Biphasic transmittance waveform in the APTT coagulation assay is due to the formation of a Ca\textsuperscript{++}-dependent complex of C-reactive protein with very-low-density lipoprotein and is a novel marker of impending disseminated intravascular coagulation.


A decrease in light transmittance before clot formation, manifesting as a biphasic waveform (BPW) pattern in coagulation assays, was previously correlated with the onset of disseminated intravascular coagulation (DIC). In this study of 1187 consecutive admissions to the intensive care unit, the degree of this change on admission predicts DIC better than D-dimer measurements. Additionally, the BPW preceded the time of DIC diagnosis by 18 hours, on average, in 56% (203 of 362) of DIC patients. The BPW is due to the rapid formation of a precipitate and coincident turbidity change on recalcification of plasma. The isolated precipitate contains very-low-density lipoprotein (VLDL) and C-reactive protein (CRP). The addition of CRP and Ca\textsuperscript{++} to normal plasma also causes the precipitation of VLDL and IDL, but not LDL or HDL. The $K_a$ of the CRP/VLDL interaction is 340 nM, and the $K_{d}$ for Ca$^{++}$ is 5.0 mM. In 15 plasmas with the BPW, CRP was highly elevated (77-398 µg/mL), and the concentration of isolated VLDL ranged from 0.082 to 1.32 mM (cholesterol). The turbidity change on recalcification correlates well with the calculated level of the CRP–VLDL complex. Clinically, the BPW better predicts for DIC than either CRP or triglyceride alone. The complex may have pathological implications because CRP can be detected in the VLDL fraction from sera of patients with the BPW, and the VLDL fraction has enhanced prothrombinase surface activity. The complex has been designated lipoprotein complexed C-reactive protein.

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Introduction

The optical profile of clot formation on simple assays of coagulation, such as the activated partial thromboplastin time (APTT), can be atypical in patients with hemostatic dysfunction.\textsuperscript{1,2} The biphasic waveform (BPW) was initially described when a decrease in plasma light transmittance before clot formation on the MDA 180 automated coagulation analyzer was shown to correlate with disseminated intravascular coagulation (DIC) in critically ill patients.\textsuperscript{3,5} In contrast to the normal sigmoidal waveform pattern that is characterized by an initial 100% light transmittance phase before clot formation, patients with a biphasic pattern had an immediate, progressive decrease in light transmittance that occurred even in the preclootting phase. The magnitude of this BPW often varied with sequential samples taken from individual patients and appeared early in samples from patients who were later diagnosed with DIC by more conventional criteria. DIC is common in many primary diseases, such as sepsis. Because its diagnosis in the acute clinical setting is largely dependent on several coagulation test changes that lack specificity on an individual basis, the BPW appeared to offer the advantage of relative simplicity, rapidity, and robustness for early diagnosis.

A subsequent prospective study,\textsuperscript{4} in which all samples received in a diagnostic laboratory over a 2-week period were examined, indicated that the sensitivity and specificity of the BPW for DIC are 97.6% and 98%, respectively. An overall positive predictive value of 74% was obtained, and this value increased with increasing falls in light transmittance. A real-time correlation was also noted between clinical events and BPW changes. Serial reductions in light transmittance invariably indicated impending death.\textsuperscript{5}

The early predictive and prognostic relevance of waveform analysis potentially provides a practical tool in assigning risk in critical care patients, in whom DIC development is known to have a worse prognosis. The identification of DIC at an early or nonovert phase could enable improved targeting of a cohort for which therapeutic modalities are now emerging.\textsuperscript{6} Findings presented below further extend the clinical diagnostic relevance of the BPW. They also show that it is caused by the precipitation of complex consisting of C-reactive protein (CRP) and very-low-density lipoprotein (VLDL).

Patients, materials, and methods

Patient samples for purification studies

Citrated plasma for isolation of the complex was obtained, with study approval from the Liverpool Research Ethics Committee, from 15 patients...
with BPW of varying severity and DIC arising from sepsis (7), pancreatitis (2), respiratory distress syndrome (2), ruptured abdominal aortic aneurysm (1), and trauma. At the time of sampling, 7 patients required assisted ventilation, 3 required enteral feeding, and 2 required total parenteral nutrition.

Materials

Human recombinant CRP, VLDL, phenylalanine-prolyl-arginyl chloromethyl ketone (PPACK), and sheep anti-human serum amylod A (SAA) immunoglobulin G (IgG) were from Calbiochem (La Jolla, CA). Sheep anti-human apolipoprotein B-100 (apoB) IgG was from Boehringer Mannheim (Laval, PQ, Canada). Infinity cholesterol reagent, cholesterol calibrator (2 g/L), rabbit anti-human CRP IgG, and mouse anti-goat/sheep IgG anti-mouse IgG conjugated to horseradish peroxidase (HRP) were from Sigma (St Louis, MO). Goat anti-mouse IgG conjugated to HRP was from Amersham (Mississauga, ON, Canada). All other reagents were of analytical grade.

Turbidity assay for complex formation

A standard curve was prepared by mixing normal and BPW-positive plasma in various proportions (50% total) with 125 μL of 20 mM HEPES/0.15 M NaCl/mM sodium citrate, pH 7.4 (HBS-cit), plus 10 μM PPACK. Turbidity at 405 nm was monitored over time. After 30 seconds, an aliquot of CaCl2 was added (50 mM final), and the turbidity change at 405 nm was monitored to completion over 20 minutes at 20°C in a microtiter plate reader. The assay was used to identify and quantify the active component or components during their isolation from patient plasma.

Isolation of active material from patient plasmas

Patient plasma (6-12 mL) was supplemented with PPACK (10 μM) and dialyzed at 4°C versus HBS-cit, pH 7.4. PPACK (10 μM) and CaCl2 (50 mM) were then added. Samples were incubated for 15 minutes at room temperature and then on ice for 30 minutes. An obvious precipitate formed. A volume of distilled water equal to the starting plasma volume was added and the mixture centrifuged at 31 000 rpm for 20 minutes at 4°C. The supernatant was removed from underneath the floating precipitate with an 18-gauge needle and syringe. The precipitate was washed 3 times by suspending in the starting plasma volume of 0.01 M HEPES, pH 7.4, containing CaCl2 (25 mM) and PPACK (10 μM) and then centrifuging. The precipitate was resuspended in HBS containing PPACK (10 μM) and trisodium citrate (10 mM), pH 7.4, and the solution was dialyzed at 4°C against 0.02 M HEPES/0.02 M NaCl containing 1.0 mM trisodium citrate, pH 7.4 (buffer A). The dialyzed solution following PPACK (10 μM) addition was applied at 22°C to a Q-Sepharose Fast Flow column (5 mL bed volume; Amersham Pharmacia, Baie d’Urfe, PQ, Canada) equilibrated with buffer A. Fractions of 2 mL were collected. Elution used a linear gradient (100 mL) of 0.02 to 0.5 M NaCl in buffer A. Fractions were subjected to the turbidity assay using normal plasma. Two peaks of material were separately pooled, concentrated to 1.0 to 1.25 mL using Millipore concentrators (10 000 MWCO, Ultrafree 15 type; Millipore, Bedford, MA), and stored at 4°C for future analyses.

SDS-PAGE and immunoblotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; nonreducing and reducing) was performed according to Neville7 using 2.5% to 5% or 4% to 15% polyacrylamide gradient gels. Transfer to Immobilon-P membranes (Millipore) at 0.25 A for 14 to 16 hours at 22°C and blotting were performed according to Towbin et al.8 Membrane blocking (1 hour, 22°C) used either 6% (wt/vol) Carnation Instant Milk powder (Toronto, ON, Canada; antiapolipoprotein B-100 antibodies) or 2% (wt/vol) bovine serum albumin (BSA; anti-CRP and anti-SAA antibodies). ApoB-100 blotting used a mouse monoclonal anti-human apoB-100 antibodies and 2% (wt/vol) bovine serum albumin (BSA; anti-CRP and anti-SAA antibodies). Anti-human IgG 1D1 from Dr Ross Milne (University of Ottawa, ON, Canada) and a sheep anti-mouse IgG conjugated with HRP. CRP blotting used a rabbit anti-human CRP IgG and goat anti-rabbit IgG conjugated with HRP. SAA blotting used a sheep anti-human SAA IgG and mouse anti-goat/sheep IgG conjugated with HRP. Detection involved mixing equal volumes of the Chemiluminescence Reagent Plus (Mandel Scientific, Guelph, ON, Canada) solutions over the blots at room temperature for 1 minute and exposure to x-ray film.

NH2-terminal sequencing

Samples were electrophoresed in 4% to 15% polyacrylamide gradient gels and transferred to an Immobilon-P membrane. The NH2-terminal sequencing of materials on the membrane was performed by Dr David Klapper (University of North Carolina School of Medicine, Chapel Hill) with automated Edman degradation.

Lipoprotein isolation

Normal human plasma lipoproteins were isolated as described previously.9 The VLDL fraction (density, 1.006 g/mL) was isolated after centrifugation at 30 000 rpm for 20 hours at 10°C in a Ti-60 fixed angle rotor (Beckman Instruments, Palo Alto, CA). The upper VLDL layer (approximately 6.0 mL) was harvested and centrifuged again. Intermediate-density lipoprotein (IDL; density, 1.019 g/mL), low-density lipoprotein (LDL; density, 1.063 g/mL), and high-density lipoprotein (HDL; density, 1.21 g/mL) were all sequentially isolated from the VLDL infranatant fraction at the same 2 centrifugation speeds and times used for VLDL, after adjusting the density in each case with solid sodium bromide. The remaining plasma was dialyzed against 0.02 M HEPES, 0.15 M NaCl, 10 mM citrate, pH 7.4 and was concentrated by ultrafiltration to the original plasma volume. This comprised lipoprotein-depleted plasma.

Turbidimetric measurement of the calcium-dependent interaction between purified VLDL and recombinant CRP

The experiments were performed in a microtiter plate. Commercially available normal VLDL in final concentrations (as cholesterol) of 0.03, 0.065, 0.1, and 0.15 mM were incubated with increasing concentrations of recombinant CRP (0-300 μg/mL) in HBS-cit. The maximal change in absorbance at 405 nm after the addition of CaCl2 (50 mM) was measured over 20 minutes at 22°C. The levels of turbidity were analyzed to determine the dissociation constant (Kd) and stoichiometry (n) of the CRP–VLDL interaction. The equations used were:

\[ \tau = \alpha [CV] \]

\[ [CV] = 0.5[K_c + [C] + n[V]] - ((K_c + [C] + n[V])^2 - 4n[C][V])^{1/2} \]

In these equations, \( \tau \) is the turbidity, \( \alpha \) is a constant relating turbidity to the concentration of the CRP/VLDL complex (CV), and C and V are the total concentrations of CRP and VLDL. \( K_c \) indicates the free concentration of CRP at which half the sites for CRP on VLDL are bound. The dependence of the CV and stoichiometry on the CRP–VLDL interaction. The equations used were:

Measurements of CRP and VLDL

VLDL fractions of 15 patient plasmas with BPW were obtained by centrifugation at 356 000g at 10°C for 2 hours in polycarbonate centrifuge tubes (11 x 34 mm) in a Beckman TL-100 tabletop ultracentrifuge (Beckman Instruments, Palo Alto, CA). Apo B-100 levels in the VLDL were measured by enzyme-linked immunosorben assay (ELISA) using LDL as the standard.9,10 Cholesterol in the isolated VLDL was determined colorimetrically using the cholesterol infinity reagent and cholesterol calibrator. Total protein concentrations were determined with the Bradford assay using BSA as a standard. CRP levels in plasma were determined by
ELISA using a rabbit anti-human CRP IgG for capture with detection by the
same antibody, conjugated to HRP, according to Tijssen and Kurstak. Elisa
was standardized with purified human recombinant CRP. Rabbit
anti-human CRP antibody was sensitive at detecting purified recombinant
CRP diluted in phosphate-buffered saline at 1.0 µg/L. Standard curves were
linear over the concentration range 1 µg/L to 60 µg/L. The antibody was
immunospecific for CRP because only a single species of 22 kDa was
 detected when plasma (0.2 µL) of intensive care unit (ICU) patients, who
had elevated levels of CRP, was immunoblotted after reducing SDS-PAGE,
and no signal was observed with normal plasma. The average standard
deviation from the mean value obtained with 3 dilutions was 10.60%. CRP
was also determined in the 1187 clinical study patients, with levels
cross-checked using the Eurogenetics kit. The Eurogenetics CRP ELISA,
which was calibrated against the World Health Organization International
Reference Standard for CRP immunoassay (85/506), had a sensitivity of 0.5
mg/L with a 5.12% coefficient variation between the assays. As a marker of
VLDL levels, triglyceride concentrations were also determined in the
same cohort.

CRP detection in isolated VLDL fractions from patient sera
Sera from 4 patients with varying degrees of the BPW were obtained. Each
of these was divided into three 0.9-ml aliquots. The first aliquot was not
manipulated. To the second was added 0.1 mL of 0.1 M EDTA (ethylenedi-
aminetetraacetic acid) to disrupt any complex that might be present. To the
third was added 0.1 mL of 0.25 M CaCl2 to optimize complex formation.
All samples were centrifuged as described above for VLDL isolation.
Isolated fractions were then measured for CRP and triglyceride (Sigma
Diagnostics Infinity Reagent) with values then expressed as CRP per
millimolar VLDL triglyceride.

Prothrombinase supporting activity of isolated VLDL
The isolated VLDL of 3 patients with BPW and a sample isolated from a pool of
20 healthy volunteers were analyzed for their ability to replace the phospholipid
component in prothrombin activation. The VLDL concentrations were adjusted
to 100, 200, and 300 µg/mL (triglyceride), and initial rates of thrombin formation
were measured by fluorescence in a fluorescence microtiter plate reader
according to a procedure similar to that described previously.

Clinical study population and design
APTT waveform analysis was prospectively evaluated in all adult patients
admitted consecutively to the intensive therapy unit (ITU) of the Royal
Liverpool University Hospital over a 24-month period. The study had the
approval of the Liverpool Research Ethics Committee, with blood samples
collected for analysis during the first hour of ITU admission and then once
daily until discharge. Clinicians performing the daily clinical assessment
and the diagnosis of DIC were masked to the results of the wave-
form analysis.

APTT waveform analysis
Plasma, derived from blood collected in 0.105 M trisodium citrate at a ratio of 1
part anticoagulant to 9 parts whole blood, was separated within 60 minutes and
analyzed immediately. The APTT, including waveform analysis, was determined
on the MDA 180 (Organon Teknika, Cambridge, United Kingdom) analyzer
using Platelet LS. The methodology has been described in detail elsewhere.
In brief, the MDA 180 uses a variable wavelength photo-optical detection system
and can chart and quantify changes in light transmission when the plasma clots
after activation and recalcification. Normal and biphasic waveforms at 580 nm
are illustrated in Figure 1. A biphasic profile occurs when light transmission
decreases before clot formation in the first part of the curve. To quantify this
abnormality, light transmission at time 0 was set to 100%, and the value recorded
18 seconds later (TL18) was taken as the index of the abnormality. As the MDA
180 blanks each specimen individually, accurate comparisons can be made
between specimens. Normal range determination studies on 20 healthy vol-
unteers (that is, persons without BPW) showed a mean TL18 value of 100%
(99.43%-100.69%), with a mean coefficient of variation of 0.15%.

DIC
The diagnosis was in accordance with the Japanese Ministry of Health and
Welfare 1988 criteria that are the basis of the International Society of
Thrombosis and Hemostasis Standardization Subcommittee definition.

Statistical analysis
APTT waveform values (TL18) and D-dimer levels in the first hour of
admission were respectively measured against DIC development using
nonlinear regression analysis with a 3-parameter logistic model fitted to the
data. The outcome association with TL18 was later contrasted with CRP
levels alone and triglyceride (TG) measurements alone, in the same cohort
of samples. All calculations were performed using S-PLUS 2000 (Math-
Soft, Seattle, WA).

Results
Biphasic waveform is due to the Ca++-dependent precipitation
of a complex of VLDL and CRP
Studies were undertaken to identify the mechanism responsible for
the BPW. The formation of a precipitate when Ca++ was added to
plasmas of patients showing the BPW was visually obvious. This
occurred even with the thrombin inhibitor hirudin (Figure 1). The
turbidity change when Ca++ was added to positive samples in the
presence of a thrombin inhibitor formed the basis of an assay. Time
courses of turbidity, as measured in a plate reader, are shown in
Figure 2. The maximum extent of turbidity change increased with
increasing proportions of BPW-positive plasmas in the assay. The
assay was standardized by the relation between turbidity and the
content of positive plasma.

Precipitated, washed material consisted of protein, phospholip-
sids (31%), cholesterol (10%), and triglycerides (50%) in propor-
tions typical of very-low-density lipoproteins. The material was
dispersed in citrate and subjected to anion exchange chromatogra-
phy (Figure 3). Two major peaks were obtained, the first of which
was very turbid (absorbance at 320 nm). Activity (turbidity
formation in a mixture of the sample and normal plasma on
recalci fi cation) was exhibited only by the material in peak 2. SDS-PAGE, immunoblotting, and amino acid sequence analysis showed that the starting material contains proteins with apparent molecular masses of 500 kDa, 22 kDa, and 10 kDa (Figure 4A). Sequence analysis identified the 22-kDa protein as C-reactive protein, starting at residue 19. The 10-kDa protein gave 2 sequences, consistent with serum amyloid A species, respectively, beginning with amino acids 19 and 20. Although the 500-kDa species did not yield a sequence, its high molecular weight was consistent with apolipoprotein B-100, the major protein component of VLDL. After fractionation, the high molecular weight band and SAA were obtained in peak 1, and CRP was obtained in peak 2. Immunoblotting (Figure 4B-D) identified the 500-kDa material as Apo B-100 (Figure 4B), the 22-kDa material as CRP (Figure 4C), and the 10-kDa material as SAA (Figure 4D). The starting material, but not isolated peak 1 or peak 2 components, formed a precipitate in buffer when recalci fi cation. The mixture of peaks 1 and 2, however, did form a precipitate (data not shown). Thus, VLDL and CRP minimally are required to form the precipitate. This procedure was repeated with at least 10 different positive plasmas. Occasionally, SAA was not recovered in the isolated peaks. Thus, SAA can be included in the precipitate but is not necessary for its formation.

Turbidity changes associated with recalci fi cated mixtures of CRP and normal lipoprotein classes of human plasma

The lipoprotein classes VLDL, IDL, LDL, and HDL, isolated from a sample of normal human plasma, were mixed with CRP isolated from patient plasma. Turbidity changes were measured after recalci fi cation in buffer. Of the total change that occurred in plasma, 63% could be attributed to the VLDL fraction, 25% to the IDL fraction, and 5% and 6%, respectively, to the LDL and HDL fractions. Although normal plasma supplemented with CRP exhibited a strong turbidity change, the lipoprotein-deficient plasma exhibited no change.

Characterization of the interaction between purified VLDL and CRP

Commercially available human VLDL was supplemented with recombinant CRP. Ca++ was then added, and the subsequent turbidity changes were measured. An approach to saturation was obtained in buffer and in lipoprotein-deficient plasma as the CRP level was increased at all 4 concentrations of VLDL (Figure 5A-B). The Kd value for the interaction between CRP and VLDL in buffer is 340 nM (7.5 mg/L). The stoichiometry is such that 178 mg CRP...
bound per liter is needed to saturate 1 mM VLDL (cholesterol concentration). In lipoprotein-deficient plasma, \(K_d\) is 440 nM, and the stoichiometry is 343 mg CRP/L per 1 mM VLDL cholesterol. The interaction is \(Ca^{++}\) dependent. The half-maximal extent of complex formation occurs at a \(Ca^{++}\) concentration of 5.0 mM (Figure 6). Complex formation was completely inhibited by 10 mM phosphorylcholine (data not shown). \(Epsilon\) aminocaproic acid inhibited formation with an IC50 value of 2.1 mM (not shown).

**Correlation between the concentrations of CRP and VLDL in several patient plasmas and the extent of complex formation on the addition of \(Ca^{++}\)**

Levels of CRP and VLDL and turbidity changes on recalci

![Image](Figure 6. Ca^{++} concentration dependence of formation of the VLDL–CRP complex. Complex formation is half maximal at 5.0 mM Ca^{++}.)

**Table 1. Turbidity changes and plasma concentrations of total CRP, total VLDL, and calculated levels of CRP in complex with VLDL from 15 patients with positive BPW**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Total CRP (mg/L)</th>
<th>VLDL cholesterol (mM)</th>
<th>Calculated CRP in complex (mg/L)</th>
<th>Turbidity Δ405</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>266</td>
<td>1.327</td>
<td>254</td>
<td>0.290</td>
</tr>
<tr>
<td>2</td>
<td>398</td>
<td>0.292</td>
<td>97</td>
<td>0.145</td>
</tr>
<tr>
<td>3</td>
<td>219</td>
<td>0.316</td>
<td>100</td>
<td>0.062</td>
</tr>
<tr>
<td>4</td>
<td>342</td>
<td>0.248</td>
<td>80</td>
<td>0.048</td>
</tr>
<tr>
<td>5</td>
<td>294</td>
<td>0.498</td>
<td>159</td>
<td>0.033</td>
</tr>
<tr>
<td>6</td>
<td>323</td>
<td>0.204</td>
<td>67</td>
<td>0.095</td>
</tr>
<tr>
<td>7</td>
<td>355</td>
<td>0.810</td>
<td>254</td>
<td>0.288</td>
</tr>
<tr>
<td>8</td>
<td>314</td>
<td>0.389</td>
<td>127</td>
<td>0.162</td>
</tr>
<tr>
<td>9</td>
<td>361</td>
<td>0.627</td>
<td>203</td>
<td>0.401</td>
</tr>
<tr>
<td>10</td>
<td>220</td>
<td>0.260</td>
<td>84</td>
<td>0.057</td>
</tr>
<tr>
<td>11</td>
<td>387</td>
<td>0.414</td>
<td>137</td>
<td>0.187</td>
</tr>
<tr>
<td>12</td>
<td>274</td>
<td>0.339</td>
<td>110</td>
<td>0.143</td>
</tr>
<tr>
<td>13</td>
<td>212</td>
<td>0.423</td>
<td>130</td>
<td>0.146</td>
</tr>
<tr>
<td>14</td>
<td>274</td>
<td>0.333</td>
<td>108</td>
<td>0.106</td>
</tr>
<tr>
<td>15</td>
<td>77</td>
<td>0.082</td>
<td>24</td>
<td>0.021</td>
</tr>
<tr>
<td>NHP</td>
<td>1.3</td>
<td>0.389</td>
<td>1.2</td>
<td>0</td>
</tr>
</tbody>
</table>

VLDL is the average value, normalized to cholesterol, based on measured levels of cholesterol, ApoB-100, and total protein in the VLDL fraction. CRP in complex was calculated using equation 2 in “Patients, materials, and methods” and parameters for the interaction between CRP and VLDL in lipoprotein-deficient plasma (\(K_d = 9.63 \text{ CRP} \text{ [mg/L]} \times \text{mM VLDL cholesterol]}. NHP indicates normal human plasma.

**Evidence for the existence of CRP–VLDL complex in the sera of patients with biphasic waveform**

These experiments were performed to identify the complex in the sera of blood not exposed to the anticoagulant. CRP was detected by ELISA in VLDL isolated from untreated sera of patients with BPW (Table 2). Prior chelation of \(Ca^{++}\) in the sample led to a loss of detectable CRP in all patients examined. Likewise, prior incubation with additional \(Ca^{++}\) led to enhanced detection of CRP within the VLDL fraction. VLDL from healthy volunteers did not have detectable CRP, and patients with high CRP without BPW had no recoverable VLDL (data not shown). From these observations, we conclude that the complex does exist to a measurable extent in the blood of patients whose plasma exhibits BPW.

**Prothrombinase supporting activity of patient and normal isolated VLDL**

Rates of thrombin formation were determined at numerous concentrations of normal VLDL and the VLDL from 3 patients with a biphasic transmittance waveform. The results (Figure 8) indicated that VLDL from patients was more potent in supporting prothrombinase activity than VLDL in healthy volunteers. Slopes of the relationships depicted in Figure 8 indicated that the 3 patients’ VLDL levels were 3.3-, 4.1-, and 7.8-fold more potent than normal VLDL. These data suggest that patient VLDL is qualitatively different from normal VLDL in a way yet to be determined. The difference is magnified when the comparison is made, not on the basis of the triglyceride contents of the VLDL but on the concentration of VLDL in plasma. Calculated the latter way, the 3 respective patient plasma VLDL levels supported prothrombinase activity 5.2-, 6.0-, and 9.0-fold more potently than VLDL from healthy volunteers. Interestingly, added C-reactive protein did not affect the results (data not shown).

**Biphasic waveform on ITU admission predicts for DIC**

A 24-month prospective clinical study of 1187 consecutive patient admissions to the ITU was carried out concurrently with the
purification studies. A biphasic waveform (BPW)—that is, a TL18 value less than 99%, was present in 346 (29%) patients on admission to the ITU. The presence of the abnormality in the waveform pattern was, as reported previously, independent of the APTT clotting time and not influenced by concurrent anticoagulant therapy. Another 412 patients subsequently acquired BPW during their ITU stay. Overall, 758 (64%) of the patients had a BPW at some time during the course of their intensive care stay.

DIC occurred in 362 (30%) patients, with sepsis as the most common primary cause. Three hundred thirty-six (93%) of these patients had BPW during their ITU period. The 26 patients without BPW, but with DIC as determined by conventional criteria, were recovering without complication from primarily major spinal procedures. Analysis of the APTT-TL18 admission values in all patients with respect to the development of overt DIC is as shown in Figure 9A. A close fit was seen between observed and predicted values with a narrow 95% confidence interval. In the absence of BPW, the fraction of patients with diagnoses of DIC was 0.21 with a stepwise increase in the patient fractions as the degree of abnormality increased. With a 25% reduction in TL18, this reached 0.76. By comparison, D-dimer, an established marker of DIC, showed a DIC fraction of 0.1 at undetectable D-dimers, to a maximal association of 0.4 for D-dimer values of 5 g/mL and above in the same cohort of patients (Figure 9B).

Biphasic waveform better predicts DIC than measurements of CRP and triglyceride

Following the identification of CRP, an established marker of inflammation, as a component of the complex underlying the BPW, it was important to determine whether CRP levels alone provided information similar to that of the BPW. Logistic regression of admission CRP values for DIC prediction is shown in Figure 10A. The most elevated results were associated with 0.46 of DIC patients, compared with 0.76 with TL18 determined by APTT waveform analysis. Comparison with triglyceride measurements in the same samples, as a marker of VLDL, showed that triglyceride levels do not allow for the prediction of DIC (Figure 10B). This suggests that the BPW provides information in addition to that obtained through measurements of CRP and triglyceride alone.

Table 2. CRP in VLDL fractions from untreated, EDTA-treated, and calcium-treated samples of serum from patients with positive BPW

<table>
<thead>
<tr>
<th>Patient</th>
<th>Serum CRP (mg/L)</th>
<th>VLDL fraction</th>
<th>VLDL fraction plus EDTA</th>
<th>VLDL fraction plus Ca²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total CRP</td>
<td>CRP/mM Trig</td>
<td>Total CRP</td>
<td>CRP/mM Trig</td>
</tr>
<tr>
<td>1</td>
<td>323</td>
<td>14.3</td>
<td>1.29</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>281</td>
<td>18.6</td>
<td>4.04</td>
<td>1.1</td>
</tr>
<tr>
<td>3</td>
<td>264</td>
<td>4.2</td>
<td>1.17</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>218</td>
<td>21.5</td>
<td>9.77</td>
<td>2.2</td>
</tr>
</tbody>
</table>

CRP levels were measured in the VLDL fraction isolated from serum. Measurements were also determined on samples from sera supplemented with either EDTA or Ca²⁺.

Figure 8. Prothrombinase-supporting activity of patient and normal isolated VLDL. Rates of thrombin formation were determined at numerous concentrations of isolated normal VLDL (□) and VLDL from 3 patients with biphasic transmittance waveform. VLDL concentrations were measured as triglycerides. A qualitative difference in potency (prothrombinase activity/VLDL) is observed in these data in that the respective patient specimens were 3.3 (A), 4.1 (B), and 7.8 (C) times more potent than normal VLDL (D).

Figure 9. Logistic regression analysis models for admission. (A) APTT TL18 values and (B) D-dimer measurements against DIC prediction. □ represents the observed fractions, and the 95% confidence limits are indicated by dashed lines.

Discussion

Initial reports of Downey et al., which indicated an association between the BPW and DIC in intensive care patients, suggest a
Distributed and highly conserved in nature. 22 Although its normal level in healthy persons is approximately 1.0 mg/L, this level rapidly increases to several hundred milligrams per liter under conditions of infection or tissue damage. 23 Thus, it is an excellent marker of, and helps to define, the acute-phase response. Although CRP was first recognized 72 years ago, 24 its physiological role is not known to date. A link to inflammation, however, is suspected because of its ability to opsonize some microorganisms and to contribute to the activation of complement.25,26 In addition, a link to atherosclerosis and cardiovascular disease has been strongly suggested.27-30

Interactions between CRP and normal or modified lipoproteins have been described by others, but not in the contexts we have described. Cabana et al31 showed that rabbit CRP binds to VLDL in a Ca \(^{++}\)-dependent manner. In the rat, CRP was shown to interact with serum components containing apolipoproteins E and A1.32 The interaction is Ca \(^{++}\) dependent and is inhibited by O-phosphoryl-ethanolamine, a compound that tightly binds specifically to rat CRP. Bhakdi et al33 showed that when LDL is modified by exposure to a combination of trypsin, cholesterol esterase, and neuraminidase, it binds CRP and has complement-activating activity.33 They proposed that LDL within the vessel wall could be enzymatically modified to a form that binds CRP and thereby activates complement, an event that might contribute to atherosclerosis.

The Ca \(^{++}\)-dependent formation of the CRP--VLDL complex explains why its formation can be detected by an increase in turbidity on the recalification of plasma samples prepared from blood collected into citrate as the anticoagulant. The complex likely would be present in vivo, though complex formation would not be optimized at the normal physiologic free Ca \(^{++}\) concentration (approximately 2.5 mM). Indeed, we show that CRP is present in the VLDL fractions extracted from the serum of these patients that had not been manipulated by chelation and recalification.

The biphasic transmittance waveform depends on a substantially elevated level of CRP and levels of VLDL or IDL adequate to rapidly form a macroscopic precipitate on recalification. Although the complex, as measured by the BPW, correlates well with DIC, the level of CRP alone does not predict DIC nearly as well. In addition, triglyceride levels alone, as a measure of VLDL, do not predict DIC.

Whether the CRP--VLDL complex contributes to, or merely reflects, DIC is a question that arises. That it might contribute to the hemostatic dysfunction of DIC is plausible, because lipoproteins have been shown to provide the equivalent of the phospholipid component in the activation of prothrombin.34,35 In addition, our studies suggest that the VLDL of some patients is qualitatively and quantitatively more active as a surface for prothrombin activation than normal VLDL. This might contribute to enhanced thrombin generation and thereby to DIC.

In summary, our work shows that the biphasic transmittance waveform observed in certain plasmas subjected to recalification in coagulation assays can be attributed to the rapid formation of a Ca \(^{++}\)-dependent, macroscopic complex between CRP and VLDL. Its formation correlates well with DIC in patients in intensive care, and its measurement has the potential to be useful for prognosis and diagnosis and for monitoring therapy. In addition, the complex might contribute to the underlying disease process in DIC.

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Figure 10. Logistic models for DIC prediction: (A) CRP and (B) triglyceride measurements on admission to the ITU. Open circles are observed fractions; dashed lines indicate 95% confidence limit.

strong potential for use of the BPW for prognosis and temporal assessment of patient status. This motivated the biochemical and clinical investigations reported here. With respect to diagnosis, the initial association with DIC was extended by demonstrating the ability of the BPW to assign risk from the first admission sample to the intensive care unit. It better predicts impending DIC than D-dimer, an established marker of this condition.6,17 The BPW also often recognizes DIC at an early, nonovert stage. Its simplicity and rapidity of performance within a frequently requested test for assessing coagulation in the ITU setting—that is, the APTT—allows the optimization of therapeutic intervention in aborting what otherwise is a serious complication of diseases frequently encountered in an ITU setting. For example, in sepsis, the most common cause of DIC in this cohort, recombinant human activated protein C treatment has been shown to improve overall survival.15 Moreover, waveform analysis can be easily repeated for monitoring purposes because quantification of the degree of abnormality provides an index of whether the patient is improving or deteriorating in response to therapy.

Precipitates isolated from 10 patients with BPW contained CRP and apoB-100. Some, but not all, also contained SAA. No isolates contained other proteins such as, for example, soluble fibrin, which might be hypothesized as potential components of the precipitate.19 Another 15 samples analyzed for the magnitude of the turbidity change on recalification showed that the CRP levels were very high compared with normal levels. By this criterion, all these patients were experiencing an acute-phase response. VLDL levels varied from below normal to above normal. Because the magnitude of the turbidity change in these samples correlated well with the calculated level of the VLDL–CRP complex, the turbidity change and the BPW can be concluded to have resulted from the formation of the complex.

CRP forms pentamers in the presence of Ca \(^{++}\) and belongs to a family of proteins known as pentraxins.14,20,21 It is ubiquitously distributed and highly conserved in nature.22 Although its normal level in healthy persons is approximately 1.0 mg/L, this level...
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Biphasic transmittance waveform in the APTT coagulation assay is due to the formation of a Ca ++-dependent complex of C-reactive protein with very-low-density lipoprotein and is a novel marker of impending disseminated intravascular coagulation