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# Time-resolved fluoroimmunoassays of the complete set of secreted phospholipases $A_2$ in human serum

Timo J. Nevalainen[a,*], Leena I. Eerola[a], Esa Rintala[b], V. Jukka O. Laine[c], Gérard Lambeau[d], Michael H. Gelb[e]

[a]*Department of Pathology, University of Turku, Kiinamyllynkatu 10, FIN-20520 Turku, Finland*
[b]*Department of Medicine, Satakunta Central Hospital, Pori, Finland*
[c]*Department of Pathology, Turku University Hospital, Turku, Finland*
[d]*Institut de Pharmacologie Moléculaire et Cellulaire, CNRS, Sophia Antipolis, Valbonne, France*
[e]*Departments of Chemistry and Biochemistry, University of Washington, Seattle, WA, USA*

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**Abstract**

Time-resolved fluoroimmunoassays (TR-FIA) were developed for all human secreted phospholipases $A_2$ ($PLA_2$), viz. group (G) IB, GIIA, GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein. Antibodies were raised in rabbits against recombinant human $PLA_2$ proteins and used in sandwich-type TR-FIAs as both catching and detecting antibodies, the latter after labeling with Europium. The antibodies were non-cross-reactive. The analytical sensitivities were 1 μg/L for the TR-FIA for GIB $PLA_2$, 1 μg/L (GIIA), 35 μg/L (GIID), 3 μg/L (GIIE), 4 μg/L (GIIF), 14 μg/L (GIII), 11 μg/L (GV), 2 μg/L (GX), 92 μg/L (GXIIA) and 242 μg/L (GXIIB). All secreted $PLA_2$s were assayed by these TR-FIAs in serum samples from 34 patients (23 men and 11 women, mean age 53.2 years) treated in an intensive care unit for septic infections, and in control samples from 28 volunteer blood donors (14 men and 14 women, mean age 57.0 years). Five serum samples (3 in the sepsis group and 2 in the blood donor group) gave high TR-FIA signals that were reduced to background (blank) levels by the addition of non-immune rabbit IgG to the sera. This reactivity was assumed to be due to the presence of heterophilic antibodies in these subjects. In all other subjects, including septic patients and healthy blood donors, the TR-FIA signals for GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein were at background (blank) levels. Four patients in the sepsis group had pancreatic involvement and elevated concentration of GIB $PLA_2$ in serum (median 19.0 μg/L, range 13.1–33.7 μg/L, $n$=4) as compared to the healthy blood donors (median 1.8 μg/L, range 0.8–3.4 μg/L, $n$=28, $P$<0.0001). The concentration of GIIA $PLA_2$ in the sera of septic patients (median 315.7 μg/L, range 15.9–979.6 μg/L, $n$=34) was highly elevated as compared to that of the blood donors (median 1.8 μg/L, range 0.8–5.8 μg/L, $n$=28, $P$<0.0001). Our current results confirmed elevated concentrations of GIB and GIIA $PLA_2$ in the sera of patients suffering from acute pancreatitis or septic infections, respectively, as compared to healthy subjects. However, in the same serum samples, the concentrations of the other secreted $PLA_2$s, viz. GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein were below the respective analytical sensitivities of the TR-FIAs. It is concluded that generalized bacterial infections do not lead to elevated serum levels of GIIE, GIIF, GIII, GV and GX $PLA_2$s above the detection limits of the current TR-FIAs.


*Keywords:* Blood donor; Immunoassay; Infection; Inflammation; Intensive care; Sepsis

## 1. Introduction

Phospholipase $A_2$ ($PLA_2$) was first identified in snake venoms and mammalian pancreas [1]. A large number of distinct $PLA_2$ types have been characterized and classified in the broad categories of intracellular and secreted forms of the enzyme [2–6]. Ten human secreted $PLA_2$s have been identified. They are group (G) IB, GIIA, GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein. GIIC $PLA_2$ found in murine testis is a pseudogene in the human [7]. Secreted $PLA_2$s are typically low

* Corresponding author. Tel.: +358 2 3337500; fax: +358 2 3337459.
*E-mail address:* timo.nevalainen@utu.fi (T.J. Nevalainen).

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molecular mass proteins (14–19 kDa) with a highly conserved catalytic site and $Ca^{2+}$-binding loop [3,8]. All 10 secreted $PLA_2$s have been sequenced, cloned and expressed, and their organ/cellular sites of expression have been variously reported by a number of authors. In these studies, gene expression has been investigated at the mRNA level by Northern blotting, RT-PCR or in situ hybridization on tissue sections, or/and at the protein level by Western blotting, immunoassays or immunohistochemistry.

GIB $PLA_2$ (pancreatic $PLA_2$) is a digestive enzyme synthesized and secreted by pancreatic acinar cells. Its function is to catalyze the hydrolysis of dietary phospholipids in the lumen of the duodenum [1]. The enzyme has been purified from human pancreas and localized by immunohistochemistry in the apical zymogen granule portion of pancreatic acinar cells [9]. The highest GIB $PLA_2$ protein concentration among human organs has been measured in the pancreas [10]. In addition, GIB $PLA_2$ is expressed at both mRNA and protein levels in non-pancreatic tissues including the lung, spleen, kidney and ovary where the enzyme has been proposed to promote cell proliferation and migration [11]. The GIB $PLA_2$ gene was the first human $PLA_2$ cloned [12].

GIIA $PLA_2$ (synovial $PLA_2$) was cloned from blood platelets and synoval fluid [13,14]. The enzyme is involved in inflammation [15] and expressed at mRNA and protein levels in Paneth cells of the small intestinal mucosa, lacrimal gland and prostatic epithelial cells and cartilage [16].

Recently cloned secreted $PLA_2$s include GIID $PLA_2$ that is expressed at the mRNA level in the pancreas, thymus, spleen, colon, skin, lung and eosinophils, and interestingly, its expression appears to be regulated by inflammatory challenges [17–19]. GIIE $PLA_2$ is expressed at the mRNA level in the brain, heart, lung and placenta, and its expression is markedly enhanced in the lung and intestine of endotoxin-challenged GIIA $PLA_2$-deficient C57BL/6J mice [20]. GIIF $PLA_2$ is expressed at the mRNA level in the placenta, testis, thymus, liver and kidney [21], and the enzyme protein has been demonstrated in synovial lining cells, capillary endothelial cells and plasma cells of rheumatoid arthritic joints [22]. GIII $PLA_2$ is a well-known component of bee and lizard venoms [2]. The human GIII $PLA_2$ gene has been cloned and its mRNA demonstrated in the kidney, heart, liver and skeletal muscle [23]. GV $PLA_2$ is expressed at the mRNA level in the heart, lung, placenta and neutrophils [24,25]. Recent studies show that the enzyme augments cytosolic $PLA_2$-mediated arachidonic acid release in mouse macrophages [26]. In the lung, the mRNA of GV $PLA_2$ has been localized to airway epithelial cells by in situ hybridization [27]. GX $PLA_2$ is expressed at the mRNA level in the spleen, thymus and peripheral blood leukocytes [25,28], and the enzyme protein has been demonstrated in lung alveolar epithelial cells and postulated to be involved in pulmonary inflammatory responses [27,29]. GXIIA $PLA_2$ is expressed at the mRNA level in the heart and skeletal muscle, kidney and pancreas with weaker expression in the brain, liver, small intestine, lung, placenta, ovaries, testis and prostate [5]. The recently cloned GXIIB $PLA_2$-like protein is expressed at the mRNA level in the liver, kidney and small intestine. The GXIIB $PLA_2$-like protein is catalytically inactive due to a mutation of the catalytic site histidine to leucine [6].

All secreted $PLA_2$ proteins have been produced by recombinant technology in quantities sufficient to produce antibodies for immunochemical investigations [6,25,30]. Time-resolved fluoroimmunoassays (TR-FIA) have been developed earlier for the measurement of the concentration GIB $PLA_2$ [31] and GIIA $PLA_2$ [32] in human serum. In the serum of healthy subjects, the concentration of both GIB and GIIA $PLA_2$ is below 10 μg/L [31,32]. Elevated serum levels of GIB $PLA_2$ are associated to acute pancreatitis [33,34], and the serum levels of GIIA $PLA_2$ increases up to 100–200-fold in various diseases involving inflammation [35]. The presence of the other secreted $PLA_2$s besides GIB and GIIA in the serum has not been reported. However, it can be hypothesized that the diversity of human secreted $PLA_2$s indicates important physiological and pathological functions for these proteins, e.g. in inflammation. As reviewed above, a number of secreted $PLA_2$s are expressed in many tissues and inflammatory cells. Therefore, we surmised that these enzymes may be secreted into the blood plasma, especially in generalized inflammatory diseases such as sepsis. In the current investigation, we developed TR-FIAs for the measurement of all human secreted $PLA_2$s and studied their levels in serum samples from patients suffering from severe septic infections, as well as in serum of healthy blood donors.

## 2. Materials and methods

### *2.1. Production of recombinant human secreted phospholipases $A_2$ and antibodies in rabbits*

Recombinant human secreted $PLA_2$s were prepared as described previously [6,30]. The proteins were pure and fully native (all disulfides formed) as judged by SDS-PAGE and mass spectrometric analyses [30]. Rabbit anti-human secreted $PLA_2$ antisera were prepared as described and were shown by Western blotting to be highly specific for each $PLA_2$ molecular species [25].

### *2.2. Time-resolved fluoroimmunoassay*

TR-FIAs for human GIB, GIIA, GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein were developed as described earlier for human GIB and GIIA $PLA_2$ [31,32], with slight modifications. The assays are based on corresponding anti-$PLA_2$ antibodies raised in rabbits. All reagents used were of analytical grade. Milli-Q-purified water (Millipore, Bedford, MA, USA) was used throughout.

IgG was isolated by passing 1–2 mL rabbit anti-$PLA_2$ antiserum through a 1 mL HiTrap Protein A HP column (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions. Twenty mM sodium phosphate buffer, pH 7 containing 0.9% NaCl (phosphate buffered saline, PBS), was used as the binding buffer and 0.1 M glycine, pH 2.5 as the elution buffer. After overnight dialysis against PBS and freeze–drying, IgG was dissolved in 300 μL of water, and 100 μL Delfia Eu-labelling reagent (Perkin Elmer Wallac, Turku, Finland) was added and incubated overnight at 4 °C according to the manufacturer's instructions. Eu-labelled IgG was isolated from the unreacted Eu-labelling reagent by size exclusion chromatography on a column 1.6 cm in diameter containing proximal 16 cm Superdex 75 (Pharmacia, Uppsala, Sweden) and subsequent 40 cm Trisacryl GF 2000 (LKB, Bromma, Sweden) gels. The column was equilibrated and eluted with 50 mM Tris–HCl buffer, pH 7.75 containing 0.9% NaCl and 0.05% sodium azide (TSA). High molecular weight fractions with time-resolved fluorescence $>5\times10^5$ counts per second (cps)/μL were pooled and stored at 4 °C until used as tracers in TR-FIAs as described below.

To coat microtitration wells (96-well Delfia microtitration plates, Wallac) with the catching antibody, 100 μL of protein A-purified IgG diluted to 10 μg/mL by TSA was added to the wells and incubated overnight at room temperature followed by washing (Delfia Platewash, Wallac) with TSA. For blocking, 300 μL of TSA containing 0.1% BSA, 6% D-sorbitol, 3.9% diethylenetriaminepentaacetic acid (DTPA, Titriplex V, Merck, Darmstadt, Germany) and 1 mM $CaCl_2$ were added and, after an overnight incubation at room temperature, the fluid was aspirated, and the plates were stored at 4 °C in a moist atmosphere until used. Protein was assayed by a spectrophotometric method by using bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) as a standard [36].

For TR-FIA, 10 μL of serum sample or standard solution and 90 μL of Delfia assay buffer (Tris–HCl buffered NaCl solution, pH 7.8 containing BSA, bovine gammaglobulins, Tween 40, DTPA and an inert red dye, Wallac) were added to the IgG-coated microtitration wells and incubated at room temperature for 30 min with shaking at 240 cycles/min (Delfia Plateshake, Wallac). After washing with TSA, 100 μL of detecting antibody (Eu-labelled IgG solution diluted to 10 μg/mL with assay buffer, 1 μg/mL in the experiments on analytical recovery and interassay variation) was added to the wells and incubated for 30 min with shaking as above. After washing, 100 μL of Delfia enhancement solution (Triton X-100, acetic acid and chelators, Wallac) was incubated in the wells for 5 min with shaking as above, and after 10 min standing at room temperature, fluorescence was measured with a 1230 Arcus or a Victor fluorometer (Wallac) at excitation and emission wavelengths of 340 nm and 615 nm, respectively.

In the preliminary experiments, high signal levels were seen in a few serum samples in all TR-FIAs. It was surmised that these signals were due to the presence of heterophilic antibodies in these sera [37]. In order to suppress this interference, IgG purified as described above from non-immunized rabbit serum was added to all serum samples to a concentration 500 μg/mL, and the mixtures were incubated at room temperature for 60 min before the assays.

Human secreted $PLA_2$ produced by recombinant techniques as described above were used as standards in the TR-FIAs. The proteins were diluted with assay buffer to the desired concentrations. Both linear and polynomial regression formulas were used to convert the cps-readings from the fluorometer to the corresponding $PLA_2$ concentration (μg/L) values. To study the analytical recovery, recombinant $PLA_2$s were added to the serum from a healthy blood donor at concentrations of 50, 100 and 200 μg/L for GIB, GIIA, GIIE, GIIF, GIII, GV and GX $PLA_2$, 125, 250 and 500 μg/L for GXIIA $PLA_2$ and 500, 1000 and 2000 μg/L for the GXIIB $PLA_2$-like protein. Recovery is expressed as [measured increase in concentration]/[predicted increase in concen-

Table 1
Absence of cross-reactivity between unrelated antisera and antibodies

| Sample | Assays | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | GIB | GIIA | GIID | GIIE | GIIF | GIII | GV | GX | GXIIA | GXIIB |
| GIB | **61.8** | 1.2 | 0.8 | 1.2 | 1.0 | 1.6 | 1.0 | 0.6 | 1.0 | 1.7 |
| GIIA | 0.9 | **582.0** | 1.1 | 1.2 | 1.0 | 1.1 | 0.8 | 0.7 | 0.9 | 0.9 |
| GIID | 0.9 | 1.0 | **3.4** | 1.0 | 0.9 | 0.9 | 0.8 | 0.6 | 1.5 | 0.8 |
| GIIE | 0.8 | 0.9 | 0.7 | **101.0** | 1.0 | 0.9 | 0.8 | 0.6 | 1.0 | 0.3 |
| GIIF | 0.9 | 0.8 | 0.7 | 1.0 | **24.3** | 0.9 | 0.8 | 0.6 | 0.9 | 0.5 |
| GIII | 1.0 | 0.9 | 1.0 | 1.0 | 1.0 | **15.8** | 1.0 | 0.8 | 0.9 | 0.6 |
| GV | 0.9 | 0.8 | 0.7 | 0.9 | 0.9 | 1.2 | **15.4** | 0.7 | 0.8 | 0.6 |
| GX | 0.9 | 0.8 | 0.8 | 1.0 | 0.9 | 0.9 | 1.0 | **136.0** | 0.9 | 0.7 |
| GXIIA | 0.8 | 0.8 | 0.7 | 1.0 | 1.0 | 1.0 | 0.9 | 1.5 | **6.8** | 0.8 |
| GXIIB | 0.8 | 0.8 | 0.7 | 1.0 | 0.9 | 0.9 | 0.8 | 1.3 | 0.8 | **10.4** |

Each of the 10 rabbit anti-$PLA_2$ IgGs purified from the respective antisera was reacted in microtitration wells with all 10 recombinant human secreted $PLA_2$s used in the immunization and, after washing and adding Europium-labeled $PLA_2$-specific detecting antibody, fluorescence was measured by time-resolved fluorometry as described in Materials and methods, sample volume 10 μL. The signals from the fluorometer were normalized by dividing the counts-per-second (cps) values by the mean zero-standard cps value of the respective TR-FIA run. Unity indicates the zero-standard (blank) signal level. The normalized signals for the specific antigen-antibody pairs are in bold.

tration]×100%. To study the reproducibility of the assays, interassay variations were determined by measuring the $PLA_2$ levels in the same standard solutions containing recombinant $PLA_2$s on 4 different days. In these experiments, the $PLA_2$ concentrations of the standards solutions were 50 and 100 μg/L for GIB, GIIA, GIIE, GIIF, GIII, GV and GX $PLA_2$, 125 and 250 μg/L for GXIIA $PLA_2$ and 500 and 1000 μg/L for the GXIIB $PLA_2$-like protein. In addition, the

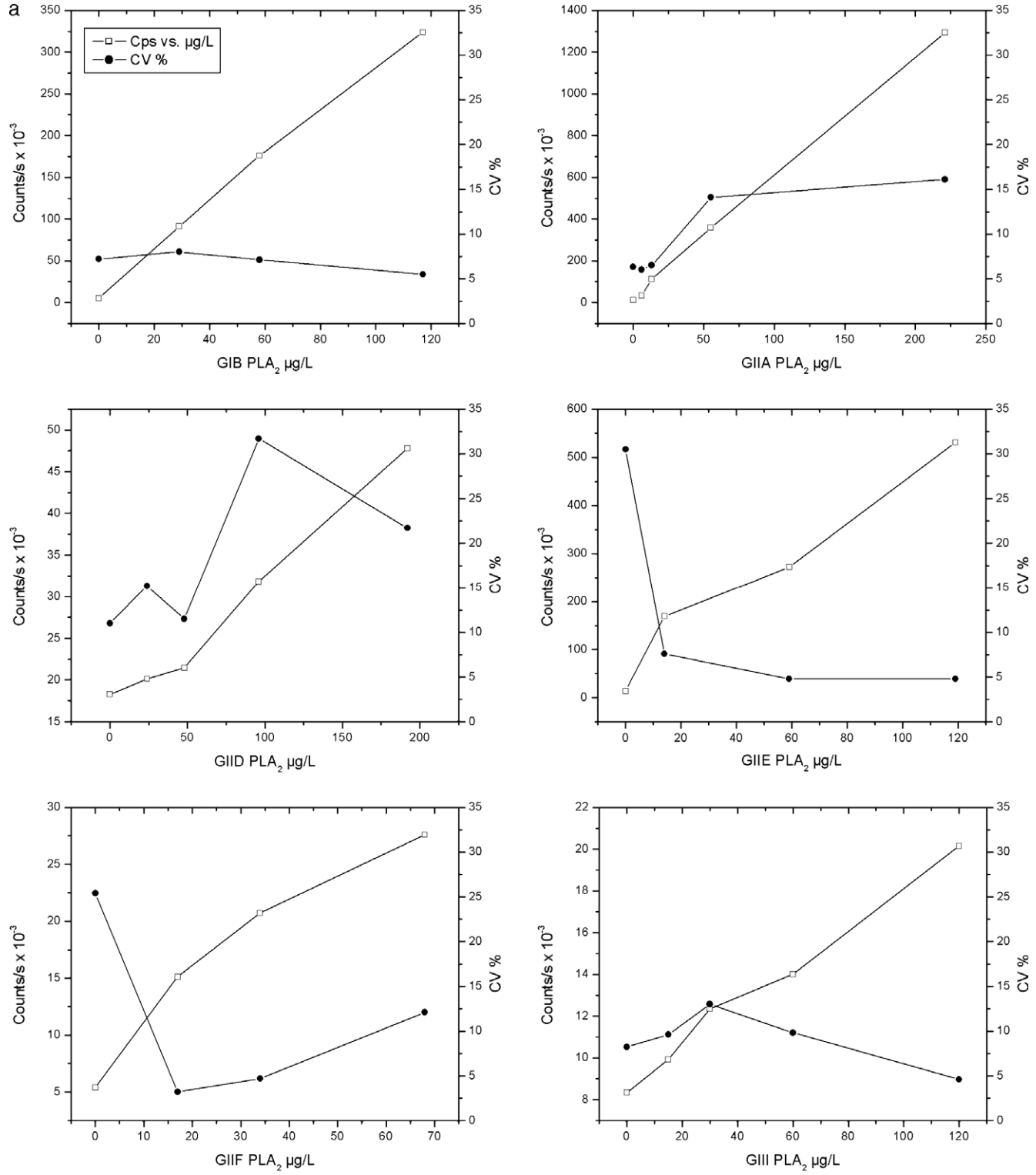


Fig. 1. Standard curves and precision profiles of TR-FIAs for human GIB, GIIA, GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein. Each data point represents the mean of at least 6 duplicates.

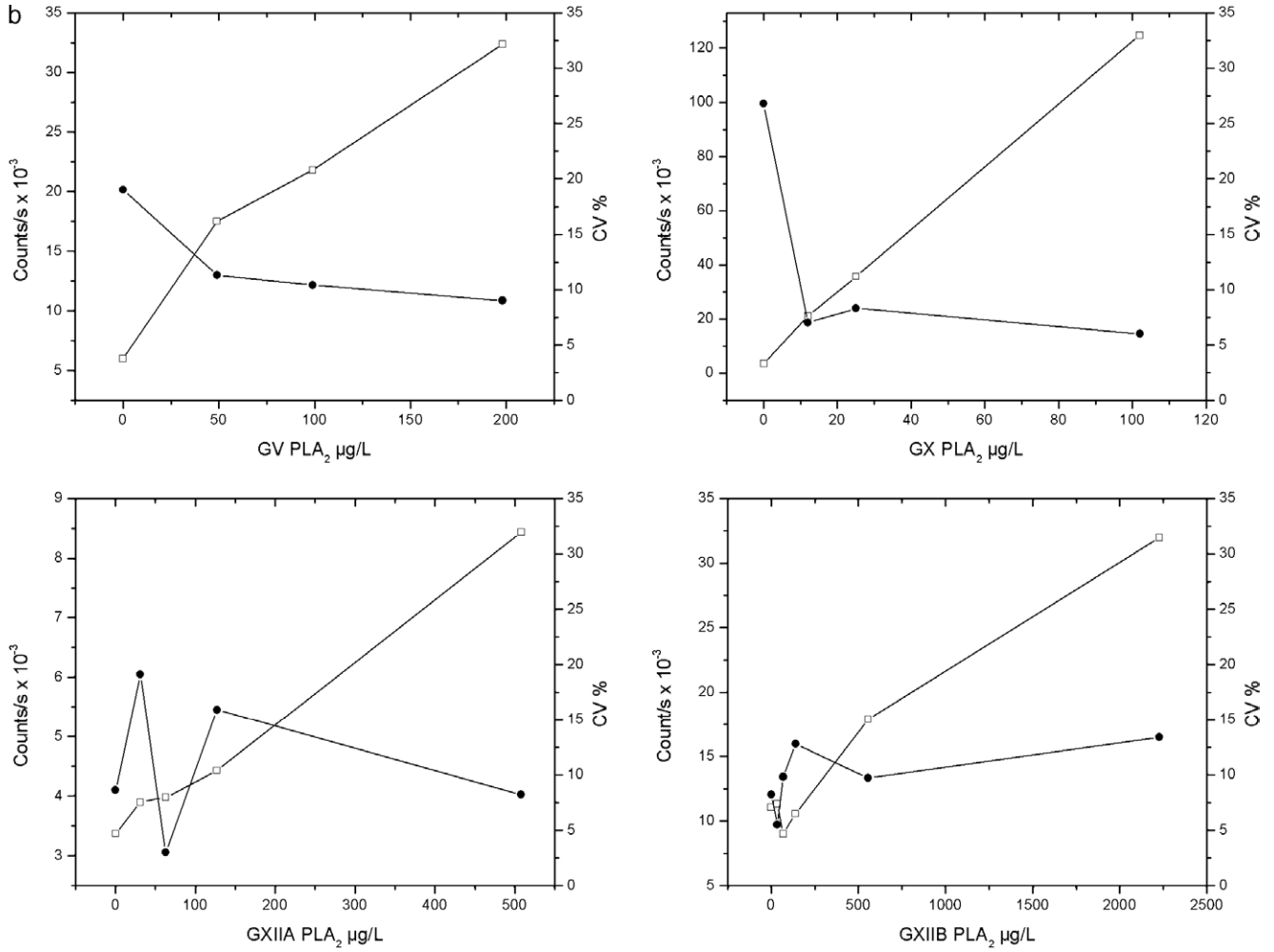


Fig. 1 (*continued*).

reproducibility of TR-FIA for GIIA $PLA_2$ was tested by assaying the same serum samples from 33 septic patients at two occasions 6 months apart. Mann–Whitney *U* test and Pearson linear regression were used for statistical analysis, and $P<0.05$ was regarded significant.

### *2.3. Serum samples from septic patients and healthy blood donors*

The cohort of septic patients consisted of 34 patients admitted to the intensive care unit of Satakunta Central Hospital because of severe sepsis. The study protocol was approved by the local ethical committee. The criteria for enrollment were: systemic inflammatory response syndrome (SIRS) and clinical or laboratory evidence of at least one organ failure related to severe sepsis [38]. The mean age (S.D.) was 53.3 (18.4) years, and there were 11 (32.3%) men and 23 (67.6%) women. Seventeen patients (50%) had septic shock. The infections included peritonitis, meningitis, pancreatitis, pneumonia, gas gangrene, and pyelonephritis. *Staphylococcus aureus*, *Escherichia coli*, *Streptococcus salivarius*, *Neisseria meningitidis*, *Klebsiella pneumoniae*, *Streptococccus pneumoniae*, and *Streptococcus acalactiae* were identified by blood cultures. Negative blood culture results were seen in 24 (70.5%) cases. Serum and plasma samples for $PLA_2$ and other analyses were taken as soon as possible after the admission before the beginning of antimicrobial therapy and subsequent samples frequently during the treatment in the intensive care unit. Plasma amylase activity and the concentration of C-reactive protein (CRP) in the serum were assayed by standard methods (Thermo Clinical Labsystems, Vantaa, Finland) in the hospital laboratory. Control serum samples were obtained from 28 healthy blood donors, 14 men and 14 women, with mean (S.D.) age of 57.0 (13.7) years. All samples were stored at −20 °C until assayed.

## 3. Results

### *3.1. Specificity of antisera*

The specificity of the rabbit antisera was tested by measuring with TR-FIA the cross-reactivity of each of the

10 anti-$PLA_2$ antisera with all 10 antigens used in the immunizations. The signals from the fluorometer were normalized for comparison by dividing the counts-per-second (cps) values by the mean zero-standard (blank) cps value of the respective TR-FIA run (signal-to-background ratio). All signals except those for the antigen–antiserum pair for each immunized rabbit were close to the blank values (signal-to-background ratio close to unity) indicating the absence of cross-reactivity (Table 1).

### 3.2. Standard curves, precision profiles and sensitivities of the assays

The TR-FIAs for different $PLA_2$s varied in their performance, most probably due to the biological variation in the process of immunization of individual rabbits that resulted in differences in the affinities of the antibodies. The standard curves and the precision profiles for each TR-FIA are illustrated in Fig. 1. The assays for GIB, GIIA and GIIE $PLA_2$s had high $PLA_2$ standard-signal responses, whereas the responses in the assays for GIIF, GIII, GV and XIIA and the XIIB $PLA_2$-like protein were relatively low. The analytical sensitivity for each assay was calculated by determining the $PLA_2$ concentration that corresponds to the mean fluorescence of zero standard (at least 6 replicates) plus 3 S.D.s. The sensitivities were 1 μg/L for GIB $PLA_2$, 1 μg/L (GIIA), 35 μg/L (GIID), 3 μg/L (GIIE), 4 μg/L (GIIF), 15 μg/L (GIII), 11 μg/L (GV) and 2 μg/L (GX). The assays for GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein were less sensitive, 92 μg/L and 242 μg/L, respectively. Since the sample volume was 10 μL, it was possible to detect the majority of the $PLA_2$s in the picogram range (from 10 pg for GIB $PLA_2$ to 350 pg for GIID $PLA_2$), and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein in the nanogram range (0.92 ng and 2.42 ng, respectively) by the current TR-FIAs.

Table 2
Analytical recoveries (%) for TR-FIAs for GIB, GIIA, GIIE, GIIF, GIII, GV and GX $PLA_2$ after adding 50, 100 and 200 μg/L of each recombinant protein to serum from a healthy blood donor

| μg/L | GIB (%) | GIIA (%) | GIIE (%) | GIIF (%) | GIII (%) | GV (%) | GX (%) | GXIIA (%) | GXIIB (%) |
|---|---|---|---|---|---|---|---|---|---|
| 50 | 74.0 | 95.7 | 79.7 | 133.3 | 82.2 | 127.9 | 80.3 | | |
| 100 | 84.8 | 95.4 | 72.3 | 116.7 | 83.6 | 111.3 | 65.9 | | |
| 200 | 78.7 | 100.0 | 82.3 | 119.5 | 85.6 | 137.3 | 61.0 | | |
| Mean | 79.2 | 97.0 | 78.1 | 123.2 | 83.8 | 125.5 | 69.1 | | |
| 125 | | | | | | | | 153.1 | |
| 250 | | | | | | | | 179.3 | |
| 500 | | | | | | | | 119.8 | |
| Mean | | | | | | | | 150.8 | |
| 500 | | | | | | | | | 216.0 |
| 1000 | | | | | | | | | 169.0 |
| 2000 | | | | | | | | | 155.0 |
| Mean | | | | | | | | | 180.0 |

For GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein, 125, 250 and 500 μg/L, and 500, 1000 and 2000 μg/L were added, respectively.

Table 3
Interassay variation (CV%) as determined by measuring on 4 four different days the $PLA_2$ levels in the same standard solutions containing 50 and 100 μg/L of recombinant $PLA_2$ for GIB, GIIA, GIIE, GIIF, GIII, GV and GX $PLA_2$, 125 and 250 μg/L for GXIIA $PLA_2$ and 500 and 1000 μg/L for the GXIIB $PLA_2$ -like protein

| μg/L | IB | IIA | IIE | IIF | III | V | X | XIIA | XIIB |
|---|---|---|---|---|---|---|---|---|---|
| | CV (%) | CV (%) | CV (%) | CV (%) | CV (%) | CV (%) | CV (%) | CV (%) | CV (%) |
| 50 | 13.7 | 14.4 | 16.7 | 7.7 | 36.0 | 17.6 | 23.3 | | |
| 100 | 6.8 | 16.6 | 13.1 | 14.3 | 50.5 | 24.4 | 32.4 | | |
| Mean | 10.2 | 15.5 | 14.9 | 11.0 | 43.2 | 21.0 | 27.8 | | |
| 125 | | | | | | | | 20.2 | |
| 250 | | | | | | | | 15.1 | |
| Mean | | | | | | | | 17.6 | |
| 500 | | | | | | | | | 21.0 |
| 1000 | | | | | | | | | 27.4 |
| Mean | | | | | | | | | 24.2 |

### 3.3. Analytical recovery and reproducibility of the assays

Analytical recovery was tested by adding recombinant $PLA_2$ proteins at three different concentrations to serum from a healthy blood donor. Mean recoveries ranged from 69.1% for GX $PLA_2$ to 125.5% for GV $PLA_2$. The recoveries for GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein gave exceedingly high readings: mean values 150.8% and 180.0% (Table 2). Interassay variations were determined by measuring the same 2 standard samples on 4 different days. The variation (mean CV%) ranged from 10.2% for GIB $PLA_2$ to 43.2% for GIII $PLA_2$ (Table 3). There was a highly significant correlation ($r$=0.967, $n$=33, $P$<0.0001) between the results when the same serum samples from septic patients were tested for GIIA $PLA_2$ at 2 different occasions 6 months apart.

### 3.4. Phospholipase $A_2$ levels in serum samples of septic patients and healthy blood donors

High TR-FIA readings were found in a few subjects for all different $PLA_2$ types when untreated serum samples were tested. Interestingly, the TR-FIA signals in these subjects were reduced to blank levels after incubating the serum samples with IgG obtained from non-immunized rabbits (Fig. 2). We assumed that the high signals in the TR-FIAs for all 10 $PLA_2$s in the serum samples of occasional subjects were due to the presence of factor(s), most probably heterophilic antibodies capable of binding rabbit IgG in general rather than just the $PLA_2$-specific rabbit IgG [37]. Non-immune rabbit IgG obviously prevented the binding of the Eu-labelled IgG tracer to these non-$PLA_2$ rabbit IgG binding factors. Subjects with putative heterophilic antibodies in their sera included septic patients (sp) 4, sp11, and sp30, and blood donors (bd) 50 and bd53. There were no consistent clinical characteristics common to these patients that would separate them from the rest of the current septic patients. The main clinical observations on these patients

can be summarized as follows. Sp4 (male, 66 years of age) suffered from epilepsy and chronic cystitis. He had *S. aureus* bacteremia and septic shock but recovered fully. Sp11 (male, 60 years) had advanced multiple sclerosis complicated by perineal gas gangrene. He developed fatal septic shock, and *E. coli* was identified in urine. Sp30 (female, 75 years of age) suffered from Crohn's disease and was operated on because of intestinal obstruction. She had a fatal myocardial infarction 17 days after the operation.

In contrast to the rest of the secreted $PLA_2$s, the TR-FIA signals for GIB and GIIA $PLA_2$ remained high after the addition of non-immune rabbit IgG to the serum samples of

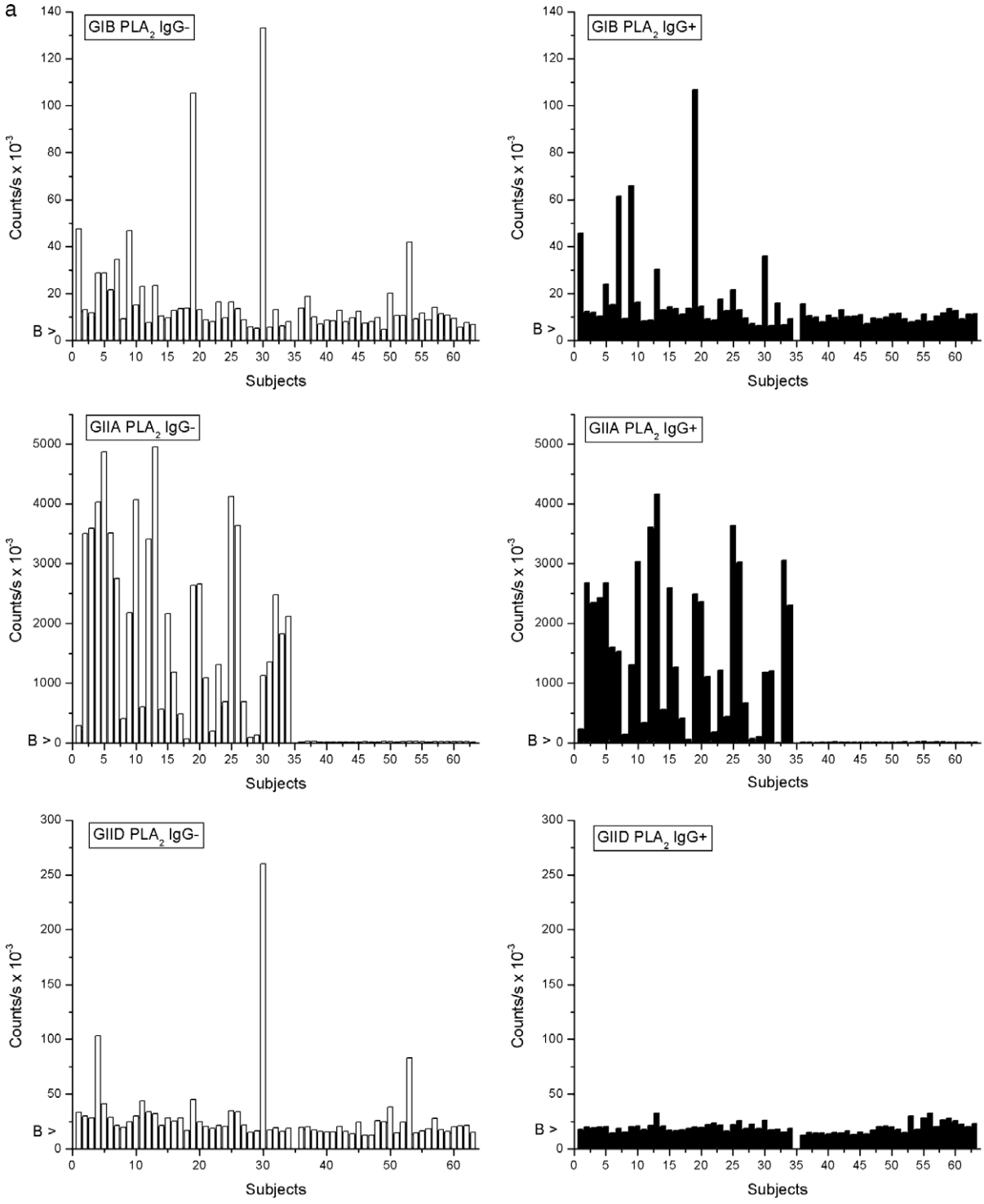


Fig. 2. TR-FIA signals (cps, counts per second as recorded by a fluorometer) in serum samples of septic patients (subjects 1–34, $n$=34) and healthy blood donors (subjects 36–63, $n$=28) before (IgG−, open columns) and after (IgG+, filled columns) the addition of non-immune rabbit IgG. "B>" indicates the blank level (cps) for each assay.

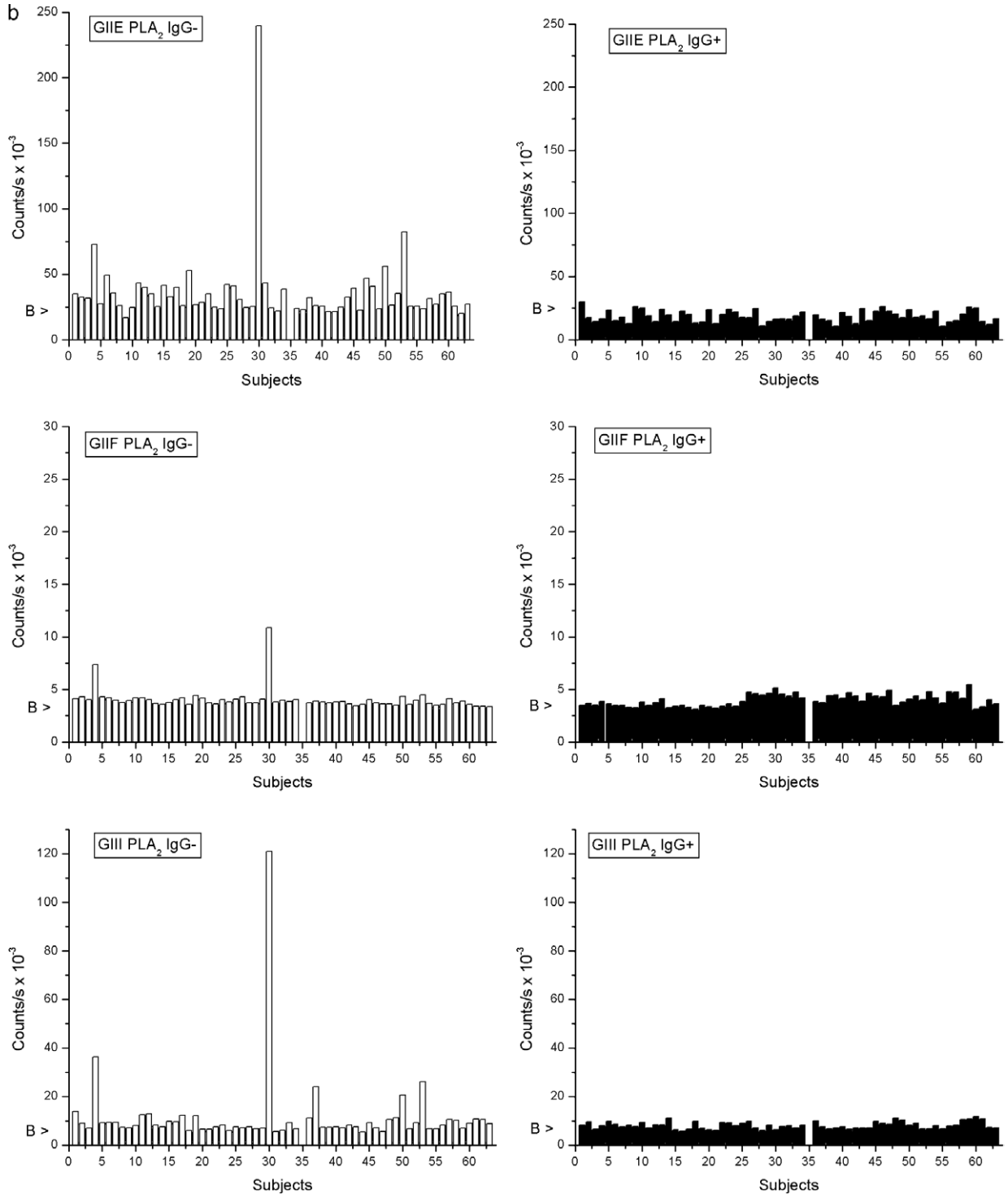


Fig. 2 (*continued*).

a number of septic patients (Fig. 2). The values referred to below are derived from assays done after adding IgG isolated from non-immune rabbit serum to the samples.

Group IB $PLA_2$ is a serum marker of pancreatic acinar cell injury [33]. In the current study (Table 4), patients with elevated serum GIB $PLA_2$ levels included sp1, sp7, sp9, and sp19. Sp1 (female, 48 years of age) suffered from alcoholic acute pancreatitis complicated by perforated intestine and fatal peritonitis. Her plasma amylase activity concentration (a commonly used clinical laboratory test for pancreatic injury) was 448 U/L (the upper limit of the reference interval 300 U/L), and the serum GIB $PLA_2$ concentration was 13.1 μg/L. Sp7 (female, 71 years) had biliary acute pancreatitis and developed fatal septic shock. Her plasma

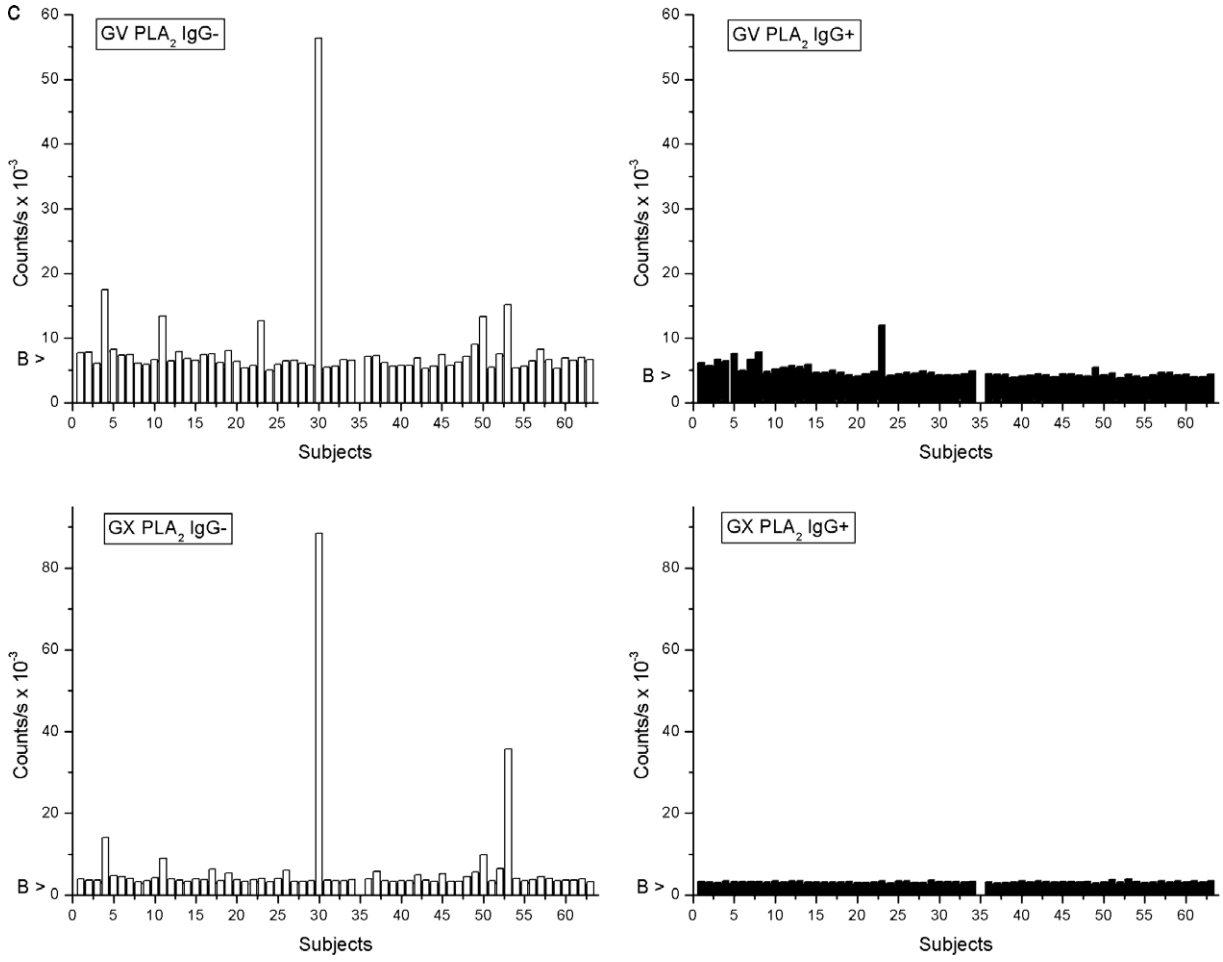


Fig. 2 (*continued*).

amylase was 2509 U/L and serum GIB $PLA_2$ concentration 18.3 μg/L. Sp9 (male, 64 years) had severe atherosclerosis, acute myocarditis and pneumonia and developed fatal shock. His plasma amylase was 5899 U/L and serum GIB $PLA_2$ concentration 19.7 μg/L. Sp19 (male, 53 years) suffered from acute appendicitis complicated by fatal peritonitis. His plasma amylase was 496 U/L and serum GIB $PLA_2$ concentration 33.7 μg/L. Collectively, the serum levels of GIB $PLA_2$ in the septic patients with pancreatic involvement (median 19.0 μg/L, range 13.1–33.7 μg/L, $n$=4) were significantly higher than those in healthy blood donors (median 1.8 μg/L, range 0.8–3.4 μg/L, $n$=28, $P$<0.0001).

The concentration of GIIA $PLA_2$ in serum is highly elevated in systemic bacterial infections and septic shock [39]. In the current study (Table 4), the concentration of GIIA $PLA_2$ in the sera of septic patients (median 315.7 μg/L, range 15.9–979.6 μg/L, $n$=34) was significantly higher than that of healthy blood donors (median 1.8 μg/L, range 0.8–5.8 μg/L, $n$=28, $P$<0.0001).

The measurement of the concentration of C-reactive protein (CRP, the upper limit of the reference interval 10 mg/L) in the serum is a commonly used laboratory test for the acute phase response caused by bacterial infection. The current septic patients (Table 4) had elevated serum CRP levels (median 181 mg/L, range 5–545 mg/L, $n$=33). There was a statistically significant positive correlation between the CRP and GIIA $PLA_2$ values ($r$=0.352, $n$=33, $P$<0.05), whereas the correlations between the CRP and GIB $PLA_2$ and between GIIA $PLA_2$ and GIB $PLA_2$ values were not significant.

## 4. Discussion

In the current study, we developed TR-FIAs for the measurement of the concentrations of all human secreted $PLA_2$s, viz. GIB, GIIA, GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein serum. By these assays, we confirmed earlier observations on the presence of GIB $PLA_2$ and GIIA $PLA_2$ at low levels in the sera of healthy subjects and elevated levels of GIB $PLA_2$ in the sera of septic patients with pancreatic involvement and highly elevated levels of GIIA $PLA_2$ in patients with septic infections [35]. Our novel observation was that the serum concentrations of GIID, GIIE, GIIF, GIII, GV, GX and

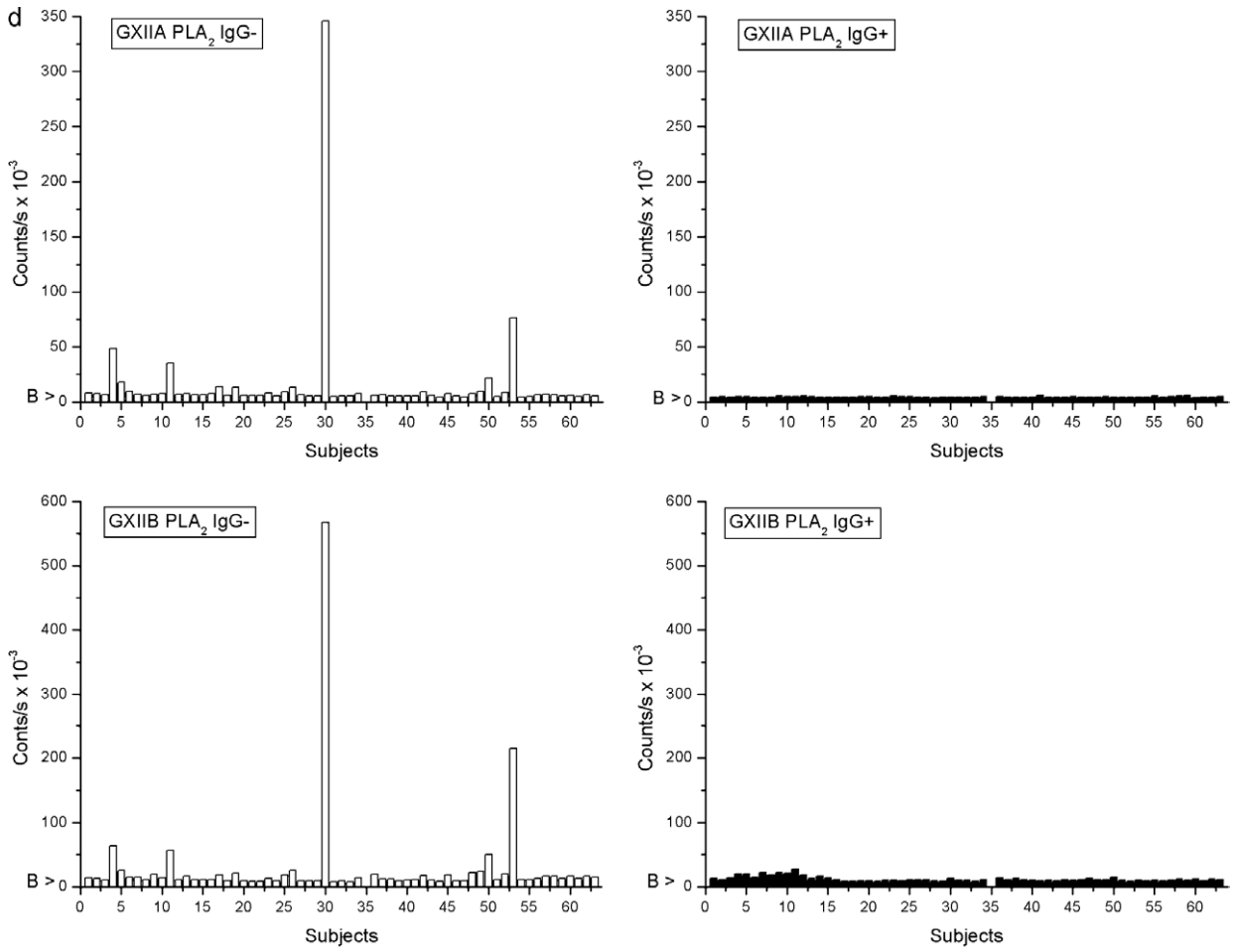


Fig. 2 (*continued*).

GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein were below the analytical sensitivities of the current TR-FIAs in both healthy subjects and patients suffering from sepsis or acute pancreatitis.

GIB $PLA_2$ is synthesized by pancreatic acinar cells and secreted via the pancreatic duct system into the duodenum where it functions as a digestive enzyme. GIB $PLA_2$ has been localized by immunohistochemistry in pancreatic acinar cells [9] that most probably serve as the cellular source of the enzyme found in circulating blood. GIB $PLA_2$ is present in the serum of healthy individuals in low (<10 μg/L) concentrations [33]. Pancreatic injury, caused e.g. by pancreatitis or pancreatic cancer, results in increased release of GIB $PLA_2$ into the blood circulation, and the detection of elevated levels of GIB $PLA_2$ in the serum is as a sensitive and specific marker of pancreatic damage [33,34].

GIIA $PLA_2$ is a mediator of inflammation [40], an acute phase protein [41] and bactericidal both in vitro [42] and in vivo [43]. GIIA $PLA_2$ is the most effective antibacterial agent against Gram-positive bacteria among secreted $PLA_2$s [44]. However, GIIA $PLA_2$ alone is ineffective against Gram-negative bacteria, whereas GXIIA $PLA_2$ is capable of killing Gram-negative bacteria in vitro [44]. The concentration of GIIA $PLA_2$ is low (<10 μg/L) in the sera of healthy individuals, but increases up to 100–200-fold in patients suffering from inflammatory diseases such as sepsis and bacterial infections [39], multiple organ failure [45], acute pancreatitis [34], in trauma victims [46], after surgical operations [47,48], as well as in chronic inflammatory diseases such as Crohn's disease [49] and rheumatoid arthritis [50].

The cellular source of circulating GIIA $PLA_2$ has not been identified unequivocally. Unlikely sources include the spleen and neutrophils, because the concentration of GIIA $PLA_2$ remained elevated in patient sera after splenectomy [51], and elevated concentrations of GIIA $PLA_2$ were measured in the serum of febrile patients suffering from hematological malignancy and neutropenia after cytotoxic treatment [52]. A recent study established the absence of the expression of the mRNA of GIIA $PLA_2$ from human blood neutrophils [25]. Putative sources of circulating GIIA $PLA_2$ include hepatocytes and blood platelets. The expression of the mRNA of GIIA $PLA_2$ has been localized by in situ hybridization in hepatocytes under pathological conditions [53,54], and cytokine-stimulated hepatoma cells secrete GIIA $PLA_2$ in vitro [41]. The GIIA $PLA_2$ protein was originally purified from platelets [13], and the mRNA of GIIA $PLA_2$ has been

Table 4
Concentrations of GIB $PLA_2$, GIIA $PLA_2$ and C-reactive protein (CRP) in sera of 34 septic patients

| Patient | Gender/age | GIB $PLA_2$ (μg/L) | GIIA $PLA_2$ (μg/L) | CRP (mg/L) |
|---|---|---|---|---|
| 1 | F 48 | 13.1 | 44.0 | 30 |
| 2 | M 20 | 2.5 | 568.3 | 139 |
| 3 | M 62 | 2.3 | 542.4 | 285 |
| 4 | M 66 | 1.8 | 604.6 | 345 |
| 5 | M 72 | 6.1 | 719.1 | 55 |
| 6 | F 73 | 3.4 | 387.0 | 187 |
| 7 | F 71 | 18.3 | 334.4 | 67 |
| 8 | M 21 | 1.5 | 40.0 | 5 |
| 9 | M 64 | 19.7 | 293.3 | 97 |
| 10 | M 75 | 3.7 | 713.6 | 5 |
| 11 | M 60 | 1.1 | 96.7 | 200 |
| 12 | F 55 | 1.3 | 813.2 | 266 |
| 13 | M 49 | 8.1 | 979.6 | 545 |
| 14 | F 61 | 2.7 | 136.1 | 187 |
| 15 | M 40 | 3.0 | 533.9 | 133 |
| 16 | F 58 | 2.8 | 275.0 | 45 |
| 17 | M 49 | 2.1 | 94.6 | 238 |
| 18 | F 78 | 2.8 | 15.9 | 202 |
| 19 | M 53 | 33.7 | 710.2 | 181 |
| 20 | M 67 | 3.1 | 599.8 | – |
| 21 | M 58 | 1.4 | 243.7 | 256 |
| 22 | F 53 | 1.3 | 36.0 | 123 |
| 23 | M 51 | 4.1 | 278.9 | 147 |
| 24 | M 45 | 2.6 | 116.1 | 112 |
| 25 | M 20 | 5.3 | 954.5 | 243 |
| 26 | M 66 | 2.7 | 716.5 | 192 |
| 27 | M 33 | 1.6 | 140.3 | 246 |
| 28 | M 63 | 0.8 | 17.5 | 62 |
| 29 | F 81 | 0.6 | 24.6 | 151 |
| 30 | F 75 | 9.9 | 271.5 | 127 |
| 31 | F 20 | 0.6 | 297.1 | 352 |
| 32 | M 17 | 3.6 | 688.4 | 57 |
| 33 | M 30 | 0.7 | 402.2 | 323 |
| 34 | M 57 | 1.4 | 573.5 | 477 |
| Mean | 53.2 | 5.0 | 390.1 | 184.2 |
| S.D. | 18.4 | 6.8 | 290.9 | 127.2 |

F, female; M, male; age, years. The figures refer to TR-FIA results obtained after adding non-immune rabbit IgG to the serum samples.

demonstrated by in situ hybridization in megakaryocytes that are the precursors of platelets [55].

In addition to GIB and GIIA $PLA_2$, a number of other secreted $PLA_2$s are expressed in various tissues and inflammatory cells, e.g. the mRNA of GIID $PLA_2$ in human eosinophils [19] and both the mRNA and protein of GV and GX $PLA_2$s in human neutrophils [25]. GV $PLA_2$ protein was localized in both azurophilic and specific granules, whereas GX $PLA_2$ was confined to azurophilic granules [25]. An interesting observation was that no other secreted $PLA_2$s besides GV and GX $PLA_2$s were expressed at the mRNA and protein levels in neutrophils [25]. In an earlier study, GIIA $PLA_2$ protein was not found by TR-FIA [32] in human neutrophils isolated from blood buffy coat but, in contrast, the enzyme protein was demonstrated in human neutrophils by immunoelectron microscopy [56]. Besides differences in the specificity of antibodies used in these studies, the discrepancies may be due to variable phagocytosis of the enzyme protein by neutrophils from the surrounding medium, e.g. blood plasma.

Enhanced expression levels of secreted $PLA_2$s in addition to GIB and GIIA $PLA_2$ have been reported in connection to inflammation. GIIF $PLA_2$ protein was found at sites of inflammation in human rheumatoid arthritic synovial and vascular cells [22]. GV and GX $PLA_2$s were found in human pulmonary epithelial cells and were postulated to be involved in lung injury [27]. the mRNA of GIIE $PLA_2$ was demonstrated in murine intestine and lung, where its expression was enhanced by lipopolysaccharide-treatment of the experimental animals [20]. The expression of GIID $PLA_2$ at the mRNA level was enhanced after treatment with endotoxin in the rat and mouse thymus [18]. However, the expression responses of secretory $PLA_2$s under inflammatory conditions may differ in the human from that seen in experimental animals and from responses recorded in various cell types in vitro.

Besides the potential role for GV $PLA_2$ in arachidonic acid metabolism [26] and bacterial killing by GIIA $PLA_2$ in human serum [57] and tears [58], the physiological and pathological functions of secreted $PLA_2$s are largely unknown. As a step towards understanding their putative involvement in the generalized inflammatory reaction in humans, we addressed in the current study the release of secreted $PLA_2$s from tissue and/or inflammatory cells into the blood circulation of patients suffering from severe generalized bacterial infections and septic shock. For this purpose, we developed specific TR-FIAs for the measurement of all human secreted $PLA_2$s, viz. GIB, GIIA, GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein. The assays were based on antibodies raised in rabbits against $PLA_2$ proteins produced by recombinant technology. The specificity of the antibodies was confirmed by excluding cross-reactivity between each antibody and all 10 $PLA_2$s used in immunizing the rabbits. The analytical sensitivities of the current TR-FIAs allowed the measurement of the $PLA_2$ concentrations down to the picogram range, except for GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein, for which assays the sensitivities were in the nanogram range. While most of the current assays were robust as indicated in their fair sensitivity and reproducibility, for unknown reasons, the assays for GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein were suboptimal both in analytical sensitivity and recovery. In addition, the interassay variation of the TR-FIA for GIII $PLA_2$ markedly exceeded that of the other assays. Furthermore, experiments on recovery and interassay variation could not be carried out on the TR-FIA for GIID $PLA_2$ because of paucity of the GIID $PLA_2$ recombinant protein available. Therefore, the current results concerning GIID, GIII and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein must be must be interpreted with caution.

A few subjects (3 in the sepsis group and 2 in the blood donor group) had substances in their serum interfering with all the current TR-FIAs. The elevated signals were reduced to the background (blank) levels by adding IgG from non-immunized

rabbits to the serum samples. The interference with immunoassays by non-analyte antibody-binding substances has been documented earlier [59]. A common source of this artifact is the presence of heterophilic antibodies in the sample [37]. This interference was effectively prevented by the inclusion of non-immunized rabbit IgG in the current TR-FIAs.

Elevated levels of circulating GIB and GIIA $PLA_2$ have been measured in various inflammatory diseases including acute pancreatitis (GIB $PLA_2$) [33] and generalized infections (GIIA $PLA_2$) [15,35]. In the current study, we confirmed significantly increased concentrations of GIB and GIIA $PLA_2$ (up to 33.7 μg/L for GIB $PLA_2$ and 979.6 μg/L for GIIA $PLA_2$) in the sera of patients suffering from acute pancreatitis or severe septic infections, respectively, as compared with the levels in the sera of healthy blood donors (up to 3.4 μg/L for GIB $PLA_2$ and 5.8 μg/L for GIIA $PLA_2$). The serum levels of CRP and GIIA $PLA_2$ correlated significantly in the current septic patients indicating an active acute phase response. The current results obtained with specific TR-FIAs for the other secreted $PLA_2$s in the same serum samples indicated serum concentrations below the analytical sensitivities of these assays, viz. 35 μg/L for GIID $PLA_2$, 3 μg/L (GIIE), 4 μg/L (GIIF), 15 μg/L (GIII), 11 μg/L (GV), 2 μg/L (GX), 92 μg/L (GXIIA) and 242 μg/L (GXIIB). However, we cannot exclude the possibility that the serum levels of some secreted $PLA_2$s were slightly elevated, but only to levels not detectable by the current assays. All secreted $PLA_2$s are encoded by different genes that are not highly similar [2,4], and their expression is driven by distinct promoters that do or do not respond to inflammatory stimuli.

In summary, our current results confirmed that, under normal conditions, GIB and GIIA $PLA_2$s were present in serum samples at low levels, and that elevated levels were found by specific TR-FIAs in sera of patients with acute pancreatitis (GIB $PLA_2$) and septic infections (GIIA $PLA_2$). However, in the same serum samples of septic patients and healthy blood donors, the concentrations of the other secreted $PLA_2$s, viz. GIID, GIIE, GIIF, GIII, GV, GX and GXIIA $PLA_2$ and the GXIIB $PLA_2$-like protein were below the analytical sensitivities of the respective TR-FIAs. Our results indicate that generalized bacterial infections that induce the acute phase response do not lead to elevated serum levels of secreted GIIE, GIIF, GIII, GV and GX $PLA_2$s. These results suggest that GIIA $PLA_2$ is the main secretory $PLA_2$ species that is likely to have a role in sepsis, e.g. as an antibacterial agent. The low serum levels of the other secreted $PLA_2$s suggest that their functions may be unrelated to septic infections. Whether these other $PLA_2$s are secreted into circulating blood under pathological conditions different from sepsis remains to be determined.

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