Epithelial cells lining human airways and cells recruited to airways participate in the innate immune response in part by releasing human neutrophil peptides (HNP). Arginine-specific ADP-ribosyltransferases (ART) on the surface of these cells can catalyze the transfer of ADP-ribose from NAD to proteins. We reported that ART1, a mammalian ADP-ribosyltransferase, present in epithelial cells lining the human airway, modified HNP-1, altering its function. ADP-ribosylated HNP-1 was identified in bronchoalveolar lavage fluid (BALF) from patients with asthma, idiopathic pulmonary fibrosis, or a history of smoking (and having two common polymorphic forms of ART1 that differ in activity), but not in normal volunteers or patients with lymphangioleiomyomatosis. Modified HNP-1 was not found in the sputum of cystic fibrosis patients or in leukocyte granules of normal volunteers. The finding of ADP-ribosyl-HNP-1 in BALF but not in leukocyte granules suggests that the modification occurred in the airway. Most of the HNP-1 in the BALF from individuals with a history of smoking was, in fact, monodi-ADP-ribosylated. ART1 synthesized in *Escherichia coli*, glycosylphosphatidylinositol-anchored ART1 released with phosphatidylinositol-specific phospholipase C from transfected NMU cells, or ART1 expressed endogenously on C2C12 myotubes modified arginine 14 on HNP-1 with a secondary site on arginine 24. ADP-ribosyltransferases (ARTs) (10). This post-translational modification occurs in viruses, bacterial, and eukaryotic cells. Most ARTs also have NAD glycohydrolase activity, generating free ADP-ribose from NAD. The best studied ADP-ribosylation reactions are those catalyzed by certain bacterial toxins, in particular, cholera toxin, which, in many cells, transfers ADP-ribose to an arginine residue in the guanine nucleotide-binding protein, Go (11). Like cholera toxin, mammalian ADP-ribosyltransferases catalyze arginine-specific transferase reactions, but specific cellular substrates differ.

In mammalian cells, the ADP-ribosyltransferases share less than 10% sequence identity, but are structurally similar in their catalytic sites. Only ART1, -3, -4, and -5 have been identified in the human genome and they exhibit restricted tissue distributions (12). Human ART1 has a polymorphism in codon 302, resulting in isoforms that differ in their capacity to ADP-ribosylate membrane-associated proteins (13). ART1, -3, and -4 appear to be glycosylphosphatidylinositol (GPI)-anchored, whereas ART5, expressed in lymphocytes, lacks the carboxyl-terminal signal sequence for addition of a GPI anchor and is predicted to be secreted (14). Both ART1 and ART5 are arginine-specific transferases.

HNP-1 is a substrate for ART. ADP-ribosylation altered its biological properties assayed in vitro, decreasing cytotoxic and antimicrobial activity of neutrophils (3) and are the major component of azurophilic granules; degranulation may be responsible for the increased defensin amount found in airway inflammation. Neutrophils and defensins have been found in airways of patients with cystic fibrosis, α,-antitrypsin deficiency, and other inflammatory lung diseases. Increased numbers of defensins were found in the airway of patients with asthma (4, 5).

α-Defensins (human neutrophil peptide 1–4) are members of a family of low molecular weight, multifunctional cationic peptides. Defensins have 29 to 35 amino acids, are arginine-rich, and contain three disulfide bridges. They are antimicrobial for Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses, cytotoxic for epithelial cells, and chemotactic for T cells. They induce cytokine production, and modulate cell proliferation (6). HNP-1, -2, and -3 are reported to have antiviral activity against human immunodeficiency virus-1 and adenovirus (7, 8) and HNP-1 was shown to inhibit lethal factor, an important component of anthrax toxin (9).

Mono-ADP-ribosylation, in which the ADP-ribose moiety of NAD is transferred to a protein substrate, is catalyzed by amino acid-specific ADP-ribosyltransferases (ARTs) (10). This post-translational modification occurs in viruses, bacterial, and eukaryotic cells. Most ARTs also have NAD glycohydrolase activity, generating free ADP-ribose from NAD. The best studied ADP-ribosylation reactions are those catalyzed by certain bacterial toxins, in particular, cholera toxin, which, in many cells, transfers ADP-ribose to an arginine residue in the guanine nucleotide-binding protein, Go (11). Like cholera toxin, mammalian ADP-ribosyltransferases catalyze arginine-specific transferase reactions, but specific cellular substrates differ.

Inflammatory cells, such as neutrophils, are recruited to the airway of patients with chronic lung disorders (1, 2). Protein mediators of innate immunity, such as defensins, are produced by these cells and epithelial cells lining the airway. Defensins constitute over 5% of the protein content of neutrophils (3) and are the major component of azurophilic granules; degranulation may be responsible for the increased defensin amount found in airway inflammation. Neutrophils and defensins have been found in airways of patients with cystic fibrosis, α,-antitrypsin deficiency, and other inflammatory lung diseases. Increased numbers of defensins were found in the airway of patients with asthma (4, 5).

α-Defensins (human neutrophil peptide 1–4) are members of a family of low molecular weight, multifunctional cationic peptides. Defensins have 29 to 35 amino acids, are arginine-rich, and contain three disulfide bridges. They are antimicrobial for Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses, cytotoxic for epithelial cells, and chemotactic for T cells. They induce cytokine production, and modulate cell proliferation (6). HNP-1, -2, and -3 are reported to have antiviral activity against human immunodeficiency virus-1 and adenovirus (7, 8) and HNP-1 was shown to inhibit lethal factor, an important component of anthrax toxin (9).

Mono-ADP-ribosylation, in which the ADP-ribose moiety of NAD is transferred to a protein substrate, is catalyzed by amino acid-specific ADP-ribosyltransferases (ARTs) (10). This post-translational modification occurs in viruses, bacterial, and eukaryotic cells. Most ARTs also have NAD glycohydrolase activity, generating free ADP-ribose from NAD. The best studied ADP-ribosylation reactions are those catalyzed by certain bacterial toxins, in particular, cholera toxin, which, in many cells, transfers ADP-ribose to an arginine residue in the guanine nucleotide-binding protein, Go (11). Like cholera toxin, mammalian ADP-ribosyltransferases catalyze arginine-specific transferase reactions, but specific cellular substrates differ.
activity while maintaining its function as a T-cell chemoattractant and stimulant of interleukin-8 release from epithelial cells (15). Consistent with a biological role for this modification, ADP-ribosylated HNP-1 was found in bronchoalveolar lavage fluid (BALF) of some, but not all smokers, possibly related to expression of an ART1 polymorphism. Epithelial cells in human airways as well as other immune response participants, e.g. polymorphonuclear leukocytes and lymphocytes, express different ARTs including ART1, -3, and -4 (16, 17). Conceivably, ARTs found on airway epithelial and leukocytes surfaces could modify the arginine-rich defensins released from polymorphonuclear leukocytes and epithelial cells in response to lung inflammation and affect anti-microbial activity.

We investigated whether modification of HNP-1 was specific for ART1 and whether free ADP-ribose generated by NAD glycohydrolase activity of ARTs might react with the cationic defensin in vitro. To determine whether the modified form of HNP-1 occurs in vivo, HNP was isolated and analyzed for modifications from the BALF of patients with several pulmonary diseases. Moreover, to assess a potential regulatory effect of HNP-1 concentration on transferase activity, we investigated the effect of HNP-1 on ART1 and -5 activities.

**MATERIALS AND METHODS**

Preparation of ART1, ART3, ART4, ART5, ART2.2, and CTA1—Rabbit ART1 (rART1) and mouse ART5 (mART5) were synthesized in *Escherichia coli* and purified as described (15, 18). Briefly, ART1, cloned from a rabbit skeletal muscle cDNA library by PCR, and mouse lymphocyte ART5 cDNAs, were subcloned into a pFLAG-MAC (Sigma) expression vector and used to transform *E. coli* BL-21(DE3) competent cells (Novagen). Cells were sonified, centrifuged, and purified on anti-FLAG M2 affinity gels (Sigma) according to the manufacturer's instructions. ART2.2 cDNA was inserted into pCMV-Neo vector (Clontech, Palo Alto, CA) and the protein was expressed as a GPI-linked protein on the surface of COS1 cells. Protein was collected following treatment of intact cells with phosphatidylinositol-specific phospholipase C (PI-PLC) as described (19). Mouse ART1 (mART1) or mART4 (20) open reading frames were subcloned in pMH vector and the hemagglutinin tag sequence (transferase domain, 100 nM, 10 nmol activity) with FAS (factor activating exoenzyme S) (100 nmol), ART2b (4 nmol) or, ART2.2 (4 nmol) were incubated with 5 nmol of HNP-1 and 5 nmol NAD in 50 mM phosphate buffer, pH 7.5, 150 µl at 30 °C overnight followed by HPLC separation of the products. To determine the extent of modification, the results were compared with the same reaction conditions with ART1 (4 nmol) incubated with HNP-1 (5 nmol) and 5 nmol NAD. In preparation for RP-HPLC, guanidine HCl was added to a final concentration of 6 M. The mixture was applied to a Vydac C18 HPLC column (Nest Group, Southboro, MA) or a Discovery BioWide Pore C18 HPLC column (Supelco, Bellefonte, PA), equilibrated with HPLC-grade water, 0.1% trifluoroacetic acid (solution A). Reaction products were separated by gradient elution, flow = 0.8 ml/min. Solution A for 20 min was followed by a linear gradient for 60 min of 0 to 60% solution B (100% isopropyl alcohol, 0.2% trifluoroacetic acid), then 95% solution B, for 25 min. Separation was monitored by absorbance at 210, 258, and 280 nm. Fractions were analyzed by MALDI-MS (Ciphergen, Biosystem). Products of NAD glycohydrolase reactions were isolated by HPLC anion exchange separation using a DuPont SAX column (4.6 x 250 mm, Thomson Instruments, Clear Brook, VA) by gradient elution. Solution A (20 mM sodium phosphate, pH 4.5), for 30 min, was followed by a linear gradient for 10 min to 100% solution B (solution A + 1.0 mM NaCl), followed by 10 min at 100% B, flow = 1.0 ml/min.

**MS Analysis of ADP-ribosylated HNP-1**—MALDI-TOF mass spectrometry and HPLC electrospray mass spectrometric mapping of ADP-riboseylation sites were performed as described (15).

**Cell Culture**—C2C12 (mouse skeletal muscle cells) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C in 5% CO2. COS1 cells were grown in Dulbecco’s modified Eagle’s medium, 55 µM 2-mercaptoethanol, 10% fetal bovine serum, with 0.45/ mg/ml Geneticin (G-418, Invitrogen Inc.). Rat mammary adenocarcinoma cells (NMU) transfected with plasmids containing mART1, or mART4 cDNA were grown at 37 °C, 5% CO2 in Eagle’s minimal essential medium (Invitrogen) with 10% fetal bovine serum (Invitrogen) with 0.5 mg/ml G-418. All cells were supplied by American Type Culture Collection (Manassas, VA).

**Isolation of ADP-riboyl-HNP-1 from Human BALF**—Clinical protocols were approved by the NHLBI, National Institutes of Health Institutional Review Board. Written informed consent was obtained for all subjects. Proteins from 10-ml samples of BALF were isolated and purified as described (15) before separation by RP-HPLC. Seven samples had HNP-1. Peaks (absorbance at 210 nm) corresponding to HNP-1 and ADP-riboyl-HNP-1 were analyzed by MALDI-MS, HNP from 8 ml of BALF from patients with biopsy-proven idiopathic pulmonary fibrosis (protocol 99-H-0068), asthma (protocol 99-H-0076), lymphangio-

**ART1-specific ADP-ribosylation of HNP-1**
ART1-specific ADP-ribosylation of HNP-1

**TABLE 1**  
Isolation and analysis of HNP-1 from patients

BALF were used for the isolation of HNP-1 from idiopathic pulmonary fibrosis patients (one of seven patients), asthma patients (one of four patients), and lymphangioleiomyomatosis patients (three patients). HNP-1 was isolated from sputum of four cystic fibrosis patients and leukocytes of four normal volunteers. BALF samples (8 ml) were applied to Sep-Pak C18 columns (Supelco) conditioned with 10% isopropyl alcohol, 0.05% trifluoroacetic acid. HNP-1 was eluted with 50% isopropyl alcohol, 0.05% trifluoroacetic acid and concentrated by evaporation of solvent. HNP-1 from sputum (23) or leukocytes was prepared as described, without size exclusion chromatography (35). HNP-1, ADP-ribosylated-HNP-1, and di-ADP-ribosylated-HNP-1 were identified by MALDI-MS.

| Sample                        | HNP-1 | ADP-ribosyl-HNP-1 | Di-ADP-ribosyl-HNP-1 | Total | Modified |
|-------------------------------|-------|-------------------|----------------------|-------|----------|
| Idiopathic pulmonary fibrosis | +     | +                 | +                    | 91    | 201      | 88       |
| Asthma (BALF)                 | +     | +                 | +                    | 87    | 149      | 88       |
| Cystic fibrosis (sputum)      | +     |                   |                      | 160   | 199      | 97       |
| Human leukocytes (granules)   | +     |                   |                      | 0     | 4        | 100      |
| Lymphangioleiomyomatosis (BALF) | -    |                   |                      | 0     | 4        | 100      |

**TABLE 2**  
Isolation of HNP-1 from patient BALF (pmol)

The amount of HNP-1, ADP-ribosylated-HNP-1, and di-ADP-ribosylated HNP-1 was determined by mass spectroscopy from the BALF of five healthy volunteers with a history of smoking. BALF (23–135 ml) was applied to a Sep-Pak C18 cartridge (Waters), equilibrated, washed, eluted, and concentrated as described (15). The BALF were concentrated to 500 μl 8 μl were analyzed and the results of that analysis are reported in the table. Data were quantified by comparison to ART1-modified HNP-1 prepared and analyzed by the same procedure.

| Sample | HNP-1 | ADP-ribosyl-HNP-1 | Di-ADP-ribosyl-HNP-1 | Total | Modified |
|--------|-------|-------------------|----------------------|-------|----------|
| A      | 25    | 85                |                      | 91    | 201      | 88       |
| B      | 18    | 44                |                      | 87    | 149      | 88       |
| C      | 6     | 33                |                      | 160   | 199      | 97       |
| D      | 0     | 4                 |                      | 0     | 4        | 100      |
| E      | 0     | 4                 |                      | 0     | 4        | 100      |

RESULTS

To determine the modification state of HNP-1 in patients with airway diseases, HNP-1 was isolated from the BALF of patients with pulmonary disease, e.g. lymphangioleiomyomatosis, idiopathic pulmonary fibrosis, and asthma, and compared with HNP isolated from BALF of smokers and leukocyte granules of normal volunteers as reported (Table 1) (15). BALF from normal volunteers did not contain HNP-1 (data not shown). In isolated HNPs, HNP-1 was the isoform present at the highest concentration; little or no HNP-2 or -3 was detected. In idiopathic pulmonary fibrosis and asthma BALF, both mono- and di-modified HNP-1 were identified suggesting that, in these patients, neutrophils were recruited to the airway where the released defensin could come in contact with epithelial cells expressing an arginine-specific ADP-ribosyltransferase. HNP was also isolated from leukocyte granules (four patients), but modified forms were not identified, consistent with the modification of HNP-1 occurring after its release from neutrophils. The extent of modification of HNP-1 was determined in individuals with a history of smoking. BALF was purified by Sep-Pak C18 and then analyzed by mass spectroscopy. Most of the HNP-1 was either mono- or di-ADP-ribosylated, based on recovery assessed with controls containing ART1-modified HNP-1 (Table 2). Sputum from cystic fibrosis patients had been reported to contain defensins at a concentration of 0.3–1.6 mg/ml (23). ADP-ribosylated HNP was not detected in HNP isolated from cystic fibrosis sputum. The absence of ADP-ribosylated HNP-1 in cystic fibrosis sputum could be because of the inhibition of ADP-ribosyltransferase activity by the high concentrations of chloride and sodium ions in cystic fibrosis airway surface liquid (24). A concentration as high as 225 mM NaCl did not, however, inhibit ADP-ribosylation of HNP-1 by ART1 in vitro (data not shown). Alternatively, HNP-1 could be cytotoxic to the epithelial cells expressing ART1 or perhaps prevented from contact with cell-associated ART1 due to the bacterial biofilm.

To determine ART specificity for modification of HNP-1, HNP-1 and NAD were incubated with ARTs from different sources, either free in solution or cell-associated (e.g. released from the cell or GPI-anchored), presuming that physical access of the peptide to the enzyme might be context-specific. The sources of bacterial and mammalian transferases included mouse C2C12 cells that endogenously express ART1 when differentiated into myotubes (Fig. 1a), undifferentiated C2C12 cells (Fig. 1d (25, 26), recombinant rabbit ART1 synthesized in E. coli (Fig. 1b), recombinant mouse ART1 (Fig. 1c) or recombinant mouse ART4 (Fig. 1g) synthesized in rat NMU cells, and two arginine-specific ARTs, recombinant mART5 synthesized in E. coli (Fig. 1e) and alkylated cholera toxin A1 subunit (Fig. 1f). Among reaction products that were purified by RP-HPLC, those identified by MALDI-MS included peptides of 3,439 Da molecular mass, consistent with unmodified HNP-1 (A); 3,983 Da, consistent with mono-ADP-ribosylated-HNP-1 (*) (ADP-ribosylated on arginine 14, based on previous analysis (15); and 4,524 Da (C), consistent with di-ADP-ribosyl-HNP-1 (ADP-ribosylated on arginine 24 in addition to arginine 14, based on MS analysis as described under “Materials and Methods”). By HPLC analysis, primarily ART1, from multiple sources, free or cell-associated, catalyzed the transfer of an ADP-ribose moiety to HNP-1; ART2b (the rat ART2 isoform, 4 nmol; data not shown), ART3 (20 ng, data not shown), ART4, and ART5 were inactive by HPLC analysis. ART2b (mouse isoform, 4 nmol) exhibited less than 10% the activity of ART1 (data not shown). Cholera toxin and P. aeruginosa exoenzyme S (transferase domain, 10 nmol), produced as a recombinant protein in E. coli, were also inactive.

ADP-ribosylated HNP-1 was not formed in the presence of NADP, consistent with an ART1 preference for NAD as substrate (Figs. 1 and 2a). HNP-1 is a basic protein containing multiple lysine and arginine residues. ADP-ribose can react non-enzymatically with lysine (27) and ARTs are known to exhibit NAD glycohydrolase activity, which produces ADP-ribose. Free ADP-ribose did not react with HNP-1, consistent with the conclusion that the modification of HNP-1 by ART1 was
not due to the non-enzymatic addition of ADP-ribose generated by ART NAD glycohydrolase activity (Figs. 1 and 2).

ADP-ribosylated-HNP-1 had already been identified in the BALF of some smokers, consistent with a role for ADP-ribosylation in modulating the activity of defensin in vivo (15). In ART1, a single nucleotide polymorphism results in proline replacement of leucine 257 with alteration of transferase activity, measured by ADP-ribosylation of membrane-associated proteins (13). To determine whether a specific ART1 isoform was responsible for the modification of HNP-1 in vivo, the genomic DNA of smokers was analyzed to identify the codon for amino

FIGURE 1. RP-HPLC separation of reaction products from the incubation of HNP-1 with mono-ADP-ribosyltransferases. a, C2C12 cells were grown in Dulbecco’s modified Eagle’s medium to 2 x 10^5 cells/cm^2. On day 6, intact differentiated cells (exhibiting myotube formation) that have mART1 (0.24 nmol/h/100-mm culture dish) on the surface were washed twice with Dulbecco’s phosphate-buffered saline and incubated with HNP-1 (10 µg) and 2 mM NAD for 1 h at 37 °C before collection of medium for analysis. b, HNP-1 (2 µg) was incubated with recombinant rART1 (1.0 nmol/h) synthesized in E. coli, and 1 mM NAD in 200 µl. c, HNP-1 (10 µg) was incubated with 5 mM NAD and recombinant mART1 (0.74 nmol/h) (synthesized in NMU cells), in 150 µl. d, undifferentiated C2C12 cells that lack mART1 were washed twice with Dulbecco’s phosphate-buffered saline and incubated as above. e, auto-ADP-ribosylated ART5 (1.8 nmol/h) synthesized in E. coli; f, alkylated CTA1 (0.74 nmol/h); or g, recombinant mART4 (expression determined by Western blot) was incubated at 30 °C with 2 µg of HNP-1 (5 µg) and either 5 mM ADP-ribose (Fig. 2a) or 5 mM NADP (Fig. 2b) in place of NAD. Reactions in 50 mM potassium phosphate, pH 7.5, with enzyme, were incubated overnight (except where noted) at 30 °C, and were terminated by adding guanidine HCl to the medium or reaction mixture at a concentration of 6 M, before separation by HPLC. Activity of the transferases was determined by quantifying ADP-ribosylation of agmatine. Reaction mixture HPLC analysis at a wavelength of 210 nm is described under “Materials and Methods.” Data shown are representative of at least two experiments.

FIGURE 2. Modification of defensin. a, effect of HNP-1 or agmatine on nicotinamide release from NAD catalyzed by ART1. mART1 (3.6 nmol/h) synthesized in NMU cells was incubated with 0.1 mM [nicotinamide-U-14C]NAD (0.05 µCi), in 50 mM potassium phosphate, pH 7.5, for 1.5 h at 30 °C, with the indicated concentrations of agmatine (●) or HNP-1 (●) in 150 µl. Two samples (50 µl) were applied to Dowex AG-1-X2 (Bio-Rad) columns (15°C) and [14C]nicotinamide was eluted with 5 ml of water for liquid scintillation counting as described (34). Data are mean ± S.D. of values from three experiments (agmatine) or one-half the ranges of two experiments (HNP-1). b, time course modification of HNP-1. mART1 (24.5 nmol/h) and HNP-1 (1.5 nmol) were incubated with 5 mM NAD in 50 mM potassium phosphate, pH 7.5, at 30 °C for the indicated times. The reactions were stopped by the addition of guanidine HCl and subjected to HPLC as described under “Materials and Methods.” Amounts of mono-ADP-ribosylated-HNP-1 (●) or di-ADP-ribosylated-HNP1 (●) were calculated from the area under the absorbance peak at 210 nm. Data are representative of those from two experiments. c, ADP-ribosylation of HNP-1 and HBD1 by ART1 and ART5. mART1 (4 nmol/h) or mART5 (4 nmol/h) were incubated with HNP-1 (1 nmol) or HBD1 (1 nmol), as indicated, in 50 mM potassium phosphate, pH 7.5, at 30 °C, followed by 5 mM NAD overnight. After trichloroacetic acid precipitation, the proteins were separated on 16% Tricine gels (Invitrogen). The gels were stained with Coomassie Blue, dried, and exposed to x-ray film (BioMax, Kodak). Data shown are representative of two experiments.

JUNE 23, 2006•VOLUME 281•NUMBER 25•JOURNAL OF BIOLOGICAL CHEMISTRY 17057
ART1-specific ADP-ribosylation of HNP-1

TABLE 3

Association of ART1 amino acid 257 polymorphism in smokers with ADP-ribosyl-HNP-1 in BALF

Genomic DNA was isolated from whole blood of smokers, using the Puregene kit (Gentra Systems, Minneapolis, MN) following the manufacturer’s directions. PCR restriction fragment length polymorphism (RFLP) analysis was performed as described (13). Briefly, genomic DNA, 50 ng, was used as template in a PCR with 20 mM Tris·HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 200 μM of each dNTP, 1 unit of Taq DNA polymerase and the primers 5′-GAGAAGGAGGTGTGTGATC-3′ (sense), and 5′-AGTGTAGGTGTGTGCTTC-3′ (antisense) in 25 μl. The PCR product was digested with HpaII (New England Biolabs), and the fragments were separated by electrophoresis in 3% agarose gels containing ethidium bromide.

| Patient No. | Position 257 | ADP-ribosyl-HNP-1 |
|-------------|--------------|-------------------|
| 1           | Leu/Leu      | +                 |
| 2           | Pro/Pro      |                  |
| 3           | Pro/Pro      |                  |
| 4           | Pro/Pro      |                  |
| 5           | Leu/Pro      |                  |
| 6           | Pro/Pro      |                  |
| 7           | Leu/Pro      |                  |

Acid 257 (Table 3). Modified HNP-1 was found in the BALF of smokers regardless of the ART1 polymorphism.

ADP-ribosyltransferase reactions proceed by an S,2-like mechanism. In the NAD glycohydrolase reaction, water serves as a nucleophilic ADP-ribose acceptor; preferred nucleophilic substrates including agmatine and arginine accelerate nicotinamide release that accompanies ADP-ribose transfer. Nicotinamide formation may be in excess of ADP-ribose transferred to acceptor due to continued access of water to the catalytic site. In agreement, HNP-1, at low concentrations, stimulated the formation of nicotinamide from NAD, by serving as an ADP-ribose acceptor. Modification of HNP-1 was coupled to nicotinamide release, with a ratio of 0.90, during incubation for 1.5 h at 30 °C for 0.1 mM NAD and 2 nmol of HNP-1. At high concentrations, HNP-1 appeared to suppress ART1 activity (Fig. 2a). Increasing the time of incubation increased the amount of both mono- and di-modified-HNP-1 (Fig. 2b). After 1 h, there was more mono- than di-ADP-ribosylated HNP-1, and the ratio of mono- to di-ADP-ribosylated product decreased with increasing time of incubation. After overnight incubation, however, most of the modified peptide contained two ADP-ribo-syl moieties (data not shown). At concentrations of HNP-1 below 5 nmol in 150 μl, ART1 activity with HNP-1 was approximately twice that seen with agmatine (Fig. 2a). ART1 activity was specific for HNP-1; human β-defensin (HBD1), which is constitutively expressed by epithelial cells in the airway (28), had no effect on nicotinamide release (data not shown). However, it was 32P]ADP-ribosylated by ART1, but the extent of modification was considerably less than that observed with HNP-1. Both HNP-1 and β-defensin were poor ART5 substrates (Fig. 2c).

The catalytic activity of ART5 in the presence of NAD and HNP-1 was evaluated by monitoring the production of ADP-ribose and ADP-ribo-syl-agmatine, as well as autoamidification of ART5. Addition of HNP-1 dramatically reduced both NAD:agmatine ADP-ribosyltransferase (Fig. 3a) and NAD glycohydrolase activities (Fig. 3b). HNP-1 also reduced automodification of ART5 (Fig. 3c). As had been reported (18), ART5 is primarily a NAD glycohydrolase; automodification decreases that activity by over 90%, whereas ADP-ribo-syltransferase activity approximately doubles. Thus, autoamidification converts ART5 from an NAD glycohydrolase to an ADP-ribosyltransferase. HNP-1, by inhibiting automodification, prevents ART5 from exhibiting transferase activity. Because HNP-1 is released by neutrophils at sites of inflammation and because HNP-1 represents greater than 5% of neutrophil protein and more than 30–50% of azurophilic granule content (5), its local concentration following neutrophil degranulation may be sufficient to inhibit ART5 and ART1 activities. ART1 transferase is apparently inhibited directly, whereas with inhibition of automodification ART5 remains a NAD glycohydrolase.

As the modification of defensin appears to be site-specific and defensins are constrained by three disulfide bridges, we questioned whether reduction of the cysteines might facilitate ADP-ribosylation. ART1-catalyzed ADP-ribosylation of HNP-1 was enhanced by reduction of the peptide with dithiothreitol, and the extent of modification was increased, suggesting that loss of the secondary structure in the presence of thiol exposed arginine residues otherwise resistant to modi-
ification. In contrast to the effects of thiol on HNP-1 modification, ART1 transferase activity with agmatine, a low molecular weight guanidine compound as ADP-ribose acceptor, was reduced slightly in the presence of dithiothreitol (data not shown). ART5 and CTA ADP-ribosylated HNP poorly compared with ART1; similar to ART1, however, modification of HNP-1 in the presence of CTA was increased slightly with dithiothreitol (Fig. 3d).

**DISCUSSION**

ADP-ribosylation of HNP-1 is ART specific. HNP-1 is a substrate for ART1, a transferase known to be expressed by airway epithelial cells; it was not ADP-ribosylated by ART3, -4, and -5, cholera toxin, and modification of HNP-1 in the presence of CTA was increased slightly in the presence of dithiothreitol (data not shown). ART5 and CTA ADP-ribosylated HNP poorly compared with ART1; similar to ART1, however, modification of HNP-1 in the presence of CTA was increased slightly with dithiothreitol (Fig. 3d).

In contrast, ART5, which is predicted to be secreted, had been shown to modify arginine in proteins, and that activity was enhanced by auto-ADP-ribosylation. As we show here, auto-ADP-ribosylation and generation of the transferase form of ART5, were suppressed by HNP-1. Even under conditions that promote ART5 transferase activity (e.g. high NAD concentrations), however, HNP-1 was not significantly modified. In this regard, another ADP-ribosyltransferase that modifies arginine residues, the bacterial product, cholera toxin, did not modify HNP-1, suggesting that the arginine-specific ADP-ribosyltransferases in vitro are substrate specific.

Amino acid target specificity of ART1 was observed in its preferential modification of arginine 14 in mono-ADP-ribosyl-HNP-1 and with a secondary site on arginine 24 in di-modified HNP-1. Both forms were isolated from airways of patients with diseases associated with inflammation, including pulmonary fibrosis and asthma as well as smokers (Tables 1 and 2). Thus, the modification appears to occur in vivo, where ART1 has been found on epithelial cells lining the airway and could be in position to use defensin as a substrate. The two other arginine residues in HNP-1 were not significantly modified in vitro. The extent of modification was constrained by the structure of the defensin, because reduction of disulfide bonds enhanced both the rate and number of modifications. Given the fact that HNP-1 is lysine-rich, we were concerned that the modification could be nonenzymatic, resulting from the covalent association of free ADP-ribose, generated through the NAD glycohydrolase activity of ART1, with the ε-amino groups of lysine. However, in studies where ADP-ribose was incubated with HNP-1, non-enzymatic covalent modification was not observed. These studies point to an arginine-specific enzymatic modification of HNP-1 by ART1, not by other ARTs. Based on our observations, the ADP-ribosylation reactions appear to be specific for ART family members, for different substrates, and for the ADP-ribosyl donor.

Epithelial cells that line the airway have a critical role in the innate immune response. They respond to pathogens by changing surface receptors, releasing cytokines, and altering regulation of key genes (29, 30). They not only provide a mucosal barrier but also produce antimicrobial peptides and recruit phagocytic cells (29). High concentrations of neutrophil defensins accumulate in airways of patients as part of the inflammatory response and may interact with airway epithelium (31, 32). α-Defensins exhibit lectin-like behavior and may bind to glycoproteins on epithelial cell surfaces (33). Such an interaction might facilitate the association of ART1 and its substrate. How these cells regulate ADP-ribosylation and modification of defensin activities may affect the inflammatory response in the airway.

It has been shown that defensins have a variety of activities including chemoattractant and antimicrobial actions at low concentrations to induction of interleukin-8 release and cytotoxicity at high concentrations. They may contribute to both epithelial cell damage and proliferation. Cell lysis may be a source of extracellular NAD as a substrate for the ADP-ribosyltransferases expressed on the epithelial surface. Of importance, ADP-ribosylation of HNP-1 alters its functional properties, which could have a regulatory role. As reported here, HNP-1 may affect transferase activity and thus regulate its own modification.

Acknowledgments—We thank Dr. Stewart Levine for providing BALF from asthma patients and Ruth Litzenberger for the cystic fibrosis sputum samples. We thank Dr. Joseph Barbieri for the generous gift of exoenzyme S and Factor Activating Exoenzyme S and Dr. Rita Bortell for ART2b. We thank Dr. Martha Vaughan and Dr. Vincent C. Manganiello for useful discussions and critical review of the manuscript. We also thank the LAM Foundation and the Tuber-
ous Sclerosis Alliance for patient referrals.

**REFERENCES**

1. Aarbiou, J., Ertmann, M., van Wetering, S., van Noort, P., Rook, D., Rabie, K. F., Litvinov, S. V., van Krieken, J. H., de Boer, W. J., and Hiemstra, P. S. (2002) J. Leukocyte Biol. 72, 167–174
2. van Wetering, S., Sterk, P. J., Rabie, K. F., and Hiemstra, P. S. (1999) J. Allergy Clin. Immunol. 104, 1131–1138
3. Rice, W. G., Ganz, T., Kinkade, J. M., Jr., Selsted, M. E., Lehrer, R. I., and Parmley, R. T. (1987) Blood 70, 757–765
4. Ordonez, C. L., Shaughnessy, T. E., Matthy, M. A., and Fahy, J. V. (2000) Am. J. Respir. Crit. Care Med. 161, 1185–1190
5. Spencer, L. T., Paone, G., Krein, P. M., Rouhani, F. N., Rivera-Nieves, J., and Brantly, M. L. (2004) Am. J. Physiol. 286, L614–L620
6. Ganz, T. (2003) Nat. Rev. Immunol. 3, 710–720
7. Chang, T. L., Vargas, J. J., Delportillo, A., and Klotman, M. E. (2005) J. Clin. Invest. 115, 765–773
8. Bastian, A., and Schafer, H. (2001) Regul. Pept. 101, 157–161
9. Kim, C., Gajendran, N., Mittrucker, H. W., Weiwald, M., Song, Y. H., Hurwitz, R., Wilmanns, M., Fischer, G., and Kaufmann, S. H. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4830–4835
10. Moss, J., Balducci, E., Cavanaugh, E., Kim, H. J., Konczalik, P., Lesma, E. A., Okazaki, I. J., Park, M., Shoemaker, M., Stevens, L. A., and Zolkiewska, A. (1999) Mol. Cell. Biochem. 193, 109–113
11. Tsai, S. C., Noda, M., Adamik, R., Moss, J., and Vaughan, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5139–5142
12. Głowacki, G., Brazen, R., Firner, K., Nissen, M., Kuhl, M., Reche, P., Bazan, F., Cetkovic-Crlej, M., Leiter, E., Haag, F., and Koch-Nolte, F. (2002) Protein Sci. 11, 1657–1670
13. Yadollahi-Farsani, M., Kefalah, P., Sassy, B. A., and MacDermot, J. (1999) Eur. J. Biochem. 262, 342–348
14. Okazaki, I. J., Kim, H. J., and Moss, J. (1996) J. Biol. Chem. 271, 22002–22007
15. Paone, G., Wada, A., Stevens, I. A., Matin, A., Hirayama, T., Levine, R. L., and Moss, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 8231–8235
16. Balducci, E., Horiba, K., Uzuiki, J., Park, M., Ferrans, V. J., and Moss, J. (1999) Am. J. Respir. Cell Mol. Biol. 21, 337–346
17. Kefalas, P., Sassy, B., Yadollahi-Farsani, M., and MacDermot, J. (1999) Eur. J. Biochem. 259, 866–871
18. Weng, B., Thompson, W. C., Kim, H. J., Levine, R. L., and Moss, J. (1999) J. Biol. Chem. 274, 31797–31803
19. Kanaitsuoka, T., Bortell, R., Stevens, I. A., Moss, J., Sardinha, D., Rajan, T. V., Zipris, D.,...
ART1-specific ADP-ribosylation of HNP-1

Mordes, J. P., Greiner, D. L., and Rossini, A. A. (1997) *J. Immunol.* **159**, 2741–2749

Koch-Nolte, F., Haag, F., Braren, R., Kuhl, M., Hoovers, J., Balasubramanian, S., Bazan, F., and Thiele, H. G. (1997) *Genomics* **39**, 370–376

Noda, M., Tsai, S. C., Adamik, R., Moss, J., and Vaughan, M. (1990) *Biochim. Biophys. Acta* **1034**, 195–199

Stevens, L. A., Bourgeois, C., Bortell, R., and Moss, J. (2003) *J. Biol. Chem.* **278**, 19991–19996

Soong, L. B., Ganz, T., Ellison, A., and Caughey, G. H. (1997) *Inflamm. Res.* **46**, 98–102

Smith, J. J., Travis, S. M., Greenberg, E. P., and Welsh, M. J. (1996) *Cell* **85**, 229–236

Zolkiewska, A., Thompson, W. C., and Moss, J. (1998) *Exp. Cell Res.* **240**, 86–94

Zolkiewska, A., and Moss, J. (1993) *J. Biol. Chem.* **268**, 25273–25276

Cervantes-Laurean, D., Minter, D. E., Jacobson, E. L., and Jacobson, M. K. (1993) *Biochemistry* **32**, 1528–1534

Singh, P. K., Jia, H. P., Wiles, K., Hesselberth, J., Liu, L., Conway, B. A., Greenberg, E. P., Valore, E. V., Welsh, M. J., Ganz, T., Tack, B. F., and McCray, P. B., Jr. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 14961–14966

Diamond, G., Legarda, D., and Ryan, L. K. (2000) *Immunol. Rev.* **173**, 27–38

van Wetering, S., Mannesse-Lazeroms, S. P., Van Sterkenburg, M. A., Daha, M. R., Dijkman, J. H., and Hiemstra, P. S. (1997) *Am. J. Physiol.* **272**, L888–L896

Hiemstra, P. S. (2001) *Pediatr. Respir. Rev.* **2**, 306–310

Bals, R. (2000) *Respir. Res.* **1**, 141–150

Wang, W., Owen, S. M., Rudolph, D. L., Cole, A. M., Hong, T., Waring, A. J., Lal, R. B., and Lehrer, R. I. (2004) *J. Immunol.* **173**, 515–520

Moss, J., Manganillo, V. C., and Vaughan, M. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 4424–4427

Harwig, S. S., Ganz, T., and Lehrer, R. I. (1994) *Methods Enzymol.* **236**, 160–172