Clinical Chemistry 43:8
1379–1385 (1997)

Enzymes and Protein Markers

Troponin I is released in bloodstream of patients with acute myocardial infarction not in free form but as complex

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Fourteen monoclonal antibodies (mAbs) against human cardiac troponin I (cTnI) were generated by commonly used experimental techniques. All these antibodies, as well as antibody 414 (HyTest), were specific for human cTnI. Fifteen antibodies thus obtained were tested in a sandwich cTnI immunofluorescence assay (altogether 196 combinations). Ten pairs giving the highest sensitivity were selected for further investigation. The effect of TnI–TnC complex formation on antibody interaction with antigen was analyzed. The formation of TnI–TnC complex results in a significant decrease of the interaction of mAbs with TnI for seven of 10 analyzed pairs of antibodies. Using two pairs of cTnI-specific mAbs, one that recognized only free cTnI but not cTnI complexed with cTnC, and another that could be used for measurement of total cTnI (free cTnI and cTnI in complex with cTnC), we demonstrated that the main part of cTnI in serum collected from acute myocardial infarction patients is presented in the complex form. We concluded that effective and reliable immunological detection of TnI is possible only when antibodies used for assay development recognize both free TnI and TnI complexed with other troponin components.

INDEXING TERMS: AMI diagnosis • immunoassay

Troponin, together with tropomyosin, belongs to the group of structural proteins involved in the regulation of striated and cardiac muscle contraction *[1, 2]*. Troponin consists of three components: troponin C (TnC), troponin I (TnI), and troponin T (TnT), each of which performs specific functions.[6] TnC is a Ca^{2+}-binding protein containing four metal-binding sites. Two sites located in the C-terminal globular domain of TnC bind both Ca^{2+} (K_{ass} $\sim 10^7$ L/mol) and Mg^{2+} (K_{ass} $\sim 10^3$ L/mol), whereas two other sites located in the N-terminal domain specifically bind Ca^{2+} with a K_{ass} of $\sim 10^5$ L/mol *[1–3]*. TnI inhibits actomyosin ATPase activity, and this inhibition is reversed by the addition of Ca^{2+}-saturated TnC *[1, 2]*. TnI and TnC tightly interact with each other and the strength of their interaction strongly depends on the saturation of Ca^{2+}-binding sites of TnC *[1, 2, 4]*. In the presence of Ca^{2+}, the K_{ass} value for the TnI–TnC complex is $\sim 10^8$–10^9 L/mol *[4]*. Multiple sites of TnI–TnC interaction have been localized *[5]*, and, supposedly, in the presence of Ca^{2+}, TnI wraps around the central helix of TnC, forming contacts with both N- and C-terminal globular domains containing Ca^{2+}-binding sites *[6]*. TnT provides proper fixation of TnC and TnI on the actin–tropomyosin filament *[1, 2]*. Although TnT interacts with both TnI and TnC, this type of interaction is not as tight as in the TnI–TnC complex *[4]*.

Ten years ago cardiac TnI (cTnI) was introduced as a marker of acute myocardial infarction (AMI) *[7, 8]*. cTnI has since proved to be one of the most specific and sensitive markers of AMI *[9–12]*, perioperative myocardial infarction *[12]*, cardiac contusion *[13]*, and other

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Received December 2, 1996; revised April 22, 1997; accepted April 30, 1997.

[6] Nonstandard abbreviations: TnT(I)(C), troponin T(I)(C); cTn, cardiac troponin; AMI, acute myocardial infarction; mAb, monoclonal antibody; CK, creatine kinase; skTnI, skeletal troponin I; HRP, horseradish peroxidase; and SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

kinds of myocardial tissue damage. Although cTnI has been used as an AMI marker for a decade and several commercial assays are currently available, many important questions remain open. The crucial question of high relevance for assay development and calibration is: In what form is cTnI released into the bloodstream? For instance, cTnI is known to be highly susceptible to proteolysis. (*a*) What, then, do differently configured immunoassays measure in serum—the whole molecule or various products of the proteolytic degradation? (*b*) What is the half-life of cTnI in the bloodstream? (*c*) cTnI has been shown to be phosphorylated by a cAMP-dependent protein kinase *[14]* and by Ca^{2+}–phospholipid-dependent protein kinase (protein kinase C) *[15, 16]* at different sites. The phosphorylation of Ser-23 and Ser-24 changes the conformation of the TnI molecule and affects interaction of TnI with certain monoclonal antibodies (mAbs) *[17, 18]*, but it is unknown in what form—phospo- or dephospho—-cTnI is released in the bloodstream. (*d*) Human cTnI contains two Cys residues (Cys-80 and Cys-97) *[19]*, and oxidation of SH-groups of TnI affects its interaction with troponin components *[20]* and may also interfere with its binding to mAbs. (*e*) Whether cTnI is released into the circulation in a free form (as an isolated protein) or as a complex with other troponin components is also unknown. mAbs are usually generated against purified TnI; therefore we can suppose that not all of these antibodies will interact with cTnI complexed with cTnC or cTnT. This means that the sensitivity and the cutoff values for cTnI will depend on the nature of the mAb used for detection of cTnI. Utilization of cTnI as AMI marker will be successful and reproducible only when cTnI circulating in blood is well determined. In this study we analyzed cTnI in the serum of AMI patients with a large collection of mAbs generated against purified cTnI and found that the predominant part of cTnI circulates not in a free form but in the form of a complex.

Materials and Methods

SAMPLES

Blood samples from AMI patients were centrifuged after collection and serum was stored at −70 °C until use. Diagnosis of AMI was confirmed by electrocardiogram and serial measurements of creatine kinase (CK), CK isoenzyme MB, and lactate dehydrogenase isoenzyme 1. All samples were collected in accordance with the Helsinki Declaration of 1975 as revised in 1983.

MAB PREPARATION

Human cTnI, human skeletal troponin I (skTnI), and human cTnC were purified by a previously described method *[21]* and provided by HyTest (Turku, Finland).

BALB/c mice were immunized with purified human cTnI and a standard protocol. Briefly, mice were injected intraperitoneally on day 1 with 0.1 mg of cTnI reconstituted in 0.1 mL of 150 mmol/L NaCl and 10 mmol/L potassium phosphate, pH 7.5 (PBS), and mixed with equal volume of complete Freund's adjuvant. On days 31 and 61, the mice were boosted intraperitoneally with 0.1 mg of cTnI in PBS mixed with incomplete Freund's adjuvant. The final boosts were administered on days 91 and 93 with 0.05 mg of the antigen in PBS delivered both intraperitoneally and intravenously. On day 96 the mice were killed, their spleens removed, and splenocytes isolated for fusion. Splenocytes were fused with a nonsecretor cell line sp2/0 and plated into Dulbecco's modified Eagle's medium containing hypoxanthine, aminopterin, and thymidine with 150 mL/L fetal bovine serum.

Microplate wells exhibiting hybridoma growth were screened for production of anti-TnI antibodies. For this purpose, hybridoma supernatants were incubated for 30 min at 37 °C in micro-ELISA plates coated with cTnI (300 ng/well) and, after washing, incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibodies. After washing, HRP activity was determined by using *o*-phenylenediamine/hydrogen peroxide as substrate. Positive hybridomas were retested for antibody specificity by using micro-ELISA wells coated with skTnI.

Hybridomas selected on the basis of specificity were cloned by two rounds of limiting dilution into aminopterin-free medium. Stable hybridoma clones were cultured as ascites tumors in BALB/c mice. The specificity of mAbs was checked once more with human cTnI- and skTnI-coated micro-ELISA plates and different dilutions of ascitic fluid. mAbs were purified from ascitic fluids by protein A–Sepharose affinity chromatography. Antibody concentrations were determined by the Lowry et al. *[22]* method with mouse serum IgG (Calbiochem, La Jolla, CA) as a calibrator.

The specificity of mAbs was confirmed by Western blotting. Human cTnI, skTnI, or crude skeletal or cardiac tissue homogenate were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) *[23]* on 7.5–15% gradient polyacrylamide gels. Proteins were blotted to the nitrocellulose *[24]* and incubated with purified mAbs (3 mg/L) for 1 h at 37 °C. After washing and 1 h of incubation with HRP-labeled goat anti-mouse antibodies at 37 °C, protein bands were visualized by incubation with 4-chloro-1-naphthol/hydrogen peroxide substrate.

LABELING ANTIBODIES WITH EU CHELATE

Stable fluorescent Eu chelate used to label the detection antibodies was obtained from Wallac (Turku, Finland). We used an Eu (III) chelate of 2,2′,2″,2‴-{{4-[4-(iodoacetamido)phenylethynyl]pyridine-2,6-diyl}bis(methylenenitrolo)}tetrakis(acetic acid) *[25]*. Labeling of mAbs with the Eu chelate was performed overnight at 4 °C with 200-fold molar excess of the chelate in 50 mmol/L sodium carbonate buffer, pH 9.8. Labeled antibodies were separated from free chelate by gel filtration on NAP-5 and NAP-10 columns (Pharmacia, Uppsala, Sweden). For different mAbs, 3–9 mol of the chelate coupled to each mole of the antibody.

BIOTINYLATION OF ANTIBODIES

Antibody solutions (1–3 g/L) in 0.1 mol/L borate buffer pH 8.8 were mixed with isothiocyanate derivative of biotin dissolved in dimethyl sulfoxide. About 100 μg of biotin ester were added per milligram of antibody. The reaction mixture was incubated for 4 h at room temperature. Twenty microliters of 1 mol/L NH_4Cl were added per 250 μg of ester and incubation was continued for another 10 min. The mixture was subjected to exhaustive dialysis against 50 mmol/L Tris-HCl (pH 7.8), 150 mmol/L NaCl, and 1 g/L sodium azide, and modified antibodies were stored at 3 °C. These antibodies were used as the coating antibodies.

IMMUNOASSAYS

cTnI calibrator preparation. cTnI is poorly soluble at neutral pH and physiological ionic strength. For the preparation of the stock solution of cTnI (0.1 g/L), we used a high concentration of urea in the diluent buffer (7 mol/L urea, 5 mmol/L EDTA, 10 mmol/L mercaptoethanol, 20 mmol/L Tris-HCl, pH 7.5). TnI calibrators were prepared by making several dilutions of the cTnI stock solution in normal male serum (Scantibodies Lab., Santee, CA). Because we used a very low concentration of cTnI in our immunological experiments (<100 μg/L), the final concentration of urea in samples was not more than 6 mmol/L and did not affect the assay.

cTnI–cTnC complex formation. cTnC was purified from human heart tissue *[21]*. cTnC migrated as a single band on SDS-PAGE and did not contain proteins detected by anti-cTnI antibodies in immunoblotting. In vitro formation of cTnI–cTnC complexes was carried out by adding human cTnC (130 μg/L) to the final dilution of cTnI (30 μg/L) in normal male serum (already containing 0.6 mmol/L $CaCl_2$) and incubating the mixture for 30 min on ice with gentle shaking. Sixfold molar excess of cTnC was added to shift the equilibrium towards TnI–TnC complex formation.

Assay protocols. A two-step fluoroimmunoassay was performed in streptavidin-coated 96-well microtitration plates (Wallac). First, biotinylated antibodies (coating antibodies, 400 ng per well) were immobilized to the streptavidin surface by incubating at room temperature in 0.2 mL of Delfia™ assay buffer (Wallac). After 30 min of incubation, the plates were washed twice with the washing solution containing 9 g/L NaCl, 0.25 g/L Tween 20, 0.5 g/L sodium azide, and 10 mmol/L Tris-HCl pH 7.4. We then added 50 μL (200 ng/well) of detection antibodies in Delfia assay buffer to the well, followed by 50 μL of cTnI calibrators or cTnI–cTnC mixture. The final concentration of cTnI in the cTnI calibrators and in the cTnI–cTnC mixture was equal. The same protocol was used in the experiments with serum samples collected from AMI patients. If necessary (to decrease the strength of cTnI–cTnC interaction) the incubation buffer contained 5 mmol/L EDTA. The mixture of antigen with Eu-labeled detection antibodies was incubated for 30 min at room temperature. After washing by Delfia wash solution (six times), Lanfia enhancement solution *[26]* (0.2 mL/well) was added, and incubation was continued for 3 min at room temperature with gentle shaking. The fluorescent signal (cps) was measured with the 1234 Delfia Plate Fluorometer (Wallac).

Results

Fifteen mAbs against cTnI were used in this investigation. Fourteen mAbs were generated by the above-described method, and antibody 414 was obtained from HyTest. Nine antibodies had no detectable cross-reactivity with skTnI, whereas six other mAbs had a detectable but <1% cross-reactivity with skTnI. None of the antibodies cross-reacted with cTnC or cTnT or other proteins from crude muscle homogenates in Western blotting (data not shown).

All antibodies (196 combinations) were tested in sandwich fluoroimmunoassay as coating and detection antibodies to find optimal pairs for TnI fluoroimmunoassay development. Ten two-site combinations of six mAbs, highly sensitive for human cTnI, were chosen for further experiments.

Interaction of TnC with TnI results in significant changes in the structure of both TnC and TnI *[1–4]*. Therefore, it was reasonable to analyze the effect of cTnC on the binding of mAbs to cTnI. Six-molar excess of cTnC was added to the TnI solution in normal human serum (already containing 0.6 mmol/L $CaCl_2$) to form cTnI–cTnC complex (see *Materials and Methods*). Isolated cTnI and cTnI–cTnC complex were tested in 10 sandwich immunoassays, utilizing six mAbs in different combinations. For cTnI–cTnC complex, the immunofluorescence experiments were performed both in the presence and absence of 5 mmol/L EDTA, thus modifying (decreasing in the presence of EDTA) the strength of cTnI–cTnC interaction. The data presented in Fig. 1 indicate that, as a rule, the addition of cTnC negatively affected the immunoreactivity of cTnI. In one case (7F4–10B11 antibodies, case 1), complexation with cTnC increased the fluorescent signal. This can be attributable to cTnC-induced exposure of antigenic determinants of cTnI interacting with 7F4 or 10B11 antibodies. In two cases (7F4–2A3, case 3 and 2A3–10B11, case 4), complexation of cTnI had practically no effect on the interaction of mAbs with cTnI. This indicates that the accessibility of the epitopes of these antibodies does not depend on the binding of cTnC to cTnI. In the remaining combinations (seven of 10 analyzed pairs of mAbs), addition of cTnC significantly reduced the fluorescent signal, reflecting changes in the interaction of mAbs with cTnI. However, addition of EDTA to the assay buffer resulted in partial (cases 2, 6, 8, 9, and 10) or complete (cases 5 and 7) reversal of the inhibitory effect of cTnC on cTnI–mAb interaction.

Assuming that a part of cTnI can be released into the

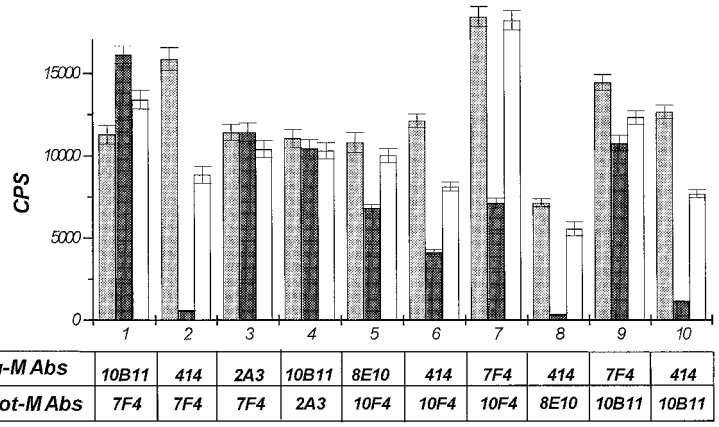

Fig. 1. Effect of cTnC on interaction of different pairs of mAbs with human cTnI.

cTnI (30 μg/L) in normal human serum was mixed with cTnC (150 μg/L) and incubated as described in *Materials and Methods*. Fluorescent sandwich technique with biotinylated coating antibody and Eu-labeled detection antibody was used for measurement of the signal level in samples containing purified protein (*light gray columns*) or TnI in the complex with cTnC without (*dark gray columns*) and with (*white columns*) 5 mmol/L EDTA. *Error bars* indicate mean ± SD.

circulation in the form of a complex with cTnC, we tested how addition of EDTA affects the cTnI measurements in the serum samples collected from AMI patients. Three assays (2A3–10B11, 10F4–7F4, and 7F4–414) were chosen for this purpose. All three systems were insensitive to addition of EDTA when tested with isolated cTnI (Fig. 2, the data for 2A3–10B11 not shown). This means that if cTnI is liberated into the bloodstream in the free form, the signal level for all three systems will not depend on the presence of EDTA in the assay. In practice it was correct only for the system 2A3–10B11 (Fig. 3). This system is insensitive to the presence of cTnC in the incubation mixture (see Fig. 1, case 4), and in the assay with serum samples from AMI patients gave identical signals independent of the presence of EDTA in the incubation mixture. In the model experiments (Fig. 1) we have shown that two other systems (7F4–414 and 10F4–7F4) are sensitive to addition of cTnC (Fig. 1, cases 2 and 7). Moreover, EDTA partially (system 7F4–414) or completely (system 10F4–7F4) reversed the inhibitory action of cTnC on the signal level. Therefore it is reasonable to use these systems to investigate the effect of EDTA on the signal level while serum samples from AMI patients are tested. As shown in Fig. 3, addition of EDTA significantly

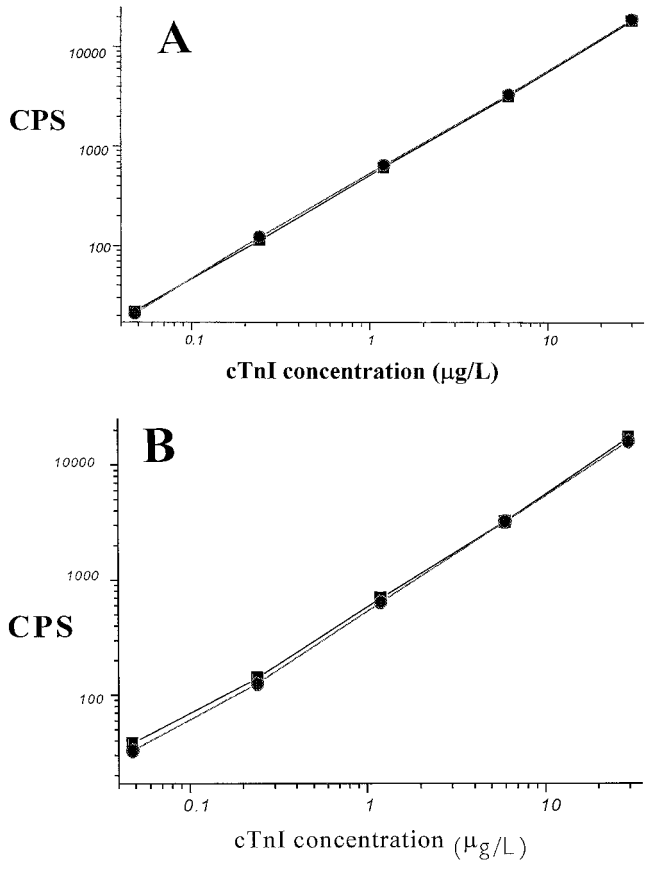

Fig. 2. Calibration curves for 10F4–7F4 (*A*) and 7F4–414 (*B*) assays used for measurement of isolated cTnI in serum.

The first antibody in each set was biotinylated and the second antibody was Eu-labeled. Experiments were run either without EDTA (●) or after addition of 5 mmol/L EDTA to the incubation mixture (■).

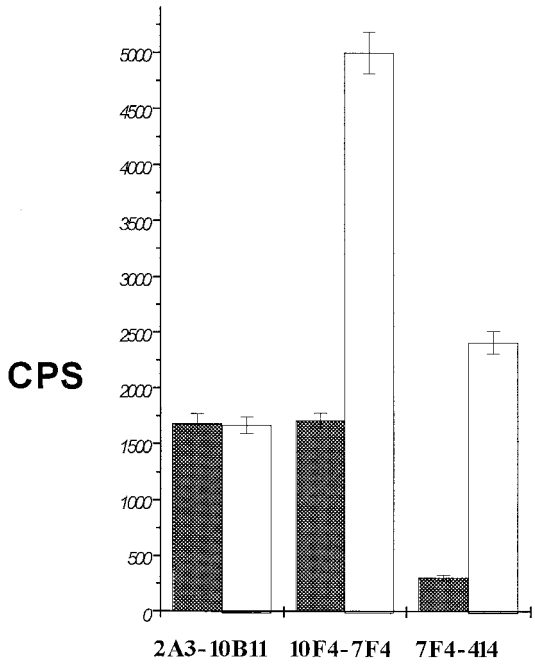

Fig. 3. Results of testing of one representative serum sample from an AMI patient by three different assays without (*black columns*) or with (*white columns*) 5 mmol/L EDTA.

increased the signal level for both assays. Addition of EDTA diminishes the strength of interaction of troponin components and by this means increases the level of the fluorescent signal. The data presented correlate with our suggestion that at least part of cTnI is released to the bloodstream in the form of a complex with cTnC.

From the data presented in Fig. 1 we may conclude that with different pairs of mAbs we can determine both the concentration of free cTnI as well as the total concentration of cTnI in the sample. Indeed, the pairs of mAbs that equally react with isolated cTnI and cTnI in the form of cTnI–TnC complex (7F4–2A3, case 3; 2A3–10B11, case 4) can be used for determination of the total cTnI concentration in the serum. The total concentration of cTnI can also be determined by using the pairs of mAbs that equally recognize isolated cTnI and cTnI complexed with cTnC in the presence of EDTA (10F4–8E10, case 5 and 10F4–7F4, case 7). For example, by using biotinylated 10F4 and Eu-labeled 7F4 and measuring in the presence of excess EDTA, we observed equal signals both for isolated TnI and its complex with cTnC (Fig. 1).

For the determination of free TnI concentration, the pairs of mAbs showing virtually complete suppression of the cTnI signal upon complexation with cTnC can be used. This requirement is satisfied by three pairs of mAbs (7F4–414, case 2; 8E10–414, case 8; and 10B11–414, case 10). But assay with 7F4–414 mAbs had the highest signal among the two combinations and >97% reduction of the signal after cTnI complexation with cTnC.

Thus, by using two assays it is possible to measure both concentration of free TnI and concentration of total TnI. In our experiments we chose 7F4–414 assay for measurement of free TnI, and 10F4–7F4 assay in the presence of EDTA for measurement of total TnI. This assay had better sensitivity, kinetics, and range of linearity than all others (7F4–2A3 and 2A3–10B11). The calibration curves of two chosen assays in the presence and absence of EDTA are shown on Fig. 2. As exemplified in Fig. 2, EDTA does not affect the signal level of both assays.

To investigate the concentrations of free and complexed TnI during AMI, we assayed serum samples from 30 AMI patients by two assays described above. The data of Fig. 4, showing profiles of free and total cTnI in four representative cases of AMI, indicate that the peak of the total TnI concentration was observed 15–25 h after onset of chest pain, and the concentration of total TnI remained increased for at least 80–100 h. The kinetics of TnI release is similar to that described in the literature *[27]*. The peak value of total TnI varied from 6 to 50 μg/L and is

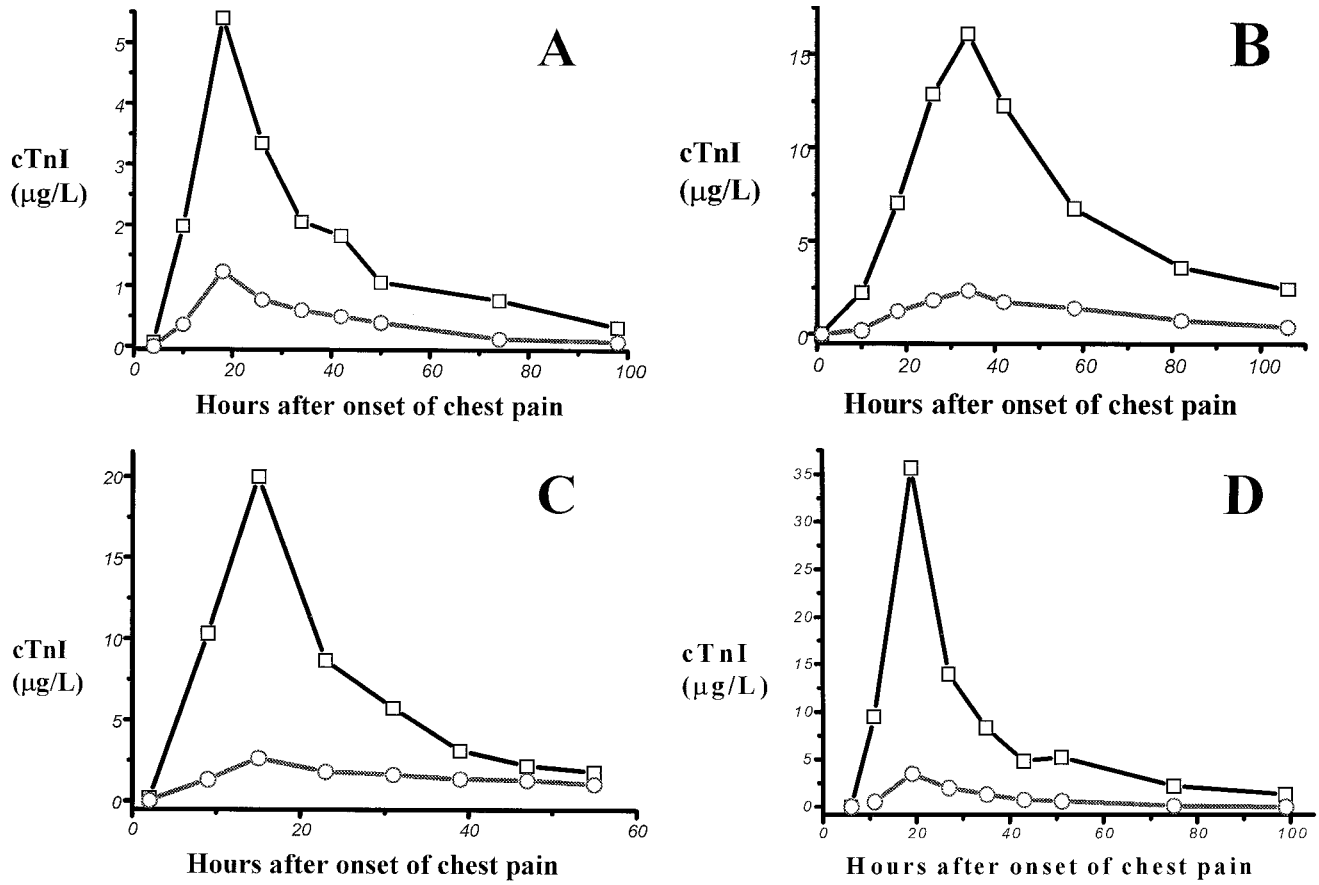

Fig. 4. Time course of liberation of total cTnI measured by 10F4–7F4 assay in the presence of EDTA (□) and free cTnI measured by 7F4–414 assay (○) in the serum of four representative AMI patients.

probably dependent on the size of infarction zone and the rate of reperfusion. In all cases the concentration of free TnI was much less than that of the total TnI. The peak of free TnI was less pronounced and, in many cases, the concentration of free TnI was only slightly changed during the whole time of observation. At the peak concentration, cTnI was 5–12 times higher than the corresponding concentration of free cTnI. The ratio $[cTnI]_{total}/[cTnI]_{free}$ was changed during the time of observation. As a rule, at the beginning of observation this ratio was low; then it increased to its maximal value and at the end of observation returned to its initial value. At the present stage of investigation we cannot give a detailed explanation for the complex kinetics of cTnI liberation.

Discussion

Highly purified proteins are commonly used for the generation of mono- or polyclonal antibodies. This approach was also used for obtaining antibodies against human cTnI *[9, 10, 12]*. As already mentioned, TnI tightly interacts with two other troponin components, and separation of these components can be achieved only in the presence of high concentration (6–8 mol/L) of urea *[28, 29]*. Although the exact structure of purified TnI in solution is unknown, several observations indicate that it is different from that of TnI in the complex with two other troponin components, especially with TnC. Thus it was demonstrated that the reactivity of SH groups of purified TnI is different from that of TnI in the whole troponin complex *[20, 30]*. TnC significantly affects phosphorylation of TnI by different protein kinases *[31]*. TnC and TnT affect the accessibility of Lys residues of TnI to the chemical modification *[32]*. All these facts mean that the interaction with two other troponin components induces significant changes in the overall structure of TnI. Therefore, antibodies raised against highly purified TnI will conceivably recognize certain epitopes that will be absent or significantly modified in the whole troponin complex or in the binary complexes of TnI with TnC or TnT. In addition, both TnC and TnT cover certain parts on the surface of TnI *[2, 5, 6]* and thus can also prevent interaction of antibodies with TnI.

We have found that cTnC does significantly modify the interaction of cTnI with mAbs. In seven of 10 analyzed pairs of antibodies, complexation with cTnC significantly diminished the fluorescent signal (Fig. 1). For certain pairs of antibodies, the effect of cTnC was so strong that the fluorescent signal was decreased by 97% (7F4–414, Fig. 1 case 2). Addition of EDTA removes Ca^{2+} and thus modifies the strength of the cTnI–cTnC interaction *[1, 2, 4]*. In the absence of Ca^{2+} the apparent dissociation constant of the cTnI–cTnC complex is increased >1000-fold *[1, 2, 4]*. Indeed, addition of EDTA to the cTnI–cTnC complex improves the binding of certain mAb combinations to cTnI (Fig. 1, cases 5, 7, and 9). Nevertheless, even in the presence of 5 mmol/L EDTA, certain pairs of mAbs interact less effectively with cTnI inside the cTnI–cTnC complex than with isolated cTnI (Fig. 1, cases 2, 8, and 10). This shows that only certain pairs of antibodies could be used for immunodetection of cTnI in the circulation if it is partly or predominantly released in the form of a complete troponin complex (TnI–TnT–TnC) or in the form of a binary cTnI–TnC complex. Until now, the form in which cTnI is released from damaged cardiac myocytes has not been known. We have tried to answer this question by using two highly sensitive immunofluorometric assays.

The data presented in Fig. 4 indicate that in all AMI patients under investigation, the largest part of cTnI is liberated in the form of a complex (probably with cTnC) and only a small part of TnI circulates in the bloodstream in a free form. The ratio of total to free TnI in the serum varies in the course of observation and is different in serum samples from different patients. In general, this means that special precautions should be taken in developing sensitive and reproducible methods for the determination of TnI in the bloodstream. First, pairs of antibodies that are used for TnI determination should equally react with free cTnI and cTnI forming complexes with other troponin components. In other words, antibodies should recognize epitopes that are not perturbed or sterically shielded by other troponin components. Second, for some pairs of antibodies for which the interaction with cTnI is affected by cTnC in the presence of Ca^{2+}, it is advisable to add EDTA to the sample or the incubation mixture. Addition of EDTA decreases the strength of interaction among troponin components *[1, 2]* and therefore increases probability of interaction of certain mAbs with TnI. Obviously, special control experiments should be performed to exclude probable effects of EDTA on the detection system.

The cutoff concentration for cTnI in published cTnI immunoassays varies from 0.2 μg/L *[12]* up to 3.1 μg/L *[9]*. This difference can at least partly be explained by different specificities of mAbs for free and complexed cTnI in the above-mentioned assays. In this investigation we analyzed only the effect of cTnC on the interaction of mAbs with cTnI. Evaluation of an effective and reliable method for cTnI determination will require further investigations directed to analyses of the effect of phosphorylation, partial proteolytic degradation, and oxidation of SH groups of TnI on its interaction with mAbs.

This investigation was supported HyTest Ltd., the Biotechnology Department of the University of Turku, and Russian Foundation for Basic Science (95-04-11030) (Moscow, Russia). We are grateful to Olga Kolosova and Elena Akinfieva for technical assistance in the course of this investigation.

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