Previous studies have shown that mast cell granules (MCG) inhibit numerous macrophage functions including tumour cytotoxicity, superoxide and nitric oxide (NO) production, and FCγR2a receptor-mediated phagocytosis. In this study, the effect of MCG on macrophage TNFα and nitric oxide synthase (iNOS) mRNA expression, and the production and fate of TNFα were examined. Upon activation with LPS+IFNγ, macrophages expressed both TNFα and iNOS mRNA and produced both TNFα and NO. Co-incubation of LPS+IFNγ-activated macrophages with MCG resulted in dose-dependent inhibition of iNOS mRNA expression. TNFα production in the activated macrophages was decreased by MCG, which was associated with a reduction in TNFα mRNA expression. MCG were also capable of degrading both macrophage-generated and recombinant TNFα. The direct effect of MCG on TNFα was partially reversed by a mixture of protease inhibitors. These results demonstrate that MCG decrease the production of NO and TNFα by inhibiting macrophage iNOS and TNFα gene expression. Furthermore, MCG post-transcriptionally alter TNFα levels via proteolytic degradation.

**Key words**: Nitric oxide, TNFα, Macrophages, Mast cell granules, Proteases

**Introduction**

Macrophages play a major role in microbicidal and tumoricidal activity, antigen presentation, and the production of a variety of cytokines and inflammatory mediators. Mast cells, on the other hand, play a key role in hypersensitivity reactions by secreting mediators such as histamine, proteoglycans, various cytokines, metabolites of arachidonate and unique proteases. The recruitment of macrophages to sites of mast cell degranulation and the subsequent phagocytosis of granules in vivo was first reported by Fawcett. We have previously shown that mast cell granules (MCG) interact with rodent macrophages and downregulate superoxide production, tumour cell killing and NO production. These reports confirm that mast cell-macrophage communication has a regulatory effect on host defence mechanisms and inflammation. A role for mast cells in tumour growth and metastasis is evident by the presence of an increased number of mast cells at the periphery of certain tumours and by the angiogetic activity of several mast cell products. Similarly, macrophage products such as NO and TNFα are implicated in tumour growth and inflammation. The fact that increased number of mast cells are found in the periphery of tumours and MCG interaction inhibited macrophage-mediated tumour cell killing prompted us to examine the mechanism of the effect of MCG on macrophage NO and TNFα production. The focus of this study was, therefore, to examine the effect of MCG on mRNA expression of iNOS and TNFα in macrophages. The results demonstrate that MCG interact with macrophages, inhibit iNOS and TNFα mRNA expression, and degrade TNFα.

**Materials and methods**

**Materials**

Lipopolysaccharide (LPS) from *Escherichia coli* cell wall, penicillin, streptomycin, HEPES, metrizamide, leupeptin, pepstatin A, aprotinin, phenylmethylsulfonyl fluoride (PMSF) and thiazolyl blue dye (MTT) were purchased from Sigma Chemical Co. (St Louis, MO). Soybean trypsin inhibitor was purchased from Boehringer Mannheim (Indianapolis, IN). Heparin solution was supplied by Elkins-Sinn, Inc. (Cherry Hill, NJ). Minimum essential medium with Earle's salts (MEM) and fetal bovine serum were obtained from Hyclone Laboratories (Logan, UT). The ELISA kit for mouse TNFα was purchased from Genzyme (Cambridge, MA). Plastek M cell culture plates were from Mat Tek Co. (Ashland, MA). The RNA Stat-60 kit was purchased from Tel-Test, Inc. (Friendswood, TX). All tissue culture reagents used in this study were free from detectable levels of endotoxin (<25 pg/ml) when tested by the limulus amoebocyte lysate gel-clot assay (Associates of Cape Cod, Inc., Woods Hole, MA).
Animals
Male Sprague-Dawley rats (350–400 g) used for harvesting mast cells, and male C57BL/6J mice (2–3 months old), the source of macrophages, were purchased from Harlan Co., Indianapolis, Indiana.

Harvesting and culture of murine peritoneal macrophages
Three days prior to harvesting peritoneal exudates, each mouse was injected intraperitoneally with 1.5 ml of a sterile solution of proteose peptone (10% w/v). The peritoneal cavity of each mouse was lavaged twice with 2.5 ml of minimum essential medium containing 15 mM HEPES, 100 units/ml of penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum (HMEM) and 5 units/ml of heparin. The pooled cell suspension was sedimented by centrifugation at 250 × g for 10 min, washed twice and resuspended in HMEM without heparin. Aliquots of the cell suspension containing 2 × 10⁵ cells were seeded in each well of a 96-well culture plate. Non-adherent cells were removed by washing after 2–4 h of incubation at 37°C. Macrophages were activated with LPS (100 ng/ml) + IFN-γ (10 units/ml) in all experiments, since this concentration has been found to induce optimum activation.

Isolation of mast cells
The method employed for the isolation of mast cells has been previously described. In brief, mast cells were collected by lavage of the peritoneal and thoracic cavities of adult rats with 50 ml HMEM containing 5 units/ml heparin. The lavaged cells from all animals were pooled, centrifuged at 250 × g for 10 min at room temperature, and washed twice with HMEM. Two ml of the cell suspension containing 6–8 × 10⁷ cells were gently layered on a 3 ml cushion of 22.5% (w/v) metrizamide (density 1.125 g/ml) in HMEM in a 15 ml centrifuge tube, and centrifuged at 200 × g for 15 min at room temperature. Mast cells were sedimented at the bottom of the conical tube while other cells (predominantly macrophages) collected at the interface. The mast cell fractions were collected, washed twice, and resuspended in HMEM without heparin. Purity and viability of mast cells isolated by this procedure exceeded 95%

Preparation of MCG and MCG sonicate
Under sterile conditions at 0–4°C, MCG were prepared from metrizamide-purified mast cells by controlled sonication and sucrose gradient centrifugation. Mast cells were suspended in 1 ml of HMEM and sonicated twice for 20 s with a microtip sonicator (Sonifier Cell Disruptor, model W140) at a power setting of 2.5 and temperature of 4°C. The disrupted cells were incubated at 30°C for 15 min and mixed vigorously for 1 min. The sonicate was layered on 2 ml of 0.34 M sucrose and centrifuged at 50 × g for 10 min at 4°C. The granules at the interface were collected and sedimented by centrifugation at 1800 × g for 20 min at 4°C. The resulting pellet consisting of a homogeneous preparation of MCG was washed twice and resuspended in the culture medium. The recovery of granules isolated by this procedure ranged from 60% to 80% based on the histamine content of the starting mast cells.

MCG-sonicate was prepared by sonicating purified MCG in HMEM three times for 30 s at maximum power. The quantity of MCG and MCG-sonicate used in each experiment was expressed as the equivalent of the starting mast cell number.

Assay of TNFα
The concentration of TNFα in the culture medium was assayed by both ELISA and bioassay. The sensitivity of ELISA is 31 pg/ml using the protocol recommended by the manufacturer. For the bioassay, the target L929 cells in RPMI-1640 medium were seeded (4.2 × 10⁴ per well) in a 96-well culture plate. After 18 h of culture, medium was replaced with 5 μg/ml of dactinomycin in RPMI. Two to 4 h after incubation with dactinomycin, serially diluted samples and TNFα standards (2–250 pg/ml) were added and incubated for an additional 18–24 h. The medium was then removed, and 1 mg/ml of MTT dye in RPMI (without serum and phenol red) was added. After 2–3 h of incubation, the medium was aspirated completely, and 100 μl of 2-propanol was added to each well. The plate was agitated for 10 min on an orbital shaker and read on a microtitre plate reader at 595 nm.

Analysis of iNOS and TNFα mRNA expression
Mouse peritoneal cells which contained > 85% macrophages were seeded in a 10 cm Petri dish and incubated for 4 h to allow for macrophage adherence. After washing three times with HMEM, the cells were treated with MCG and activated with LPS + IFN-γ. At selected time points after activation, culture medium was removed, and the total cellular RNA was extracted using an RNA extraction kit. Total RNA (10 μg) was electrophoresed on 0.8% agarose-formaldehyde gel and then transferred to Nytran nylon membrane. After 2 h of prehybridization, the membrane was hybridized with ³²P-labelled cDNA probe specific for murine iNOS, TNFα or β-actin. The probes were labelled by random hexamer priming method using [α-³²P] dCTP as previously described. The membrane was then washed four times and autoradiographed on a Kodak X-OMAT-AR film. A scanner was used to determine the density of the mRNA band and the relative density of each band was normalized to β-actin.
Treatment of MCG with protease inhibitors

MCG were incubated with a mixture of protease inhibitors containing PMSF 2.5 mM, pepstatin A 5 μg/ml, trypsin inhibitor 50 μg/ml, leupeptin 50 μg/ml, and aprotinin 50 μg/ml, at 37°C for 2 h. The protease inhibitor-treated MCG were then evaluated for their effects on macrophages.

Statistical analysis

Whenever applicable, the data were analysed by one-way analysis of variance with subsequent Student-Newman-Kuels’ test. All results were expressed as means ± SEM and P<0.05 was considered significant.

Results

Effects of MCG on macrophage iNOS mRNA expression

Earlier work demonstrated that MCG inhibit macrophage NO production. To investigate the mechanism, the effect of MCG on macrophage iNOS mRNA expression was analysed at selected mast cell-to-macrophage ratios. The addition of MCG simultaneously with LPS+IFNg to macrophage monolayer inhibited iNOS mRNA expression in a dose-dependent manner (Fig. 1). Maximal inhibition was noted at a MCG dose equivalent to a mast cell-to-macrophage ratio of 1:5 and the inhibition was evident even at a ratio of 1:40. When macrophages were pre-incubated with MCG for 3 h prior to activation, the inhibitory effect of MCG decreased compared with simultaneous addition of MCG and the activators. Furthermore, if macrophages were incubated with MCG for more than 6 h prior to activation, there was no inhibition of the expression of iNOS transcript (Fig. 2).

Effects of MCG on TNFα production

When activated with LPS+IFNg, macrophages generated large amounts of TNFα. Addition of MCG or MCG-S at the time of LPS+IFNg stimulation resulted in dose-dependent decrease in TNFα levels and more than a 90% decrease was noted at a MCG dose equivalent to a mast cell-to-macrophage ratio of 1.5 to 2 (Fig. 3). The MCG-induced decrease in TNFα levels was seen when determined by ELISA (Fig. 3A) and by bioassay (Fig. 3B). Although the assayed values differed for the same samples depending on the assay method employed, the decrease of TNFα levels was evident. There was no statistically significant difference between the effects of MCG and MCG-S. Therefore, in later experiments only the intact MCG were used. Unactivated macrophages or those...
exposed only to MCG or MCG-S did not produce detectable levels of TNFα.

The production of TNFα by macrophages was evident as early as 1.5 h after activation. The cumulative TNFα level increased for up to 24 h while the rate of production appeared to decrease (Fig. 4). The presence of MCG decreased the level of TNFα by approximately 40% at 1.5 and 6 h in contrast to a 95% decrease at 24 h. This effect was also evident when TNFα was assayed by bioassay (data not shown).

**Effects of MCG on TNFα mRNA expression**

In order to assess the effect of MCG on TNFα mRNA expression by activated macrophages, the transcripts were analysed at 2, 6 and 24 h of incubation after activation. The result demonstrates that TNFα mRNA was rapidly expressed to a maximal level within 2 h after activation and then declined thereafter (Fig. 5). Addition of MCG to LPS+IFNγ-stimulated macrophages inhibited TNFα mRNA expression by 60% at a mast cell-to-macrophage ratio of 1:3.

**Effect of MCG proteases on TNFα degradation**

Rat mast cell granules contain a variety of proteases including tryptase, chymase, and carboxypepti-
To investigate if TNFα is susceptible to these enzymes, conditioned media with known levels of TNFα from LPS+INFγ-activated macrophages or recombinant mouse TNFα were incubated with MCG for 24 h at 37°C. The TNFα levels, assayed by ELISA, indicate that both recombinant and macrophage-secreted TNFα were degraded by MCG (Fig. 6). This effect was partially reversed by the pre-treatment of MCG with a mixture of protease inhibitors containing PMSF, pepstatin A, trypsin inhibitor, leupeptin and aprotinin (Fig. 7). The complete abrogation of TNFα degradation by MCG could not be achieved even after extending the pre-treatment of MCG with these protease inhibitors to 24 h at 4°C.

Discussion

The present study demonstrates that incubation of macrophages with MCG during activation with LPS+INFγ results in the inhibition of iNOS and TNFα mRNA expression with a consequent decrease in NO and TNFα production. This corroborates our previous report documenting MCG inhibition of NO production and tumour cell killing by LPS+INFγ-activated macrophages. The MCG effect was evident even at an estimated mast cell to macrophage ratio of 1:40, a relationship likely to occur in vivo. The inhibitory effect of MCG on macrophage iNOS mRNA expression seems transient because this effect disappears when the macrophages were pre-incubated with MCG for 6 h or more. The same phenomenon was observed with the inhibitory effect of MCG on TNFα mRNA expression (data not shown). Our earlier studies have ruled out the possibilities that MCG may inactivate LPS or INFγ. Further investigation is needed to clarify the mechanism by which MCG inhibit macrophage iNOS and TNFα mRNA expression in a transient manner. The expression of TNFα by activated macrophages is of particular significance since the genes encoding iNOS and TNFα can be coordinately regulated. In vivo studies have suggested a role for TNFα in the positive regulation of iNOS following LPS injection. Anti-TNFα antibodies or soluble TNFα receptor antagonists partially block LPS-induced pulmonary iNOS activity or hepatic iNOS mRNA expression respectively. Furthermore, the involvement of TNFα in both the induction and maintenance of iNOS mRNA in macrophages was recently reported using an in vivo mouse model. These studies indicate that TNFα is an autocrine regulator of iNOS expression. The TNFα mRNA expression begins as early as 0.5 h, whereas iNOS mRNA cannot be detected until 8 h after activation. Our data also show that TNFα mRNA expression by macrophages is optimal at 2 h after activation and then progressively decreases. Based on those data we conclude that the depletion of macrophage-secreted TNFα by MCG could contribute to inhibition of LPS-induced iNOS mRNA expression. It is clear that the MCG to macrophage ratio that completely inhibits the expression of iNOS mRNA only partially inhibits the expression of TNFα mRNA. We do not yet have an explanation for this phenomenon. It is possible that the iNOS expression needs a finite amount of TNFα to trigger the gene.

Rodent mast cells contain histamine, serotonin, and proteases including serine proteases, neutral PMSE, pepstatin A, trypsin inhibitor, leupeptin and aprotinin. This is representative of four experiments. *P<0.05 vs. no MCG.
proteases, and carboxypeptidase A.\textsuperscript{10–23} Histamine and serotonin at concentrations present in mast cells (20 μg and 2 μg per million cells respectively) may induce immunomodulatory effects. For instance, histamine up-regulates macrophage synthesis of IL-1.\textsuperscript{29} However, histamine at concentrations in the range of 10^{-6}–10^{-3} M (equalling and exceeding that present in MCG used in this study) failed to affect TNFα and NO production by macrophages (data not shown). These results are in agreement with our earlier report of MCG inhibition of macrophage superoxide production, which showed that unlike MCG, histamine and serotonin were ineffective in modulating the respiratory burst.\textsuperscript{9} The effect of serotonin on macrophage NO and TNFα was not tested in this study. It is also noteworthy that histamine and serotonin are short-lived and their effects are rapidly lost after mast cell degranulation. Heparin is an important constituent of MCG and murine macrophages possess heparin receptors.\textsuperscript{30} A previous report has shown that commercial heparin and MCG are capable of inhibiting Fcγ2a receptor-mediated phagocytosis in macrophage cell line.\textsuperscript{31} Although commercial heparin differs from rat MCG-heparin, it is possible that MCG-heparin may have some modulatory effects on macrophage iNOS and TNFα proteases, and carboxypeptidase A.

Y. Li

1. Adams DO, Hamilton TA. The cell biology of macrophage activation. \textit{Ann Rev Immunol} 1984; 2: 283–318.

2. Adams DO, Hamilton TA. Molecular basis of macrophage activation. In: Lewis CE, McGregor JOD, eds. \textit{The Macrophages}. New York: Oxford University Press, 1993; 77–114.

3. Ishizaka T, Ishizaka K. Activation of mast cells for mediator release through IgE receptors. \textit{Prog Allergy} 1984; 34: 188–235.

4. Schwartz LB, Austen KF. Structure and function of the chemical mediators of mast cells. J Immunol 1984; 34: 271–321.

5. Galli SJ, Lichtenstein LM. Biology of mast cells and basophils. In: Middleton E Jr, ed. \textit{Allergy: principles and practices}, 3rd edn. St Louis: Mosby, 1988; 160.

6. Holgate ST, Robinson C, Church MK. Mediators of immediate hypersensitivity. In: Middleton E Jr, ed. \textit{Allergy: principles and practices}, 3rd edn. St Louis: Mosby, 1988; 135.

7. Fawcett DW. An experimental study of mast cell degranulation and regeneration. \textit{Agent Acta} 1955; 121: 29–43.

8. Dileepan KN, Stechschulte DJ. Influence of MCG on macrophage function. \textit{Biochem Soc Trans} 1986; 14: 913–914.

9. Dileepan KN, Simpson KM, Stechschulte DJ. Modulation of macrophage superoxide-induced cytochrome c reduction by mast cells. \textit{J Lab Clin Med} 1989; 113: 577–585.

10. Shah BA, Li Y, Stechschulte DJ, Dileepan KN. Phagocytosis of mast cell granules results in decreased macrophage superoxide production. \textit{Med Inflamm} 1995; 4: 406–412.

11. Dileepan KN, Lorsbach R, Stechschulte DJ. Mast cell granules inhibit macrophage-mediated lysis of mastocytesoma cells (PBl5) and nitric oxide production. \textit{J Leukoc Biol} 1993; 53: 446–453.

12. Cawley EF, Hoch-Ligeti C. Association of tissue mast cells and skin tumors. \textit{Arch Dermatol} 1961; 83: 146–150.

13. Bowers HM, Compans RW. Regulation of mast cell numbers in the proximal tubules of the early postnatal rat. \textit{J Lab Clin Med} 1984; 13: 57–64.

14. Roche WR. Mast cells and tumors. The specific enhancement of tumor proliferation in vitro. \textit{Am J Pathol} 1985; 119: 57–64.

15. Qu Z, Liebler JM, Powers MR, Galey T, Mahdini P, Huang XN, Ansel JC, Butterfield JH, Planck SR, Rosenbaum JT. Mast cells are a major source of basic fibroblast growth factor in chronic inflammation and cutaneous neoplasms. \textit{J Exp Med} 1990; 171: 115: 428–431.

16. Kaarinen M, Penttila A, Kovanen PT. Mast cells accompany microvessels in human coronary atherosomas: implications for intimal neo-vascularization and atheromogenesis. \textit{Atherosclerosis}. 1996; 123: 123–131.

17. Li Y, Stechschulte AC, Smith DD, Lindsey HB, Stechschulte DJ, Dileepan KN. Mast cell granules potentiate endotoxin-induced interleukin-6 production by endothelial cells. \textit{J Leukoc Biol} 1997; 62: 210–216.

18. Raphael GD, Henderson WR, Kaliner M. Isolation of membrane-bound rat mast cell granules. \textit{Exp Cell Res} 1978; 115: 289–300.

19. Li Y, Ito N, Suzuki T, Stechschulte DJ, Dileepan KN. Dexamethasone inhibit nitric oxide-mediated cytotoxicity via effect on both macrophages and target cells. \textit{Immunopharmacology} 1995; 30: 177–186.

20. Yu Y, Austen KF. Preparative purification of the rat mast cell chymase: characterization and interaction with granule components. \textit{J Exp Med} 1977; 146: 1405–1419.

21. Woodbury RG, Everitt MT, Neurath H. Mast cell proteases. \textit{Anat Rec} 1995; 269: 577–583.

22. Kokkonen JO, Vartiainen M, Kovanen PT. Low density lipoprotein degradation by secretory granules of rat mast cells. Sequential degradation of apolipoprotein B by granule chymase and carboxypeptidase A. \textit{J Biol Chem} 1995; 270: 578–583.

23. Schwartz LB, Irani AM, Roller K, Castells MC, Schechter NM. Quantitation of histamine, tryptase and chymase in dispersed human T and TC mast cells. \textit{J Immunol} 1987; 138: 2611–2615.

24. Thiemermann C, Wu CC, Stahb C, Pietrini M, Vane JR. Role of tumour necrosis factor in the induction of nitric oxide synthase in a rat model of endotoxin shock. \textit{Br J Pharmacol} 1993; 110: 177–182.

25. Geller DA, de Vera ME, Russell DA, Shapiro RA, Nussler AK, Simmons RL, Billiar TR. A central role for IL-1β in the in vitro and in vivo regulation of hepatic nitric oxide synthase. \textit{J Immunol} 1995; 155: 4890–4898.

26. Salkowski CA, Detore G, McNally R, van Rooijen N, Vogel SN. Regulation of inducible nitric oxide synthase messenger RNA expression and nitric oxide production by lipopolysaccharide in macrophages, endogenous IFNγ, and TNF receptor-1-mediated signaling. \textit{J Immunol} 1997; 158: 905–912.

27. Merchaut A, Gueydan C, Houzet L, Amraoui Z, Sels A, Huez G, Goldman M, Kruys V. Defective translation of tumor necrosis factor mRNA in lipopolysaccharide-activated mouse macrophages. \textit{J Immunol} 1997; 158: 905–912.

28. Lorsbach RB, Murphy WP, Lowenstein CJ, Snyder SH, Russell SW. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. \textit{J Biol Chem} 1993; 268: 1908–1913.

29. Okamoto H, Nakano K. Regulation of interleukin-1 synthesis by histamine produced by mouse peritoneal macrophages per se. \textit{Immunology} 1979; 69: 162–165.

30. Bleiberg I, MacGregor I, Aronson M. Heparin receptors on mouse macrophages. \textit{Thorax} 1983; 29: 53–61.

31. Yamada A, Dileepan KN, Stechschulte DJ, Suzuki T. Regulation of Fcγ2a receptor-mediated phagocytosis by a murine macrophage like cell line,
ACKNOWLEDGEMENTS. This study was supported by grants from the American Heart Association (Kansas affiliate) and the University of Kansas Medical Center Research Institute, and by the Joseph and Elizabeth Carey Arthritis Funds, Hinman Fund, and Jones Fund from the KU Endowment Association.

Received 16 August 1998
accepted 1 September 1998