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Original Article

Stability of interleukin 8 and neutrophil elastase in bronchoalveolar lavage fluid following long-term storage

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Abstract

Background: Interleukin-8 (IL-8) and neutrophil elastase (NE) are commonly measured markers of inflammation in bronchoalveolar lavage (BAL) fluid from patients with cystic fibrosis. Longitudinal analysis assumes uniform stability during storage, however the effect of extended low-temperature storage on these markers remains unclear.

Methods: BAL fluid from 104 children with cystic fibrosis was assayed for IL-8 and NE after storage at 4 °C for 7 days and −80 °C for up to 6 years and compared with the initial assays performed soon after collection.

Results: IL-8 levels were stable after any measured length of time at −80 °C or 4 °C. NE levels were stable for 6 months at −80 °C but decreased beyond that or after 7 days at 4 °C.

Conclusions: Our data support the stability of IL-8 in BAL stored at −80 °C for prolonged periods. NE in BAL decreases with storage and should be assayed as soon as practical after collection.

Keywords: Cystic fibrosis; Inflammation; Children; Bronchoalveolar lavage

1. Introduction

Cytokines are produced by immune cells such as lymphocytes and macrophages and are responsible for immune cell signalling. Structurally, the majority are composed of low molecular weight polypeptides which may be distinguished by their function and origin. One cytokine of clinical relevance is interleukin-8 (IL-8), a chemotactic cytokine that recruits and activates neutrophils to assist in the inflammatory response. IL-8 has been shown to be elevated in selected tissues from patients with conditions such as psoriasis, periodontal disease and cystic fibrosis [1–3]. As a relatively short and simple protein, it is potentially less susceptible to environmental degradation than larger polypeptides.

Activated neutrophils release a variety of products, including enzymes. Neutrophil elastase (NE) is a protease capable of breaking down bacterial membrane proteins and contributes to bacterial killing. NE can also contribute to lung disease if the amount exceeds the lung's anti-protease defences due to the indiscriminate destruction of elastin and other such proteins present in connective tissue. In cystic fibrosis, NE exaggerates the effects of IL-8 to produce a more aggressive neutrophilic response to inflammation [4]. Like IL-8, it is uncomplicated in structure, and likely to exhibit integrity under storage conditions.

IL-8 and NE both serve as reliable markers of inflammation in patients with cystic fibrosis [3], and can readily be detected in samples of bronchoalveolar lavage (BAL) fluid. As part of the

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Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST CF: www.arestcf.org) program in Perth and Melbourne, infants and children undergo annual bronchoscopy and bronchoalveolar lavage for microbiological surveillance and to assess inflammation. Samples of BAL fluid are analysed for correlations between inflammatory marker levels and other clinical and laboratory readings.

The accurate comparison of analyte concentrations over time is best achieved by simultaneous measurement under identical conditions. The nature of the laboratory setting and the infrequency of clinical sampling mean that this is usually not an effective use of time or resources. Freezing at the time of collection allows for uniform treatment of samples and stable preservation of their content until they can be assayed in larger batches to reduce inter-assay variation. Refrigerated storage, while not a suitable long-term option, can provide more flexibility for analysis by slowing the degradation of a sample and allowing any work to be done over the course of several days. Previous studies have demonstrated that some cytokines are more susceptible to degradation in storage than others. Cytokine degradation has been reported in serum samples stored at 4 °C, −20 °C and −70 °C for such markers as IL-10, while TNF-α and IL-6 are stable at 4 °C for up to 6 hours and 21 days respectively [5,6]. To date, no studies have examined cytokine degradation in BAL, or degradation for time periods greater than a few weeks.

The aim of the present study was to determine the effects of storage conditions on IL-8 and NE levels, with the hypothesis that storage of BAL supernatants would not cause significant degradation. Samples were stored, after routine collection, in both refrigerated (4 °C) and deep-freeze (−80 °C) conditions prior to assaying. Refrigeration at 4 °C reflected the use of transitional storage conditions typical of laboratory practice requiring continuous work across several days, whereas freezing at −80 °C better represented long-term archival collection.

2. Methods

2.1. Study population

Children attending the cystic fibrosis clinics at Princess Margaret Hospital for Children, Perth or the Royal Children's Hospital, Melbourne, Australia provided samples for this study. These children were participating in the AREST CF early disease surveillance program which consists of assessment soon after diagnosis (average age 3 months) and annually until the age of 6 years [7]. At each assessment lung function is measured using age-appropriate techniques and a Chest computed tomography scan and BAL are performed under general anaesthesia. All BALs were performed while the child was clinically stable and fit for general anaesthesia. Details about the AREST CF program are available from www.arestcf.org.

The AREST CF surveillance program was approved by the Human Ethics Committees at the Princess Margaret Hospital for Children, Perth and at the Royal Children's Hospital, Melbourne. Informed written consent was obtained prior to each procedure.

2.2. Sample collection

Flexible bronchoscopy was performed via a laryngeal mask under general anaesthesia. Three separate aliquots of normal saline (1 ml/kg) were instilled and retrieved from the right middle lobe bronchus. The first aspirate was sent for microbiological analysis with the second and third sample pooled for inflammatory analysis. At the time of original collection BAL was kept on ice and processed within one hour. Samples were centrifuged at 300 g after which cell-free supernatants were aliquoted and stored at −80 °C. Melbourne samples were transported frozen to Perth for storage and subsequent assay.

Aliquots from 146 BAL samples, collected from 104 children with CF over a six year period, were available for this study. Limits on individual sample volumes restricted a complete investigation of all archival aliquots.

2.3. Cytokine and elastase assays

IL-8 levels were determined using a commercial assay kit in accordance with the manufacturers' protocol (BD OptEIA, BD Biosciences, San Diego, CA, USA). Levels were measured as previously described [8]. Briefly, 50 µl of each sample was added in duplicate to a 96-well plate, along with 50 µl of a human neutrophil elastase standard (Calbiochem, Merck Group, Darmstedt, Germany) serially diluted from 25 u/ml to 0.02 u/ml in 0.2 M Tris pH 8.0. A 100 mM solution of n-methoxysuccinyl-ala-ala-pro-val p-nitroanilide (Sigma Aldrich Australia, Castle Hill, NSW, Australia) in 1-methyl-2-pyrrolidinone was diluted to 1 mM in 0.2 M Tris pH 8.0, and 5 µl was added to each well. Absorbance was read immediately on a Wallac Victor 3 spectrophotometer (PerkinElmer, Shelton, CT, USA) at 405 nm before incubation at 37 °C. Subsequent readings were taken every 10 min and subtracted from the initial to determine enzyme activity. All ELISA and activity assay plates included a sample from a separate subject with known elevated IL-8 and NE levels as a positive control. Each sample was assayed for IL-8 and NE within one month of initial storage as part of routine testing (t=0). All assays were performed in the same laboratory (Perth) by qualified laboratory personal following standardized operating procedures. For evaluation of the stability of IL-8 at −80 °C, a second aliquot of each sample was thawed and assayed after t=6 months, 2, 4 and 6 years of storage at −80 °C. No samples had previously been thawed. Neutrophil elastase was assayed after t=6 months, 2 and 4 years storage at −80 °C. For evaluation of stability at 4 °C, all thawed samples with sufficient volume remaining were re-assayed after one week of refrigerated storage. The IL-8 assays were sensitive to 0.02 ng/ml with an inter-assay coefficient of variation of 10.22%. The NE assays were sensitive to 0.05 µg/ml with an inter-assay coefficient of variation of 7.99%. Samples below the detection limit of either assay were assigned a value equal to half the minimum limit.

2.4. Statistical analysis

The mean bias and limits of agreement (1.96 * SD of difference between 2 measurements) were calculated for samples tested 6 months, 2, 4 and 6 years apart according to the methods of Bland and Altman with comparisons made using Wilcoxon signed rank tests. Graphs and analyses were performed using GraphPad. Statistical significance was considered to be a p-value less than or equal to 0.05.

3. Results

3.1. Study demographics

Samples were selected for this study if they fell into the time frame, i.e. the initial test date allowed for re-analysis after 6 months, 2, 4 or 6 years of storage, and if sufficient volume was available for repeat testing. 146 samples collected from 104 children (34.6% male), with a mean age of 3.3 years (range 0.1–8.4 years) met these criteria.

3.2. Storage at −80 °C

Storage at −80 °C for a period of 2, 4 or 6 years did not result in clinically significant alterations in the concentrations of IL-8 in BAL (Table 1). In particular, no sample went from having a detectable level of IL-8 when first tested to being below the limit of detection after storage. At each time point the group median concentration was similar to the values obtained on the initial analysis. The only storage interval associated with a statistically significant decrease in IL-8 was 6 months [1.05 (0.02–5.75) ng/ml and 1.03 (0.05–7.40) ng/ml, $p=0.005$]. No systematic bias was observed for IL-8 after any storage interval at −80 °C (Fig. 1).

Levels of NE in BAL appeared to be affected more by long-term storage with a significant decrease after storage at −80 °C for 2 and 4 years ($p=0.017$ and $p<0.0001$ respectively), but not after storage for 6 months ($p=0.151$) (Table 2). All paired samples were concordant for the presence of NE activity. Twenty-six of the samples were at or below the minimum detection limit of the assay at both measured time points. No sample that had detectable levels of NE when first analysed became "undetectable" after storage and none that were initially "undetectable" became "datable" after storage. No systematic bias in between test repeatability for NE levels after any storage interval at −80 °C was observed (Table 2) (Fig. 2).

3.3. Storage at 4 °C

The median concentration of IL-8 was similar between the initial measurement and after storage for 7 days at 4 °C (Table 1). The Bland–Altman analysis showed a bias of −0.24 ng/ml, with 95% limits of agreement of −4.95 to 4.46 for repeated analysis of IL-8 after storage at 4 °C (Fig. 3). NE appeared to be more influenced by storage at 4 °C for 7 days, with a significant decrease in group mean concentration (Table 2) and 25 samples that had detectable levels initially falling below the limit of detection. The Bland–Altman analysis showed a bias of 0.44 ng/ml, with 95% limits of agreement of −1.17 to 2.06 (Fig. 3).

Table 1
Interleukin-8 (IL-8) concentration in BAL fluid after storage at −80 °C and 4 °C.

Follow up time	Storage time	Initial	Follow up	p value
6 years [a]	n	10	10	
	IL-8 ng/ml [median]	1.55	2.08	0.93
	IL-8 ng/ml [range]	(0.05, 66.0)	(0.18, 21.5)	
	IL-8 ng/ml [25–75%]	[0.85–3.11]	[0.33–3.82]	
	Mean difference	−0.33		
	95% LOA [b]	−6.04, 5.38		
4 years [a]	n	32	32	
	IL-8 ng/ml [median]	1.47	1.37	0.36
	IL-8 ng/ml [range]	0.02, 29.5	0.02, 29.60	
	IL-8 ng/ml [25–75%]	0.50–3.88	0.60–4.12	
	Mean difference	−0.24		
	95% LOA [b]	−3.55, 3.06		
2 years [a]	n	32	32	
	IL-8 ng/ml [median]	0.49	0.94	0.52
	IL-8 ng/ml [range]	0.02, 107.50	0.03, 74.0	
	IL-8 ng/ml [25–75%]	0.15–17.75	0.32–4.80	
	Mean difference	3.45		
	95% LOA [b]	−8.89 15.8		
6 months [a]	n	32	32	
	IL-8 ng/ml [median]	1.05	1.03	0.005
	IL-8 ng/ml [range]	0.02, 5.75	0.05, 7.40	
	IL-8 ng/ml [25–75%]	0.20–2.08	0.29–2.56	
	Mean difference	−0.27		
	95% LOA [b]	−1.55, 1.02		
7 days [c]	n	91	91	
	IL-8 ng/ml [median]	1.16	1.31	0.16
	IL-8 ng/ml [range]	0.02, 74.00	0.03, 65.00	
	IL-8 ng/ml [25–75%]	0.37–3.20	0.47–3.26	
	Mean difference	−0.24		
	95% LOA [b]	−4.95, 4.46		

Data after each storage interval were compared to the values obtained from an aliquot of the same sample measured approximately one month after collection.
[a] Samples stored at −80 °C.
[b] LOA, limits of agreement.
[c] Samples stored at 4 °C.

4. Discussion

The results from the present study demonstrate that IL-8 concentrations in BAL do not change substantially after long-term storage at −80 °C for periods up to 6 years. NE in BAL is more vulnerable to long-term storage but storage for up to 6 months at −80 °C does not result in substantial loss of activity. Short-term storage of BAL at 4 °C for 7 days does not result in a decrease in IL-8 concentrations but does result in a decrease in levels NE.

Previous studies have demonstrated the robust nature of protein markers in biological samples to handle storage conditions. Desmosines in urine exhibit appreciable recovery after storage and repeated freeze–thaw cycles at −70 °C [9], as do lipocalins [10]. Urinary markers of styrene exposure have

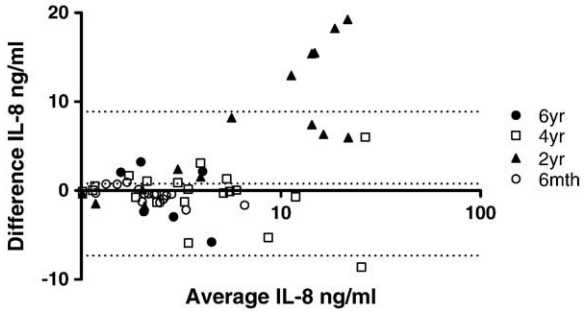

Fig. 1. Bland–Altman plot of IL-8 concentrations for samples stored at −80 °C. Samples stored at −80 °C for 6 months, 2, 4 and 6 years. The mean bias (middle broken line) was −0.27, 3.45, −0.24 and −0.33 for each group respectively. The limits of agreement (upper and lower broken lines) were (1.02, −1.55), (15.8, −8.89), (3.06, −3.55) and (5.38, −6.04) for each group respectively.

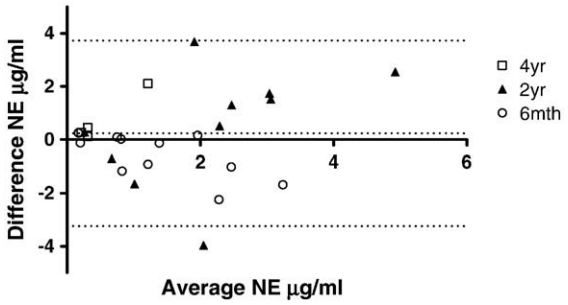

Fig. 2. Bland–Altman plot of NE concentrations for samples stored at −80 °C. Samples stored at −80 °C for 6 months, 2 and 4 years. No data for NE was available beyond four years. The mean bias (middle broken line) was −0.11, 0.06 and 0.13 for each group respectively. The limits of agreement (upper and lower broken lines) were (1.15, −1.37), (3.16, −3.05) and (0.94, −0.68) for each group respectively.

also been shown to maintain stability for up to 4 days at 4 °C [11]. Similarly, markers in cerebrospinal fluid [12], pleural fluid [13] and sera [5,9,14] are also suitable for safe storage at−80 °C, while the reverse seems true of those in cervical mucous, where snap-freezing can cause significant degradation [15].

Inflammatory cytokines in serum such as TNF-α and IFN-γ have been shown to remain stable for up to 20 days in both refrigerated and frozen conditions [16]. Others such as IL-6, IL-10 and CC16 have significant stability estimated for several years when stored at −70 °C [5]. The present study is the first to examine the effect of storage on the stability of IL-8 and NE in BAL. We have found IL-8 to be stable for up to 6 years in storage at −80 °C, though there is a conflicting trend suggested by the 6 month group. This can be attributed to the high number of samples that were close to the minimum detection limit at both the initial and 6 month measurement.

The cytokines IL-6, IL-10 and CC16 in serum have all been shown to maintain on or about perfect recovery after 4 °C storage for up to 21 days [5]. Our data demonstrate that IL-8 in BAL is also stable at 4 °C for 7 days. Taken together, these data

Table 2
Neutrophil elastase (NE) concentration in BAL fluid after storage at −80 °C and 4 °C.

Follow up time	Storage time	Initial	Follow up	*p* value
4 years [a]	n	28		
	NE µg/ml [median]	0.10	0.08	<0.0001
	NE µg/ml [range]	0.10, 4.12	0.08, 3.90	
	NE µg/ml [25–75%]	0.10–0.10	0.08–0.08	
	Mean difference	0.13		
	95% LOA [b]	−0.68, 0.94		
2 years [a]	n	32		
	NE µg/ml [median]	0.19	0.13	
	NE µg/ml [range]	0.07, 102.80	0.13, 70.80	
	NE µg/ml [25–75%]	0.19–2.98	0.07–1.98	
	Mean difference	0.06		
	95% LOA [b]	−3.05, 3.16		
6 months [a]	n	32		
	NE µg/ml [median]	0.30	0.04	
	NE µg/ml [range]	0.14, 2.40	0.04, 4.08	0.151
	NE µg/ml [25–75%]	0.14–0.78	0.02–1.11	
	Mean difference	−0.11		
	95% LOA [b]	−1.37, 1.15		
7 days [c]	n	36		
	NE µg/ml [median]	0.35	0.16	<0.0001
	NE µg/ml [range]	0.20, 3.86	(0.16, 3.65	
	NE µg/ml [25–75%]	0.20–0.79	[0.16–0.16	
	Mean difference	0.44		
	95% LOA [b]	−1.17, 2.06		

Data after each storage interval were compared to the values obtained from an aliquot of the same sample measured approximately one month after collection.
[a] Samples stored at −80 °C.
[b] LOA, limits of agreement.
[c] Samples stored at 4 °C.

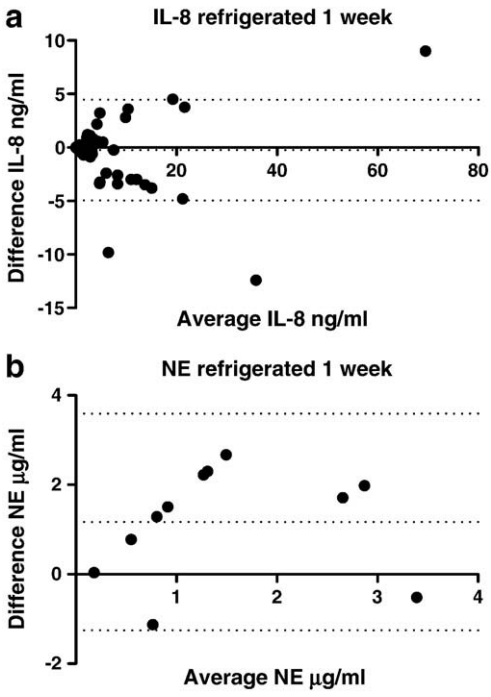

Fig. 3. Bland–Altman plot of IL-8 and NE concentration for samples stored at −4 °C. Samples were tested after one week at 4 °C. a Indicates the change in IL-8 concentration with a mean bias (middle broken line) of −0.24, and limits of agreement (upper and lower broken lines) of 4.46 and −4.96 respectively. b Indicates the change in NE concentration. The mean bias was 1.17 (middle broken line), while the limits of agreement (upper and lower broken lines) were 3.59 and −1.25 respectively.

suggest that short-term storage at 4 °C does not affect cytokine levels. However, our analyses were performed on samples that had been previously frozen and thawed prior to storage at 4 °C. At the time of original collection BAL was put immediately onto ice for transport to the laboratory and processed within 1 h. The processing included centrifugation, removal and aliquoting of the supernatant and freezing at −80 °C. The results of the present study should not be taken to indicate that a BAL sample could be stored at 4 °C before processing.

A previous report has shown that NE levels in gingival crevicular fluid are unaffected for at least three days in storage at −80 °C [17]. In BAL fluid, the data from the present study show that NE levels remain stable for up to 6 months at −80 °C but decreases after storage for 2 and 4 years. This may not reflect the true nature of the enzymes integrity, as many of the samples in the tested sets fell below the minimum detection limit, similar to the issue with the 6 month group of IL-8 samples at −80 °C mentioned above. Inter-assay variations are exaggerated the most at the upper and lower limits of detection where values are extrapolated, and with the majority of the NE results calculated at these limits, some of the interpretations may be spurious. In addition, no sample that initially had detectable NE activity fell below the limit of detection after storage at −80 °C.

Neutrophil elastase has been shown to remain relatively stable at 4 °C for up to 24 h in blood samples [11] but no studies exist for stability beyond this point or in BAL fluid. The present study indicates a significant decrease in NE after seven days at 4 °C, although once again the majority of the samples tested were below the minimum detection limit of the assay at both the initial and follow up time point. In addition, 6 samples that had detectable NE when first tested fell below the limit of detection after storage at 4 °C. A fall in elastase activity in storage is not surprising. Since we only measured the concentration by the level of activity, it is possible that the NE itself may still be present in each sample intact, but lacking any enzymatic functions.

We must place a caveat on the interpretation of the data from the present study. None of our samples were assayed for either IL-8 or NE fresh when originally collected. All samples had been frozen at −80 °C for approximately one month prior to the initial analysis. We cannot therefore exclude the possibility that some initial degradation occurred.

In summary, for long-term archiving and longitudinal analysis, researchers measuring IL-8 in BAL can confidently keep samples at −80 °C for 6 years with no loss of integrity, and the trends in our data suggest it may be possible for the storage limit to extend beyond even this. IL-8 is also stable under refrigerated conditions, presenting more flexibility of time with a safe short-term storage option. The stability of NE in BAL at −80 °C is less certain, but storage for 6 months at −80 °C seems reasonable. Clearly BAL samples kept at 4 °C are at risk of degradation. The safest option for analysis would be a prompt measurement on or about the time of collection.

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