The Journal of Immunology

RESEARCH ARTICLE | JANUARY 15 2001

Flagellin, a Novel Mediator of *Salmonella*-Induced Epithelial Activation and Systemic Inflammation: IκBα Degradation, Induction of Nitric Oxide Synthase, Induction of Proinflammatory Mediators, and Cardiovascular Dysfunction [1]

Tonyia Eaves-Pyles; ... et. al

J Immunol (2001) 166 (2): 1248–1260.
https://doi.org/10.4049/jimmunol.166.2.1248

Flagellin, a Novel Mediator of *Salmonella*-Induced Epithelial Activation and Systemic Inflammation: IκBα Degradation, Induction of Nitric Oxide Synthase, Induction of Proinflammatory Mediators, and Cardiovascular Dysfunction[1]

Tonyia Eaves-Pyles,[†] Kanneganti Murthy,[*] Lucas Liaudet,[2§¶] László Virág,[*] Gary Ross,[‡] Francisco Garcia Soriano,[3¶] Csaba Szabó,[*¶] and Andrew L. Salzman[4*†§]

Gram-negative sepsis is mediated by the actions of proinflammatory genes induced in response to microbes and their products. We report that flagellin, the monomeric subunit of flagella, is a potent proinflammatory species released by *Salmonella.* Flagellin (1 μg/ml) induces IκBα degradation, NF-κB nuclear translocation, and inducible NO synthase expression in cultured intestinal epithelial cells (IEC). Aflagellic *Salmonella* mutants do not induce NF-κB activation or NO production by cultured IEC. Antiserum to flagellin blocks NO production in IEC induced by medium conditioned by a variety of motile Gram-negative enteric pathogens (*Escherichia coli, Salmonella muenchen, Serratia marcescens, Proteus mirabilis,* and *Proteus vulgaris*). Flagellin, when injected systemically (~10 μg/mouse), induces systemic inflammation characterized by the systemic expression of a range of proinflammatory cytokines and chemokines and of inducible NO synthase. At higher doses (~300 μg/mouse), flagellin induces shock, characterized by hypotension, reduced vascular contractility in mice, and death. The effects of flagellin do not diminish in C3H/HeJ LPS-resistant mice, indicating that the Toll-like receptor-4 receptor is not involved in flagellin's actions. In LPS-resistant mice, i.p. injection of *S. dublin* flagellin or medium conditioned by wild-type *S. dublin* induces serum IFN-γ and TNF-α, whereas medium conditioned by aflagellic mutants has no effect. Flagellin can be detected in the blood of rats with septic shock induced by live bacteria at approximately 1 μg/ml. We propose that flagellin released by Gram-negative pathogens may contribute to the inflammatory response by an LPS- and Toll-like receptor-4-independent pathway. ***The Journal of Immunology,*** **2001, 166: 1248–1260.**

Salmonellae are Gram-negative, pathogenic micro-organisms that gain entrance to the human host by penetrating enterocytes in the intestinal mucosa. Enteroinvasion elicits a complex genetic response in the gut mucosa, inducing the expression of free radicals, cytokines, and chemokines associated with inflammation, tissue injury, and shock (1–5). Translocation of *Salmonellae* into the mesenteric lymphatics and the systemic circulation further activates proinflammatory gene expression in mononuclear cells, resulting in constitutional symptoms and hemodynamic alterations of septic shock (6). These effects are mediated by a broad range of cytotoxins, among them NO, a reactive free radical synthesized from L-arginine by inducible NO synthase (iNOS)[5] (7). At nanomolar concentrations, NO functions as an endogenous antimicrobial factor, inhibiting oxygen consumption by disrupting the catalytic activity of the iron center in aconitase (8). Bacterial resistance to NO is dependent upon the inducible up-regulation of NO dioxygenase, a microbial flavohemoglobin that oxidizes NO via a peroxynitrite-like intermediate (9, 10).

De novo expression of iNOS in gut epithelial cells occurs in response to direct microbial interaction (1) and indirectly through the actions of proinflammatory cytokines induced by microbes and their products (11). Despite marked species variation in the organization of proinflammatory *cis* response elements of the iNOS promoter, the transcription of both murine and human iNOS genes is dependent upon the nuclear translocation of the cytosol-sequestered heterodimer p50/p65 (NF-κB) of the Rel family (11–13). Intestinal epithelial cells (IEC) stimulated by Gram-negative bacteria readily induce degradation of the cytosolic inhibitor IκBα, resulting in the nuclear translocation and activation of NF-κB (14) and the transcriptional up-regulation of nuclear iNOS mRNA (15). We now report that flagellin, a monomeric constituent of bacterial flagella, represents a crucial proinflammatory activity released by *Salmonella dublin*, a monophasic serotype making flagella (and so flagellin) of antigenic type *g,p*.

Materials and Methods

In vitro experiments

Bacterial culture. *Salmonella dublin* (SD)*, Salmonella typhimurium, Salmonella muenchen, Proteus vulgaris, Proteus mirabilis,* and *Serratia marcescens* (American Type Culture Collection, Manassas, VA), *Pseudomonas*

*Inotek Corporation, Beverly, MA 01915; [†]Critical Care Medicine, [‡]Pulmonary Biology, and [§]Pulmonary Medicine, Allergy, and Clinical Immunology Division, Children's Hospital Medical Center, Cincinnati, OH 45229; and [¶]Department of Surgery, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, NJ 01703

Received for publication April 20, 2000. Accepted for publication October 11, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by National Institutes of Health Grant R01GM57407 and a grant from the American Heart Association (to A.L.S.), National Institutes of Health Grant R29GM54773 (to C.S.), a fellowship (to F.G.S.) from Fundaçao de Amparo à Pesquisa do Estado de Sao Paulo (Brazil). Part of this work represents a Ph.D. thesis by T.E.-P. in fulfillment of her graduate degree at the Department of Cell Biology, Neuroscience, and Anatomy, University of Cincinnati.

[2] On leave from the Critical Care Division, Department of Internal Medicine, University Hospital (Lausanne, Switzerland).

[3] On leave from Department of Critical Care Medicine, Hospital das Clinicas da Universidade de Sao Paulo (Sao Paulo, Brazil).

[4] Address correspondence and reprint requests to Dr. Andrew L. Salzman, Inotek Corporation, Suite 419E, 100 Cummings Center, Beverly, MA 01915. E-mail address: alsalzman@aol.com

[5] Abbreviations used in this paper: iNOS, inducible NO synthase; IEC, intestinal epithelial cell; SD, *Salmonella dublin*; BHI, brain heart infusion broth; STE buffer, 10 mM Tris (pH 8), 50 mM NaCl, and 1 mM EDTA; MIP-1α, macrophage inflammatory protein-1α; CM, medium conditioned by SD; TLR, Toll-like receptor; NFDM, nonfat dry milk.

0022-1767/01/$02.00

aeruginosa, and *Escherichia coli* (clinical isolates provided by Ian Holder, Ph.D., of the Shriners Burns Institute, Cincinnati, OH) were inoculated into 25 ml of brain heart infusion broth (BHI; Difco, Detroit, MI) and incubated overnight at 37°C in a shaking incubator (New Brunswick Scientific, Edison, NJ) to a stationary phase (10^{12} CFU/ml). The cultures were centrifuged at 10,000 rpm for 10 min, and the broth was sterile-filtered through a 0.45-μm pore size filter.

Cell culture and infection. DLD-1 and Caco-2BBe cells (American Type Culture Collection) between passages 5 and 15 were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 1% nonessential amino acids, and antibiotics. RAW cells (American Type Culture Collection) between passages 5 and 15 were cultured in DMEM supplemented with 10% FBS, 4 mM glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/L glucose, 1.0 mM sodium pyruvate, and antibiotics. Cells were seeded onto 6- and 96-well plates and 10-cm tissue culture plates and cultured to confluence. The growth medium was replaced by DMEM without FBS or antibiotics immediately before studies.

NO_2^-/NO_3^- determination. The combined concentration of nitrite and nitrate, the degradation products of NO in the culture medium, was determined by the Griess reaction following nitrate reduction as previously described (11). Total nitrite/nitrate production is referred to in the text as NO production.

Western blotting. Cells grown in six-well culture plates were washed once in PBS and lysed in cold buffer containing 50 mM Tris (pH 8.0), 110 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 0.1 mM PMSF. The Bradford assay (Bio-Rad, Hercules, CA) was used to determine protein concentrations of each sample. Cell lysates were boiled in an equal volume of loading buffer (4% SDS, 20% glycerol, 125 mM Tris-HCl (pH 6.8), and 10% 2-ME), and 50 μg of each protein sample was loaded per lane on an 8–16% Tris-glycine gradient gel (NOVEX, San Diego, CA). Electrophoresed proteins were transferred to a nitrocellulose membrane (NOVEX) using the NOVEX Xcell MiniGel system. Membranes were blocked with 10% nonfat dried milk in TBS for 1 h before exposure to rabbit polyclonal anti-IκBα antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-iNOS antiserum (gift from Robert Webber, Research Diagnostic Antibodies, San Francisco, CA) at a dilution of 1/200 for 45 min. Blots were washed twice in TBS containing 0.1% Tween-20, followed by the addition of peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO) at a dilution of 1/10,000 for 30 min. Blots were washed three times for 5 min per wash in TBS containing 0.1% Tween-20, incubated in commercial ECL reagents (Amersham, Aylesbury, U.K.), and exposed to photographic film.

Nuclear protein extraction. Nuclear protein extracts were prepared after cells were incubated with medium conditioned by SD (CM). Cells were washed twice with ice-cold PBS and harvested by scraping into 1 ml of PBS. After pelleting at 6,000 rpm for 5 min, cells were washed twice with cold PBS, resuspended in one cell pellet volume of lysis buffer (1.5 mM $MgCl_2$, 0.2% (v/v) Nonidet P-40, 10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF), and incubated on ice for 5 min with intermittent vortexing. Nuclei were collected from the cell lysates by centrifugation (6,000 rpm for 5 min), then resuspended in one cell pellet volume of extraction buffer (1.5 mM $MgCl_2$, 25% (v/v) glycerol, 20 mM HEPES (pH 7.9), 420 mM NaCl, 0.1 M EDTA, 1 mM DTT, and 0.5 mM PMSF). After incubation on ice for 15 min with intermittent vortexing, nuclear proteins were collected by centrifugation (14,000 rpm for 15 min). Supernatants also were collected to ensure the elimination of nuclear debris.

EMSA. An NF-κB oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG-3′; Santa Cruz Biotechnology) was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Life Technologies, Gaithersburg, MD) and then purified on a Bio-Spin chromatography column (Bio-Rad, Hercules, CA). Ten micrograms of nuclear protein extracts were preincubated with EMSA buffer (1 mM EDTA, 1 mM DTT, 12 mM HEPES (pH 7.9), 4 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 25 mM KCl, 12% glycerol, 50 ng/ml poly[d(I-C)], and 0.2 mM PMSF) on ice for 10 min, followed by the addition of the radiolabeled probe for 20 min. The specificity of the binding reaction was determined by incubating duplicate nuclear protein samples with a 100-fold molar excess of unlabeled probe. Samples were resolved on a nondenaturing polyacrylamide gel containing 5% acrylamide and run in 0.5× TBE (1 mM EDTA, 45 mM boric acid, and 45 mM Tris-HCl) at a constant current (30 mA) for 1 h. Gels were transferred to Whatman 3M paper (Clifton, NJ) and dried under a vacuum at 80°C for 1 h, followed by film exposure to using an intensifying screen at −70°C for approximately 3 h.

Purification and preparation of flagellins. Flagellin was purified from SD and a congenic flagellin-deficient mutant according to well-established methods (16). Bacterial isolates were used to incubate 1-l Erlenmeyer flasks containing BHI infusion broth. Flasks were incubated at 35°C in an orbital shaker incubator at 80 rpm for 16 h. Bacterial cells were harvested by centrifugation at 5,000 × *g* for 30 min and then mixed with saline solution to form a moderately thick suspension. The suspension was adjusted to pH 2.0 with 1 M HCl and maintained at that pH under constant stirring for 30 min at room temperature. The bacterial cells, devoid of flagella, were separated by centrifugation at 5,000 × *g* for 30 min. The supernatant, which contained detached flagellin in monomeric form, was further centrifuged at 100,000 × *g* for 1 h at 4°C to sediment the pH 2.0-insoluble material. The pH of the supernatant was adjusted to 7.2 with 1 M NaOH. Ammonium sulfate was added slowly with vigorous stirring to achieve two-thirds saturation (2.67 M). The mixture was centrifuged at 15,000 × *g* for 15 min at 4°C. The precipitate was dissolved in approximately 5 ml of distilled water and then transferred to dialysis tubing (m.w. cutoff, 50,000). Dialysis was conducted for 18 h at 4°C with constant stirring in 4 l of distilled water. The dialyzed flagellin preparations were then lyophilized and stored at −70°C.

Recombinant flagellins of *S. muenchen*, *E. coli*, *P. vulgaris*, *P. mirabilis*, and *S. marcescens* were prepared as follows. The gene fragment corresponding to aa 1–156 of the flagellin gene of *S. muenchen* was generated by PCR amplification with a sense primer designated 1S (5′-CGCGGATCCCAATGGCACAAGTCATTAATACAAACA) and an antisense primer designated 468A (5′-TCCGCTCGAGTTAAATAGTTTCACCGTCGTTGGCACC). The entire flagellin gene was amplified with primers 1S and 1530A (5′-TCCGCTCGAGTTAACGCAGTAAAGAGAGGACGTTTTG). Underlined nucleotides represent adaptor sequences added to the ends of primers to maintain proper reading frame and facilitate cloning (*Bam*HI recognition sites on sense primers and *Xho*I sites on antisense primers). Template DNA for PCR reactions was plasmid CL402, a clone of pBR322 containing a 3.8-kb *Eco*RI fragment of *S. muenchen* chromosomal DNA

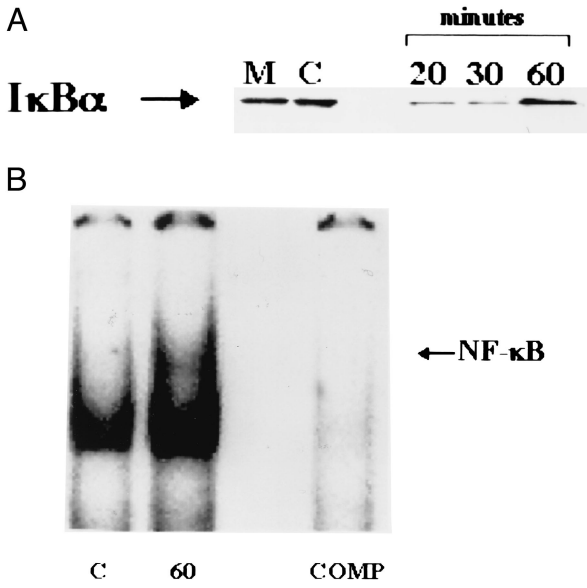

FIGURE 1. Salmonella-conditioned medium induces IκBα degradation and NF-κB activation in Caco-2BBe cells. *A*, IκBα degradation was induced in a time-dependent manner in Caco-2BBe cells stimulated with CM. Confluent Caco-2BBe cells were stimulated by CM for 20, 30, or 60 min. Total protein was extracted, and equal amounts of protein were subjected to SDS-PAGE gel electrophoresis, followed by immunoblotting for IκBα as previously described (14). Blots were probed with a lapine polyclonal anti-IκBα antiserum (Santa Cruz Biotechnology). Unconditioned medium (M) and unstimulated control cells (C) showed no degradation of IκBα at 30 min. Cells stimulated with CM showed a loss of immunoreactive IκBα at 20 and 30 min. At 60 min poststimulation, IκBα is resynthesized by the activation of NF-κB released through the degradation of IκBα protein. *B*, Representative EMSA demonstrating NF-κB activation in Caco-2BBe cells stimulated for 60 min with CM. Nuclear extracts from unstimulated cells showed no NF-κB activation (C). NF-κB activation was demonstrated in cells stimulated with flagellin for 60 min. To confirm the specificity of the NF-κB signal, a 100-fold excess of cold oligonucleotide was used as a competitor to inhibit the binding activity of NF-κB (COMP). Gels representative of three to five independent determinations are shown.

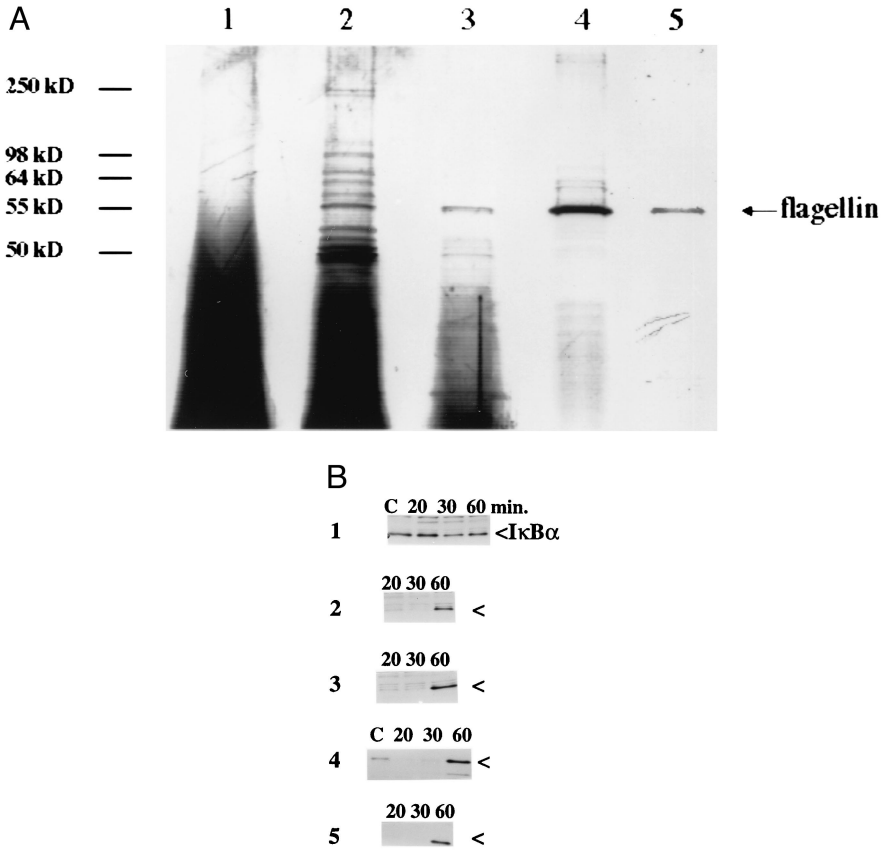

FIGURE 2. Purification of proinflammatory activity of *Salmonella*-conditioned media. *A*, SDS-PAGE gel demonstrating silver-stained SD-released protein(s) purified by standard protein purification methods. Equal amounts of total protein were loaded in each lane. *B*, IκBα degradation in Caco-2BBe cells stimulated with SD-released protein(s). Confluent Caco-2BBe cells were exposed for 20, 30, or 60 min to 1/200 dilution of purified fractions of CM. Unstimulated cells served as controls (C). Cells were washed, and total eukaryotic protein was extracted and equal amounts of protein were subjected to SDS-PAGE gel electrophoresis, followed by immunoblotting of IκBα as described in Fig. 1*A*. *Lane 1*, Sterile BHI. *Lane 2*, BHI medium inoculated with SD and grown for 18 h. Conditioned medium was filtered to eliminate intact bacteria and medium containing secreted proteins from SD was collected. *Lane 3*, Conditioned medium was run over an anion exchange column (fast protein liquid chromatography), and fractions that induced NO production in IFN-γ (100 U/ml)-primed DLD-1 cells were pooled and electrophoresed. *Lane 4*, Conditioned medium fractionated by fast protein liquid chromatography, followed by gel filtration. Fractions that induced NO production were pooled and electrophoresed. *Lane 5*, Purified SD flagellin as previously described (16). Gels representative of three to five independent determinations are shown.

that harbors the 1.5-kb flagellin gene. PCR-generated flagellin DNA was digested with *Bam*HI plus *Xho*I, gel purified, and subcloned into the *Bam*HI/*Xho*I sites at the 3′ end of the GST gene in expression vector pGEX-5X-2 (Pharmacia Biotech, Piscataway, NJ) and pET 30C (Novagen, San Diego, CA). Similarly DNA sequences corresponding to flagellin coding region of *E. coli*, *P. vulgaris*, *P. mirabilis*, and *S. marcescens* were amplified by PCR and cloned into pRSET C vector (Invitrogen, San Diego, CA). The correct reading frame and integrity of subcloned DNA were verified by DNA sequence analysis.

A single colony of *E. coli* DH5α (Life Technologies) containing the desired plasmid was grown at 37°C in Luria broth containing 100 μg of ampicillin/ml to an A600 of 0.5 and then induced for 3 h with 0.5 mM isopropyl-β-D-thiogalactoside. Following induction, bacteria were harvested and washed with STE buffer (10 mM Tris (pH 8), 50 mM NaCl, and 1 mM EDTA). Cells were suspended in STE buffer containing 100 μg/ml lysozyme, incubated 45 min on ice, and then adjusted to 5 mM DTT. After two freeze-thaw cycles, the cell suspension was sonicated and clarified by centrifugation at 10,000 $\times$ *g*. The soluble lysate was applied to a glutathione-agarose column, washed with STE plus 5 mM DTT, and then eluted with STE plus 5 mM DTT buffer containing 10 mM reduced glutathione. The purity of each eluted fusion protein was analyzed by SDS-PAGE. Purified proteins were dialyzed against PBS, quantified by the Bradford method, and stored at −70°C.

Antiserum. Polyclonal lapine antiserum directed against a recombinant *S. muenchen* flagellin was prepared by Protein Express (Cincinnati, OH) as follows. Serum was collected from New Zealand rabbits immunized with a GST fusion of the N-terminus of recombinant *S. muenchen* flagellin, corresponding to aa 1–156. The Ag was prepared by expression of a cDNA clone obtained by PCR amplification of DNA from *S. muenchen* using a sense primer designated 1S (5′-CGCGGATCCCAATGGCACAAGT CATTAATACAAACA) and an antisense primer designated 468A (5′-TC CGCTCGAGTTAAATAGTTTCACCGTCGTTGGCACC). Underlined nucleotides represent adaptor sequences added to the ends of primers to maintain proper reading frame and facilitate cloning (*Bam*HI recognition sites on sense primers and *Xho*I sites on antisense primers). The template DNA for PCR was plasmid CL402, a clone of pBR322 containing a 3.8-kb *Eco*RI fragment of *S. muenchen* chromosomal DNA that harbors the 1.5-kb flagellin gene. PCR-generated flagellin DNA were digested with *Bam*HI plus *Xho*I, gel purified, and subcloned into the *Bam*HI/*Xho*I sites at the 3′ end of the GST gene in expression vector pGEX-5X-2 (Pharmacia Biotech). The correct reading frame and integrity of subcloned DNA was verified by DNA sequence analysis.

In vivo and ex vivo experiments

Effect of flagellin and conditioned medium on inflammatory mediator and iNOS expression in vivo. To test whether flagellin is capable of inducing the systemic inflammatory alterations characteristic of Gram-negative sepsis, C57BL/6 mice were challenged with 10 μg (~400 μg/kg i.p.) of purified SD flagellin. Mice were sacrificed at various time points (2, 4, 8, and 24 h postflagellin), and serum concentrations of TNF-α, IL-6, IL-12p40, macrophage inflammatory protein-1α (MIP-1α), and IL-10 were measured with ELISA (see below), and nitrite/nitrate levels were measured with the modified Griess reaction (see above). In addition, at 24 h ileal samples were obtained and processed for iNOS immunohistochemistry. In an additional set of experiments, LPS-resistant C3H/HeJ mice were treated with vehicle, 10 μg/mouse purified flagellin, SD-conditioned medium (0.3 ml), or conditioned medium from aflagellic SD mutants (0.3 ml). Mice were sacrificed at 3 h, and serum TNF-α and IFN-γ levels were measured. The presence of serum cytokines was detected by enzyme immunoassay. Enzyme immunoassay plates (Corning Costar, Cambridge, MA) were coated with 100 μl of 5 μg/ml goat anti-murine cytokine neutralizing Abs (R&D Systems, Minneapolis, MN) diluted in 0.1 M carbonate buffer (pH 9.6) and incubated at 4°C for 16 h. The plates were washed three times with washing buffer (1× PBS (pH 7.4) and 0.5% Tween-20). After washing, plates were incubated with blocking buffer (1× PBS (pH 7.4), 0.5% Tween-20, and 2% BSA) at 37°C for 2 h. To prepare a standard curve, serial dilutions of standard purified recombinant cytokines (R&D Systems) were prepared in blocking buffer and added to the plates. Samples (100 μl) or standards were added to the plates followed by incubation for 2 h at 37°C, washing three times, and blocking for 15 min at room temperature. Plates were incubated with 100 μl of 10 μg/ml rabbit anti-murine cytokine Ab at 37°C for 2 h. After incubation, plates were washed three times with washing buffer and incubated with 100 μl of 2 μg/ml goat anti-rabbit IgG (whole molecule) HRP-conjugated Ab (Calbiochem, San Diego, CA) at

37°C for 2 h. Plates were washed three times with washing buffer and then incubated with 150 μl of K Blue Substrate (Neogen, Lexington, KY), and the color was developed for 20 min at room temperature. Following color development, the plates were read using an automated microplate reader (Molecular Devices, Menlo Park, CA) at a wavelength of 650 nm. For iNOS immunohistochemistry, ileum was fixed and processed for immunohistochemistry as previously described (17). Tissue samples were fixed for 3 h in 4% paraformaldehyde and cryoprotected by a 1-h incubation in 10, 20, and 30% sucrose in 0.1 M sodium cacodylate buffer. The tissues were then placed on tissue holders covered with a thin layer of OCT (a water soluble glycol & resin compound; Tissue-Tek) and frozen in distilled freon 22 in liquid nitrogen. Eight-micron thin sections were placed onto slides and stained with the anti-nitrotyrosine Ab. The blocking solution consisted of 4% fatty acid-free BSA, 10% goat serum, and 3% Triton X-100 in 0.1 M PBS, pH 7.2. The iNOS immunoreactivity was detected using iNOS mAb (provided by Robert Webber, Research Diagnostic Antibodies). Samples were incubated with Ab at 2 μg/ml for 3 h. The Ab binding on tissue sections was visualized by a 1-h incubation with an anti-murine IgG conjugated to Texas Red (1/100 dilution in PBS). Slides were examined under a Nikon Diaphot-TND epifluorescence inverted microscope (Melville, NY).

Hemodynamic studies. Male C3H/OuJ mice and C3H/HeJ (25 g) were anesthetized i.p. with ketamine (50 mg/kg) and xylazine (5 mg/kg). Maintenance doses of ketamine (10 mg/kg) and xylazine (1 mg/kg) were given when necessary (interdigital reflex). Under deep anesthesia, polyethylene catheters (PE 10) were inserted into the left jugular vein for the i.v. administration of compounds and into the right carotid artery. The carotid artery catheter was connected to a pressure transducer for blood pressure measurement. Pressure signals were recorded using a MacLab A/D converter and were continuously displayed on a Macintosh computer (Cupertino, CA). Following a 20-min stabilization period, baseline hemodynamic parameters were recorded. The animals were then randomized to the i.v. injection of recombinant *S. muenchen* flagellin (10 mg/kg), *E. coli* LPS (10 mg/kg), or vehicle (10 mM PBS), given as a slow bolus over 1 min (200 μl), and were then observed for the next 4 h. At this time point, mice were euthanized by exsanguination, immediately followed by thoracolaparotomy to allow excision of the aorta. The aorta was cleared from periadventitial fat and cut into 1- to 2-mm-wide rings, mounted in organ baths filled with warmed (37°C) and oxygenated (95% O_2 and 5% CO_2) Krebs' solution. Isometric forces were measured with isometric transducers (Kent Scientific, Litchfield, CT), digitized using a MacLab A/D converter, and stored and displayed on a Macintosh computer. A tension of 1 g was applied, and the rings were equilibrated for 60 min, followed by measurements of the concentration-response curves to phenylephrine (10^{-10} to 3×10^{-5} M) and acetylcholine (10^{-9} to 3×10^{-5} M).

Induction of bacterial sepsis in rats and quantification of serum levels of flagellin by enzyme immunoassay. *S. marcescens* was grown overnight in nutrient broth and then harvested by centrifugation. The cell pellet was washed twice with PBS and suspended in PBS at a concentration of 3.3×10^5 CFU/ml. Three milliliters of bacteria (1×10^9 CFU/ml) were injected into male 250-g Wistar rats (i.p.). Blood samples were collected at 0, 4, and 8 h. Serum was collected after centrifugation of blood at 14,000 rpm for 15 min. The centrifugation step was repeated three times to remove any contaminating bacteria. Plasma samples were tested negative for colony-forming viable bacteria after centrifugation. Serum samples were screened by ELISA using Immulon 2HB Microtiter plates (Dynex, Chantilly, VA). The plate wells were coated overnight at 4°C with 0.5 μg of murine anti-flagellin Abs in 50 mM sodium carbonate buffer (pH 9.3). After each step, unbound reagents in the wells were removed by repeated washing with PBS and 0.05% Tween 20. Wells were blocked with 5% nonfat dry milk (NFDM) and 1% BSA in PBS for 1 h. Following blocking, serum samples were diluted 1/1 with 1% BSA in PBS, and then 100 μl was added to each well. Bacterially expressed and purified recombinant *Serratia marcescens* flagellin protein serially diluted in 1% BSA was used as standard for quantitative measurements. Plates were incubated overnight at 4°C with gentle shaking. Rabbit anti-flagellin polyclonal Abs (1/2000) were added to the wells and incubated at room temperature with gentle shaking for 2 h. To detect Ag-Ab complexes, the wells were treated with 100 μl of peroxidase-conjugated goat anti-rabbit IgG used at a 1/2000 dilution in 1% NFDM/PBS. The peroxidase enzyme substrate 3,3′,5,5′-tetramethylbenzidine was used at a volume of 100 μl/well. After 5 min at room temperature the reaction was stopped by adding 25 μl of 0.1 M H_2SO_4, and the absorbance was measured at 450 nm.

Statistical Analysis. Numerical results are reported as the mean ± SEM of three or more independent experiments. Gels or immunohistochemical pictures shown are representative of results obtained in three or more independent experiments. For analysis of the numerical data, ANOVA followed by Tukey's test was used to compare mean values. Statistical differences were declared significant for $p < 0.05$.

Results

Flagellin influences inflammatory gene expression by Salmonella

Sterile-filtered CM grown overnight to a stationary phase (10^{12} CFU/ml) was added to cultured human IEC, Caco-2BBe, and DLD-1. CM (1/100 dilution) induced IκBα degradation (Fig. 1*A*) and the nuclear translocation of NF-κB (Fig. 1*B*) at 30 and 60 min poststimulation, respectively. Addition of CM (1/100–50,000 dilution) to DLD-1 cells primed for 2 h with IFN-γ (100 U/ml) induced high levels of NO production (64 μM nitrate/nitrite; EC_{50} = 1/1,600 dilution) over 16 h of incubation.

Hexane extraction of CM revealed that the NO-inducing and IκBα-degrading activities resided in the aqueous phase. Proteolysis of the CM abrogated the NO-inducing activity, suggesting the contribution of a protein species. Protein components of CM were concentrated by 55–60% ammonium sulfate precipitation and dialyzed against 10 mM NaOH at 4°C for 72 h using a m.w. cutoff of 10,000. The retained proteins (Fig. 2*A*, *lane 2*) were added to IFN-γ-primed DLD-1 cells, analyzed for NO production (EC_{50} = 3 μg/ml), then separated by anion exchange chromatography. Eluted fractions were added to IFN-γ-primed DLD-1 cells and analyzed for their ability to induce NO production. SDS-PAGE analysis of the peak NO-inducing fraction (EC_{50} = 850 ng/ml) revealed a partial purification (Fig. 2*A*, *lane 3*). Samples were further separated by Sephadex-75 gel filtration, and fractions were analyzed following exposure to IFN-γ-primed DLD-1 cells. NO-inducing activity in the CM was concentrated in a single peak (EC_{50} = 660 ng/ml). SDS-PAGE analysis disclosed a single 55-kDa band (Fig. 2*A*, *lane 4*). At each stage of the purification, the fractions of peak NO-inducing activity also retained the ability to stimulate IκBα degradation (Fig. 2*B*).

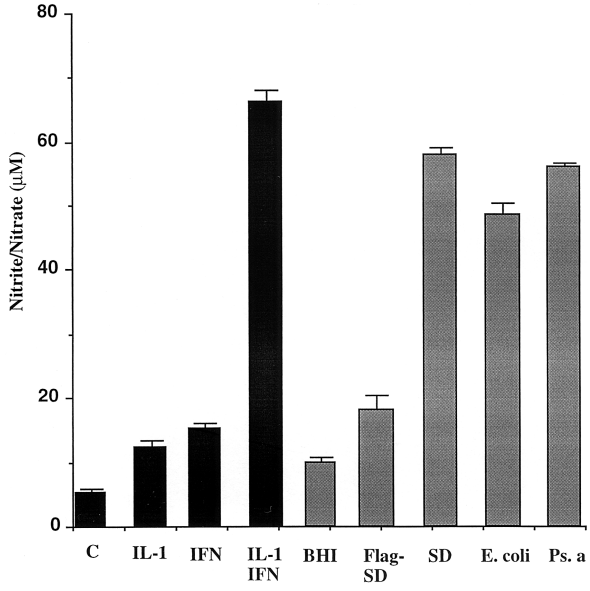

FIGURE 3. Bacteria-conditioned medium induces NO production in DLD-1 cells. Addition of CM (1/200 dilution) to DLD-1 cells primed for 2 h with IFN-γ (100 U/ml) induced NO production over 16 h of incubation, comparable to the level of induction produced by the cytokine combination of IL-1β and IFN-γ. Medium conditioned by an aflagellic SD mutant did not induce NO production. A similar induction of NO production was obtained with sterile-filtered medium conditioned by *E. coli* and *P. aeruginosa (Ps. a.).* The combined concentration of nitrite and nitrate was determined by the Griess reaction following nitrate reduction. There were seven to nine determinations per experimental group or time point.

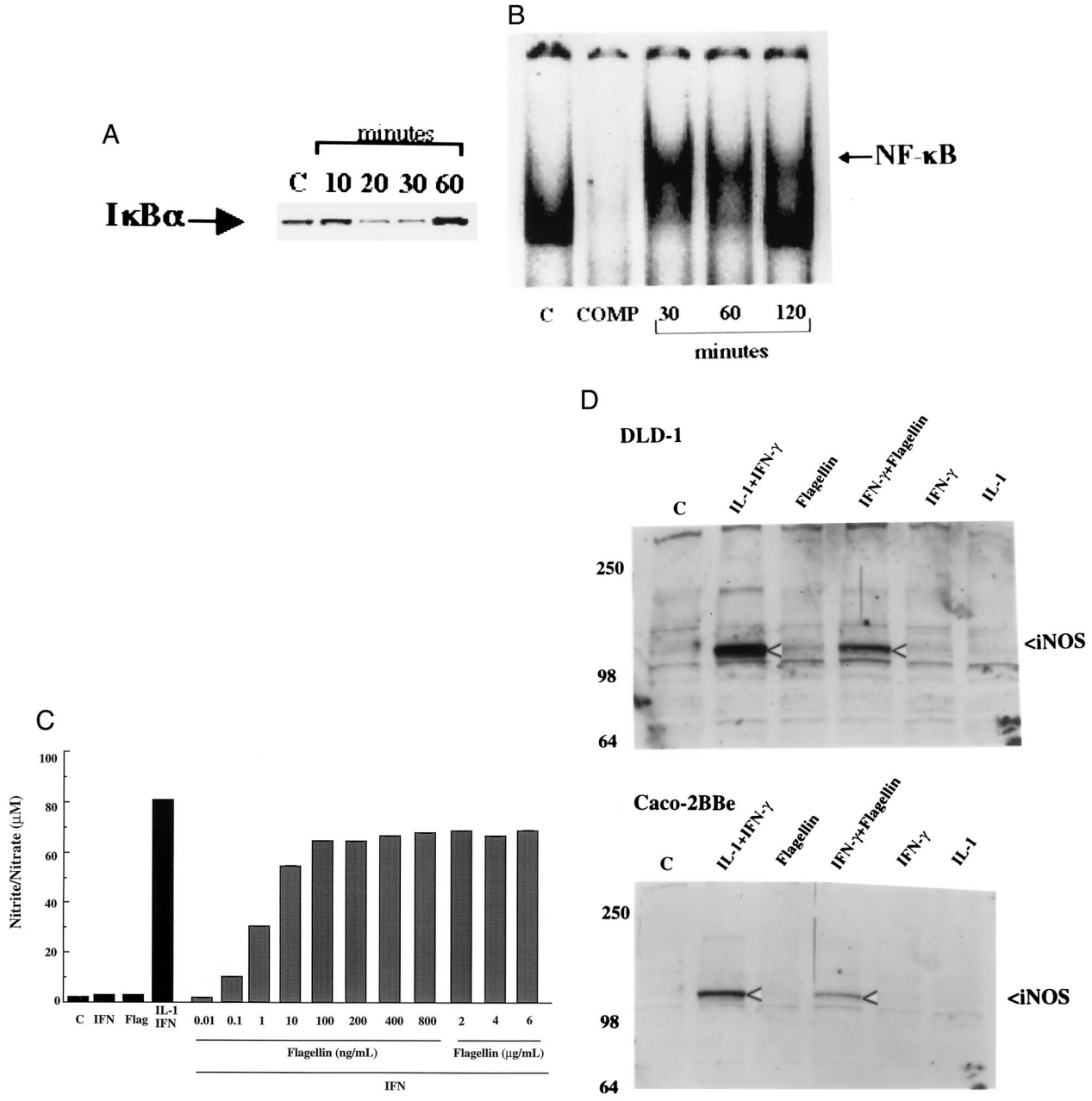

FIGURE 4. Purified flagellin induces IκBα degradation, NF-κB activation, and NO synthesis in Caco-2BBe cells. *A*, IκBα degradation was induced in a time-dependent manner in Caco-2BBe cells stimulated with SD-purified flagellin. Confluent Caco-2BBe cells were exposed for 10, 20, 30, or 60 min to 1 μg/ml of SD-purified flagellin, purified as previously described (16). Total protein was extracted, and equal amounts of protein were subjected to SDS-PAGE, followed by immunoblotting of IκBα as described in Fig. 1*A*. Unstimulated cells showed no loss of IκBα immunoreactivity (C). Cells exposed to SD-purified flagellin demonstrated IκBα degradation at 20 and 30 min, with a reappearance at 60 min. *B*, Representative EMSA demonstrating NF-κB activation in Caco-2BBe cells stimulated for 30, 60, or 120 min with SD-purified flagellin. Nuclear extracts from cells stimulated with flagellin were incubated with ^{32}P-labeled NF-κB oligonucleotides to determine NF-κB activation as described in Fig. 1*B*. Nuclear extracts from unstimulated cells showed no NF-κB activation (C). NF-κB activation was demonstrated in cells stimulated with flagellin at 30, 60, or 120 min. To confirm the specificity of the NF-κB signal, a 100-fold excess of cold oligonucleotide was used as a competitor to inhibit the binding activity of NF-κB (COMP). *C*, Addition of purified flagellin to Caco-2BBe cells primed for 2 h with IFN-γ (100 U/ml) dose-dependently induced NO production over 16 h of incubation. The combined concentration of nitrite and nitrate was determined by the Griess reaction following nitrate reduction. There were seven to nine determinations per experimental group or time point. *D*, Purified SD flagellin induces iNOS protein expression in cultured human intestinal epithelial cells. DLD-1 and Caco-2BBe cells were primed for 2 h with 0.5 ng/ml IL-1β or 100 U/ml IFN-γ followed by the addition of 1 μg/ml purified SD flagellin for approximately 16 h. *Upper*, IFN-γ-primed DLD-1 cells exposed to flagellin or IL-1β showed induction of iNOS expression. Individual cytokines and flagellin alone did not up-regulate iNOS protein expression in either cell line. *Lower*, IFN-γ-primed Caco-2BBe cells exposed to flagellin or IL-1β showed induction of iNOS expression. Individual cytokines and flagellin alone did not up-regulate iNOS protein expression in either cell line. There were three to five determinations per experimental group.

Microsequence analysis of the 55-kDa band identified the N-terminal sequence as (K)TASGVSTLINEDAAAAK. A GenBank database search produced an exact match with 14 identified forms of SD flagellin. Purified flagellin migrated by SDS-PAGE analysis in an identical fashion as the purified activity in the conditioned medium (Fig. 2*A*, *lane 5*) and induced IκBα degradation (Fig. 2*B*, *lane 5*).

Aflagellic Salmonella *mutants do not induce NO production by cultured IEC*

To establish the relative importance of flagellin to the CM-mediated induction of NO by IFN-γ primed DLD-1 cells, we compared the effect of CM obtained from wild-type SD (SL7276) and an aflagellic SD mutant (SL7276; Fig. 3). CM produced by wild-type SD potently induced synthesis of NO comparable to that stimulated by the cytokine combination of IL-1β and IFN-γ. In contrast, CM produced by the aflagellic SD mutant induced only slightly more NO than BHI alone. We also observed a high level of induction of NO production by CM obtained from clinical isolates of other flagellated Gram-negative bacillary pathogens, such as *E. coli* and *P. aeruginosa* (Fig. 3).

Effect of purified flagellin on IκBα stability, NF-κB activation, and NO production

Because NF-κB activation regulates iNOS transcription (11–13), we tested the effect of SD CM and purified flagellin on the stability of IκBα expression. Caco-2BBe cells stimulated by 1 μg/ml of SD-purified flagellin underwent a loss of immunoreactive IκBα 20 and 30 min poststimulation, followed by its reappearance at 60 min (Fig. 4*A*). The loss of IκBα preceded NF-κB binding activity, which was detected 30, 60, and 120 min after exposure of cells to flagellin (Fig. 4*B*). As predicted by the replenishment of IκBα protein at 60 min (Fig. 4*A*), NF-κB binding activity was diminished at 120 min (Fig. 4*B*). The specificity of the binding activity was confirmed by competition with a 100-fold excess of cold oligonucleotide competitor (Fig. 4*B*).

IFN-γ primed DLD-1 and Caco-2BBe cells incubated for 18 h with purified SD flagellin or recombinant *S. muenchen* flagellin dose-dependently induced NO production (EC_{50} = 2.5 ng/ml), with a threshold-inducing concentration of 100 pg/ml (Fig. 4*C*). Maximal NO production plateaued at a flagellin concentration of 5 ng/ml ($p < 0.05$) and was comparable to the levels of NO induced by the cytokine combination of IL-1β and IFN-γ, potent inducers of iNOS expression (15). Similar to the action of IL-1β (1, 11, 15), flagellin did not induce detectable levels of NO in cultured intestinal epithelial cells in the absence of IFN-γ. The requirement for IFN-γ is probably secondary to the presence of IFN-γ-inducible Stat1 and IRF-1 consensus elements in the 5′ human iNOS enhancer, a region located 10.3 kb upstream of the transcription initiation site that is required to support iNOS expression in a transient reporter assay in DLD-1 cells (15).

The effect of flagellin on iNOS protein expression was determined by Western analysis of extracts from DLD-1 and Caco-2BBe cells exposed to purified flagellin derived from SD. Stimulation by IFN-γ, IL-1β, or flagellin alone did not induce the expression of detectable immunoreactive iNOS protein (Fig. 4*D*). In IFN-γ-treated cells, in contrast, IL-1β and flagellin elicited increased iNOS expression in both cell lines. In addition, ELISA measurements of the supernatants of Caco-2BBe cells stimulated with purified SD flagellin (1 μg/ml) revealed secretion of IL-6 (from undetectable levels to 216 ± 12 pg/ml at 24 h). Purified SD flagellin, in the presence of IFN-γ (but not without IFN-γ costimulation), induced the production of IL-8 (from undetectable levels to 87 ± 3 pg/ml at 24 h). Thus, flagellin is able to elicit a general proinflammatory response from human epithelial cells (18, 19).

To establish the overall contribution of flagellin to the induction of IκBα degradation in intestinal epithelial cells, we compared the effect of CM obtained from wild-type SD (SL7276) and the aflagellic SD mutant (SL7276; Fig. 5). CM produced by wild-type SD (1/200 dilution) potently induced IκBα degradation by Caco-2BBe cells. In contrast, CM produced by the aflagellic SD mutant

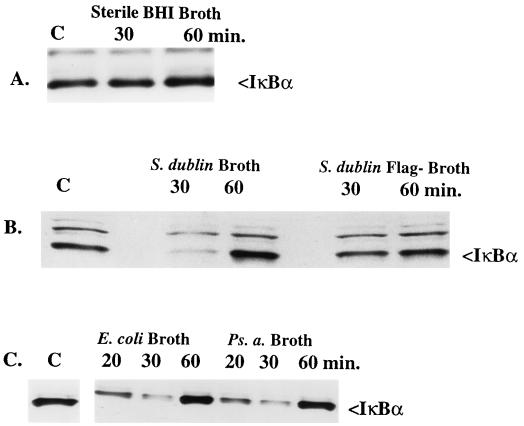

FIGURE 5. Conditioned bacterial medium induces IκBα degradation in Caco-2BBe cells. IκBα degradation was induced in a time-dependent manner in Caco-2BBe cells exposed for 30 or 60 min to BHI (*lane A*), medium conditioned by wild-type SD or aflagellic SD (*lane B*), and medium conditioned by *E. coli* or *P. aeruginosa* (*lane C*). Total protein was extracted, and equal amounts of protein were subjected to SDS-PAGE, followed by immunoblotting of IκBα as described in Fig. 1*A*. BHI-treated cells showed no loss of IκBα immunoreactivity (*lane A*). Cells exposed to CM demonstrated IκBα degradation at 30 min, with a reappearance at 60 min, whereas aflagellic SD did not induce IκBα degradation (*lane B*). Medium conditioned by *E. coli* and *P. aeruginosa* demonstrated a similar pattern as wild-type SD (*lane C*). Gels representative of three to five independent determinations are shown.

(1/200 dilution) had no effect on IκBα expression. We also observed that CM produced by *E. coli* and *P. aeruginosa* induced IκBα degradation comparable to the effect of CM from wild-type *S. dublin* (Fig. 5).

Flagellin induces inflammatory genes independent of LPS

Media conditioned by the aflagellic mutant of SD (SL7276) failed to induce NO production in IFN-γ-primed DLD-1 cells, suggesting that flagellin may be the predominant proinflammatory exotoxin released by SD. Since LPS is a well-established Gram-negative endotoxin, we conducted the following series of investigations to exclude its contribution to the flagellin-induced inflammatory responses. 1) The level of immunoreactive IκBα was unaltered by exposure of Caco-2BBe cells to purified *S. typhimurium* LPS (1 μg/ml) (20), in contrast to the effect of purified SD flagellin. 2) Cotreatment of IFN-γ primed DLD-1 cells with an LPS binding agent, polymyxin B (10 μg/ml), blocked NO production induced by 1 μg/ml of LPS purified from *S. typhimurium* (Sigma, catalogue L-7261), but not by 1 μg/ml of purified SD flagellin (Fig. 6*A*). 3) The content of LPS in 1 μg of purified SD flagellin, as measured by the chromogenic *Limulus* amebocyte assay (1), was <20 pg. As we previously reported, *S. typhimurium* LPS at a concentration of <1 μg/ml is unable to induce iNOS expression and NO production in cultured human intestinal epithelial cells (1). 4) In C3H/HeJ LPS-resistant mice, i.p. injection of purified SD flagellin or conditioned medium of the wild-type parent strain of SL7276 induced serum IFN-γ and TNF-α, whereas conditioned medium from SL7276 aflagellic mutants had no effect (Fig. 6*B*). In further support of the LPS-independent role of flagellin on proinflammatory gene expression, addition of recombinant *S. muenchen* flagellin (purified over a polymyxin B column) to IFN-γ-primed DLD-1 and Caco-2BBe cells dose-dependently induced NO production (data for Caco-2BBe cells shown in Fig. 7*A*). 5) Full-length, but not truncated, recombinant N-terminal *S. muenchen* flagellin (aa 1–156) induced NO synthesis in IFN-γ-primed DLD-1 cells (data not shown).

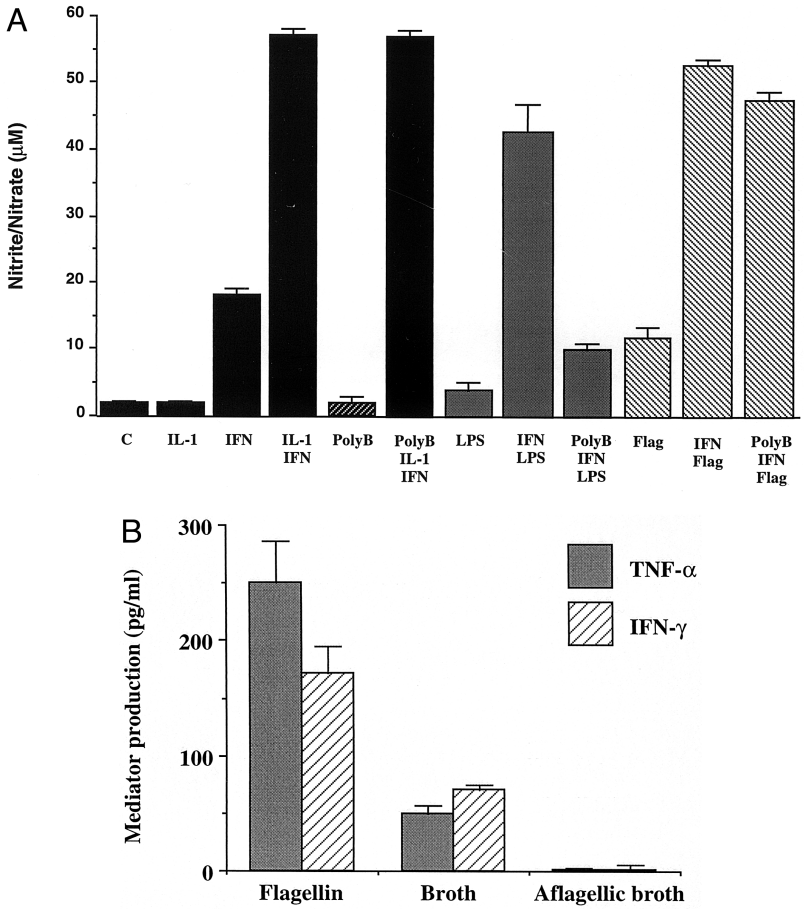

FIGURE 6. The proinflammatory effect of flagellin is independent of LPS. *A*, Polymyxin blocks LPS-induced, but not flagellin-induced, production of NO in Caco-2BBe cells. Cotreatment of IFN-γ (100 U/ml)-primed DLD-1 cells with an LPS-binding agent, polymyxin B (10 μg/ml), blocked NO production induced by 1 μg/ml of LPS purified from *S. typhimurium*, but not by 1 μg/ml of purified SD flagellin ($n = 12$; $p < 0.01$). *B*, Systemic flagellin injection induces serum cytokine expression in LPS-resistant mice. C3H/HeJ mice were treated with vehicle, 10 μg/mouse purified flagellin, SD-conditioned medium (broth), or conditioned medium from aflagellic SD mutants (aflagellic broth). Mice were sacrificed at 3 h, and serum TNF-α and IFN-γ levels were measured. Purified flagellin and SD-conditioned medium, but not conditioned medium from aflagellic SD mutants, induced the production of IFN-γ and TNF-α. There were three to five determinations per experimental group.

Taken together, the above data support the conclusions that 1) the proinflammatory activation induced by flagellin is not an artifact secondary to LPS contamination, and 2) flagellin derived from *Salmonella sp.* is a potent inflammatory stimulus of NO production by cultured intestinal epithelial cells.

Anti-flagellin antiserum blocks induction of NO production induced by flagellins of various motile Gram-negative bacteria

To further implicate flagellin as the relevant NO-inducing species, we next tested whether anti-flagellin Abs would neutralize the production of NO induced by conditioned medium or recombinant flagellin. As a positive control, lapine antiserum raised against the N-terminus of *S. muenchen* flagellin abolished the NO-inducing activity of recombinant *Salmonella muenchen* flagellin in IFN-γ primed Caco-2BBe cells (Fig. 7*A*). The antiserum also abolished the NO-inducing activity of recombinant flagellins (Fig. 7*B*) or conditioned medium (Fig. 7*C*) derived from a series of Gram-negative motile bacterial species (*E. coli*, *S. muenchen*, *S. marcescens*, *P. mirabilis*, and *P. vulgaris*). The latter data imply that flagellin may represent the crucial factor in the induction of NO production by CM obtained from flagellated Gram-negative bacilli.

Flagellin induces inflammatory gene expression in mice

Retention of the flagellin monomer within the growing polymeric filament is dependent upon the presence of the HAP2 cap protein, which is located at the distal terminus of the intact flagellum. HAP2 gene-deficient mutants produce and excrete flagellin monomers into the surrounding media at a high rate (>1/s) (21). Release of flagellin also appears to occur in wild-type *Salmonella*, as shown by its purification from SD CM.

To test whether flagellin, independent of live bacteria, is capable of inducing the systemic inflammatory alterations characteristic of Gram-negative sepsis, C57BL/6 mice were challenged with 10 μg (~400 μg/kg i.p.) of purified SD flagellin. Flagellin induced a prototypic inflammatory response, demonstrated by up-regulated serum concentrations of TNF-α, IL-6, IL-12p40, MIP-1α, IL-10, and nitrite/nitrate (Fig. 8). Similar results were obtained in BALB/c mice injected with recombinant *S. muenchen* flagellin (data not shown).

The production of TNF-α, MIP-1α, IL-6, and IL-10 peaked within 2 h of purified SD flagellin injection, whereas IL-12p40 production and NO peaked at approximately 4 h and 8 h post-flagellin, respectively (Fig. 8, *A–F*). The threshold dose of purified SD flagellin for induction of the inflammatory response was 0.3 μg/mouse (~12 μg/kg), with a dose-dependent increase in the production of the mediators between 0.3–10 μg/mouse (i.e., ~12–400 μg/kg; Fig. 9, *G* and *H*). The degree and time course of the cytokine, chemokine, and NO production induced by 10 μg of flagellin were comparable to the inflammatory response elicited by *E. coli* LPS (30–100 μg/mouse) or by staphylococcal enterotoxin B (100 μg/mouse) (22–24). Immunohistochemical studies of the ileum of flagellin-treated C57BL/6 mice localized iNOS expression to the apex of the villi and, to some extent, the crypt (Fig. 9).

Flagellin induces shock in mice

To demonstrate that flagellin induces hemodynamic instability characteristic of septic shock, endotoxin-responsive C3H/OuJ

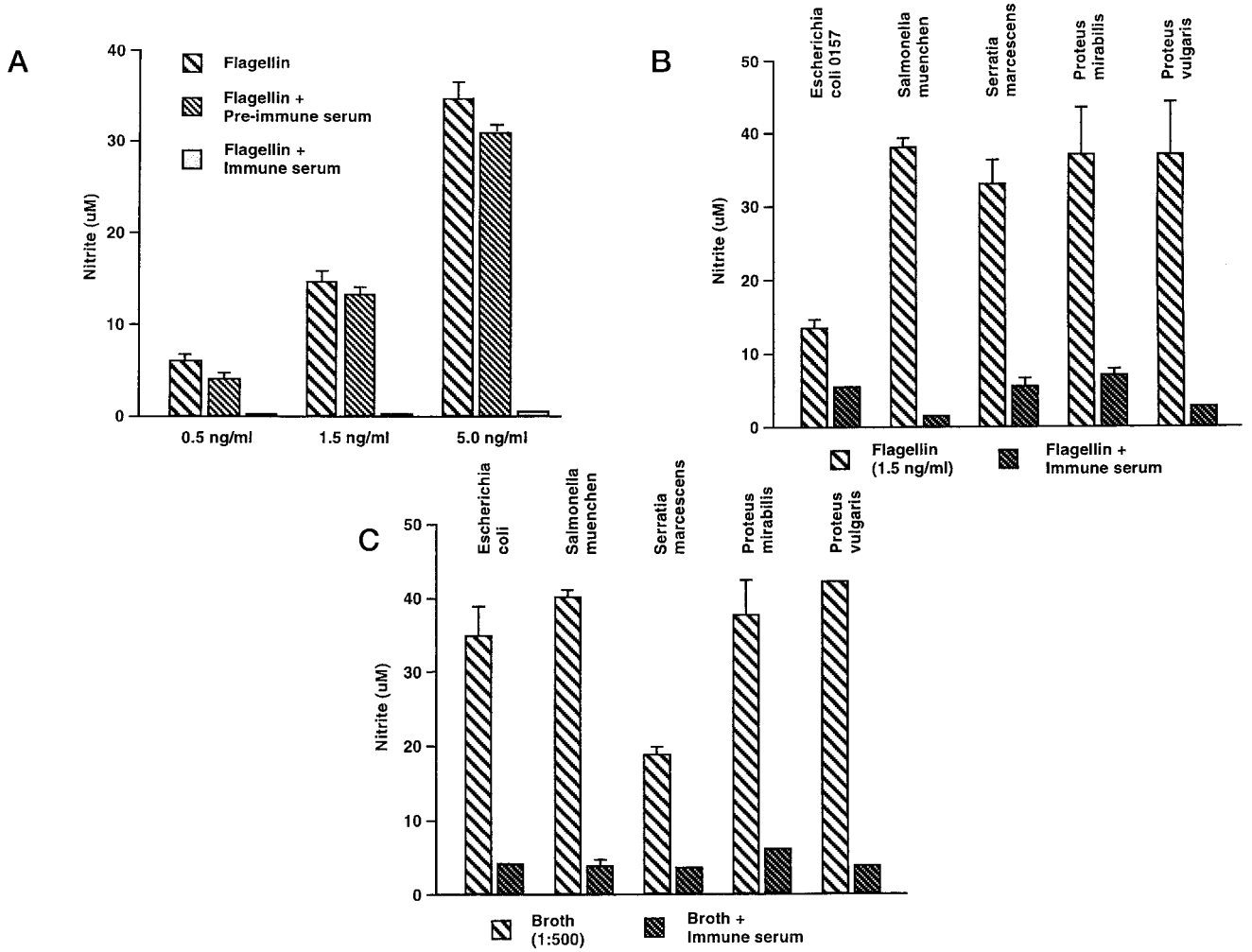

FIGURE 7. Antiserum to flagellin inhibits NO production induced by Caco-2BBe cells exposed to bacteria-conditioned medium or recombinant flagellin. *A*, Recombinant *S. muenchen* flagellin induced a concentration-dependent increase in NO production in IFN-γ-primed (100 U/ml) Caco-2BBe cells. *B*, Medium conditioned by various motile Gram-negative pathogens (*E. coli*, *S. muenchen*, *S. marcescens*, and *P. mirabilis*) induced NO production in IFN-γ primed (100 U/ml) Caco-2BBe cells. *C*, Recombinant flagellins cloned from various motile Gram-negative pathogens (*E. coli*, *S. muenchen*, *S. marcescens*, and *P. mirabilis*) induced NO production in IFN-γ primed (100 U/ml) Caco-2BBe cells. NO production was inhibited by preincubation (1 h at room temperature) of recombinant flagellin (*A* and *C*) or conditioned medium (*B*) with a 1/100 dilution of lapine polyclonal antiserum generated against recombinant *S. muenchen* flagellin. Incubation with preimmune serum was not inhibitory. There were three to five determinations per experimental group.

mice were injected i.v. with recombinant *S. muenchen* flagellin (10 mg/kg; i.v.; $n = 10$) or a saline vehicle control ($n = 5$). In response to a challenge of 10 mg/kg flagellin (i.e., ~300 μg/mouse), the mean arterial pressure remained at baseline levels for 2 h, followed by severe hypotension, respiratory distress, cyanosis, and death between 2–4 h (Fig. 10). Saline-treated vehicle controls, in contrast, were normotensive and free of respiratory distress for the duration of the 4 h period of observation. Thoracic aortae from flagellin-treated mice demonstrated an ex vivo loss of vascular contractility, a classic feature of Gram-negative bacilli induced systemic inflammation and shock (Fig. 10). A potential confounding effect of endotoxin contamination was investigated by also testing the flagellin responses in the LPS-resistant C3H/HeJ mice. This latter strain of mice is resistant to the LPS effects because it lacks functional Toll-like receptor-4 (TLR-4) receptors (25). In agreement with previous studies, i.v. injection of *E. coli* LPS (10 mg/kg) in the C3H/OuJ mice caused a time-dependent fall in mean arterial blood pressure (e.g., from 102 ± 4 to 78 ± 3 mm Hg at 4 h; $n = 6$), whereas no significant drop in blood pressure was observed in the C3H/HeJ mice (mean arterial blood pressure at 0 and 4 h after LPS injection, 89 ± 5 and 86 ± 7 mm Hg, respectively; $n = 4$). However, i.v. flagellin injection (10 mg/kg) induced similar effects on blood pressure, vascular responsiveness, and mortality, in both the LPS-responsive (C3H/OuJ) and the LPS-resistant (C3H/HeJ) mice (Fig. 10), indicating that 1) the effect of flagellin was not mediated by LPS contamination, and 2) the hypotensive and shock-inducing effects of flagellin are not mediated by activation of the TLR-4 receptor. Reduction of the flagellin challenge to 1 mg/kg i.v. produced moderate progressive hypotension without mortality and significant ex vivo vascular hypocontractility at 6 h (data not shown).

Flagellin circulates in the blood of septic rats

The proinflammatory and shock-inducing effects of flagellin suggested that free circulating flagellin might mediate some of the septic responses to Gram-negative infection. To establish that free flagellin circulates in the early phase of sepsis, rats were challenged with a lethal i.p. dose of *S. marcescens* (3×10^8 CFU). Before infection, the blood was sterile (Fig. 11*A*), and circulating serum flagellin was undetectable (Fig. 11*B*). Four hours after challenge, rats became bacteremic (Fig. 11*A*), and the serum flagellin concentration increased to approximately 400 ng/ml (Fig. 11*B*).

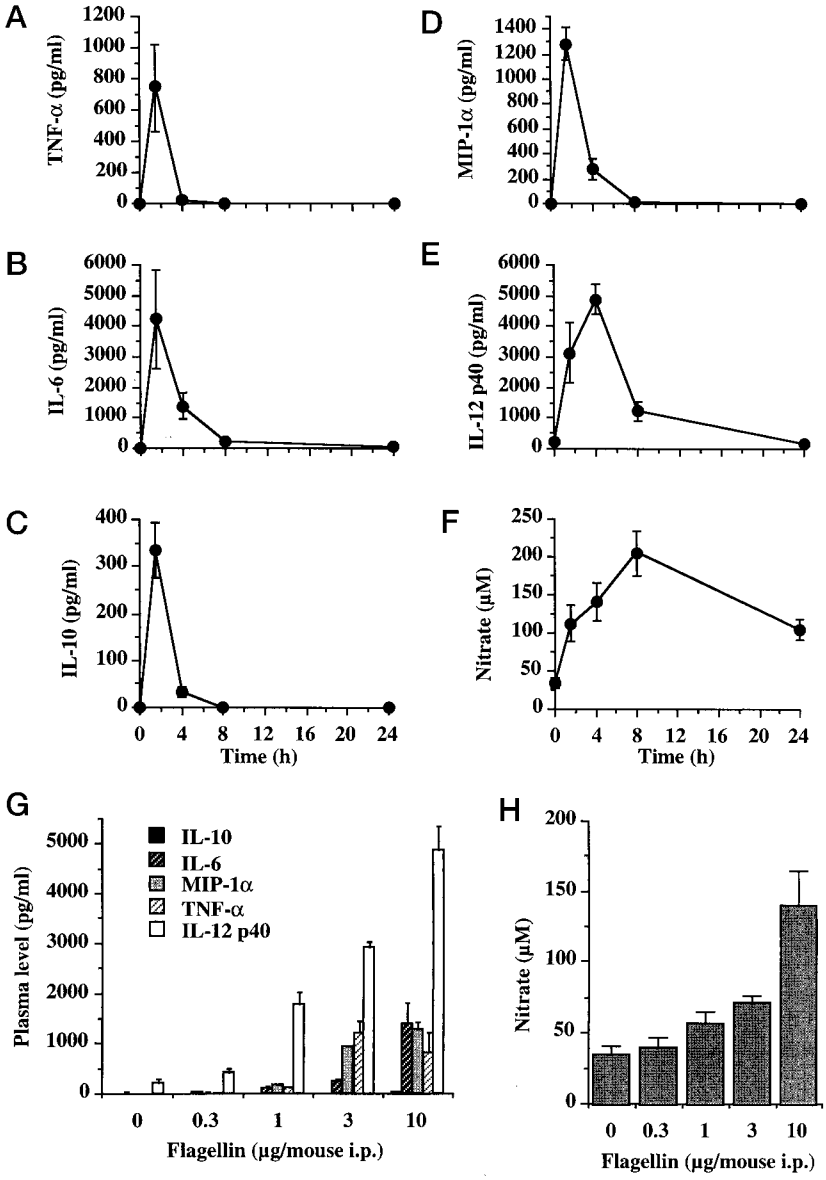

FIGURE 8. *A—H,* Flagellin induces the production of inflammatory mediators in vivo. *A–F,* C57BL/6 mice were treated with vehicle or 10 μg/mouse flagellin at time zero, and plasma TNF-α, MIP-1α, IL-6, IL-12p40, IL-10, and nitrate levels were measured at 0–24 h. *G* and *H,* Dose-dependency of the flagellin-induced inflammatory response (0.3–10 μg/mouse flagellin). Dose-responses were determined at 4 h, except for TNF-α and MIP-1α, where dose-responses were determined at 90 min postflagellin injection. There were four to six mice per time point or experimental group.

Rats became morbidly ill at 8 h, with a 50% mortality rate at 12 h. Parallel to these observations, the level of bacteremia and flagellemia increased sharply at 8 h postchallenge.

Discussion

We have identified a major proinflammatory activity in CM as flagellin. We demonstrate that purified and recombinant *Salmonella* flagellins play an unexpected, but powerful, role in activating inflammatory responses in human intestinal epithelial cells in vitro and in eliciting a systemic activation of proinflammatory cytokines and NO and shock in vivo. The mechanism of action of flagellin involves the induction of IκBα degradation and NF-κB activation. Conditioned broth obtained from a flagellin-deficient mutant had no effect on NO production, IκBα degradation, and NF-κB activation, indicating that flagellin is the major, if not exclusive, proinflammatory stimulus released by *Salmonella*.

Flagellin is a 55-kDa monomer obtained from bacterial flagella, a polymeric rod-like appendage extending from the outer membrane of Gram-negative bacteria that propels the organism through its aqueous environment (26). In addition to their role in motility, flagella may also act as a virulence factor. Flagella aid bacteria in attaching to epithelial cells, assisting bacterial invasion (27). Flagellin-deficient mutants of *P. mirabilis* invade renal epithelial cells less efficiently and fail to induce pyelonephritis, unlike wild-type strains (28). Flagella may also play a key role in up-regulating IL-8 secretion in gastric epithelial cells stimulated by *Helicobacter pylori* (29).

It is generally accepted that LPS released from the bacterial cell wall is the trigger of the inflammatory response to Gram-negative pathogens (30). This conclusion is based on substantial data revealing that 1) parenteral administration of LPS produces a lethal shock state in experimental animals; 2) LPS can be measured in the circulation of the majority of patients with symptomatic Gram-negative infection; and 3) LPS induces proinflammatory responses in cultured cells. However, several lines of recent evidence suggest that LPS may not be the sole proinflammatory stimulus of clinical inflammation induced by motile enteric Gram-negative bacilli. First, incontrovertible differences exist between LPS-induced shock and bacterial sepsis. LPS does not faithfully reproduce the kinetics, magnitude, and distribution of proinflammatory gene expression typical of experimental models of live Gram-negative infection or clinical sepsis (31, 32). Second, the passive immunization of anti-LPS antisera has not increased survival from sepsis

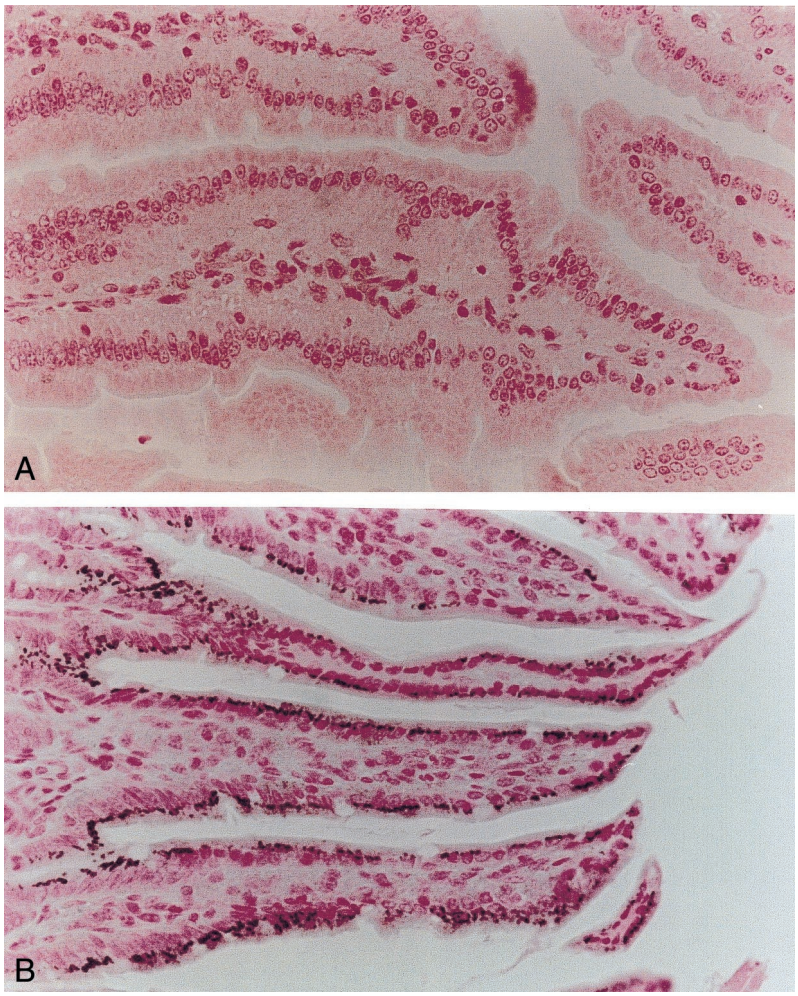

FIGURE 9. Systemic flagellin injection induces iNOS expression in the intestinal epithelium in mice. C57BL/6 mice were treated with vehicle or 10 μg/mouse flagellin for 24 h, followed by immunohistochemical staining for iNOS of intestinal samples as previously described (43). While no iNOS staining was localized in vehicle-treated control animals (*upper panel*), flagellin induced a massive expression of iNOS, which almost exclusively localized to the basal intestinal epithelial cells (*lower panel*). Magnification, $\times$100. Immunohistochemical pictures shown are representative of four to six slides per group.

in pivotal clinical trials (33, 34). Increasingly, it is proposed that inflammatory injury and shock in Gram-negative infection may also be triggered by microbial stimuli other than LPS.

Our data suggest that flagellin, the subunit of bacterial flagella, may be an additional proinflammatory factor induced by motile Gram-negative bacilli. This hypothesis is supported by the following observations. 1) Flagellin is a potent stimulus of inflammation and shock in LPS-resistant mice. 2) Flagellin appears to act as an exotoxin; free flagellin is released by motile Gram-negative bacteria in vitro and appears in the blood of septic rats. The concentration of flagellin detected in vivo in septic rats was several orders of magnitude in excess of that required to induce NF-κB activation and inflammatory mediator production in cultured cells in vitro. 3) Flagellin induces alterations in key proinflammatory signaling pathways and in the expression of free-radical generating enzymes. 4) Flagellin injection potently induces the expression of iNOS and proinflammatory cytokines in vivo and at higher doses induces a shock-like state, associated with hypotension, vascular dysfunction, and mortality. 5) Flagellated bacteria, purified flagellin, and medium conditioned by flagellated bacteria all readily induce IκBα degradation, NF-κB activation, and iNOS expression in transformed human epithelial cells and murine macrophages. 6) Medium conditioned by a flagellin-deficient mutant has no effect on IκBα stability, NF-κB activation, and iNOS expression. 7) Parenteral administration of medium conditioned by aflagellic mutants has no proinflammatory effect in LPS-resistant mice. 8) Polyclonal antiserum to flagellin eliminates the NO-inducing activity of medium conditioned by multiple motile Gram-negative bacillary pathogens.

The eukaryotic receptors responsible for flagellin-induced NF-κB activation and proinflammatory gene expression remain to be identified. Based on the results of the study in the C3H/HeJ mice, TLR4 (a crucial receptor involved in LPS signaling) does not play a role in flagellin signaling. Recent data show that a broad variety of Gram-positive and Gram-negative bacterial proteins stimulate proinflammatory signaling pathways in IECs in a TL4-independent manner via binding to TLR2 (35–41). Studies in our laboratory are currently underway to assess the role of TLR2 in flagellin-induced NF-κB degradation and iNOS expression in human cells.

The majority of infections in the critical care setting are produced by flagellated Gram-negative bacilli, such as *E. coli*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *S. marcesens*, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Citrobacter freundii*, and *P. mirabilis*. The relative contribution of flagellin to sepsis-induced shock and mortality induced by these organisms remains to be determined. Presumably, multiple virulence factors, in particular LPS, trigger the systemic proinflammatory host response to motile Gram-negative pathogens. It should be additionally noted that sepsis is readily induced by nonflagellated infectious species, in which flagellin clearly does not play a role, including *Rickettsia*, fungi, yeast, *Babesia*, Gram-positive bacteria, hemorrhagic viruses, *P. falciparum*, and Gram-negative diplococci and nonmotile Gram-negative bacilli.

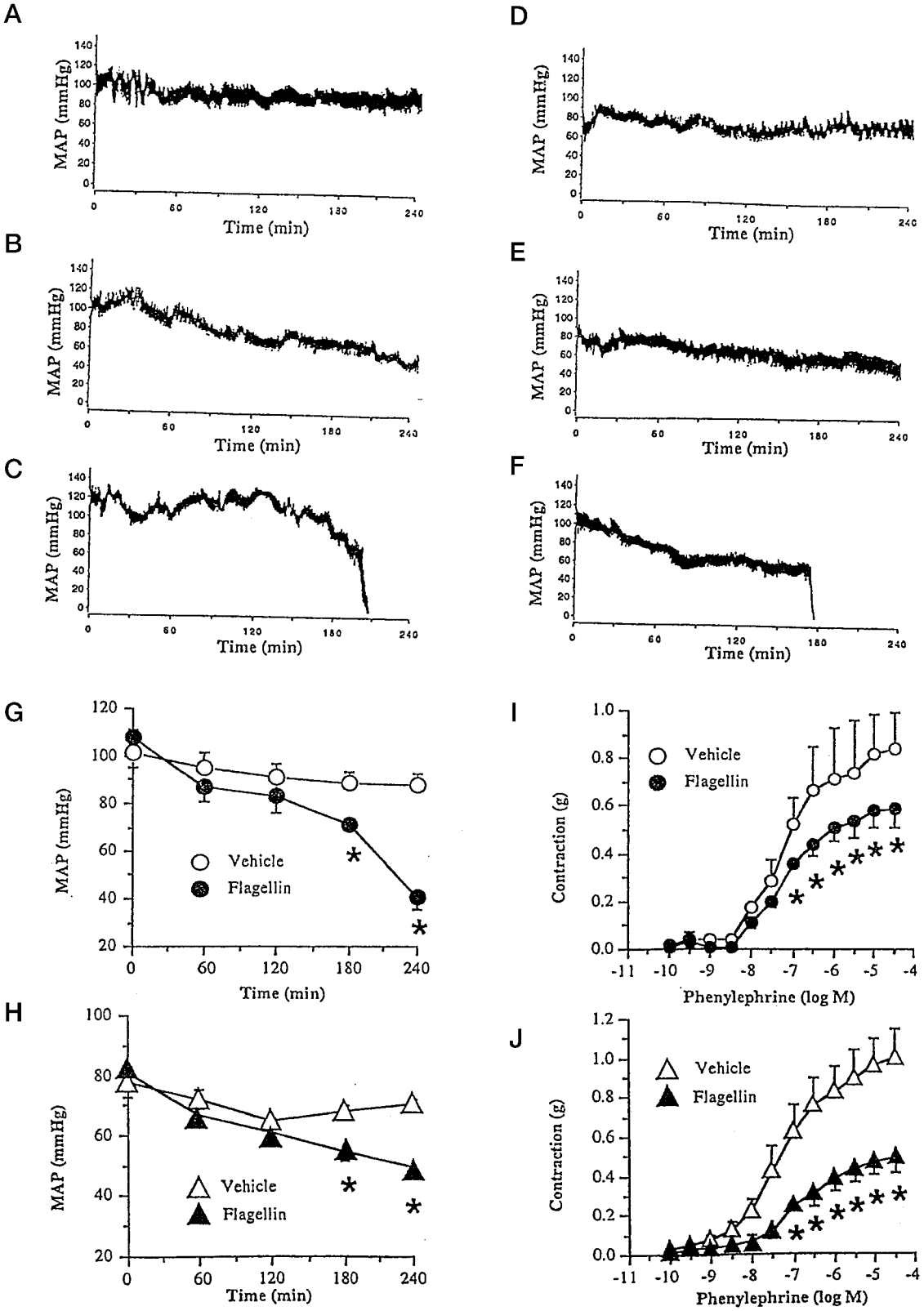

FIGURE 10. Flagellin induces shock in mice. Anesthetized, 8-wk-old male C3H/OuJ (*A–C* and *G–H*) and LPS-resistant C3H/HeJ (*D–F* and *I–J*) mice were treated with recombinant *S. muenchen* flagellin (10 mg/kg i.v.) or saline vehicle control (0.2 ml). Treatment with 10 mg/kg flagellin produced severe hypotension within 3 h. Representative tracings are shown of vehicle-treated mice (*A* and *D*) and flagellin-treated mice demonstrating hypotension (*B* and *E*) and mortality (*C* and *F*). *g*, Flagellin induces progressive hypotension ($n = 10$) and mortality (7 of 10 animals at 4 h) in BALB/c mice compared with saline-treated controls ($n = 5$; $*$, $p < 0.05$). *H*, Flagellin induces progressive hypotension ($n = 7$) and mortality (four of seven animals at 4 h) in C3H/HeJ mice compared with saline-treated controls ($n = 4$; $*$, $p < 0.05$). Hypotension was associated with a severe reduction in the ex vivo contractility of thoracic aortic rings harvested at 220–240 min from mice surviving flagellin treatment (10 mg/kg i.v.). Flagellin induced hypocontractility in C3H/OuJ (*I*) and C3H/HeJ (*J*) mice compared with saline-treated controls ($p < 0.05$). In the vascular ring studies, four or five rings per group were used.

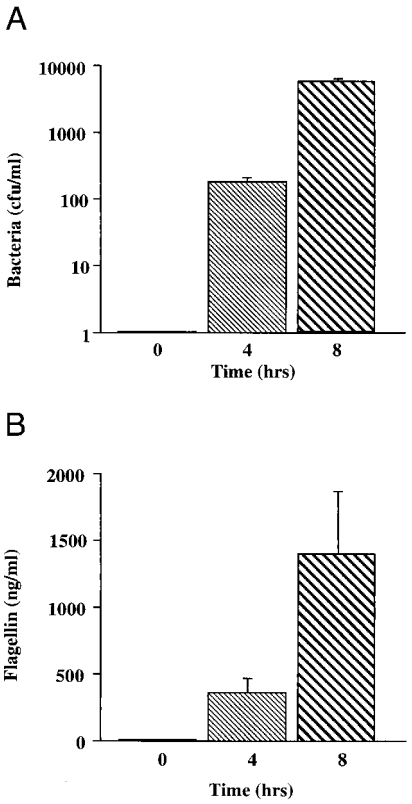

FIGURE 11. Free flagellin circulates in septic rats. Wistar rats were injected i.p. with *S. marcescens* (3×10^9 CFU/rat). *A*, Quantitative blood cultures were obtained at 4 and 8 h postinfection. *B*, Serum flagellin concentrations were determined at 4 and 8 h postinfection. Before infection, the blood was sterile, and circulating serum flagellin was undetectable. Four hours after challenge, rats became bacteremic, and the serum flagellin concentration was about 400 ng/ml. Rats became morbidly ill at 8 h, with a 50% rate of mortality at 12 h. In parallel to these observations, the level of bacteremia and flagellemia increased sharply at 8 h postchallenge. There were four animals per determination.

Given its considerable potency and its probable release in the host during active infection, flagellin may be an important stimulus of in vivo inflammation and shock induced by motile Gram-negative enteric bacilli. However, the doses of flagellin needed to induce hypotension and shock were orders of magnitude higher than the doses of flagellin required to induce proinflammatory mediator expression. In this respect, flagellin behaves similar to LPS. Nevertheless, the causative role of flagellin in bacteremic shock remains to be clarified. The possibility that flagellin induces systemic inflammation and shock in synergy with LPS or other bacterial components must also be investigated in further studies. We hypothesize that differences in the rate of flagellin release at sites of infection might represent a clinically relevant virulence factor. It may also be expected that antibiotics might influence the rate of release of monomeric flagellin, akin to their well-established effect on LPS release. Further work must determine whether neutralization of flagellin represents a novel anti-inflammatory or anti-sepsis strategy.

While our current manuscript was in review, a related study appeared, identifying flagellin as a novel factor of enteroaggregative *E. coli* (EAEC)–induced epithelial cell activation (42). Similar to our findings, the soluble, heat-stable, IL-8-inducing factor released by these EAEC bacteria in culture was identified as flagellin. It was found that flagella purified from EAEC isolates potently induce IL-8 release from Caco-2 cells, while an engineered aflagellar mutant did not induce the release IL-8. Finally, it was found that cloned EAEC flagellin expressed in nonpathogenic *E. coli* as a polyhistidine-tagged fusion protein maintained its proinflammatory activity. These findings coupled with our present findings demonstrate that flagellin production and release may be a novel mechanism by which various bacteria induce intestinal and systemic inflammation. Our findings have obvious implications for diagnosis and vaccine development.

Acknowledgments

We acknowledge Paul Hake (Children's Hospital Medical Center, Cincinnati, OH) for assistance with in vivo studies, Dr. Carol Beach (University of Kentucky, Lexington. KY) for assistance with microsequence analysis, and Alvin Denenberg (Children's Hospital Medical Center, Cincinnati, OH), Dr. Al Comb, and Dr. Kenneth Blumenthal (University of Cincinnati College of Medicine, Cincinnati, OH) for assistance with analytical separations. We thank Prof. Bruce Stocker (Department of Microbiology, Stanford University, Stanford, CA) for providing the aflagellic *S. dublin* mutant strains. Animal care was conducted in accordance with institutional guidelines at Children's Hospital Medical Center (Cincinnati, OH) and Inotek Corp. (Beverly, MA).

References

1. Salzman, A. L., T. Eaves-Pyles, S. Linn, A. Denenberg, and C. Szabó. 1998. Bacterial induction of inducible nitric oxide synthase in cultured human intestinal epithelial cells. *Gastroenterology 114:93.*
2. Eckmann, L., M. F. Kagnoff, and J. Fierer. 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infect. Immun. 61:4569.*
3. Huang, G. T., L. Eckmann, T. C. Savidge, and M. F. Kagnoff. 1996. Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 expression and neutrophil adhesion. *J. Clin. Invest. 98:572.*
4. Jung, H. C., C. L. Eckmann, S. Yang, A. Panja, E. Fierer, F. Morzycka-Wroblewska, and M. F. Kagnoff. 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J. Clin. Invest. 95:55.*
5. Fiocchi, C. 1991. Production of inflammatory cytokines in the intestinal lamina propria. *Immunol. Res. 10:239.*
6. Salzman, A. L. 1995. Nitric oxide in the gut. *New Horizons 3:352.*
7. Xie, Q., and C. Nathan. 1994. The high-output nitric oxide pathway: role and regulation. *J. Leukocyte Biol. 56:576.*
8. Gardner, P. R., G. Constantino, C. Szabó, and A. L. Salzman. 1997. Nitric oxide sensitivity of the aconitases. *J. Biol. Chem. 272:25071.*
9. Gardner, P., G. Costantino, and A. L. Salzman. 1998. Constitutive and adaptive detoxification of nitric oxide in *Escherichia coli*: evidence for a role of nitric oxide dioxygenase. *J. Biol. Chem. 273:26528.*
10. Gardner, P. R., A. M. Gardner, L. A. Martin, and A. L. Salzman. 1998. Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. *Proc. Natl. Acad. Sci. USA 95:10378.*
11. Salzman, A. L., A. G. Denenberg, I. Ueta, M. O'Connor, S. Linn, and C. Szabó. 1996. Induction and activity of nitric oxide synthase in cultured human intestinal epithelial monolayers. *Am. J. Physiol. 270:G565.*
12. Taylor, B. S., M. E. de Vera, R. W. Ganster, Q. Wang, R. A. Shapiro, S. M. Morris, T. Billiar, and D. Geller. 1998. Multiple NF-κB enhancer elements regulate cytokine induction of the human inducible nitric oxide synthase gene. *J. Biol. Chem. 273:15148.*
13. Xie, Q., Y. Kashiwabara, and C. Nathan. 1994. Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem. 269:4705.*
14. Eaves-Pyles, T., C. Szabó, and A. L. Salzman. 1999. Bacterial invasion is not required for activation of NF-κB in enterocytes. *Infect. Immun. 67:800.*
15. Linn, S. C., P. J. Morelli, I. Edry, S. E. Cottongim, C. Szabó, and A. L. Salzman. 1997. Transcriptional regulation of the human inducible nitric oxide synthase gene in an intestinal epithelial cell line. *Am. J. Physiol. 272:G1499.*
16. Ibrahim, G. F., G. H. Fleet, M. J. Lyons, and R. A. Walker. 1985. Method for the isolation of highly purified *Salmonella* flagellins. *J. Clin. Microbiol. 22:1040.*
17. Szabó, C., A. L. Salzman, and H. Ischiropoulos. 1995. Peroxynitrite-mediated oxidation of dihydrorhodamine 123 occurs in early stages of endotoxic and hemorrhagic shock and ischemia-reperfusion injury. *FEBS Lett. 373:229.*
18. Parikh, A., A. L. Salzman, J. E. Fischer, C. Szabo, and P. O. Hasselgren. 1997. Interleukin-1β and interferon-γ regulate interleukin-6 production in cultured human intestinal epithelial cells. *Shock 8:249.*
19. DiMango, E., H. J. Zar, R. Bruan, and A. Prince. 1995. Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. *J. Clin. Invest. 96:2204.*
20. Wilson, L., C. Szabó, and A. L. Salzman. 1999. PKC-dependent activation of NF-κB in enterocytes is independent of IκB degradation. *Gastroenterology 117: 106.*
21. Ikeda, T., S. Yamaguchi, and H. Hotani. 1993. Flagellar growth in a filament-less *Salmonella* fliD mutant supplemented with purified hook-associated protein 2. *J. Biochem. 114:39.*

22. Szabó, C., G. Haskó, B. Zingarelli, Z. Németh, A. L. Salzman, V. Kvetan, S. Pastores, and E. Vizi. 1997. Isoproterenol regulates tumor necrosis, interleukin-10, interleukin-6, and nitric oxide production and protects against the development of vascular hyporeactivity in endotoxemia. *Immunology 90:95.*
23. Haskó, G., C. Szabó, Z. Németh, A. L. Salzman, and E. Vizi. 1998. Suppression of IL-12 production by phosphodiesterase inhibition in murine endotoxemia is IL-10 dependent. *Eur J Immunol. 28:468.*
24. Haskó, G., L. Virág, G. Egnaczyk, A. L. Salzman, and C. Szabó. 1998. The crucial role of interleukin-10 in the suppression of the immunological response in mice exposed to staphylococcal enterotoxin B. *Eur. J. Immunol. 28:1417.*
25. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science 282:2085.*
26. Nmaba, K., I. Yamashita, and F. Vonderviszt. 1989. Structure of the core and central channel of bacterial flagella. *Nature 342:648.*
27. Penn, C. W., and C. J. Luke. 1992. Bacterial flagellar diversity and significance in pathogenesis. *FEMS Microbiol. Lett. 100:331.*
28. Mobley, H. L., R. Belas, B. R. Lockatell, G. Chippendale, A. L. Trifillis, D. E. Johnson, and J. W. Warren. 1996. Construction of a flagellum-negative mutant of *Proteus mirabilis*: effect on internalization by human renal epithelial cells and virulence in a mouse model of ascending urinary tract infection. *Infect. Immun. 64:5332.*
29. Ohta-Tada, U., A. Takagi, Y. Koga, S. Kamiya, and T. Miwa. 1997. Flagellin gene diversity among *Helicobacter pylori* strains and IL-8 secretion from gastric epithelial cells. *Scand. J. Gastroenterol. 32:455.*
30. Henderson, B., S. Poole, and M. Wilson. 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiol. Rev. 60:316.*
31. Remick, D. G., S. J. Garg, D. E. Newcomb, G. Wollenberg, T. K. Huie, and G. L. Bolgos. 1998. Exogenous interleukin-10 fails to decrease the mortality or morbidity of sepsis. *Crit. Care Med. 26:818.*
32. Zhou, M., P. Wang, and I. H. Chaudry. 1998. Cardiac contractility and structure are not significantly compromised even during the late, hypodynamic stage of sepsis. *Shock 9:352.*
33. Bone, R. C., R. A. Balk, A. M. Fein, T. M. Perl, R. P. Wenzel, H. D. Reines, R. W. Quenzer, T. J. Iberti, N. Macintyre, and R. M. Schein. 1995. A second large controlled clinical study of E5, a monoclonal antibody to endotoxin: results of a prospective, multicenter, randomized, controlled trial. The E5 Sepsis Study Group. *Crit. Care Med. 23:994.*
34. Abraham, E., A. Anzueto, G. Gutierrez, S. Tessler, G. San Pedro, R. Wunderink, A. Dal Nogare, S. Nasraway, S. Berman, R. Cooney, et al. 1998. Double-blind randomised controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock: NORASEPT II Study Group. *Lancet 351:929.*
35. Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, et al. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem. 274:33419.*
36. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J. Biol. Chem. 274:17406.*
37. Takeuchi, O., A. Kaufmann, K. Grote, T. Kawai, K. Hoshino, M. Morr, P. F. Muhlradt, and S. Akira. 2000. Cutting edge: preferentially the R-stereoisomer of the mycoplasmal lipopeptide macrophage-activating lipopeptide-2 activates immune cells through a toll-like receptor 2- and MyD88-dependent signaling pathway. *J. Immunol. 164:554.*
38. Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis. 1999. Inflammatory signaling by *Borrelia burgdorferi* lipoproteins is mediated by toll-like receptor 2. *J. Immunol. 163:2382.*
39. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol. 163:1.*
40. Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. USA 96:14459.*
41. Aliprantis, A. O., R. B. Yang, M. R. Mark, S. Suggett, B. Devaux, J. D. Radolf, G. R. Klimpel, P. Godowski, and A. Zychlinsky. 1999. Cell activation and apoptosis by bacterial lipoproteins through toll- like receptor-2. *Science 285:736.*
42. Steiner, T. S., J. P. Nataro, C. E. Poteet-Smith, J. A. Smith, and R. L. Guerrant. 2000. Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. *J. Clin. Invest. 105:1769.*
43. Virág, L., G. Haskó, A. L. Salzman, and C. Szabó. 1998. NADPH diaphorase histochemistry detects inducible nitric oxide synthase activity in the thymus of naive and *Staphylococcal* enterotoxin B-stimulated mice. *J. Histochem. Cytochem. 46:787.*