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Partial development of a radioimmunoassay for horse metallothionein*

CHRISTER HOGSTRAND, PER-ERIK OLSSON and CARL HAUX†

Department of Zoophysiology, University of Göteborg, P.O. Box 25059, S-400 31 Göteborg, Sweden

Abstract: The partial development and evaluation of a radioimmunoassay for horse metallothionein (MT) with low amounts of antigen is described. Factors that affect the yield of the conjugation reaction between horse MT and bovine IgG are discussed. Dot-blot has been used as a simple, rapid and inexpensive test for antibody screening.

Keywords: *Radioimmunoassay; metallothionein; horse; polyclonal antibodies; dot-blot.*

Introduction

Metallothioneins (MT) are low molecular weight (6000 daltons), cysteine-rich, heavy metal binding proteins that lack aromatic amino acids and histidine. MT or MT-like proteins have been demonstrated in a wide variety of eucaryotes, including vertebrates and plants [1]. Available information suggests a function of MT in zinc and copper homeostasis and there is also evidence for involvement of MT in the detoxification of heavy metals [2, 3].

Rapid, specific and sensitive quantification of MT has been a problem and few effective and practical immunoassays have been successfully developed [4–8]. Problems encountered during development of antibodies against MT are related to the tendency of MT to polymerize and the changes that occur if metals are stripped of the protein. Moreover, MT seems to be a weak immunogen even if it is coupled to carrier molecules. Vander Mallie and Garvey [5] made high molecular weight polymers of rat-MT, which were found to be more immunogenic than monomeric MT. Antibodies produced by this method were bound to two antigenic determinants near the NH_2-terminal [9]. The regions involved are highly conservative and differ only in one amino acid between rat and rabbit [10]. It has been proposed that the poor immunogenicity of the protein could be due to the high degree of structural similarity between MT from different species [7].

Several methods have been used to increase the antigenicity of MT [5–7]. Highest avidity and antibody titre have been achieved with a MT–IgG conjugate that was injected into sheep [7].

Production of polyclonal antibodies against MT has involved injection of substantial amounts of MT. High amounts of immunogen may induce immunological tolerance

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† Correspondence to: Carl Haux, Department of Zoophysiology, P.O. Box 25059, 400 31 Göteborg, Sweden.

rather than immunoresponse [11]. Moreover, the use of low doses of immunogen is more likely to give rise to high-avidity antibodies [11].

The aim of the present study was to evaluate and optimize methods to create a specific radioimmunoassay (RIA) for MT with low amounts of antigen.

Experimental

Preparation of antigen

Antigen was prepared by conjugating horse-MT (Sigma) to bovine IgG with glutaraldehyde. The reaction conditions were optimized in respect of buffer type, ionic strength, pH, temperature, reaction time and proportions of the involved reactants. The amount of glutaraldehyde added, the molar ratio MT : IgG and the total protein concentration were found to be the most important variables influencing the yield of the reaction. In its final version, 200 μg of MT and 400 μg of IgG (molar ratio 11 : 1) were conjugated with 25 μl of 25% (*m/m*) glutaraldehyde and diluted to 250 μl with 50 mM phosphate buffer, pH 7.5. The mixture was allowed to react for 2 h before it was prepared for injection into rabbits. The yield of the reaction was monitored by measuring the amount of Cd that was incorporated in the IgG peak of a Sephadex G-25 column.

Immunization

Conjugated horse-MT (200 μg MT), dissolved in 2 ml of 50 mM phosphate buffer, pH 7.5, was emulsified with an equal volume of Freund's complete adjuvant and injected into two male NZW-rabbits with multiple-site intradermal injections. Booster injections were given after 6, 15, 22 and 28 weeks in accordance with the same procedures.

Iodination of MT

Metallothionein was labelled with 18.5 MBq [^{125}I]Bolton–Hunter reagent (Amersham), which had a specific activity of 74 TBq mmol^{-1}.

The benzene–dimethylformamide solvent, in which the reagent is supplied, was evaporated with N_2 (H_2O < 1/50,000). Five micrograms of horse-MT in 10 μl of 0.1 M borate buffer, pH 9.0, was added to the dried iodinated ester and the reaction mixture was stirred for 1 h on ice. Unreacted ester was reacted with 0.5 ml of 0.2 M glycine for 5 min and 0.5 ml of 50 mM Tris-HCl, pH 8.0, containing 0.1% (*m/v*) gelatin and 0.1% (*m/v*) NaN_3, was added. The labelled MT was separated from other labelled products on a 10-ml Sephadex G-25 column, equilibrated with the same buffer.

The yield of the iodination was normally 50% and the specific activity of the labelled protein was typically 2.2 MBq μg^{-1} MT.

Detection of antibodies

Sera from the treated rabbits were regularly checked for content of MT-antibodies by specific binding to labelled MT. Since no labelled MT was found to bind to the sera 13 weeks after the primary injection (Fig. 1), dot-blot was used at the next serum check as a supplementary antibody screening method.

Titre curves of the antisera were prepared with double serial dilutions, ranging from 1 : 250 to 1 : 8000 in 50 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, 0.1% (*m/v*) gelatin, 0.1% (*m/v*) NaN_3, 0.4% (*v/v*) normal rabbit serum and 30,000 cpm ^{125}I-MT. After 24 h incubation at 4°C, the antigen–antibody complex was precipitated with goat anti-rabbit IgG, which was allowed to react with rabbit IgG for 24 h at 4°C. The

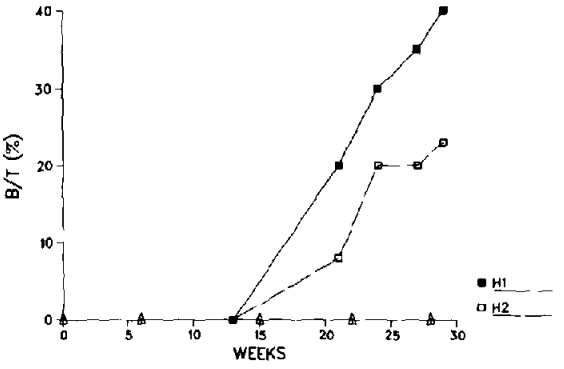

Figure 1
Development of the ability of rabbit antisera H1 and H2 (in 1 : 500 dilution) to bind ^{125}I-MT up to 30 weeks after the primary injection. Each point represents the mean of two samples and the arrows denote MT injections. The diluted antisera were allowed to react with 30,000 cpm ^{125}I-hMT, and the antigen–antibody complex was precipitated with goat-anti-rabbit IgG after 24 h in 4°C.

precipitates were centrifuged at 1700 *g* and 4°C for 1 h, whereupon the supernatants were decanted and ^{125}I in the precipitates counted in a gamma counter.

Dot-blot was performed, with Bio-Rad immun-blot (GAR-HRP) assay kit, using a first antibody dilution of 1 : 100.

Results

The yield of the optimized conjugation reaction between MT and IgG was 80–90%, determined by the distribution of the MT-peak on a Sephadex G-75 column (Fig. 2).

The amount of glutaraldehyde added, the molar ratio MT : IgG and the total protein concentration were found to affect the yield of the conjugation reaction between horse MT and bovine IgG. An increase in glutaraldehyde and protein concentrations resulted in an increased amount of MT bound to IgG (data not shown). However, at higher glutaraldehyde and protein concentrations, the conjugate started to form precipitating complexes. Optimum levels of glutaraldehyde (2.5%) and the total protein concentration (2.4 $\mu g\ \mu l^{-1}$) were chosen to give maximal incorporation of MT into IgG without any precipitation of the product. A decreased MT/IgG ratio increased the yield of the reaction. Since highly substituted carriers are usually most effective as immunogens [12], a molar ratio MT : IgG of 11 : 1 was chosen.

The specific activity of MT labelled with [^{125}I]Bolton–Hunter reagent was typically 2.2 MBq μg^{-1} MT and the yield of the iodination was normally 50%. A Sephadex G-25 chromatographic pattern of the labelled reaction products from iodination is shown in Fig. 3.

The titres of the antisera raised against hMT increased from week 13 after the primary injection throughout the rest of the period (Fig. 1). In a 1 : 250 dilution one antiserum was found to bind 50% of the labelled hMT one week after the last booster injection. The other serum bound 31% of the total activity at the same dilution (Fig. 4).

Both antisera were able to detect a minimum of 0.5 ng hMT by dot-blot 21 weeks after the primary injection.

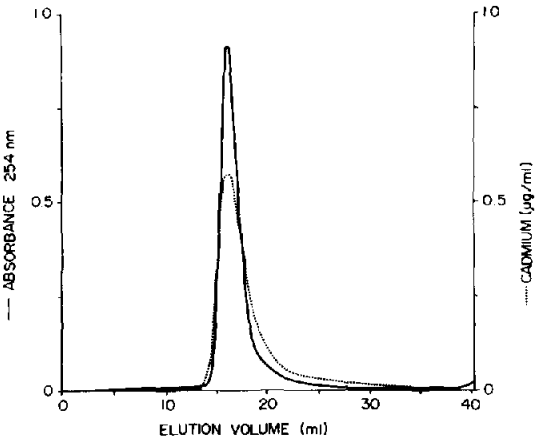

Figure 2
Sephadex G-75 chromatography of the MT–IgG conjugation mixture after 2 h reaction at room temperature. 200 μg of MT was added to 400 μg of IgG in 250 μl of 50 mM phosphate buffer, pH 7.5, containing 2.5% glutaraldehyde. The MT–IgG conjugate is eluted in the void volume of the column; free MT is eluted after 28–33 ml.

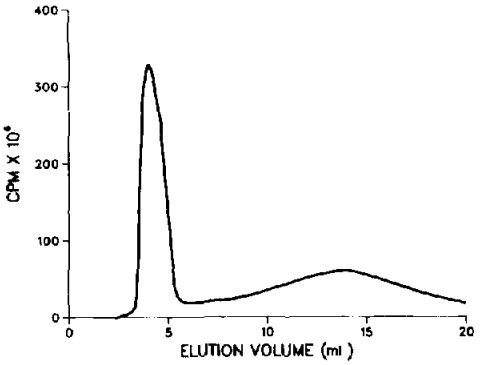

Figure 3
Separation of ^{125}I-MT from other iodinated products on Sephadex G-25 after conjugation of [^{125}I]Bolton–Hunter reagent to MT. The column was eluted with 50 mM Tris-HCl, pH 8.0, containing 0.1% gelatin and 0.1% NaN_3.

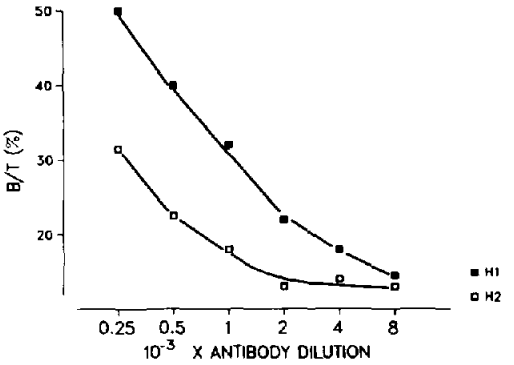

Figure 4
Titration curves of the two antisera against hMT, which were collected one week after the last booster injection. The diluted antisera were allowed to react with 30,000 cpm ^{125}I-hMT and the antigen–antibody complex was precipitated with goat-anti-rabbit IgG after 24 h in 4°C.

Discussion

A hapten conjugate between MT and IgG has been shown to be an effective inducer of MT-antibodies with high avidity [7]; 35% incorporation of MT to IgG was reported. With the optimized reaction conditions used in the present study, a 80–90% yield of the conjugation was achieved.

The rat-MT antibodies produced with MT–IgG conjugate as immunogen were raised in sheep and each immunized sheep gave high-titre antibodies [7]. In the present study both rabbits immunized with MT–IgG conjugate developed antibodies against MT; this may indicate that rabbits could be equally good as sheep in MT-antibody production.

In RIAs, tracers with high specific activity are desirable in order to increase the sensitivity of the assay. Since MT lacks aromatic amino acids as well as histidine, it cannot be iodinated by a direct method. Brady and Kafka [4] tried several different approaches to label rat-MT, including incorporation of ^{109}Cd, ^{65}Zn, [^{35}S]cysteine and [^{125}I]Bolton–Hunter reagent into the protein. Of these methods, labelling with ^{109}Cd was found to give the highest yield and specific activity. A disadvantage of this tracer is that ^{109}Cd is not covalently bound to MT and therefore may be lost to other compounds during the assay. Other authors [5–7] have successfully iodinated MT with Bolton–Hunter reagent as a coupling agent. This method seems superior to ^{109}Cd-labelling since it usually gives higher specific activity and ^{125}I is covalently bound to the tracer. In the present study, horse-MT was labelled with [^{125}I]Bolton–Hunter reagent. This agent reacts predominantly with the side-group of lysine residues. Since the side-chain of lysine has a pK_a value of 10.8, pH 9 was used in the reaction buffer instead of pH 8.5 as recommended by the manufacturer. Both the yield of the iodination and the specific activity of labelled MT increased markedly after this change in pH. The specific activity of labelled MT (2.2 MBq μg^{-1}) obtained in the present study confirms that Bolton–Hunter reagent is highly suitable for iodination of MT.

In the present study, dot-blot was used as a complementary method for antibody screening. Dot-blot was found to be a simple and rapid test for antibody screening. In addition this method is also inexpensive since no instrumentation is needed.

The titres of antibodies raised against horse-MT showed a progressive increase throughout the study. A preliminary standard curve indicated that the 50% bound ($B_0/B \times 100$) intercept value after the last booster injection was 10 ng horse MT with serum H1. This is about the same value as presented by Vander Mallie and Garvey [5] and Tohyama and Shaikh [6], while the 50% intercept value in the RIA published by Mehra and Bremner [7] was 1.2 ng rat MT I.

The methods used to produce polyclonal antibodies for horse MT reported in the present study have in this laboratory been found to be equally suitable for perch MT. Thus these methods may be useful in the development of RIAs for MT from other species.

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