American Journal of Pathology, Vol. 150, No. 6, June 1997

Sublytic Concentrations of the Membrane Attack Complex of Complement Induce Endothelial Interleukin-8 and Monocyte Chemoattractant Protein-1 through Nuclear Factor-κB Activation

Kenneth S. Kilgore,* Elisabeth Schmid,† Thomas P. Shanley,* Craig M. Flory,‡ Vipul Maheswari,* Nicole L. Tramontini,* Hillary Cohen,* Peter A. Ward,* Hans P. Friedl,† and Jeffrey S. Warren*

From the Department of Pathology, University of Michigan Medical School, and Pharmaceutical Research Division,‡ Warner Lambert-Parke Davis, Ann Arbor, Michigan, and the Department of Surgery,† University Hospital, Zurich, Switzerland*

Activation of the complement cascade and subsequent assembly of the membrane attack complex (MAC) occur in a number of pathophysiological settings. When formed on the surface of endothelial cells in sublytic concentrations, the MAC can induce a number of proinflammatory activities, including the secretion of soluble mediators (eg, interleukin (IL)-8 and monocyte chemoattractant protein (MCP)-1) and the up-regulation of cell surface adhesion molecules. Available data indicate that MAC-induced cell activation may occur through several complex signal transduction pathways, but little is known about the intranuclear mechanisms by which complement-derived products promote the up-regulation of inflammatory mediators. Using purified distal complement proteins (C5–9) to assemble functional MAC on early-passage human umbilical vein endothelial cells (HUVECs), we examined mechanisms of MCP-1 and IL-8 induction. Formation of sublytic concentrations of MAC promoted an increase in nuclear factor (NF)-κB DNA binding activity within 60 minutes as determined by serial electrophoretic mobility shift assay. Cytosolic to nuclear translocation of NF-κB was confirmed by Western immunoblot and immunocytochemical analyses. Formation of the C5b-8 complex also promoted NF-κB translocation but to a lesser degree than observed in HUVECs containing complete MAC. No cytosolic to nuclear translocation of the p65 NF-κB subunit was observed in unstimulated HUVECs or in cells incubated with the MAC components devoid of C7. Preincubation of HUVECs with pyrrolidine dithiocarbamate prevented MAC-induced increases in IL-8 and MCP-1 mRNA concentrations and protein secretion. A direct cause and effect linkage between MAC assembly and NF-κB activation was established through examination of the pharmacological effect of the peptide SN50 on IL-8 and MCP-1 expression. SN50 is a recently engineered 26-amino-acid peptide that contains a lipophilic cell-membrane-permeable motif and a nuclear localization sequence that specifically competes with the nuclear localization sequence of the NF-κB p50 subunit. This study provides direct* in vitro *evidence that the distal complement system (MAC) can promote proinflammatory endothelial cell activation, specifically, increases in IL-8 and MCP-1 mRNA concentrations and protein secretion, and that cytosolic to nuclear translocation of NF-κB is necessary for this response. (Am J Pathol 1997, 150:2019–2031)

A large body of evidence suggests that both soluble and cell surface mediators coordinately regulate the events that characterize the acute inflammatory response.[1] Activation of the complement system can modulate leukocyte and endothelial cell functions through the actions of the anaphylatoxins, C3b and

Supported in part by the National Institutes of Health (HL 48287 and 5T 32-HL 07517). K. S. Kilgore was supported, in part, by the American Heart Association of Michigan (21F956 fellowship).

Accepted for publication February 13, 1997.

Address reprint requests to Dr. Jeffrey S. Warren, Department of Pathology, Box 0602, University of Michigan Medical School, 1301 Catherine Road, Ann Arbor, MI 48109-0602.

its derivatives, and the heteropolymeric membrane attack complex (MAC) and related complexes (eg, C5b-7 and C5b-8).[2,3] Assembly of the MAC on some cell surfaces can result in a number of proinflammatory actions. Nucleated cells exhibit membrane-associated mechanisms that protect them from lysis by the C5b-9 complex.[3,4] Injury or death of nucleated cells occur when there is a high density of pores formed by MAC, whereas lower densities of cell surface MAC may induce changes in cellular activity.[3] Assembly of MAC in sublytic concentrations can modulate cell function in a variety of ways, including through alteration of the expression of cellular adhesion molecules and through up-regulation of the secretion of proinflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, basic fibroblast growth factor, and platelet-derived growth factor.[5–7] Using purified C5-C9, we recently reported that assembly of sublytic densities of functional MAC on endothelial cells results in the up-regulation and secretion of the neutrophil and monocyte chemotactic cytokines IL-8 and monocyte chemoattractant protein (MCP)-1, respectively.[8] Furthermore, Torzewski et al[9] have reported that MCP-1 is also released by smooth muscle cells, suggesting that the proinflammatory actions of the MAC are not limited to endothelial cells.

The mechanisms by which assembly of the MAC promotes increased expression of these proinflammatory mediators have yet to be fully ascertained. Assembly of the MAC (and C5b-7 and C5b-8) has been associated with a number of signal transduction events. For example, MAC formation on endothelial cell membranes results in a rapid increase in intracellular cyclic AMP concentration, suggesting that the MAC may interact directly with pertussis-toxin-sensitive G proteins.[10] Intracellular calcium, derived from both intracellular and extracellular stores, increases after MAC deposition.[11] A rise in intracellular calcium concentration allows cell activation via Ca^{2+}-sensitive proteins or through direct activation of various protein kinases.[12] Finally, MAC deposition is associated with the appearance of phosphatidylcholine breakdown products and the activation of protein kinase C.[12]

Although it is apparent that the MAC may modulate cell function through several signal transduction pathways, the mechanisms through which it mediates IL-8 and MCP-1 production by endothelial cells remain to be established. A large body of evidence suggests that nuclear factor (NF)-κB (and other members of the Rel family) plays a key role in the inflammatory response through its capacity to modulate the production of proinflammatory cytokines and the up-regulation of adhesion molecule expression.[13,14] Among genes that encode proinflammatory mediators and contain promoter elements that are regulated by members of the NF-κB family of transcription factors are E-selectin, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, IL-6, IL-8, and MCP-1.[15–17] NF-κ B, a transcription factor associated with rapid activation responses, is composed of two subunits, p50 and p65.[18] Within inactive cells, the p50 and p65 subunits are bound within the cytoplasm to the inhibitor protein, IκB-α.[19,20] When the cell is appropriately stimulated, IκB-α is phosphorylated, ubiquitinated, and proteolytically degraded, thus allowing the liberation of NF-κB and its translocation from the cytosol to the nucleus, where it binds to specific promoter elements and activates target genes.[21]

We examined the role of NF-κB activation in MAC-induced endothelial activation, specifically, the up-regulation of IL-8 and MCP-1 mRNA concentrations and protein secretion. Assembly of sublytic concentrations of functional MAC resulted in the translocation of NF-κB from the cytosol to the nucleus. These responses were inhibited in the presence of pyrrolidine dithiocarbamate (PDTC) and SN50, which have previously been shown to inhibit NF-κB-dependent cell activation. Recent reports suggest that PDTC may interrupt NF-κB-activated translocation by preventing inducible phosphorylation of IκB-α.[22,23] Inhibition of IL-8 and MCP-1 production with SN50, a 26-amino-acid peptide that contains a lipophilic cell-membrane-permeable motif and a nuclear localization sequence that specifically competes with the nuclear localization sequence of the NF-κB p50 subunit,[24] strongly supports the conclusion that MAC may serve to activate proinflammatory mediator genes (IL-8 and MCP-1) through the activation and translocation of NF-κB. The data contained in the present study suggest that NF-κB translocation/activation is necessary for MAC-induced IL-8 and MCP-1 secretion by human umbilical vein endothelial cells (HUVECs). It remains unclear whether NF-κB activation/translocation is sufficient.

Materials and Methods

Reagents

Purified complement proteins (C5-C9) were purchased from Quidel (San Diego, CA). Lipopolysaccharide (LPS) concentrations in purified complement protein stock solutions were assayed with the E-Toxate (*Limulus* amoebocyte lysate) assay (Sigma Chemical Co., St. Louis, MO; catalog item 210-A1).

Peptide-specific rabbit antiserum (product SC-372) to NF-κB p65 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Additional chemicals and reagents, unless otherwise indicated, were purchased from Sigma.

Endothelial Cell Culture

HUVECs were isolated from umbilical veins by treating with 0.1% collagenase in Dulbecco's modified Eagle's medium (Whittaker Bioproducts, Walkersville, MD) as previously described.[25] Cells were grown and maintained in M199 medium (Whittaker Bioproducts), supplemented with 20% heat-inactivated fetal calf serum, L-glutamine (4 mmol/L), penicillin (100 U/ml), streptomycin (100 ug/ml), endothelial growth supplement (25 μg/ml; Collaborative Research, Bedford, MA), and bovine lung heparin (15 U/ml). Cells were grown on gelatin-coated 150 × 25 mm plates and allowed to grow to confluence at 37° with 5% CO_2 before harvesting for extract preparation. Cells were characterized as previously described and used between the first and third passages.[8]

Non-Enzymatic Formation of C5b-Like C5C6 Complex (C5C6)*

Purified proteins C5 and C6 were suspended in serum-free HUVEC medium. Formation of the C5b-like activation product was accomplished by oxidation with chloramine-T as described previously.[26–28] Briefly, C5 (10 μg) was added to veronal-buffered saline (10 μl) (Sigma) and incubated in the presence of 10 μl of 0.32 mmol/L chloramine-T (Sigma) in water for 10 minutes at room temperature. At the end of the incubation period, methionine (1 mmol/L; 10 μl; Sigma) was added to the mixture to inactivate the remaining chloramine-T. To form the modified C5b-like C5C6 complex (C5C6*), purified C6 (20 μg) in 300 μl of serum-free medium was incubated (24 hours at 37°C) with the chloramine-T-treated C5. The functional (lytic) and structural (reactivity with monoclonal antibody directed against MAC neoantigen) integrity of chloramine-T-generated MAC on HUVECs was verified as previously described.[8]

Assembly of the Membrane Attack Complex

HUVECs were plated in 150 × 25 mm dishes 24 hours before use. Cells were then washed with serum-free medium (see above), and the MAC was assembled for 30 minutes. Assembly of the MAC was initiated by a 15-minute preincubation (37°C) with the modified C5C6* activation product (5 μg/ml) and C7 (10 μg/ml).[8] Endothelial monolayers were then washed with serum-free medium to remove excess C5C6* activation product and C7. Complement components C8 (10 μg/ml) and C9 (10 μg/ml) were then added to the monolayers and allowed to incubate for an additional 30 minutes. Monolayers were then washed with serum-free medium to remove excess noncomplexed complement components and incubated with serum-free medium for the times indicated. The total volume per dish was 10 ml. Where indicated, individual complement components were heat inactivated (100°C for 30 minutes) or polymyxin B (5 to 50 μg/ml) was added to the medium.

Extraction of Cell Nuclei

Nuclear extraction was carried out as described previously.[29,30] Briefly, HUVECs (5×10^6 to 5×10^7 cells) were harvested by scraping and centrifuging (200 × *g* for 5 minutes) in phosphate-buffered saline (PBS; 4°C) containing a protease inhibitor cocktail (10 μmol/L aprotinin, 1 μmol/L leupeptin, 1 μmol/L bestatin, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF); Sigma). Cells were prewashed in PBS (4°C; 2 ml) and pelleted (200 × *g* for 5 minutes at 4°C). The supernatant was removed and the pellet was washed twice with 2 ml of ice-cold buffer A (10 mmol/L HEPES, pH 7.9, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol (DTT), and 0.5 mmol/L PMSF) and centrifuged as described above. The supernatant was aspirated and the pellet resuspended in 80 μl of buffer A plus 0.1% Nonidet P-40. The resulting suspension was then transferred to a 1.7-ml Eppendorf tube and incubated on ice for 5 minutes, followed by centrifugation at 450 × *g* for 12 minutes at 4°C. The crude nuclear pellet was resuspended in 60 μl of buffer C (20 mmol/L HEPES, pH 7.9, 0.42 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L DTT; 0.5 mmol/L PMSF, 25% glycerol (v/v)) and incubated on ice for 15 minutes. Nuclei were sonicated twice at 15% power output (10 seconds) and microcentrifuged at 14,000 rpm for 2 minutes. The nuclear fraction was again centrifuged at 14,000 rpm for 12 minutes (4°C) to collect the supernatant containing the nuclear protein extracts. These extracts were subsequently diluted with 60 μl of modified buffer D (20 mmol/L HEPES, pH 7.9, 0.05 mol/L KCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L PMSF). Protein concentrations were determined by Folin's assay with bovine serum albumin (BSA) as a standard (Sigma).

Electrophoretic Mobility Shift and Supershift Assays

Electrophoretic mobility shift assays (EMSA) were carried out using a gel shift assay system kit (catalog item E-3050, Promega Corp., Madison, WI).[31] Double-stranded NF-κB consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was end-labeled with [^{32}P]ATP (3000 Ci/mmol at 10 mCi/ml; Amersham Life Science, Arlington Heights, IL). After T4 kinase end-labeling, oligonucleotide (35 fmol; ~1 × 10^5 dpm) probe and nuclear protein (5 μg) were incubated for 30 minutes at room temperature in binding buffer (50 mmol/L Tris/HCl, pH 7.5, 250 mmol/L NaCl, 2.5 mmol/L DTT, 20% glycerol (v/v)), and 0.5 μg of poly dI.dC (Pharmacia, Piscataway, NJ). The reaction volumes were held constant at 10 μl. Where indicated, unlabeled competitive oligonucleotide (NF-κB) or irrelevant oligonucleotide (AP1) was added 10 minutes before the addition of radiolabeled probe.[31] Samples were run on a nondenaturing 4% polyacrylamide electrophoretic gel in 0.25X TBE buffer (10 mmol/L Tris/HCl, pH 8.0, 1 mmol/L EDTA) at 100 mV/15 mA for 4 hours. Gels were vacuum dried and visualized by Kodak X-OMAT film exposure to the gel at −70°C for 1 hour. For supershift assays, the reaction mixture was incubated with anti-p65 antibody (Santa Cruz Biotechnology; sc-372) for 20 minutes at room temperature. The oligonucleotide probe was added and the incubation carried out as described above.

Western Blot Analysis

Nuclear and cytosolic extracts from stimulated HUVECs were subjected to electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose in 25 mmol/L Tris, 192 mmol/L glycine, 5% methanol at 100 V for 1.5 hours at 4°C as described previously.[32] Blots were analyzed for the p65 NF-κB subunit using primary antibody (Santa Cruz Biotechnology) at a 1:1000 dilution. After incubation with the primary antibody, blots were washed and incubated with a 1:500 dilution of peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA). Western blot detection was achieved using a colorimetric detection system (Bio-Rad).

Immunocytochemical Staining

Immunocytochemical analysis was carried out to assess subcellular NF-κB p65 subunit localization in HUVECs activated with MAC.[31] HUVECs were grown for 24 hours in eight-well microchamber slides (Nunc, Naperville, IL). After assembly of the MAC, monolayers were fixed with 4% paraformaldehyde in PBS for 20 minutes at room temperature and then for 6 minutes with methanol at −20°C. Rabbit polyclonal antibody to the p65 subunit of NF-κB (Santa Cruz Biotechnology) was layered onto slides for 1 hour at room temperature and then incubated with a biotinylated goat anti-rabbit secondary antibody (1:1000 dilution; Vector Laboratories, Burlingame, CA). Detection of the primary antibody was accomplished using a Vectastain ABC kit (Vector Laboratories) with 3-amino-9-ethyl-carbazole as the substrate. Controls included chambers in which the primary antibody was omitted and chambers in which nonspecific rabbit immunoglobulin was substituted for the anti-p65 antibody. To rule out the possibility that sublytic concentrations of MAC induce artifactual association of cytosolic NF-κB with nuclear extracts, we examined the localization of an irrelevant cytosolic protein, lactate dehydrogenase (LDH), in HUVECs exposed to MAC. These studies were conducted as described above but employed a monoclonal antibody directed against LDH (Accurate Chemical and Scientific Corp., Westbury, NY).

Effects of PDTC and SN50 on MAC-Mediated MCP-1 and IL-8 Secretion

Chemokine-specific enzyme-linked immunosorbent assay (ELISA) analyses were used to quantify IL-8 and MCP-1 in conditioned media after stimulation by the MAC in the presence or absence of either PDTC or SN50 at the indicated concentrations. The sandwich ELISAs used in this study are modifications of previously described methods that used goat polyclonal and mouse monoclonal antibodies directed against each chemokine under investigation.[8] Microtiter plates (Nunc, Roskilde, Denmark) were coated with either anti-IL-8 or MCP-1 polyclonal antibody in coating buffer (0.05 mol/L H_3BO_3, 0.120 mol/L NaCl, pH 8.6) overnight at 4°C (100 μl/well final volume). The wells were washed three times with washing buffer (PBS, 0.02% Tween-20) and incubated for 60 minutes at 37°C with washing buffer containing 2% BSA. Samples of HUVEC culture supernatants (50 μl/well) were added to the wells and incubated for 90 minutes at 37°C. Standard curves for each chemokine were prepared by diluting recombinant human IL-8 or MCP-1 (Genzyme, Cambridge, MA) in washing buffer at concentrations ranging from 0.01 to 1000 ng/ml. After repeated washings, a mouse

monoclonal anti-human antibody (Genzyme) was added to the wells and allowed to incubate at 37°C for 60 minutes. The wells were washed and incubated with a peroxidase-conjugated rabbit anti-mouse IgG (1:1000 dilution; Dako Corp., Carpinteria, CA) for 1 hour at room temperature followed by addition of the chromogenic substrate (*o*-phenylenediamine hydrochloride) for 30 minutes. The reaction was quenched with 3 mol/L H_2SO_4, and the optical density was determined at 490 nm using an EL340 automated microplate reader (Bio-Tek Instruments, Winooski, VT). The sensitivity limits for the ELISAs were 0.01 ng/ml for IL-8 and 0.1 ng/ml for MCP-1.

Northern Hybridization Analysis of IL-8 and MCP-1 mRNA after PDTC Pretreatment of MAC-Stimulated Cells

Northern hybridization was performed as described previously.[8] Briefly, RNA was isolated from HUVEC monolayers stimulated with the MAC or MAC-stimulated HUVECs that had been pretreated with PDTC (10 μmol/L, 2 hours). Monolayers stimulated with LPS (10 μg/ml) were used as positive controls. Five hours after assembly of the MAC, HUVECs were prepared for Northern hybridization analysis. Total RNA extraction was carried out using TRIzol reagent (Gibco, Gaithersburg, MD). After removing insoluble debris by centrifugation, the RNA was precipitated and washed with isopropyl alcohol and ethanol. After resuspension of the pellet, the RNA concentration of a 1:250 dilution was determined by measuring absorbance at 260 nm.

Total RNA (12 μg) was electrophoretically separated in 1% agarose, 2.2 mol/L formaldehyde denaturing gel, followed by capillary transfer to nylon membrane (Zetabind, Cuno, Meridien, CT). The membrane was dried for 2 hours *in vacuo* at 80°C, prehybridized for 5 to 6 hours at 65°C in 10 ml of 7% SDS, 0.5 mol/L NaH_2PO_4 (pH 7.0), and 1 mmol/L EDTA, and 1% BSA. Approximately 1×10^7 to 2×10^7 of ^{32}P-labeled IL-8 or MCP-1 probe (see below) or β-actin cDNA (gift of Paul Killen, University of Michigan Medical School) was mixed with 300 μl of a 10 mg/ml solution of salmon sperm DNA (0.1 mg/ml final), boiled for 10 minutes, and placed on ice before adding to the hybridization bag. The membranes were hybridized overnight at 65°C, after which they were washed once at room temperature, twice at 65°C (10 minutes each wash) in 5% SDS, 10 mmol/L NaH_2PO_4 (pH 7.0), 1 mmol/L EDTA, 0.5% BSA, and then twice in 1% SDS, 10 mmol/L NaH_2PO_4, 1 mmol/L EDTA at 65°C. The membranes were then exposed to XAR-5 film (Kodak) at −70°C.

The sequence of the IL-8 cDNA probe was 5′-GTT-GGC-GCA-GTG-TGG-TCC-ACT-CTC-AAT-CAC-3′. The probe sequence for the MCP-1 was 5′-TTG-GGT-TTG-CTT-GTC-CAG-GTG-GTC-CAT-GGA-3′. To confirm equal loading of the RNA, the amount of total RNA per lane was assessed by monitoring the amount of β-actin mRNA.

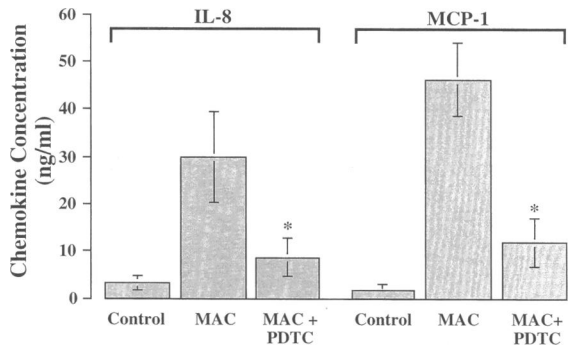

Figure 1. *Inhibition of MAC-mediated IL-8 and MCP-1 secretion from HUVECs after pretreatment with PDTC (10 μmol/L for 2 hours). HUVECs were exposed to MAC for 30 minutes and washed, with medium then collected at 24 hours. These data represent the mean ± SEM from one experiment (of two) performed in quadruplicate.* *$P < 0.05$ versus *response of HUVECs exposed to MAC alone (one-way ANOVA).*

Results

PDTC Pretreatment of MAC-Stimulated HUVECs Inhibits IL-8 and MCP-1 Secretion

We have previously observed that sublytic concentrations of MAC can induce IL-8 and MCP-1 secretion by HUVECs.[8] Conditioned medium from PDTC-treated HUVECs bearing sublytic concentrations of MAC were examined by ELISA 24 hours after MAC deposition (Figure 1). Assembly of MAC on HUVECs resulted in IL-8 and MCP-1 secretion substantially above that observed in unstimulated cells. Pretreatment of HUVECs with PDTC (10 μmol/L for 2 hours) inhibited the MAC-induced secretion of both IL-8 and MCP-1. Given the inhibitory effect of PDTC in NF-κB-mediated cell activation,[33] these data suggest that the MAC-induced increase in IL-8 and MCP-1 requires functional NF-κB.

PDTC Pretreatment of HUVECs Inhibits MAC-Mediated Increases in IL-8 and MCP-1 mRNA

RNA was isolated from unstimulated HUVEC monolayers, monolayers stimulated with the MAC, and

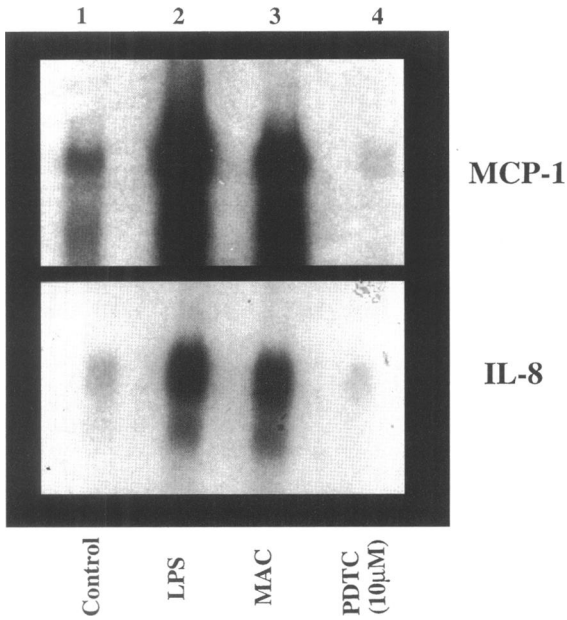

Figure 2. *Northern blot analysis of IL-8 and MCP-1 mRNA after stimulation of HUVECs by MAC. Cells were pretreated for 2 hours with either PDTC (10 μmol/L) or vehicle. Total cellular mRNA was isolated 5 hours after stimulation.* Lanes 1 *to* 4, *unstimulated control cells, LPS-stimulated cells, cells stimulated with MAC, and cells pretreated with PDTC before MAC deposition, respectively. Equal loading of RNA was confirmed as assessed by densitometric comparisons of β-actin mRNA. There was less than 11% lane-to-lane variation in RNA loading in the depicted experimental result (data not shown). The depicted data represent a single, representative blot from three experiments.*

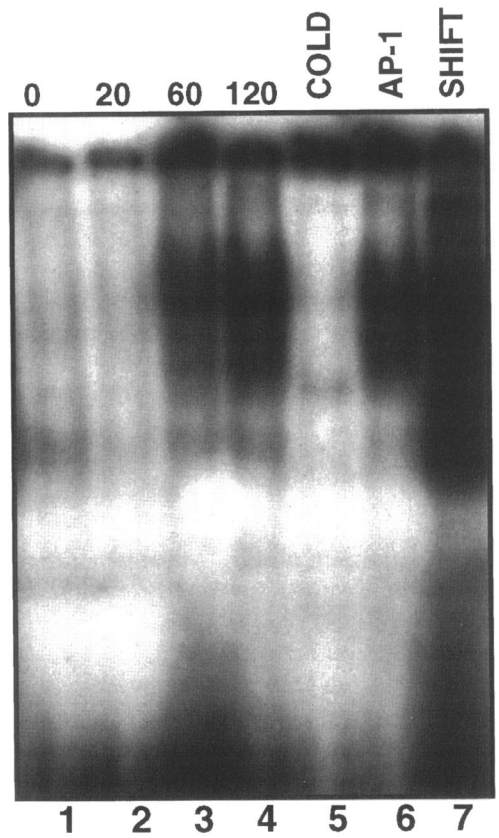

Figure 3. *Temporal characteristics of MAC-mediated cytosolic to nuclear NF-κB translocation. HUVECs were exposed to functionally intact, sublytic concentrations of MAC (see Materials and Methods) for 0, 20, 60, and 120 minutes as indicated* (lanes 1 *to* 4). *Preparations shown in* lanes 5 *to* 7 *were from HUVECs exposed to MAC for 120 minutes. NF-κB consensus oligonucleotide probe (35 fmol; ~1 × 10^5 dpm) and nuclear protein (5 μg) were incubated for 30 minutes in binding buffer. Where indicated, unlabeled competitive oligonucleotide (NF-κB) or irrelevant oligonucleotide (AP-1) was added 10 minutes before the addition of radiolabeled probe* (lanes 5 *and* 6). *Samples were run on a nondenaturing 4% polyacrylamide gel. No nuclear NF-κB activity was noted at 0 or 20 minutes after MAC formation* (lanes 1 *and* 2). *Assembly of the intact MAC was associated with a gradual increase in nuclear NF-κB activity beginning within 60 minutes* (lane 3) *after activation of endothelial cells. Maximal NF-κB activity was observed at 2 hours* (lane 4). *NF-κB activity was not found in samples incubated with 100-fold excess of unlabeled probe* (lane 5) *whereas the presence of the irrelevant oligonucleotide AP-1 probe did not affect NF-κB detection* (lane 6). *Supershift assays were conducted in the presence of anti-p65 antibody, which induced a shift in the band for NF-κB* (lane 7). *The depicted results represent one of three separate experiments.*

monolayers incubated with with PDTC (10 μmol/L/ml, 2 hours) before MAC deposition. Monolayers stimulated with LPS (10 μg/ml) were used as a positive control. As illustrated in Figure 2, low concentrations of IL-8 and MCP-1 mRNA were detected in unstimulated monolayers. Marked increases in IL-8 and MCP-1 mRNA were observed in monolayers stimulated by the MAC as compared with the amounts observed in HUVEC monolayers pretreated with PDTC. These results indicate that MAC-mediated increases in IL-8 and MCP-1 mRNA are inhibited by PDTC and, in conjunction with the data above, suggest a potential role for NF-κB (see below).

Temporal Analysis of MAC-Mediated NF-κB Translocation in HUVECs

To directly determine whether MAC can induce cytosolic to nuclear translocation of NF-κB and to ascertain the temporal characteristics of MAC-induced NF-κB translocation, EMSA analysis was carried out using nuclear extracts from HUVECs exposed to sublytic concentrations of MAC for varied lengths of time (Figure 3). No nuclear NF-κB activity was noted 20 minutes after MAC deposition (lane 2). However, deposition of the intact MAC was associated with a gradual increase in nuclear NF-κB activity beginning within 60 minutes after activation of endothelial cells (lane 3). Maximal nuclear NF-κB activity was observed at 2 hours (lane 4). Incubation of samples with 100-fold excess of unlabeled probe inhibited detection of NF-κB (lane 5) whereas the presence of the irrelevant probe (AP-1) did not alter NF-κB detection (lane 6). Samples preincubated with anti-p65 antibody induced a supershift in the NF-κB band (lane 7). These data indicate that assembly of sub-

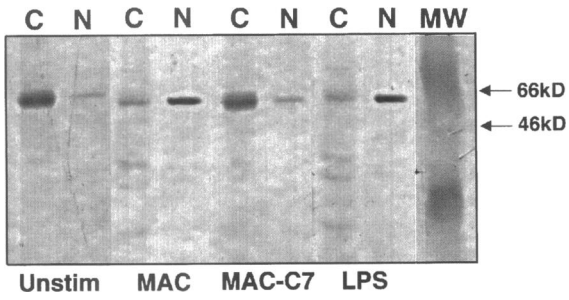

Figure 4. *Immunoblot analysis of MAC-induced cytosolic (C) to nuclear (N) translocation of NF-κB p65 unit. Endothelial cells were stimulated with intact MAC for 30 minutes and allowed to incubate for 2 hours at 37°C. NF-κB translocation was visualized by addition of a polyclonal antibody to the p65 subunit followed by a biotin-labeled secondary antibody. Deposition of intact MAC on HUVECs resulted in an increase in nuclear p65 and a corresponding decrease of the protein in the cytosolic fraction. Prevention of MAC formation through omission of C7 resulted in failure of cytosolic to nuclear translocation of NF-κB p65. LPS-stimulated (10 μg/ml) cells revealed pronounced cytosolic to nuclear translocation of NF-κB p65. The depicted results represent one of four separate experiments.*

lytic concentrations of the MAC on HUVECs results in the rapid translocation (1 hour) of NF-κB to cell nuclei. The requirement for intact MAC in NF-κB translocation is addressed below.

Western Blot Analysis of p65 Translocation

Immunoblot analysis of nuclear and cytosolic extracts from unstimulated HUVECs revealed the presence of the NF-κB p65 subunit in the cytosolic fraction whereas little p65 was detected in the nuclear fraction (Figure 4). Assembly of the intact MAC on HUVECs resulted in a marked increase in nuclear p65 and a corresponding decrease in cytosolic p65. These data are indicative of p65 translocation. It should be noted that the translocation of p65 to the nucleus did not entirely deplete the cytosolic p65 pool. Identical treatment of HUVECs with MAC components devoid of C7 resulted in failure of cytosolic to nuclear p65 translocation. As expected, cytosolic and nuclear fractions derived from LPS-stimulated (10 μg/ml) cells, used as a positive control, revealed pronounced cytosolic to nuclear translocation of p65.

Intact MAC Is Required for NF-κB DNA Binding

EMSA analysis of nuclear extracts derived from HUVECs stimulated with either intact (functional) MAC or identical concentrations of MAC components devoid of either C7 or C9 (ie, nonfunctional) is shown in Figure 5. Assembly of intact MAC (lane 3) promoted a marked shift in the binding of the

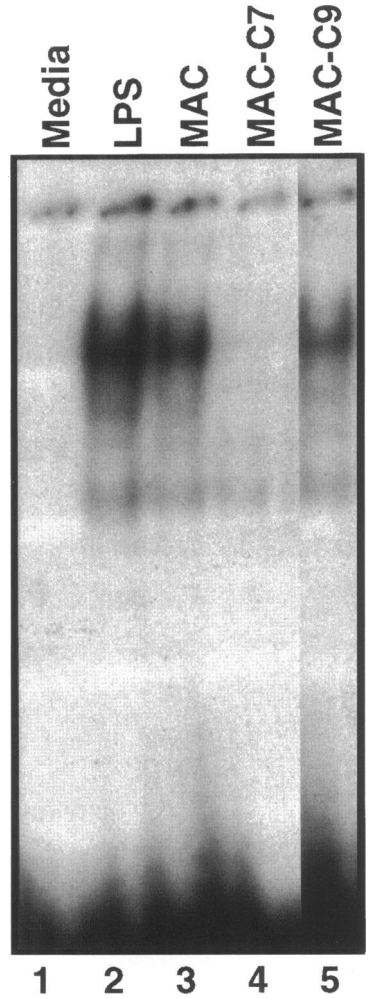

Figure 5. *EMSA analysis of nuclear extracts derived from HUVECs activated with the intact MAC or MAC components in the absence of either C7 or C9. Unstimulated cells are noted in* lane 1. *The presence of intact MAC* (lane 3) *promoted increased band shift of the NF-κB oligonucleotide fragment as compared with that noted for cells stimulated with MAC components in the absence of C9* (lane 5). *Omission of C7 from the MAC* (lane 4) *abrogated the band shift. Stimulation of HUVECs for 1 hour with LPS (10 μg/ml) was used as a positive control* (lane 2). *These results are representative of three separate experiments.*

NF-κB oligonucleotide fragment band, similar to that observed in cells incubated with equivalent MAC components but no C9 (lane 5). Exclusion of C7 from the MAC (lane 4) resulted in a markedly reduced band shift as compared with that seen in HUVECs exposed to the complete MAC or C5b-8 (MAC components without C9). Stimulation of HUVECs with media alone or 1 hour with LPS (10 μg/ml) were used as negative and positive controls, respectively (lanes 1 and 2). These results suggest that intact MAC is required for maximal NF-κB DNA binding and that C5b-8 may also induce NF-κB DNA binding.

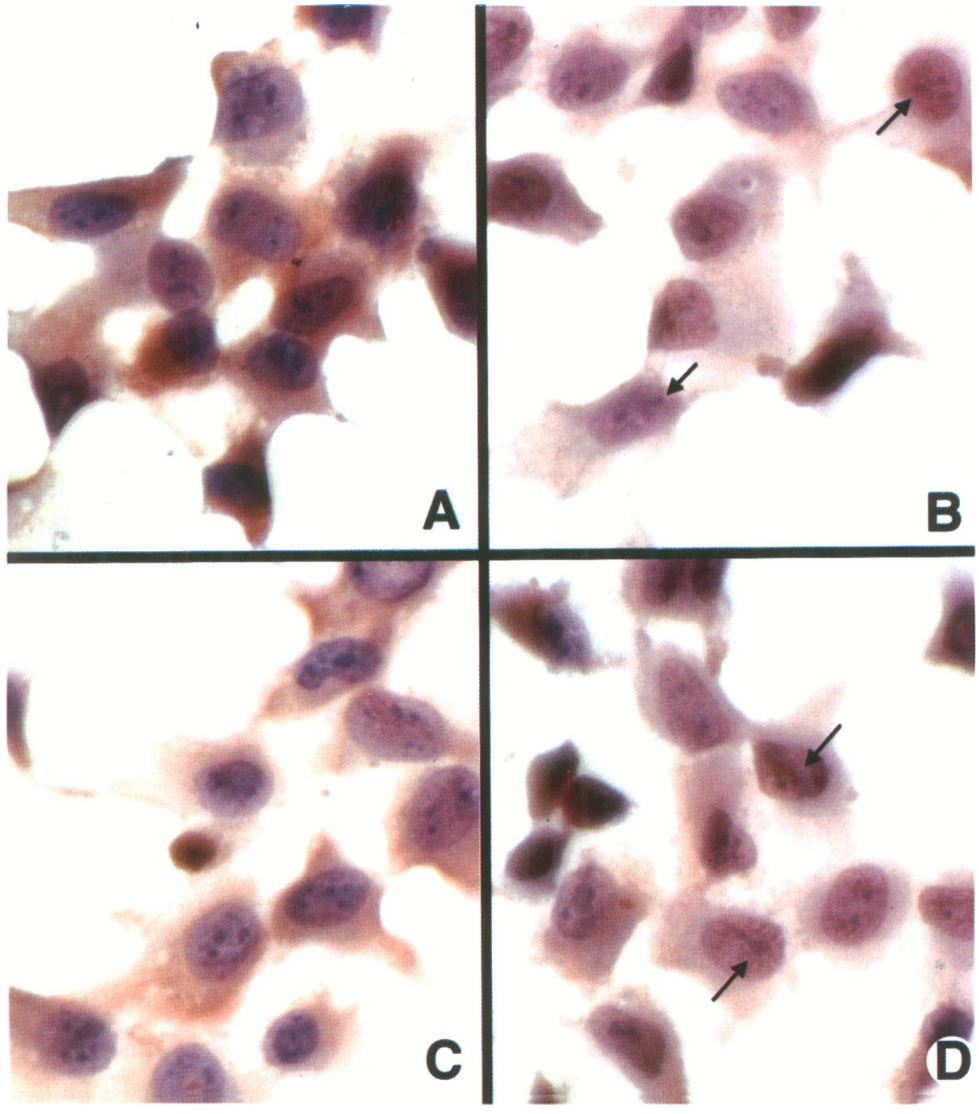

Figure 6. *Immunocytochemical analysis of MAC-induced translocation of NF-κB p65 from cytosol to nucleus.* **A:** *In unstimulated HUVECs, the p65 subunit was localized primarily in the cytoplasm.* **B:** *In contrast, deposition of intact MAC induced a marked increase in nuclear staining and a corresponding loss of cytoplasmic NF-κB p65 staining).* **C:** *Nuclear staining was not observed in cells incubated with MAC components in the absence of C7.* **D:** *LPS-stimulated cells (10 μg/ml) were used as a positive control for NF-κB translocation.*

Immunocytochemical Evidence that the Intact MAC Specifically Induces Translocation of NF-κB p65 from Cytosol to Nucleus

Immunocytochemical analysis of MAC-induced cytoplasmic to nuclear translocation of NF-κB p65 is depicted in Figure 6. Consistent with the results derived from Western blot and EMSA analysis, p65 is detected primarily in the cytoplasm of unstimulated cells (Figure 6A). Little nuclear staining is observed (Figure 6A). In contrast, assembly of intact MAC induced a marked increase in the amount of nuclear staining and a corresponding decrease in cytoplasmic staining (Figure 6B). These data confirm the ability of the MAC to promote NF-κB translocation. Nuclear staining was not observed in cells incubated with equivalent concentrations of MAC components minus C7 (Figure 6C). LPS-stimulated cells (10 μg/ml) were used as a positive control for NF-κB translocation (Figure 6D). These results confirm that deposition of intact MAC results in translocation of NF-κB from the cytosol to the nucleus of HUVECs. Assembly of MAC on HUVECs did not induce a cytosolic to nuclear shift in LDH (Figure 7), suggesting that MAC-induced NF-κB translocation is specific and not an artifactual phenomenon (eg, osmotic effect).

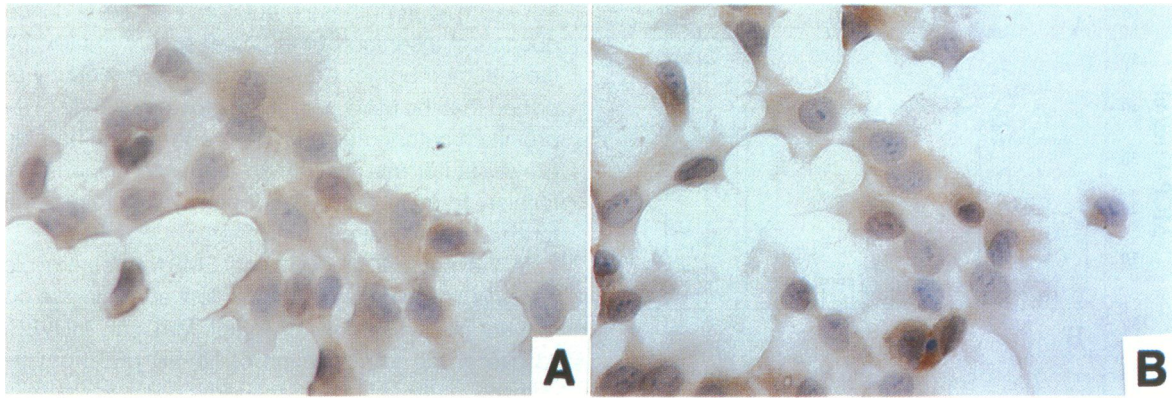

Figure 7. *Immunocytochemical analysis of the cytosolic protein LDH after MAC deposition. LDH is localized to the cytoplasm in unstimulated cells* (A) *and MAC-stimulated cells* (B). *Nuclear localization of LDH was not observed in either of the treatment groups.*

MAC-Stimulated HUVECs Produce IL-8 and MCP-1 via NF-κB: SN50 and LPS Specificity Studies

Because of recent reports that suggest that PDTC may interrupt NF-κB activation/translocation through prevention of inducible phosphorylation of IκB-α (as a kinase inhibitor),[20,21] we used SN50[22] to more specifically address the role of NF-κB in MAC-induced HUVEC activation. In addition, studies using heat-inactivated complement components (C5-C9), MAC devoid of either C7 or C8, and intact MAC in the presence of polymyxin B were carried to address the potential role of endotoxin contamination in endothelial cell activation. As shown in Figure 8, preincubation of HUVECs with SN50 resulted in concentration-dependent reductions in both IL-8 and MCP-1. Heat inactivation of the complement components used to form MAC (C5-C9) reduced IL-8 and MCP-1 produced by HUVECs to concentrations produced by unstimulated cells.

Addition of polymyxin B to HUVECs exposed to MAC did not reduce IL-8 or MCP-1 production (compared with that observed in media from HUVECs exposed to MAC in the absence of polymyxin B). The inhibitory effect of SN50 on MAC-induced IL-8 and MCP-1 production, in conjunction with the EMSA, immunocytochemical, and Western blot data presented in the preceding sections, strongly support the conclusion that sublytic concentrations of MAC induce HUVECs through NF-κB-dependent mechanisms. The failure of heat-inactivated MAC components and MAC devoid of C7 or C8 to trigger IL-8 and MCP-1 production argue against LPS contamination of MAC as the explanation for these findings. The latter conclusion is further supported by the lack of effect of polymyxin B on MAC-induced IL-8 and MCP-1 production by HUVECs. It should be noted that direct measurements of endotoxin in C5-C9 and medium alone, at the dilutions used in these studies, were all less than or equal to 0.027 EU/ml.

Discussion

Assembly of sublytic concentrations of intact MAC can modulate cell function in a variety of ways. For example, Hattori et al[34,35] reported that activation of complement in close proximity to the surface of endothelial cell monolayers results in the rapid up-regulation of P-selectin. The MAC has been observed to interact in a synergistic fashion with TNF-α to promote increased neutrophil adhesion to endothelial cells through the up-regulation of E-selectin and ICAM-1.[28] Deposition of the MAC on endothelial cells is also associated with the up-regulation and secretion of soluble proinflammatory mediators. Schonermark et al[5] and Lovett et al[6] reported that the MAC promotes the secretion of TNF-α and IL-1β, respectively. We recently reported that assembly of intact MAC on endothelial cells is associated with the up-regulation and secretion of the chemotactic cytokines IL-8 and MCP-1.[8] However, the mechanisms by which assembly of the MAC promotes increased expression of selectins, ICAM-1 and various soluble proinflammatory mediators have yet to be fully elucidated.

A large body of evidence suggests that the expression of various cytokines and leukocyte adhesion molecules is regulated through several signal transduction pathways including activation of protein kinase C (PKC), cholera-toxin-sensitive G-proteins, and the generation of oxygen-derived metabolites.[12] The MAC has been reported to interact directly and indirectly with a number of these second messenger pathways. For example, complete (C5b-9) or partial

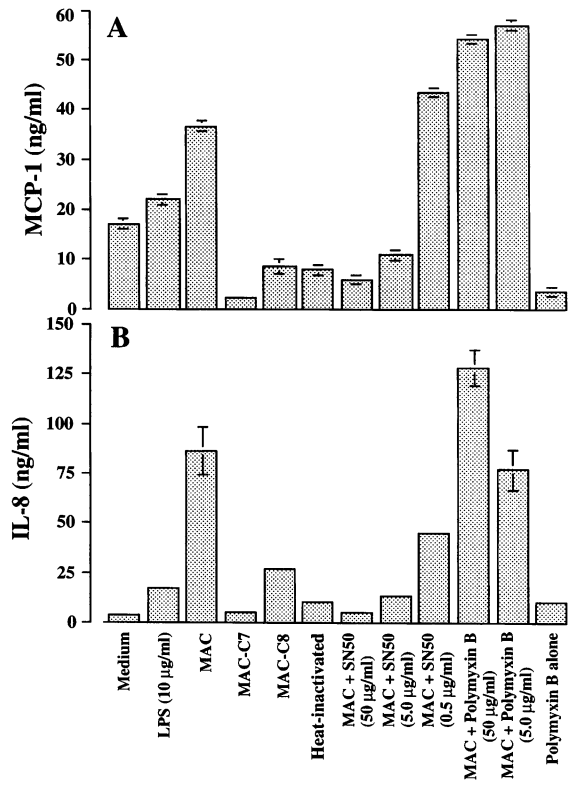

Figure 8. *IL-8 and MCP-1 secretion from HUVECs incubated with either MAC, MAC devoid of C7 or C8, heat-inactivated MAC (100°C for 30 minutes), SN50 (added 15 minutes before MAC assembly), or polymyxin B. HUVECs were exposed to MAC (or indicated treatment) for 30 minutes and washed, with medium then collected at 24 hours. These data represent the means ± SEM from one experiment (of two) performed in quadruplicate.*

(C5b-8) formation of the MAC is associated with increased intracellular levels of *sn*-1,2-diacylglycerol and activation of a variety of protein kinases including PKC and protein kinase A.[11,12] Niculescu et al[10] have observed that the MAC may interact directly with pertussis-toxin-sensitive guanine nucleotide binding proteins (G-proteins) in a receptor-independent manner. Although not a specific signal transduction pathway, oxygen-derived metabolites are generated after MAC deposition.[36] Activation of these second messenger systems may afford an explanation as to the capacity of the MAC to promote nuclear translocation of NF-κB. The signal transduction pathways that interact with the MAC, including activation of G-proteins and PKC, share in the ability to mediate the activation of NF-κB.[37,38] Interaction of the MAC with multiple signal transduction pathways that correlate with those that affect NF-κB suggests a possible link between MAC formation and the subsequent up-regulation of proinflammatory mediators via NF-κB. Although the expression of IL-8 and MCP-1 may be mediated via activation of signal transduction pathways, the possibility remains that the expression of these mediators is a direct consequence of insertion of the C5b-9 pore complex into the cell membrane. Acosta et al[39] have recently reported that the MAC pore allows for the release of the proinflammatory mediators IL-1 and basic fibroblast growth factor that may, in an autocrine or paracrine fashion, form a positive feedback system to promote further endothelial cell activation.

In the present study, using EMSA, Western blot analysis, and immunocytochemical analysis, we observed that assembly of intact MAC on HUVECs results in the translocation of NF-κB p65 from the cytosol to the nucleus. Initial NF-κB translocation was noted 60 minutes after MAC deposition with the maximal degree of NF-κB activity noted at 120 minutes. In addition, we observed that the maximal MAC-induced nuclear translocation requires the presence of all of the proteins that constitute MAC (C5-C9) or at least C5b through C8. This conclusion is based on the observation that MAC proteins devoid of C7, but not C9, do not promote translocation of the heterodimers to the nucleus to the degree observed after assembly of intact MAC. To confirm that the translocation of NF-κB to the nucleus is specific and not a random or artifactual event, the intracellular localization of the cytosolic protein LDH was determined by immunocytochemistry. LDH is a cytosolic protein that is not associated with nuclear translocation. In the present study, LDH was not detected in association with nuclei after MAC deposition, as determined by immunohistochemistry, indicating that the MAC does not promote the nonspecific movement of cytosolic proteins (eg, LDH) from the cytosolic compartment to the nuclear compartment. These data suggest that the nuclear translocation of p65 is a specific intracellular signaling event and not simply due to MAC-mediated loss of nuclear membrane integrity.

We previously reported that deposition of the MAC on HUVECs promotes the expression of the chemotactic cytokines IL-8 and MCP-1, both of which are regulated by the NF-κB family of transcriptional regulators.[8,15–17] To address a potential direct linkage between MAC deposition and NF-κB activation, we examined the effect of the NF-κB inhibitors PDTC and SN50 on the production of MCP-1 and IL-8. PDTC has been previously reported to selectively inhibit NF-κB activity, presumably by functioning in an antioxidant capacity.[33] Pretreatment of HUVECs with PDTC (10 μmol/L for 2 hours) resulted in significant decreases in the concentration of both IL-8 and MCP-1 as demonstrated by ELISA and Northern blot analysis. More recent studies suggest that PDTC may interrupt NF-κB activation/translocation by pre-

venting inducible phosphorylation of IκB-α.[22,23] Therefore, to more directly address the cause and effect linkage between sublytic MAC assembly and NF-κB activation/translocation, we employed SN50, which specifically inhibits entry of NF-κB into cell nuclei[24]. The failure of heat-inactivated MAC proteins, in aggregate, and MAC devoid of C7 or C8, in conjunction with the lack of effect of polymyxin B on MAC-induced IL-8 and MCP-1 production, argue strongly against LPS contamination of MAC components as an explanation for our conclusions.

In addition to defined interactions with second messenger systems, it has also been reported that the MAC can elicit the production of oxygen metabolites including O_2^- and H_2O_2.[36] Numerous studies indicate that reactive oxygen intermediates influence intracellular redox status, which in turn modulates cell activation and proinflammatory cytokine gene expression.[38,40] Studies that have addressed lymphocyte proliferation and activation of latent viral genomes (eg, HIV-1-infected T lymphocytes) suggest that intracellular redox potential is important in the modulation of cell activation.[31,32] Changes in cytosolic redox potential have been paralleled by changes in NF-κB binding and correlate inversely with intracellular thiol content. Increased intracellular thiol content is thought to reflect increased intracellular glutathione concentrations. Studies by Israel et al[41] and Staal et al[42] suggest that cell activators such as TNF-α and phorbol esters can efficiently exert their effects only when target cells are in appropriate redox status. This is converse to the interpretation that TNF-α, phorbol ester, and other mediators lead to NF-κB activation through changes in cellular redox status *per se*. In the case of some inducers of cell activation (phorbol ester), NF-κB appears to be activated via a PKC pathway, whereas in the cases of other activators (eg, TNF-α and IL-1β), PKC is not involved.[43] It appears from these studies that an intracellular redox equilibrium tending toward oxidation is required for full activation of transduction pathways regulating the activity of NF-κB-dependent genes. Studies by Schreck et al[33,44] indicate that H_2O_2, and other reactive oxygen intermediates that are produced in the microenvironment of an inflammatory process, can induce the expression and replication of HIV-1 in human T-lymphocyte cell lines. Again, this effect is mediated by the NF-κB transcription factor and can be abrogated by either noncytotoxic antioxidants such as *N*-acetyl cysteine or compounds that block NF-κB activation in intact cells (eg, dithiocarbamates and metal chelators such as desferrioxime).[33,45]

The premise that cellular redox status and NF-κB are potentially important in inflammation is further supported by recent *in vitro* studies that indicate that the expression of some inflammatory cytokines is regulated by intracellular redox status and NF-κB.[46–48] Alterations in ambient oxygen tension affect IL-1 production in mononuclear cells[46] and IL-8 expression in endothelial cells.[48] TNF-α production in phorbol-ester-stimulated U937 cells can be partially blocked by the antioxidant butylated hydroxyanisole.[47] The ability of the MAC to promote the generation of reactive oxygen intermediates supports the premise that the MAC may be an important mediator of proinflammatory gene expression via NF-κB activation. Regulation of NF-κB by the MAC affords a potential mechanism by which complement-derived products potentiate the inflammatory response. For example, we have recently observed that MAC deposition promotes the up-regulation and secretion of IL-8 and MCP-1.[8] Alterations in ambient oxygen tension affect IL-8 expression in endothelial cells, fibroblasts, pulmonary epithelial cells, and hepatocytes after direct exposure of the cells to H_2O_2.[48] Furthermore, *in vitro* studies employing isolated human mesangial cells have shown that either exogenous or endogenous (intracellular) oxidants can induce MCP-1 expression.[47] The present study offers a potential mechanism by which the up-regulation of these chemokines may be influenced by the MAC.

The ability of the MAC to promote NF-κB translocation represents a potential mechanism by which the MAC participates in the induction and maintenance of the inflammatory response. In that NF-κB is involved in the regulation and expression of adhesion molecules and cytokines, it is likely that this regulatory mechanism is intimately involved in mediating the secretion of these proinflammatory mediators after MAC deposition. Consequently, interactions between the MAC and multiple signal transduction pathways and regulators of gene expression may play an important role in amplification and maintenance of the inflammatory response.

References

1. Carlos TM, Harlan JM: Leukocyte-endothelial adhesion molecules. Blood 1994, 84:2068–2085
2. Hugli RE: The chemistry and biology of C3a, C4a and C5a and their effects on cell. Biological Response Mediators and Modulators. Edited by JT August. New York, Academic Press, 1983, pp 99–116
3. Nicholson-Weller A, Halperin JA: Membrane signaling by complement C5b-9, the membrane attack complex. Immunol Res 1993, 12:244–257

4. Asghar SS: Biology of disease: membrane regulators of complement activation and their aberrant expression in disease. Lab Invest 1995, 72:254–271
5. Schonermark M, Deppisch R, Riedasch G, Rother GK, Hansch GM: Induction of mediator release from human glomerular mesangial cells by the terminal complement components C5b-9. Int Arch Allergy Appl Immunol 1991, 96:331–337
6. Lovett D, Hansch GM, Resch K, Gemsa D: Activation of glomerular mesangial cells by the terminal membrane attack complex of complement. J Immunol 1987, 138: 2473–2479
7. Benzaquen LR, Nicholson-Weller A, Halperin JA: Terminal complement proteins C5b-9 release basic fibroblast growth factor and platelet-derived growth factor from endothelial cells. J Exp Med 1994, 179:985–992
8. Kilgore KS, Flory CM, Miller BF, Evans VM, Warren JS: The membrane attack complex of complement induces interleukin-8 and monocyte chemoattractant protein-1 secretion from human umbilical vein endothelial cells. Am J Pathol 1996, 149:953–961
9. Torzewski J, Oldroyd R, Lachmann P, Fitzsimmons C, Proudfoot D, Bowyer D: Complement-induced release of monocyte chemotactic protein-1 from human smooth muscle cells. Arterioscler Thromb Vasc Biol 1996, 16: 673–677
10. Niculescu F, Rus H, Shin ML: Receptor-independent activation of guanine nucleotide-binding regulatory proteins by terminal complement complexes. J Biol Chem 1994, 269:4417–4423
11. Carney DF, Lang TJ, Shin ML: Multiple signal messengers generated by terminal complement complexes and their role in terminal complement complex elimination. J Immunol 1990, 45:623–629
12. Wiedmer T, Ando B, Sims PJ. Complement C5b-9-stimulated platelet secretion is associated with a Ca^{2+}-initiated activation of cellular protein kinases. J Biol Chem 1987, 262:13674–13681
13. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T: Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. FASEB J 1995, 9:899–909
14. Collins T: Biology of disease: endothelial nuclear factor-κB and the initiation of the atherosclerotic lesion. Lab Invest 1993, 68:499–508
15. Hou J, Baichwal V, Cao Z: Regulatory elements and transcription factors controlling basal and cytokine-induced expression of the gene encoding ICAM-1. Proc Natl Acad Sci USA 1994, 91:1641–11645
16. Jahnke A, Johnson JP: Synergistic activation of intercellular adhesion molecule-1 (ICAM-1) by TNF-α and IFN-γ is mediatied by p65/p50 and p65/c-Rel and interferon-responsive factor Stat 1-α (p91) that can be activated by both IFN-γ and INF-α. FEBS Lett 1994, 354:220–226
17. Parry GC, Mackman N: A set of inducible genes expressed by activated human monocytic and endothelial cells contain κB-like sites that specifically bind c-Rel-p65 heterodimers. J Biol Chem 1994, 269: 20823–20825
18. Pober JS, Cotran RC: Cytokines and endothelial cell biology. Physiol Rev 1990, 70:427–451
19. Baeuerle PA: The inducible transcription activator NF-κB: regulation by distinct protein subunits. Biochim Biophys Acta 1991, 107:63–80
20. Lenard MJ, Baltimore D. NF-κB: a pleiotropic mediator of inducible and tissue-specific gene control. Cell 1988, 7:318–332
21. Zabel U, Baeuerle PA: Purified human IκB can rapidly dissociate the complex of the NF-κB transcription factor with its cognate DNA. Cell 1990, 61:255–265
22. Finco TS, Beg AA, Baldwin AS: Inducible phosphorylation of IκBα is not sufficient for its dissociation from NF-κB and is inhibited by protease inhibitors. Proc Natl Acad Sci USA 1994, 91:11884–11888
23. Miyamoto S, Maki M, Schmitt MJ, Hatanaka M, Verma IM: Tumor necrosis factor α-induced phosphorylation of IκBα is a signal for its degradation but not dissociation from NF-κB. Proc Natl Acad Sci USA 1994, 91: 12740–12744
24. Lin Y-Z, Yao SY, Veach RA, Torgerson TR, Hawiger J: Inhibition of nuclear translocation of transcription factor NF-κB by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. J Biol Chem 1995, 270:14255–14258
25. Jaffe EA: Culture and identification of large vessel endothelial cells. Biology of Endothelial Cells. Edited by EA Jaffe. The Hague, Martinus Nijoff, p 13
26. Vogt W, Hesse D: Activation of the fifth component of human complement by oxygen-derived free radicals, and by methionine oxidizing agents: a comparison. Immunobiology 1992, 184:384–391
27. Vogt W, Zimmerman B, Hesse D, Nolte R: Activation of the fifth component of human complement, C5, without cleavage, by methionine oxidizing agents. Mol Immunol 1992, 29:251–256
28. Kilgore KS, Shen JP, Miller BF, Ward PA, Warren JS: Enhancement by the complement membrane attack complex of tumor necrosis factor-α induced endothelial cell expression of E-selectin and ICAM-1. J Immunol 1995, 155:1434–1441
29. Kravchenko VV, Pan, Z, Han J, Herbert JM, Ulevitch RJ, Ye RD: Platelet-activating factor induces NF-κB activation through a G protein-coupled pathway. J Biol Chem 1995, 270:14928–14934
30. Menon SD, Qin S, Guy GR, Tan YH: Differential induction of nuclear NF-κB by protein phosphatase inhibitors in primary and transformed human cells. J Biol Chem 1993, 268:26806–26812
31. Molitor JA, Walker WH, Doerre S, Ballard DW, Greene WC. NF-κB: a family of inducible and differentially expressed enhancer-binding proteins in human T cells. Proc Natl Acad Sci USA 1990, 87:10028–10032
32. Osborn K, Kunkel S, Nabel GJ: Tumor necrosis factor and interleukin 1 stimulate the human immunodefi-

ciency virus enhancer by activation of nuclear κB. Proc Natl Acad Sci USA 1989, 86:2336–2340
33. Schreck R, Meier B, Mannel DN, Drogew W, Baeuerle PA: Dithiocarbamates as potent inhibitors of nuclear factor κB activation in intact cells. J Exp Med 1992 175:1181–1194
34. Hattori R, Hamilton KK, Fugate RD, McEver RP, Sims PJ: Stimulated secretion of endothelial von Willebrand factor is accompanied by rapid redistribution to the cell surface of the intracellular granule membrane protein GMP-140. J Biol Chem 1989, 264:7768–7771
35. Hattori R, Hamilton, KK, McEver RP, Sims PJ: Complement proteins C5b-9 induce secretion of high molecular weight multimers of endothelial von Willebrand factor and translocation of granule membrane protein GMP-140 to the cell surface. J Biol Chem 1989, 264: 9053–9060
36. Adler S, Baker PJ, Johnson RJ, Ochi F, Pritzi P, Couser WG: Complement membrane attack complex stimulates production of reactive oxygen metabolites by cultured rat mesangial cells. J Clin Invest 1986, 77:762–767
37. Baeuerle PA, Baltimore D: Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-κB transcription factor. Cell 1988, 53:211–217
38. Hirano M, Hirai S, Mizuno K, Osada S, Hosaka M, Ohno S: A protein kinase C isozyme nPKCε is involved in the activation of NF-κB by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in rat 3Y1 fibroblasts. J Biol Chem 1995, 206:429–436
39. Acosta JA, Benzaquen LR, Goldstein DJ, Tosteson MT, Halperin JA: The transient pore formed by homologous terminal complement complexes functions as a bidirectional route for the transport of autocrine and paracrine signals across human cell membranes. Mol Med 1996, 2:755–765
40. Read MA, Whitley MZ, Williams AJ, Collins T: NF-κB and IκBα: an inducible regulatory system in endothelial activation. J Exp Med 1994, 179:503–512
41. Isral N, Gougerot-Pocidalo MA, Aillet F, Virelizier JL: Redox status of cells influences constitutive or induced NF-κB translocation and HIV long terminal repeat activity in human T and monocytic cell lines. J Immunol 1992, 149:3386–3393
42. Staal JT, Roederer M, Herzenberg LA, Herzenberg LA: Intracellular thiols regulate activation of nuclear factor κB and transcription of human immunodeficiency virus. Proc Natl Acad Sci USA 1990, 87:9943–9947
43. Meichle A, Schutze S, Hensel G, Brunsing D, Kronke M: Protein kinase C-independent activation of nuclear transcription factor κB by tumor necrosis factor. J Biol Chem 1990, 265:8339–8343
44. Schreck R, Rieber P, Baeuerle PA: Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB and transcription factor and HIV-1. EMBO J 1991, 10:2247–2258
45. Ghezzi P, Dinarello CA, Bianchi M, Rosandich ME, Repine JE, White CW: Hypoxia increases production of interleukin-1 and tumor necrosis factor by human mononuclear cells. Cytokine 1991, 3:189–194
46. Metinko AP, Kunkel SL, Standiford TJ, Strieter RM: Anoxia-hyperoxia induces monocyte-derived interleukin-8. J Clin Invest 1992, 90:791–798
47. Satriano JA, Shuldiner, Hora MK, Xing Y, Shan Z, Schlondorff D: Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein JE/MCP-1 and the monocyte colony-stimulating factor CSF-1 in response to tumor necrosis factor-α and immunoglobulin G. J Clin Invest 1993, 92:1564–1571
48. DeForge LE, Preston AM, Takeuchi E, Kenney J, Boxer LA, Remick DG: Regulation of interleukin 8 gene expression by oxidant stress. J Biol Chem 1993, 268: 25568–25576