Assay Development of a Serum Marker for Myocardial Injury: Experiences with an Avian anti-Troponin I Antibody

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Summary
The diagnosis of acute myocardial infarction is still difficult in certain cases. The measurement of human cardiac Troponin I, a new marker for heart muscle cell necrosis, can help to support current procedures of diagnosis. The detection is usually done with antibodies in a sandwich immunoassay. For development of such a test for human cardiac Troponin I on the automated analyser Technicon Immuno I avian egg yolk antibodies were tested. Antibody preparations from two immunised chickens were tested for content of specific anti-Troponin I antibodies. Following affinity purification against a peptide from the Troponin I sequence the antibodies were conjugated with alkaline phosphatase and used as detector antibodies. The combination with different monoclonal antibodies resulted in standard curves for Troponin I, which were in terms of sensitivity inferior to a commercial polyclonal antibody from goat.

Keywords: Troponin I, myocardial infarction, avian antibody, immunoassay, diagnostics

1 Introduction

1.1 Diagnostic problems of Acute Myocardial Infarction
Acute myocardial infarction (AMI) is one of the main health problems in western countries. According to the American Heart Association, approximately 1.5 million heart attacks occur each year in the United States, resulting in 400,000 deaths (Spectrum Diagnostics, Medical Equipment, and Supplies Division Resources, Inc., 1995). Traditionally, AMI is diagnosed using patient history, electrocardiography (ECG), and assays for the serum enzymes creatin-kinase, creatin-kinase MB isoenzyme, lactate dehydrogenase (LDH), and glutamic-oxaloacetic transaminase (GOT) (Rotenberg, 1989; Vaidya, 1988; Thompson et al., 1988).

ECG alone is insufficient for diagnosis as it is inconclusive in 1 out of 4 cases, particularly in small infarcts and unstable angina. Enzyme assays lack specificity and require a certain time after onset of chest pain to give positive results. Early and accurate detection of myocardial infarction is not only important because of earliest application of therapy but also has cost reducing aspects. 70 % of patients can be released from intensive care units rapidly because they do not suffer from myocardial infarction (Lee and Goldman, 1986).

1.2 Troponin I (TnI) as a marker for myocardial injury
Troponin I (TnI) is a component of the troponin complex on the thin filament of the muscle myofibril. Together with troponin T (TnT) and troponin C it is involved in the regulation of muscle contraction. TnT is the tropomyosin-binding subunit, TnI is the actomyosin ATPase-inhibiting subunit, and troponin C is the calcium binding subunit (Potter, 1982). In case of cell necrosis following embolism as a result of myocardial infarction the troponin subunits are released into circulation rapidly. TnI and TnT have cardiac specific isoforms not being expressed in other tissue (Larue et al., 1993; Kattus et al., 1991). This is why they are useful markers for cardiac cell injury. The sensitivity of both markers can be compared to that of the gold-standard CK-MB (Adams et al., 1994; Apple et al., 1995). Regarding specificity, TnI is apparently the only marker specific for cardiac events (Bhayana et al., 1995). CK-MB is known to be elevated in skeletal muscle trauma and in renal failure patients. Similarly, several reports show increased TnT levels in patients with renal insufficiency or renal failure (Bhayana et al., 1995; Li...
et al., 1995) and TnT expression in both healthy and regenerating skeletal muscle (Cummins et al., 1987; Bodor et al., 1995).

1.3 Assay development for cardiac Troponin I

In order to help physicians with certain diagnostic problems of AMI, biochemical markers are applied. They are used to confirm myocardial infarction diagnosis, for monitoring of reperfusion, and to aid decisions in special cases e.g. when the ECG is ambiguous. In addition TnI provides the possibility to detect an infarction in patients coming late to hospital after onset of chest pain, as TnI is still measurable days after the incident.

TnI assay development requires antibodies with a high specificity and affinity. Generally, fully automated machines like Immuno1 need higher quantities than conventional ELISA techniques. Additionally, the antigen is phylogenetically conserved, so that TnI antibody production in chickens promised to have better success than immunisation of mammals.

2 Laboratory animals, materials and methods

2.1 Animals

Chickens were housed in groups of two animals in cages appropriate to allow a species-specific behaviour. A brown (LB) and a white (LSL) chicken (Geflügelvermeh rungsbetriebe Speenhagen GmbH, Speenhagen) were kept together to identify the eggs laid. The animals were housed under a constant L:D (12:12 hours) regimen with food and water ad libitum. The animals were fed with a standard pellet food with additional shell-lime and wheat grains to allow a normal repertoire of behaviour.

2.2 Antigen

Human cardiac TnI was purchased from Genzyme, West Mailing, UK. Recombinant material was provided by Dr. Trayer, University of Birmingham, UK.

Both kinds of material were stored either lyophilised or reconstituted in Urea-buffer containing 50 mM Tris-HCl pH 8.0, 6 M urea, 1 mM EDTA, 0.1 mM DTT.

2.3 Antibodies

Polyclonal goat anti TnI was purchased from BiosPacific (Emeryville, CA, USA). Monoclonals against TnI were used from different companies.

2.4 Production of avian egg yolk antibodies against cardiac Troponin I

Chickens were immunised with an emulsion in Freund's complete (first immunisation) or incomplete (booster-injections) adjuvants (FCA/FIA, Difco) and the antigen solution. Two chickens were immunised with rTnI and one chicken with human cardiac TnI. FCA was used in this case to have conditions comparable with the immunisation procedures used to produce corresponding antibodies in mammals. A volume of maximal 1 ml was injected i.m. (each 0.5 ml right and left) in the musculus pectoralis. 100–200 μg protein was given per immunisation. Booster injections were performed in monthly intervals. The antibodies were extracted from egg yolk (chicken) as described previously (Schade et al., 1994).

Further purification of the avian egg yolk anti-TnI antibodies was performed at Biogenes (Berlin, D). For the affinity purification the following peptide from the amino acid sequence of the TnI molecule was used: MADGSS-DAAREPRPAPAPRRSSNY. An amount of 2–3 mg of the TnI peptide per ml CNBr-Sepharose (Pharmacia) was incubated 4 hours at room temperature. After washing (once with 1M Ethanolamine pH 9.0, twice with Bicarbonate buffer pH 8.8 and twice with Acetate buffer pH 4.75) the material was applied to a 5 ml column.

10 ml of an IgY solution was applied to the column. Non specific binding antibodies were eluted by washing with 50 mM Tris/HCl. Specifically bound IgY were eluted with 0.2 M Glycine/HCl buffer pH 2.2. Antibodies were ammonium acetate precipitated, dialysed against PBS, and ultrafiltrated to a concentration of 100 μg IgY/ml.

2.5 TnI Assay on the Technicon Immuno 1

The TnI assay described here is designed for the Technicon Immuno 1, a fully automated, true random access immunoassay system. The machine makes use of a magnetic separation technique. For TnI as an emergency marker, the fastest protocol for a sandwich immunoassay on the Immuno 1 was chosen: the results are available after 23 min.

The Immuno 1 cTnI Assay is an enzyme labelled sandwich assay using magnetic particles coated with anti-fluorescein antibodies for immobilisation. The monoclonal capture-antibody is fluoresceinated, and the goat polyclonal detection antibody is conjugated with alkaline phosphatase. The enzyme catalyses the reaction of p-Nitropheno

![Sandwich Assay Format on the Technicon Immuno 1](Figure 1: Sandwich immuno assay format on the Technicon Immuno 1)
directly proportional to the concentration of cTnI in the samples. The calibration curve is produced by six standards covering an assay range from 0 to 200 ng/ml with a detection limit of 0.3 ng/ml.

2.6 ELISA to test avian egg yolk anti-Troponin I antibodies

The antigen (human cardiac TnI, Genzyme, West Malling, UK, or peptides from the TnI sequence) was coated to an ELISA plate (Greiner, Erlangen) for one hour at 37 °C or over night at 4 °C in a concentration of 1 μg/ml in coating buffer (200 mM NaHCO₃, pH 9.0) after 5 times washing with PBS/Tween (8 mM K₂HPO₄, 150 mM NaCl, 2 mM NaH₂PO₄, pH 7.4, 0.01% Tween 20) the anti TnI antibodies were added in concentrations of 0.5 to 3 μg/ml in ELISA buffer (0.1 M Tris(hydroxy-methyl-aminomethan, 242 mM NaCl, pH 7.3). After one hour of incubation at 37°C washing was repeated and alkaline phosphatase conjugated to anti chicken IgY (Sigma, Deisenhofen, D), or anti goat IgG (Sigma, Deisenhofen, D), respectively, was added in 1:1000 dilution in ELISA buffer. After another incubation (1 hour, 37°C) subsequent washing, and substrate addition optical density was measured at 405 nm by a microplate reader (SLT, Crailsheim, D).

2.7 Conjugation to alkaline phosphatase (ALP)

ALP-Conjugation was performed as described elsewhere (Wong, 1991). Activation of the antibody was done with 2-IT (2-Iminothiolanhydrochlorid, Pierce, Rockford, IL, USA), the enzyme was activated with the cross-linker SMCC (N-Succinimidyl-4-(N-maleimidomethyl)cyclohexan-1-carboxylate, Pierce, Rockford, IL, USA).

3 Results

3.1 Evaluation of avian egg yolk antibodies against human cardiac Troponin I

Avian egg yolk antibodies from two different animals were tested with the ELISA method described above. The reactivity of both antibodies against human cardiac TnI was nearly equally high. A sheep serum showed much lower reactivity.

Figure 2: ELISA for detection of anti-Troponin I antibodies. Avian egg yolk antibodies from two different chickens were compared to a sheep serum. Control 1: antigen was emitted, Control 2: alkaline phosphatase anti chicken IgY was emitted.

Figure 3: Reactivity of avian egg yolk antibodies raised against human cardiac Troponin I with the antigen and two different peptides from the Troponin I sequence.

Sequence of Peptide 1: MADGSSDAARPPAPAPRRSSNY
Sequence of Peptide 2: RGEKRALSTRCQPLEL
Control: no antigen coated to the ELISA plate.
lower reaction against the antigen in this system (figure 2). All three anti Tnl antibodies were also tested on an immunoblot (data not shown). Antigen recognition was good in all three cases, but the avian antibodies showed a significant lower background staining. There was no cross reactivity to Troponin C and skeletal muscle Tnl with the avian and goat antibodies.

The reactivity of the avian anti Tnl antibodies with two peptides from the Tnl sequence was investigated (figure 3). The binding of the antibodies to peptide 2 was at background level (Control). The binding of peptide 1 was slightly increased. In this experiment antibodies isolated from chicken 9 showed better reactivity than those of chicken 10.

3.2 Results with an avian egg yolk antibody affinity purified against a Troponin I peptide

After affinity purification with the Tnl peptide 1 reactivity of the antibody preparation towards the peptide increased significantly. Reactivity towards peptide 2 remained at background level. Nevertheless, the antibodies recognised the complete Tnl molecule much better.

3.3 Troponin I standard curves with avian egg yolk antibodies on the Analyser Technicon Immuno I

Polyclonal antibodies against Tnl were conjugated to the enzyme alkaline phosphatase and used as detection antibody in an immuno assay for human cardiac Tnl on the Technicon Immuno I. Different behaviour of the antibodies during this conjugation procedure is shown in the table.

Figure 5 shows differences in the sensitivity of the assay depending on the combination of different monoclonal antibodies to the avian anti Tnl. The pair of mAb 4 and the avian anti Tnl gave best results. These antibodies were used for the assay optimisation shown in figure 6.

4 Discussion

The purpose of the present study was to evaluate the possibility of using an avian egg yolk antibody for a diagnostic test to measure human cardiac Tnl in patient serum or plasma samples. The first results (figure 2 and 3) were produced with IgY from different chickens prepared as described in material and methods. We could demonstrate good reactivity with Tnl and very good specificity of these IgY in ELISA and Western Blot tests. Nevertheless, this total IgY fraction could not match our requirements in terms of assay sensitivity and low background level on the Immuno 1. This finding is comparable to IgG-fractions of sheep or goat sera, which also could not perform necessary sensitivity without further purification. Consequently, an affinity purification was applied. We did not use the whole Tnl molecule for this procedure, but a peptide out of its amino acid sequence. The advantage of this method is that no human cardiac Tnl was required which is expensive and difficult.
cult to handle because of its limited solubility and stability in physiological solutions. Additionally, we preferred to have more homogeneous antibodies with a known binding sequence.

The affinity of the purified IgY antibodies towards the peptide was increased compared to the IgY fraction before purification (figure 4). Surprisingly, reactivity with the whole TnI molecule was still better after affinity purification. An explanation for this finding may be that during affinity purification very high affinity antibodies were lost which is a well known disadvantage of affinity purification. Another reason might include the isolation method using peptides for affinity chromatography. Those antibody species recognising epitops at the peptide's periphery are possibly isolated resulting in antibodies directed against only a very small part of the peptide. This could lead to lower affinity towards the peptide, but unchanged (high) affinity to the whole molecule.

A problem which was visible during work with IgY antibodies was unspecific adherence to different matrix materials. The consequence is shown in the table where the yield of alkaline phosphatase labeled product of different antibodies was shown. This procedure includes concentration and ultrafiltration through a Centricon membrane with an exclusion limit of 50,000 kd (Amicon, Witten, D), gel filtration through Sephadex G-25 M columns (Pharmacia, Upsala, Sweden) and Superdex 200 HR 10/30 columns (Pharmacia, Upsala, Sweden). All steps led to a loss of material much higher than observed with monoclonals, sheep or goat antibodies. This is a very important point which can produce severe problems during large scale industrial production of antibody conjugates. The problem might be solved by buffer optimisation or addition of detergents.

The assay optimisation with the use of the chicken anti TnI resulted in reasonable standard curves for human cardiac TnI (figures 5 and 6). They were comparable to most other polyclonal anti TnI antibodies tested. Nevertheless, assay formulation with IgY could not reach sensitivity of the best commercial goat anti TnI found after extensive antibody screening.

Therefore, in principle, we think it is possible to use avian yolk antibodies for diagnostic test development. In case of TnI more chickens should have been immunised and a more extensive antibody screening performed in order to produce a high affinity anti TnI IgY. Additionally, more experience with assays making use of IgY could help to solve problems such as unspecific adherence.
Die elektrisierende Welt der Nervenzellen

Jacques-Michel Robert

**Nervenkitzel**


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**References**


