TABLE 2. In vitro susceptibilities of *P. aeruginosa* IMCJ2.S1 and *P. aeruginosa* ATCC 27853 to various antimicrobial agents

Antibiotic	MIC (μg/ml) for:	
	P. aeruginosa IMCJ2.S1	*P. aeruginosa* ATCC 27853
Piperacillin	>128	<4
Piperacillin-tazobactam	64	4
Cefotaxime	>128	8
Ceftazidime	>128	<1
Cefepime	>64	2
Cefoxitin	>64	>64
Flomoxef	>128	>128
Moxalactam	>128	16
Imipenem	128	4
Meropenem	128	1
Aztreonam	128	2
Amikacin	128	2
Arbekacin	2	<0.5
Dibekacin	>128	<0.5
Gentamicin	16	<1
Isepamicin	128	<4
Kanamycin	>128	>128
Netilmicin	>128	<0.5
Sisomicin	>128	<0.5
Streptomycin	>64	<4
Tobramycin	64	<0.5
Tetracycline	32	16
Sulfamethoxazole-trimethoprim	128	32
Levofloxacin	64	<0.5
Ciprofloxacin	32	<0.5
Polymyxin B	2	2
Silver sulfadiazine	64	64

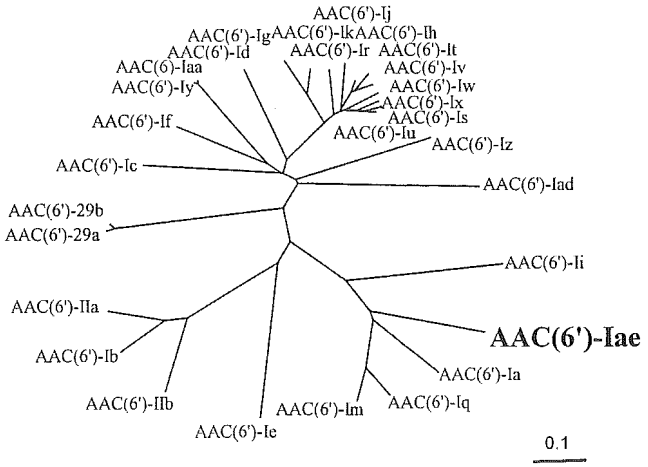

FIG. 4. Dendrogram of aminoglycoside 6′-*N*-acetyltransferases for comparison with AAC(6′)-Iae. The dendrogram was calculated with the CLUSTAL W program. Branch lengths correspond to the number of amino acid exchanges for AAC proteins. EMBL/GenBank/DDBJ accession numbers of AAC proteins are as follows: AAC(6′)-Ia, M18967-1; AAC(6′)-Ib, M23634; AAC(6′)-Ic, M94066; AAC(6′)-Id, X12618; AAC(6′)-Ie, M13771; AAC(6′)-If, X55353; AAC(6′)-Ig, L09246; AAC(6′)-Ih, L29044; AAC(6′)-Ii, L12710-1; AAC(6′)-Ij, L29045; AAC(6′)-Ik, L29510; AAC(6′)-Il, Z54241 and U13880; AAC(6′)-Im, Z54241-2; AAC(6′)-Iq, AF047556-1; AAC(6′)-Ir, AF031326; AAC(6′)-Is, AF031327; AAC(6′)-It, AF031328; AAC(6′)-Iu, AF031329; AAC(6′)-Iv, AF031330; AAC(6′)-Iw, AF031331; AAC(6′)-Ix, AF031332; AAC(6′)-Iy, AF144880; AAC(6′)-Iz, AF140221; AAC(6′)-Iaa, NC_003197; AAC(6′)-Iad, AB119105; AAC(6′)-IIa, M29695; AAC(6′)-IIb, L06163; AAC(6′)-29a, AF263519; AAC(6′)-29b, AF263519.

referred to as AAC(6′)-Ip, by Centrón and Roy (4)] (61.7% identity in a 149-aa overlap) and to AAC(6′)-Ii (9) (40.3% identity in a 166-aa overlap) (Fig. 4). On the basis of the work of Neuwald and Landsman (34), four motifs in the amino acid sequences of the subfamily proteins belonging to AAC(6′)-Iae were designated motifs C, D, A, and B (Fig. 5). Comparison of amino acid sequences of members of the AAC(6′)-I subfamily with that of AAC(6′)-Iae revealed that motifs C, D, A, and B, which are found in most GCN5-related *N*-acetyltransferases (GNATs) (12, 34), were conserved in AAC(6′)-Iae (Fig. 5). A large motif at the C terminus, motif B (12), was 63.3% identical between AAC(6′)-Im (19) and AAC(6′)-Iae. The third cassette was 856 nt long and contained the aminoglycoside 3″-adenyltransferase gene *aadA1* (18, 22) and a 60-nt 59-be site (Fig. 3). This cassette was similar to one reported previously (30, 36) except for a silent C-to-T substitution at nt 135.

The 3′-CS included *qacEΔ1* (39), *sul* (47), and orf5 (30, 37). There were three inserted sequences (IS), IS*1326* (3), IS*1353* (3), and IS*26* (38), in the region downstream of the 3′-CS (Fig. 1). IS*26* is known to be inserted into the *tniA* coding region of the *tni* transposition module (30).

Drug resistance mediated by the AAC(6′)-Iae enzyme. To examine the role of AAC(6′)-Iae in aminoglycoside resistance, a recombinant plasmid, pAAC6, carrying *aac(6′)-Iae* from strain IMCJ2.S1 was transformed into *E. coli* DH5α. *E. coli* harboring pAAC6 showed significantly lower susceptibility to

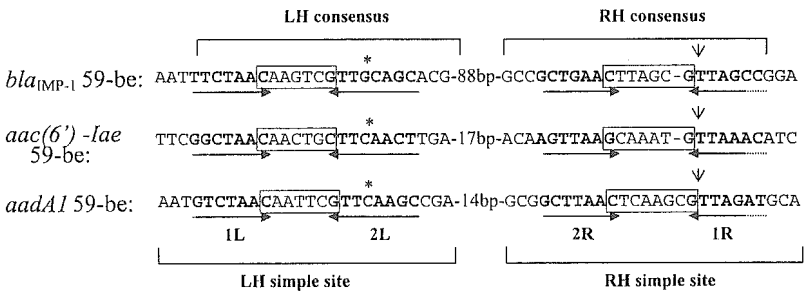

FIG. 3. Structures of 59-be of In113. Seven-base-pair putative core sites in the left-hand (LH) and right-hand (RH) consensus sequences were designated 1L and 2L and 2R and 1R, respectively. The putative recombination event occurs between the G and the first T in the 1R core site and is indicated by vertical arrows (see reference 45). The relative orientations of 1L, 2L, 2R, and 1R are indicated by arrows under the sequence. An extra base in 2L is marked with an asterisk. Inverted repeats are underscored with arrows.

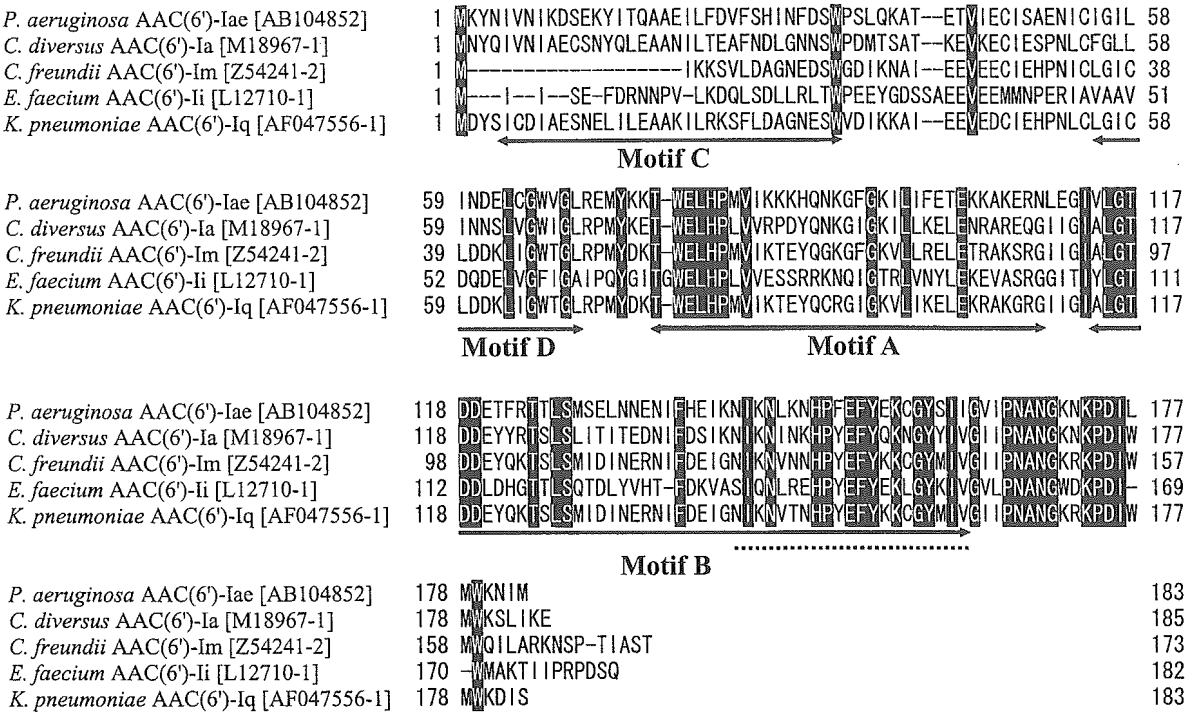

FIG. 5. Alignment of the AAC(6′)-Iae amino acid sequence with those of four members of the AAC(6′)-I subfamily. Identical residues are marked with black boxes. Four motifs, including the highly conserved motif B, are underlined. A conserved region of 21 amino acids, described by Shmara et al. (44), is indicated by a dotted line. GenBank accession numbers are given in brackets to the right of AAC names. *C. diversus*, *Citrobacter diversus*; *C. freundii*, *Citrobacter freundii*.

amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin than the parent strain and the negative control. MICs for other aminoglycosides, including arbekacin, gentamicin, and streptomycin, were unchanged (Table 3). These results indicate that *aac(6′)-Iae* is involved in aminoglycoside resistance.

To examine potential acetylase activity of AAC(6′)-Iae, we assessed the purified recombinant AAC(6′)-Iae against aminoglycosides by thin-layer chromatography (53). As shown in Fig. 6, kanamycin, amikacin, tobramycin, netilmicin, sisomicin, isepamicin, arbekacin, neomycin, and gentamicin were acetylated by AAC(6′)-Iae and AAC(6′). Acetylation by AAC(6′)-Iae was complete for all of these aminoglycosides except gentamicin, which showed incomplete acetylation. These aminoglycosides all have 6′-NH_2. The present results, therefore, suggest that AAC(6′)-Iae is a functional acetyltransferase that modifies the 6′-NH_2 position of aminoglycosides.

Location of In113. Clinical isolates of *P. aeruginosa* frequently possess the R plasmid, which carries a class 1 integron. Therefore, we screened our seven *P. aeruginosa* clinical isolates for the presence of this plasmid. *P. aeruginosa* GN17203 was used as a positive control for bla_{IMP-1}, since it has been shown to harbor pMS350, which contains a bla_{IMP-1} gene. Genomic DNA from IMCJ2.S1 was used as a control for *aac(6′)-Iae* and bla_{IMP-1}.

The extracts from the seven clinical isolates and *P. aeruginosa* GN17203 were separated by agarose gel electrophoresis, and Southern blotting with *aac(6′)-Iae* or bla_{IMP-1} as a probe was performed. A plasmid that contained bla_{IMP-1} but not *aac(6′)-Iae* was detected in *P. aeruginosa* GN17203. Despite

TABLE 3. Aminoglycoside resistance patterns of *E. coli* DH5α alone or harboring plasmids with or without *aac(6′)-Iae*

Strain	MIC (μg/ml)[a] of:										
	AMK	ABK	DIB	GEN	ISE	KAN	NEO	NET	SIS	STR	TOB
E. coli DH5α(pAAC6)[b]	8	0.5	32	0.25	8	64	4	32	16	4	8
E. coli DH5α(pREVAAC6)[c]	0.5	0.5	0.5	0.25	0.25	1	2	0.25	0.25	4	0.5
E. coli DH5α(pCRT7/NT)[d]	0.5	0.25	0.5	0.25	0.25	1	2	0.25	0.25	4	0.25
E. coli DH5α	0.5	0.5	0.5	0.25	0.25	1	2	0.25	0.25	4	0.25

[a] AMK, amikacin; ABK, arbekacin; DIB, dibekacin; GEN, gentamicin; ISE, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; SIS, sisomicin; STR, streptomycin; TOB, tobramycin.
[b] Recombinant plasmid constructed by cloning *aac(6′)-Iae* into pCRT7/NT.
[c] Recombinant plasmid constructed by insertion of DNA fragment with reverse sequence of *aac(6′)-Iae* into pCRT7/NT.
[d] Cloning vector (ABPC).

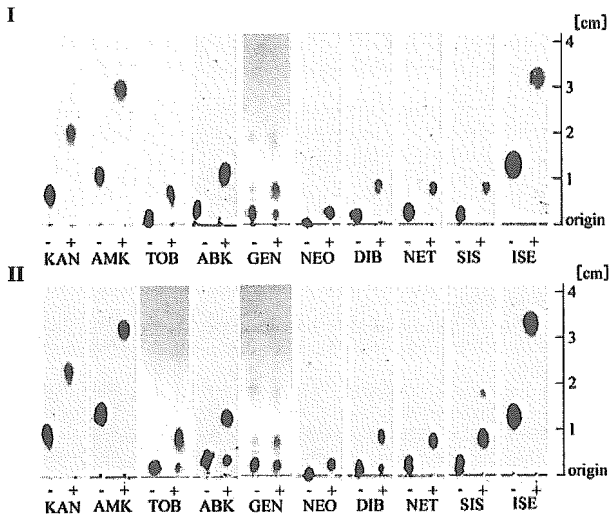

FIG. 6. Thin-layer chromatogram of aminoglycosides incubated with AAC(6′)-Iae protein (I) or with AAC(6′) from *Streptomyces lividans* TK21 as a control (II) (53) in the presence (+) or absence (−) of acetyl coenzyme A. KAN, kanamycin; AMK, amikacin; TOB, tobramycin; ABK, arbekacin; GEN, gentamicin; NEO, neomycin; DIB, dibekacin; NET, netilmicin; SIS, sisomicin; ISE, isepamicin.

repeated attempts (three times per procedure), we did not detect this plasmid by ethidium bromide staining or Southern blotting in any of the clinical isolates (data not shown). In contrast, Southern hybridization of SpeI-, XbaI-, and HpaI-digested genomic DNAs of the seven clinical isolates revealed 50-kb, 250-kb, and 60-kb *aac(6′)-Iae*-positive fragments, respectively (Fig. 2). These fragments were also positive for bla_{IMP-1} (data not shown). To examine whether the drug-resistant phenotype of *P. aeruginosa* IMCJ2.S1 can be transferred by conjugation, IMCJ2.S1 was incubated with *P. aeruginosa* ATCC 27853 RFP^r. Carbapenem resistance was transferred from *P. aeruginosa* GN17203 to *P. aeruginosa* ATCC 27853 RFP^r, consistent with the results reported by Watanabe et al. (51). In contrast, resistance to amikacin or carbapenem was not transferred from IMCJ2.S1 to ATCC 27853 RFP^r. These results suggest that In113 is located in the chromosome, and not on a plasmid, of *P. aeruginosa* IMCJ2.S1.

Resistance of IMCJ2.S1 to fluoroquinolones. IMCJ2.S1 was highly resistant to fluoroquinolones (Table 2). This resistance is typically associated with mutations in the QRDR within *gyrA*, *gyrB*, *parC*, and *parE*, which encode DNA gyrase or topoisomerase IV in *P. aeruginosa* (1, 21, 26, 31). Therefore, we screened IMCJ2.S1 mutations within the QRDR. Compared to the *gyrA* sequence of strain PAO1 (46), the *gyrA* sequence of IMCJ2.S1 contained an ACC-to-ATC mutation in codon 83 that causes a Thr-to-Ile change in the A subunit of DNA gyrase. IMCJ2.S1 also had a TCG-to-TTG mutation in codon 87 of *parC* that causes a Ser-to-Leu substitution in the C subunit of topoisomerase IV. IMCJ2.S1 had four mutations in *gyrB*: CGC to CGT in codon 396, AAA to AAG in codon 408, GAA to GAG in codon 484, and TTG to CTG in codon 513. There were four mutations in *parE*: GAA to GAG in codon 448, GGT to GGC in codon 472, AGT to AGC in codon 474, and GCC to GCT in codon 477. These mutations in *gyrB* and *parE* did not lead to amino acid changes in the proteins encoded (1, 31). Identical results were obtained with the other six clinical isolates. Together, these results indicate that IMCJ2.S1 contains mutations in *gyrA* and *parC* that are associated with its fluoroquinolone resistance.

DISCUSSION

A variety of aminoglycoside 6′-*N*-acetyltransferases have been described (Fig. 4) and classified into three subgroups (42, 50). Recently, a new enzyme, AAC(6′)-Iad, which is a member of the largest subfamily, was isolated from an *Acinetobacter* genospecies 3 strain in Japan (10). In the present study, we identified AAC(6′)-Iae, which shows considerable phylogenetic distance from members of the largest subfamily, which includes AAC(6′)-Iad and its divergents (Fig. 4). AAC(6′)-Iae belongs to the subfamily comprising AAC(6′)-Ia, -Ii, -Im, and -Iq (4, 9, 19, 48). There was only a low level of homology between the 59-be site of *aac(6′)-Iae* and those of the genes encoding other members of the *aac(6′)-I* family. Furthermore, *aac(6′)-Iae* has a low G+C content (26.8%) (data not shown), whereas the average G+C content of the *P. aeruginosa* PAO1 genome is 66.6% (46). Therefore, *aac(6′)-Iae* may be derived from an environmental species with an intrinsically low G+C content.

AAC(6′)-Iae from *P. aeruginosa* strain IMCJ2.S1, which was responsible for an outbreak of catheter-associated urinary tract infections, acetylated all of the aminoglycosides with 6′-NH_2, and acetylation of arbekacin and neomycin appeared to be complete (Fig. 6I). However, *E. coli* DH5α(pAAC6), expressing exogenous AAC(6′)-Iae, was sensitive to arbekacin and did not show reduced susceptibility to neomycin. Arbekacin and neomycin were shown to retain their antibiotic effects even after they were acetylated by AAC(6′) from an arbekacin-resistant actinomycete strain at the 6′ positions (53). *Enterococcus faecium* producing AAC(6′)-Ii was susceptible to neomycin even though AAC(6′)-Ii acetylated neomycin (52). These results suggest that acetylation of arbekacin and neomycin at 6′ positions does not affect the antimicrobial activities of these drugs. We cannot exclude the possibility that the antimicrobial activity observed after treatment with AAC(6′)-Iae is due to residual arbekacin or neomycin that was not acetylated.

E. coli DH5α expressing AAC(6′)-Iae was sensitive to gentamicin (Table 3), although AAC(6′)-Iae showed only partial acetylation of gentamicin (Fig. 6I). The sensitivity of these bacteria to gentamicin appears to be due to incomplete acetylation of gentamicin, which was observed with AAC(6′) from an arbekacin-resistant actinomycete strain (53)(Fig.6II). Commercially available gentamicin is a mixture of a number of derivatives of gentamicin, such as gentamicin C_1, C_{1a}, C_2, and C_{2b}, that have modifications of position 6′. Gentamicin C_1 and C_{2b} carry a methyl group on N-6′ and are refractory to AAC(6′)-I enzymes (42, 50). We cannot exclude the possibility that acetylated gentamicin components, which are more susceptible to AAC(6′)-I enzymes, retain antibiotic activity.

In the present study, we identified In113, a class 1 integron that contains a novel aminoglycoside resistance gene, *aac(6′)-Iae*. Several classes of integrons have been categorized on the basis of the structure of integrase (15, 40). The most common integrons in *P. aeruginosa* are those of class 1 (27, 28, 37).

Because their structures are very similar to each other, the direct origin of In113 could be from In2 (30), which was originally isolated from *Shigella flexneri* in Japan in the late 1950s (32) (Fig. 1).

IMCJ2.S1 was resistant to all antibiotics tested except arbekacin and polymyxin B (Table 2). However, the presence of In113 and the mutations in *gyrA* and *parC* of the QRDR are not sufficient to explain the multidrug resistance of this strain. Alterations of *gyrA* and *parC* are known to contribute to fluoroquinolone resistance (1, 21, 26, 31). The bla_{IMP-1} gene cassette, which encodes the IMP-1 metallo-β-lactamase, confers resistance to all β-lactams except monobactams (2, 27, 35). The *aac(6')-Iae* gene cassette, which encodes AAC(6')-Iae, confers resistance to amikacin, dibekacin, isepamicin, kanamycin, netilmicin, sisomicin, and tobramycin (Table 3). The variant *aadA1* gene cassette, which encodes aminoglycoside 3″-adenylyltransferase, confers resistance to streptomycin (18, 22). The *sul1* gene, which encodes dihydropteroate synthetase type I, confers resistance to sulfamethoxazole (47). Thus, the resistance of IMCJ2.S1 to aztreonam, gentamicin, tetracycline, trimethoprim, and silver sulfadiazine appears to be related to another, unidentified resistance factor(s).

In conclusion, we describe here a novel aminoglycoside 6′-*N*-acetyltransferase gene contained on a class 1 integron in a *P. aeruginosa* strain that caused a nosocomial outbreak of urinary tract infections. In113 may spread across Japan, because β-lactams, including carbapenems and aminoglycosides, are frequently used as therapeutic agents against *P. aeruginosa* and methicillin-resistant *Staphylococcus aureus* (20, 23). Surveillance for multidrug-resistant *P. aeruginosa* containing In113 is under way at several medical care facilities in the Sendai area of Japan.

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Rapid and Simple Method for Detecting the Toxin B Gene of *Clostridium difficile* in Stool Specimens by Loop-Mediated Isothermal Amplification

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We applied the loop-mediated isothermal amplification (LAMP) assay to the detection of the toxin B gene (*tcdB*) of *Clostridium difficile* for identification of toxin B (TcdB)-positive *C. difficile* strains and detection of *tcdB* in stool specimens. *tcdB* was detected in all toxin A (TcdA)-positive, TcdB-positive (A^+B^+) and TcdA-negative, TcdB-positive (A^-B^+) *C. difficile* strains but not from TcdA-negative, TcdB-negative strains. Of the 74 stool specimens examined, A^+B^+ or A^-B^+ *C. difficile* was recovered from 39 specimens, of which 38 specimens were LAMP positive and one was negative. Amplification was obtained in 10 specimens that were culture negative, indicating that LAMP is highly sensitive. The LAMP assay was applied to detection of *tcdB* in DNA extracted by a simple boiling method from 47 of those 74 specimens, which were cultured overnight in cooked-meat medium (CMM). Twenty-two of 24 culture-positive specimens were positive for LAMP on DNA from the culture in CMM. Four specimens were culture negative but positive by LAMP on DNA from CMM cultures. The LAMP assay is a reliable tool for identification of TcdB-positive *C. difficile* as well as for direct detection of *tcdB* in stool specimens with high sensitivity. Detection of *tcdB* by LAMP from overnight cultures in CMM could be an alternative method of diagnostic testing at clinical laboratories without special apparatus.

Clostridium difficile is well known as a cause of pseudomembranous colitis and a principle causative agent of antibiotic-associated diarrhea. Rapid and sensitive laboratory diagnostic testing is highly desirable for appropriate treatment of *C. difficile*-associated diarrhea (26). Two toxins, toxin A (TcdA) and toxin B (TcdB), are involved in the pathogenicity of this organism. The cell culture assay with the neutralization test is still used as a sensitive and specific method to detect TcdB, although the method is not easy to perform, cost-effective, or highly standardized.

A number of commercial tests are available for rapid and simple immunological detection of TcdA alone or of both TcdA and TcdB. However, these tests were found not to be as sensitive as the cell culture assay (6, 19, 22), and the infection caused by TcdA-negative, TcdB-positive (A^-B^+) *C. difficile* could not be diagnosed by using only the TcdA detection kit (1, 3, 16, 17, 18). Culture of *C. difficile* is a sensitive and specific method when cultured isolates are tested for TcdA and TcdB production (7). The PCR assay for detecting the toxin genes has been widely used for identification of types of toxin produced by recovered isolates (3, 12, 14, 27). Detection of *tcdA* and/or *tcdB* in stool specimens by PCR (9, 15), nested PCR (2), and real-time PCR (5) has also been developed and evaluated. Although reported to be rapid and sensitive diagnostic methods, they are not necessarily of practical use in clinical laboratories, where special equipment such as a thermal cycler or detection systems are not available.

Recently, loop-mediated isothermal amplification (LAMP) has been developed as a novel method that amplifies DNA with high specificity and simplicity (20, 21). In this study, we evaluated a LAMP method for identification of TcdB production by recovered isolates as well as for direct detection of *tcdB* in fecal specimens. DNA extraction from stool specimens requires some tedious steps to remove amplification inhibitors, making it difficult to do routinely in clinical laboratories. To further simplify the methods, the LAMP method was applied to detection of *tcdB* in DNA extracted by a simple and quick boiling method from stool specimens which were cultured overnight in cooked meat medium (CMM).

MATERIALS AND METHODS

Bacterial strains. The 40 *Clostridium difficile* strains used in this study were clinically isolated at various hospitals in Japan and previously classified into toxinotypes (Table 1) (24). Strains of *Clostridium* species other than *C. difficile* were obtained from the Japan Collection of Microorganisms except for an enterotoxin-positive *Clostridium perfringens* strain, MRY 05-0166, which was recovered from a case of antibiotic-associated colitis (Table 1).

Stool specimens. Stool specimens were obtained with the informed consent of patients admitted to five hospitals in Japan, who were given the diagnosis of antibiotic-associated diarrhea or colitis. The stool specimens were frozen at −80°C until transported and tested at the National Institute of Infectious Diseases. All tests were performed in batches on the same day, after a single thawing of the stored specimen.

Culture. *C. difficile* was isolated on cycloserine-cefoxitin-mannitol agar (CCMA) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) from stool specimens, which were treated with alcohol for spore selection. *C. difficile* was identified by colony morphology on CCMA and cell morphology after Gram staining. The latex agglutination test detecting glutamate dehydrogenase (Shionogi Pharmaceutical Co., Ltd., Tokyo, Japan) was used to confirm the identification.

The nonrepeating and repeating sequences of *tcdA* were amplified by PCR with primer sets NK3-NK2 (14) and NK9-NK11-NKV011 (11, 12), respectively. The presence of *tcdB* was examined by PCR with primer set NK104 and NK105 (12).

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TABLE 1. Strains used in this study and results of detection of *tcdB* by PCR and LAMP

Species (strain no.)	Tcd type	Toxinotype[a]	Detection[b] of *tcdB* by:		No. of strains studied
			PCR	LAMP	
Clostridium difficile	A^+B^+	0	+	+	20
	A^+B^+	I	+	+	1
	A^+B^+	III	+	+	1
	A^+B^+	IV	+	+	1
	A^+B^+	IX	+	+	1
	A^+B^+	XII	+	+	1
	A^+B^+	XVIII	+	+	1
	A^+B^+	XIX	+	+	1
	A^+B^+	XX	+	+	1
	A^-B^+	VIII	+	+	5
	A^-B^+	XVI	+	+	1
	A^-B^+	XVII	+	+	1
	A^-B^-	NA	−	−	5
Clostridium absonum (JCM 1381)	−	NA	−	−	1
Clostridium bifermentans (JCM 1386)	−	NA	−	−	1
Clostridium beijerinckii (JCM 1390)	−	NA	−	−	1
Clostridium histolyticum (JCM 1403)	−	NA	−	−	1
Clostridium novyi (JCM 1406)	−	NA	−	−	1
Clostridium perfringens (JCM 1290)	−	NA	−	−	1
Clostridium perfringens (MRY 05-0166)	−	NA	−	−	1
Clostridium ramosum (JCM 1298)	−	NA	−	−	1
Clostridium septicum (JCM 8144)	−	NA	−	−	1
Clostridium sordellii (JCM 3814)	−	NA	−	−	1
Clostridium sordellii (JCM 11011)	−	NA	−	−	1
Clostridium sporogenes (JCM 1416)	−	NA	−	−	1
Clostridium tertium (JCM 6289)	−	NA	−	−	1

[a] NA, not applicable.
[b] +, positive; −, negative.

Fecal TcdB assay. Stool specimens were tested for TcdB using a Vero cell cytotoxicity assay with a neutralization test with anti-*C. difficile* TcdB serum (TechLab, Blacksburg, VA). The final dilution of stool specimens in each microtiterplate well was 1:100. The cells were examined after both 24 h and 48 h of incubation.

Detection of *tcdB* by LAMP. DNA extraction from cultured isolates for a LAMP assay was performed in the same manner as previously described for PCRs of the toxin genes (12). DNA was directly extracted from stool specimens using the QIAamp DNA stool minikit (QIAGEN, Hiden, Germany) according to the manufacturer's instructions. LAMP was also applied to detection of *tcdB* in DNA which was extracted from overnight cultures of stool specimens with cooked meat medium (Becton Dickinson, Sparks, MD). One swab of stool specimens was inoculated into 5 ml of CMM and incubated at 35°C overnight; 1 ml of inoculated broth was centrifuged at 15,000 × *g* for 2 min, and 500 μl of TES (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 50 mM NaCl) was added to the pellet. The suspension was heated at 95°C for 15 min and centrifuged at 15,000 × *g* for 2 min, and the resultant supernatant was used as the template DNA for the LAMP assay.

The six primers used for the LAMP were derived from *tcdB* (4) (Fig. 1). The outer primers were HK101-F3 (5′-GTATCAACTGCATTAGATGAAAC-3′) and HK101-B3 (5′-CCAAAGATGAAGTAATGATTGC-3′); the inner primers were primer HK101-FIP, consisting of HK101-F1c and HK101-F2 (5′-CTGCACCTA AACTTACACCATCTATCTTCCTACATTATCTGAAGGATT-3′), and primer HK101-BIP, consisting of HK101-B1c and HK101-B2 (5′-GAGCTAAGTGAA ACGAGTGACCCGCTGTTGTTAAATTTACTGCC-3′). The loop primers were primers HK101-FL (5′-AATAGTTGCAATTATAGG-3′) and HK101-BL (5′-AGACAAGAAATAGAAGCTAAGATAGG-3′) (Fig. 1).

The LAMP reaction was performed using the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. We added 2 μl of DNA template to a total volume of 25 μl buffer consisting of 5 pM of each of the outer primers, 40 pM of each of the inner primers, and 20 pM of each of the loop primers. Amplification was performed at 62°C for 60 min, followed by incubation at 80°C for 2 min to terminate the reaction. The increased turbidity was monitored by a real-time turbidimeter, LA-320C (Eiken Chemical Co., Ltd., Tokyo, Japan). The turbidity was calculated based on the ratio of light intensity (intensity of light received by the photodiode/emitted light intensity). A ratio of 0.1 was defined as positive for the LAMP assay (20).

Detection of *tcdB* and *tcdA* by nested PCRs in stool specimens. DNA extracted from stool specimens for the LAMP assay was also used as the template for a nested PCR. The primers used for the nested PCR detecting *tcdB* were NK201 (5′-TTTAGATACTACACACGAAG-3′) and NK202 (5′-GCCATTATACCT ATCTTAGC-3′) for the outer primer set and NK104 and NK105 (12) for the inner primer set (Fig. 1), which were derived from *tcdB* (4). A nested PCR

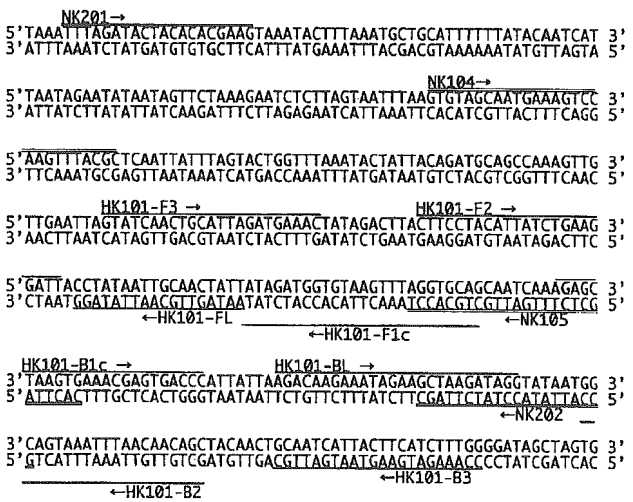

FIG. 1. Oligonucleotide primers used for amplification of *tcdB*. Single-underlined and double-underlined letters indicate the sequences of primers for LAMP and for nested PCR, respectively.

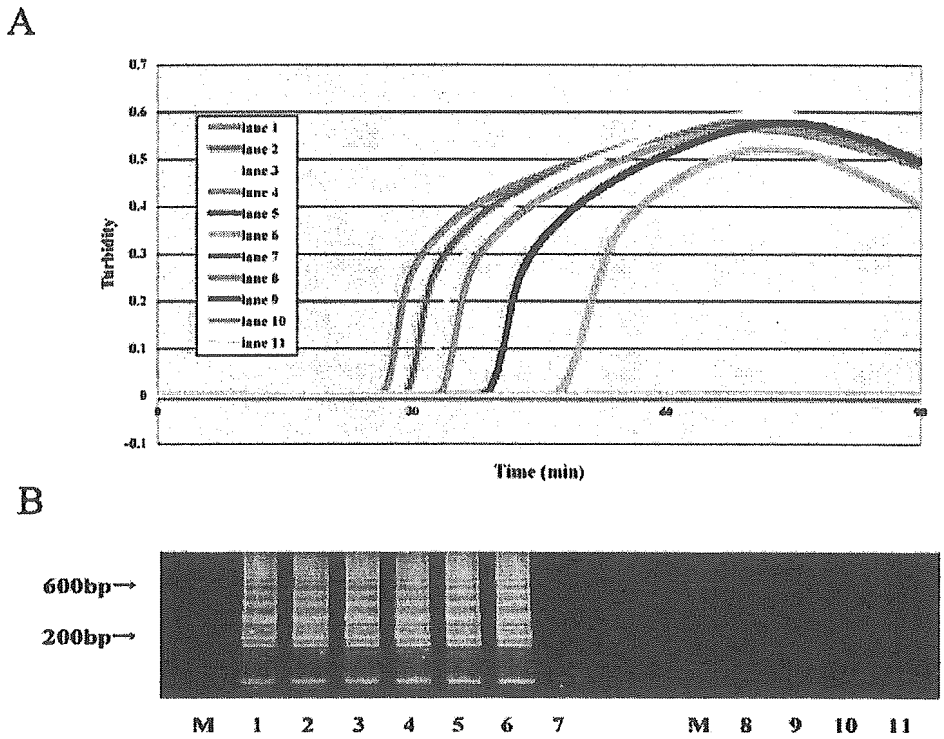

FIG. 2. Real-time detection of turbidity (A) and 5% polyacrylamide gel electrophoresis (B) of amplification products by LAMP. The template extracted from strain VPI 10463 in 10-fold serial dilutions from 50 ng to 50 ag per reaction tube (lanes 1 to 10) was added. Lane 11, negative control; lanes M, 100-bp ladder as a molecular size marker.

detecting the nonrepeating sequences of *tcdA* was performed with primer sets HK5 and HK6 for the outer primer and NK3 and NK2 for the inner primer (13). The nested PCR assay on DNA extracted from stool specimens was performed as described previously (13).

RESULTS

Sensitivity and specificity of LAMP. A total of 40 clinical isolates of *C. difficile* were examined for detection of *tcdB* by LAMP (Table 1). *tcdB* was detected in all 28 A^+B^+ and seven A^-B^+ isolates representing nine and three different toxinotypes, respectively (23). All five A^-B^- isolates examined were LAMP negative for *tcdB*. The test results by LAMP completely agreed with those by PCR detecting *tcdB* with primer set NK104 and NK105. The LAMP was performed in a 90-min reaction to confirm the specificity in 13 strains of 11 *Clostridium* species other than *C. difficile*, with negative results (Table 1).

DNA was extracted from strain VPI 10463 (A^+B^+), and 10-fold serial dilutions from 50 ng to 50 ag of DNA were added to each reaction tube for the LAMP and the nested PCR. Amplification by LAMP was obtained in reaction tubes containing from 50 ng to 0.5 pg of DNA template within 60 min (Fig. 2). Electrophoretic analysis (Fig. 2B) of the final products showed stem-loop DNAs with several inverted repeats of the target DNA and cauliflower-like structures with multiple loops (20, 21). On the basis of the results, the LAMP assay was performed in a 60-min reaction for the following tests. The same serial dilutions of DNA were applied to the nested PCR; the single PCR by primer set NK201 and NK202 was 10-fold less sensitive and the nested PCR was 100-fold more sensitive than the LAMP method (data not shown).

Direct detection of *tcdB* by LAMP in stool specimens. The results of detection of *tcdB* by the LAMP assay in DNA extracted directly from stool specimens compared with those of other tests are shown in Table 2. Of 74 stool specimens examined, 68 were available for detection of TcdB by cell culture assay; 32 were positive for the detection of fecal TcdB and 35 were negative, and the test result was nonspecific in the remaining 1. Amplification of *tcdB* by LAMP was obtained in all stool specimens that were positive for fecal TcdB. All 74 stool specimens were cultured for *C. difficile*; 40 were culture positive and 34 were negative. Of 40 isolates recovered from those specimens, 38 were A^+B^+, one was A^-B^+, and the remaining one was A^-B^-. *tcdB* was detected by LAMP on DNA extracted from 38 of 39 stool specimens from which an A^+B^+ or A^-B^+ *C. difficile* strain was recovered. Direct detection of *tcdB* by LAMP was positive in 10 stool specimens that were negative for *C. difficile* by culture.

The results of direct detection of *tcdB* by LAMP were compared with those by a nested PCR assay. All of the specimens that were positive by LAMP were also positive by nested PCR. The nested PCR detecting *tcdB* generated a PCR product on DNA extracted from 16 stool specimens that were culture negative, of which 10 specimens were positive for LAMP and 6 were negative. A nested PCR detecting *tcdA* was performed on 15 of these 16 specimens, all of which were positive. No specimens were negative for the nested PCR but positive for other tests.

TABLE 2. Results of LAMP assay detecting *tcdB* in DNA extracted from stool specimens and overnight culture in CMM and comparison to other tests

TcdB detection[a] in stool specimen by cell culture	*C. difficile* culture		Direct detection of *tcdB* in stool specimen by:		Detection of *tcdB* from overnight culture in CMM by LAMP	No. of stool specimens
	TcdB detection	Tcd type of isolate	Nested PCR	LAMP		
+	+	A^+B^+	+	+	+	16
+	+	A^+B^-	+	+	−	2
+	+	A^+B^+	+	+	ND[c]	13
Invalid	+	A^+B^-	+	+	+	1
+	+	A^-B^+	+	+	+	1
−	+	A^+B^+	+	+	+	3
−	+	A^+B^+	+	−	+	1
−	+	A^-B^-	−	−	−	1
−	−	NA[b]	+	+	+	2
−	−	NA	+	+	−	5
−	−	NA	+	+	ND	1
−	−	NA	+	−	+	2
−	−	NA	+	−	−	3
−	−	NA	+	−	ND	1
−	−	NA	−	−	−	10
−	−	NA	−	−	ND	6
ND	+	A^+B^+	+	+	ND	2
ND	−	NA	+	+	ND	2
ND	−	NA	−	−	ND	2

[a] +, positive; −, negative. Invalid, test result invalid because of atypical cytotoxic effect.
[b] NA, not applicable.
[c] ND, not done.

Detection of *tcdB* by LAMP from overnight cultures of stool specimens in CMM. A total of 47 stool specimens were available for evaluation of the LAMP assay detecting *tcdB* on DNA extracted from overnight cultures of stool specimens in CMM (Table 2). Of the 47 stool specimens examined, 24 were positive for fecal TcdB and/or for culture of *C. difficile* of A^+B^+ or A^-B^+ on CCMA, of which 22 specimens were positive by LAMP for overnight cultures in CMM and 2 were negative. The two specimens that were LAMP negative in overnight cultures were positive for direct detection of *tcdB* in stool specimens by both LAMP and nested PCR. Amplification by LAMP was obtained on DNA extracted from overnight cultures of four specimens that were negative for both fecal TcdB and *C. difficile* culture on CCMA. The four CMM tubes in which *tcdB* was detected by LAMP were inoculated, and A^+B^+ *C. difficile* could be recovered from all four specimens. Of those four specimens, two were positive for direct detection of *tcdB* from stool specimens by both LAMP and nested PCR, and two were positive only by nested PCR.

DISCUSSION

LAMP is a novel nucleic acid amplification method using DNA polymerase with strand displacement activity and six primers that recognize eight regions on the target nucleic acid, leading to extremely high specificity (20, 21). In the present study, we successfully identified TcdB-positive (A^+B^+ and A^-B^+) *C. difficile* strains with various toxinotypes by LAMP. Recent reports (1, 11, 16, 17, 18) have demonstrated the clinical significance of A^-B^+ strains. Most of the A^-B^+ strains are known to belong to toxinotype VIII (23, 24) and produce a variant toxin B ($TcdB_{1470}$) (12, 25). The primers used for the LAMP assay here could detect $tcdB_{1470}$ as well as other variant types of *tcdB* produced by toxinotypes III and IV (24) that could not be detected by real-time PCR (5). No amplification was observed from TcdB-negative (A^-B^-) *C. difficile* strains or 13 strains of other *Clostridium* species, including two *Clostridium sordellii* strains, which produce the lethal toxin (TcsL), indicating the specificity of the LAMP. Although the LAMP assay used here cannot distinguish A^-B^+ strains from A^+B^+ strains, identification of TcdB-positive *C. difficile* should be important for clinical diagnosis.

The LAMP detecting *tcdB* in DNA extracted directly from stool specimens proved to be a reliable assay when the test results were compared with those for detection of fecal TcdB and *C. difficile* culture. Furthermore, amplification was obtained by direct LAMP in 10 specimens that were negative for both fecal TcdB and culture, indicating the LAMP is more sensitive than culture for some specimens. Positive results in nested PCRs for not only *tcdB* but also *tcdA* in LAMP-positive but culture-negative specimens indicate the presence of PaLoc sequences in specimens and the specificity of the LAMP assay. Two of 10 patients from whom the LAMP-positive but culture-negative specimens were obtained were on vancomycin therapy when the specimens were tested, which should be one of the reasons for the lack of *C. difficile* growth. Although the nested PCR proved its high sensitivity, it is not of practical use in clinical laboratories because the procedure is time-consuming and tedious and due concern must be paid to contamination of PCR products.

Although the QIAamp DNA stool minikit is useful for extraction of DNA from stool specimens (2, 9, 13), it is not always practical for clinical laboratories. The LAMP method was applied to the detection of *tcdB* in DNA extracted by a simple

and quick boiling method from stool specimens which were cultured overnight in CMM, and a positive LAMP reaction was successfully obtained for 22 of 24 culture-positive specimens. Two specimens were culture positive but LAMP negative on DNA extracted from CMM culture. This discrepancy might be explained by the existence of amplification inhibitors in samples, because DNA was extracted without any steps for removing inhibitory substances. The heterogeneity of stool specimens also might cause the discrepancy when the specimens contain a low number of *C. difficile* or contain mucus.

Interestingly, four stool specimens that were negative for culture on CCMA were LAMP positive in overnight-cultured CMM. Recovery of *C. difficile* from of these four specimens in CMM indicated that the results of the LAMP with CMM cultures were not false-positives. This simple method using CMM requires neither tedious steps for DNA extraction from stool specimens nor anaerobic incubation equipment, such as an anaerobic chamber, jar, or pouch, making it possible to perform the test at clinical laboratories without special apparatus. In addition, the hands-on time of the procedure is very short, even though the test results are provided on the day after specimen collection.

The LAMP assay is a novel method to amplify DNA under isothermal conditions (20, 21) and has been applied to the identification or detection of some bacteria with high sensitivity and specificity (8, 10). The method is more rapid and easier to perform than a PCR assay and does not require any special equipment, such as a thermal cycler or electrophoresis system. The turbidimeter used in the present study is not needed when the fluorescent detection reagent (Eiken Chemical Co., Ltd., Tokyo, Japan) and UV lamp are available.

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Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation

Wei Xiao, Yao-Pi Hsu, Akitoshi Ishizaka, Teruo Kirikae and Richard B. Moss
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Sputum Cathelicidin, Urokinase Plasminogen Activation System Components, and Cytokines Discriminate Cystic Fibrosis, COPD, and Asthma Inflammation*

Wei Xiao, MD; Yao-Pi Hsu, MS; Akitoshi Ishizaka, MD; Teruo Kirikae, MD, PhD; and Richard B. Moss, MD

Background: **Interest in airways inflammatory disease has increasingly focused on innate immunity. We investigated several components of innate immunity in induced sputum of patients with cystic fibrosis (CF), COPD, and asthma, and healthy control subjects.**
Methods: **Twenty eight patients with mild CF lung disease (age ≥ 12 years; FEV_1, 74 ± 3% predicted [mean ± SE]), 74 adults with COPD (FEV_1, 55 ± 2% of predicted), 34 adults with persistent asthma (FEV_1, 66 ± 2% of predicted), and 44 adult control subjects (FEV_1, 85 ± 1% of predicted) were studied while in stable clinical condition. Levels of sputum interleukin (IL)-8, IL-10, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, human cationic antimicrobial protein 18 (CAP18), urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), and plasminogen activator inhibitor (PAI)-1 were determined. Cell sources were investigated by flow cytometry and immunohistochemistry. Spirometry was performed prior to sputum induction.**
Results: **CF patient sputum showed greatest increase in IL-8 compared to that of patients with COPD and asthma (which were also greater than control subjects), and elevated levels of TNF-α and IL-10 compared to other groups. There were no differences in IFN-γ. CAP18 levels were elevated in CF and COPD patients compared to control subjects, while asthma patients had reduced CAP18 levels. uPA levels were similar but uPAR was elevated in CF and COPD patients more so than in asthma patients, while PAI-1 levels were elevated in all three disease groups. CAP18 localized to neutrophil secondary granules; neutrophils were also sources of IL-8 and PAI-1. CAP18 and PAI-1 negatively correlated with pulmonary function.**
Conclusion: **Induced-sputum innate immune factor levels discriminate inflammatory changes in CF, COPD, and asthma, suggesting potential roles in pathophysiology and as well as providing disease-specific biomarker patterns.** ***(CHEST 2005; 128:2316–2326)***

Key words: cathelicidin; cystic fibrosis; cytokine; innate immunity; urokinase plasminogen activator system

Abbreviations: BSA = bovine serum antigen; CAP18 = human cationic antimicrobial protein 18; CF = cystic fibrosis; ELISA = enzyme-linked immunosorbent assay; FITC = fluorescein isothiocyanate; IFN = interferon; IL = interleukin; PAI = plasminogen activator inhibitor; PBS = phosphate-buffered saline solution; PE = phycoerythrin; TNF = tumor necrosis factor; uPA = urokinase-type plasminogen activator; uPAR = uPA receptor

The pathogenesis of cystic fibrosis (CF) lung disease is characterized by compromised local innate immunity, which permits microbial colonization and chronic infection.[1] Current thinking emphasizes the primary role of volume depletion of airway surface liquid and resulting compromise of mucociliary clearance.[2] Additional innate defense mechanisms may also be involved, as a primary proinflam-

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Drs. Moss, Ishizaka, and Kirikae have assigned entire right, title, and interest in the CAP18 immunoassay described here to Seikagaku Corporation, Tokyo, Japan.
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matory bias of the CF epithelia has been posited,[3-4] and activity of endogenous antimicrobial peptides produced by airway epithelia and glands may be altered in CF.[5-6] While some epithelial antimicrobials have received much attention in CF, in particular the β-defensins, others have not.[7-8] In particular, the role of the only human cathelicidin, the 18-kd, 140 amino acid cationic antimicrobial protein (human cationic antimicrobial protein 18 [CAP18]), has not been investigated in sputum from patients with CF. It is produced by respiratory epithelia as well as stored in secondary (specific) granules of polymorphonuclear leukocytes, and in a model system restores deficient antimicrobial activity in the CF airway milieu.[9-10] Proteolytic cleavage of CAP18 by proteinase 3 yields a potent antimicrobial peptide (carboxy terminal 37 amino acid fragment of CAP18).[11] Via its action on the formyl peptide receptor-like 1 expressed on several cell types, carboxy terminal 37 amino acid fragment of CAP18 is also an important regulator of macrophage function; has potent chemotactic activity for neutrophils, monocytes, and T cells; and possesses angiogenesis activity.[12-14] Abnormalities in CAP18 could therefore profoundly affect the pathophysiology of CF by its ability to link innate to adaptive immunity and its neovascularizing effect.

Another innate defense pathway recently found active in the airway is the plasminogen activator system constituted by its local serine protease activator urokinase-type plasminogen activator (uPA), the uPA-specific cell surface receptor (uPA receptor [uPAR]) [CD87], and an arginine-specific serine protease inhibitor (serpin), plasminogen activator inhibitor (PAI)-1.[15-16] uPA binding to uPAR results in enhanced activation of cell-bound plasminogen with subsequent effects on cell adhesion, chemotactic migration, and tissue remodeling.[16-20] The uPA system is involved in a number of pathologic states, including inflammation after tissue injury as key participants in the enzymatic modification of the extracellular matrix, resulting in cell recruitment, migration, adhesion, and mitogenesis.[15,19] In addition to proteolytic activity, plasmin activates metalloproteinases that degrade extracellular matrix.[20] To maintain normal lung function and integrity in the host response, tight control of the proteolytic enzymes and their inhibitors is needed to maintain proper function of neutrophils, macrophages, and mesenchymal cells. The uPA system is one of the components that act on neutrophils and macrophages to facilitate the interaction between cells and matrix.[21-22] A critical innate defense role for the uPA/uPAR/PAI-1 system has recently been described in murine models of acute pulmonary infection as well as in the pathogenesis of interstitial lung diseases.[23-26] The role of this system in human chronic airway inflammatory lung diseases is just emerging.[26-27]

CF, COPD, and asthma are chronic airway diseases with different causes that share some features in pathologic changes and clinical syndrome. In these diseases, there is chronic mucosal and airway inflammation with distinct pathophysiologic features in each but a common increase in the infiltration of neutrophils and a variety of inflammatory mediators including interleukin (IL)-8. The pathologic processes in these diseases all seem to involve progressive inflammatory responses with elements of tissue remodeling, airway obstruction, and reduction in expiratory flow rates. In the present study, we determined and compared the levels and sources of uPA/uPAR/PAI-1, CAP18, and several cytokines (IL-8, tumor necrosis factor [TNF]-α, interferon [IFN]-γ, and IL-10) in induced sputum of CF, COPD, and asthma patients, and healthy control subjects to see if differing local inflammatory patterns can be discerned noninvasively and related to airflow obstruction as measured by expiratory flow rates.

Materials and Methods

Subjects

We recruited 28 patients with CF (age, 12 to 50 years) followed up at the CF Center clinic at Stanford University Medical Center. CF diagnoses in all patients were made by positive (> 60 mEq/L) pilocarpine iontophoresis sweat test results, with homozygous or compound heterozygous for ΔF508 CF transmembrane conductance regulator mutations. All patients had chronic infection with *Pseudomonas aeruginosa* by serial sputum culture and were in stable clinical condition (no pulmonary exacerbation within previous month). CF patients were also excluded for FEV_1 values < 40% predicted, oxyhemoglobin saturation < 92% on room air, pneumothorax, hemoptysis, or history of *Burkholderia cepacia* in sputum. None were receiving regular inhaled or systemic corticosteroids. Seventy-four patients (age 38 to 79 years) with previously diagnosed COPD were recruited from the Respiratory Clinic of the Hospital of Shandong Medical University, Peoples Republic of China. All were in stable condition and not receiving antibiotics for at least 2 weeks prior to testing. Thirty-four patients with asthma (age, 14 to 75 years) followed up in Shandong were also studied. Finally 44 healthy nonsmoking subjects without reported respiratory symptoms (age, 20 to 60 years; 20 patients at Stanford and 24 patients at Shandong) were also studied. All participants gave written informed consent with protocols approved by the Institutional Review Boards at Stanford and Shandong.

Pulmonary Function

Pulmonary function tests were performed according to American Thoracic Society guidelines for performance and acceptance prior to sputum induction.

Sputum Induction and Processing

Sputum was collected from each patient as previously described using 3% hypertonic saline solution (at Stanford) or 3.5% hypertonic saline solution (at Shandong) via an ultrasonic nebulizer with 2-min collections of sputum, which were pooled for analysis.[28–29] All subjects underwent sputum induction regardless of history of ability to expectorate. Subjects were encouraged to cough, and sputum was collected into polypropylene cups. The induced-sputum samples were weighed, and an equal volume of Sputolysin (Calbiochem-Novabiochem; San Diego CA) diluted 10% in normal saline solution was added. Samples were vortexed 3 seconds and incubated for 5 min at 37°C in a water bath with vigorous shaking (160 rotations per minute). Samples were further mixed by aspirating up and down 20 times in a transfer pipette. Five-minute incubations were then repeated two more times. Finally, the samples were centrifuged at 2,000 revolutions per minute (800g) for 5 min at 4°C, and the sol phase was used for analysis.

Soluble Mediators of Innate Immunity

Human IL-8, IL-10, IFN-γ, TNF-α, CAP18, uPA, uPAR, and PAI-1 levels in the supernatant sol phase of sputum were determined by enzyme-linked immunosorbent assay (ELISA) using a standardized format. Wells of microtiter plates (polypropylene 96-well culture clusters, Catalog No. 3598; Costar; Pleasanton CA) were coated with 50 μL per well capture antibody (see below for specific reagents) diluted in phosphate-buffered saline solution (PBS) [P-4417; Sigma Chemical; St. Louis, MO], incubated overnight at 4°C, and washed three times with wash buffer (PBS 0.01%, Thimersol; Sigma Chemicals; 0.05% Tween 20 [polyoxyethylene sorbitan mono-oleate]; Sigma Chemical). Blocking solution (1% bovine serum albumin [BSA], A-2153; Sigma Chemical; 5% sucrose PBS) 200 μL per well was added, incubated at room temperature for 1 h, and the wells were washed three times with washing buffer. Samples and standards diluted in diluting solution (0.1% BSA-0.05% Tween 20-Tris-buffered saline solution) were then added (50 μL per well), incubated overnight at 4°C, and washed three times with washing buffer. Biotinylated detection antibody (see below for specific reagents) in dilution buffer was then added (50 μL per well) and incubated 2 h at room temperate with gentle mixing. Plates were then washed four times with washing buffer. Avidin-peroxidase-conjugated secondary antibody (see below for specific reagent) diluted in dilution buffer was then added (50 μL per well), and incubated 1 h at room temperature with gentle mixing. Plates were then washed four times with washing buffer. Developing solution (75 μL per well *o*-phenylenediamine [P-6912; Sigma Chemical] in citrate-phosphate buffer pH 6.0 with 4 μL 30% H_2O_2 [H-1009; Sigma Chemical]) per 10-mL buffer was added, the reaction stopped with 25 μL per well 2 N H_2SO_4, and the well color was read at an optical density of 492 with an automated microplate reader (Molecular Devices; Mountain View, CA).

Specific Reagents for the ELISA

IL-10: For IL-10, the capture antibody was from Pharmingen (Catalog No. 18551D; BD Pharmingen; San Diego, CA), the primary antibody was purified rat anti-human IL-10 diluted to 4 μg/mL, the detection antibody was biotinylated rat anti-human IL-10 (Catalog No. 18562D; BD Pharmingen) diluted to 4 μg/mL, the detector was horseradish peroxidase-streptavidin (Catalog No. 43–4323; Zymed Laboratories; San Francisco, CA) diluted 1:1000, and the standard was recombinant human IL-10 (Catalog No. 19701N; BD Pharmingen) diluted to 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156, 78, and 39 pg/mL.

IFN-γ: For IFN-γ, the capture antibody was purified mouse anti-human INF-γ (N1B4, Catalog No. 18891D; BD Pharmingen) diluted to 2 μg/mL, the detection antibody was biotinylated mouse anti-human IFN-γ (4SB3, Catalog No. 18902D; BD Pharmingen) diluted to 2 μg/mL, and the standard was recombinant human IFN-γ (Catalog No. 19751N; BD Pharmingen), diluted to 10,000, 5,000, 2,500, 1,250, 625, 312.5, 156, 78, and 39 pg/mL.

IL-8: For IL-8, the capture antibody was purified monoclonal antibody to human IL-8 (Catalog No. MAB208; R&D Systems; Minneapolis, MN) diluted to 4 μg/mL, the detection antibody was biotinylated goat anti-human IL-8 (Catalog No. BAF208; R&D Systems) diluted to 200 ng/mL, and the standard was recombinant human IL-8 (Catalog No. 208-IL; R&D Systems) diluted to 5,000, 2,500, 1,250, 625, 312.5, 156, and 78 pg/mL.

TNF-α: For TNF-α, the capture antibody was mouse anti-human TNF-α (Part 840119; R&D Systems) diluted to 4 μg/mL, the detection antibody was biotinylated goat anti-human TNF-α (Part 840120; R&D Systems) diluted to 300 ng/mL, the detector was horseradish peroxidase-streptavidin (Part 89080; R&D Systems) diluted to 1:200, and the standard was recombinant human TNF-α (Part 840121; R&D Systems) diluted to 2,000, 1,000, 500, 250, 125, 62.5, 31.25, and 16 pg/mL.

uPA, PAI-1, and uPAR: For uPA, PAI-1, and uPAR, ELISA kits (Imubind; American Diagnostics; Greenwich, CT) were used according to the instructions of the manufacturer (Catalog Nos. 894, 821, and 893, respectively).

CAP18: For CAP18, the capture antibody was rabbit polyclonal antibody to human lipopolysaccharide-binding domain of CAP18 diluted to 1:200; the detection antibody was a mouse IgG1 monoclonal antibody to CAP18 diluted to 1:1000; the detector was horseradish peroxidase-conjugated goat anti-mouse IgG (Catalog No. 115-367-5296; Jackson Immuno Research Lab Inc.; West Grove, PA) diluted 1:2500; and the standard was a synthetic 27 amino acid peptide fragment of CAP18 (amino acids 109–135) diluted to 5,000, 2,500, 1,250, 625, 312.5, 156, 78, 39, and 20 ng/mL. The capture antibody, detection antibody and standard were obtained from Dr. Yoshikazu Naiki and Teruo Kirikae, Japan. In a pilot study, the same CAP18 ELISA was used to measure CAP18 levels in serum, BAL fluid, and expectorated sputum of patients with CF (see "Results").

Immunhistochemistry of Induced-Sputum Cells

Adherence of Sputum Cells to Glass Slides: After removal of the supernatant of centrifuged sputum samples, cell pellets were each suspended in 20 mL of PBS (P-4417; Sigma Chemical) and centrifuged at 1,200 revolutions per min for 10 min. After removal of the supernatant, the cell pellet was resuspended in 1% BSA (A-2153; Sigma Chemical)-PBS. Dead cells were excluded by Trypan Blue (T-9520; Sigma Chemical) cell count. Cells were diluted to approximately 2.5×10^5 in 1% BSA-PBS. Following cytocentrifuge (700 revolutions per minute for 5 min) 100 μL of cell suspension per slide were air dried and freezer stored at −20°C.

Staining: Frozen slides were thawed at room temperature. Cells were fixed by incubating slides in 4% formaldehyde (Catalog No. 16220; Electron Microscopy Sciences; Fort Washington PA)-PBS for 15 min at room temperature to fix cells. After three washes in PBS, slides were incubated in 1% H_2O_2 (H-1009; Sigma Chemical)-PBS 10 min at room temperature, washed three times in PBS, and stained. Slides were permeabilized by incubation with 0.5% saponin (S-2149; Sigma Chemical)-PBS for 10 min at room temperature. Slides were then washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal (T-5125; Sigma). Once the cells were permeabilized, the same buffer was used after this step. According to the protocol of the Vectastain Elite ABC kit

(Catalog No. PK-6102; Vector Laboratories; Burlingame, CA), slides were incubated for 20 min at room temperature in blocking solution (5% horse serum-PBS for uPAR, PAI-1, CAP18, IL-8 CD14, and cytokeratin; 5% goat serum for CD15) and washed thrice with PBS. Slides were then incubated in avidin blocking solution (Catalog No. SP-2001; Avidin/Biotin Blocking Kit; Vector Laboratories) for 15 min at room temperature, washed thrice with PBS for surface staining, and then washed in PBS-0.05% Tween 20–0.01% Thimerosal for cytoplasmic staining. Slides were then incubated with biotin blocking solution for 15 min at room temperature and washed thrice with PBS-0.05% Tween 20–0.01% Thimerosal. Following this preparation, slides were incubated with primary monoclonal antibody (for uPAR, Catalog No. 3936; American Diagnostica; Greenwich, CT; for PAI-1, Catalog No. 3785; American Diagnostica; for CAP18, see primary antibody, ELISA methods above; for IL-8, Catalog No. 554717; BD Pharmingen mouse IgG2b clone G265–8; for CD14, Catalog No. 555396; BD Pharmingen; and for cytokeratin, Catalog No. 349205; BD Pharmingen) each diluted in 5% horse serum-1% BSA-0.05% Tween 20-PBS. For CD15, the antibody (Catalog No. 555400; BD Pharmingen) was diluted in 5% normal goat serum-1%BSA-0.05% Tween 20-PBS. Slides were incubated with primary antibody for 60 min at room temperature, and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. An isotype control antibody was run with each experiment (mouse IgG isotype control; Catalog No. I-2000; Vector Laboratories). For staining uPAR, PAI-1, CAP18, IL-8, CD14, and cytokeratin, reactions were developed with biotinylated horse anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% horse serum-1% BSA-0.05% Tween 20-PBS. For staining CD15, the reaction was developed with biotinylated goat anti-mouse antibody (Catalog No. PK-6102; Vector Laboratories) diluted in 5% goat serum-1% BSA-0.05% Tween 20-PBS. Slides were incubated for 30 min at room temperature and washed five times with PBS-0.05% Tween 20–0.01% Thimerosal. For development, slides were incubated for 30 min at room temperature with Vector ABC reagent (Catalog No. PK-6102; Vector Laboratories) prepared in PBS 30 min before use and then washed thrice in PBS. Color was developed (3,3'-diaminobenzidine peroxidase substrate; Catalog No. SK-4100; or Vector VIP [peroxidase substrate, Catalog No. SK-4600; Vector Laboratories]) according to manufacturer's instruction. Color development was stopped by washing in distilled water, the slides were air dried, and the slides were viewed under microscope to assess the positive staining cells and determine cell type by comparing with Wright-Giemsa–stained slides (Hema 3 set, Catalog No. 122–911; Biochemical Science; Swedesboro, NJ).

Flow Cytometry

Five $\times 10^5$ Sputolysin-treated (Calbiochem Corporation; San Diego, CA), PBS-washed sputum cells were added to each assay tube, incubated in 5% BSA-PBS for 10 min at room temperature to block nonspecific binding, washed once in PBS, fixed in 4% formaldehyde-PBS for 15 min at room temperature, and washed once with PBS. The cell samples were permeabilized in 0.5% saponin-PBS for 10 min at room temperature and washed once in 0.1% saponon-0.5% BSA-PBS. Monoclonal anti-human IL-8, uPAR, PAI-1, and CAP18 antibodies, and mouse IgG1, G2a, and G2b isotype control antibodies were labeled with fluorescein isothiocyanate (FITC) according to the zenon complex formation protocol (Zenon Alexa Fluor 488 mouse IgG1, IgG2a and IgG2b labeling kits, Catalog Nos. Z-25002, Z-25202, and Z-250102; Molecular Probes; Eugene, OR). Phycoerythrin (PE) labeled anti-human CD14, CD15, and cytokeratin were obtained from BD Pharmingen (Catalog Nos. 555398, 555402, and 347204, respectively). FITC- and PE-labeled anti-human antibodies (IL-8/FITC, uPAR/FITC, PAI-1/FITC, CAP18/FITC, CD15/PE, CD14/PE, and CK/PE) were added to respective tube with 1% mouse serum (Sigma Chemical), to reduce nonspecific staining. Mouse IgG1, IgG2a, IgG2b (Southern Biotech; Birmingham, AL), and IgM (Catalog No. 555584; BD Pharmingen) isotype-matched controls were included in each experiment. Tubes were incubated for 30 min at room temperature in the dark with slowing mixing followed by one wash with PBS and resuspension of the pellet in 300 μL of 1% formaldehyde. Samples were analyzed using a flow cytometer (FACSCalibur E3139; Becton Dickinson; Franklin Lakes, NJ).

Statistical Analysis

Data are expressed as mean ± SE unless otherwise indicated. Statistical comparisons were made using Student t test for unpaired two-group samples, analysis of variance for multiple group comparisons, and Pearson correlation coefficient. Two-tailed tests were performed, and a p value of < 0.05 was considered significant.

RESULTS

Subject Groups

The CF patients (17 women and 11 men) were generally young adults (mean ± SD age, 23.7 ± 11.1 years) with well-preserved pulmonary function (mean ± SD FEV_1, 74.0 ± 17.4% predicted). The patients with COPD (24 women and 50 men) were, as expected, older (59.2 ± 9.9 years) with generally greater airflow obstruction (FEV_1, 54.6 ± 13.7% predicted). The patients with asthma (19 women and 15 men) were intermediate between these other groups in age (47.4 ± 13.9 years) and airflow obstruction (FEV_1, 65.9 ± 13.0% predicted). All but two patients with asthma were receiving regular bronchodilator medication (β_2-adrenergic aerosols and/or theophylline), while only seven patients were receiving regular inhaled corticosteroids.

CAP18 in Serum, BAL Fluid, and Sputum

To study the biology of CAP18 in CF, in a pilot study we first measured serum CAP18 levels by ELISA in patients with CF in stable condition (n = 15) and compared these to samples obtained from CF patients on admission to hospital for treatment of pulmonary exacerbation (n = 15) and 15 healthy adult control subjects. The ELISA showed linear parallelism in the range of 3 to 3,000 ng/mL with interassay coefficient of variation < 15%. Levels of CAP18 were similar in the three subject groups (stable CF, 966 ± 980 ng/mL; exacerbation CF, 1,137 ± 685 ng/mL; and control, 1,012 ± 648 ng/mL). We next compared CAP18 levels in BAL fluid of 23 patients with CF and 12 control subjects. CF patients had significantly higher levels of BAL fluid CAP18 than control subjects (189.7 ± 18.7 ng/mL vs

120.7 ± 24.7 ng/mL, p = 0.036, by two-sided unpaired *t* test). As this suggested increased local production and/or reduced metabolism within the pulmonary compartment of inflamed CF airways, we proceeded to measure CAP18 levels in expectorated sputum of 30 patients with CF. Expectorated CF sputum levels (177.4 ± 14.7 ng/mL) were quite similar those in BAL, suggesting that sputum is an easily accessible and representative sample of airway secretions for measurement of CAP18 levels.

In order to obtain sputum samples from CF patients with mild disease severity and little or no sputum productivity (including nonexpectorators), we then proceeded to study CAP18 levels in induced-sputum samples using a validated standardized methodology. All nonexpectorators were able to produce adequate sputum for analysis after induction. CF patients were compared to patients with COPD, patients with asthma, and healthy control subjects. CF and COPD patients had comparable levels (79.6 ± 93 ng/mL vs 75.3 ± 38.9 ng/mL, respectively) and significantly elevated levels of sputum CAP18 when compared to control subjects (39.9 ± 24.2 ng/mL, $p < 0.009$ for either group vs control), while asthmatics had significantly reduced CAP18 levels (13.6 ± 9.8 ng/mL) compared to control subjects ($p < 0.0001$) or patients with CF or COPD ($p < 0.0001$) [Table 1].

Cytokines and Plasmin Activation Component Levels in Induced Sputum of CF, COPD, and Asthma Patients, and Control Subjects

To see if this pattern was similar to other innate defense factors, we compared levels of IL-8, IL-10, INF-γ, TNF-α, uPA, uPAR, and PAI-1 in these subjects. As also shown in Table 1, levels of sputum IL-8 were significantly higher in patients with CF than in patients with COPD or asthma ($p < 0.001$ for each) or control subjects ($p < 0.0001$); COPD and asthma patients also had higher IL-8 levels than control subjects ($p < 0.05$). IL-10 levels were also higher in the CF patients than patients with COPD or asthma, or control subjects ($p < 0.05$), without differences noted between the latter three groups. TNF-α levels showed a similar pattern as IL-10 with elevation in patients with CF compared to patients with COPD or asthma, or control subjects ($p < 0.001$). INF-γ levels were similar among all four groups.

uPA levels were similar among the four groups. In contrast, uPAR levels were elevated and similar in patients with CF and COPD compared to patients with asthma and control subjects ($p < 0.001$ for each); uPAR levels were also higher in asthma than controls ($p < 0.05$). PAI-1 levels were comparably elevated in patients with CF, COPD, and asthma, compared to control subjects ($p < 0.05$; Table 1).

Neutrophil Origin of Sputum CAP18, IL-8, and PAI-1

In order to examine the cellular sources of these sputum substances in patients with CF, we examined cells obtained from induced-sputum samples by immunohistochemical staining and independently by flow cytometry. Most sputum cells were either intact or more commonly degenerated polymorphonuclear neutrophils with variable mucus component and occasional other cell types such as monocytes, macrophages, eosinophils, or squamous epithelial cells; ciliated epithelial cells were not seen (Fig 1, *top*). CAP18 heavily stained the cytoplasmic granules of these neutrophils (Fig 1, *center* and *bottom*), suggesting that a major source of sputum CAP18 was infiltrating neutrophils. CAP18 is known to be localized to secondary or specific neutrophil granules. Using flow cytometry, CAP18 expression was markedly increased in airway cells that coexpressed CD15 (3-fucosyl-N-acetyl-lactosamine), which is present in the intracellular secondary granules of neutrophils,

Table 1—*Levels of Innate Immune Factors in Sputum of Patients With CF, COPD, and Asthma, and Healthy Control Subjects

Factors, pg/mL	CF (n = 28)	COPD (n = 74)	Asthma (n = 34)	Healthy (n = 44)
CAP18	79,623 ± 18,597†	75,262 ± 4,583†	13,646 ± 1,678†	39,936 ± 3,644
IL-8	29,880 ± 4,226†	11,852 ± 1,880†	11,683 ± 3,707†	1,713 ± 317
IL-10	386 ± 87†	53 ± 14	36 ± 4	72 ± 14
INF-γ	794 ± 137	392 ± 65	627 ± 225	684 ± 117
TNF-α	74 ± 31†	10 ± 4	8 ± 2	12 ± 6
uPA	59 ± 26	76 ± 24	64 ± 30	18 ± 6
uPAR	803 ± 112†	583 ± 87†	399 ± 103†	85 ± 11
PAI-1	3,812 ± 469†	6,410 ± 1039†	4,802 ± 1127†	592 ± 97

*Data are presented as mean ± SEM. pg/mL
†Significant at ≥ 95%. See text for p values.

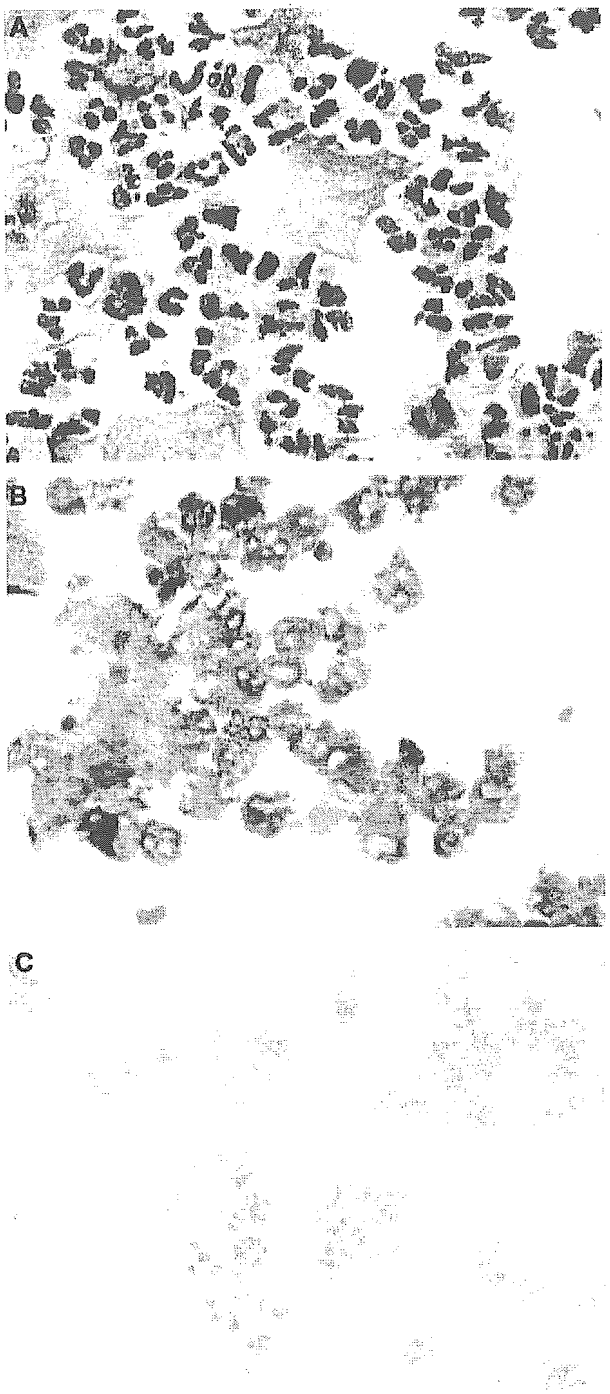

FIGURE 1. *Top*: Wright-Giemsa stain (original × 200) of induced-sputum sample from a CF patient showing neutrophil predominance. *Middle*: CF specimen stained for presence of CAP18 showing neutrophil granule localization. *Bottom*: isotype-negative control antibody result.

confirming the importance of neutrophil specific granules as a major source of airway CAP18 in CF (Fig 2).

Another neutrophil product important in CF pathobiology, IL-8, was also identified by flow cytometry in sputum cells that expressed CD15 (Fig 3). Similarly, PAI-1 was found, albeit at lower levels of expression, in CD15+ sputum cells (Fig 4).

Sputum levels of innate immune factors were correlated with each other in order to examine potential relationships in production or regulation. As shown in Table 2, levels of the three plasmin activation system components correlated with each other ($p < 0.0001$) but also with several cytokine responses (*eg*, uPAR and uPA with IL-8, $p < 0.0001$; uPAR and uPA with TNF-α, $p \leq 0.002$; and uPAR with IL-10, $p = 0.006$). In addition, uPAR was weakly correlated with CAP18 ($p = 0.01$). Among cytokines, IL-8 levels correlated with TNF-α and IL-10 ($p < 0.0001$), and IL-10 with IFN-γ ($p = 0.001$). CAP18 levels were correlated not only weakly with uPAR but also weakly with IL-8 ($p = 0.04$) and strongly with IL-10 ($p < 0.0001$).

To examine the potential role of these factors in disease activity or progression, innate immune factors were correlated with level of pulmonary function as determined by spirometric assessment of airflow obstruction (Table 2). Among all study subjects, FEV_1 percentage of predicted showed a strong negative correlation with sputum CAP18 and PAI-1 levels ($p \leq 0.0009$) and a weak negative correlation with uPAR levels ($p = 0.01$; Table 2). The overall negative correlation between pulmonary function and CAP18 was due primarily to results in patients with CF ($r = -0.40$, $p = 0.06$) and to a slightly lesser extent COPD ($r = -0.19$, $p = 0.11$). A similar negative correlation trend was seen between FEV_1 percentage of predicted and IL-8 in patients with CF ($r = -0.39$, $p = 0.056$) and patients with COPD ($r = -0.21$, $p = 0.08$). However, when innate factors were correlated with each other in patients with CF, CAP18 and IL-8 did not correlate with each other; instead, CAP18 levels correlated strongly with IL-10 ($r = 0.78$, $p = 0.001$) and IFN-γ ($r = 0.59$, $p = 0.002$), suggesting that elevated CAP18 levels in CF may represent a counterinflammatory response along with IL-10 and IFN-γ (IL-10 correlated strongly with IFN-γ, $r = 0.58$, $p = 0.001$). In contrast, levels of IL-8, a known proinflammatory factor in CF and COPD that we confirmed correlated negatively with pulmonary function (see above), correlated strongly with levels of uPAR ($r = 0.59$, $p = 0.002$), PAI-1 ($r = 0.5$, $p = 0.009$), and TNF-α ($r = 0.5$, $p = 0.007$). TNF-α correlated strongly with uPA ($r = 0.65$, $p = 0.0004$).

DISCUSSION

Research[1–2,30–31] has focused on the role of deranged aspects of innate immunity in the pathobiol-

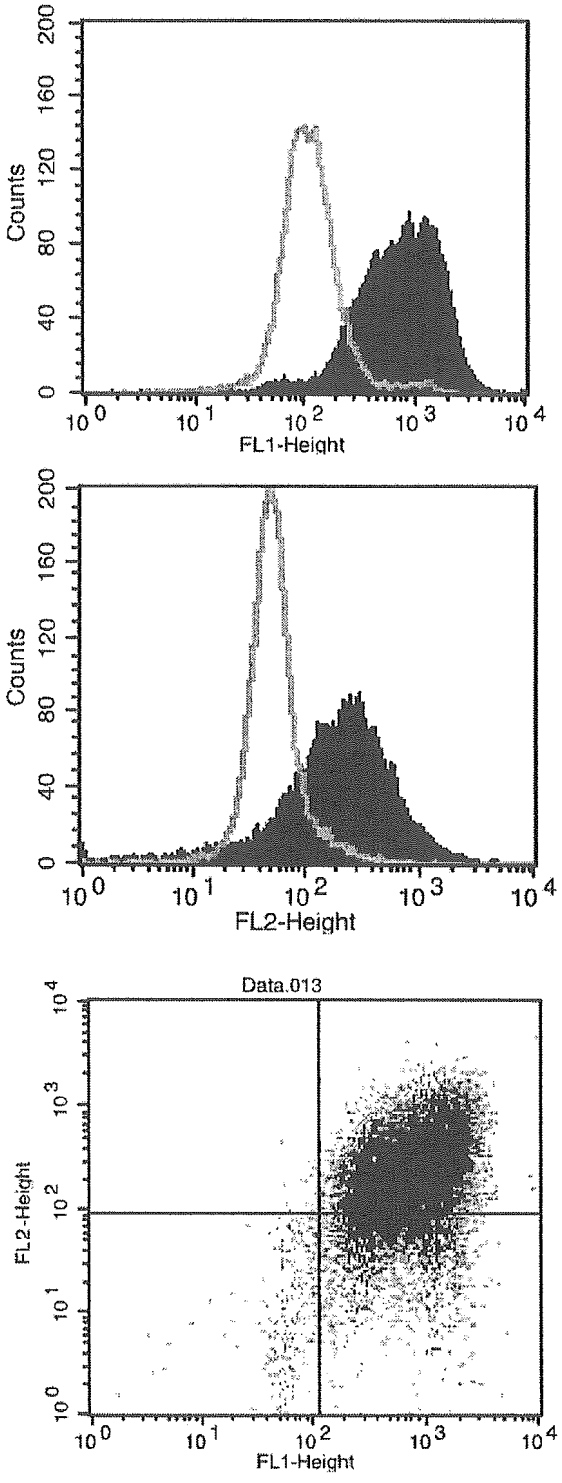

FIGURE 2. Flow cytometry for presence of CAP18 and CD15 in nonapoptotic (propidium iodide-negative) cells. *Top*: CF sample stained for CAP18 (purple) with isotype control antibody (open green) shown. *Middle*: same sample stained for CD15 with control. *Bottom*: two-color flow cytometry double-positive CAP18/CD15 cells are shown in upper right quadrant of gated cells (67.4% of total). Isotype control staining resulted in 1.0% double-positive cells (not shown). FL1 = fluorescence emissions wavelength 515 to 545 nm; FL2 = fluorescence emissions wavelength 564 to 606 nm.

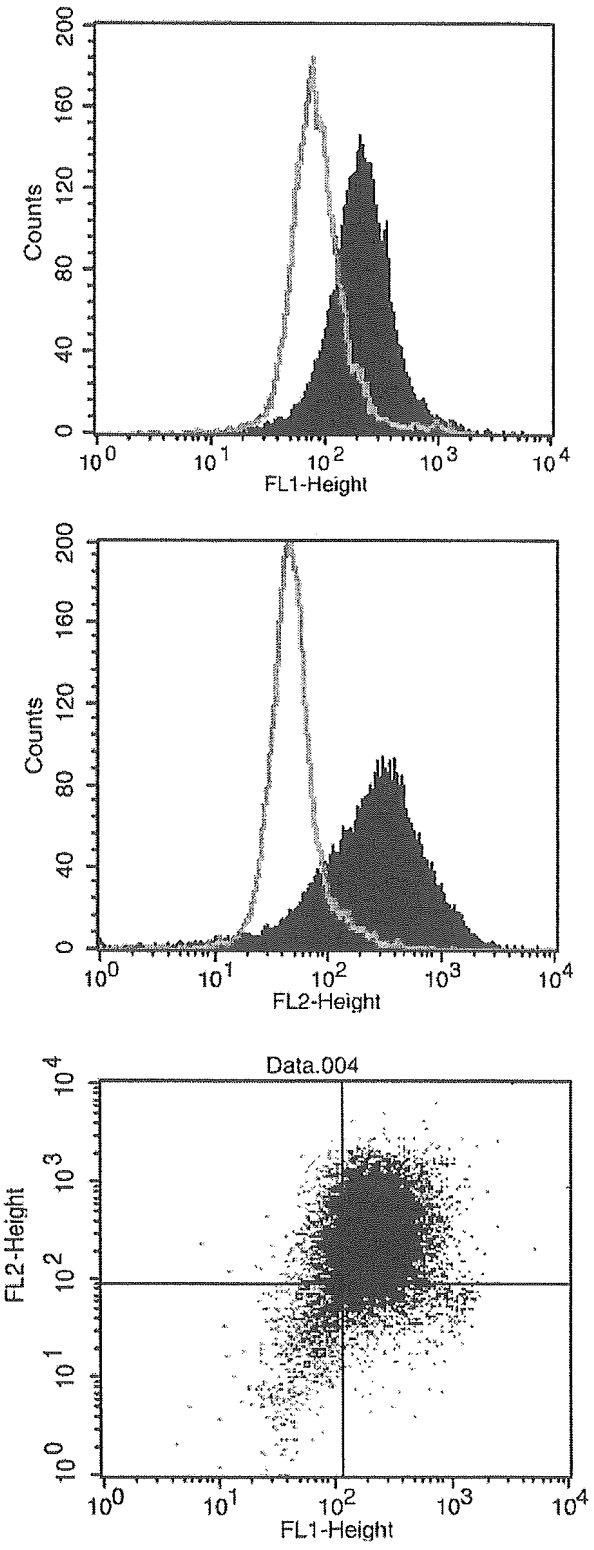

FIGURE 3. Flow cytometry for presence of IL-8 and CD15. *Top*: CF sample stained for IL-8 with isotype control shown as for Figure 2. *Middle*: same sample stained with CD15 and control. *Bottom*: double-positive IL-8/CD15 cells shown in upper right quadrant (64.4% of total). See Figure 2 legend for expansion of abbreviations.

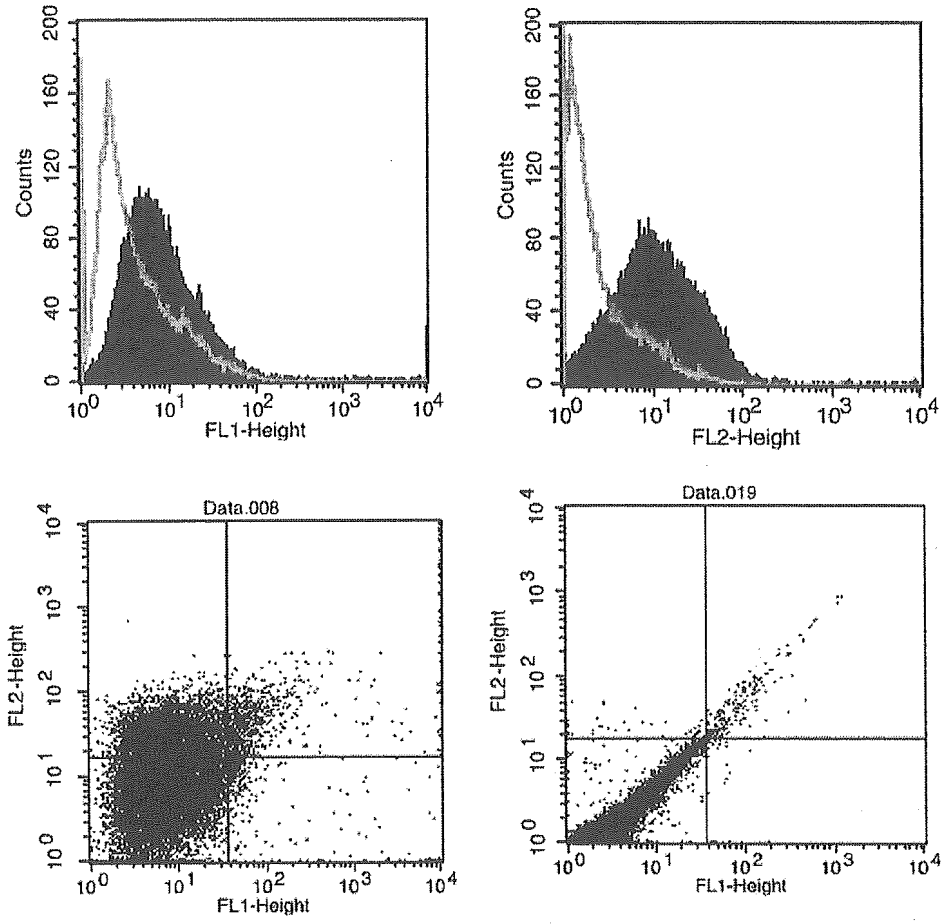

FIGURE 4. Flow cytometry for presence of PAI-1 and CD15. *Top left*: CF sample stained for PAI-1 with isotype control shown. *Top right*: same sample stained for CD15 and control. *Bottom left*: double-positive cells (upper right quadrant) representing 4.16% of total gated cells. *Bottom right*: double-positive cells in upper right quadrant (1% of total) using isotype control antibodies. See Figure 2 legend for expansion of abbreviations.

ogy of chronic inflammatory airways diseases such as CF, asthma, and COPD. In the present study, we were interested in aspects of the innate immune response in CF, in particular the following: (1) the role of the uPA system and the antimicrobial cathelicidin CAP18 that have not received much attention, and (2) how any abnormalities in CF compared to COPD and asthma as well as healthy persons. In order to study this local response noninvasively, we employed a standardized, validated sputum-induction protocol.[29]

The pulmonary plasminogen activator system involves multiple components including uPA, uPAR, and PAI-1. A saturable, specific binding of uPA to uPAR has been demonstrated in a number of cell types including monocytes, macrophages, mast cells, lymphocytes, fibroblasts, endothelial cells, and pulmonary airway epithelial cells.[32–33] Mature uPAR lacks the hydrophobic transmembrane and intracellular domains. Instead, a glycosylphosphatidylinositol moiety is added to the C-terminus providing the anchorage to the outer leaflet of the plasma membrane. Soluble uPAR has been isolated from normal and diseased plasma. By interacting with cell surface adapter molecules and extracellular proteins, uPAR acts beyond the traditional role of localizing and activating cell surface uPA and extends its effects to cellular adhesion and signal transduction.[32] The expression of uPAR is enhanced in various tumors and by a number of proinflammatory agents such as growth factors and cytokines.[32,34] Intraperitoneal injection of endotoxin to mice increased the expression of uPAR in many tissues.[35] Both membrane and soluble forms of uPAR were up-regulated in monocyte cultures by several bacterial surface proteins.[34] There is thus reason to suspect that chronic airways infection, such as seen in CF and COPD, may up-regulate uPAR, and indeed we found increased

Table 2—*Significant Correlations of Induced Sputum Innate Immune Factors With Each Other and With Pulmonary Function Among All Study Subjects*

Factors	Correlates	r	p Value
CAP18	IL-10	0.53	0.0001
CAP18	IL-8	0.16	0.04
uPA	uPAR	0.61	0.0001
uPA	PAI-1	0.40	0.0001
uPA	IL-8	0.34	0.0001
uPA	TNF-α	0.24	0.001
uPAR	PAI-1	0.58	0.0001
uPAR	IL-8	0.47	0.0001
uPAR	TNF-α	0.22	0.002
uPAR	IL-10	0.20	0.006
uPAR	CAP18	0.19	0.01
PAI-1	IL-8	0.53	0.0001
IL-8	TNF-α	0.31	0.0001
IL-8	IL-10	0.30	0.0001
IL-10	IFN-γ	0.24	0.001
FEV_1	CAP18	− 0.31	0.0001
FEV_1	PAI-1	− 0.26	0.0009
FEV_1	uPAR	− 0.20	0.01

levels in sputum in CF and COPD, and to lesser degree in asthma, compared to normal. Interestingly, local uPA itself does not appear to be elevated in any of the disease groups, but its effects could be augmented by the up-regulated receptor levels seen.

PAI-1, a serine protease inhibitor belonging to the serpin family, is a single-chain 45- to 50-kd glycoprotein secreted by many cell types.[36–37] It binds to uPA to modulate the activity of uPA beyond plasmin activation to involve cell adhesion and tissue remodeling. PAI-1 may be inactivated by binding to uPA or forming complexes with uPA and uPAR that are internalized and digested in lysosomes. PAI-1 is secreted by many cell lines, and its expression can be regulated by hormones, growth factors, cytokines, and endotoxin in cell cultures.[37] Depending on the presence of specific regulatory agents, the expression of PAI-1 can either be enhanced or reduced. We found comparably increased levels of PAI-1 in all three chronic inflammatory airways diseases studied (Table 3). Marshall and Shute[38] reported quantitatively similar elevations of PAI-1 in CF sputum (mean, 5.7 ng/mL vs 1.0 ng/mL in control subjects, as compared with our mean of 3.8 ng/mL vs 0.6 ng/mL). During the inflammatory processes, autocrine and paracrine chemokine and cytokine secretion by neutrophils, monocytes, lymphocytes, and airway epithelial cells is enhanced. This may result in increased PAI-1 in sputum by different paths in these diseases.

Table 3—*Schematic Representation of Disease-Specific Patterns of Innate Immune Factors in Sputum as Compared to Healthy Control Subjects**

Factors	CF	COPD	Asthma
CAP18	↑	↑	↓
IL-8	↑↑	↑	↑
IL-10	↑	⟷	⟷
INF-γ	⟷	⟷	⟷
TNF-α	↑	⟷	⟷
uPA	⟷	⟷	⟷
uPAR	↑↑	↑↑	↑
PAI-1	↑	↑	↑

* ↑ = elevated; ↓ = depressed; ↔ = equivalent.

Increases of the neutrophil chemotactic cytokine IL-8 level in airways of CF and COPD patients have been previously reported and related to pathogenesis.[4,26,39] IL-8 is produced by a variety of cells including neutrophils, monocytes, T-cells, and endothelial and airway epithelial cells.[39–42] IL-8 and uPAR both are chemotactic with monocyte and airway epithelial cell expression induced by bacterial products.[34,43] The interaction between leukocytes and endothelial cells is regulated by IL-8 via changes in integrin expression that also involve uPAR.[44–47] Therefore, integrins are potential mediators connecting the functions of IL-8 and uPAR (sputum levels of which we found to be highly correlated). Marshall et al[48] have also related IL-8 activity to PAI-1 by demonstrating that PAI-1 enhances IL-8 activity via inhibition of shedding IL-8/heparan sulfate/syndacan-1 complexes from endothelium. Indeed, IL-8 levels were highly correlated in our study not only with uPAR but also with PAI-1, suggesting a coordinate response in these diseases. The plasminogen activator system may play a prominent role in sustaining airway inflammation by increasing β_2-integrin–mediated leukocyte adhesion and also leukocyte adhesion to extracellular matrix vitronectin.[49–51]

With regard to cathelicidin CAP18, we found comparably elevated levels in CF and COPD patients and subnormal levels in asthmatics. These differences were due to local production, as systemic CAP18 levels in CF patients and control subjects were similar to each other and previously reported plasma levels (approximately 1 μg/mL).[52] CAP18 levels inversely correlated with pulmonary function, a relationship seen most strongly in CF patients but also in COPD. These changes have not been previously reported, although recently Chen et al[53] found increased CAP18 in BAL fluid from patients with CF; CAP18 levels correlated with neutrophilia and decreased lung function. Under normal conditions, CAP18 is secreted by airway epithelial cells and alveolar macrophages, but in conditions of neutrophilia this cell type would be expected to be the dominant source, as we found. The antimicrobial characteristics of CAP18 are related to its α-helical