

Original Article

Neutrophil Activation Subsequent to Cardiopulmonary Bypass: A Sensitive Method for Determining Neutrophil Activation by Quantification of Lactoferrin Concentrations

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ABSTRACT

Neutrophils have been implicated as contributors to the pathogenesis of cardiopulmonary bypass (CPB). We report a method to quantify the extent of neutrophil activation during CPB through measuring the serum concentrations of lactoferrin. We developed a modified enzyme-linked immunosorbent assay (ELISA) for the quantification of lactoferrin that is sensitive to 1 ng/ml. The preliminary data acquired to support this method confirm that there is a time-dependent increase in neutrophil activity during CPB. This was determined by measuring lactoferrin serum concentrations, and the lactoferrin concentrations appear to be higher in the pediatric patient compared to the adult patient.

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INTRODUCTION

Numerous complications related to cardiopulmonary bypass (CPB) can be attributed to neutrophil activation (1-3). In order to quantify the extent of neutrophil activation during CPB, we developed a modified assay for measuring the serum concentrations of the neutrophil secretory product, lactoferrin. This paper reports our method for an enzyme-linked immunosorbent (ELISA) for the determination of lactoferrin serum concentrations. Our objective is to report a simple and reproducible method that provides the research perfusionist the ability to study questions related to neutrophils and CPB.

The neutrophil is the most abundant leukocyte, comprising 50 to 70% of all white blood cells. The neutrophil possesses two sets of cytoplasmic granules that contain substances that, through enzymatic activities or chemical denaturation, destroy injured tissues or pathogens. These granules are classed as the primary granules and the specific granules. The degradative substances of specific granules include lactoferrin in addition to lysozyme, transcobalamin III, collagenase, and C5-cleaving protease (4). The degranulation of the neutrophil specific granules is a direct result of activation and in our case due in part to the CPB system. It has been reported that neutrophil activation and the resulting degranulation is responsible for the systemic inflammatory response during and after CPB (1-3, 5-7). As a means to specifically characterize the neutrophil response, we developed a rapid and simple ELISA assay.

Lactoferrin is considered one of the main components of the specific granules of the neutrophil. Lactoferrin concentrations in normal serum have been reported to be 84.7 ng/ml in normal females and 97.8 ng/ml in normal males (8). Most importantly, measuring the concentration of lactoferrin in blood has become a standard method for measuring neutrophil activation (1,9-11) because the only source of serum lactoferrin is the neutrophil (12). Biochemically, lactoferrin is a single chain polypeptide with a molecular mass of approximately 75,000 Daltons. (9) Its role in the immune system is considered primarily antibacterial due to its ability to reversibly bind two atoms of iron that otherwise would be used for bacterial metabolism (13,14).

We report that our ELISA method is sensitive to 1 ng/ml of lactoferrin. Furthermore, we found that there is a time-dependent increase in lactoferrin concentrations in the blood during CPB. These increases in lactoferrin concentrations will be further characterized in subsequent clinical studies and related to the mortality and morbidity of the open-heart surgical patient.

MATERIALS AND METHODS

Reagents: The phosphate buffered saline (PBS) was prepared with 1000 ml of double distilled water to which was added 0.3 g (2.5 mmol) of NaH_2PO_4 , 1.06 g (7.5 mmol) of $\text{Na}_2\text{HPO}_4^a$, and 8.47 g (145.0 mmol) NaCl^a , and the pH was adjusted to 7.2 ± 0.2 . PBS-bovine serum albumin (BSA) solution was made with the addition of 2.0 g of BSA^a to 1000 ml of PBS. PBS-Tween 20

(0.05%) was prepared with the addition of 0.50 ml of Tween 20^a to 1000 ml of PBS. Carbonate buffer was prepared by the addition of 5.3 g (15.85 mol) of NaHCO_3 to 500 ml of distilled water at a pH of 9.6. Citrate-phosphate buffer was prepared by the addition of 9.47 g (66.7 mmol) NaH_2PO_4 , 7.29 g (34.7 mmol) citric acid and the pH was adjusted to 5.0 with 1 M NaOH. The color reagent was made in 15 ml volumes with 0.008 g (44 mmol) of 1,2-phenylenediamine dihydrochloride^a, 5 μl of 30% hydrogen peroxide. The 96 well microtiter plate wells were coated with a dilution of 1:3000 of antihuman lactoferrin sheep antibody^b diluted in the carbonate buffer. The secondary antibody was a peroxide labeled antihuman lactoferrin sheep antibody diluted to 1:1000 with PBS-BSA. The reaction was stopped with 1 M sulfuric acid.

ASSAY PROCEDURE

As shown in Figure 1, 100 μl of the diluted sheep-antihuman lactoferrin was pipetted into each of the wells of a microtiter plate—Corning 96 well high binding flat bottom plates^c. The plate was then rotated with a Lab Line 3-D plate rotator^d for 60 minutes at room temperature, and then incubated overnight at 4°C. Within 24 hours the plate was washed with the PBS-Tween 20 solution using a Corning ELISA plate washer^e. The wash pressure was maintained at a constant 125 mmHg. The plates were washed three times for four seconds and all liquid was aspirated out of each well. Into the washed plate was added 200 μl of each patient's serum sample in triplicate. These were rotated for 10 minutes at room temperature and incubated for 90 minutes at 37°C. The plate was then washed as above. One hundred μl of peroxide conjugated antibody was then added to each well and the plate was rotated for 60 minutes at room temperature. The plate was washed three times and 100 μl of the color reagent was added to each well and allowed to rotate for 15 minutes at room temperature. The reaction was then stopped by adding 100 μl of sulfuric acid to each well. The plate was immediately read spectrophotometrically in a microtiter plate reader^e at 490 nm. A standard curve was created by plotting a known serial dilution of human lactoferrin^f diluted in 0.5% PBS-Tween 20.

BLOOD SAMPLES

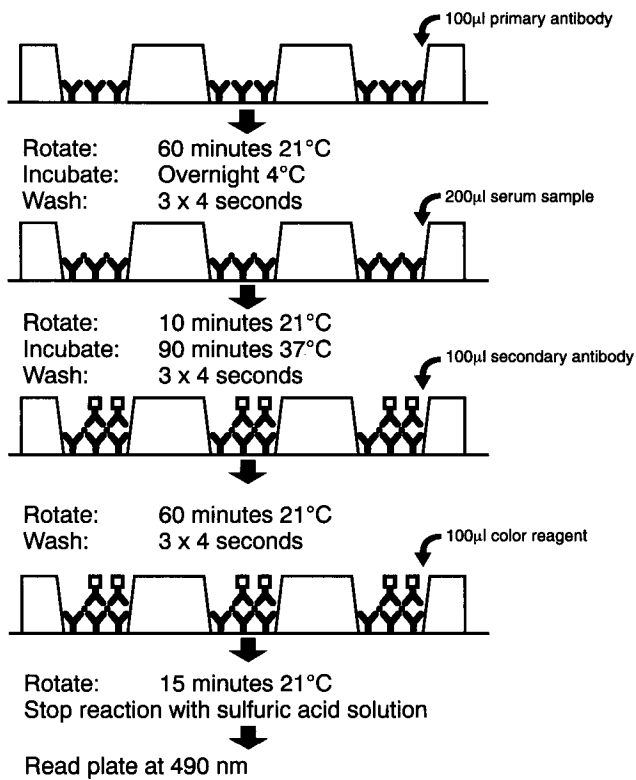
Blood samples were taken at 5 minutes on CPB and every 15 minutes until the termination of CPB. With the pediatric patients blood samples of the blood prime were also taken. Samples were compared between the three adult patients and five pediatric patients. Heparinized samples were taken directly from the CPB system and immediately immersed in ice. The samples were stored at -70°C.

PATIENT POPULATION

- a Sigma, St. Louis, MO
- b Binding Site, Birmingham, UK
- c Corning, NY
- d Melrose Park, IL
- e BIO-RAD 3550-uv, Richmond, CA
- f Calbiochem, La Jolla, CA

Figure 1. Lactoferrin ELISA procedure.

Schematic representation of the ELISA assay procedure using the microtiter plate and double-antibody technique.



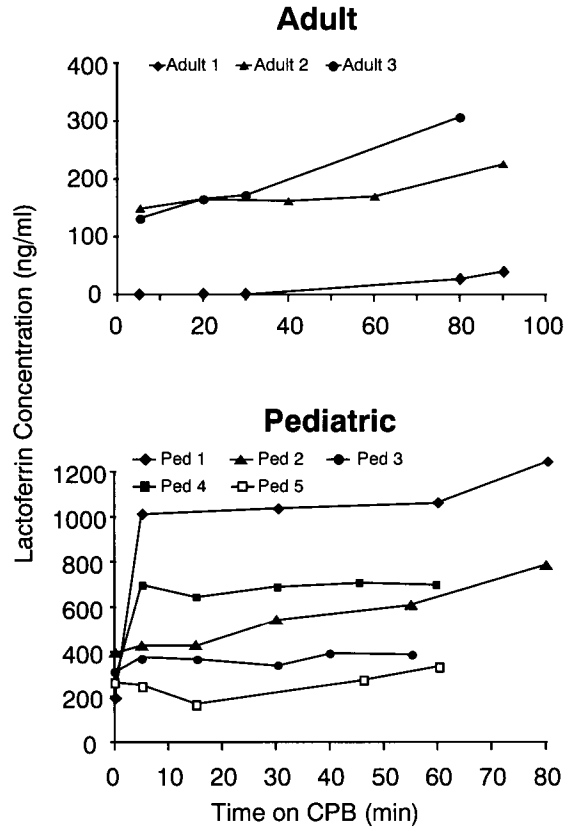
The pediatric patients were 1.9 ± 2.7 years old and 8.5 ± 5.2 kg in weight and were being corrected for congenital heart malformations. The adults were 67 ± 22 years old and 77 ± 23 kg in weight and having coronary bypass grafting.

RESULTS

The standard curves achieved with this method yielded a significant and reproducible curve with $r^2 = 0.98$ with a range of 1 ng/ml. Serum samples were diluted to fall within the range of the standard curve. Furthermore, Figure 2 shows that there is a temporally related increase in the lactoferrin serum concentrations while on CPB. There appeared to be a marked increase in the lactoferrin serum concentrations in pediatric patients ranging from 186 ng/ml at the beginning of CPB to 1504 ng/ml at the end of CPB. It also appeared that there was no relationship between the use of whole blood prime to packed red blood cell prime in the initial lactoferrin concentrations. The first levels measured in the pediatric patients ranged from 186 to 392 ng/ml and after 60 minutes of CPB the concentrations increased to a range of 332 to 1244 ng/ml. In this very limited study no correlations to clinical variables could be made. However, the base line lactoferrin

Figure 2. Serum lactoferrin concentrations in relationship to time on cardiopulmonary bypass.

The top graph illustrates three adult patients where crystalloid prime was used in conjunction with a hollow fiber oxygenator. The bottom graph illustrates the serum lactoferrin concentrations from several pediatric patients where a blood prime was used with a pediatric hollow fiber oxygenator.



concentrations in the adult patients were much lower compared to the pediatric patients and the increase during one-hour of CPB was 220% for the pediatric patients compared to 180% for the adults.

DISCUSSION

The development of ELISA methodology is an ongoing process. Other methods for the quantification of lactoferrin in biological fluids and blood have been reported (15,16). This method achieved an increase in sensitivity to 1 ng/ml (< 1 pM) compared to other reported methods. This increased sensitivity could be accounted for by several important method changes. There is an extensive array of microtiter plates available and the process of how the first antibody binds to the plates is not fully understood (16). We chose the "high bond" microtiter plates, which could be responsible for our increased sensitivity. Additionally, no other method has reported using the plate rotator. We

believe this provided a uniformity of binding of antibodies to the plate wells. Also our wash technique reduced cross well contamination with uniform wash and vacuum pressures. These methodological improvements in ELISA-based quantification can also be applied to other neutrophil secretory products.

Studies that use techniques such as this are fundamentally responsible for changing the face of perfusion. Modification of the perfusion system or techniques have been shown to decrease the systemic inflammatory response in CPB patients (11,17). One of the benefits of membrane oxygenation rather than bubble oxygenation is related to less neutrophil activation and associated morbidity (7,11,18,19). The recent development of heparin-coated circuits has also been shown to be beneficial. While the surface interaction is not completely understood, the heparin-coated circuits are believed to be more "endothelial-like," thus increasing the biocompatibility (10,19-21). A third "equipment upgrade" that has a strong potential in reduction of the systemic inflammatory process is leukocyte filters (22). This study showed that the hearts perfused with leukocyte (neutrophil)-depleted blood had less structural damage and improved myocardial function when compared to non-filtered blood. Therefore, one would predict that neutrophil inhibition techniques will prove to be extremely beneficial to the open-heart surgical patient, especially the pediatric patient.

This report describes a very sensitive methodology for the quantification of the neutrophil secretory product, lactoferrin, and a trend toward increasing lactoferrin concentrations during CPB. This preliminary data underscores the need to perform a controlled study comparing the modalities to modulate neutrophil activation during CPB.

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