

OPTIMUM LABELLING OF MONOCLONAL ANTIBODY 3E1.2 WITH  $^{111}\text{In}$   
USING A BIFUNCTIONAL CHELATE

R.M. Reilly\*, G.N. Ege  
Department of Nuclear Medicine,  
The Princess Margaret Hospital,  
Toronto, Canada M4X 1K9

SUMMARY

Monoclonal antibody 3E1.2 was coupled with the bifunctional chelate, cyclic DTPA anhydride, at molar ratios (cDTPAA:3E1.2) of 1:1, 10:1, 100:1 and 1000:1. The desired substitution level of less than one mol of DTPA/mol of 3E1.2 was achieved at a molar ratio of 10:1 and a coupling efficiency of  $9.9 \pm 3.38$  percent. The DTPA-coupled antibody was purified by dialysis before labelling with  $^{111}\text{In}$  acetate. The labelling efficiency was  $38.7 \pm 3.59$  percent. The radiolabelled antibody was purified by PD-10 Sephadex G-25M chromatography then sterilized by filtration through a 0.22  $\mu\text{m}$  filter (Millex-GS or Millex-GV). A substantial adsorption effect ( $87.4 \pm 3.84$  percent) occurs with the Millex-GS filter but to a much smaller extent ( $26.8 \pm 5.58$  percent) with the Millex-GV filter. The radiochemical purity of the product was 80-90 percent depending on the method of analysis. The product was tested and found to be sterile and non-pyrogenic.

Key Words: Monoclonal antibodies,  $^{111}\text{In}$ , Bifunctional Chelates

INTRODUCTION

Radiolabelled monoclonal antibodies directed against tumour-specific antigens have undergone considerable study in recent years as tumour imaging radiopharmaceuticals (1). Using bifunctional chelates it is now possible to label monoclonal antibodies with metallic radionuclides such as  $^{111}\text{In}$  (2-6). The use of  $^{111}\text{In}$  as the radiolabel offers many advantages: efficient detection by the gamma camera, a physical half-life sufficiently long to permit delayed imaging and a relatively low radiation dose to the patient. In addition, the in-vivo stability of monoclonal antibodies labelled with  $^{111}\text{In}$  using bifunctional chelates is excellent (2,4). There is also negligible loss of immunoreactivity after labelling provided the substitution level of the

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\*To whom correspondence should be addressed.

antibody does not exceed one mol of bifunctional chelate/mol of antibody (3,4,6).

In this paper, we describe our approach to  $^{111}\text{In}$  labelling of a new murine monoclonal antibody to human breast carcinoma (3E1.2, an IgM) using the bifunctional chelate, cyclic DTPA anhydride.

#### EXPERIMENTAL

##### Materials

Monoclonal antibody 3E1.2 was generously supplied as murine ascites by Professor I. McKenzie, Research Center for Cancer and Transplantation, University of Melbourne. The antibody was extracted from the ascites using the method supplied by Professor McKenzie then dissolved in 0.05 M bicarbonate buffer pH 7.5 to give a concentration of 8.45 mg/ml.

Bicarbonate buffer 0.05 M in 0.9% sodium chloride pH 7.5 and acetate buffer 1 M pH 6.0 were prepared from analytical grade chemicals and Sterile Water for Injection USP. Trace-metal contamination was minimized by use of acid-washed glassware and by passing the prepared buffers through a column of Chelex-100<sup>®</sup> cation exchange resin (Bio-Rad). The buffers were sterilized by filtration.

Cyclic DTPA anhydride (diethylenetriamine pentaacetic acid anhydride) was purchased from Sigma. Cellulose dialysis tubing (Spectrapor No. 7, Spectrum Medical Industries) was sulphur-free and EDTA treated and had a molecular weight cut-off of 1000. Chromatography supplies included Sephadex G-50 and PD-10 sterile, disposable G-25M columns (Pharmacia) and ITLC-SG instant thin layer chromatography strips (Gelman). Pipette tips (trace-metal free) and micro test tubes were obtained through Brinkman. Sterilizing 0.22  $\mu\text{m}$  filter units were Millex-GS and Millex-GV (Millipore). The limulus amoebocyte lysate reagent (Pyrogen<sup>®</sup>, Mallinckrodt) had a titrated sensitivity of 0.24 E.U./ml.

$^{111}\text{In}$  chloride (370 MBq/ml in 0.04 N hydrochloric acid) was obtained from Amersham. The acid normality was increased to 0.4 N by the addition of 1 N hydrochloric acid.  $^{111}\text{In}$  acetate (in 0.5 M acetate buffer pH 6.0) was prepared by mixing equal volumes of  $^{111}\text{In}$  chloride and 1 M acetate buffer pH 6.0.

## METHOD

A. Determination of the Optimum Molar Ratio of Cyclic DTPA Anhydride : 3E1.2

The coupling efficiency of 3E1.2 with cyclic DTPA anhydride was studied at molar ratios (cyclic DTPA anhydride:3E1.2) of 1:1, 10:1, 100:1 and 1000:1.

Cyclic DTPA anhydride was suspended in chloroform (0.1 - 10.0 mg/ml) and an aliquot (0.24 - 240 ug,  $6.76 \times 10^{-4}$  -  $6.76 \times 10^{-1}$  umols) was dispensed into a 200 ul glass Reacti-Vial<sup>®</sup> (Pierce). The chloroform was then evaporated under a stream of nitrogen. Monoclonal antibody 3E1.2 (507ug,  $6.76 \times 10^{-4}$  umols) in 0.05 M bicarbonate buffer pH 7.5 was added and 30 minutes were allowed for coupling. <sup>111</sup>In acetate (10 MBq) was then added and a further 30 minutes allowed for labelling.

The labelling mixture was analysed by Sephadex G-50 column chromatography (2 cm ID x 20 cm) eluted with 0.9% sodium chloride. Twenty 1.2 ml/minute fractions were collected and the activity of each measured using a radioisotope calibrator (Capintec Model CRC-5). The radiolabelled antibody (<sup>111</sup>In-DTPA-3E1.2) elutes in the void volume of the column (fractions 4-6), <sup>111</sup>In-DTPA in fractions 7-14 and free <sup>111</sup>In binds to the column and is not eluted. The coupling efficiency is equal to the percentage of activity present as <sup>111</sup>In-DTPA-3E1.2. The substitution level of the antibody is obtained by multiplying the coupling efficiency by the molar ratio of cyclic DTPA anhydride :3E1.2.

B. Pre-Labeling Purification of the Coupled Antibody

Monoclonal antibody 3E1.2 (12.67 mg,  $1.69 \times 10^{-2}$  umols) in 0.05 M bicarbonate buffer pH 7.5 was coupled with cyclic DTPA anhydride (60.4 ug,  $1.69 \times 10^{-1}$  umols) at a molar ratio (cyclic DTPA anhydride/:3E1.2) of 10:1 as previously described but under aseptic conditions. Coupling efficiency was determined as before. The coupled antibody was then transferred to dialysis tubing and dialysed against Sterile Water for Injection USP at 4°C for 96 hours with four changes of the water. The precipitated coupled antibody was re-dissolved in 0.05 M bicarbonate buffer pH 7.5 and the concentration determined spectrophotometrically. Aliquots (100 ul, 500 ug of DTPA-3E1.2) were then dispensed into sterile micro test tubes and frozen at -10 °C until required.

Labelling was carried out by allowing a 100  $\mu$ l aliquot of DTPA-3E1.2 to thaw, then adding  $^{111}\text{In}$  acetate (37 MBq) in 0.5 M acetate buffer pH 6.0. The labelling efficiency was determined by Sephadex G-50 chromatography as before.

#### C. Purification and Sterilization of $^{111}\text{In}$ -DTPA-3E1.2

The labelling mixture was diluted to 2.5 ml with Sodium Chloride Injection USP then purified on a sterile, disposable PD-10 Sephadex G-25M column. Twelve 1 ml fractions were collected. Fractions 4-6 contained the purified  $^{111}\text{In}$ -DTPA-3E1.2 and were pooled for sterilization.

The fractions were sterilized by filtration through a 0.22  $\mu$ m filter unit (Millex-GS or Millex-GV). The percentage of  $^{111}\text{In}$  activity remaining bound to the filter was determined.

#### D. Quality Control

Radiochemical purity of  $^{111}\text{In}$ -DTPA-3E1.2 was determined by Sephadex G-50 chromatography as previously described or by instant thin layer chromatography on ITLC-SG strips developed in 85% methanol/15% water. Instant thin layer chromatography separates  $^{111}\text{In}$ -DTPA-3E1.2 ( $R_f=0.0$ ) from  $^{111}\text{In}$ -DTPA ( $R_f=0.4-0.5$ ) and free  $^{111}\text{In}$  ( $R_f=1.0$ ).

The sterility of the product was determined by incubating a sample on agar at 35 °C for 48 hours. The agar plate was then visually inspected for bacterial colonies, which if present were subsequently identified.

The product was tested for the presence of pyrogens by the Limulus Amebocyte Lysate technique (7). Dilutions (1/100 and 1/1000) were made with Sterile Water for Injection USP for testing.

### RESULTS AND DISCUSSION

#### A. Determination of the Optimum Molar Ratio of Cyclic DTPA Anhydride:3E1.2

The coupling efficiency of 3E1.2 with cyclic DTPA anhydride at molar ratios of 1:1, 10:1, 100:1 and 1000:1 is shown in Table 1-A. In Table 1-B are the values obtained for human IgG coupled under identical conditions. For all molar ratios except 1000:1 the coupling efficiency of 3E1.2 is much lower than that of IgG. In addition there seems to be a different relationship between molar ratio and coupling efficiency for 3E1.2 than for IgG. As the molar ratio

**Table 1-A: The Effect of Molar Ratio (cDTPAA:3E1.2) on Coupling Efficiency and Substitution Level for Monoclonal Antibody 3E1.2<sup>1</sup>**

MOLAR RATIO cDTPAA:3E1.2	n	COUPLING EFFICIENCY <sup>2</sup> PERCENT (MEAN ± SD)	MEAN SUBSTITUTION LEVEL (MOLS DTPA / MOL 3E1.2)
1:1	5	3.1 ± 1.20	0.03
10:1	8	9.9 ± 3.38	0.99
100:1	5	26.7 ± 5.32	26.70
1000:1	6	37.2 ± 5.90	372.00

<sup>1</sup> Monoclonal Antibody 3E1.2 8.45 mg/ml in 0.05M bicarbonate buffer pH 7.5

<sup>2</sup> Determined by Sephadex G-50 chromatography using 0.9% sodium chloride after labelling with <sup>111</sup>In.

**Table 1-B: The Effect of Molar Ratio (cDTPAA: IgG) on Coupling Efficiency and Substitution Level for Human IgG<sup>3</sup>**

MOLAR RATIO cDTPAA:IgG	n	COUPLING EFFICIENCY <sup>4</sup> PERCENT (MEAN ± SD)	MEAN SUBSTITUTION LEVEL (MOLS DTPA / MOL IgG)
1:1	2	63.3 ± 5.73	0.63
10:1	3	69.4 ± 3.41	6.94
100:1	3	49.4 ± 3.47	49.40
1000:1	3	2.6 ± 1.27	26.00

is increased from 1:1 to 1000:1, an increase in coupling efficiency is observed for 3E1.2 whereas a decrease is observed for IgG. This could possibly be explained by differences in the ability of IgM (3E1.2) and IgG to couple with bifunctional chelates such as cyclic DTPA anhydride. Although the relationship between molar ratio and coupling efficiency has been previously described for IgG (2), no comparable data is available in the literature for IgM. This is one area for further investigation.

In order to preserve immunoreactivity, a substitution level of no more than one mol of DTPA/mol of antibody is desirable (3,4,6). This substitution level is achieved for 3E1.2 at molar ratios of 1:1 or 10:1. To obtain as high

<sup>3</sup> Human IgG 8.45 ml/ml in 0.05 M bicarbonate buffer pH 7.5

<sup>4</sup> Determined by Sephadex G-50 chromatography using 0.9% sodium chloride after labelling with <sup>111</sup>In.

a specific activity as possible for the radiolabelled antibody, a molar ratio of 10:1 which results in a substitution level of approximately one mol of DTPA/mol of 3E1.2 was chosen.

#### B. Pre-Labeling Purification of the Coupled Antibody

When labelling with  $^{111}\text{In}$ , a competition exists between free and antibody-coupled DTPA for the radioisotope. If the coupled antibody is not purified prior to labelling, then the percentage of  $^{111}\text{In}$  labelled antibody and  $^{111}\text{In}$ -DTPA simply reflects the proportions of free and antibody-coupled DTPA in the preparation. In such a case, the labelling efficiency is equal to the coupling efficiency.

However, if the proportion of free DTPA in the coupled antibody preparation is reduced through purification, then a labelling efficiency greater than the coupling efficiency is possible. Table 2 clearly illustrates this point for DTPA-coupled 3E1.2.

Table 2: Effect of Pre-Labeling Purification<sup>5</sup> of DTPA-Coupled 3E1.2 on the Labelling Efficiency and Levels of Radiochemical Impurities

DTPA-3E1.2	n	PERCENT (MEAN $\pm$ SD)		
		FREE $^{111}\text{In}$	$^{111}\text{In}$ -DTPA	$^{111}\text{In}$ -DTPA-3E1.2
UNPURIFIED	8	11.8 $\pm$ 2.21	78.4 $\pm$ 4.16	9.9 $\pm$ 3.38
PURIFIED	5	18.7 $\pm$ 2.90	42.6 $\pm$ 3.42	38.7 $\pm$ 3.59

The dialysis-purified preparation shows an almost four-fold increase in labelling efficiency (percentage of  $^{111}\text{In}$ -DTPA-3E1.2) over the unpurified preparation. At the same time the percentage of  $^{111}\text{In}$ -DTPA has decreased by almost one-half. The slight increase in the percentage of free  $^{111}\text{In}$  in the purified preparation may be due to a decrease in the total number of DTPA molecules (free + antibody-coupled) available for chelation.

Interference by trace metal contamination in the labelling of antibodies with  $^{111}\text{In}$  using bifunctional chelates has been previously described (2). This

<sup>5</sup> Purified by dialysis against Sterile Water for Injection USP for 96 hours.

was also our experience during initial <sup>111</sup>In labelling trials of 3E1.2. The problem was very effectively overcome however, by the use of Chelex-100<sup>®</sup> cation exchange resin to purify the buffers.

C. Purification and Sterilization of <sup>111</sup>In-DTPA-3E1.2

Although the labelling efficiency of the purified DTPA-coupled antibody was substantially increased in the purified preparation, the resulting radiochemical purity of <sup>111</sup>In-DTPA-3E1.2 was still less than 40 percent. Therefore post-labelling purification by PD-10 Sephadex G-25M chromatography was performed. Using this purification method, 43.5±6.90 percent of the <sup>111</sup>In activity was collected in fractions 4-6 ( <sup>111</sup>In-DTPA-3E1.2), 46.5±6.90 percent in fractions 7-12 (<sup>111</sup>In-DTPA) and 9.8±2.45 percent remained bound to the column and could not be eluted (free <sup>111</sup>In). These values are similar to those observed earlier by Sephadex G-50 analysis. The selection of the particular type of 0.22 um filter to be used for sterilization of the purified <sup>111</sup>In-DTPA-3E1.2 proved especially crucial. The labelled antibody was almost quantitatively adsorbed (87.4±3.84%) by the commonly used Millex-GS filter. The adsorption phenomenon was much less (26.5±5.53%) with the Millex-GV filter which is designed for low binding sterilization of proteins.

D. Quality Control

Table 3: Radiochemical Purity of <sup>111</sup>In-DTPA-3E1.2

ANALYTICAL METHOD	n	PERCENT (MEAN ± SD)		
		FREE <sup>111</sup> In	<sup>111</sup> In-DTPA	<sup>111</sup> In-DTPA-3E1.2
SEPHADEX-G-50 using 0.9% Sodium Chloride	6	11.2 ± 1.25	10.2 ± 5.46	78.5 ± 5.62
ITLC-SG using 85% Methanol/15% Water	5	0.6 ± 0.51	7.0 ± 3.53	91.4 ± 1.28

The radiochemical purity of <sup>111</sup>In-DTPA-3E1.2 was found to be greater than 90 percent by instant thin layer chromatography but only about 80 percent by Sephadex G-50 analysis (Table 3). While the levels of <sup>111</sup>In-DTPA were similar when determined by either method, there was a discrepancy in the percentage of free <sup>111</sup>In. The higher values for free <sup>111</sup>In determined by Sephadex G-50 chromatography could be explained by non-specific binding of a proportion of

the radiolabelled antibody to the resin. It is possible that the actual radiochemical purity may be more accurately measured by the instant thin layer chromatography method.

Results of sterility tests (n=6) of the product showed no microorganisms present. Limulus amebocyte lysate tests (n=6) for bacterial endotoxin were also negative.

#### CONCLUSIONS

The coupling of monoclonal antibody 3E1.2 with cyclic DTPA anhydride was optimized to achieve a substitution level of one mol of DTPA/mol of 3E1.2. The DTPA-coupled antibody was then purified by dialysis to yield a preparation which could be labelled with  $^{111}\text{In}$  with a labelling efficiency of approximately 40 percent. The radiolabelled antibody was then purified on a PD-10 Sephadex G-25M column to give a product which was 80-90 percent radiochemically pure. The product was most effectively sterilized by filtration through a 0.22  $\mu\text{m}$  Millex-GV filter designed for low-binding sterilization of proteins. The product was tested and found to be sterile and non-pyrogenic.

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