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IMMUNOAFFINITY METHODS IN RADIOLABELLING OF ANTIBODIES

A critical assessment using I-125-anti-β-chorionic gonadotropin as an example.

Fu Li-cheng* and Roger Y. Andres+

* National Institute for the Control of Pharmaceuticals and Biological Products
Temple of Heaven
Beijing, China

+ Swiss Federal Institute of Reactor Research
CH - 5303 Würenlingen, Switzerland

SUMMARY

The usefulness of immunoaffinity methods for the preparation of biologically fully active radiolabelled antibodies was investigated, using anti-β-chorionic gonadotropin as a model substance. Immunoaffinity purification increased the fraction of antigen binding antibody after previous labelling from 25 % to 81 %. Radiolabelling of anti-β-chorionic gonadotropin while bound to the antigen resulted in biologically highly active material, possibly due to the protection of the immunologically active sites on the antibody during the labelling procedure. Immunoaffinity purification prior to labelling did not result in biologically active material. Elution yields from immunoaffinity supports were generally low.

Key Words: Immunoaffinity purification
Radiolabelling of antibodies
Anti- β -chorionic gonadotropin

INTRODUCTION

In modern medical diagnostic procedures, radiolabelled monoclonal antibodies are increasingly used, e.g. in vitro for radioimmunoassay or in vivo for the localization of tumors (1), myocardial infarction (2) or infectious lesions (3). No general labelling technique for these materials exists, and an individual approach has to be found and optimized in each case. This is due to the fact that antibodies are differentially sensitive to labelling conditions or to the structural modifications caused by label incorporation (4). Occasionally it is impossible to label an antibody with complete retention of biological activity and a purification procedure is necessary to yield a optimal radiolabel. Among the most powerful purification procedures known are the immunoaffinity methods based on the strong and specific binding interaction between antibody and antigen. A model system was therefore established in order to test the efficiency of these methods for the preparation of labelled antibodies with intact biological activity. As a model antibody anti-β-chorionic gonadotropin

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Time of labelling relative to affinity purification	Step 1	Step 2	Step 3
Before	Label AB + — I-125 Label AB − — I-125	Bind AG — AB + — I-125 Discard AB − — I-125	Elute AB + — I-125
After	Bind AG — AB + Discard AB −	Elute AB +	Label AB + — I-125
During	Bind AG — AB + Discard AB −	I-125 Label AG — AB + — I-125	Elute AB + — I-125

Figure 1

Schematic representation of the immunoaffinity methods used in this study. AB+: antibody capable of binding to the antigen; AB- immunologically inactive material; AG: Antigen (β-HCG) coupled to Sepharose.

(anti-β-HCG) was chosen because of the easy access to substantial amounts of antigen (β-HCG). Although not all results found here can be expected to apply to all other antibodies, this model system proved to be useful to draw some general conclusions.

In principle, three alternative ways exist for the application of immunoaffinity methods (see Fig. 1): The antibody can be affinity purified **before** or **after** labelling, or it can be labelled **while** bound to the antigen. With the model system, all three possibilities have been investigated, using two different labelling techniques: Iodogen or N-bromosuccinimide. Despite the fact that both of these procedures rely on oxidation of the iodide, there are clear differences in their performance: iodogen labelling proceedes along the surface of the vessel only, while N-bromosuccinimide is active in the bulk of the solution.

MATERIALS AND METHODS

Materials:

The materials used in this study were purchased from the following sources: Anti-β-HCG (clone 5005, lot 013, 1 mg/ml in 0.9 % $^{w}/_{v}$ sodium chloride, affinity purified) from Medix Biochemica, Kauniainen, Finnland; β-HCG (3918 IU/mg) and N-bromosuccinimide from Sigma, St. Louis Mo. USA; iodogen and BCA-protein assay reagent from Pierce, Rockford Ill. USA; human serum albumin from the Swiss Red Cross, Berne, Switzerland; CNBr-activated Sepharose-4B and Sephadex G-25 from Pharmacia, Uppsala, Sweden; I-125 (sodium iodide) from Atomic Energy of Canada Ltd., Ontario, Canada. All other reagents, of analytical grade or better, were purchased from local suppliers.

Methods:

The antigen (β-HCG, 20 mg) was covalently coupled to CNBr-activated Sepharose (2 g) according to the manufacturers instructions (5), resulting in 4.8 ml of settled gel containing 8 mg of β-HGG (representing a coupling yield of 40 %).

The biological activity of labelled antibody preparations was determined by incubation of 4 µl, and serial dilutions thereof (6), of β-HCG-Sepharose with I-125-anti-β-HCG (0.5 - 10 nCi) in a total volume of 500 µl of 10 mmol/l potassium phosphate, pH 7.5 containing 0.15 mol/l sodium chloride and 1 g/l human serum albumin (PBS/HSA). After reaching binding equilibrium (18 hrs, shaking at room temperature) the β-HCG-Sepharose was washed twice with 2 ml PBS/HSA. The pellet was counted and maximal binding was calculated by extrapolation to infinite

antigen concentration (6). The fraction of protein bound radioactivity was determined by precipitation of the protein with trichloracetic acid (final concentration: 12.5 % $^{w}/_{v}$) at 4°C. After centrifugation at 2000 x g for 10 min, the supernatant was removed and the pellet was counted.

For radiolabelling of the antibody a reaction glas tube was prepared by drying 200 µl of a solution of idogen (0.1 mg/ml in ethyl acetate) under a stream of nitrogen. Prior to the labelling reaction, all materials were cooled to 0 °C. To the reaction tube 100 µl of 0.1 mol/l potassium phosphate, pH 7.5, was added, followed by 120 µg of antibody (1 mg/ml) and 0.5 mCi I-125. After 10 min at 0°C with shaking the reaction was stopped by transfer to 200 µl of PBS/HSA. Unbound radioiodine was removed by passage over a Dowex 1 x 8 column (0.5 x 2 cm), equilibrated and eluted with PBS. Radiolabelling with N-bromosuccinimide was performed by mixing 120 µg of antibody (1 mg/ml) with 100 µl of 0.1 mol/l potassium phosphate pH 7.5 and 10 µl N-bromosuccinimide (1 mg/ml) in the same buffer. After 30 sec the reaction was quenched with tyrosine (100 µl, 0.2 mg/ml in the same buffer), and unbound iodine was removed as above.

Antigen bound anti- β-HCG was labelled as follows: 100 µl of β-HCG-Sepharose was washed with 0,5 ml of 3 mol/l potassium iodide containing 10 % $^{w}/_{v}$ of glycerol and 4 times with 5 ml of PBS in order to remove loosely bound material. Anti-β-HCG was then added (200 µg in 200 µl). After 2 hrs at room temperature, unbound material was removed by washing 3 times with 5 ml of PBS. After the addition of 100 µl of 0.1 mol/l potassium phosphate, pH 7.5, the slurry was labelled with N-bromosuccinimide as described above. After labelling, the Sepharose was washed 3 times with 5 ml PBS/HSA.

Elution of antigen bound antibody was performed by incubation of the Sepharose slurry (usually 100 µl) with 5 volumes of the eluant (see Table 1). The whole material was then transferred to the top of a Sephadex G-25 column (0.5 x 4 cm), equilibrated and eluted in PBS/HSA (PBS without HSA, if the affinity procedure was performed before labelling). The protein fraction (void volume) was collected for further use.

RESULTS AND DISCUSSION

Basic experiments

The antigen binding capacity (=biological activity) of the unlabelled antibody was tested by the determination of the fraction of protein incapable of binding to β-HCG- Sepharose. In multiple determinations it was found that 61.5 % ± 3 % (mean ± 1 SD, n=9) of the protein is not bound to the solid phase antigen. According to information from the manufacturer, the antibody was purified by

Table 1: Evaluation of various immunoaffinity eluants

Eluant	Biological activity (%)
Potassium iodide (3 mol/l)* containing 10 % w/v glycerol	83
Potassium iodide (3 mol/l)*	71
Potassium thiocyanate (4 mol/l)*	47
Magnesium chloride (4 mol/l)*	54
Guanidine hydrochloride (6 mol/l)*	28

* in water, pH adjusted to 7.

Biological activity of I-125-anti-β-HCG after binding to and elution from β-HCG-Sepharose using various elution media (for details see METHODS). The biological activity represents triplicate determinations of a single elution experiment.

affinity chromatography. The immunologically inactive material is therefore an antibody, presumably damaged by harsh elution conditions (see INTRODUCTION) used for antibody purification.

Labelling of the untreated antibody with I-125 was then performed. Using two different oxidants, iodogen and N-bromosucciminide, biological activities of 25 % (see Table 2) were found. The specific radioactivity of the label was approximately 3μCi/μg (111 kBq/μg) for iodogen labelling and 1.5 μCi/μg (56 kBq/μg) when using N-bromosuccinimide. In addition to this low antigen binding capacity it was found that in the final preparation the amount of radioactivity which could not be precipitated by TCA was 10 % to 20 % (Table 2). This free or loosely bound radionuclide could not be removed by passage over Sephadex G-25.

After the first experiments the following preliminary conclusions were drawn: Firstly, the antibody preparation is contaminated with more than 60 % of inactive protein, and secondly, oxidation labelling results in some structural damage. The latter conclusion is supported by results from labelling with Bolton-Hunter reagent. Biological activities close to the maximally expected value for unpurified material (approximately 40 %) have been found. The reduction to 25 % (see above) when using oxidative labelling could therefore be caused by unwanted side reactions or introduction of the radionuclide into a tyrosine at or close to the antigen binding site (4).

Table 2: Summary of labelling results

Labelling method / Processing of the antibody	Iodogen			N-Bromosuccinimide		
	Biological activity %	Proteinbound radioactivity %	Yield %	Biological activity %	Proteinbound radioactivity %	Yield %
No affinity procedure used:						
mean	25	90	81*	24	81	32*
standard deviation	± 1	± 2	± 7	± 3	± 4	±11
Affinity purification after labelling:						
mean	81	98	11+	55	98	6+
standard deviation	± 2	± 1	± 2	± 5	± 1	± 2
Labelling of antibody during affinity purification:						
mean	not possible			83	98	< 5+
standard deviation				± 2	± 1	-

The data represents the mean and standard deviations of three separate iodinations

* based on the radioactivity used for labelling

\+ based on radioactivity added to the β-HCG-Sepharose

Due to the multiple problems encountered with this radiolabelling procedure and because of the virtually unrestricted availability of antigen, this system was chosen as a model for testing affinity procedures to improve the quality of the labelled product.

Affinity purification of labelled antibody

The tight binding of the antibody to the antigen necessitates the use of very harsh conditions in order to elute the antibody from immunoaffinity supports. Low pH and high concentration of chaotropic salts have been used (7), possibly resulting in inactivated side products. The eluants used often represent a compromise between maximal yield of elution and minimal damage to the protein. Several chaotropic solutions were tried for the elution of I-125-anti-β-HCG from the immunoaffinity support (Table 1). The best eluant of the ones tested proved to be 3 mol/l potassium iodide containing 10 % $^{w}/_{v}$ glycerol, and this was used in all further studies. Purification of anti-β-HCG, when labelled with iodogen, results in biological activities higher than 80 %. All the eluted radioactivity is protein bound. Based on radioactivity a yield of approximately 10 % is found. In light of the fact that maximally 25 % of the original labelled material is biologically active, a yield of 44 % can be calculated with respect to biologically active antibody. Using antibody labelled by N-bromosuccinimide biological activity and yield are inferior.

Affinity purification prior to labelling

Using this method, biologically inactive components can be expected to be removed. Any material damaged by the labelling process, however, is found in the final preparation (Fig. 1).

Based on protein determinations after elution from immunoaffinity columns, a yield of less than 5 % was calculated. Labelling of this material by either iodogen or N-bromosuccinimide did not result in biologically active labelled antibody. A possible explanation is that despite chromatography on Sephadex G-25 (see METHODS) some iodide remains associated with the protein. Even if only a minimal fraction of the high concentration of iodide used for elution is present during labelling, the specific radioactivity of the label is expected to be very low. Alternatively, increased labelling damage could occur because of the dilute nature of these prepurified protein solutions. This procedure was not further evaluated.

Labelling of anti-β-HCG bound to the antigen

This method has the potential advantage that the biologically active site is not freely accessible to nuclide incorporation (see Fig. 1), possibly yielding a radio

labelled antibody with essentially intact biological activity. In fact, labelling of anti-β-HCG bound to the antigen followed by elution with 3 mol/l potassium iodide containing 10 % w/v glycerol results in the highest biological activity found in this study. During the procedure, also β-HCG is labelled resulting in unavoidable loss of radionuclide. This is also reflected in the low radioactive labelling yield (< 5 %). Labelling of antigen bound anti-β-HCG with iodogen was unsuccessful, probably due to the fact that in this labelling method the reaction proceeds along the surface of the vessel only. Antibody bound to the interior of the Sepharose bead would presumably not be labelled.

Conclusions

Immunoaffinity methods can be powerful tools for the improvement of the quality of radiolabelled antibodies. Careful adaptation of the labelling and elution method to the individual characteristics of the antibody is necessary, however. Labelling in the antigen-bound state offers the potential to protect the biologically active site from chemical or structural damage. This method seems particularly useful for the preparation of tracers for radioimmunoassays, where a cheap nuclide is used (I-125) and only small quantities (μCi) are used in an experiment.

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