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Time-resolved fluoroimmunoassay for bactericidal/permeability-increasing protein

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BACTERICIDAL/permeability-increasing protein (BPI) is a cationic antimicrobial protein produced by polymorphonuclear leukocytes, that specifically interacts with and kills Gram-negative bacteria. BPI competes with lipopolysaccharide-binding protein (LBP) secreted by liver cells into blood plasma for binding to lipopolysaccharide (LPS) and thus reduces the proinflammatory effects of LPS. We have developed a time-resolved fluoroimmunoassay for BPI and measured the concentration of BPI in human serum and plasma samples. The assay is based on a rabbit antibody against recombinant BPI. This antibody specifically adheres to polymorphonuclear leukocytes in immunostained human tissues. The difference in the serum concentration of BPI between unselected hospitalized patients with and without an infection was statistically significant. The mean concentration of BPI in serum samples was 28.3 μg/l (range 1.64–132, S.D. 26.8, $n=83$). In contrast, there was no difference between the two groups in the BPI levels in plasma samples. For all individuals tested, BPI levels were consistently higher in plasma samples compared to the matched serum samples. The mean concentration of BPI in plasma samples was 52.3 μg/l (range 0.9–403, S.D. 60.6, $n=90$). There was a positive correlation between the concentration of BPI and the white blood cell count as well as between the BPI concentration and C-reactive protein (CRP) in serum samples. In conclusion, the present study demonstrates that BPI can be quantified reliably by time-resolved fluoroimmunoassay in human serum samples.

Key words: Bactericidal/permeability-increasing protein, ELISA, Polymorphonuclear leukocytes, Serum/plasma protein, Time-resolved fluoroimmunoassay

Introduction

The bactericidal/permeability-increasing protein (BPI) is a cationic antimicrobial protein produced by polymorphonuclear leukocytes. BPI specifically interacts with and kills Gram-negative bacteria. BPI binds to the lipopolysaccharide (LPS) component of the outer membrane of Gram-negative bacteria, increases membrane permeability to hydrophobic substances and causes irreversible loss of bacterial cell homeostasis.[1–6] BPI competes with lipopolysaccharide-binding protein (LBP) secreted by liver cells into the blood plasma for binding to LPS.[7–10] In this way BPI reduces the proinflammatory effects of LPS.[11–19]

Because there is potential anti-infectious therapeutic use for recombinant BPI,[20–25] a sensitive assay which can measure BPI in body fluids is needed. The purpose of the present study was to develop a time-resolved fluoroimmunoassay (TR-FIA) for the measurement of the concentration of BPI in human serum.

Materials and Methods

Instrumentation: Time-resolved fluorescence was measured with an Arcus fluorometer (Wallac, Turku, Finland). The plate washer (Wellwash) and plate shaker (Delfia Plateshake) used in the fluoroimmunoassay were from Denley (Billinghurst, England) and Wallac (Turku, Finland), respectively. Data were handled with MultiCalc data management software (Wallac, Turku, Finland).

Serum and plasma samples: Serum and plasma samples were collected from unselected hospital-

ized patients with and without an infection (42 women and 48 men). The average age was 61 years (range 14–93 years). Samples were stored frozen at −20°C until assayed.

BPI standards: The BPI cDNA was cloned and expressed in a Chinese hamster ovary cell line as described elsewhere.[26] BPI standards were prepared from recombinant human BPI (kindly donated by Dr Marian Marra, Incyte Pharmaceuticals, Inc., Palo Alto, CA, USA) stock solution into assay buffer (Wallac, Turku, Finland) to give five concentrations (4.07, 9.76, 48.8, 122 and 305 µg/l).

Preparation of antibodies to recombinant human BPI: Antiserum to recombinant BPI was raised in a rabbit. The rabbit was immunized four times at 3-week intervals subcutaneously with 0.05–0.2 mg of human recombinant BPI (Incyte, Palo Alto, CA, USA) in Freund's complete adjuvant at the first immunization and in Freund's incomplete adjuvant on later occasions. Serum was collected 2 weeks after the last booster injection.

Labelling of anti-BPI antibody: Protein A-purified anti-recombinant BPI antibody was labelled with an isothiocyanate derivative of a europium chelate (Eu^{3+}-*N*-(*p*-*iso*thiocyanatobenzyl)-diethylene-tri-amine-N^1,N^2,N^3,N^3-tetra-acetate) by using an Eu-labelling kit (Wallac, Turku, Finland) according to the manufacturer's instructions.

Time-resolved fluoroimmunoassays: For the TR-FIA, microtitre plates were coated overnight with protein A-purified anti-BPI antibody (25 µg/ml in 50 mmol/l Tris-HCl, pH 7.75/0.15 mmol/l NaCl/0.05% NaN_3, 200 µl/well) treated with three volumes of HCl/water (125 µl of 11.6 M HCl in 50 ml of water) for 5 min. Coated plates were washed two times and 25 µl of BPI standard (0, 4.07, 9.76, 48.8, 122 and 305 µg/l) or sample were pipetted into the wells containing 175 µl of assay buffer. After 1 h incubation, with shaking, at room temperature and washing six times, Eu-labelled anti-BPI antibody (2.5 µg/ml in assay buffer, 200 µl/well) was added. The washing step was repeated after 1 h and 200 µl of enhancement solution (Wallac, Turku, Finland) was added. Fluorescence was measured after a further 5 min shaking and 10 min standing. Microtitre plates were from Eflab (Helsinki, Finland) and assay buffer for TR-FIA was from Wallac (Turku, Finland).

Immunostaining: Sections of formalin-fixed, paraffin-embedded human tissues from the files of the Department of Pathology, University of Turku were reacted with an IgG fraction of polyclonal rabbit anti-BPI antiserum, and the primary immunoreaction was localized as described previously[27] by using a Vectastain ABC kit (Vector Laboratories, Burlinghame, CA, USA) according to manufacturer's instructions. The intensity of immunostaining improved when the sections were heated for 2 × 5 min in a microwave oven before staining. For controls, the primary antibody was replaced by preimmune rabbit serum. The sections were counterstained by haematoxylin.

Statistical analysis: Student's *t*-test and Pearson's linear regression were used for statistical analysis.

Results

The mean concentration of BPI in plasma ($n = 90$) was 52.3 µg/l (range 0.9–403, S.D. 60.6) and in serum samples ($n = 83$) 28.3 µg/l (range 1.64–132, S.D. 26.8). The linear range for the BPI standard curve was 5–500 µg/l (Fig. 1). The detection limit of the assay was 1.6 µg/l corresponding to the mean ± 3 S.D. of the zero standard (blank) fluorescence counts. The difference in the serum concentration of BPI between unselected hospitalized patients with and without infection was statistically significant ($p < 0.0001$, Fig. 2). The mean concentration of BPI in serum samples for all measured patients was 28.3 µg/l (range 1.64–132, S.D. 26.8, $n = 83$). In contrast, there was no difference between the two groups in plasma samples. For all individuals tested, BPI

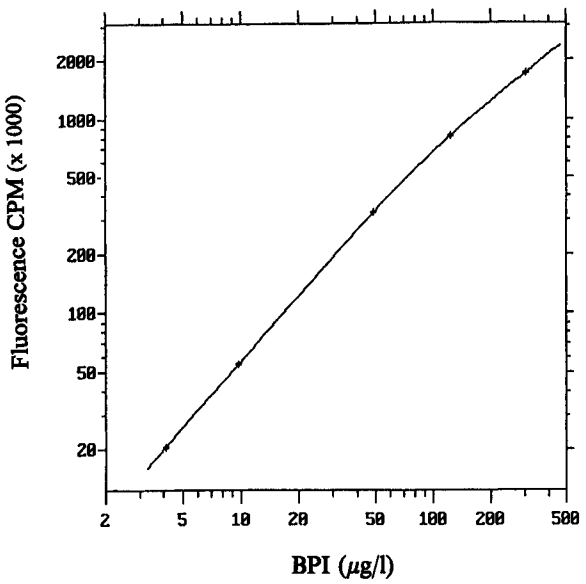

FIG. 1. The standard curve of time-resolved fluoroimmunoassay for BPI three separate assays.

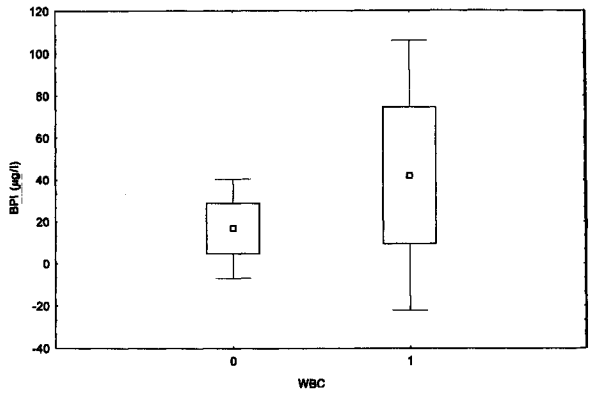

FIG. 2. Difference in the BPI concentrations between unselected hospitalized patients without an infection (group 0, $n=45$) and patients with an infection (group 1, $n=38$, $p < 0.0001$). Student's *t*-test was used for statistical analysis.

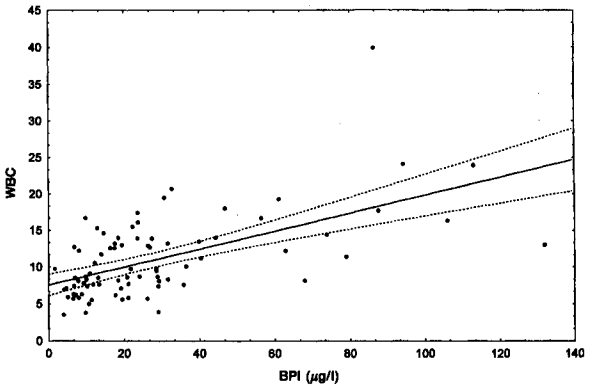

FIG. 3. Linear regression with 95% confidention intervals between blood white cell count (WBC, $\times 10^9$/l) and serum BPI concentration ($r=0.589$, $p < 0.0001$, $n=83$).

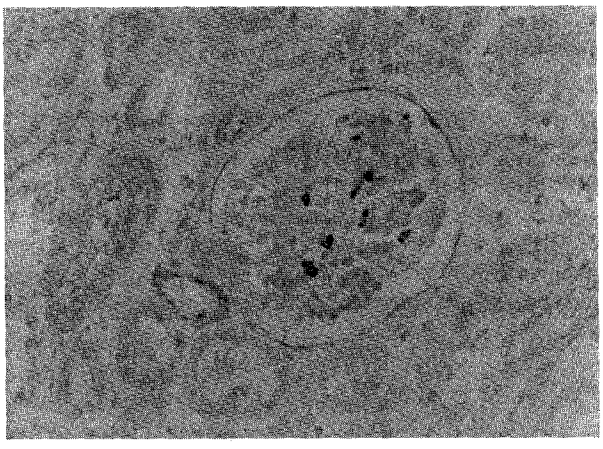

FIG. 4. Immunoreaction for BPI in polymorphonuclear leukocytes in the vascular compartment of a glomerulus of human kidney. Anti-BPI antibody. Avidin-biotin-peroxidase complex, haematoxylin counterstaining. Magnification 330×.

levels were consistently higher in plasma samples compared to the matched serum samples. The mean concentration of BPI in plasma samples was 52.3 µg/l (range 0.9–403, S.D. 60.6, $n = 90$).

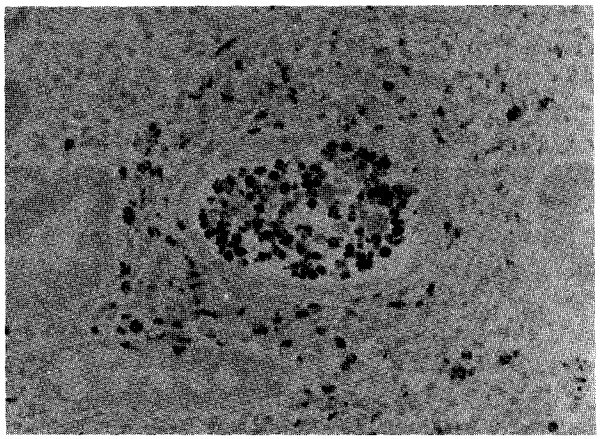

FIG. 5. Immunoreaction for BPI in polymorphonuclear leukocytes in the vascular compartment of colonic mucosa. Anti-BPI antibody. Avidin-biotin-peroxidase complex, haematoxylin counterstaining. Magnification 330×.

There was a positive correlation between the concentration of BPI in serum and the white blood cell count ($r = 0.589$, $p < 0.0001$, $n = 83$) (Fig. 3). There was also a positive correlation between serum BPI and CRP levels ($r = 0.39$, $p < 0.05$, $n = 59$). However, in plasma samples there was no correlation between BPI and white blood cell count or CRP. Intense immunoreaction was seen in polymorphonuclear leukocytes at numerous locations, e.g. in the vascular compartment of kidney glomeruli (Fig. 4) and colonic mucosa (Fig. 5). Control sections reacted with preimmune serum were devoid of immunoreaction.

Discussion

The present paper describes a new immunoassay using time-resolved fluorescence technology for measuring the concentration of BPI. An enzyme immunoassay (ELISA) for determining the concentration of BPI was recently developed by White and coworkers.[28] The mean concentration of BPI in serum as measured by the ELISA[28] and the current TR-FIA are very similar (27.1 µg/l and 28.3 µg/l, respectively). As determined by the current TR-FIA, there was a positive correlation between the concentration of BPI and the white blood cell count as well as between the serum BPI and CRP values. A statistically significant difference was found in the serum BPI levels between patients with and without manifest infections by the current TR-FIA. Furthermore, the presence of BPI in polymorphonuclear leukocytes was confirmed by immunohistochemistry in the current study. Thus, the serum concentration of BPI seems to reflect the intensity of the inflammatory process in the body.

The mean concentration of BPI in heparinized plasma samples was markedly higher than that in serum samples as determined by the current assay. However, no correlations was found between BPI values and white blood cell counts in plasma samples. Furthermore, BPI concentrations varied randomly in plasma samples when ammonium-heparin, sodium citrate and EDTA were used as anticoagulants in preliminary tests (data not shown). Thus, the detection of BPI in plasma calls for further studies.

In conclusion, the concentration of BPI can be measured reliably in human serum by time-resolved fluoroimmunoassay.

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