Negative Interference in Cardiac Troponin I Immunoassays from a Frequently Occurring Serum and Plasma Component

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Background: Cardiac troponin I (cTnI) is a sensitive marker of cardiac injury, but cTnI assays, like other immunoassays, are susceptible to interferences. We evaluated the presence of interfering substances by measuring the recovery of cTnI added to samples from volunteers and from patients with acute coronary syndromes (ACS).

Methods: We added a ternary complex of human cardiac troponin (30–500 µg/L) or cTnI from serum to samples from healthy volunteers and ACS patients. We measured cTnI with a two-site sandwich time-resolved immunofluorometric assay using two antibodies against epitopes in the central stable part of cTnI. We also analyzed 108 heparin-plasma samples from 16 ACS patients with this assay, with an assay based on four antibodies, and with two commercial cTnI assays, AxSYM and ACS:180.

Results: In samples from both healthy persons and ACS patients, recoveries for our assay were 1–167% (range). Recoveries were increased by addition of an antibody with an epitope in the N-terminal region of cTnI to the solid phase and an antibody with an epitope against epitopes in the central stable part of cTnI. We also analyzed 108 heparin-plasma samples from 16 ACS patients with this assay, with an assay based on four antibodies, and with two commercial cTnI assays, AxSYM and ACS:180.

Results: In samples from both healthy persons and ACS patients, recoveries for our assay were 1–167% (range). Recoveries were increased by addition of an antibody with an epitope in the N-terminal region of cTnI to the solid phase and an antibody with an epitope against epitopes in the C-terminal region as a second detection antibody. In 2 of 16 patients with ACS, normal cTnI concentrations found when measured with the original assay demonstrated clinically abnormal (up to 10-fold higher) results with the additional N- and C-terminal antibodies in the early phase of infarction. Both commercial cTnI assays also demonstrated clinically misleading, falsely low cTnI concentrations.

Conclusions: Some yet unidentified, variable component, present in the blood from healthy volunteers and ACS patients, interferes with the binding of antibodies against epitopes in the central part of cTnI used in two commercial assays. Our approach to supplement the mid-fragment cTnI antibodies with antibodies in the N- and C-terminal parts of the molecule in an experimental assay represents a step in resolving this interferent.

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Cardiac troponin I and T (cTnI and cTnT) are sensitive and reliable markers of myocardial damage, and their use has been recommended by scientific committees for the diagnosis of acute coronary syndromes (ACS) (1, 2). The complex molecular nature of cTnI complicates measurement by immunoassays, producing large discrepancies in cTnI concentrations as measured with differently configured commercial and research assays (3, 4). This is conceivably a compound effect of the lack of an international standard and the divergent recognition of the numerous cTnI forms by the antibodies used in the assays (5, 6).

Several forms of the cTnI molecule can occur, and this fact needs to be considered when designing cTnI assays. Because cTnI is a component of the troponin complex responsible for the regulation of muscle contraction, it interacts with the other components of the complex, cTnT and troponin C (TnC) with a strong interaction with TnC in the presence of intracellular calcium (7). It has been shown that the major form of cTnI in the blood of patients with acute myocardial infarction (AMI) is the binary cTnI-TnC complex and that only a small part is in the free
The antibodies used in immunoassays should ideally recognize both free and complexed cTnI in an equimolar manner (10), but more specifically, unrestricted recognition of the complex is of vital importance. Moreover, the use of antibodies against epitopes in the central part of cTnI has been generally recommended because the N- and C-terminal ends are susceptible to proteolytic degradation both in vivo and after sampling (10–12).

The cTnI molecule contains two serine residues in positions 23 and 24 that can be phosphorylated in tissue, and it has been reported that ~50% of the cTnI in the blood of AMI patients is in the phosphorylated form (13). The structural and conformational changes following phosphorylation can significantly affect the binding of some antibodies to cTnI (10, 14). Furthermore, the two cysteine residues in positions 80 and 97 can form a disulfide bond, allowing cTnI to be found in oxidized and reduced forms (15), again affecting the antigen recognition of different commercially available assays (6). Because the cTnI molecule has a high positive charge (pl 9.87), it will attract negatively charged molecules such as heparin, which in turn can interfere with the antibody-antigen interaction (10). Heparinized plasma samples are widely used in clinical laboratories; thus discrepant results compared with serum will occur if the antibodies are affected by heparin (16). EDTA used as anticoagulant can also cause discrepancies, especially in assays utilizing antibodies that differentially recognize free and complexed cTnI (17), because EDTA can cause partial unfolding of the calcium-dependent troponin complex. All of the characteristics of cTnI mentioned above should be thoroughly considered in the design and development of cTnI immunoassays.

Analytical interference from many more nonspecific causes is a common problem that affects almost all immunoassays to some extent, producing false-positive or negative results (18). The most frequently described interfering mechanisms are those caused by heterophilic antibodies, human anti-mouse antibodies, rheumatoid factors, and complement [reviewed in Refs. (19, 20)]. Introduction of blocking agents (e.g., mouse serum or IgG) is often useful in eliminating this type of interference (21, 22). False-positive results for cTnI have been reported frequently, whereas the number of false-negative results reported is much lower. The false-positive results have been attributed to the presence of heterophilic antibodies (23), rheumatoid factor (24, 25), or an unknown macromolecular complex (26). Unrepeatable falsely high results have also been attributable to the presence of fibrin after incomplete separation of serum (27). They may also result from fluid therapy given to patients (28). One report of a false-negative cTnI result was attributed to circulating cTnI autoantibodies (29), and bilirubin and hemoglobin have been reported to cause negative interference in certain cTnI assays (30).

Remarkably little attention has been paid to reporting the analytical recovery of cTnI when evaluating new cTnI immunoassays, despite the well-recognized problems associated with these assays. We therefore studied the analytical recovery of tissue-derived cTnI (ternary troponin complex) in different sample matrices obtained from healthy individuals or patients with symptoms of chest pain, using antibodies that recognize different cTnI epitopes. We also measured the cTnI concentration in admission and monitoring samples from ACS patients with two investigative and two commercial cTnI assays. The results indicate the frequent occurrence of an as yet unidentified serum and plasma component that can severely interfere with the binding of antibodies, especially those that bind to epitopes of the stable mid-fragment of cTnI.

**Materials and Methods**

**REAGENTS**

Human cardiac troponin complex (native, tissue-derived cTnI-cTnT-TnC complex) and monoclonal antibodies (Mabs) specific for cTnI were obtained from HyTest Ltd. We used five Mabs: three with epitopes in the stable part of cTnI (Mab 1, epitope in the region of amino acid residues 35–55; Mab 2 and Mab 5, epitopes in the region of amino acid residues 80–95), one with an N-terminal epitope in the region of residues 20–35 (Mab 3), and one with a C-terminal epitope in the region of residues 185–200 (Mab 4). All five antibodies recognize both free cTnI and cTnI in complex with TnC or TnT or both. Calibrators were prepared by diluting the cardiac troponin complex in a buffer containing 75 g/L bovine serum albumin, 50 mmol/L Tris-HCl (pH 7.75), 15 mmol/L NaCl, and 0.5 g/L NaN₃. The cTnI concentration of the troponin complex stock reported by the manufacturer was used to assign the cTnI concentrations of the dilutions. The calibrators were stored at −20 °C until use, and a new set of calibrators was taken for every assay.

**SAMPLES**

We obtained 10 paired EDTA-plasma and serum samples from apparently healthy volunteers at the Department of Biotechnology, University of Turku. Additional serum or heparin-plasma samples from patients for whom a cTnI or cTnT measurement had been requested (total of 475 samples) were obtained from Turku University Central Hospital and from the Department of Clinical Chemistry, Helsinki University Central Hospital (referred to as cohort 1). We also obtained 575 serum samples from patients without cardiac-related symptoms from Turku University Central Hospital (referred to as cohort 2). All identification labels were removed at the hospitals to retain anonymity of the patients. Heparin-plasma samples from 16 chest pain patients were taken at admission and for monitoring up to 97 h at the Department of Internal Medicine, Oulu University Hospital. The corresponding serum samples were initially analyzed at the Laboratory of Oulu University Hospital by AxSYM troponin I (Abbott...
Laboratories) and ACS:180 cTnl (Bayer Diagnostics) assays (31). Informed consent was obtained from the volunteers and from the 16 chest pain patients. The procedures followed were in accordance with the Helsinki Declaration of 1975 as revised in 1996. All samples were stored at −20 or −70 °C for long-term storage.

LABELING OF ANTIBODIES WITH LANTHANEIDE CHELATE AND BIOTIN

The intrinsically fluorescent europium chelate used for labeling of the detection antibodies was kindly provided by Jaana Rosenberg (Department of Bio-Organic Chemistry, University of Turku, Turku, Finland). The chelate was a europium(III) chelate of 2,2',2''-[4-((4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]bis(methylene-nitrilo)tetrakis(acetic acid) (32). The antibodies were labeled overnight (16–20 h) at room temperature with a 100-fold molar excess of chelate in 50 mmol/L sodium carbonate buffer (pH 9.8). The labeled antibodies were separated from excess free label on a Superdex 200 HR 10/30 gel filtration column equilibrated and run with 50 mmol/L Tris-HCl (pH 7.75), 15 mmol/L NaCl, 0.5 g/L NaN3 at 25 mL/h, and 0.5-mL fractions were collected. The fractions containing labeled protein were pooled, the protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad), and the degree of labeling was determined with a europium calibration solution. Bovine serum albumin was added to a final concentration of 1 g/L, and the solution was filtered through a 0.22 μm pore size filter and stored at 4 °C. The labeling degrees of the antibodies were 6–12 Eu3+ chelates per molecule of IgG.

Biotin-isothiocyanate (a gift from J. Rosenberg) was used for the biotinylation of antibodies. The antibody was biotinylated with a 30-fold molar excess of biotin-isothiocyanate in 50 mmol/L sodium carbonate buffer (pH 9.8) at room temperature for 4 h. The biotinylated antibody was separated from free biotinylation reagent by passing the reaction mixture twice through disposable NAP-5 or NAP-10 columns (Amersham Biosciences AB) with 50 mmol/L Tris-HCl (pH 7.75), 15 mmol/L NaCl, 0.5 g/L NaN3 as eluent. Bovine serum albumin was added to a final concentration of 1 g/L, and the solution was filtered through a 0.22 μm pore size filter and stored at 4 °C.

IMMUNOASSAYS

To attach the capture antibody, we incubated 400 ng of biotinylated Mab 1 (in 25 μL of DELFIA® Assay Buffer; Perkin-Elmer Life Sciences, Wallac Oy) per well in streptavidin-coated microtiter wells (Innotrac Diagnostics Oy) for 1 h at 35 °C without shaking. Unbound capture antibody was removed by washing the wells, and 20 μL (100 ng) of Eu3+-labeled antibody (Mab 2, 3, 4, or 5) was added, followed by 20 μL of calibrator or sample. The wells were incubated for 1 h at 36 °C with slow shaking, washed, and dried for 5 min under a stream of hot dry air. The time-resolved europium fluorescence was measured directly from the dry surface in a Victor™ 1420 Multilabel Counter (Perkin-Elmer Life Sciences, Wallac Oy). The four-antibody assay using Mabs 1 and 3 as capture antibodies and Mabs 2 and 4 as detection antibodies (Mabs 1 + 3/Mabs 2 + 4) was performed in the same way except that 200 ng of each biotinylated capture antibody was used. The lower detection limits (2 SD of blank) were −0.03 μg/L for the Mab 1/Mab 2 assay and 0.02 μg/L for the Mabs 1 + 3/Mabs 2 + 4 assay.

ANALYTICAL RECOVERY

Unless otherwise stated, the analytical recovery of cTnl calibrator was measured after the addition of ternary troponin complex corresponding to 30 μg/L of cTnl (final concentration) to the samples. The signal from the sample containing added cTnl calibrator was compared with the signal of a buffer [75 g/L bovine serum albumin, 50 mmol/L Tris-HCl (pH 7.75), 15 mmol/L NaCl, and 0.5 g/L NaN3] to which calibrator had been added, and the recovery was calculated based on the assumption that the recovery in buffer was 100%. The recovery of endogenous cTnl was tested by the addition of a small volume of a patient sample with a high cTnl concentration to the samples. The cTnl concentration in the patient sample was measured with the Mab 1/Mab 2 assay against calibrators prepared from the ternary troponin complex stock solution.

STATISTICAL ANALYSIS

Statistical analysis was performed using Microcal™ Origin®, Ver. 6.0. Significant differences between means were calculated using the paired t-test, and significant differences from the mean for samples 3 and 10 were calculated using the one-population t-test.

Results

The analytical recoveries of cTnl calibrator, added as ternary troponin complex corresponding to final cTnl concentrations of 30 and 500 μg/L, and of cTnl from a serum sample with high cTnl concentration, added to a final concentration of 9 μg/L, in serum and EDTA plasma from 10 healthy volunteers were determined with the original Mab 1/Mab 2 assay. For 30 μg/L cTnl added to samples, the mean recoveries were 43.8% (range, 7.0–71.2%) in serum samples and 27.1% (range, 5.5–38.6%) in EDTA-plasma samples. For 500 μg/L cTnl added to samples, the mean recoveries were 81.3% (range, 66.1–88.8%) in serum and 74.0% (range, 61.6–80.9%) in EDTA-plasma samples. In samples with endogenous cTnl added to a final concentration of 9 μg/L, the mean recoveries were 48.1% (range, 22.4–66.2%) in serum and 38.5% (range, 23.6–51.0%) in EDTA plasma. The mean recovery was significantly different between serum and plasma regardless of the amount of added cTnl and its form (P ≤0.0062, paired t-test; Fig. 1). The box-and-whisker plot in Fig. 1 also illustrates that the recovery was significantly increased when 500 μg/L cTnl was added com-
The mean (SD) recovery was 84.1 (26.8)% with individual recoveries ranging from 1.0% to 166.8%; median recovery was 87.4%. An additional 575 serum samples from patients who had blood samples taken for noncardiac reasons (cohort 2) were analyzed both before and after the addition of cTnI to investigate whether any differences existed in the recovery distribution between the two patient categories. The mean (SD) recovery was 80.0 (24.6)%, with individual recoveries between 0.8% and 143.2% and a median recovery of 85.6%. Recovery rates <10% were observed in 3.2% of patients in cohort 1, compared with 3.5% in cohort 2. The portion of patients with recoveries <50% was 10.5% in cohort 1 and 10.3% in cohort 2.

On the basis of our observation that recovery improved when more cTnI was added, we examined the relationship between original cTnI concentrations and cTnI recovery. Fig. 2 shows the original cTnI concentrations in 475 samples from patients in cohort 1 plotted against the analytical recovery of cTnI. As expected from the first results, recoveries were lower in samples containing no or low concentrations of cTnI. Only cTnI concentrations ≤30 μg/L and recoveries <160% are shown to better visualize the recovery in samples with low cTnI concentrations.

Different antibodies (Mabs 2, 3, 4, and 5) were tested as detection antibodies with Mab 1 as capture antibody to determine whether the recovery of cTnI was dependent on which antibodies were used. cTnI was added to EDTA-plasma samples 8 (“normal” recovery) and 10 (low recovery) to final concentrations of 0.3, 3, and 30 μg/L. The recoveries were ~10% in sample 10 and 20–40% in sample 8 with both Mab 2 and Mab 5, which share the same epitope in the central part of cTnI. When we used Mab 3, which has an epitope in the N-terminal region of cTnI, as the detection antibody, recovery improved sig-

To further investigate the recovery of cTnI in a larger population, we assayed serum or heparin-plasma samples from 475 patients with chest pain (cohort 1, assigned to have a cTnI or cTnT test) before and after cTnI was added.

Fig. 1. Box-and-whisker plots showing cTnI recovery in different sample matrices with cTnI calibrator (ternary troponin complex) or endogenous cTnI.

Plot 1, serum with cTnI calibrator added to 30 μg/L. Plot 2, EDTA plasma with cTnI calibrator added to 30 μg/L. Plot 3, serum with cTnI calibrator added to 500 μg/L. Plot 4, EDTA plasma with cTnI calibrator added to 500 μg/L. Plot 5, serum with endogenous cTnI added to 9 μg/L. Plot 6, EDTA plasma with endogenous cTnI added to 9 μg/L. The boxes indicate the 25th–75th percentiles; the whiskers indicate the minimum and maximum recoveries; the horizontal line indicates the median; and the dashed boxes indicate the mean recovery. The arrows indicate P values for significant differences between means (paired t test).

Fig. 2. Original cTnI concentrations measured in 475 samples from patients in cohort 1 plotted against the analytical recovery of cTnI in the same sample with cTnI added to a final concentration of 30 μg/L.
significantly, and the difference in recovery between sample 8 and 10 was considerably decreased. Mab 4, with a C-terminal epitope, also gave improved recovery compared with Mabs 2 and 5, but with sample 10, the recovery still remained <30%. The recoveries obtained with the different antibodies are shown in Fig. 3.

The recovery results with different antibodies provided the basis for development of a new cTnI assay format with two capture Mabs and two detection Mabs. On the basis of the finding that recovery with Mab 3 as the capture and Mab 4 as the detection antibody was close to 100% even with the low-recovery sample (data not shown), we tested different combinations of Mabs 1, 2, 3, and 4. Because it is known that the N- and C-terminal parts of the cTnI molecule, to which Mabs 3 and 4 bind, may be rapidly degraded, we decided to retain Mabs 1 and 2 so that detection of cTnI would be possible in samples containing degraded cTnI. The four combinations tested were Mabs 1 + 3 (capture) with Mabs 2 + 4 (tracer), Mabs 1 + 4 (capture) with Mabs 2 + 3 (tracer), Mabs 2 + 3 (capture) with Mabs 1 + 4 (tracer), and Mabs 2 + 4 (capture) with Mabs 1 + 3 (tracer). The final combination was selected based on a compromise between high specific signals, background signals in samples from healthy individuals, and increased recovery of cTnI calibrator in samples showing low recovery with the Mab 1/Mab 2 combination. The final assay format with two capture and two tracer antibodies was Mabs 1 + 3/Mabs 2 + 4.

The cTnI results from 16 patients presenting with chest pain (admission sample plus 3–8 follow-up samples; total of 108 samples) with the Mab 1/Mab 2 and Mabs 1 + 3/

Mabs 2 + 4 assays were compared with each other and with the serum sample results from the AxSYM and ACS:180 cTnI assays. Two patients (patients 1 and 5) had clearly higher relative results with the Mabs 1 + 3/Mabs 2 + 4 assay than with any of the other assays, whereas the results from all assays were in good agreement for the other patients (Fig. 4). As can be seen in Fig. 4, the differences between the four assays were considerable because of differences in calibration and in antibody specificity. Our investigational Mab 1/Mab 2 and Mabs 1 + 3/Mabs 2 + 4 assays, which use the same calibrators, gave quite similar results, with slightly lower concentrations with the Mabs 1 + 3/Mabs 2 + 4 assay. The Mabs 1 + 3/Mabs 2 + 4 assay gave much lower results than the commercial assays, generally <10% of the concentration measured by AxSYM and ~20% of that measured by ACS:180. At the time the analyses were performed, the manufacturers’ recommended clinical MI cutoffs were 2.0 µg/L for AxSYM cTnI and 1.5 µg/L for ACS:180 cTnI. The clinical cutoffs for our investigational assays have not been finally established, but an estimation based on correlation with the two commercial assays would suggest a cutoff around 0.2 µg/L for both assays (e.g., AxSYM with a cutoff of 2.0 µg/L gives ~10-fold higher cTnI concentrations than our assays).

When the data were normalized using the above cutoff values for patients 1 and 5 (Fig. 5), it was evident that, especially for patient 1, the new Mabs 1 + 3/Mabs 2 + 4 assay would suggest a MI diagnosis earlier than any of the other assays. For patient 5, the Mabs 1 + 3/Mabs 2 + 4 assay also gave concentrations above the cutoff starting from the first sample. The difference between the assays was largest in the first samples with lower cTnI concentrations, and the relative differences decreased in samples obtained later in the monitoring of the patients. On the basis of the electrocardiographic results and measurements of cardiac markers (creatine kinase MB, myoglobin, and cTnI), patient 1 was diagnosed with unstable angina with ST-segment depression, and patient 5 was classified as having a ST-segment elevation infarction.

Discussion

This study is the first report of a frequently occurring serum and plasma component that seriously interferes with the determination of circulating cTnI. Although at this point we have not been able to identify this component, some essential characteristics have been clarified regarding its occurrence and interference with cTnI measurements. More importantly, based on these observations, an alternative way of selecting antibodies for cTnI assays can be recommended, whereby the inhibiting effect of the interfering factor can be avoided, or at least alleviated. This finding could be of central importance for the development of new cTnI assays with improved and standardized performance.

![Fig. 3. Analytical recovery of cTnI calibrator with use of different tracer antibodies together with Mab 1 as capture antibody.](image-url)
Our recovery tests with different antibodies indicate that the antibodies against mid-fragment epitopes are most affected by the interfering factor, especially Mabs 2 and 5 with epitopes around amino acids 80–95. Mab 1, which is a mid-fragment antibody with an epitope around amino acids 35–55, is clearly less affected, but it was difficult to fully estimate the influence of the interfering factor on individual antibodies because the tests were always performed with a combination of two antibodies. The N- and C-terminal epitopes were affected to a much smaller extent, and the N-terminal epitope in particular seemed almost unaffected. Our results indicate that cTnI assays that use antibodies against epitopes situated in the
central, stable part of cTnI give falsely low cTnI concentrations in samples containing the interfering factor, and because the recommendation to use such antibodies is generally accepted, it is likely that most commercially available cTnI assays will be affected by this unknown component.

According to Collinson et al. (33), the epitopes of the antibodies used in the ACS:180 assay are in the region of amino acids 30–40 and 70–88, whereas the epitopes for the antibodies in the AxSYM assay comprise amino acids 20–39 and 87–91. From the cTnI results for patients 1 and 5, both assays appear to be seriously affected by the interfering component. This is in good agreement with our observation that epitopes around amino acids 80–95 are most severely affected by the interfering factor. The number of antibodies tested in this study was small; only five antibodies against four separate epitopes were tested. Further tests with more antibodies against additional epitopes should be performed to get a more complete picture of the parts of cTnI that are blocked by the interfering factor. Such studies are currently underway in our laboratory.

The recoveries in a large number of samples from patients assigned to have a cTnI or cTnT test and from patients not presenting with cardiac symptoms showed that the interfering factor is a frequently occurring phenomenon. Intriguingly, it seemed to be present to some extent in most people, with recoveries =10% for \(-3.5\%\) of the tested samples. Because the effect is more pronounced as the added amount of cTnI is decreased, it is conceivable that the consequence for detecting small increases early after onset of chest pain is even more severe. Further investigations with more well-defined patient groups with full clinical documentation are necessary to verify the occurrence of this interfering component in healthy individuals and in patients with various cardiac complications.

We suggest that the mechanism of action of the interfering factor is via blocking of certain mid-fragment epitopes on cTnI, presumably by noncovalent binding to the central part of cTnI. The effect was somewhat more prominent with tissue-derived ternary troponin complex (calibration material) than with endogenous cTnI forms and more evident in EDTA plasma than in serum. From our recovery experiments we know that the interfering factor binds very rapidly to cTnI, causing low recoveries even if cTnI is added immediately before the assay, indicating that it has a high affinity for cTnI. The interfering factor appears to be present in small and limited amounts because the addition of cTnI to samples significantly improved recoveries, apparently by saturating the interfering factor. We also observed lower recoveries in samples containing no or low original concentrations of cTnI compared with samples with higher cTnI concentrations (Fig. 2). Because cTnI interacts with many proteins of the thin filament, such as cTnT, TnC, and actin (34), it is conceivable that the interfering component could belong to the thin-filament proteins. The region of cTnI comprising amino acid residues 33–80 binds to TnC, and this region also contains sites of interaction with cTnT (34). The epitope of Mab 1 is within this region (residues 35–55), and the epitopes of Mabs 2 and 5 (residues 80–95) are close to this region. The cTnI inhibitory peptide (residues 129–150) has been shown to interact with both TnC and actin (34), but the fact that the ternary complex was used in the recovery studies makes it unlikely that the interfering factor would be any of the other troponin subunits. When we tested the addition of potential interfering components to samples, neither actin nor TnC (slow-twitch skeletal muscle/cardiac isoform) caused any decrease in cTnI recovery (data not shown). There are two isoforms of TnC, one isoform is characteristic for fast-twitch skeletal muscle fibers and the other for slow-twitch skeletal muscle fibers and heart (35,36). The possibility that free cTnI could bind to residual free TnC in serum has been considered previously (6). Our results from addition of the slow-twitch/cardiac TnC isoform and the high homology reported between the fast- and slow-twitch isoforms (37) does not support the presumption that TnC would cause the interference. Furthermore, we have also tested the recovery of cTnI in three samples taken after surgery from patients with bone fractures. The amount of TnC in the samples was not known, but all samples had highly increased myoglobin concentrations, indicating substantial skeletal muscle damage. The recoveries were normal in all three samples (data not shown), which additionally reduces the probability of TnC (and perhaps of many other thin-filament proteins) being the interfering component. Recently, interactions have been reported between skeletal TnI and the receptor for human basic fibroblast growth factor (38) and between cTnI and polycystin-2 (39), which shows that cTnI also interacts with proteins other than those of the thin filament.

The lower recoveries in EDTA plasma than in serum might be a consequence of changes in the three-dimensional structure of the troponin complex when calcium ions are bound to EDTA. Partial unfolding of the cTnI-TnC complex in the absence of calcium would thus facilitate blockage of some cTnI epitopes by the interfering factor. In the presence of calcium, the cTnI-TnC complex will stay more tightly together, reducing the interaction of the interfering factor with cTnI. In addition, we recently found that the Mab 1/Mab 2 immunoassay recognizes the cTnI-TnC complex somewhat better than free cTnI, which would explain why the recovery generally was lower in EDTA plasma.

Böhner et al. (29) previously reported false-negative immunoassay results for cTnI in one patient who had undergone elective coronary artery bypass graft and who did not have perioperative myocardial infarction. When increasing amounts of cTnI up to 38.5 µg/L were added to perioperative serum samples from this patient, no added cTnI was detected. After treatment of the patient’s serum with protein A or specific anti-human IgG antisera,
97% of the added cTnI was recovered. The authors conclude that the interfering IgG worked as an analyte-binding antibody and prevented the recognition of cTnI by the two-site immunoassay used because it did not interfere with creatine kinase isoenzyme MB or cTnT results, which were both positive (29). We also tried to detect human antibodies toward cTnI in low-recovery samples with use of labeled anti-human IgG as a detection antibody with the cTnI in the sample attached to the solid phase by an antibody that is not affected by the interfering factor. No specific signal was obtained from either low- or normal-recovery samples (data not shown). It also seems quite unlikely that such a large proportion of the population would have anti-cTnI antibodies present in their circulation. We therefore conclude that the interfering factor described here is not human IgG autoantibodies. The interference also does not seem to be related to the IgG subclasses of the tested antibodies because of three antibodies in the same subclass, two showed considerable interference and the third did not.

In two recent reports, cTnI was detected by Western Blot analysis, whereas the commercial immunoassay used did not detect any cTnI or detected much lower concentrations (14, 40). The authors of the first publication hypothesized that some unidentified modification (other than degradation) was responsible for altering the immunogenicity of cTnI in the serum (14). This report also presented Western blots with eight degradation products and three products of higher molecular weight. The second publication reported that a Western blot method could detect cTnI in samples that were negative by the Technicon Immuno1 and that in most cases cTnI could be detected even in the admission sample (40). On the basis of our results, the question arises whether the detection of cTnI may not have been so much dependent on the better sensitivity of the Western blot (as proposed by the authors), but rather was dependent on differences in the antibodies that were used in the two methods or, alternatively, on the fact that an interfering factor-cTnI complex was denatured, releasing cTnI, which was then detectable in the Western blot method.

Our results indicate that the consequences of the interfering factor on cTnI measurements should be taken into careful consideration. This factor can cause variable inhibition of cTnI immunoreactivity, from mild to very severe, depending on the amount of interfering factor in the sample. The preliminary results obtained with patients 1 and 5 showed that the interfering factor had the greatest effect on cTnI measurements when there were only small amounts of cTnI present in the sample, such as in the early hours of a MI or unstable angina event. Samples taken early after a cardiac event might give a negative result because all of the released cTnI will be masked by the interfering factor. Later in the progression, when more cTnI is released, more cTnI will be measured because the interfering factor is present in only a limited amount and will be saturated by the released cTnI. This means that in patients with high concentrations of the interfering factor, the MI or unstable angina event might be totally overlooked, especially if a patient is discharged after the first negative cTnI measurements.

Our present, and perhaps temporary, solution to the described interference problem is a multiantibody assay approach to ensure detection of cTnI in the presence of the interfering factor. Because the most “interference-free” antibodies are against epitopes in the terminal parts of the molecule, which are not present in fragmented cTnI molecules, combinations with mid-fragment antibodies provide a viable option, which allows altogether four possibilities for sandwich formation. Although the Mabs 1 + 3/Mabs 2 + 4 assay gave better recoveries in samples containing the interfering factor, there are still some factors that can distort the true cTnI concentration. The first is the remaining disturbance from the interfering factor. If the interfering factor is bound to cTnI in the sample, little or no signal will be obtained from the antibody against the epitope in the central region (Mab 2). On the other hand, the reduced concentration is not so critical because it is still better to get half of the “correct” signal than no signal at all, which could be the case with an assay design similar to that of the Mab 1/Mab 2 assay.

The second factor is that problems with quantification also arise when cTnI is degraded because the second tracer antibody has its epitope in the C-terminal region, which may be lost on degradation, and thus no signal will be obtained from this antibody. This could be one reason for the somewhat lower results obtained with the Mabs 1 + 3/Mabs 2 + 4 assay compared with the Mab 1/Mab 2 assay. If a sample contains both large amounts of the interfering factor and cTnI that is extensively degraded, the cTnI concentration would apparently be grossly underestimated. Our impression is that the interfering factor to some extent protects cTnI from extensive degradation, but this hypothesis needs more investigation. For an ideal cTnI assay, the preferred solution would be to find antibodies that bind to regions of the cTnI molecule that are not affected by the interfering factor but still have epitopes in the stable part of the molecule. None of the antibodies used in this work fulfills both of these criteria.

Another approach to lessen the consequences of the interfering factor is the development of analytically more sensitive cTnI assays, a request that has been frequently presented in recent years (41–43). A more sensitive assay would be able to better detect very low cTnI concentrations despite interference. This could avoid misclassifications, such as false negatives, but it would still be incapable of avoiding the serious underestimations caused by the interfering factor. Our study helps to explain some of the discrepancies observed between assays and also gives some guidelines concerning the development of new generations of cTnI assays with improved performance, especially in the early detection of cTnI in ACS.
This study was supported financially by the National Technology Agency in Finland (TEKES) as part of the Diagnostics 2000 Technology Program. We gratefully acknowledge Drs. Alexei Katrukha and Maria Severina for helpful discussions and HyTest Ltd. for supplying reagents.

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