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Transferrin and Lactoferrin Undergo Proteolytic Cleavage in the *Pseudomonas aeruginosa*-Infected Lungs of Patients with Cystic Fibrosis

BRADLEY E. BRITIGAN,[1,2,3]* MICHAEL B. HAYEK,[1] BRADLEY N. DOEBBELING,[2,3] AND ROBERT B. FICK, JR.[1,2,3]†

Research Service[1] and Department of Internal Medicine,[2] Department of Veterans Affairs Medical Center, and Department of Internal Medicine, University of Iowa College of Medicine,[3] Iowa City, Iowa 52242

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Bacterium- and neutrophil-derived proteases have been suggested to contribute to tissue injury at sites of *Pseudomonas aeruginosa* infection. Pseudomonas elastase cleavage of transferrin enhances in vitro iron removal from this protein by the *P. aeruginosa* siderophore pyoverdin. This cleavage also generates new iron chelates which, in contrast to iron bound to transferrin, are able to catalyze formation of the highly cytotoxic hydroxyl radical from neutrophil-derived superoxide and hydrogen peroxide via the Haber-Weiss reaction. In order to determine whether this cleavage occurs in vivo, a chemiluminescence immunoblot system was developed to detect the presence of proteolysis products of transferrin or the related iron-binding protein, lactoferrin. Using this immunoblot system, we detected transferrin and lactoferrin cleavage products in bronchoalveolar lavage (BAL) samples from 21 of 22 and 20 of 21 cystic fibrosis (CF) patients, respectively. Three of eleven and two of nine BAL samples from individuals with other forms of chronic inflammatory lung disease had transferrin and lactoferrin cleavage products, respectively. Each patient in whom such products were detected was also infected with *P. aeruginosa*. No such products were detected in normal individuals. In the CF patients, there was no clear correlation between the extent of transferrin or lactoferrin cleavage and BAL neutrophil or *P. aeruginosa* concentration or the disease status of the patient. In contrast, in the non-CF patients with chronic inflammatory lung disease, transferrin and lactoferrin cleavage products were detected only in those BAL samples which contained the greatest concentration of both neutrophils and *P. aeruginosa*. These data provide evidence that *P. aeruginosa*- and/or human-derived protease cleavage of transferrin and lactoferrin occurs in vivo in the airways of individuals with CF and other forms of chronic lung disease, suggesting that this process could contribute to *P. aeruginosa*-associated lung injury in these patients.

Pseudomonas aeruginosa plays a major role in the chronic progressive bronchiectatic lung disease which is now responsible for more than 90% of deaths in patients with cystic fibrosis (CF) (11, 16). Although a number of *P. aeruginosa* secretory products have been implicated in this process, the primary pathogenic mechanism(s) through which the organism contributes to this pulmonary injury remains unclear. Much work has focused on the abilities of *Pseudomonas*- and neutrophil-derived proteases to damage the lung through digestion of extracellular matrix and to direct cytotoxicity (11). We and others have obtained in vitro data suggesting the possibility that both pseudomonas- and neutrophil-derived proteases may also contribute to lung injury in CF patients via their ability to cleave the host iron-binding proteins transferrin and lactoferrin (4, 7).

Transferrin and lactoferrin are normal constituents of airway secretions (24). Both proteins appear to contribute to host defense by limiting the availability of iron for use by microbial pathogens (13). In addition, they may decrease the potential for oxidant-mediated tissue injury via their ability to bind iron in such a way so as to prevent iron-dependent formation of hydroxyl radicals (Haber-Weiss reaction) (1). Cleavage of diferric transferrin by pseudomonas elastase has been previously shown by Doring et al. to enhance the ability of the *P. aeruginosa* siderophore pyoverdin to obtain iron from this iron chelate (7). We found that cleavage of ferritransferrin by pseudomonas elastase generates an iron chelate(s) which is a highly effective catalyst of the Haber-Weiss reaction (4). This process was enhanced by the simultaneous presence of other proteases, particularly human neutrophil elastase (4). Similarly, pseudomonas elastase cleavage of apotransferrin markedly decreased its ability to inhibit hydroxyl radical formation (4). Bacterial or neutrophil protease-mediated cleavage of diferric lactoferrin or apolactoferrin resulted in minimal alteration of lactoferrin function (4).

These in vitro data suggested that cleavage of transferrin by pseudomonas elastase could contribute to pseudomonas-associated tissue injury by both making iron more available for bacterial growth and metabolism and increasing the potential for local hydroxyl radical formation. In order to provide further support for this hypothesis, evidence that such cleavage products were generated under in vivo conditions was sought. The present work provides evidence that cleavage of transferrin and lactoferrin occurs in the airways of patients with CF and other chronic lung diseases associated with coexistent *P. aeruginosa* infection.

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MATERIALS AND METHODS

BAL samples. A retrospective study of bronchoalveolar lavage (BAL) samples obtained over a 9-year time period

* Corresponding author.
† Present address: Genentech, Inc., South San Francisco, CA 94080.

from three separate groups was performed by standard techniques (12). The first group (n = 22) consisted of patients with CF; *P. aeruginosa* was cultured from BAL samples from all but three CF patients and *Pseudomonas cepacia* was cultured from one CF patient. All *P. aeruginosa* isolates had a mucoid phenotype. The mean age (and standard deviation) of these patients was 25 ± 7.3 years. The second group (n = 11) was composed of patients with chronic inflammatory lung disease (bronchiectasis) who had been previously shown not to have cystic fibrosis by pilocarpine iontophoresis (14). Of these 11 individuals, 6 were culture positive for *P. aeruginosa*, all of a nonmucoid phenotype. The mean age of these individuals was 48 ± 11 years. These patients were initially evaluated because their clinical condition closely resembled adult CF but without the well-recognized cellular electrolyte transport defect. Thus, these individuals clearly represent a distinct subset of individuals with idiopathic bronchiectasis. The final group was that of normal individuals (n = 7) who lacked any history of lung disease or *P. aeruginosa* infection. The mean age of this final group was 32 ± 12 years. The severity of lung disease of each individual was scored by the pulmonary portion of the standard prognostic and clinical CF scoring system of Taussig et al. (23). Although scores were assigned to those individuals in the non-CF bronchiectatic lung disease group and normal individuals, the use of this scoring system has not been validated for such individuals.

After each individual lavage, the concentration (CFU per milliliter) of *P. aeruginosa* and neutrophils (cells per milliliter) in each BAL sample was determined. Each BAL sample was diluted in Dulbecco's phosphate-buffered saline (PBS) (pH 7.2) containing 2% *N*-acetylcysteine and then inoculated with a calibrated dilution loop onto blood agar, chocolate, and eosin-methylene blue plates. After overnight incubation in 5% CO_2, colony counts were determined and organisms were identified to species level by standard methodology of the Vitek system. Total cell counts from the BAL pellet were determined with a hemocytometer and the number of neutrophils was calculated on the basis of the percentage of such cells present upon microscopic examination of Wright-stained slides prepared by cytospin (15). All BAL samples were frozen at −70°C. The period of time which elapsed before study ranged from 2 days to 9 years. There was no correlation between the levels of cleavage products detected and duration of freezing, either when samples from different patients were examined or when the same sample was repeatedly examined over a 6-month interval. In a few cases, fresh samples were treated with the metalloprotease inhibitor EDTA (10 mM).

Generation of transferrin and lactoferrin cleavage products in vitro. Human transferrin and lactoferrin (apo or diferric form; Sigma Chemical Co., St. Louis, Mo.) were suspended in PBS (20 mg/ml) and were then incubated in the presence of pseudomonas elastase (200 μg/ml), pseudomonas alkaline protease (200 μg/ml), neutrophil elastase (200 μg/ml), or trypsin (50 μg/ml) for 48 to 72 h at 37°C as previously described (4). Pseudomonas elastase (53.7 milliprotease units/mg of protein) and pseudomonas alkaline protease (3 to 5 milliprotease units/mg of protein) which had been purchased from Nagase Biochemicals Ltd., Fukuchiyama, Japan, were kindly provided by Charles Cox, Department of Microbiology, University of Iowa, Iowa City. Human neutrophil elastase was purchased from Calbiochem, San Diego, Calif. (22 U/mg of protein). Trypsin (bovine pancreas, type XIII, 13,000 Nα-benzoyl-L-arginine ethyl ester units/mg of protein) was purchased from Sigma. Control transferrin and lactoferrin samples were incubated in parallel in PBS in the absence of added proteases.

Immunoblot analysis. Samples were solubilized in Laemmli solubilizing buffer (2.3% sodium dodecyl sulfate [SDS], 0.06 M Tris, 1% glycerol) for 5 min at 100°C and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (10% running and 4.5% polyacrylamide stacking gels) at a constant current with a 1.5-mm-thick MINI-PROTEIN gel system (Bio-Rad, Hercules, Calif.). Gels were then transferred to a nitrocellulose membrane overnight at 4°C (300 V-h). The nitrocellulose was then blocked in 4% bovine serum albumin (BSA) for 1 h at 37°C. For lactoferrin immunoblot analysis, blocking was followed by incubation with a 1:400 dilution (10 μg/ml) of immunoglobulin G (IgG) fraction rabbit anti-human lactoferrin (4 mg/ml; Organon Teknika, West Chester, Pa.) in Tris-buffered saline (TBS) containing 2% BSA for 1.25 h at 25°C. For transferrin immunoblot analysis, after the initial blocking step, the blot was incubated in a 1:4,000 dilution of IgG fraction rabbit anti-human transferrin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) in 2% BSA–TBS for 1.25 h at 25°C. Both lactoferrin and transferrin blots were then washed three times in TBS containing 0.1% Tween 20 for 10 to 15 min per wash. Blots were then incubated with a 1:4,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (whole molecule; Sigma) in 2% BSA–TBS for 1.25 h at 25°C. Following an additional three washes in TBS–0.1% Tween 20 as described above, immunoreactive protein was detected by enhanced chemiluminescence using 5- to 60-s exposures on Kodak X-OMAT/AR scientific imaging film (Kodak, Rochester, N.Y.) according to the manufacturer's (Amersham, Arlington Heights, Ill.) instructions.

The two primary antibodies (antilactoferrin and antitransferrin) are affinity purified as part of their commercial preparation. As assessed by their manufacturer, both antibodies are free from cross-reactivity with other human serum proteins. Consistent with this non-cross-reactivity, we found that as employed above, the transferrin immunoblot system did not recognize human lactoferrin and the lactoferrin system did not show cross-reactivity with human transferrin. Similarly, no proteins were recognized when either purified human neutrophils or *P. aeruginosa* (strain PAO1) was examined with the transferrin immunoblot system. In the case of neutrophils, the lactoferrin system detected only neutrophil lactoferrin. A faint approximately 40-kDa band was detected upon lactoferrin immunoblot analysis of 10^9 *P. aeruginosa* (PAO1) per ml, although a band of similar molecular mass was not detected in the BAL samples. With serial dilutions of human transferrin and lactoferrin, the transferrin and lactoferrin detection systems were shown to be able to detect 0.01 and 0.1 ng of protein, respectively.

For purposes of data analysis, the relative cumulative magnitude of detectable immunoreactive protein bands of lower molecular weight than transferrin or lactoferrin were graded as 0 (none), 1 (trace), 2 (low), 3 (moderate), or 4 (large) by two evaluators blinded to the nature of the sample. Examples of each of these grades are shown in Fig. 1. The high-molecular-mass bands (>200 kDa) sometimes seen with both protein standards and BAL samples, which are believed to represent aggregated transferrin or lactoferrin, were not considered in this evaluation. The agreement between observers was ~95%. Different scores for a sample never exceeded one grade step and were resolved by using the lower of the two scores.

Statistical analysis. Descriptive statistics were calculated

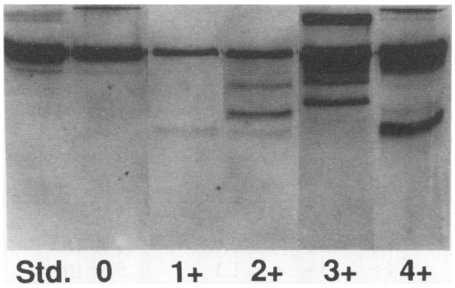

FIG. 1. Immunoblot analysis for transferrin cleavage products showing transferrin standard (Std.) (10 ng) or BAL samples from patients which demonstrate the extent of cleavage representative of each of the grading categories of transferrin cleavage. A similar scale was employed for lactoferrin.

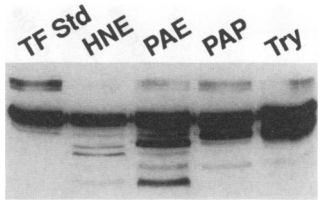

FIG. 2. Immunoblot analysis for transferrin cleavage products using the enhanced chemiluminescence detection system described in Materials and Methods in which the gel was loaded with transferrin (40 μg/ml) (TF Std) or the same amount of transferrin which had been incubated in the presence of human neutrophil elastase (HNE), pseudomonas elastase (PAE), pseudomonas alkaline protease (PAP), or trypsin (Try) for 48 h at 37°C as described in Materials and Methods (4).

for each of the study variables by group. For purposes of statistical calculations, missing data were excluded test by test. For values which were less than a minimum cutoff value, the next lowest value was assumed. Kendall's tau b correlation coefficient was calculated to compare the correlations between each of the study variables. Kendall's correlation coefficient is used to test for order association with ordinal, interval, or ratio scale data (25). Correlations were calculated for the two largest study groups individually (CF and other inflammatory lung disease). The number of subjects in the control group was not adequate to allow calculation of correlation coefficients in this group alone. Differences between the concentrations of degradation products in CF patients and in the other two groups were compared individually by a Wilcoxon (Mann-Whitney) rank sum test. The Wilcoxon (Mann-Whitney) rank sum test is a nonparametric test for two independent groups, in which the sums of the ranked values in each group are compared (25). Statistical calculations were performed by SPSS for Windows (SPSS, Inc., Chicago, Ill.). Alpha was set at 0.05. All *P* values are two tailed.

RESULTS

Detection of transferrin and lactoferrin cleavage products by immunoblot analysis. In preparation for analyzing biological samples for evidence of transferrin or lactoferrin degradation by bacterial and human proteases under in vivo conditions, we first established the ability of our immunoblot system to detect such products. Therefore, human transferrin and lactoferrin were incubated in vitro in the presence of various proteases: pseudomonas elastase, pseudomonas alkaline protease, human neutrophil elastase, or trypsin. Incubation conditions were those we had previously found (4) to lead to protease-mediated degradation of these iron-binding proteins. They were not chosen necessarily to represent in vivo conditions but rather to assure that transferrin or lactoferrin cleavage occurred. As shown in Fig. 2 and 3, immunoblot analysis was readily able to detect lower-molecular-weight products resulting from the exposure of transferrin or lactoferrin to each of the four proteases. Sensitivity of the detection system for cleavage products appeared similar regardless of whether the apo or diferric form of transferrin or lactoferrin were employed (data not shown). In addition, the relative ratio of immunoreactive bands detected with each sample appeared to be similar to those observed with silver staining of a companion SDS-PAGE sample (data not shown). These data indicated that the immunoblot system was capable of providing semiquantitative analysis of the presence of specific proteolysis products of transferrin and lactoferrin.

Detection of transferrin and lactoferrin degradation products in BAL samples. Having established the ability of our immunoblot system to detect protease-mediated degradation products of human transferrin and lactoferrin, the presence of such products in human pulmonary secretions was examined. BAL samples were examined from three different subject types: patients with CF, all of whom were chronically infected with *P. aeruginosa*; patients with chronic inflammatory lung disease (*P. aeruginosa* was recovered from BAL samples for 6 of the 11 patients); or normal individuals who had no history of pulmonary infection or other lung disease. All of the *P. aeruginosa* isolates from the CF patients were mucoid, whereas none of the six *P. aeruginosa* isolates from non-CF patients were mucoid. BAL samples from 21 of 22 individuals with CF demonstrated lower-molecular-weight proteins which were detected with the antitransferrin sera (Fig. 4 and Table 1). Similarly, BAL samples from 20 of 21 individuals with cystic fibrosis contained immunoblot evidence of lactoferrin cleavage products as well (Fig. 5 and Table 1). A high-molecular-weight band(s) was seen for some protein standards as well as BAL samples. This band(s) was felt to represent aggregates of transferrin or lactoferrin, depending on the immunoblot system employed. BAL samples from two individuals could be examined only for evidence of transferrin cleavage products because of the limited amount of BAL fluid available for study. The pattern and magnitude of protein breakdown products detected in BAL samples from CF patients

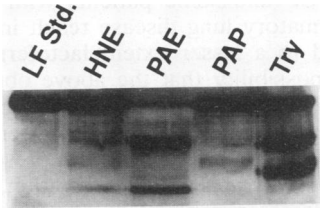

FIG. 3. Immunoblot analysis for lactoferrin cleavage products using the enhanced chemiluminescence detection system described in Materials and Methods in which the gel was loaded with lactoferrin (8 μg/ml) (LF Std.) or the same amount of lactoferrin which had been incubated in the presence of human neutrophil elastase (HNE), pseudomonas elastase (PAE), pseudomonas alkaline protease (PAP), or trypsin (Try) for 48 h at 37°C as described in Materials and Methods.

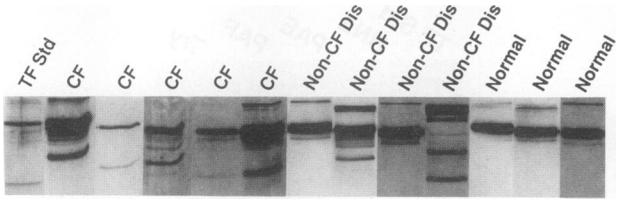

FIG. 4. Representative immunoblot analysis for transferrin cleavage products of BAL samples from CF patients infected with *P. aeruginosa*, non-CF patients with chronic inflammatory lung disease, and normal individuals. Lane TF Std contains purified human transferrin as the standard. Immunoreactive proteins consistent with transferrin cleavage products are apparent in CF BAL samples, some BAL samples from non-CF patients with chronic inflammatory lung disease, and none of the normal controls.

with the transferrin or lactoferrin immunoblot system varied from individual to individual (Fig. 4 and 5 and Table 1). In spite of the absence of detectable ($>10^4$ CFU/ml) pseudomonas in their BAL samples, two subjects nevertheless had small amounts of detectable transferrin and lactoferrin degradation products (Table 1). The one BAL sample in which evidence for neither transferrin nor lactoferrin degradation products could be observed was from an individual who had undergone a BAL washout procedure as part of a clinical trial 2 weeks prior to the BAL.

Of the BAL samples examined from individuals with chronic inflammatory lung disease not associated with CF, 3 of 11 and 2 of 9 showed evidence of transferrin and lactoferrin cleavage products, respectively (Fig. 4 and 5 and Table 1). The sample demonstrating the largest amount of lactoferrin cleavage also showed the greatest amount of transferrin degradation products (Table 1). In contrast, none of the BAL samples from normal subjects showed evidence of transferrin ($n = 7$) or lactoferrin ($n = 6$) cleavage products (Fig. 4 and 5 and Table 1). It is clear that the amounts of transferrin and lactoferrin varied from sample to sample, with greater concentrations of transferrin and lactoferrin generally present in the CF patients. Altering the amount of BAL sample loaded on the gel such that the amounts of transferrin (or lactoferrin) loaded from CF patients and those subjects in which cleavage products were not seen (i.e., normal controls) were similar did not indicate that the above results were simply related to the amount of the protein loaded. BAL samples from normal subjects still did not contain detectable cleavage products whereas these products remained readily detectable in the CF patients.

These data suggest that conditions in the airways of patients with CF and some patients with other forms of chronic inflammatory lung disease result in degradation of transferrin and to a lesser extent lactoferrin. In order to eliminate the possibility that the above observations were the result of ex vivo proteolysis of the proteins, we attempted to correlate the extent of transferrin degradation with the length of time specimens were preserved at −70°C. No relationship was observed. In addition, no increase in transferrin or lactoferrin cleavage was observed when the same BAL sample was studied sequentially over several months of storage at −70°C. Finally, samples to which the metalloprotease inhibitor EDTA (10 mM) had been added at the time they were obtained also demonstrated similar evidence of transferrin and lactoferrin degradation products.

Correlation between transferrin and lactoferrin degradation product formation and other factors in airways. The airways of patients with CF have been shown to possess enhanced protease activity (5, 6, 12, 18, 20–22). Such proteases are probably derived from many sources, with neutrophils and other phagocytes and *P. aeruginosa* most often suggested as the major contributors (5, 6, 10, 12, 18, 20–22). In order to

TABLE 1. Data on BAL samples and lungs of the three study groups

Group and patient no.	Relative level of cleavage products		Neutrophil concn (10^6/ml)	*Pseudomonas* concn (CFU/ml)[a]	Pulmonary clinical score[b]
	TF	LF			
CF patients					
1	4+	3+	0.78	1×10^7	63
2	4+	4+	1.1	5×10^7	70
3	4+	4+	0.25	1×10^6	38
4	4+	1+	0.39	5×10^6	40
5	4+	3+	0.18	1×10^5	75
6	3+	2+	0.35	1×10^7	62
7	3+	3+	0.18	1×10^5	54
8	3+	3+	0.79	1×10^7	72
9	3+	1+	0.89	1×10^7	50
10	3+	2+	1.35	1×10^9	71
11	3+	2+	0.31	ND[c]	29
12	3+	3+	0.58	1×10^6	33
13	3+	1+	0.44	8×10^6	53
14	3+	3+	0.67	1×10^6	45
15	2+	ND	1.5	5×10^8	58
16	2+	1+	1.1	4×10^6	65
17	2+	1+	0.45	ND	ND
18	2+	1+	0.26	2×10^5	28
19	2+	1+	0.31	2×10^5[d]	42
20	2+	2+	0.27	$<10^4$	<15
21	1+	2+	0.52	$<10^4$	<15
22[e]	0	0	0.5	$<10^4$	76
Non-CF patients with inflammatory lung disease					
23	3+	ND	1.2	1×10^9	57
24	4+	4+	1.5	1×10^9	32
25	1+	0	0.34	0	25
26	0	0	0.19	0	46
27	0	ND	0.81	0	26
28	0	0	1.0	1×10^6	41
29	0	0	0.8	5×10^5	35
30	0	0	0.73	0	26
31	0	0	0.50	0	30
32	0	2+	1.11	1×10^8	38
33	0	0	0.58	2×10^5	45
Normal subjects					
34	0	0	0.15	ND	<15
35	0	ND	0.13	ND	<15
36	0	0	0.08	ND	<15
37	0	0	0.08	ND	<15
38	0	0	0.09	ND	<15
39	0	0	0.21	ND	<15
40	0	0	0.24	ND	<15

[a] All *Pseudomonas* isolates were *P. aeruginosa* with one exception. All of *P. aeruginosa* isolates from the CF patients were of a mucoid phenotype, whereas none of the six isolates from the non-CF patients were mucoid.
[b] The pulmonary clinical score system used was described previously (23).
[c] ND, not done.
[d] *P. cepacia* was isolated from this patient.
[e] This patient underwent lung washout procedure 2 weeks prior to lavage.

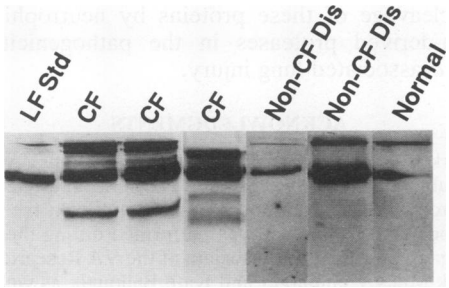

FIG. 5. Representative immunoblot analysis for lactoferrin cleavage products of BAL samples from CF patients infected with *P. aeruginosa*, non-CF patients with chronic inflammatory lung disease, and normal individuals. Lane LF Std contains purified human lactoferrin as the standard. Immunoreactive proteins consistent with lactoferrin cleavage products are detectable in the CF patients, to a small extent in some non-CF patients with chronic inflammatory disease, and none of the normal controls.

provide insight into the mechanism(s) responsible for the transferrin and lactoferrin degradation products observed in the BAL fluids of patients with CF and other chronic pulmonary inflammatory disease, we attempted to correlate the extent of protein cleavage with the concentrations of neutrophils and *P. aeruginosa* in the BAL samples and the clinical status of each patient, using a clinical scoring index previously standardized for CF patients (23). The amount of transferrin or lactoferrin degradation observed on immunoblots was graded from 0 (none) to 4 (large) by two blinded evaluators (M.B.H. and B.E.B.). Figure 1 shows samples representative of each of the various categories. Agreement between the two observers was ~95%. For most samples, the extent of transferrin cleavage product formation exceeded that of lactoferrin (Table 1). There was a statistically significant correlation between the extent of transferrin and lactoferrin cleavage ($P < 0.003$). No correlation ($P > 0.3$) was apparent between the neutrophil or *P. aeruginosa* concentration or clinical status of patients and the extent of transferrin and lactoferrin cleavage in the CF patients (Table 1). In contrast, the only patients with other chronic inflammatory lung diseases in which major amounts of such products were detected had high concentrations of both *P. aeruginosa* and neutrophils in their BAL samples compared with samples from the same group of patients in whom no such products were detectable (Table 1). This correlation reached statistical significance for lactoferrin ($P < 0.05$) but not transferrin.

DISCUSSION

The airways of CF patients contain increased levels of protease activity, potentially overwhelming local factors responsible for preventing protease-mediated lung injury and contributing to CF-associated lung disease (5, 6, 10, 12, 18, 20–22). Most of the protease activity appears to be derived from local neutrophils as well as *P. aeruginosa* infecting the airway, with most studies suggesting that the greatest contribution was by neutrophil elastase (5, 6, 10, 12, 18, 20–22). Recent in vitro studies from our laboratory (4) and that of Doring (7) have shown that the iron-binding proteins transferrin and lactoferrin are susceptible to cleavage by both neutrophil- and *P. aeruginosa*-derived proteases. Furthermore, these studies suggested that cleavage of these proteins in the airways of CF patients could contribute to lung disease in CF patients via two mechanisms: (i) increasing the capacity of *P. aeruginosa* siderophores to obtain iron from transferrin, thereby allowing it to grow more readily (7), and (ii) generating new iron chelates capable of interacting with neutrophil-derived oxidants to generate the cytotoxic hydroxyl free radical (4).

In order to provide greater insight into the biological relevance of these in vitro observations, sensitive immunoblot systems which are capable of detecting cleavage products resulting from the interaction of transferrin or lactoferrin with a variety of eukaryotic and prokaryotic proteases were developed. When this technique was utilized to examine BAL samples from patients with CF, nearly all of them had detectable transferrin and lactoferrin degradation products. Consistent with earlier reports of the relative resistance of lactoferrin to proteolysis relative to transferrin (3, 4), greater amounts of transferrin degradation products appeared to be present in most samples. Although we cannot totally eliminate the possibility that the protein cleavage observed occurred ex vivo, a number of factors suggest that this was not the case. All samples were stored at −70°C which should have minimized protease activity. Detection of cleavage products appeared to be independent of the length of time samples were preserved prior to immunoblot analysis. In addition, sequential analysis performed prospectively on samples over 6 months of preservation did not reveal an increase in cleavage products, which should have occurred had ongoing protease activity been occurring under our storage conditions. Finally, cleavage of both transferrin and lactoferrin was still detected in those BAL samples which had been treated with the metalloprotease inhibitor EDTA. This compound does not inhibit neutrophil-derived serine proteases such as neutrophil elastase. Nevertheless, had our observations been the result of ex vivo neutrophil protease activity, we might have expected a correlation between the BAL neutrophil concentration and the extent of lactoferrin or transferrin cleavage. This was not the case. Nevertheless, although we believe that this possibility is unlikely, the retrospective nature of our study does not allow its definitive exclusion.

The presence of transferrin and lactoferrin degradation products in airways does not appear to reflect a normal physiologic process, because no such products were detectable in BAL samples from healthy controls. However, the events leading to transferrin and lactoferrin cleavage do not appear to be unique to the airways of CF patients, as significant amounts of these products were detectable in a few BAL samples from a selected group patients with non-CF chronic inflammatory lung diseases. Neutrophils and to a lesser extent secretory products of *P. aeruginosa* have been considered to be the major contributors to the increased protease activity of the airways in CF patients (5, 6, 10, 12, 18, 20–22). No pattern of transferrin or lactoferrin cleavage similar to that observed when these proteins were singularly exposed to pseudomonas elastase, pseudomonas alkaline protease, human neutrophil elastase, or trypsin under in vitro conditions was routinely observed in the BAL samples we examined. On the basis of the variability in the pattern of transferrin and lactoferrin cleavage products observed in the BAL samples from CF patients, it is difficult to attribute the cleavage to one specific type of protease alone. In the BAL samples from the CF patients, we found no correlation between the extent of transferrin or lactoferrin cleavage product formation and the concentration of either neutrophils or *P. aeruginosa* present at the time of lavage.

However, in the patients with the non-CF form of chronic

inflammatory lung disease, the only two patients whose BAL samples demonstrated large amounts of transferrin and lactoferrin degradation products were the ones with the highest concentrations of both neutrophils and *P. aeruginosa*. The limited number of these patients from whom samples were available makes it difficult to make definitive conclusions from these data. Nevertheless, they suggest that the presence of *P. aeruginosa*- and/or neutrophil-derived proteases contribute to transferrin and lactoferrin cleavage. As expected, all of the *P. aeruginosa* isolates from CF patients were mucoid, whereas those from the non-CF patients were not. The implication of these two phenotypes with regard to cleavage of host iron-binding proteins remains to be examined. In addition, since cleavage products were observed in CF patients but not non-CF patients with similar numbers of neutrophils and low concentrations of *P. aeruginosa*, there may be other factors related to CF itself or the airways of CF patients which promote transferrin and lactoferrin cleavage. The fact that pseudomonas was not recovered from BAL samples of two CF patients who did have low levels of detectable transferrin and lactoferrin cleavage products is consistent with this possibility. The technique employed with BAL samples, however, would not have detected $<10^4$ CFU ml. The possible role of the duration of *P. aeruginosa* infection as well as the mean concentration of neutrophils and organisms in the airway over extended time periods in these two patient populations may be worthy of study.

The findings of transferrin and lactoferrin proteolysis products in BAL samples from patients with CF is not entirely surprising, given the in vitro susceptibility of these proteins to a variety of proteases (2–4, 7–9, 17) as well as previous evidence of proteolysis of other airway proteins in the setting of CF (5, 12). Elastin degradation products have been reported in autopsy specimens from the lungs of patients with CF (5). In addition, one of us (R.B.F.) has previously demonstrated the presence of C3 and IgG fragments in BAL samples from CF patients (12). The findings of transferrin and lactoferrin degradation products in similar samples reported in this communication provides further evidence for increased protease activity in the lungs of CF patients. More importantly, these data provide clinical support for the hypothesis of Doring and colleagues (7) that transferrin cleavage may contribute to the pathogenicity of CF lung disease by increasing iron availability for *P. aeruginosa* growth and metabolism. Similarly, it supports our earlier suggestion that such products could contribute to *P. aeruginosa*-associated lung injury via their ability to act as catalysts of the Haber-Weiss reaction.

The work performed to date has examined only BAL samples from individuals with chronic *P. aeruginosa* pulmonary infection. Acute pulmonary infection (i.e., pneumonia) with *P. aeruginosa* is also an important medical problem. *P. aeruginosa* ranks in the top three gram-negative organisms causing nosocomial pneumonia in most clinical series (19). Pseudomonas pneumonia is particularly deadly, with a mortality approaching 70%, even with appropriate antibiotic therapy (11, 19). We have not yet had the opportunity to examine pulmonary secretions from patients with acute *P. aeruginosa* infections of the lung.

In conclusion, our data demonstrate that in vivo the airways of chronically *P. aeruginosa*-infected patients with CF and a selected group of other patients with inflammatory lung diseases contain protease cleavage products of the iron-binding proteins transferrin and lactoferrin. These results support earlier in vitro data (4, 7) suggesting a possible role for cleavage of these proteins by neutrophil- and/or bacterium-derived proteases in the pathogenicity of *P. aeruginosa*-associated lung injury.

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