Clinical Chemistry 51:5
839–847 (2005)

Proteomics and
Protein Markers

Negative Interference in Cardiac Troponin I Immunoassays by Circulating Troponin Autoantibodies

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Background: **There are numerous potential sources of interference in immunoassays. Our aim was to identify the blood component that causes negative interference in cardiac troponin I (cTnI) immunoassays based on antibodies against the central part of cTnI.**

Methods: **We isolated an interfering factor (IF) from a sample with low recovery of added cTnI, using several consecutive purification steps: caprylic acid precipitation, ammonium sulfate precipitation, and purification on Cibacron Blue gel and protein G columns. Purified IF was identified by gel electrophoresis and mass spectrometric analysis of protein bands. For the direct detection of human antibodies to cardiac troponin in serum samples, we developed immunoassays using three different anti-human immunoglobulin antibodies and measured troponin antibodies in samples with low and normal cTnI recovery.**

Results: **Treatment with caprylic acid did not precipitate IF, but IF precipitated at 40% ammonium sulfate saturation. IF bound to a Cibacron Blue gel column, from which it was eluted with a linear salt gradient; it also bound to protein G. Gel electrophoresis of purified IF showed two major bands with molecular masses corresponding to the heavy (~50 kDa) and light chains (~25 kDa) of immunoglobulin, and their identities were confirmed by mass spectrometry. The presence of troponin-specific autoantibodies was confirmed in samples with low recoveries of cTnI by three different immunoassays. The median signals were significantly higher in 10 samples with low recovery than in 10 samples with normal recovery of cTnI ($P \leq 0.007$).**

Conclusions: **Circulating autoantibodies to cTnI or other proteins of the troponin complex can be a source of negative interference in cTnI immunoassays.**

During the four decades that have passed since immunoassays were first introduced, numerous improvements in assay design, detection systems, and instrumentation have emerged. State-of-the-art immunoassays can quantify very small amounts of analytes in complex biological fluids such as serum and whole blood. However, immunoassays are still not faultless, because several interfering substances may compromise their accuracy *(1, 2)*. The interference can be either positive or negative, thus causing either falsely high or low test results. Interference caused by antibodies, such as heterophilic antibodies *(3)* and human anti-species antibodies *(4)*, is a well-recognized phenomenon, and this interference can usually be eliminated by introduction of blocking agents *(5, 6)*. Because the true concentration of the analyte in a sample is rarely known, detection of interference can be extremely difficult. During evaluation of an immunoassay, interference can be detected by comparing different assays, testing for linearity, recovery, or commonly known interferents, e.g., lipemia, icterus, hemolysis, and drugs *(7)*. For immunoassays in routine use, there is usually no indication of interference other than that the result does not fit the clinical picture. When discrepancies between clinical and biochemical data occur, clinicians should be aware of the possibility of interference in immunoassays.

Cardiac troponin I and T (cTnI and cTnT)[3] are released

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Received July 13, 2004; accepted February 1, 2005.
Previously published online at DOI: 10.1373/clinchem.2004.040063

[3] Nonstandard abbreviations: cTnI and cTnT, cardiac troponin I and T, respectively; IF, interfering factor; LR, low recovery; NR, normal recovery; TnC, troponin C; Mab, monoclonal antibody; TSA, Tris-saline-azide; and SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

to the bloodstream after myocardial necrosis, and they are the preferred biochemical markers for detection of myocardial damage *(8)*. Many analytical issues are associated with cTnI immunoassays in particular *(9–11)*, partly as a result of the heterogeneous molecular nature of cTnI *(12–14)*. In view of the major role that troponin measurements play in diagnosing myocardial infarction *(8, 15)* and their increasing use for treatment guidance *(16)*, it is of utmost importance to minimize any interference in these assays. Because cardiac troponins are not detectable or are found at very low concentrations in healthy individuals, negative or positive interferences can easily lead to missed or incorrect diagnoses. Numerous assay-dependent false-positive results for troponin assays have been reported, the majority of which were caused by heterophilic antibodies *(17)* or rheumatoid factor *(18–20)*. False-negative results have been attributed to bilirubin, hemoglobin *(21)*, and in one case, to circulating cTnI autoantibodies *(22)*. When performing recovery studies with a cTnI immunoassay, we previously observed frequent negative interference from a serum- and plasma-derived component found in both healthy individuals and patients with acute coronary syndromes *(23)*. Further investigation showed that this interfering factor (IF), with an apparent molecular mass of 50–200 kDa, blocks the binding of antibodies to the central part of cTnI *(24)*. Consequently, there is a risk of obtaining false-negative results if the immunoassay uses antibodies against this region of cTnI. Many commercial cTnI immunoassays may be prone to this type of interference because use of antibodies directed against the most stable region of cTnI (amino acids 30–110) is recommended *(25)*. In the present study, we describe the isolation and identification of IF and describe immunoassays for its direct detection.

Materials and Methods

SAMPLES

Serum samples from one volunteer with constantly low recovery (LR) of added cTnI (<10%) and serum samples for which cTnI concentrations were correctly measured [normal recovery (NR); mean (SD) recovery, 96 (3.1)%] were obtained from apparently healthy volunteers and from patients without cardiac-related symptoms from Turku University Central Hospital *(23)*. Ten selected samples with apparently high amounts of IF on the basis of divergent results with differently configured cTnI immunoassays and a low recovery of cTnI were from a sample panel obtained from 541 consecutive chest pain patients who presented to Turku University Central Hospital *(26)*.

REAGENTS

Human cardiac troponin complex [native, tissue-derived cTnI-cTnT-troponin C (TnC) complex] and four monoclonal antibodies (Mabs) specific for cTnI with epitopes at amino acid residues 41–49 (Mab 1), 87–91 (Mab 2), 23–29 (Mab 3), and 190–196 (Mab 4) were obtained from HyTest Ltd. Two monoclonal anti-human IgG antibodies, clones 3D3 and 5A9, which recognize the Fc part of the immunoglobulin γ-chain, and one monoclonal anti-human IgA, IgG, and IgM antibody, clone 7A9, which recognizes light chains of immunoglobulins, were also kindly provided by HyTest Ltd. All reagents used were of analytical grade.

LABELING OF ANTIBODIES AND STREPTAVIDIN

Antibodies were labeled with biotin and europium as described previously *(23)*. Streptavidin (BioSpa, Società Prodotti Antibiotici) was labeled with europium in 50 mmol/L sodium carbonate buffer (pH 8.4) containing 9 g/L NaCl, with a 75-fold excess of {2,2′,2″,2‴-{[2-(4-isothiocyanatophenyl)ethylimino]bis(methylene)bis{4-{[4-(α-galactopyranoxy)phenyl]ethynyl}pyridine-6,2-diyl}bis(methylenenitrilo)} tetrakis(acetato)} europium(III) chelate *(27)*. The concentration of streptavidin was 2.8 g/L in the reaction, which was carried out overnight at room temperature. Purification was performed as described previously *(23)*, except that 144.5 g/L glycerol was added to the buffer [Tris-saline-azide (TSA); 50 mmol/L Tris-HCl (pH 7.75), 15 mmol/L NaCl, 0.5 g/L NaN_3] used for purification by gel filtration and an additional purification step was performed with a PD-10 column (Amersham Biosciences). The labeling degree was 8 Eu^{3+} per streptavidin molecule.

RECOVERY OF cTnI

The troponin complex was serially diluted in TSA buffer containing 75 g/L bovine serum albumin. The cTnI concentration in the troponin complex stock reported by the manufacturer was used to assign the cTnI concentrations of the dilutions. Aliquots of the dilutions were stable for more than 1 year when stored at −70 °C. Fresh aliquots were taken for every assay. We determined the recovery of cTnI by adding ternary troponin complex corresponding to a final cTnI concentration of 3 μg/L to samples or fractions. To estimate the amount of IF present in a sample, we added troponin complex corresponding to final cTnI concentrations of 0.3–500 μg/L. We measured cTnI recovery with the Mab 1/Mab 2 assay, which is strongly affected by the presence of IF, as described previously *(23)*. All samples were analyzed in duplicate or triplicate. The imprecision (CV) of the Mab1/Mab 2 assay was <10%. To avoid any possible matrix effects attributable to differences in buffer composition, the signals from fractions obtained during purification of the LR sample were compared with the corresponding fractions from the NR sample.

ISOLATION OF IF

Precipitation with caprylic acid. Caprylic acid (octanoic acid) was used to precipitate albumin and other proteins according to a modified protocol of McKinney and Parkinson *(28)*. Briefly, 4 mL of serum was diluted with 4 volumes of 60 mmol/L sodium acetate buffer (pH 4.0), and the pH was adjusted to 4.5 with 0.1 mmol/L NaOH if necessary. Caprylic acid (25 μL/mL of diluted sample)

was slowly added dropwise with continuous mixing. The solution was then stirred for 40 min. Insoluble material was removed by centrifugation (4000*g* for 30 min at room temperature). The supernatant was filtered through a 0.8 μm MilliFil filter (Millipore), and 10 parts of supernatant were mixed with 1 part of 10× concentrated phosphate-buffered saline [80 g/L NaCl, 2 g/L KCl, 11.5 g/L Na_2HPO_4, 2 g/L KH_2PO_4, and 2 mmol/L EDTA, (pH 7.4)].

Precipitation with ammonium sulfate. Approximately 20 mL of the supernatant from the caprylic acid precipitation was fractionated at 4 °C with stepwise addition of 100% saturated ammonium sulfate to give 20%, 30%, 40%, 50%, or 60% saturation. The sample was stirred for 30 min and then centrifuged (4000*g* for 15 min at 4 °C). Additional ammonium sulfate was then added to the supernatant for precipitation at the next concentration. The pellets were resuspended in a small volume of 1× phosphate-buffered saline, placed inside a bag made from Dialysis Spectra/Por 1 dialysis membrane (molecular weight cutoff, 6000–8000; Spectrum Laboratories Inc.), and dialyzed overnight against 1× phosphate-buffered saline. The recovery of cTnI was measured in each dialyzed pellet to determine at which ammonium sulfate concentration IF precipitated.

Purification on Blue gel column. Affi-Gel Blue gel (Bio-Rad), a beaded, cross-linked agarose gel with covalently attached Cibacron Blue F3GA dye, was packed into columns (5 mL of gel). Each column was equilibrated with 20 mmol/L sodium phosphate buffer (pH 7.1) at 4 °C. The samples obtained by ammonium sulfate precipitation were dialyzed against the same buffer to remove NaCl. After application of sample (~500 μL), the column was washed with 10 mL of equilibration buffer at 0.2 mL/min, after which a linear 60-mL NaCl gradient (equilibration buffer containing 0–1.5 mol/L NaCl) was applied. Finally, the column was washed with 6 mL of equilibration buffer containing 1.5 mol/L NaCl. The recovery of cTnI was measured in fractions (1 mL) collected during this process to determine at which NaCl concentration the IF eluted.

Purification on protein G column. We used a 1-mL HiTrap Protein G HP column (Amersham Biosciences) for affinity purification of immunoglobulins. The fractions containing IF from chromatography on Blue gel were pooled and dialyzed overnight at 4 °C against 20 mmol/L sodium phosphate buffer (pH 7.1) to remove the NaCl. The column was equilibrated with 20 mmol/L sodium phosphate buffer (pH 7.1), and the dialyzed sample (5.5 mL) was applied at 0.4 mL/min at 4 °C. The column was washed with 6 mL of buffer before the immunoglobulins were eluted with 0.1 mol/L glycine-HCl buffer (pH 2.7). Fractions of 0.6 mL were collected, and acidic fractions were immediately neutralized with 1 mol/L Tris-HCl buffer, pH 9.0.

Gel electrophoresis and in-gel digestion of proteins. Fractions from different purification steps were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to standard procedures *(29)*. A set of low-molecular-weight calibrators for SDS electrophoresis (Amersham Biosciences) was used for molecular weight estimation. The samples were mixed with SDS-PAGE sample buffer (100 mL/L glycerol, 2 g/L sodium dodecyl sulfate, 50 mL/L β-mercaptoethanol, 0.04 g/L bromphenol blue, 62.5 mmol/L Tris-HCl, pH 6.8) and boiled for 5 min. The samples were then subjected to SDS-PAGE on a 4% stacking gel and 13% resolving gel. Protein bands were visualized either by silver staining according to a protocol developed by O'Connell and Stults *(30)* or by Coomassie staining according to the procedure of Scheler et al. *(31)*. Protein bands were excised from the Coomassie-stained gel, treated, and in-gel digested with trypsin (sequencing grade modified trypsin from Promega) according to a protocol by Shevchenko et al. *(32)* as modified by O'Connell and Stults *(30)*.

Mass spectrometry. After trypsin digestion, the samples were desalted with C_{18} ZipTips (Millipore) and directly eluted to the sample plate with 600 mL/L acetonitrile, 1 mL/L trifluoroacetic acid containing a saturating amount of α-cyano-4-hydroxycinnamic acid. Digested protein samples were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry on a Voyager-DE Pro instrument (Applied Biosystems) according to the manufacturer's instructions. The obtained spectra were calibrated internally by use of the trypsin autolysis products, *m/z* 842.50 and *m/z* 2211.10, or by use of close external calibration. Proteins were identified by the Mascot mass fingerprinting program (http://www.matrixscience.com).

IMMUNOASSAYS FOR DETECTION OF HUMAN ANTI-TROPONIN ANTIBODIES

Maxisorp microtitration plates (Nunc A/S) were coated with 500 ng each of Mab 3 and Mab 4, which are capable of binding cTnI in the presence of IF *(24)*, in 50 μL/well of 0.2 mol/L sodium phosphate buffer (pH 7.8) for 4 h at 35 °C. After the wells were washed twice, 100 μL of a saturation solution containing 50 mmol/L Tris-HCl (pH 7.2), 1 g/L bovine serum albumin, 1 g/L Germall II, and 30 g/L D-sorbitol was added, and the plates were incubated for 1 h with slow shaking at room temperature. The solution was aspirated, and the plates were either used immediately or dried at room temperature for 1 h in a fume hood and stored with desiccant at 4 °C until use. Serum samples were fivefold diluted with TSA buffer, unless otherwise stated. Troponin complex corresponding to 30 μg/L cTnI (15 μL of 600 μg/L stock) was added to diluted serum samples (285 μL) to allow binding of IF to cTnI, and the samples were incubated for 1 h at 4 °C. For background controls, the same volume of TSA buffer

containing 75 g/L bovine serum albumin was added to an aliquot of the diluted sample. Diluted samples (30 μL) with added troponin complex or buffer were then added to plates coated with Mab 3 and Mab 4 and incubated for 1 h at 36 °C. The plate was washed twice; 10 ng of biotinylated 3D3, 5A9, or 7A9 in 30 μL of buffer was added; and the plate was incubated for 1 h at 36 °C. After two washes, 10 ng of Eu^{3+}-labeled streptavidin in 30 μL of buffer was added, and the plate was incubated for 1 h at 36 °C. After six washes, the plate was dried for 5 min under a stream of hot dry air. After cooling, the time-resolved fluorescence was measured directly from the dry surface in a Victor 1420 Multilabel Counter (PerkinElmer Life and Analytical Sciences, Wallac Oy). All samples were measured in triplicate, and the mean signal was calculated. The specific signal was calculated by subtracting the fluorescence signal obtained from the background control samples without added troponin complex from the signals for samples to which troponin complex was added. Samples with specific signals <0 were reported as 0. The assay principle is shown in Fig. 1 of the Data Supplement that appears with the online version of this article at http://www.clinchem.org/content/vol51/issue5/.

IMMUNOASSAYS FOR cTnI

Samples from 541 chest pain patients were measured with a new investigational cTnI assay and a first-generation cTnI assay *(26)*. The new cTnI assay is an immunofluorometric sandwich assay that uses two cTnI monoclonal antibodies for capture and one cTnI monoclonal antibody for detection. One capture antibody detects an epitope at amino acid residues 41–49, whereas one capture antibody and the detection antibody detect separate epitopes after amino acid residue 110 in the C-terminal region of cTnI. The first-generation Innotrac Aio cTnI assay (Innotrac Diagnostics Oy) is an immunofluorometric sandwich assay *(33)* that uses Mabs against amino acid residues 41–49 (Mab 1) and 87–91 (Mab 2). This assay is no longer commercially available and has been replaced by a more sensitive second-generation Aio cTnI assay *(34)*, which in essence is identical to the investigational cTnI assay *(26)*.

Results

ESTIMATION OF RELATIVE cTnI RECOVERY

We estimated the relative cTnI recovery in one LR sample (the same as used for isolation of IF) by comparing the signals obtained for samples containing increasing amounts of added troponin complex with the signals in one NR sample. As shown in Table 1, when ≤30 μg/L cTnI was added, only ≤2.1% was detected in the LR sample by the Mab 1/Mab 2 assay compared with the NR sample. When 100–500 μg/L cTnI was added, the recovery gradually increased from 13.6% to 75.5%, but still remained clearly below full recovery. When 300 and 500 μg/L cTnI were added, ~120 μg/L of cTnI remained undetected, suggesting that this was the amount of cTnI that the IF was able to block.

Table 1. Relative cTnI recovery in the studied LR serum sample with different concentrations of troponin complex added.[a]

Added troponin complex as cTnI, μg/L	Mean (SD) recovery, %	Mean (SD) undetected[b] cTnI, μg/L
0.3	0.0 (1.7)	0.30 (0.005)
3	1.5 (0.1)	2.95 (0.003)
30	2.1 (0.1)	29.4 (0.03)
100	13.6 (0.9)	86.4 (0.9)
300	60.7 (1.3)	117.9 (3.9)
500	75.5 (3.4)	122.3 (17.0)

[a] The signal from the LR sample was compared with the signal from a NR sample to which the same amount of troponin complex was added (n = 3).
[b] Refers to the theoretical amount of cTnI that is not detected by the Mab 1/Mab 2 assay.

ISOLATION OF IF

The same LR serum sample was used for isolation of IF, and a NR serum sample was used as a control. As a first purification step, we selected precipitation with caprylic acid for separation of the two major protein components of serum, albumin and immunoglobulin. After neutralization of the supernatant, the mean (SD) signal from added cTnI (3 μg/L) in the LR sample was 46.7 (5.5)% of the NR sample (n = 3), which indicated that the IF had not precipitated, although the recovery had increased compared with the starting material as a result of dilution. We then attempted precipitation with increasing concentrations of ammonium sulfate. Stepwise addition of ammonium sulfate revealed that IF precipitated at 40% saturation. The mean (SD) signal (n = 2) from added cTnI (3 μg/L) in the resuspended and dialyzed LR pellet obtained with 40% ammonium sulfate was 10.5 (0.0)% of the signal in the corresponding NR pellet. The dialyzed and resuspended pellet from the 40% ammonium sulfate precipitation was applied to the Blue gel column, and a salt gradient was used for elution of IF. On the basis of the recovery of cTnI added to fractions obtained during the purification of LR and NR samples, the IF eluted mainly in fractions 25–27 (Fig. 1), in which the NaCl concentration was ~0.4 mol/L.

The previous purification steps had primarily favored the enrichment of immunoglobulins, as can be seen from SDS-PAGE (Fig. 2, lane 1). Therefore, for the next fractionation step, we used protein G chromatography, which is commonly used for purification of immunoglobulins, to investigate whether IF would also bind to protein G. We observed no significant decrease in the recovery of cTnI in fractions containing unbound material, meaning that the IF was bound to protein G. In the first acidic fraction of the LR sample, the recovery was decreased, as shown in Fig. 3. In fractions containing IF, the bands corresponding to the light and heavy chains of immunoglobulin were still present (Fig. 2, lane 2).

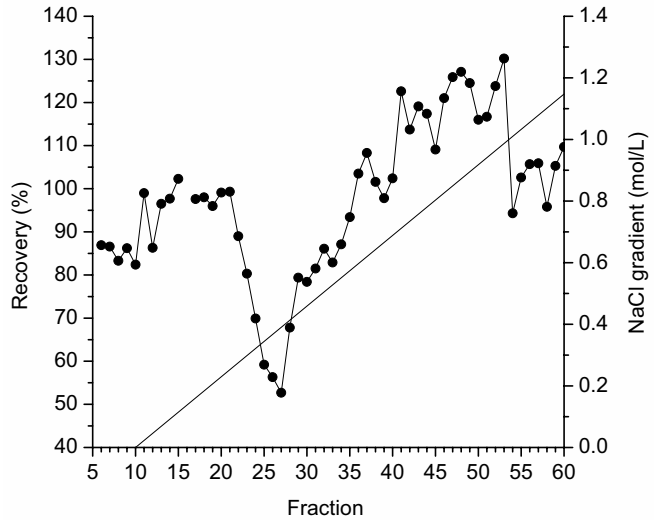

Fig. 1. Relative cTnI recovery in fractions collected after purification on a Blue gel column.

The signal from troponin complex (3 μg/L cTnI) added to fractions obtained during purification of a LR sample was compared with the signal from corresponding NR sample fractions. The *line* indicates the NaCl concentration in the fractions (units on the *right-hand y axis*).

A sample from the fractions containing IF obtained after purification on protein G was subjected to SDS-PAGE, and the protein bands were visualized by Coomassie staining. Proteins bands corresponding to the bands seen in lane 2 of Fig. 2 were excised from the gel and analyzed. After in-gel digestion and mass spectrometric analysis, the obtained tryptic peptide mass values were used for searching against protein databases with the Mascot program. The larger band obtained by SDS-PAGE yielded several significant matches to human immunoglobulin heavy chains, of which gi 2765421 ranked highest (score, 119; sequence coverage, 32%). For the lower molecular weight band, we obtained several significant matches to human immunoglobulin light chains, of which gi 11275326 ranked highest (score, 88; sequence coverage, 31%). Consistently, the matching peptides were situated in the constant regions of the heavy and light chains, respectively.

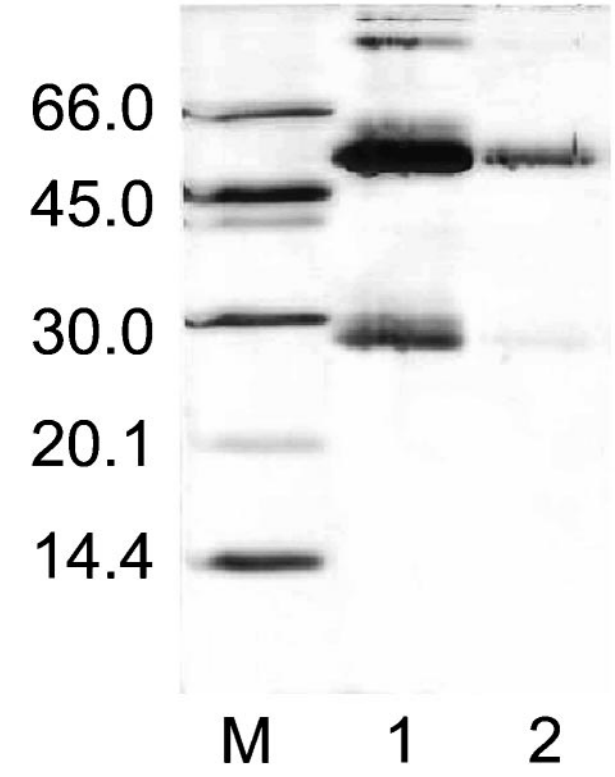

Fig. 2. SDS-PAGE analysis of fractions containing IF after purification on Blue gel (*lane 1*) and protein G (*lane 2*), visualized by silver staining. *Lane M*, molecular weight markers.

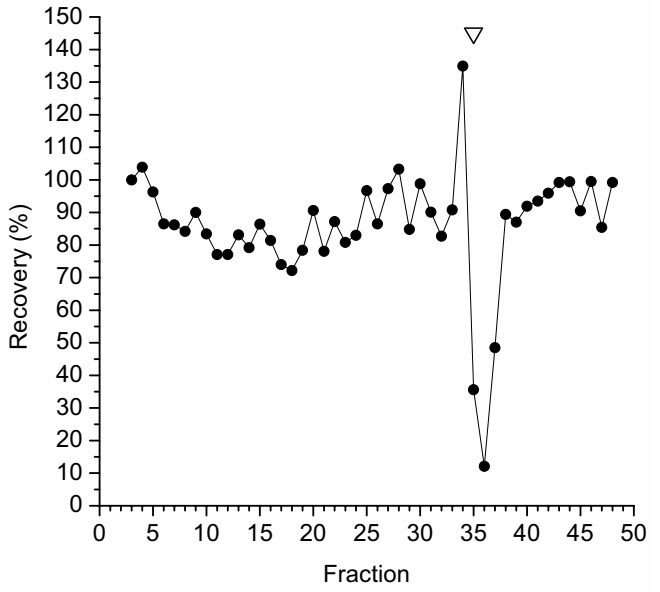

Fig. 3. Relative cTnI recovery in fractions collected after chromatography on protein G column.

The signal from troponin complex (3 μg/L cTnI) added to fractions obtained during purification of a LR sample was compared with the signals from corresponding NR sample fractions. The first acidic fraction is indicated by ▽.

DIRECT DETECTION OF TROPONIN AUTOANTIBODIES

To better demonstrate troponin-specific autoantibodies in a larger selection of samples, we developed immunoassays using three different monoclonal anti-human immunoglobulin antibodies. One antibody, 7A9, is specific for the light chains of IgA, IgG, and IgM, whereas the two other, 3D3 and 5A9, are specific for the heavy chain of IgG. Because of quite high and variable background in individual serum samples, we prepared background control samples in which no troponin complex was added, but to which the same volume of bovine serum albumin in TSA buffer was added. After addition of the diluted samples to the wells, immobilized immunoglobulins were detected by biotinylated anti-human immunoglobulin antibodies and Eu^{3+}-labeled streptavidin (Fig. 1 in the online Data Supplement).

Because no controls or calibrators were available for the immunoassays detecting troponin autoantibodies, we performed initial validation tests with different dilutions of LR and NR samples and various amounts of added troponin complex. When troponin complex corresponding to 30 μg/L cTnI was added to 5- to 80-fold dilutions of the LR sample used for isolation of IF, the specific signal decreased. When we used Mab 3D3 for detection, the mean (SD) specific signal was 128 790 (3692) counts for the 5-fold dilution of the LR sample (n = 3) and decreased to 36.9 (3.8)%, 16.6 (1.3)%, 5.3 (1.1)%, and 3.8 (1.2)% of the counts for the 5-fold dilution in the 10-, 20-, 40-, and 80-fold dilutions, respectively (n = 3). We obtained similar results with the two other Mabs. The NR sample gave no specific signal at any dilution. When increasing amounts of troponin complex were added (0.3–30 μg/L cTnI) to the fivefold-diluted LR sample, the specific signal increased when any of the anti-immunoglobulin Mabs was used (results obtained with 3D3 are shown in Fig. 4). Again, the NR sample gave no specific signal at any cTnI concentration.

We measured troponin autoantibody titers in 10 admission samples selected from the sample panel obtained from 541 consecutive chest pain patients. Samples were selected on the basis of their low recovery of cTnI (range; 0.0–27.2%) and divergent results between the Innotrac Aio first-generation cTnI assay (antibody pair, Mab 1/Mab 2) and a new cTnI assay that was designed to be minimally affected by IF *(26)*. Ten samples with 91–101% recovery of cTnI were used as controls. As shown in Fig. 5, the median specific signal from the LR samples was significantly higher than that from the NR samples for all three detection antibodies ($P \leq 0.007$, Mann–Whitney *U*-test). Within the LR group, there was also a correlation between specific counts in the autoantibody assay and the percentage of inhibition of recovery (calculated as 100% − recovery, %) as shown with detection antibody 3D3 (Fig. 5D). The Pearson correlation coefficient was 0.717 (95% confidence interval, 0.158–0.928; $P = 0.020$), giving a coefficient of determination (R^2) of 0.514. Thus, if we assume a linear relationship between troponin autoantibody titers and recovery inhibition, ~50% of the recovery inhibition would remain unexplained when correlation with the amount troponin autoantibodies is considered. The sample with the lowest recovery (0%) and with more than 10-fold higher cTnI concentration when measured with the new cTnI assay compared with the Innotrac Aio first-generation cTnI assay (0.645 vs 0.051 μg/L) gave the highest specific signal with all three detection antibodies.

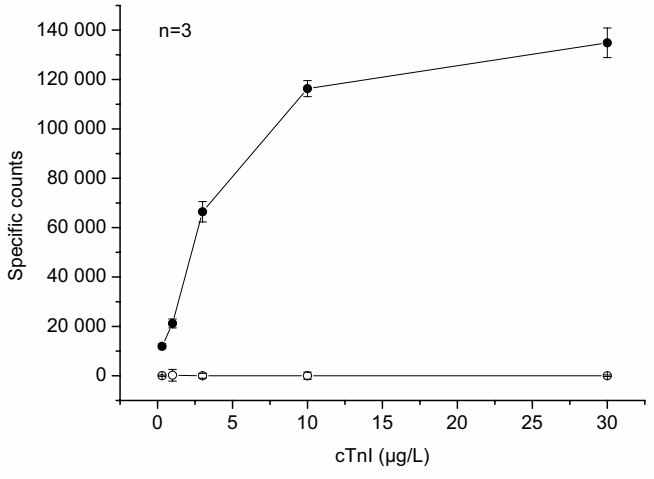

Fig. 4. Specific signals measured by the immunoassay for troponin autoantibodies in samples containing increasing amounts of added cTnI.

Mab 3 and Mab 4 were used for capture of cTnI (troponin complex) added to fivefold dilutions of serum samples, and troponin autoantibodies were detected by Mab 3D3. ●, LR sample; ○, NR sample. *Error bars*, SD.

Discussion

We have identified the component causing negative interference in some cTnI immunoassays based on antibodies against midfragment epitopes of cTnI *(23)* as circulating autoantibodies to cTnI or the troponin complex. The fairly small amount of IF present in samples with low analytical recovery of cTnI together with the limited supply of IF-containing sera complicated its isolation. Another challenge was that the component had to stay in active form throughout the purification process because the only method originally available for its detection was measurement of the analytical recovery of cTnI. Because the previously determined molecular mass range was so broad, 50–200 kDa *(24)*, and coinciding with the most abundant serum proteins, initial gel filtration was not considered helpful. After performing several other purification steps, we noticed that IF was eluted together with immunoglobulins in every step. Caprylic acid is known to precipitate albumin and most other proteins under acidic concentrations, whereas IgG, IgA, and the major part of IgM are not precipitated *(35)*. For precipitation of antibodies, 40% ammonium sulfate saturation is typically used *(36)*. Cibacron Blue F3GA dye has also been reported to bind immunoglobulins *(37)*. We made an attempt to further isolate troponin-specific antibodies, using biotinylated troponin complex attached to streptavidin-coated magnetic particles. Despite extremely harsh washing conditions with urea-containing wash buffer, nonspecific binding of antibodies to the particles occurred both in the control sample with no IF and in the absence of troponin complex (data not shown).

The isolation of autoantibodies was laborious and required large sample volumes; unfortunately, it did not provide complete isolation of troponin-specific antibodies. Therefore, after identifying IF as autoantibodies in one individual with consistently low cTnI recovery, we instead attempted a direct demonstration of troponin autoantibodies in other samples with low cTnI recovery. For this purpose, we designed three separate serologic assays using different monoclonal anti-immunoglobulin antibodies for detection. The results show that in the majority

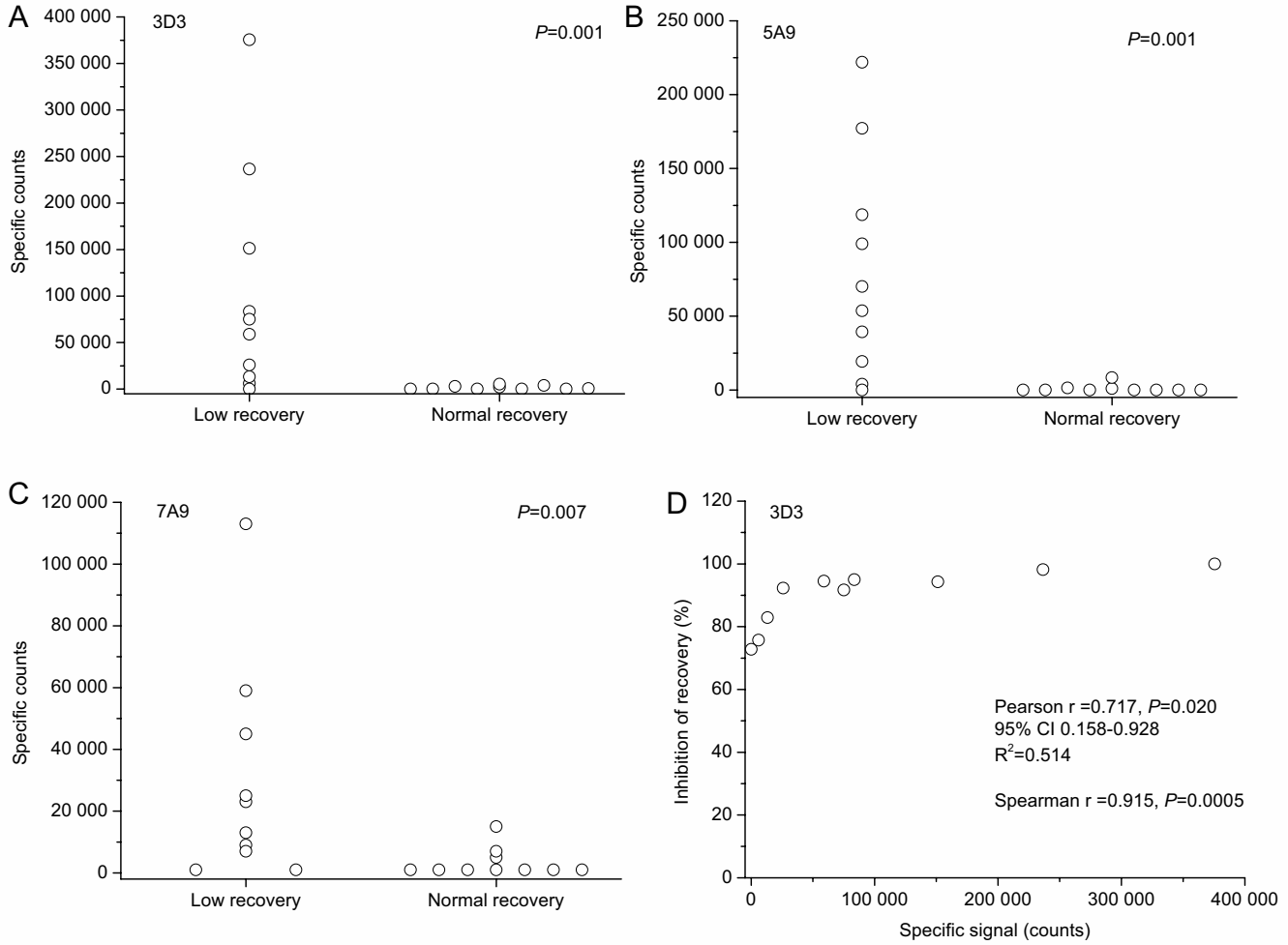

Fig. 5. Specific signals from LR and NR serum samples measured by immunoassays for detection of troponin autoantibodies, and correlation between specific signals from LR samples and percentage of inhibition of recovery.

Mabs 3 and 4 were used to capture cTnI (troponin complex) added to dilutions of samples, and troponin autoantibodies were detected by anti-human immunoglobulin antibodies 3D3 (*A*), 5A9 (*B*), or 7A9 (*C*). *P* values for significant differences between median specific signals of LR and NR samples are shown (Mann–Whitney *U*-test). (*D*), correlation between specific signals in the troponin autoantibody assay (detection antibody 3D3) and the percentage of inhibition of recovery (calculated as 100% − recovery, %). *CI*, confidence interval.

of tested samples, there was a significant positive relationship between low recovery of cTnI and the presence of troponin-specific autoantibodies (Fig. 5). The control samples (NR) gave mostly undetectable specific signals, with a few exceptions mainly with antibody 7A9, which had the broadest specificity (IgA, IgG, and IgM) of the three detection antibodies. Other sources of negative interference cannot be completely excluded, because according to the coefficient of determination (R^2), only ~50% of the recovery inhibition can be explained by correlation with troponin autoantibody titers (Fig. 5D). However, in this case, it may be more appropriate to use the Spearman correlation coefficient, which is based on ranks, rather than the Pearson correlation coefficient, which assesses a linear association. Immunoassays detecting autoantibodies can differ in many respects from immunoassays detecting relatively homogeneous epitopes. The autoantibody assay result can be affected by large variations in affinity and concentration of the autoantibodies, and there may also be pronounced epitope heterogeneity. The situation is further complicated by the nonspecific background, which can vary substantially among individuals, although we determined the background for each sample to limit this source of error. Furthermore, the immunoassays for detection of troponin autoantibodies have not been validated with respect to, e.g., detection limits and imprecision, so that the obtained estimation of troponin autoantibodies should be considered only a qualitative categorization. A few LR samples gave no specific signal (Fig. 5), and in these samples the source of interference might consequently be some other component, or alternatively, it may simply be a reflection of insufficient sensitivity of the serologic assays.

The high incidence of autoantibodies affecting the measurement of cTnI is certainly an unexpected and novel finding. We previously found cTnI recoveries <10% in

3.2% of patients with chest pain and 3.5% of patients with noncardiac symptoms *(23)*. Still more surprising is the fact that this relatively common interference in existing cTnI assays has not been reported previously despite recovery experiments being a central part of immunoassay development and validation. It should also be remembered that the remarkably high frequency of decreased recovery of cTnI is related to the fact that cTnI assays aim to measure very small amounts of the antigen, so that even minute autoantibody titers may exert a noticeable inhibitory effect. Only a single case of cTnI autoantibodies causing false-negative results has been reported previously *(22)*. False-negative results are seen when the autoantibody titer is high relative to the released cTnI amount. Partial inhibition causing saturation of autoantibodies can be seen when the autoantibody titers are lower or the autoantibodies have a lower affinity and when the amount of released or added cTnI is relatively high (as shown in Table 1).

The N- and C-terminal ends of human cTnI have been shown to be the strongest antigenic regions when mice were immunized with free cTnI *(38–40)*, whereas antibodies raised against the troponin complex often interact with the N-terminal region of cTnI *(40)*. The human troponin autoantibodies, on the other hand, interfere with the binding of antibodies to the central region of cTnI *(24)*. It should be noted that the autoantibodies do not necessarily bind directly to cTnI because we have used the troponin complex for measurement of recovery and also for detection of autoantibodies by immunoassays. The N-terminal region of TnI that interacts with TnC appears to be localized within residues 33–53 *(41)*, and additional regions binding to TnC are found at residues 129–150 and 161–181 of cTnI *(41, 42)*. The region including residues 33–80 is likely to contain sites of interaction with cTnT *(41)*. Thus, it is possible that autoantibodies binding to TnC or cTnT render the central region of cTnI unavailable for antibodies to these epitopes. Consequently, autoantibodies could also react with the other troponin subunits in a way that hinders binding of analytical antibodies to cTnI.

Recovery measurements and immunoassays for detection of autoantibodies were performed with the tissue-derived ternary troponin complex, which is conceivably not completely identical to endogenous circulating cTnI. Consequently, the inhibiting effect of autoantibodies cannot be automatically transferred to measurement of endogenous cTnI. However, both our previous *(23)* and recent results *(26)* from measurements of patient samples show that immunoassays based on antibodies with epitopes outside the central region are capable of detecting cTnI in some samples that give very low or undetectable cTnI concentrations when measured with midfragment antibodies. In view of our present and previously published results, the current recommendation to use only cTnI antibodies directed to stable, midfragment epitopes *(25)* may not be optimal for the unrestricted recognition of cTnI in the presence of autoantibodies against this region. Clinical chemists and cardiologists need to test whether commercially available immunoassays are affected by autoantibodies against troponin.

This study was supported in part by grants from the Paulo Foundation and the Aarne and Aili Turunen Foundation. We thank Johanna Hellström for excellent technical assistance and Pirjo Laaksonen for labeling of streptavidin and drawing blood samples. We gratefully acknowledge HyTest Ltd. for supplying immunoreagents.

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