an inflammatory reaction. The time kinetics of the number of cells in the pouch fluid after challenge with LPS is shown in Fig. 3. In the control rats that had been injected with the vehicle, few leukocytes had infiltrated into the pouch by 24 h (data not shown). On the other hand, LPS injection caused severe infiltration of leukocytes into the pouch. Almost all of the infiltrating leukocytes were neutro-

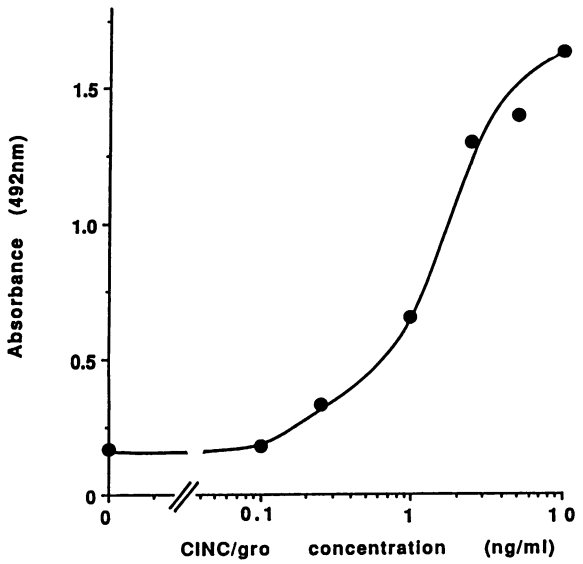

FIG. 2. Titration in ELISA of rat CINC/gro dissolved in PBS containing 0.05% Tween 20 and 0.05% BSA.

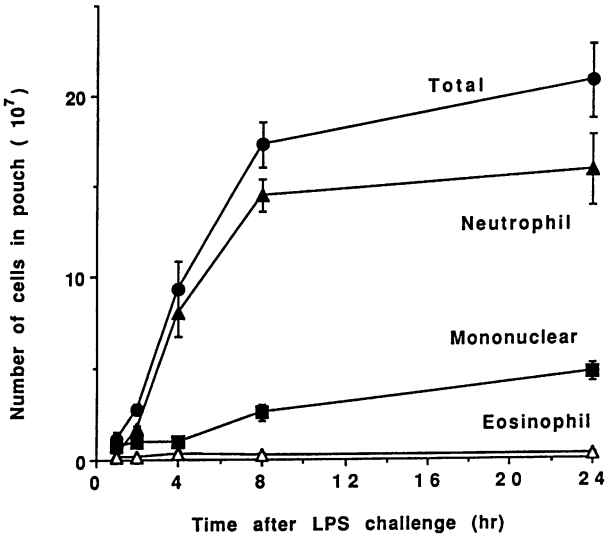

FIG. 3. Infiltration of inflammatory cells into the exudate induced by LPS injection. The numbers of total cells, neutrophils, mononuclear cells, and eosinophils in the exudate were counted. Each point represents the mean ± standard error (number of rats = 6).

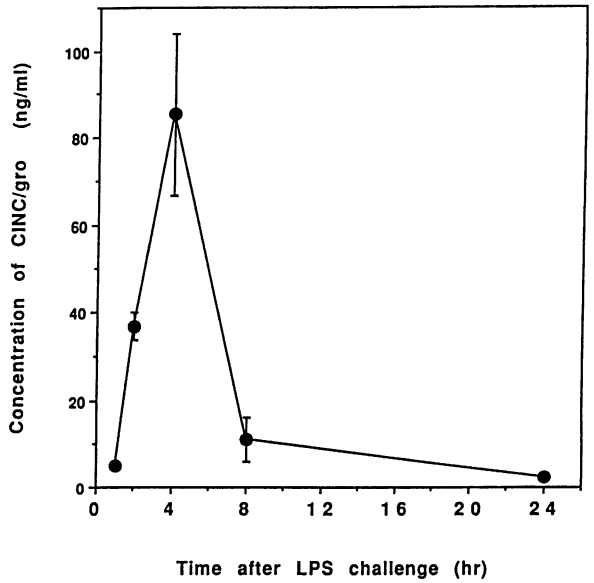

FIG. 5. Sandwich ELISA for CINC/gro in the LPS-induced inflammatory exudates. Concentrations of CINC/gro in the supernatant of the LPS-induced inflammatory exudates were determined by the ELISA described in Materials and Methods. Each point represents the mean ± standard error (number of rats = 6).

phils. Eight hours after the injection, 84% of the cells were neutrophils, 15% were mononuclear cells, and 1% were eosinophils (Fig. 3). Neutrophils accumulated in the inflammatory fluid after a lag phase of about 2 h.

Chemotactic activity in the pouch fluid. The time kinetics of the chemotactic activity of the pouch fluid is shown in Fig. 4. Chemotactic activity for neutrophils in the exudates significantly increased at 1 h and reached a maximum at 4 h after stimulation with LPS and then decreased gradually. An increase in chemotactic activity in the exudate preceded the accumulation of neutrophils in the pouch. Chemotactic activity in the exudate at 24 h after the sham injection was not determined, because sufficient exudate could not be collected without LPS stimulation.

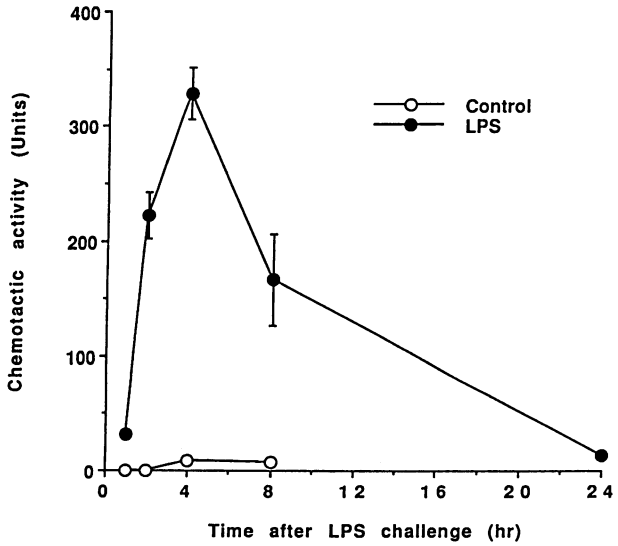

FIG. 4. Time course of chemotactic activity in the exudates. Seven milliliters (each) of LPS-CMC (1 μg of LPS per ml dissolved in 1% CMC solution in saline; number of rats = 6) and the vehicle (1% CMC solution in saline; number of rats = 4) was injected into a preformed air pouch. Appropriately diluted supernatants of pouch fluid were placed in the lower portion of the Boyden chamber. Rat neutrophils were loaded into the upper chambers. Each point represents the mean ± standard error of the migration rate.

Concentration of CINC/gro in the pouch fluid. The ELISA established in this study revealed the time kinetics of the concentration of CINC/gro in the LPS-stimulated inflammatory exudates (Fig. 5). The concentration of CINC/gro increased rapidly at 2 h and reached a maximum (85 ng/ml) at 4 h after the LPS injection and then decreased gradually. This change coincided closely with the change in chemotactic activity in the pouch fluid (Fig. 4).

Neutralization by rabbit anti-CINC/gro antiserum. To determine the capacity of anti-CINC/gro antiserum to neutralize rat CINC/gro, antiserum and the antigen were mixed and incubated for 1 h at 37°C. As shown in Fig. 6, a 100-fold dilution of the antiserum neutralized the chemotactic activity of 6×10^{-8} M (480 ng/ml) CINC/gro. On the other hand, the antiserum did not neutralize the chemotactic activity of ZAS containing C5a (data not shown). The antiserum had sufficient neutralizing capacity to neutralize about half of the chemotactic activity of LPS-induced inflammatory exudates obtained at 2, 4, and 8 h after stimulation, but it failed to completely abolish the chemotactic activity (Fig. 7).

DISCUSSION

A potent antiserum to rat CINC/gro was raised by immunizing rabbits with chemically synthesized pure rat CINC/gro as an antigen. A sandwich ELISA for the quantification of rat CINC/gro was established with the polyclonal IgG antibody purified from the antiserum. The ELISA was sensitive enough to measure 250 pg of CINC/gro per ml, as shown in Fig. 2. Although we also tried to obtain a murine monoclonal antibody to rat CINC/gro, mouse KC, which is

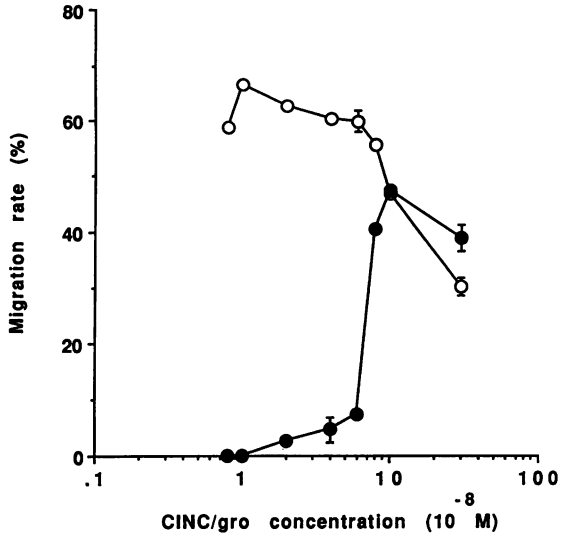

FIG. 6. Inhibition of chemotactic activity of CINC/gro by polyclonal rabbit anti-CINC/gro antiserum. Serially diluted CINC/gro solutions were incubated with 100-fold diluted polyclonal anti-CINC/gro antiserum (●) or 0.1% human serum albumin (○) for 1 h at 37°C, and then the residual chemotactic activities were measured by Boyden's method as described in Materials and Methods. Each point represents the mean ± standard error (n = 3).

the equivalent of rat CINC/gro (7), is too homologous in its amino acid sequence to sufficiently immunize the mice; rat CINC/gro, consisting of 72 amino acid residues, differs from mouse KC at only six amino acid residues in its amino acid sequence (17). Furthermore, four of the six residues are located in the beta sheet structure by which CINC/gro forms a homodimer. As those four amino acid residues are situated at the inner space of the molecule, CINC/gro appears to have only two residues acting as outer epitopes to the mice.

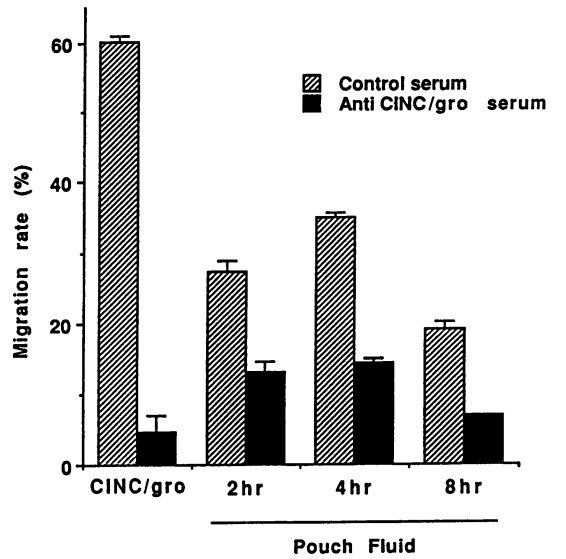

FIG. 7. Inhibition of pouch fluid and CINC/gro chemotactic activity by polyclonal anti-CINC/gro serum. CINC/gro (4×10^{-8} M) or 30-fold diluted exudates obtained 2, 4, and 8 h after the LPS injection were incubated with 100-fold diluted anti-CINC/gro serum and control serum for 1 h at 37°C. Residual chemotactic activities were tested by Boyden's method as described in Materials and Methods. Each point represents the mean ± standard error (n = 3).

Application of LPS inside the air pouch, which was preformed by subcutaneous injection of air, provoked an inflammatory reaction, resulting in vigorous infiltration of neutrophils into the air pouch. Infiltration of mononuclear cells was also observed, though they were the minority in the air pouch fluid (Fig. 3). The neutrophil infiltration into the pouch fluid occurred after a time lag of about 2 h, while chemotactic activity in the pouch fluid had already reached a submaximal level at 2 h after LPS injection (Fig. 4). A maximum level of chemotactic activity was observed at 4 h. The activity declined and reached a noninflammatory level at 24 h. On the other hand, a marked increase in the number of neutrophils occurred between 2 and 8 h. In other words, the increase in chemotactic activity preceded the neutrophil infiltration. Consequently, it is natural to conclude that the rise and fall in chemotactic activity plays a decisive role in neutrophil accumulation in the inflammatory site.

The sandwich ELISA established in this study enabled us to determine CINC/gro levels in the air pouch fluids. After the injection of LPS, CINC/gro levels increased rapidly between 1 to 4 h, reached a maximum of 85 ng/ml at 4 h, and then decreased to 11 ng/ml at 8 h (Fig. 5). The changes coincided closely with the time kinetics of chemotactic activity in the exudate (Fig. 4). The relationship between CINC/gro concentration and chemotaxis of neutrophils is not always parallel. The dose-response curve was bell-shaped, as previously described (16). This is the reason why CINC/gro levels fell from 85 to 11 ng/ml at 8 h after the injection, whereas about 50% of the chemotactic activity still remained in the exudate. In fact, 10 ng of CINC/gro per ml sufficiently attracts neutrophils in vitro (16). The maximum level of CINC/gro in the pouch fluid, 85 ng/ml, was very close to the optimal concentration, 10^{-8} M, at which CINC/gro caused maximal neutrophil infiltration in vivo and chemotaxis in vitro as previously described (16). These data indicate a significant role for CINC/gro as a chemotactic factor in neutrophil infiltration in LPS-induced inflammation in rats.

The antiserum raised against CINC/gro was capable of neutralizing the chemotactic activity of the antigen. Antiserum diluted 100-fold abolished the chemotactic activity of CINC/gro at 480 ng/ml. On the other hand, the potent antiserum with strong neutralizing activity failed to exert complete blocking of chemotactic activity in the LPS-induced inflammatory exudates (Fig. 6), in which the maximum CINC/gro level was only 85 ng/ml (Fig. 5). This suggests the existence of another neutrophil chemotactic factor in the LPS-induced inflammatory exudates. As no significant neutrophil chemotactic activity was detectable in the Sep-pak C-18 column-bound fraction in the exudate, neither leukotriene B_4 nor platelet-activating factor could be involved as a possible second factor. Furthermore, complement-derived C5a was not detected in the basic protein fraction collected from the exudate (data not shown).

Human cells and tissues are known to synthesize at least two molecular species of chemotactic cytokines, IL-8 and gro, for neutrophils. Generation of IL-8 is predominant, and gro appears as a minor component (10, 11). Therefore, it is very likely that IL-8 is the major chemotactic cytokine in humans. On the other hand, no rat equivalent of human IL-8 has yet been identified, despite the efforts in this laboratory. We report here that CINC/gro is responsible for neutrophil recruitment as a major chemoattractant in LPS-induced

inflammation in rats. These data suggest that expression of the gro-related gene in the inflammatory locus in rats is by far higher than that in humans. It is likely that CINC/gro in rats is the functional equivalent of IL-8 in humans.

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