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

Margit Doth[1], Rüdiger Schade[2], Silke Claas[1], Rolf Matysiak[1]

[1] Bayer AG, Zentrale Forschung und Entwicklung, D-Krefeld, [2] Institut für Toxikologie und Pharmakologie des Universitätsklinikums Charité der Humboldt-Universität, D-Berlin

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 1 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

Zusammenfassung: Testentwicklung eines Serummarkers für Myokardinfarkt: Erfahrungen mit einem aviären anti-Troponin I Antikörper.

Die Diagnose des akuten Myokardinfarktes ist in vielen Fällen immer noch mit großen Unsicherheiten verbunden. Zu deren Klärung kann das humane kardiale Troponin I, ein neuer Marker für Nekrosen von Herzmuskelzellen beitragen. Der Nachweis erfolgt in der Regel über Antikörper in Form eines Sandwich-Immunoassays. Zur Entwicklung eines solchen Test für das humane kardiale Troponin I auf dem Analyseautomaten Technicon Immuno1 sollten u.a. aviäre, vitelline Antikörper zum Einsatz kommen. Antikörperpräparationen von zwei immunisierten Hühnern wurden auf ihren Gehalt an spezifischen anti-Troponin I Antikörpern getestet. Nach einer Affinitätsreinigung gegen ein Peptid aus der Sequenz des Troponin I wurden die gewonnenen Antikörper mit alkalischer Phosphatase konjugiert und als Detektorantikörper eingesetzt. In Kombination mit verschiedenen monoklonalen Antikörpern ergaben sich Standardkurven für Troponin I, die jedoch in der Sensitivität nicht vergleichbar mit einem polyklonalen kommerziellen Antikörper aus der Ziege waren.

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 Resourses, 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 has also 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 (Geflügelvermehrungsbetriebe 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 Malling, UK. Recombinant material was provided by Dr. Trayer, University of Birmingham, UK.

Both kinds of material was 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) adjuvans (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-DAAREPRPAPAPIRRRSSNY. 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 Bicarbonat buffer pH 8.8 and twice with Acetat buffer pH 4.75) the material was applicated to a 5 ml column.

10 ml of an IgY solution was applied to the column. Non specific binding antibodies were eluated by washing with 50 mM Tris/HCl. Specifically bound IgY were eluated 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 Immuno1 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-Nitrophenolphosphate to p-Nitrophenolate. The speed of p-Nitrophenolate formation that is measured spectroscopically is

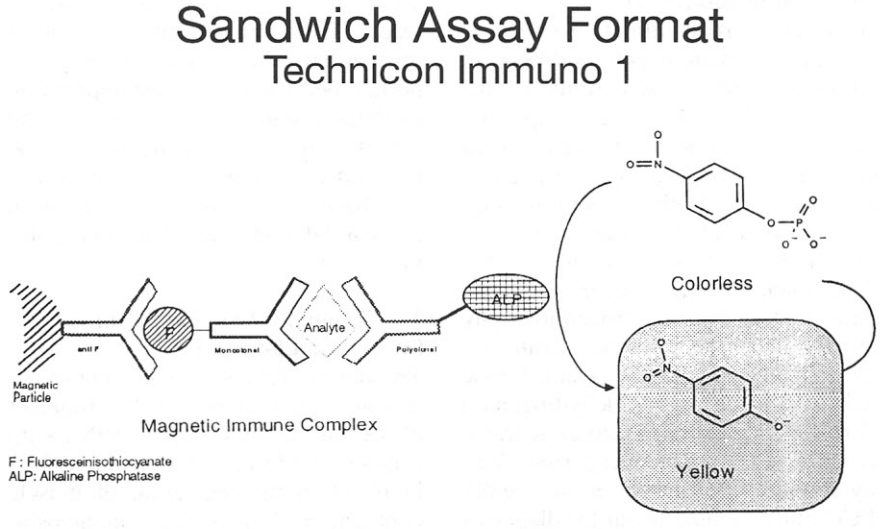

Figure 1: Sandwich immuno assay format on the Technicon Immuno 1

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 $NaHCO_3$, pH 9.0) after 5 times washing with PBS/Tween (8 mM K_2HPO_4, 150 mM NaCl, 2 mM NaH_2PO_4, 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(hydroxymethl-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 crosslinker SMCC (N-Succinimidyl-4-(N-maleimidomethyl)cyclohexan-1-carbonate, 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

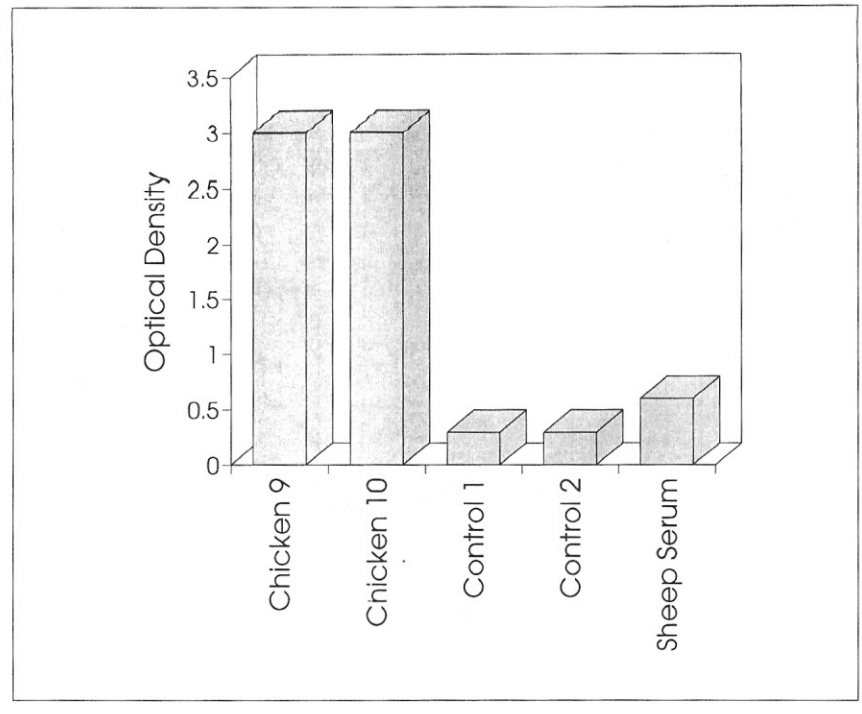

Figure 2: ELISA for detection of anti-Troponin I antibodies. Avian egg yolk antibodies from two different chickens were compared to a sheep serum. Control 1: antigen was emitted, Control 2: alkaline phosphatase anti chicken IgY was emitted.

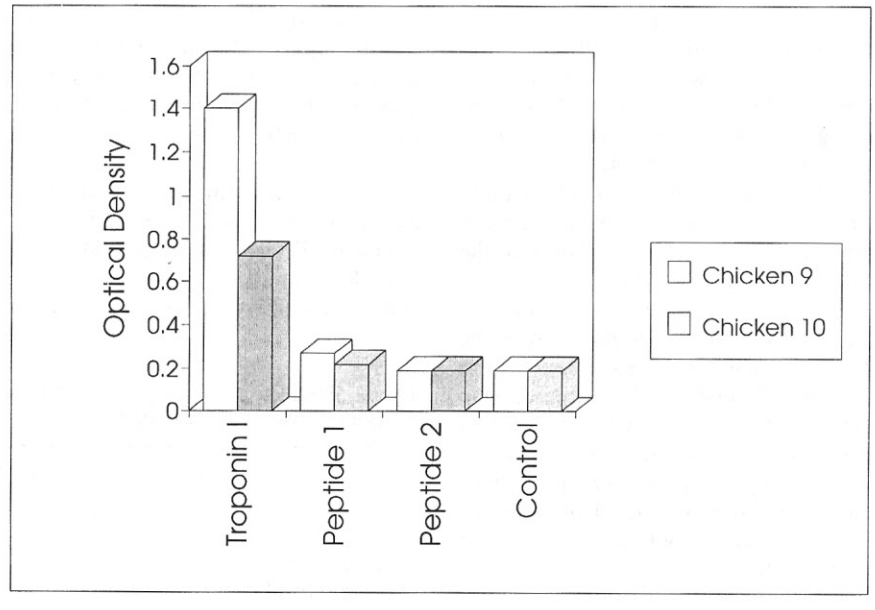

Figure 3: Reactivity of avian egg yolk antibodies raised against human cardiac Troponin I with the antigen and two different peptides from the Troponin I sequence.
Sequence of Peptide 1: MADGSSDAA REPRPAPAPIRRRSSNY
Sequence of Peptide 2: RGEKGRALSTRCQPLEL
Control: no antigen coated to the ELISA plate

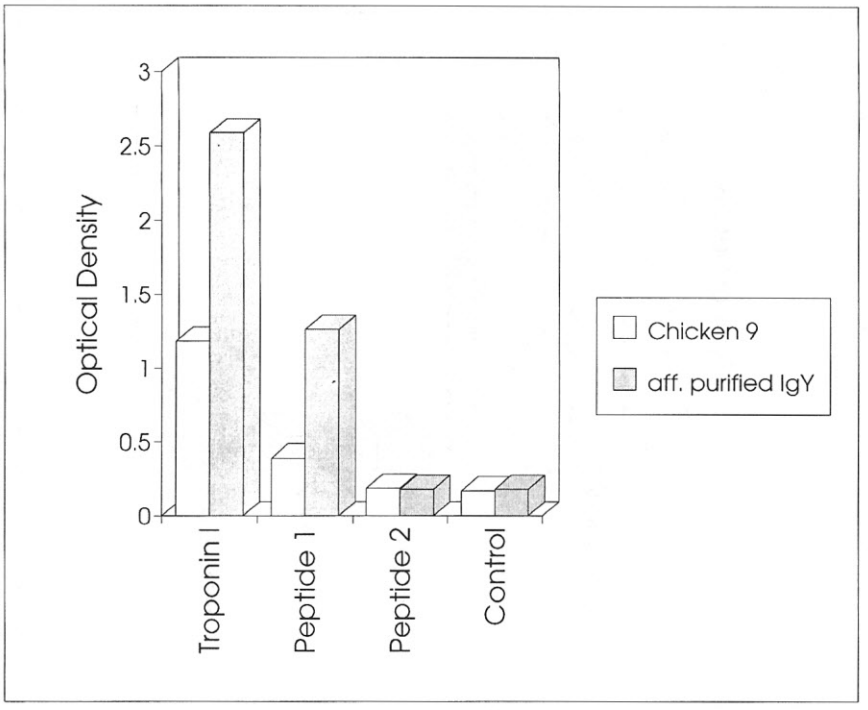

Figure 4: Reactivity of avian egg yolk antibodies raised against human cardiac Troponin I and affinity purified against Peptide 1 with the antigen and two different peptides from the Troponin I sequence.
Sequence of Peptide 1: MADGSSDAA REPRPAPAPIRRRSSNY
Sequence of Peptide 2: RGEKGRALSTRCQPLEL
Control: no antigen coated to the ELISA plate

lower reaction against the antigen in this system (figure 2). All three anti TnI 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 TnI with the avian and goat antibodies.

The reactivity of the avian anti TnI antibodies with two peptides from the TnI 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 TnI 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 TnI molecule much better.

3.3 Troponin I standard curves with avian egg yolk antibodies on the Analyser Technicon Immuno1

Polyclonal antibodies against TnI were conjugated to the enzyme alkaline phosphatase and used as detection antibody in an immuno assay for human cardiac TnI on the Technicon Immuno1. 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 TnI. The pair of mAb 4 and the avian anti TnI 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 TnI in patient serum or plasma samples. The first results (figure 2 and 3) were produced with IgY from different chikkens prepared as described in material and methods. We could demonstrate good reactivity with TnI 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 TnI molecule for this procedure, but a peptide out of its amino acid sequence. The advantage of this method is that no human cardiac TnI was required which is expensive and diffi-

Table: Results of conjugation of polyclonal antibodies against human cardiac Troponin I with alkaline phosphatase

	Antibodies against human cardiac Troponin I from indicated animals			
	Chicken 9	Chicken 10	Goat[1]	Goat[1]
Initial amount of protein [mg]	4.84	5.6	2	2
Yield [mg]	0.3	0.3	0.83	0.77
[%]	6	5.5	42	39
Activity of alkaline phosphatase [Units/nmol]	247	273	175	223

[1] Two preparations of the same antibody

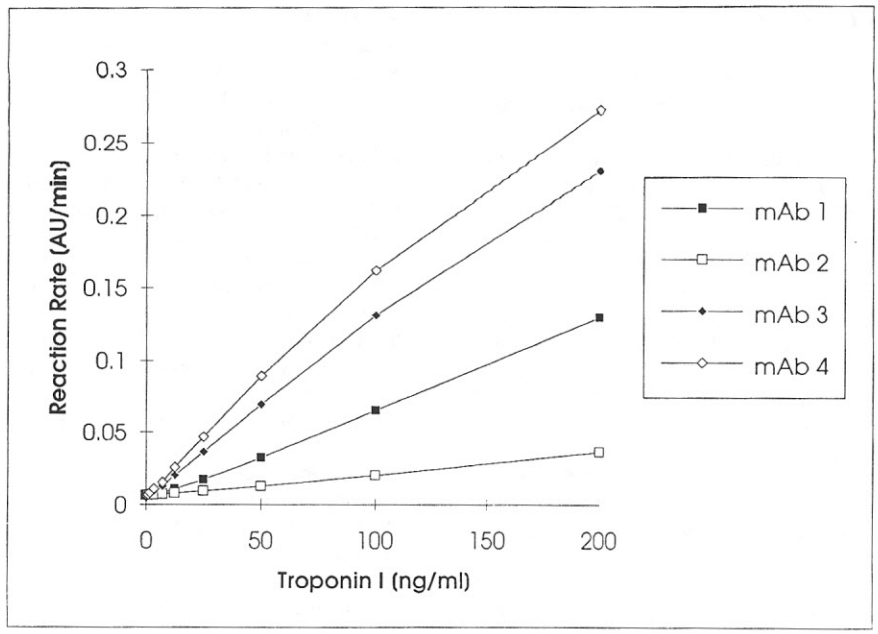

Figure 5: Standard curves with increasing concentrations of human cardiac Troponin I. Detection antibody: alkaline phosphatase conjugate of an avian egg yolk affinity purified anti Troponin I antibody, different monoclonal anti Troponin I capture antibodies: mAb 1, mAb 2, mAb 3, mAb 4. AU: Absorption Units

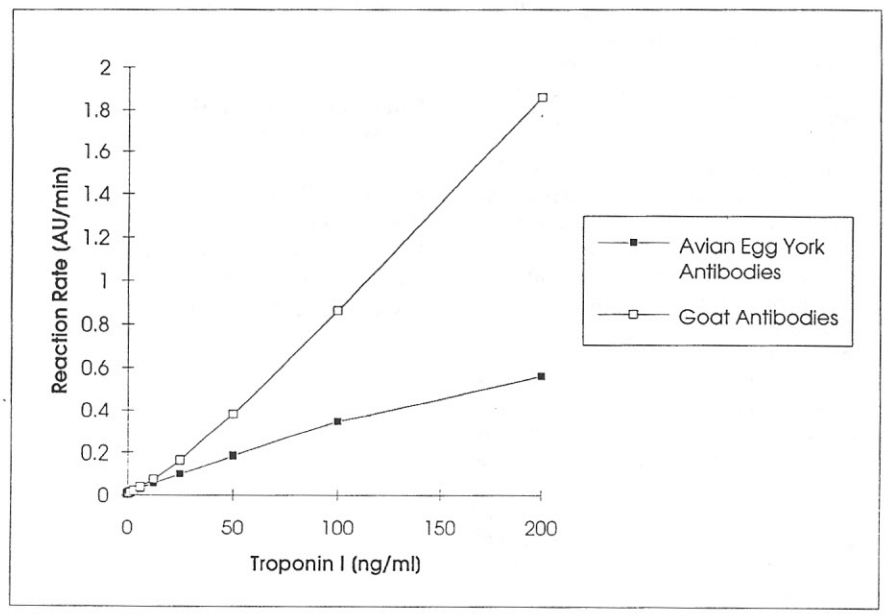

Figure 6: Optimised standard curves for human cardiac Troponin I using different detector antibodies. AU: Absorption Units

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), gelfiltration 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.

References

Adams, J. E. III, Schechtman, K. B., Landt, Y., Ladenson, J. H. and Jaffe, A. S. (1994). Comparable detection of acute myocardial infarction by creatin kinase MB isoenzyme and cardiac troponin I. *Clin. Chem. 40*, 1291–1295.

Apple, F. S., Voss, E., Lund, L., Preese, L., Berger, C. R. and Henry, T. D. (1995).Cardiac troponin, CK-MB and myoglobin for the early detection of acute myocardial infarction and monitoring of reperfusion following thrombolytic therapy. *Clinica Chimica Acta 237*, 59–66.

Bhayana, V., Gougoulias, T., Cohoe, S. and Henderson, A. R. (1995). Disconcordance between results for serum troponin T and troponin I in renal disease. *Clin. Chem. 41*, 312–318.

Bodor, G. S., Porterfield, D., Voss, E. M., Smith, S. and Apple, E. S. (1995). Cardiac troponin-I is not expressed in fetal and healthy or diseased adult human skeletal muscle tissue. *Clin. Chem. 41*, 1710–1715.

Cummins, P., Young, A., Ackland, M., Michie, C. A., Stone, P. C. W. and Shepstone, B. J. (1987). Comparison of serum cardiac specific troponin-I with creatin kinase, creatin kinase-MB isoenzyme, tropomyosin, myoglobin and C-reactive protein release in marathon runners: Cardiac or skeletal muscle trauma. *Eur. J. Clin. Invest. 17*, 317–324.

Kattus, H. A., Schoeppenthau, M., Tanzeem, A., Bauer, H. G., Saggau, W., Diederich, K. W., Hagl, S. and Kuebler, W. (1991). Non-invasive assessment of perioperative myocardial cell damage by circulating cardiac troponin T. *Br. Heart J. 65*, 259–264.

Larue, C., Calzolari, C., Bertinchant, J.-P., Leclercq, F., Grolleau, R. and Pau, B. (1993). Cardiac-specific immunoenzymomatric assay of troponin I in the early phase of acute myocardial infarction. *Clin. Chem. 39*, 972–979.

Lee, T. and Goldman, L. (1986) Serum enzyme assays in the diagnosis of acute myocardial infarction. *Ann. of Intern. Med. 105*, 221–233.

Li, D., Keffer, J., Corry, K., Vazquez, M. and Jialal, I. (1995). Nonspecific elevation of troponin T levels in patients with chronic renal failure. *Clin. Biochem. 28*, 474–477.

Potter J. D. (1982). Preparation of Troponin and its subunits. *Meth. Enzymol. 85*, 241–263.

Rotenberg, Z. (1989). Does determination of serum sapartate amoniotransferase contribute to the diagnosis of acut myocardial infarction. *Am. J. Clin. Pathol. 91*, 91–94.

Schade, R., Bürger, W., Schöneberg, T., Schniering, A., Schwarzkopf, C., Hlinak, A. und Kobilke, H. (1994). Aviäre vitelline Antikörper (Dotterantikörper). Ergebnisse zur Beeinflussung der Legeleistung von Hühnern nach Immunisierung mit Antigenen unterschiedlichster Art und Herkunft sowie zur Leistungsfähigkeit aviärer vitelliner Antikörper im Vergleich zu mammären Antikörpern. *ALTEX 2*, 75–84.

SPECTRUM Diagnostics, Medical Equipment, and Supplies Division Resourses, Inc. (1995). Biochemical markers for the diagnosis of myocardial infarction and other myocardial injuries: market trends and opportunities.

Thompson, W. G, Mahr, R. G., Johannan, W. S. and Pincus, M. R. (1988). Use of creatin kinase MB isoenzyme for diagnosing myocardial infarction when total creatine kinase activity is high. *Clin. Chem. 34*, 2208–2210.

Vaidya, H. C. (1988). Quantitation of lactate dehydrogenase-1 in serum with use of an M-subunit-specific monoclonal antibody. *Clin. Chem. 34*, 2410–2414.

Wong, S. S. (1991). *Chemistry of protein conjugation and cross-linking*. Boca Raton, Florida: CRC Press Inc.

Correspondence address
Dr. Margit Doth
Bayer AG
ZF-FDM, R79
Rheinuferstr. 7-9
D-47829 Krefeld Germany
Phone: +49-2151-883732
FAX: +49-2151-887703