

THE exposure of the macrophage cell line, J774 to mast cell granules (MCG) led to the formation of altered nuclear transcription factor proteins (NF- κ Bx), which had faster electrophoretic mobility than the p50 homodimer of NF- κ B, but retained comparable DNA binding capacity. Antibodies to N-terminal peptides of p50, p52, p65 or c-Rel supershifted only a fraction of NF- κ Bx. Western blot analyses revealed that nuclear p65 and c-Rel were progressively degraded after exposure to MCG, whereas nuclear p50 appeared to be unaffected. In contrast, cytoplasmic p50, p65, c-Rel as well as I κ B α remained intact after MCG treatment, although p52 was clearly degraded. In comparison to J774 cells, incubation of mouse peritoneal macrophages with MCG resulted in more extensive alterations to NF- κ B proteins. The alterations in NF- κ B proteins did not affect the expression of inducible nitric oxide synthase (iNOS) or TNF- α mRNA in J774 cells. These data indicate that exposure of J774 cells to MCG leads to generation of altered nuclear p52, p65 and c-Rel, which retain intact N-terminal peptides, specific oligonucleotide binding and transactivating activity. On the other hand, in peritoneal macrophages, MCG induce more extensive modifications to NF- κ B proteins with associated inhibition of iNOS or TNF- α mRNA expression.

Key words: Macrophages, Mast cells, Mast cell granules, NF- κ B, Nitric oxide synthase, TNF- α

Transient degradation of NF- κ B proteins in macrophages after interaction with mast cell granules

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Introduction

Macrophages play a key role in microbicidal and tumoricidal activity associated with the production of a variety of cytokines and inflammatory mediators.^{1,2} Mast cells induce hypersensitivity reactions by secreting mediators such as histamine, proteoglycans, various cytokines, metabolites of arachidonate and unique proteases.³⁻⁶ The cytoplasmic granules of mast cells are membrane-bound organelles, which contain biogenic amines, proteoglycans, proteases and superoxide dismutase.³⁻⁸ The interaction between mast cells and macrophages, and the uptake of mast cell granules (MCG²) by macrophages both *in vivo*⁹ and *in vitro*^{8,10} is well documented. Studies in mast cell deficient animals have indicated the important role of mast cells in modulating inflammatory response.¹¹ *In vitro* studies demonstrated that phagocytosis of MCG by macrophages resulted in suppression of macrophage functions such as Fc γ 2a receptor-mediated phagocytosis,¹⁰ PMA-triggered superoxide generation,¹² LPS-induced nitric oxide production and tumoricidal activity,¹³ and LPS-induced transcription of mRNA for nitric oxide synthase (iNOS) and

TNF- α .¹⁴ The transcription factor NF- κ B has been shown to be involved in LPS-induced activation of the iNOS gene in mouse macrophages.¹⁵ Although the mechanisms of MCG regulation of macrophage function are unknown, it is possible that phagocytosed MCG alter the structure and function of NF- κ B. We therefore investigated the effect of MCG on NF- κ B proteins and the expression of iNOS and TNF- α mRNA in the mouse macrophage cell line J774, with and without LPS stimulation. The data presented in this paper indicate that exposure to MCG results in transient degradation of multiple transcription factors including nuclear NF- κ B proteins in J774 cells. The degraded NF- κ B proteins retain DNA binding domains and nuclear localizing signal peptides resulting in translocation to nuclei. Although the modified NF- κ B proteins translocated to the nuclei are of lower molecular weight (designated as NF- κ Bx) they did not lose the ability to promote transcription of iNOS and TNF- α mRNA in J774 cells. In mouse peritoneal macrophages, on the other hand, MCG exposure resulted in more extensive modulation in NF- κ B proteins, and resulted in the inhibition of iNOS and TNF- α mRNA expression.

Materials and Methods

Cells

The mouse macrophage-like cell line, J774, was derived from a tumor of a female BALB/c mouse and possesses characteristics typical of macrophages.¹⁶ Protease peptone-elicited macrophages were harvested from two to three month old male C57Bl/6 mice as described.¹³ The culture media and other reagents used in the cell culture were ascertained to contain <0.025 ng of LPS/ml by the Limulus amoebocyte lysate assay, and the cells were maintained in culture as described.¹⁷⁻²⁰

Mast cell granule preparation

Briefly, mast cells were obtained by lavage of the peritoneal cavity of male Sprague Dawley rats with minimum essential medium containing 15 mM HEPES, penicillin (100 U/ml), streptomycin (100 µg/ml), 10% fetal bovine serum and 50 µg/ml heparin (HMEM) as described.^{12,13} Cells were pooled, centrifuged for 15 min at 400×g at 25°C, washed twice and resuspended in HMEM. The cell suspensions (5 to 10 × 10⁷/2 ml) were layered over 4 ml cushions of 22.5% metrizamide (density of 1.125 g/ml) in HMEM and centrifuged at 200×g for 15 min. Mast cells in the pellet were collected, washed twice and resuspended in HMEM. Mast cells isolated in this manner exceeded 90% in purity and viability. To prepare MCG, the cell suspension (in 2 ml of HMEM) was sonicated for 15 s, cooled for 30 s on ice and re-sonicated for 15 s at a power setting of 2.5 with a microtip sonicator to release granules.^{12,13} The disrupted cells were incubated for 15 min at 30°C, vortexed for 1 min, layered over 2 ml of 0.34 M sucrose and centrifuged at 50×g for 10 min at 4°C to remove debris. The resulting supernatant was centrifuged at 1800×g for 20 min at 4°C. The pellet consisting of a highly purified, homogeneous preparation of granules was washed and resuspended in HMEM. The quantity of MCG used in each experiment was expressed as the equivalent of the starting mast cell number.

Assay for protease activity in MCG

Chymotrypsin-like (chymase) activity was assayed by monitoring the hydrolysis of N-succinyl-L-phenylalanine-*p*-nitroaniline at 405 nm²¹ in a reaction mixture (1 ml) containing 100 mM Tris-HCl (pH 7.6), 100 µM substrate and MCG sonicate equivalent of 2 to 4 × 10⁵ mast cells. The specificity of the enzymatic activity was confirmed by the ability of TPCK to inhibit the reaction. Trypsin-like (trypsin) activity was assayed by measuring the hydrolysis of benzoyl-L-arginine-*p*-nitroaniline at 410 nm²² in a reaction mixture (1 ml) consisting of 50 µM Tris-HCl (pH 8.2), 0.02 µM CaCl₂, 100 µM substrate and MCG

sonicate equivalent to 2 to 4 × 10⁵ mast cells. Commercially available trypsin (Sigma, St Louis, MO) served as control. Carboxypeptidase A activity was assayed by monitoring the hydrolysis of hippuryl-L-phenylalanine at 254 nm²³ in a reaction mixture (1 ml) containing 50 mM Tris-HCl (pH 7.5), 1 mM substrate and MCG sonicate equivalent to 2 to 4 × 10⁵ mast cells. In all cases, one unit of enzyme activity was defined as the enzyme required for hydrolyzing 1 µmole of substrate per min at 25°C.

Antisera

Antisera directed against various NF-κB and IκBα proteins were raised in rabbits by immunizing against synthetic peptides coupled to keyhole limpet hemocyanin (generous gift from Dr Nancy Rice of NCI-Frederick Cancer Research and Development Center). The peptide sequences are as follows: ADDDPYGTGQMFHLC (#1263, N-terminus of mouse p50); CADMDFSALLSQISS (#1226, C-terminus of mouse p65); CEQLSDPPTYGFFKI (#1266, C-terminus of mouse c-Rel); FQPAGHGQDWAMEGRC (#751, N-terminus of mouse IκBα); and DELPYDDCVFGGQRLTL (#1258, C-terminus of mouse IκBα).²⁴ These peptides were coupled to hemocyanin either through the free amino group at the peptide N-terminus or through an added cysteine at the N or C terminus. Each antiserum is capable of immunoprecipitation of the specific protein and does not cross-react with other family members. Additional antisera directed against N-terminus of p52 and p65 were purchased from Santa Cruz (Santa Cruz, CA).

Western blot analysis

The proteins separated by a standard SDS-PAGE were electrophoretically transferred to nitrocellulose membrane (0.2 µm pore, Schleicher and Schuell, Keene, NH) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA) in 36 min at 5.5 mA/cm². The membrane was then immersed for 10 min at 25°C in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 22% non-fat dry milk, washed 3 times with TBST, and incubated for 1 h at 25°C with rabbit antisera directed against various NF-κB proteins in the absence or presence of corresponding antigen peptides. The membranes were then washed 3 times with TBST and incubated for 30 min at 25°C with peroxidase-tagged goat antibody against rabbit IgG (Sigma). The membrane was washed 5 times with TBST and developed by ECL system (Amersham, Arlington Heights, IL).

Nuclear protein extraction

Nuclear protein extracts were prepared by the method of Dignam *et al.*,²⁵ as described.¹⁷⁻²⁰ Briefly,

cells (2.5×10^6) were washed and lysed with a Dounce homogenizer at 4°C in 0.5 ml of buffer I (10 mM HEPES-KOH, 10 mM KCl, and 1.5 mM MgCl₂, pH 7.9) containing 0.5% Nonidet P-40. All subsequent procedures were carried out at 4°C. The lysate was centrifuged for 5 min at 1000g. The pellet obtained was washed twice with buffer I containing 0.5% Nonidet P-40, and centrifuged at $10,000 \times g$ for 5 min to collect the nuclear pellet. Nuclear proteins were extracted from the pellet for 10 min with 40 μ l of buffer II (20 mM HEPES, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol, pH 7.9). After vigorous mixing, the nuclear suspension was centrifuged ($10,000 \times g$ for 5 min) and the resultant supernatant was diluted with 60 μ l of dilution buffer (20 mM HEPES, 50 mM KCl, 0.2 mM EDTA, and 20% glycerol, pH 7.9). The following reagents were added to all buffers just before use: 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin A, and 10 μ g/ml each of aprotinin, leupeptin (all from Sigma) and soybean trypsin inhibitor (Boehringer-Mannheim, Indianapolis, IN).

Electrophoretic mobility shift assay (EMSA)

The NF- κ B-specific oligonucleotide, containing two tandemly arranged NF- κ B sites (underlined) of the HIV-1 enhancer (5'-ATCAGGGACTTTCGCTGGG-GACTTTCGG-3'),¹⁷ the ISRE-specific oligonucleotide (5'-ACTGTCAATATTTTCACTTTCATAATGGAAA-3'),²⁶ an oligonucleotide containing a H2TF1 binding site of H2K^b promoter (5'-GCCCTAGGGCTGGGGATTCCCC-ATCTCCACA-3'), an unrelated oligonucleotide lacking a κ B site (5'-AGGATGGGAGTGTGATATATATCCTTGAT-3')¹⁷ and their respective complimentary oligonucleotides were synthesized in the Biotechnology Support Facility of the University of Kansas Medical Center.^{17,18} The oligonucleotides specific for AP-1, SP-1, OCT-1 and CREB and their respective complimentary strands were purchased from Promega (Madison, WI). Each oligonucleotide and its complimentary strand were annealed and then end-labelled using γ -[³²P]ATP (6000 Ci/mmol; New England Nuclear, Boston, MA) and T4-polynucleotide kinase (Amersham), as described.¹⁷⁻²⁰ Nuclear protein extracts (1 μ g) with 3 μ g poly (dI-dC).poly (dI-dC) (Pharmacia) and ~2 ng (100,000–400,000 cpm) of end-labelled DNA (which was added last) were mixed in 30 μ l of EMSA buffer and incubated for 30 min at 25°C after mixing. The EMSA buffer for NF- κ B consisted of 10 mM Tris-HCl buffer, pH 7.5, containing 40 mM NaCl, 1 mM EDTA, 1 mM β -mercaptoethanol, 4% glycerol, 0.1% NP-40 and 1 μ g/ μ l bovine serum albumin. Following this initial binding reaction, 20 μ l of the mixture were electrophoresed at 15 V/cm for 1–1.5 h at 25°C through a native 6% polyacrylamide gel, which was prepared in 45 mM Tris-borate buffer

containing 1 mM EDTA. Gels were then processed for autoradiography.

UV crosslinking

Briefly, bromodeoxyuridine substituted HIV NF- κ B oligonucleotide was made by the primer extension method¹⁸ using HIV-enhancer template and the primer, 5'-ATCAGGGAC-3', Klenow fragment of DNA polymerase I (25 units, Gibco BRL) and [α -³²P]-dCTP (6000 Ci/mmol, Amersham). Nuclear extract (10 μ g) incubated with 1×10^6 cpm of the probe was subjected to EMSA. The wet EMSA gel was UV-irradiated at a distance of 5 cm with a trans-illuminator (312 nm, 8000 μ W/cm²) for 30 min at 0°C and autoradiographed at 4°C. The bands corresponding to NF- κ B proteins were cut out, crushed and equilibrated for 16 h at 4°C in 100 mM Tris-HCl, pH 6.8, 50 mM DTT and 2% SDS. The samples were then microfuged and the resultant supernatants were subjected to electrophoresis on a SDS-10% polyacrylamide gel.

Northern blot analyses of nitric oxide synthase and TNF- α mRNA expression

J774 cells (1×10^7) were incubated with or without MCG for 3 h and were activated with LPS (100 ng/ml) for 6 h. Total cellular RNA was extracted using the guanidinium thiocyanate procedure.²⁷ RNA (10 μ g) was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a Nytran nylon membrane which had been prehybridized with salmon sperm DNA. The membranes were then incubated for 20 h at 42°C with ³²P-labelled cDNA probes specific for iNOS or TNF- α labelled by the random hexamer priming method using [α -³²P]dCTP. The membranes were then washed twice at 25°C in 0.1% SDS and 2xSSPE for 30 min and twice at 42°C in 0.1% SDS and 0.1xSSPE. The membrane was autoradiographed at -80°C with intensifying screens. After stripping, the same membranes were rehybridized with β -actin ³²P-labelled cDNA probe to control for RNA loading.

Results

Effect of MCG on constitutive and LPS-activated NF- κ B in J774 cells

The results presented in Fig. 1 show that the exposure of J774 cells to LPS substantially increased the nuclear levels of NF- κ B1 (p50 homodimer) and B2 (p50/p65 heterodimer) and induced a third component, NF- κ B3 (p65/c-Rel heterodimer) (lane 5 vs 1). The exposure of J774 cells to MCG for a total of 1.5 h at the J774 cell to mast cell ratio of 500 to 1 (lane 2) or 50 to 1 (lane

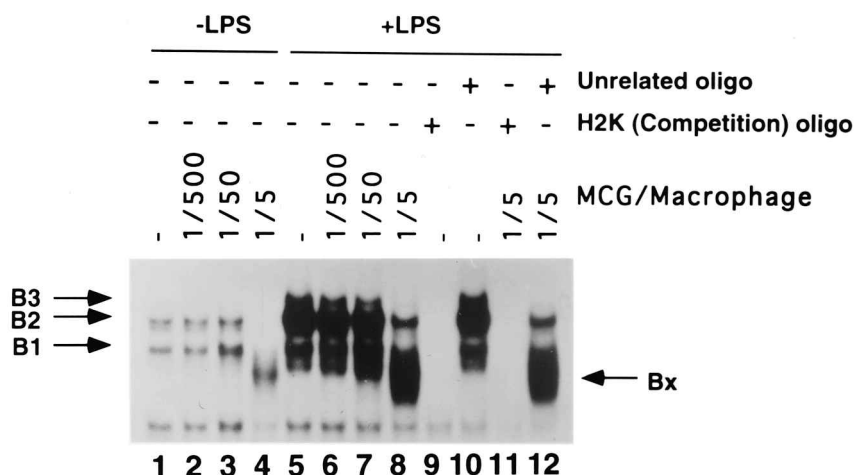


FIG. 1. Effect of MCG exposure on NF-κB proteins in J774 cells. J774 cells were incubated for 1 h at 37°C without MCG (lanes 1, 5, 9 and 10) or with MCG (lanes 2 to 4, 6 to 8, 11 and 12) at J774 cells to mast cell ratios of 500 to 1 (lanes 2 and 6), 50 to 1 (lanes 3 and 7) or 5 to 1 (lanes 4, 8, 11 and 12). They were further incubated for 30 min at 37°C without (lanes 1 to 4) or with LPS (10 ng/ml) (lanes 5 to 12). The nuclear proteins extracted from the cells were incubated with [³²P]-labeled HIV NF-κB motif-containing oligonucleotide in the absence (lanes 1 to 8) or presence of 100-fold excess of either competitor H2K^b (lanes 9 and 11) or unrelated (lanes 10 and 12) oligonucleotide, and were subjected to EMSA. The autoradiograph shown is the representative of three separate experiments, which gave very similar results.

3) did not affect the constitutively expressed levels of NF-κB1 and B2. The cells exposed at a J774 cell and mast cell ratio of 5 to 1 (lane 4) had decreased B1 and B2 levels and contained a low level of a new species of nuclear proteins (designated as NF-κBx) which bound to NF-κB oligonucleotide and had faster electrophoretic mobility than NF-κB1. The exposure of MCG-pretreated cells to LPS also caused substantial activation of NF-κB proteins (lanes 6–8). The intensity of NF-κBx gradually increased as the concentration of MCG was increased (lanes 6–8), whereas the bands corresponding to NF-κB2 and B3 decreased or disappeared. The binding of LPS-induced NF-κB proteins (including NF-κBx) present in both MCG-treated and untreated cells to NF-κB probe was specific, as the presence of the 100-fold excess of competitor H2K^b (lane 9 and 11), but not unrelated oligonucleotides (lane 10 and 12), completely blocked the binding. Based on these results, the ratio of J774 cells to mast cell equivalents of 5 to 1 was routinely used.

Detection of the degradation of NF-κB proteins by supershift analysis

Further characterization of NF-κBx proteins activated in MCG-pretreated J774 cells was carried out by supershift analysis using antibodies specific for the N-terminal peptide of p50, p52, p65 or c-Rel. These antibodies supershifted various NF-κB proteins from LPS-treated J774 cells (Fig. 2 top) as expected from previous studies.¹⁸ The preincubation of nuclear extract from MCG and LPS-treated cells with the same antibodies resulted in the creation of the supershifted bands indicated by arrows (Fig. 2 bottom). However,

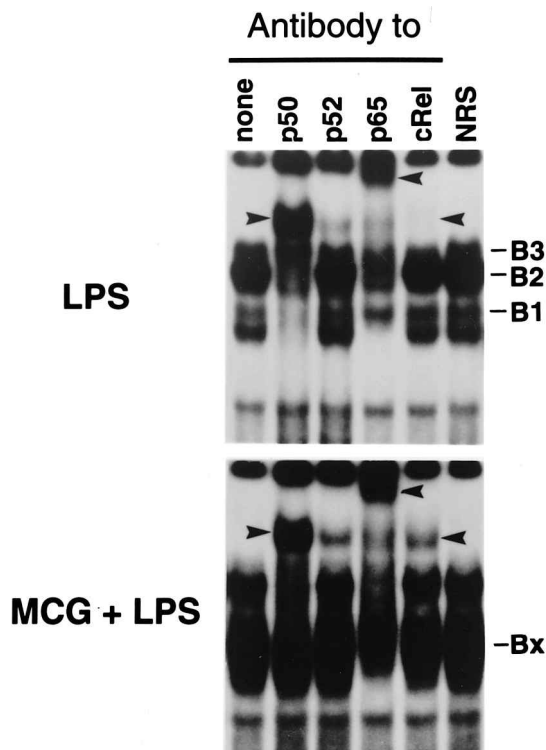


FIG. 2. Supershift analysis of NF-κBx proteins generated following phagocytosis of MCG. J774 cells were incubated for 3 h at 37°C without MCG (top) or with MCG (bottom) at a macrophage to mast cell ratio of 5 to 1. They were then exposed to LPS (10 ng/ml) for 30 min at 37°C. The nuclear proteins extracted from the cells at the end of incubation period were incubated at 25°C for 30 min with antibodies against the N-terminals of p50, p52, p65 or c-Rel or with control non-immune rabbit serum, and then subjected to EMSA as in Fig. 1. Autoradiographs were prepared by exposing to X-ray films for 14.5 h. Arrows indicate the supershifted bands. Similar experiments carried out using antibodies against the C-terminal peptides of p65 or c-Rel did not show any supershifted band (Data not shown).

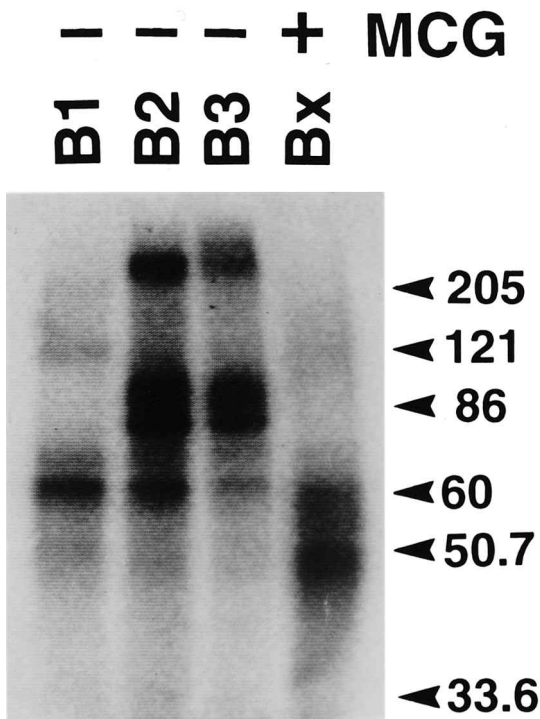


FIG. 3. UV-crosslinked NF-κB proteins of J774 cells. J774 cells were incubated for 3 h at 37°C without or with purified MCG at the ratio of J774 cell to mast cell equivalent of 5 to 1. They were then exposed to LPS (10 ng/ml) for 30 min at 37°C. The nuclear proteins extracted from the cells were subjected to UV crosslinking using ³²P-labelled bromodeoxyuridine substituted HIV NF-κB oligonucleotide as described in the Materials and Methods section. UV-crosslinked NF-κB1, B2, B3 and Bx eluted from the EMSA gel were analyzed by SDS-PAGE. The arrows indicating 60 and 50.7 are UV-crosslinked NF-κBx proteins. A similar result was obtained by an additional experiment.

the majority of NF-κBx proteins were not supershifted with any antibodies.

The approximate size of NF-κBx proteins present in the nuclear extracts of the MCG-exposed cells was then estimated by UV-crosslinking method. The

autoradiograph of the gel (Fig. 3) revealed that UV-crosslinked NF-κB proteins migrated during SDS-PAGE primarily as two bands of p45–50 and p55–65 (lane Bx, indicated by arrows). UV-crosslinked-NF-κB1 (lane B1), κB2 (lane B2) and κB3 (lane B3) proteins consisted, as previously reported,¹⁸ of p55, p55 plus two closely moving p75 and p80, and p75–p80 proteins, respectively. Various amounts of high molecular weight proteins seen in NF-κB2 and κB3 proteins probably represent aggregated forms which are deleted by exposure to MCG.

MCG degradation of other transcription factors

A possibility that the exposure of J774 cells to MCG also affects transcription factors other than NF-κB was examined. Nuclear extracts from the cells were analyzed by EMSA for NF-κB, AP-1, CREB, OCT-1, SP-1 and ISRE using radiolabelled oligonucleotide specific for each transcription factor. Results (Fig. 4) showed that the constitutive levels of transcription factors other than NF-κB were not markedly affected by the exposure of the cells to LPS and that the exposure of the cells to MCG resulted in disappearance (AP-1) or substantial changes in electrophoretic mobilities (CREB and SP-1) of these nuclear associated transcription factors. In contrast, the levels of OCT-1 and ISRE present in the nuclear extract remained unaffected by the treatment of cells with either LPS and/or MCG.

Kinetics of the effect of MCG on NF-κB degradation in J774 cells

The question of whether MCG-triggered degradation of NF-κB proteins is transient was next investigated. To this end, five matched groups of J774 cells were cultured for 24 h before exposure to LPS. During this pre-incubation period, J774 cells were exposed to MCG for 24, 6, 3 or 0 h. One half of each group of

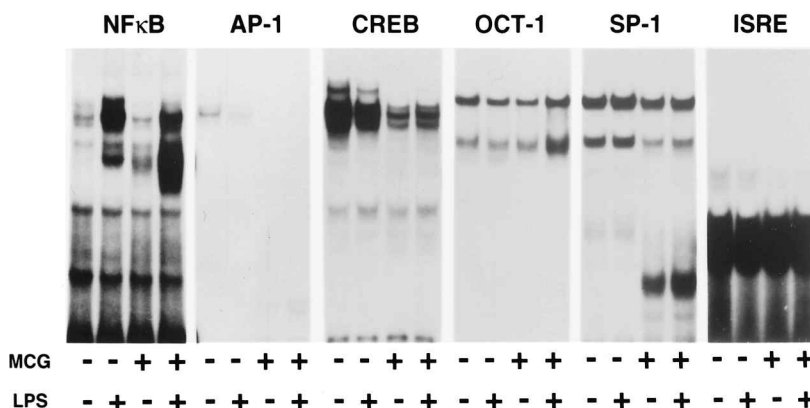


FIG. 4. Effect of MCG phagocytosis on the DNA-binding activities of various transcription factors. J774 cells were incubated for 3 h at 37°C without (lanes 1 and 2) or with (lanes 3 and 4) MCG at J774 cell to mast cell ratio of 5 to 1. They were then incubated for an additional 30 min at 37°C with (lanes 2 and 4) or without (lanes 1 and 3) LPS (10 ng/ml). Nuclear extracts prepared at the end of the incubation period were then subjected to EMSA using ³²P-labelled oligonucleotides specific for NF-κB, AP-1, CREB, Oct-1, SP-1 or ISRE. An additional experiment gave a similar results to that shown.

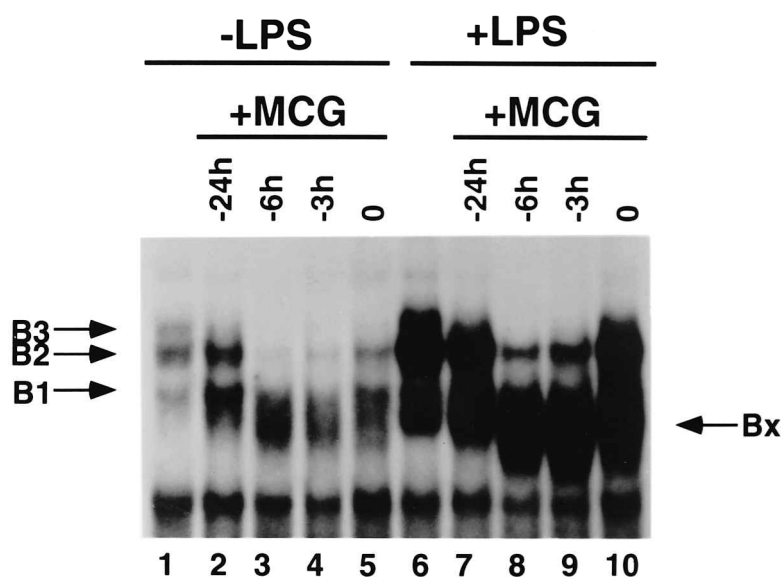


FIG. 5. Kinetics of generation of NF- κ Bx proteins after phagocytosis of MCG. J774 cells were divided into 5 matched groups (lanes 1 to 10). They were cultured without MCG (lanes 1 and 6) or with MCG for 24 h (lanes 2 and 7), 6 h (lanes 3 and 8), 3 h (lanes 4 and 9) or 0 h (lanes 5 and 10) at a macrophage to mast cell ratio of 5 to 1. One of each matched group of MCG-untreated (lane 6) or -treated cells (lanes 7 to 10) was then exposed for 30 min to LPS (10 ng/ml), whereas the other half was not. Thus, all groups of the cells were cultured for a total of 24.5 h. After washing the cells, nuclear proteins were extracted from each group of cells and were subjected to EMSA using radiolabelled NF- κ B oligonucleotide as ligand as described in the Experimental section. An additional experiment gave a similar result to that shown.

MCG-treated cells was then exposed for 30 min to LPS (10 ng/ml), whereas the other half was not exposed to LPS. Thus, all groups of the cells were cultured for a total of 24.5 h with variable time of exposure to MCG. Nuclear proteins were extracted from each group of cells and were then subjected to EMSA using radiolabelled NF- κ B oligonucleotide as ligand. Nuclear proteins as well as cytosolic proteins were separately subjected to western analysis using antisera specific for the N- or C-terminus of p50, p52, p65, c-Rel and I κ B α .

Results of the EMSA (Fig. 5) showed that NF- κ B proteins constitutively expressed in the nuclei (lane 1) were substantially increased upon LPS stimulation as three discernible bands, NF- κ B1, κ B2 and κ B3, respectively (lane 6), as noted above. Treatment of the cells for 30 min with MCG alone (lane 5) caused a disappearance of NF- κ B3 and an increase in the intensity of the bands corresponding to NF- κ B1 and κ Bx. Treatment of these cells with LPS substantially increased the levels of NF- κ B2, κ B1 and κ Bx (lane 10). The nuclear proteins obtained from the cells which were incubated for 6.5 or 3.5 h with MCG alone (lanes 3, 4) contained low levels of NF- κ B2 and κ B1 and a substantial level of NF- κ Bx, but no NF- κ B3. Treatment of these cells with LPS resulted in the marked increase of the levels of these NF- κ B proteins other than NF- κ B3 (lanes 8, 9). The cells incubated for 24.5 h with MCG alone (lane 2) contained NF- κ B1 and κ B2, but neither NF- κ B3 nor NF- κ Bx. Treatment of these cells with LPS (lane 7) substantially enhanced the levels of both NF- κ B2 and κ B1, but did not cause the activation

of NF- κ B3 or κ Bx. These data thus suggested that phagocytosis of MCG led to a gradual loss of NF- κ B3 and NF- κ B2 and a progressive generation of NF- κ Bx in J774 cells by 6.5 h. Judged by the results with cells which were incubated for 24.5 h with MCG alone or MCG plus LPS, the degradation of NF- κ B proteins was transient but at 24.5 h after the initiation of phagocytosis, recovery was not complete. The samples in lanes 2 and 7 contained no NF- κ B3 and possibly a reduced level of NF- κ B2 (compare lane 7 to lane 6) after LPS stimulation.

The effect of MCG on NF- κ B proteins in mouse peritoneal macrophages

The effect of MCG on the NF- κ B profile in control and LPS-activated murine peritoneal macrophages was also examined. Four matched samples of macrophages were cultured for 6 h prior to LPS addition. During this pre-incubation period, macrophages were exposed to MCG for 6, 3 or 0 h. One half of each group of MCG-treated cells was then exposed for 30 min to LPS (10 ng/ml), whereas the other half served as control. Thus, all groups were cultured for a total of 6.5 h with variable time of exposure to MCG. Nuclear proteins were then extracted from each group of cells and were subjected to EMSA using radiolabelled NF- κ B oligonucleotide as ligand.

The results (Fig. 6) showed that all NF- κ B proteins constitutively expressed in the nuclei were markedly increased upon LPS stimulation. Treatment of the cells with MCG in the absence of LPS caused in virtual

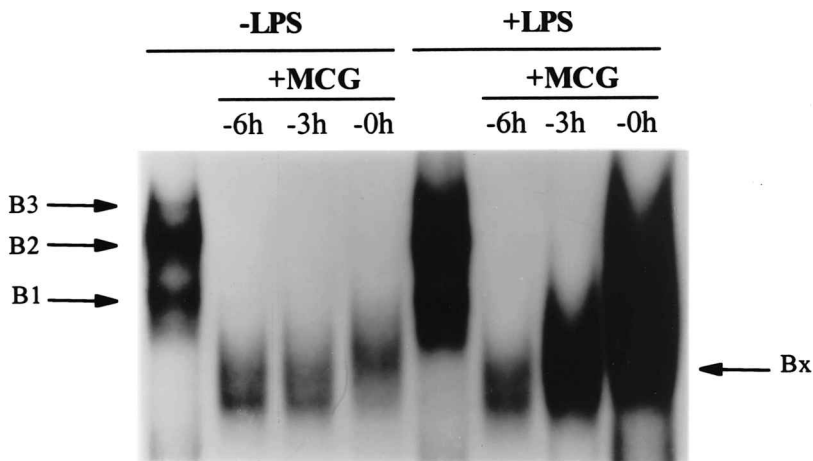


FIG. 6. Effect of MCG exposure on NF- κ B proteins in mouse peritoneal macrophages. Murine peritoneal macrophages were cultured with MCG for 6, 3 or 0 h at a macrophage to mast cell ratio of 5 to 1 prior to a 30 min incubation in the absence or presence of LPS (10 ng/ml). After incubation, the cells were washed, nuclear proteins were extracted from each group of cells and were subjected to EMSA using radiolabelled NF- κ B oligonucleotide as described in the Experimental section.

disappearance of nuclear NF- κ B1, B2 and B3, with the appearance of a κ Bx population at all times studied. As expected, treatment of macrophages with LPS resulted in the marked increase of the levels of the NF- κ B proteins. Significantly higher amounts of nuclear NF- κ B proteins including NF- κ Bx were noted in macrophages treated with MCG and LPS simultaneously. The nuclear NF- κ B1, B2 and B3 were absent by 3.5 h with a progressive loss of κ Bx at 6.5 h in LPS-stimulated cells.

Location and mechanism of NF- κ B protein degradation following MCG exposure

The question of whether the progressive degradation of NF- κ B proteins following MCG phagocytosis occurs in nuclei or in cytosol was investigated by western analysis of nuclear and cytosolic proteins extracted from J774 cells.

Identification of nuclear fractions with anti-N-terminus antibodies (Fig. 7a) showed that phagocytosis of MCG for 0.5, 3.5 and 6.5 h did not cause noticeable changes with time in the levels of immunoreactive nuclear p50 or p65 from the constitutive levels (lane 1 vs lanes 2–5). The exposure of MCG-untreated cells to LPS for 30 min noticeably increased the levels of immunoreactive p50, p65 and c-Rel in nuclear proteins (lane 6), suggesting LPS-induced nuclear translocation of these proteins. The exposure of the cells treated with MCG for 0, 3 or 6 h to LPS for 30 min appeared to result in a very slight reduction of immunoreactive nuclear-translocated p50 (lanes 8–10). The nuclear proteins extracted from the cells, which were incubated with MCG for 24 h and then exposed to LPS for 30 min (lane 7) contained about the same level of p50 as that seen in MCG-untreated and LPS-exposed cells (lane 6). Under any condition, these experiments did not demonstrate production of

any additional immunoreactive proteins of mol. wt smaller than p50. The level of immunoreactive p52 in nuclear proteins of MCG- and LPS-untreated cells very slightly increased in response to 30 min exposure to LPS (lane 1 vs lane 6), suggesting a probable occurrence of LPS-induced nuclear translocation of this protein. The constitutive as well as LPS-activated levels of p52 progressively declined for up to 6 h after exposure to MCG, with the simultaneous, progressive

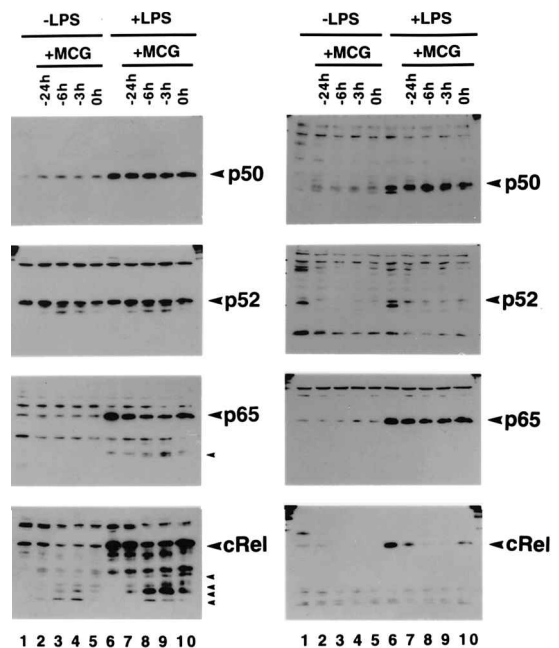


FIG. 7. Western analyses of nuclear proteins using antibodies against N (left) or C (right) terminal peptides of p50, p52, p65, or c-Rel. J774 cells were cultured for varying periods of time, as in Fig. 5, without MCG (lane 1 and 6) or with MCG (lanes 2 to 5 and 7 to 10) at a macrophage to mast cell ratio of 5 to 1. After the pretreatment, cells were cultured for an additional 30 min without (lanes 1 to 5) or with (lanes 6 to 10) LPS. Nuclear proteins were then prepared and Western analyses were carried out as described in the Experimental section.

generation of immunoreactive fragments which migrated as p51 and p48 (lanes 3–5 and 8–10). The constitutive (lane 2) as well as LPS-activated (lane 7) levels of p52 in the nuclear proteins extracted from the cells exposed to MCG for 24 h were close to those seen in MCG-untreated cells (lanes 1 and 6, respectively), which did not contain any immunoreactive material smaller than p52. Constitutive expression of c-Rel in nuclei of J774 cells was found to be progressively lost from nuclei upon treatment of cells with MCG up to 6.5 h (lane 1 vs lanes 3–5) and returned to the MCG-untreated level between 6.5 and 24.5 h (lane 2). The disappearance of the constitutive form of c-Rel from nuclei was clearly associated with the generation of numerous immunoreactive fragments which are smaller than c-Rel and are indicated by arrows. The exposure of MCG-treated cells to LPS led to a progressive disappearance of p-65 or c-Rel from nuclei with simultaneous generation of immunoreactive fragments smaller than parent proteins up to 6.5 h of MCG treatment (lanes 8–10). The level of p65 or c-Rel returned, at 24.5 h after MCG treatment, almost but not completely, to the level in the nuclei of MCG-untreated and LPS-treated cells (lane 7 to 6).

Western blot analysis using antibodies against the C-terminus of p50, p52, p65 or c-Rel (Fig. 7b) showed quantitative changes to nuclear p50, p52, p65 or c-Rel in response to LPS activation in both control and MCG-treated cells. LPS-induced translocation and MCG-induced breakdown of these NF- κ B proteins are clearly evident. However, the antibodies against C-terminus of p65 or c-Rel failed to recognize smaller molecules which were readily recognized by antibodies against N-terminus of p65 or c-Rel, suggesting that MCG caused the loss of antigenic determinants in the C-terminal regions of p65 and c-Rel.

The question of whether phagocytosis of MCG causes proteolytic degradation of cytosolic NF- κ B and/or I κ B α proteins was also investigated by Western analysis. The cytosolic fractions were prepared from J774 cells, which were treated with MCG alone, or MCG plus LPS under identical conditions as utilized for recovery of nuclear fractions. Western analyses carried out using anti-N-terminus or anti C-terminus antibodies revealed that the cytosolic p50, p52, p65,

c-Rel and I κ B α were minimally affected by MCG treatment (data not shown). LPS treatment of control or MCG-treated cells reduced the amounts of p50, p52, p65, and c-Rel, most likely due to nuclear translocation of these proteins. The levels of cytosolic I κ B α were also reduced following LPS treatment, most likely due to LPS-induced proteolytic degradation.^{24,26}

Extracellular degradation of cytosolic NF- κ B proteins by MCG

Rat MCG contain several proteases, including chymase and carboxypeptidase A.^{21,23,28,29} In order to examine whether MCG-proteases could act on isolated cytosolic NF- κ B/I κ B α proteins, the cytosolic fractions of J774 cells were prepared, incubated for 1 h at 37°C, with or without MCG, at a J774 cell and mast cell ratio of 5 to 1, and were then subjected to Western analysis using antibodies directed against the N-terminus of p50, p52, p65, c-Rel, or I κ B α . The results (Fig. 8) showed that the incubation of the isolated cytosolic fraction with MCG caused substantial loss of all components of NF- κ B/I κ B α proteins, suggesting their degradation by MCG-derived proteases.

The observed generation of NF- κ Bx proteins following phagocytosis of MCG could be due to proteolysis of NF- κ B proteins by proteases known to be present in MCG.^{21,23,28,29} The levels of MCG-associated trypsin, chymase and carboxypeptidase A in MCG-sonicates were therefore examined by spectrophotometric methods using a substrate specific for each enzyme. The data indicated that MCG contained 3.4 units of chymase and 788 units of carboxypeptidase activity/10⁶ mast cell equivalent. There was no detectable trypsin activity in MCG.

MCG effects on LPS-induced transcription of mRNA for iNOS and TNF- α in J774 and peritoneal macrophages

Previous studies have shown that MCG treatment of murine peritoneal macrophages results in the inhibition of LPS-induced tumor cell killing, nitric oxide

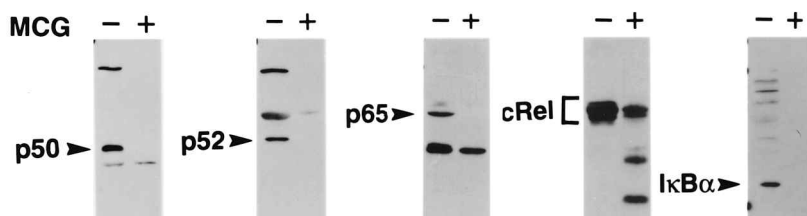


FIG. 8. Western analysis of the effect of MCG on NF- κ B/I κ B α in the isolated cytosolic fraction. The cytosol fractions prepared from J774 cells were incubated without (–) or with MCG (+) for 1 h at 37°C at J774 cell to mast cell equivalent ratio of 5 to 1. At the end of the incubation period, they were subjected to Western analysis using anti-N-terminal peptides of p50, p52, p65, c-Rel or I κ B α .

production,¹³ and iNOS and TNF- α mRNA expression.¹⁴ The virtual disappearance of nuclear NF- κ B proteins in peritoneal macrophages exposed to MCG for 6 h (Fig. 6) implicated this transcription factor in the MCG effect. In the present study, we also investigated whether or not the transient and low level degradation of NF- κ B proteins in J774 cells, compared to peritoneal macrophages, altered the expression of iNOS and TNF- α mRNA in J774 cells. Northern blot analysis (Fig 9) show that J774 cells which were pre-treated with MCG for 3 h and then stimulated with LPS for 6 h, expressed the same amounts of iNOS and TNF- α mRNA as LPS-stimulated control cells. LPS-induced NO production by J774 cells was also not affected by MCG treatment (data not shown). TNF- α mRNA levels were also unchanged when analyzed after 30 min of LPS activation of J774 cells treated with MCG for 0, 3, 6 and 24 h (data not shown).

Discussion

The data presented in Figures 1–6 collectively show that nuclear NF- κ B proteins (particularly p65 and c-Rel) in J774 cells (Figs 1–5) and in mouse peritoneal macrophages (Fig. 6) are proteolytically modified upon exposure of the phagocytes to MCG at a macrophage to mast cell ratio of 5 to 1. The alterations to nuclear NF- κ B proteins in mouse peritoneal macrophages is extensive when compared to J774 cells and is in agreement with the previously observed inhibition of iNOS and TNF- α mRNA expression.^{13,14} The results of Western blotting experiments (Fig. 6) clearly show that nuclear p52, p65 and c-Rel, in either their constitutive or LPS-activated forms, undergo pro-

gressive degradation for up to 6.5 h after the initiation of MCG phagocytosis, as seen by the decrease in the amounts of intact forms and by the increased generation of fragments smaller than the parent molecules. Degradation of these proteins is transient with the maximal effect noted at 3–6 h and some residual effects were noted even at 24 h after the addition of MCG. Nuclear p50 appears to be most resistant to degradation by MCG compared to the other NF- κ B proteins. I κ B α protein in the cytosol of J774 cells remains intact following phagocytosis of MCG, but is degraded upon exposure of the cells to LPS, as previously noted.³⁰

Mast cell granules used in these experiments were found to contain at least two different active proteases, carboxypeptidase A and chymase. If the contents of MCG are released into cytosol following phagocytosis, these proteases are capable of degrading cytoplasmic proteins such as the NF- κ B/I κ B α complex (Fig. 8). However, Western analysis (Figs. 7) clearly showed that phagocytosis of MCG leads to more extensive proteolytic degradation of nuclear NF- κ B proteins than cytosolic NF- κ B/I κ B α proteins. This could be due to the protection of cytosolic NF- κ B/I κ B α proteins from proteolytic digestion, because these enzymes are contained within the phagolysosome following phagocytosis of MCG by J774 cells. A possibility exists, however, that proteolytic enzymes in MCG which leak out into the cytoplasmic compartment from the phagolysosome may degrade NF- κ B proteins complexed with intact I κ B α in cytosol. Subsequent LPS treatment activates cellular TPCK-sensitive protease which degrades I κ B α .³⁰ Proteolytically altered and LPS-activated NF- κ B proteins then translocate to nuclei and bind to NF- κ B motifs, if NF- κ B proteins retain their nuclear localizing signal peptides and DNA-binding domains. Alternatively, the association of NF- κ B, I κ B α and the I κ B α -associated PKAc proteins³¹ in the cytosol may protect the components from proteolysis by MCG enzymes if present in the cytoplasm. The observation that NF- κ B proteins translocated to the nucleus constitutively or by LPS stimulation and are transiently degraded by exposure of the cells to MCG, suggests that components of the MCG may also be translocated to the nuclei. The results of Western blot analyses are consistent with the idea of intra-nuclear degradation of NF- κ B proteins. This intriguing question and identification of the possible mechanisms by which MCG induces alterations to the nuclear NF- κ B proteins requires further investigation. Another MCG component, superoxide dismutase^{3,7} may also participate in the regulation of NF- κ B. However, the presence of superoxide dismutase does not explain the appearance of NF- κ Bx, because the changes in the pattern of NF- κ B in macrophages over-expressing superoxide dismutase³² are totally different from those noted after phagocytosis of MCG.

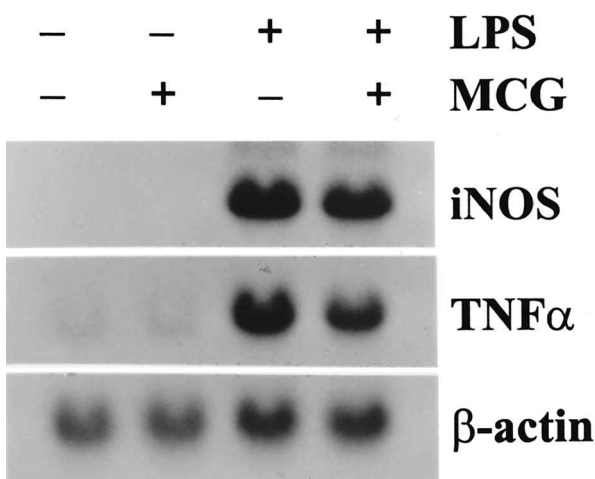


FIG. 9. Effect of MCG exposure on LPS-induced iNOS and TNF- α mRNA expression in J774 cells. J774 cells were pre-treated with MCG at a J774 cell to mast cell ratio of 5 to 1 for 3 h and were then stimulated with LPS for 6 h. Total RNA extracted at the end of the incubation were electrophoresed on 1% agarose gel, transferred to a Nytran nylon membrane, and sequentially hybridized with specific ³²P-labelled cDNA probes.

The data of Fig. 5 show that the effects of MCG phagocytosis on NF- κ B proteins are transient. The electron microscopic studies previously demonstrated that the majority of phagocytosed MCG are eliminated from peritoneal macrophages through a phagolysosomal pathway within 45 min after the initiation of phagocytosis.⁸ However, our data demonstrate that predominant NF- κ B species in the nuclear extract from J774 cells at 6.5 h after phagocytosis of MCG are the proteolytically modified NF- κ Bx proteins. This is possible if the proteases derived from MCG after phagocytosis remain active and are associated with J774 cells at 6 h after granules are no longer intact in J774 cells. Alternatively, NF- κ B proteins fragmented following phagocytosis of MCG may remain for at least 6.5 h and may not be replaced by newly synthesized NF- κ B proteins, if the constitutive synthesis of NF- κ B is inhibited by the occupation of NF- κ B sites with p65 or c-Rel which retains DNA binding domains but have lost C-terminal regions. The finding that NF- κ B proteins extracted from the cells at 24.5 h after phagocytosis are mostly intact forms of p50, p52, p65 and c-Rel could be explained by inactivation of phagocytosed MCG-derived proteases and the synthesis of new NF- κ B proteins.

The proteolysis of NF- κ B proteins is limited to sites distal to the nuclear localizing signal peptide sequences of these proteins, leaving DNA-binding domains intact, as evident by the ability of NF- κ Bx to specifically bind to NF- κ B oligonucleotide (Fig. 1). Thus, the proteolytically altered p65 and c-Rel retains DNA binding and transactivating domains in spite of proteolytic degradation from their C-terminal ends (Fig. 7). This may be due to the action of carboxypeptidase A derived from MCG. It is interesting to note that p50 which does not possess a transactivating domain but possesses both nuclear localizing signal peptide and DNA-binding domain is relatively unaffected by phagocytosis of MCG. The lack of inhibition of iNOS and TNF- α messenger RNA expression in MCG-exposed J774 cells may be explained by the transient nature of NF- κ B degradation and retention of the DNA binding and transactivating domains. The extensive proteolytic modifications to NF- κ B proteins in peritoneal macrophages by MCG (Fig. 5 vs Fig. 6) as compared to the relatively moderate alterations in J774 cells may explain the inhibition of iNOS and TNF- α gene expression in the former and the lack of inhibition in the latter. Although the underlying mechanism for the difference in MCG effect in these two cell types is unknown, the association between the magnitude of proteolytic alterations to NF- κ B and the functional changes are interesting. It is noteworthy that in spite of the ability of MCG proteases to degrade all NF- κ B proteins when incubated with cytosol preparations (Fig. 7), MCG minimally affected the NF- κ B proteins in the cytosol of intact cells after their phagocytosis. This suggests

that in intact cells the cytosolic proteins are protected from MCG action. The MCG proteases may also activate other unidentified proteases in the host cell which function at the nuclear level to modify translocated NF- κ B proteins.

Mast cell degranulation is known to generate and release a number of factors that directly stimulate a variety of cells such as epithelial cells,³³ fibroblasts³⁴ and endothelial cells.³⁵ This is the first evidence which demonstrates that the uptake of MCG by cells leads to alterations in the structure and possibly the functions of transcription factors of the NF- κ B/Rel family which are important regulators of inflammation, cell proliferation and apoptosis. The explanation for the variable functional responses of peritoneal macrophages and J774 cells to MCG requires further study.

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