INFECTION AND IMMUNITY, July 1997, p. 2648–2655 Vol. 65, No. 7
0019-9567/97/$04.00+0

Nitrite Reductase from *Pseudomonas aeruginosa* Induces Inflammatory Cytokines in Cultured Respiratory Cells

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Received 27 November 1996/Returned for modification 16 January 1997/Accepted 12 April 1997

Persistent infection with *Pseudomonas aeruginosa* increases interleukin-8 (IL-8) levels and causes dense neutrophil infiltrations in the airway of patients with chronic airway diseases. To investigate the role of *P. aeruginosa* infection in IL-8 production in the airway of these patients, we examined whether cell lysates of *P. aeruginosa* could cause IL-8 production from human bronchial epithelial cells. Diluted sonicated supernatants of *P. aeruginosa* (SSPA) with a mucoid or nonmucoid phenotype stimulated human bronchial epithelial (BET-1A) cells to produce IL-8. In this study, we have purified a 59-kDa heat-stable protein with IL-8-inducing activity from the SSPA by sequential ion-exchange chromatography. The N-terminal sequence of this purified protein completely matched a sequence at the N-terminal part of the mature protein of nitrite reductase from *P. aeruginosa*. In addition, immunoblotting with a polyclonal immunoglobulin G (IgG) against recombinant *Pseudomonas* nitrite reductase demonstrated a specific binding to the purified protein. Furthermore, the immunoprecipitates of the SSPA with a polyclonal IgG against recombinant nitrite reductase induced a twofold-higher IL-8 production in the BET-1A cell culture than did the immunoprecipitates of the SSPA with a control IgG. These lines of evidence confirmed that *Pseudomonas* nitrite reductase was responsible for IL-8 production in the BET-1A cells. The purified nitrite reductase induced maximal expression of IL-8 mRNA in the BET-1A cells at 1 to 3 h after stimulation, and the IL-8 mRNA expression declined by 8 h after stimulation. New protein translation was not required for nitrite reductase-mediated IL-8 mRNA expression in the BET-1A cells. Nitrite reductase stimulated the BET-1A cells, as well as human alveolar macrophages, pulmonary fibroblasts, and neutrophils, to produce IL-8. In contrast, nitrite reductase induced significant levels of tumor necrosis factor alpha and IL-1β protein only in human alveolar macrophages. These data support the notion that nitrite reductase from *P. aeruginosa* induces the production of inflammatory cytokines by respiratory cells and may contribute to the pathogenesis of chronic airway diseases and persistent *P. aeruginosa* infection.

Inflammation in the airway of patients with chronic airway diseases (CAD), such as cystic fibrosis and diffuse panbronchiolitis (DPB), is characterized by dense neutrophil infiltrations (11, 17, 24). Mucoid strains of *Pseudomonas aeruginosa* often arise as a chronic infection in the late stage of these diseases (7, 10, 28). We previously reported that persistent *P. aeruginosa* infection increased the levels of the neutrophil chemoattractant interleukin-8 (IL-8) and induced dense neutrophil infiltrations in the airway of patients with CAD (28). In addition, a significant role for IL-8 in neutrophil accumulation in the airway of patients with CAD and persistent *P. aeruginosa* infection was clearly demonstrated (28, 32). These observations confirm that a perpetual cycle of IL-8 production and neutrophil accumulation caused by persistent *P. aeruginosa* infection plays an important role in the pathogenesis of CAD. Recent studies show that *Pseudomonas* products strongly stimulate neutrophils to produce IL-8 in vitro and in vivo (14, 19, 28). In addition, bronchial epithelial cells are regarded as one of the major sources of airway IL-8 in patients with CAD and *P. aeruginosa* infection (15, 28). A variety of stimuli, including tumor necrosis factor alpha (TNF-α), IL-1β, phorbol 12-myristate 13-acetate, and neutrophil elastase, induce IL-8 production or IL-8 gene expression in bronchial epithelial cells (25, 26). It has been shown recently that several products of *P. aeruginosa* similarly stimulate bronchial epithelial cells to induce IL-8 in vitro (5, 22). Triggering of IL-8 production in bronchial epithelial cells by these *Pseudomonas* products may enhance neutrophil accumulation in the airway of patients with CAD.

In the present study, we demonstrate that a heat-stable protein purified from the supernatants of sonicated *P. aeruginosa* (SSPA) stimulates human bronchial epithelial cells to produce IL-8. To investigate the biochemical and functional properties of the IL-8-inducing protein, we purified the protein to homogeneity, determined its N-terminal amino acid sequence, and evaluated the ability of the purified protein to induce inflammatory cytokines in human respiratory cells, including human bronchial epithelial cells. We report here that a well-known enzyme, nitrite reductase from *P. aeruginosa*, is an inflammatory cytokine inducer in human respiratory cells.

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MATERIALS AND METHODS

Bacteria. A serum-sensitive strain with a mucoid phenotype, *P. aeruginosa* 5276, was isolated from a patient with DPB (11). The bacteria were grown overnight in 3 liters of Mueller-Hinton broth (Difco Laboratories, Detroit,

TABLE 1. Purification of IL-8-inducing protein from the SSPA

Purification step	Vol (ml)	Total amt of protein (mg)	Total activity (ng)[a]	Sp act (ng/mg)	Yield (%)	Purification factor
SSPA	200	408	27,813	68	100	1
50% SAS[b]-precipitated SSPA	20	87.5	10,888	124	39.1	1.82
26/10 Q Sepharose	44	0.74	682	922	2.5	13.6
Mono-Q HR 5/5	1	0.05	105	2,177	0.4	32.0

[a] Total activity is defined as the activity that induces IL-8 production in confluent BET-1A cells for 24 h.
[b] Saturated ammonium sulfate.

Mich.). When the bacteria reached the postlog phase, they were harvested in 200 ml of sterile normal saline. The culture supernatant of *P. aeruginosa* (CSPA) was obtained by centrifugation at 9,000 × *g* for 20 min and filtered through a 0.2-μl-pore-size filter. Harvested bacterial cells were sonicated 10 times with an ultrasonifier (cell disruptor 185; Branson Ultrasonics Co., Danbury, Conn.) at 1-min intervals. The SSPA was obtained after ultracentrifugation at 80,000 × *g* for 60 min at 4°C and filtration through a 0.2-μl filter. The SSPAs were similarly prepared from four mucoid and three nonmucoid clinical isolates of *P. aeruginosa*. The CSPAs and SSPAs were stored at −80°C until use.

IL-8, IL-1β, and TNF-α assay. The IL-8 levels were determined by an enzyme-linked immunosorbent assay (ELISA) with monoclonal antibody WS 4 as the capturing antibody and polyclonal rabbit anti-IL-8 antibody as the second antibody; both of these antibodies were raised against human recombinant IL-8 as previously described (18). The detection limit of this assay was 31.1 pg of IL-8 per ml. IL-1β and TNF-α levels in culture medium supernatants were measured with ELISA kits provided by Otsuka Pharmaceutical Co., Tokushima, Japan. The detection limits of IL-1β and TNF-α were 20 and 30 pg/ml, respectively.

Cell culture. BET-1A, which is a human cell line transformed by simian virus 40 (a kind gift from C. C. Harris, National Cancer Institute, Bethesda, Md.), was cultured in serum-free LHC-9 medium (Biofluids, Rockville, Md.) with 25 μg of amphotericin B per ml, 25 U of penicillin per ml, and 25 μg of streptomycin per ml in a 24-well plate coated with fibronectin and collagen (25, 26). After confluent cultures were washed with HEPES-buffered saline, the BET-1A cells were incubated with the SSPA, CSPA, or filtered samples following chromatography, diluted with LHC-9 medium. To assess cell viability, lactate dehydrogenase release was measured with an in vitro toxicology assay kit (Sigma Chemical Co., St. Louis, Mo.); it never exceeded 5% under these conditions in the absence or presence of cycloheximide (40 μg/ml; Sigma Chemical Co.). The uniformity of the monolayer was also determined by quantifying the cell number per well. Cell-free supernatants of culture media were harvested after incubation for the indicated times. All supernatants of culture media were stored at −80°C for less than 1 week until tested by ELISA for IL-8, IL-1β, and TNF-α. In preliminary experiments, the BET-1A cells were incubated with different dilutions (1:5, 1:50, and 1:500) of SSPA or CSPA for 24 h. The 1:50 dilution for the SSPA and the 1:5 dilution for the CSPA were confirmed to be capable of inducing more than 3 ng of IL-8 per ml for 24 h in the BET-1A cells. IL-8 production by the BET-1A cells was dependent on protein synthesis, because the addition of cycloheximide at 40 μg/ml completely inhibited IL-8 release for 3 h in response to the SSPA (data not shown). Lipopolysaccharide (LPS) from *P. aeruginosa* immunotype 1 (List Biological Laboratories, Campbell, Calif.) at 1 μg/ml did not induce IL-8 production by the BET-1A cells. Each value represents the mean ± standard deviation (SD) of three determinations.

Gel filtration. A 0.5-ml volume of the SSPA solution was loaded onto a Superose 12 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) at a flow rate of 1.0 ml/min in PBS. Each filtered effluent was tested on the BET-1A cell line. Each active fraction, concentrated fivefold with a Vivapore concentrator (Vivascience), was treated with trypsin (40 μg of trypsin type IX per ml; Sigma Chemical Co.) or pronase (200 μg of pronase type E XIV per ml; Sigma Chemical Co.) for 120 min at 37°C and subsequently boiled for 10 min to inactivate the enzymes. Untreated samples were also boiled for 10 min and subsequently tested on the BET-1A cell culture.

Purification of IL-8-inducing protein. The purification steps for the IL-8-inducing protein from the SSPA are shown in Table 1. A 200-ml sample of the SSPA was precipitated with 50% saturated ammonium sulfate, and the precipitates were obtained by ultracentrifugation at 80,000 × *g* and 4°C. The precipitates were dissolved in 1/10 of the original volume of phosphate-buffered saline (PBS) and dialyzed against 20 mM Tris-HCl buffer (pH 8.1) containing 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Chemical Co.). The dialyzed sample was applied to a PD-10 column (Pharmacia Biotech) for further desalting. Then 20 ml of the desalted sample was applied to a 26/10 Q Sepharose Fast Flow column (Pharmacia Biotech) at a flow rate of 3.0 ml/min in 20 mM Tris-HCl (pH 8.1) containing 1 mM PMSF and eluted with a 0 to 200 mM NaCl gradient. Sample from each fraction was subjected to the BET-1A cell assay. Fractions containing IL-8-inducing activity were pooled and dialyzed against 20 mM Tris-HCl containing 1 mM PMSF. Then 44 ml of the dialyzed sample was loaded onto a Mono-Q HR 5/5 column (Pharmacia Biotech) at a flow rate of 1 ml/min in 20 mM Tris-HCl containing 1 mM PMSF and eluted with a 0 to 200 mM NaCl gradient. A 5-ml sample of the Mono-Q HR 5/5 and Superose 12 HR 10/30 eluate was applied to a μBondasphere reversed-phase high-performance liquid chromatography (HPLC) column (3.9 by 150 mm; Millipore Ltd., Tokyo, Japan) with a linear concentration gradient of acetonitrile from 20 to 60% (vol/vol) in 0.5% (vol/vol) trifluoroacetic acid at a flow rate of 0.5 ml/min at 37°C. Each eluted sample of 1 ml was evaporated by an automatic environmental Speedvac (Savant Instruments Inc., Farmingdale, N.Y.), resuspended in 100 μl of distilled water, and tested on the BET-1A cells.

SDS-PAGE. Purified protein, the SSPA or the 50% saturated ammonium sulfate-precipitated SSPA was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5% stacking gel and a 10% separating gel. The gels were fixed by incubation in 40% ethanol and 5% acetic acid and were then silver stained.

N-terminal amino acid sequencing. The protein purified by ion-exchange chromatography was separated by SDS-PAGE and then electrically blotted onto a polyvinylidene difluoride membrane as specified by the manufacturer (LKB Instruments, Stockholm, Sweden). After the membrane was stained with Coomassie brilliant blue, the protein band was cut out and analyzed with a PSQ-1 gas-phase protein sequencer (Shimadzu, Kyoto, Japan).

Expression of recombinant *Pseudomonas* nitrite reductase and preparation of a polyclonal antibody against recombinant nitrite reductase. *P. aeruginosa* 5276 was grown as described above, and total DNA was isolated (21). Nitrite reductase cDNA was obtained by PCR with a set of specific primers, 5′-GCCCATG GCGAAGGACGACATGAAAGCCGCCGAGCAA-3′ and 5′-TTGTGGGTC GTGCTGCACATGACTACTCTTAAGCG-3′, and *P. aeruginosa* 5276 total DNA as a template. The PCR product was cloned into the *Nco*I and *Eco*RI sites of the pSCFV1 vector and transduced into *Escherichia coli* HB101. The transformant was cultured in modified M9 medium with 3-indoleacrylic acid at 37°C for 24 h. The culture fluid was centrifuged at 4,000 × *g* for 5 min, and the cell pellet was sonicated with distilled water for 1.5 min. The pellet was solubilized with distilled water and an equal volume of SDS sample buffer. The equivalent of 0.4 ml of bacterial culture was loaded onto an SDS-PAGE gel (8% polyacrylamide). After electrophoresis and staining for 5 min with aqueous Coomassie brilliant blue (1 volume of 3% Coomassie brilliant blue in methanol, 4 volumes of 0.1 M Tris-HCl [pH 7.4]), the nitrite reductase band was cut out. The gel was fragmented, and 0.5 ml of 25% isopropanol was added. After being stirred at 4°C for 12 h, this isopropanol solution was aspirated and replaced by a fresh solution. After further stirring, 25% isopropanol was aspirated and exchanged for 10% methanol and then finally for PBS. For immunization, the remaining gel, containing recombinant nitrite reductase, was ground with PBS and emulsified with an equal volume of complete Freund's adjuvant. A 16-week-old male New Zealand White rabbit (2.5 kg; Sankyo Laboratory Service Co. Ltd., Toyama, Japan) was given recombinant nitrite reductase (equivalent to 0.53 ml of bacterial culture) with the gel twice at an interval of 1 week. After two further immunizations with incomplete Freund's adjuvant at intervals of 1 to 2 weeks, antiserum against recombinant nitrite reductase was obtained. The immunoglobulin G (IgG) fraction was purified from the antiserum on a protein A-Sepharose column (Pharmacia Biotech). The purified nitrite reductase or the SSPA was separated by SDS-PAGE (10% polyacrylamide), transferred to the nitrocellulose membrane, analyzed by immunoblotting with a polyclonal IgG against recombinant nitrite reductase at 1 μg/ml and horseradish peroxidase-conjugated streptavidin (Amersham, Little Chalfont, United Kingdom), and subjected to detection by enhanced chemiluminescence (Amersham).

Immunoprecipitation. A 400-μl volume of the 10-fold-concentrated SSPA was incubated with 0.66 mg of a polyclonal IgG against recombinant nitrite reductase or the same amount of control normal rabbit IgG (Cappel, West Chester, Pa.) at 4°C overnight. A 100-μl volume of protein G-Sepharose (Pharmacia Biotech) in 20 mM sodium phosphate (pH 7.0) was added to the reaction mixture, which was then incubated at 35°C for 30 min. Immunoprecipitates were washed three times with 0.5 ml of PBS and were incubated with 0.2 ml of glycine-HCl (pH 2.7) for 5 min. The reaction mixtures were centrifuged at 10,000 × *g* for 3 min, and the supernatants containing immunoprecipitates released from protein G-Sepharose were separated and dialyzed against PBS at 4°C. The dialyzed samples were analyzed on the BET-1A cell culture and by immunoblotting.

Inflammatory cytokine induction by the purified protein in different respiratory cells. IL-8, TNF-α and IL-1β production by different human respiratory cells in response to the purified protein was determined. Alveolar macrophages and neutrophils were collected from healthy nonsmokers as previously described (28). Alveolar macrophages (1×10^5 cells/ml) were cultured in Eagle minimal

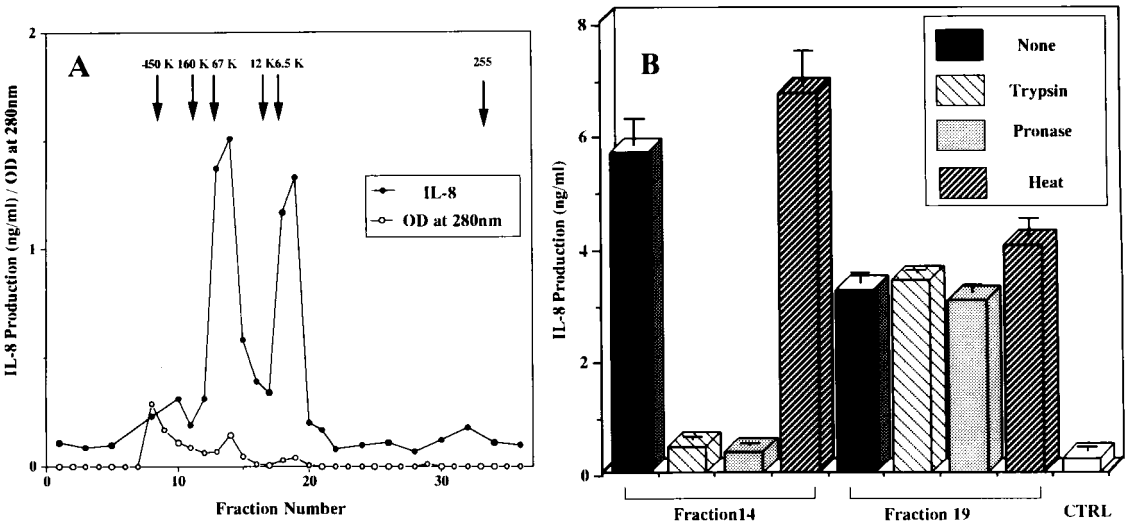

FIG. 1. (A) Gel chromatography of the SSPA and IL-8 production by BET-1A cells in response to each fraction. A 0.5-ml volume of the SSPA solution was applied to a Superose 12 HR 10/30 column at a flow rate of 1.0 ml/min in PBS. Then 1:5-diluted filtered samples from each fraction in LHC-9 medium were tested on the BET-1A cells, and IL-8 production at 24 h poststimulation in the cell-free supernatants of culture media were measured by ELISA. The molecular mass was calibrated with standard markers (ferritin, 450 kDa [450 K]; aldolase, 160 kDa [160 K]; bovine serum albumin, 67 kDa [67 K]; cytochrome *c*, 12 kDa [12 K]; aprotinin, 6.5 kDa [6.5 K]; DNA-alanine, 255 Da [255]). (B) Effects of proteolytic digestion and heating on IL-8 inducing activity of the two major IL-8 inducing fractions. Fivefold-concentrated samples from fractions 14 and 19 were filtered and pretreated with trypsin (40 μg/ml) or pronase (200 μg/ml) for 2 h or heated at 100°C for 10 min, and the reaction mixtures (diluted 1:5 in LHC-9 medium) were tested on the BET-1A cells. IL-8 production was measured as described for panel A. Each value represents the mean ± SD of three determinations. OD, optical density.

essential medium (Nissui, Tokyo, Japan) containing 25 mM HEPES and 1% nonessential amino acid (Gibco, Grand Island, N.Y.). Neutrophils (4×10^6 cells/ml) were cultured in RPMI 1640 (Nissui) containing 1 mM L-glutamine and 25 mM HEPES. A human lung fibroblast cell line (CCD-18Lu) was cultured in Eagle minimal essential medium containing 10% fetal calf serum (Gibco) (33). The purified protein (0 to 10 μg/ml) was added to the culture medium, and the cell-free supernatants were harvested for determination of inflammatory cytokines by ELISA at 24 h poststimulation.

RNA preparation and RT-PCR. Total cellular RNA was extracted from the BET-1A cells with Isogen LS (Wako Pure Chemical Co. Ltd., Osaka, Japan), and purified RNA was quantitated by measuring the absorbance at 260 nm. cDNA was synthesized from 2 μg of total RNA from the BET-1A cells by priming with 2.5 μM of oligo(dT) primer, 1 mM each deoxynucleoside triphosphate, and reverse transcriptase. cDNA equivalent to 80 ng of starting RNA was used for each PCR with primers for human IL-8 or control human glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers were as follows: for IL-8 cDNA, 5′-ATTTCTGCAGCTCTGTGTGAAGGTGC-3′ (sense) and 5′-TTGT GGATCCTGGCTAGCAGAC-3′ (antisense) (23); and for GAPDH cDNA, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ (sense) and 5′-CATGTGGGC CATGAGGTCCACCAC-3′ (antisense) (34). PCRs were performed with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM deoxynucleoside triphosphates, and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer, Branchburg, N.J.) in a final volume of 100 μl. The primers were added at a final concentration of 0.1 μM. The reactions were carried out in a DNA thermal cycler (Perkin-Elmer) for 45 cycles, each including denaturing at 95°C for 1 min, annealing at 56°C for 2 min, and extension at 74°C for 2 min. The PCR products were analyzed on ethidium bromide-stained 1% agarose gels. Reverse transcription PCR (RT-PCR) was performed on total RNA isolated from confluent cultures at the indicated times after stimulation.

RESULTS

Induction of IL-8 production by SSPA. The IL-8 levels in the culture supernatants of unstimulated bronchial epithelial cells at 24 h poststimulation were negligible (0.2 ± 0.1 ng/ml). While the 1:5-diluted CSPA-stimulated cells produce a relatively small amount of IL-8 (3.6 ± 0.6 ng/ml) at 24 h, as previously described (22), treatment with the 1:50-diluted SSPA from *P. aeruginosa* 5276 resulted in an increase of IL-8 production in the same cells at 6 h (11.9 ± 1.7 ng/ml), reaching 16.9 ± 2.1 ng/ml at 24 h poststimulation. The SSPAs (diluted 1:50) from four mucoid and three nonmucoid clinical isolates of *P. aeruginosa* similarly stimulated bronchial epithelial cells to produce IL-8 (9.2 to 16.0 ng/ml for 24 h poststimulation). These results confirmed that the SSPAs contained a potent IL-8-inducing activity for the BET-1A cells.

Gel filtration of SSPA. To characterize the substances of SSPA which induce IL-8, we performed gel filtration chromatography of the SSPA on a Superose 12 column and determined the IL-8-inducing activity of each eluted fraction. We detected two separate fractions showing IL-8-inducing activity (Fig. 1A). Compared to elutions of marker proteins, these separable fractions were estimated chromatographically to have molecular masses of 39 to 61 kDa and 3 to 4 kDa, respectively. We identified two fractions (fractions 14 and 19) as the high- and low-molecular-mass substance and examined the effects of proteolytic digestion and heat treatment on their IL-8-inducing activities (Fig. 1B). The IL-8-inducing activity of fraction 14 (5.7 ± 0.5 ng/ml) was significantly reduced after treatment with trypsin (0.5 ± 0.1 ng/ml) or pronase (0.4 ± 0.1 ng/ml) but was not influenced by heat treatment (6.2 ± 0.6 ng/ml). In contrast, treatment with trypsin (3.4 ± 0.1 ng/ml), pronase (3.1 ± 0.1 ng/ml), or heat (4.1 ± 0.3 ng/ml) did not alter the activity of fraction 19 (3.3 ± 0.2 ng/ml). These data indicate that the SSPA contains two heat-stable IL-8-inducing factors, a protein with a molecular mass of 39 to 61 kDa and a nonprotein factor with a molecular mass of 3 to 4 kDa.

Purification of IL-8-inducing protein. We next subjected the dialyzed sample of ammonium sulfate-precipitated SSPA to 26/10 Q Sepharose Fast Flow chromatography. A single broad peak showing IL-8-inducing activity was eluted at approximately 150 mM NaCl (Fig. 2A). After collecting these fractions, we loaded the dialyzed sample onto a Mono-Q HR 5/5 column and eluted it. A single sharp peak showing IL-8-inducing activity was eluted at around 150 mM NaCl (Fig. 2B). The SDS-PAGE (10% polyacrylamide) of the SSPA and the saturated ammonium sulfate-precipitated SSPA is shown in Fig. 3, lanes 2 and 3, respectively, and the purified protein containing a single band with a molecular mass of 59 kDa is shown in lane 4 (Fig. 3). The IL-8-inducing fraction from the Mono-Q HR

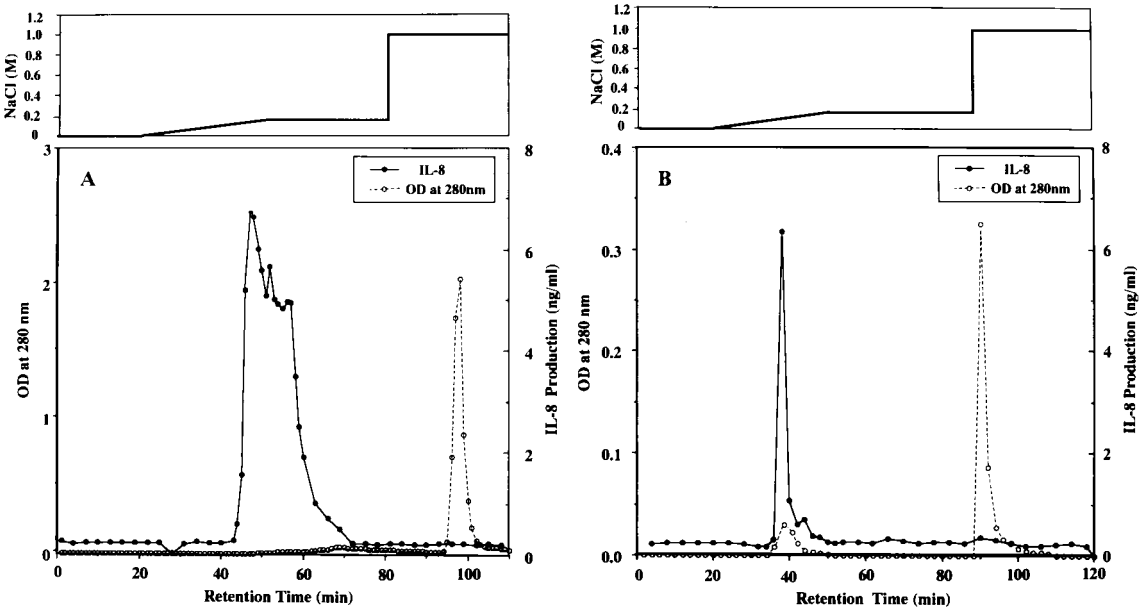

FIG. 2. Purification of IL-8-inducing protein by sequential ion-exchange chromatography. (A) A 20-ml volume of saturated ammonium sulfate-precipitated SSPA, dialyzed against 20 mM Tris-HCl (pH 8.1) and further desalted on the PD-10 column, was loaded onto a 26/10 Q Sepharose Fast Flow column at a flow rate of 3.0 ml/min in 20 mM Tris-HCl (pH 8.1) and eluted with 0 to 200 mM NaCl. (B) The fraction containing IL-8-inducing activity dialyzed against 20 mM Tris-HCl was next applied to a Mono-Q HR 5/5 column and eluted with a 0 to 200 mM NaCl gradient. The filtered samples following the chromatography steps, diluted 1:10 in LHC-9 medium, were tested on the BET-1A cells, and IL-8 production was measured as described above. OD, optical density.

5/5 chromatography was concentrated fivefold with a Vivapore concentrator and was reapplied to a Superose 12 HR 10/30 column as described above. After gel filtration, a single IL-8-producing activity with an estimated molecular mass of 61 kDa was found in fraction 13 (data not shown). A 5-ml volume of sample from this fraction was then subjected to reversed-phase HPLC. This protein was eluted as a single peak, which was associated with a high IL-8-inducing activity (Fig. 4). Similarly, the fraction containing the high IL-8-inducing activity following reversed-phase HPLC showed a single band with a molecular mass of 59 kDa, as shown in Fig. 3, lane 5. The concentrations of purified protein were estimated by the Bradford assay (Bio-Rad Laboratories, Hercules, Calif.) as specified by the manufacturer. The protein solution following elution from the Mono-Q HR 5/5 column contained 30 ng of LPS per ml when examined by a sensitive Toxicolor test (Seikagaku Kogyo Co., Tokyo, Japan) (27).

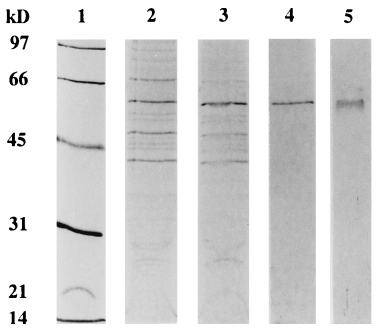

FIG. 3. SDS-PAGE of molecular mass markers (lane 1), the SSPA (lane 2), the saturated ammonium sulfate-precipitated SSPA (lane 3), the protein purified on a Mono-Q HR 5/5 column (lane 4), and the protein purified by sequential gel chromatography and reversed-phase HPLC (lane 5).

Amino acid sequences of the purified protein. Automated Edman degradation of the purified protein with a Shimadzu PSQ-1 gas-phase protein sequencer yielded an N-terminal sequence of 14 amino acid residues (Lys-Asp-Asp-Met-Lys-Ala-Ala-Glu-Glu-Tyr-Gln-Gly-Ala-Ala-). Fast homology analysis indicated that the N-terminal sequence completely matched a

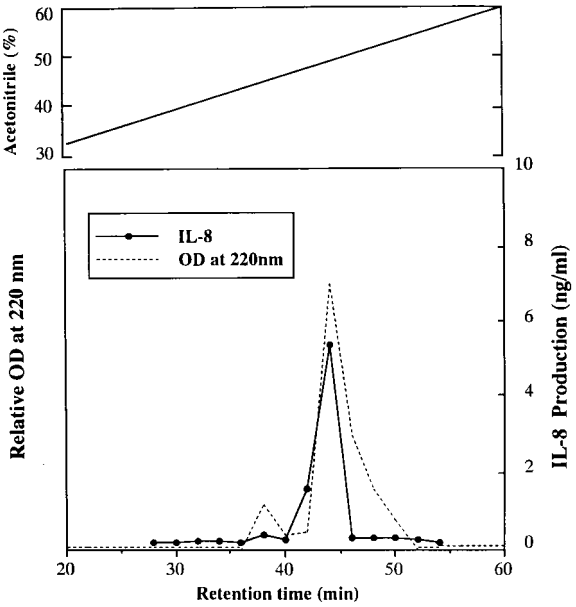

FIG. 4. Reversed-phase HPLC of the protein purified by sequential ion-exchange chromatography and gel filtration chromatography. OD, optical density.

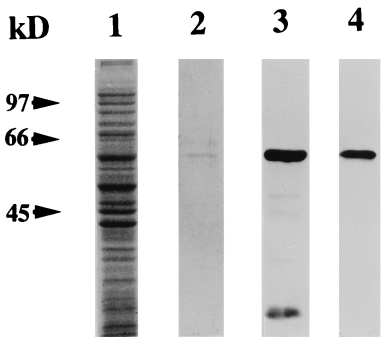

FIG. 5. Coomassie brilliant blue-stained polyacrylamide gel of the SSPA (lane 1), the protein purified on a Mono-Q HR 5/5 column (lane 2), and the immunoblot analysis of the SSPA (lane 3) and the purified protein (lane 4) with a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase at 1 μg/ml. The protein bands of the SSPA and the purified protein were transferred from an identical and unstained companion gel.

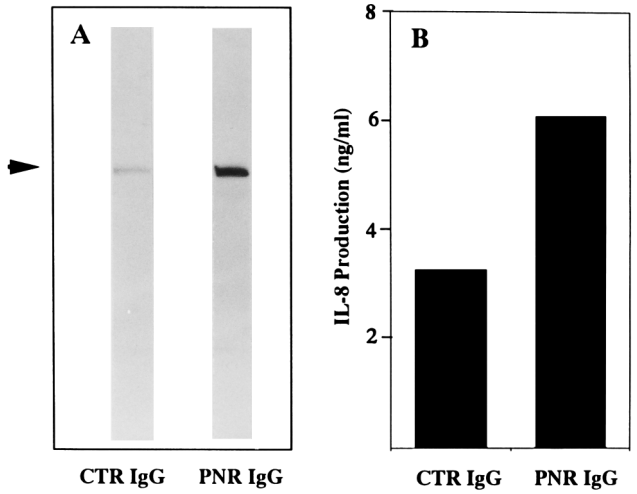

FIG. 6. (A) Immunoblot analysis of immunoprecipitates of the SSPA with a control polyclonal IgG (CTR IgG) or a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase (PNR IgG) with a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase. Immunoprecipitates were prepared as described in Materials and Methods. The arrowhead on the left shows the position of purified *Pseudomonas* nitrite reductase. (B) IL-8 production in BET-1A cells in response to the immunoprecipitates of the SSPA with a control IgG (CTR IgG) or a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase (PNR IgG). These samples (diluted 1:10 in LHC-9 medium) were tested on the BET-1A cells, and IL-8 production was measured as described above.

sequence at the N-terminal part of the mature nitrite reductase protein from *P. aeruginosa* (35).

Immunoblotting. The anti-recombinant nitrite reductase IgG reacted with a major antigen with a molecular mass of around 60 kDa, which corresponded to the purified nitrite reductase, and a minor band with low molecular mass in the SSPA (Fig. 5, lane 3). The latter band appeared to be a proteolytic product of *Pseudomonas* nitrite reductase. A polyclonal IgG against recombinant *Pseudomonas* nitrite reductase demonstrated specific binding to the purified IL-8-inducing protein with a molecular mass of 59 kDa (lane 4). In contrast, the same amount of a control normal rabbit IgG gave only a trace band (data not shown).

Immunoprecipitation. To confirm whether *Pseudomonas* nitrite reductase is responsible for IL-8 production in the BET-1A cells, immunoprecipitates of the SSPA with a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase or a control polyclonal IgG were separated by SDS-PAGE and visualized by immunoblotting with a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase (Fig. 6A). The immunoblot analysis with anti-recombinant *Pseudomonas* nitrite reductase IgG exhibited a broad band with a molecular mass of 59 kDa in the immunoprecipitates of the SSPA with a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase but exhibited a faint band with the same molecular mass in the immunoprecipitates of the SSPA with a control IgG. *Pseudomonas* nitrite reductase in the SSPA appeared to be showing a strong and specific binding to anti-recombinant *Pseudomonas* nitrite reductase IgG and a weak binding to a control IgG. The weak recognition of *Pseudomonas* nitrite reductase by a control IgG may suggest the existence of natural antibody in the control IgG. Furthermore, the dialyzed immunoprecipitate of SSPA with a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase stimulated the BET-1A cells to produce a higher level of IL-8 (6.1 ng/ml), while the dialyzed immunoprecipitate with a control IgG induced 3.3 ng of IL-8 per ml for 24 h poststimulation (Fig. 6B). The higher production of IL-8 in response to the immunoprecipitate of SSPA with anti-recombinant *Pseudomonas* nitrite reductase IgG confirmed that *Pseudomonas* nitrite reductase was responsible for IL-8 production in the BET-1A cells.

IL-8 mRNA expression by BET-1A cells. We used RT-PCR to evaluate IL-8 mRNA expression in response to the purified nitrite reductase (10 μg/ml) in the BET-1A cells. The RT-PCR products obtained with GAPDH were included for comparison. Increases in IL-8 cDNA levels were first detected after 1 h of nitrite reductase stimulation (10 μg/ml), and maximum expression of IL-8 mRNA was seen at 3 h poststimulation. The expression of IL-8 mRNA diminished at 8 h and disappeared at 24 h poststimulation (Fig. 7). We also assessed whether nitrite reductase-induced IL-8 gene expression in the BET-1A cells at 3 h requires new protein synthesis. Treatment with cycloheximide suppressed IL-8 protein synthesis by nitrite reductase-stimulated BET-1A cells at 3 h poststimulation (2.2 ng/ml in the absence of cycloheximide; 0.1 ng/ml in the presence of cycloheximide) but did not inhibit the nitrite reductase-mediated increase in steady-state IL-8 mRNA expression. In addition, treatment of BET-1A cells with cycloheximide (40 μg/ml) alone did not alter the steady-state IL-8 mRNA expression levels (data not shown).

Induction of inflammatory cytokines. Addition of *Pseudomonas* nitrite reductase at 10 μg/ml stimulated the BET-1A cells to produce IL-8 in a time-dependent manner up to 24 h poststimulation (Fig. 8A). A dose-dependent production of IL-8 in response to the nitrite reductase at a concentration range of 0 to 10 μg/ml was also observed (Fig. 8B). The IL-8-inducing activity (2.4 ng/ml) of the purified protein (5 μg/ml) in the BET-1A cells for 24 h poststimulation was significantly reduced after treatment with trypsin (0.14 ng/ml) or pronase (0.12 ng/ml). However, the heat treatment did not influence

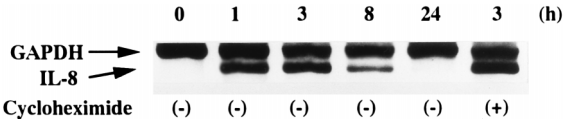

FIG. 7. Time course of IL-8 mRNA expression by the BET-1A cells after stimulation with *Pseudomonas* nitrite reductase (10 μg/ml). IL-8 mRNA expression induced by *Pseudomonas* nitrite reductase (10 μg/ml) at different times is shown. To investigate the effect of cycloheximide on IL-8 mRNA expression, the BET-1A cells were also simultaneously treated with the same concentration of *Pseudomonas* nitrite reductase and cycloheximide (40 μg/ml) for 3 h.

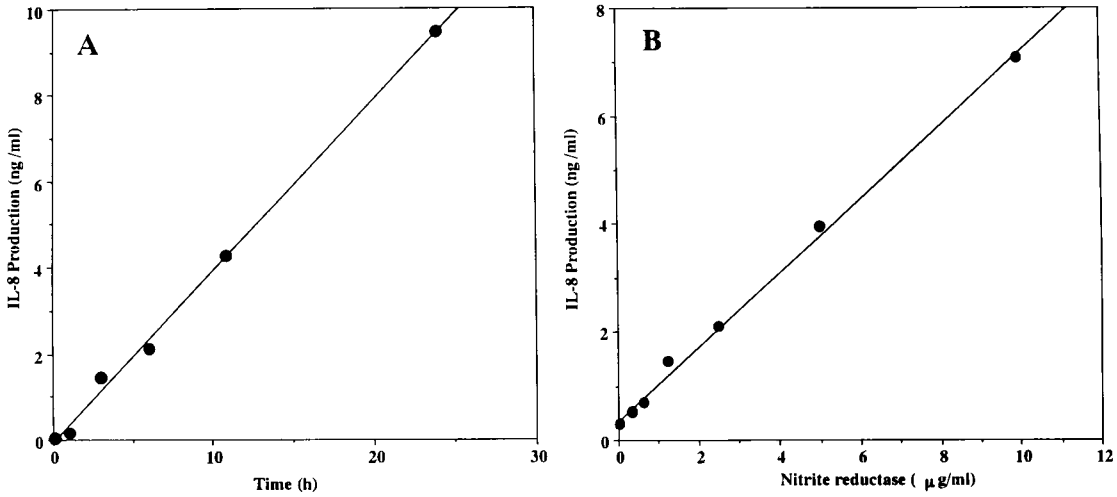

FIG. 8. Time- and dose-dependent IL-8 production by BET-1A cells in response to the nitrite reductase from *P. aeruginosa*. (A) The BET-1A cells were stimulated with 10 μg of *Pseudomonas* nitrite reductase per ml, and the cell-free supernatants were harvested at various times. (B) The BET-1A cells were stimulated with various concentrations (0 to 10 μg/ml) of *Pseudomonas* nitrite reductase for 24 h, and the cell-free supernatants were harvested. IL-8 levels in the culture supernatants were determined by ELISA. Each value represents the mean of two determinations.

the IL-8-inducing activity (2.7 ng/ml). The biochemical characteristics of the purified *Pseudomonas* nitrite reductase were similar to those of fraction 14 after gel filtration chromatography of the SSPA. The purified nitrite reductase (10 μg/ml) similarly induced IL-8 production by human pulmonary fibroblasts (CCD-18Lu cells) and neutrophils at 24 h poststimulation, compared to the levels produced by unstimulated controls (Table 2). However, significant production of TNF-α or IL-1β was not detected in the culture supernatants of human bronchial epithelial cells, human pulmonary fibroblasts, or neutrophils. For human alveolar macrophages, adherence to the plastic dish induced a high level of IL-8 production at 24 h (36). Purified nitrite reductase at 5.0 μg/ml increased IL-8 production by adherent alveolar macrophages at 24 h compared to the level produced by unstimulated adherent alveolar macrophages (Table 2). Interestingly, nitrite reductase at 5.0 μg/ml induced significant levels of TNF-α or IL-1β production by alveolar macrophages at 24 h, whereas unstimulated adherent alveolar macrophages produced negligible levels of TNF-α or IL-1β. LPS at 30 ng/ml did not induce significant levels of inflammatory cytokine production (mean IL-8 level, 133.2 ng/ml; mean TNF-α level, 0.1 ng/ml; mean IL-1β level, 0.1 ng/ml) by alveolar macrophages at 24 h, compared to the levels of unstimulated controls.

DISCUSSION

There is increasing evidence that the bronchial epithelium participates in the inflammation of CAD, including cystic fibrosis, DPB, and asthma (13, 15, 28). *P. aeruginosa* infection is closely associated with the progression of these diseases (7, 10, 28). *Pseudomonas* products appear to stimulate bronchial epithelial cells to induce IL-8 production, causing neutrophils to accumulate in the airway of patients with CAD and *P. aeruginosa* infection. DiMango et al. have recently reported that the binding of *Pseudomonas* pilin or flagellin to their asialo-GM_1 receptors on respiratory epithelial cells results in IL-8 production (5). They have also shown that *Pseudomonas* autoinducer, an exoproduct from *P. aeruginosa*, induces IL-8 protein in respiratory epithelial cells. This homoserine lactone derivative is a diffusible molecule, which acts as a cofactor for the transcriptional activator LasR. Similarly, the CSPA contains a substance which induces IL-8 in bronchial epithelial cells, since Massion et al. demonstrated that a nonprotein factor of less than 1 kDa in CSPA could induce IL-8 production (22). In the present study, we found IL-8-inducing activity in bronchial epithelial cells in the SSPA from clinical isolates of *P. aeruginosa* and focused on an IL-8-inducing protein in the SSPA. The elution pattern of the heat-stable, nonprotein factor with a low molecular mass in the SSPA was identical to that of the

TABLE 2. Production of inflammatory cytokines by different types of respiratory cells in response to nitrite reductase from *P. aeruginosa*[a]

Cytokine	Cytokine production (ng/ml)[b] by nitrite reductase in:							
	BET-1 A cells		CCD-18Lu cells		Neutrophils		Alveolar macrophages	
	0 μg/ml[c]	10 μg/ml	0 μg/ml	10 μg/ml	0 μg/ml	10 μg/ml	0 μg/ml	5 μg/ml
IL-8	0.3 ± 0.1	7.1 ± 0.7	0.1	1.7	0.1	1.3	123.3 ± 7.9	260.3 ± 28.9
TNF-α	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1 ± 0.1	2.8 ± 0.1
IL-1β	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1 ± 0.1	2.3 ± 0.3

[a] The cell-free supernatants were harvested at 24 h poststimulation for ELISA determination of inflammatory cytokine levels.
[b] Each value represents the mean ± SD of three determinations or the mean of two determinations.
[c] Nitrite reductase concentrations.

nonprotein IL-8-inducing factor in the CSPA, being characterized by a single IL-8-inducing peak with a molecular mass of 3 to 4 kDa on gel filtration chromatography (data not shown). Thus, the intracellularly located, nonprotein IL-8-inducing factor of *P. aeruginosa* may be released into the CSPA during bacterial growth in culture media. On the other hand, the heat-stable IL-8-inducing protein with a molecular mass of 39 to 61 kDa was purified by two sequential ion-exchange chromatography steps and was demonstrated to have a molecular mass of about 59 kDa on SDS-PAGE. This purified protein was similarly eluted by sequential gel filtration and reversed-phase HPLC as a single band with the same molecular mass, associated with a potent IL-8-inducing activity in the BET-1A cells.

More importantly, the purified protein was identified as nitrite reductase from *P. aeruginosa* by analysis of the N-terminal amino acid sequence. Furthermore, a polyclonal IgG against recombinant *Pseudomonas* nitrite reductase recognized the purified protein with a molecular mass of 59 kDa. Therefore, this purified protein was proved to be *Pseudomonas* nitrite reductase. This nitrite reductase, containing hemes *c* and d_1, was originally isolated and purified by Horio et al. (12) and cloned by Silvestrini et al. (35). Functionally, this enzyme aerobically oxidizes reduced *Pseudomonas* cytochrome c_{551} and also anaerobically reduces nitrite to nitric oxide by using reduced *Pseudomonas* cytochrome c_{551} (38, 39). Thus, this bifunctional enzyme has also been called *Pseudomonas* cytochrome oxidase. This enzyme has been recognized as a periplasmic component of energy generation (4) and has been identified most often in denitrifying isolates such as *Alcaligenes faecalis* and *Pseudomonas fluorescens* (3). A recent study of the 1.55-Å crystal structure of this enzyme from *Thiosphaera pantotropha* suggests that the d_1 heme was the mononuclear center where both nitrite and oxygen reduction occurred (9). The mature *Pseudomonas* nitrite reductase protein has been demonstrated to consist of 543 amino acid residues and to have a molecular mass of 60,204 Da (35). Therefore, the calculated value of a molecular mass (59 kDa) was shown to be close to the molecular mass of this mature protein. In the present study, we demonstrated a dose- and time-dependent IL-8 production in human bronchial epithelial cells. The studies of immunoprecipitations of the SSPA with anti-*Pseudomonas* nitrite reductase IgG confirmed that *Pseudomonas* nitrite reductase was responsible for the IL-8-inducing activity in the BET-1A cells. The purified *Pseudomonas* nitrite reductase also stimulated alveolar macrophages, pulmonary fibroblasts, and neutrophils to produce IL-8. Moreover, the purified *Pseudomonas* nitrite reductase also induced TNF-α or IL-1β only in alveolar macrophages. These lines of evidence strongly support the notion that *Pseudomonas* nitrite reductase is an inflammatory cytokine inducer in human respiratory cells.

Nitric oxide has recently been supposed to play major roles in the expression of inflammatory cytokines, such as TNF-α, IL-1β, or IL-6 (6, 29). Recent studies also showed that nitric oxide regulated IL-8 mRNA expression in melanoma cells or endothelial cells (1, 37). In addition, Francoeur and Denis demonstrated that exposure to *P. aeruginosa* bacteria elicited significant nitrite release and IL-8 production by human airway epithelial cells (8), although the authors did not clarify if nitric oxide regulated IL-8 production in upper airway epithelial cells. However, it seems that the purified *Pseudomonas* nitrite reductase did not stimulate respiratory cells to produce inflammatory cytokines by released nitric oxide through its enzymatic reaction, because in vitro cultures were under aerobic conditions without electron donors such as *Pseudomonas* cytochrome c_{551} (38). Alternatively, we propose that a direct stimulation of respiratory cells by the purified cytochrome nitric reductase is a mechanism for IL-8 gene induction. Our preliminary studies with reduced *Pseudomonas* cytochrome c_{551} under aerobic conditions showed a potent cytochrome oxidase activity in the SSPA but not in the purified *Pseudomonas* nitrite reductase (data not shown). Therefore, the cytochrome oxidase activity appears not to be essential for the IL-8-inducing activity by the purified *Pseudomonas* nitrite reductase. Further studies of the relationship between the IL-8-inducing activity and the enzymatic activity of *Pseudomonas* nitrite reductase are under way.

If the *Pseudomonas* nitrite reductase, which is located in the periplasmic space (3), is released from *Pseudomonas* cells in the airway of patients with CAD and *P. aeruginosa* infection, what is the mode of release for this protein? Lysis of bacterial cells due to exposure to complement may play an important role in the release of *Pseudomonas* nitrite reductase in the airway (16), because up to 80% of strains of mucoid *P. aeruginosa* isolated from patients with CAD are sensitive to low concentrations of normal human serum (30). Another possible mechanism for the release of this enzyme might involve administration of bactericidal antibiotics to these patients (2, 20). We have previously reported that a variety of respiratory pathogens induce IL-8 production in the airway of patients with lower respiratory tract infections (31). Therefore, further investigations to determine which respiratory pathogens possess *Pseudomonas* nitrite reductase and induce inflammatory cytokines, including IL-8, in human respiratory cells are required.

Pseudomonas nitrite reductase induces inflammatory cytokines in human respiratory cells and may contribute to the pathogenesis of CAD and *P. aeruginosa* infection. More precise molecular mechanisms of nitrite reductase-mediated induction of inflammatory cytokine production by respiratory cells remain to be elucidated. If the nitrite reductase from *P. aeruginosa* actually persists and induces inflammatory cytokines contributing to the pathogenesis of CAD and *P. aeruginosa* infection, neutralization of *Pseudomonas* nitrite reductase might be a novel target of intervention in CAD.

ACKNOWLEDGMENTS

We are very grateful to K. Kubo and T. Nakatsuru for their technical support and to M. Nakamura, D. W. Nielsen, and H. Young for their helpful comments during the preparation of the manuscript.

This work was supported in part by a grant (06454273) from the Ministry of Education, Science and Culture, Japan.

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Editor: R. E. McCallum