

A RAPID AND EFFICIENT METHOD FOR PREPARING  
PURIFIED RADIO-LABELED ANTIBODY:  
Use in Solid-Phase Radioimmune Assays\*

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## INTRODUCTION

Radiolabeled anti-immunoglobulin antibody preparations used to measure immunoglobulin on cell surfaces or bound to solid phase immunoabsorbants must contain a high proportion of labeled antibody molecules capable of specifically combining with the determinant(s) being measured. Antibody to be radiolabeled is specifically purified before labeling, usually by binding to antigen coupled to an insoluble matrix and subsequent elution in the absence of carrier protein, i.e., by affinity chromatography. This procedure, however, has two drawbacks for labeling small amounts of antibody. First, elution in the absence of carrier frequently results in the loss of a large fraction of the eluted antibody due to non-specific adsorption on container surfaces; and second, iodination of the free antibody molecule destroys a further fraction of the antibody (particularly mouse antibody) probably because iodine binds to tyrosine residues in or near the active site.

These problems are overcome by radiolabeling the antibody while it is specifically bound to insolubilized antigen ( 1 ). Using this method, the antibody combining site is protected during iodination and the antibody may be eluted afterward, in the presence of carrier protein to avoid non-specific adsorption losses. Data is presented here on the isolation and radiolabeling of antibody from a mouse anti-allotype antiserum (anti-Ig-1a allotype on IgG<sub>2a</sub> immunoglobulins) and the utilization of the antibody in highly sensitive binding assays which measure the Ig-1a allotype levels in mouse sera, and the Ig-1a allotype representation in anti-dinitrophenyl (anti-DNP) antibodies in sera from DNP immunized mice.

## MATERIALS AND METHODS

### Insoluble Antigen Matrix

GPC-8 (Ig-1a), C.BPC101 (Ig-1b), S8 (Ig-4a), and MOPC-245 (Ig-4b) myeloma proteins were individually conjugated to cyanogen bromide-activated Sepharose. The quantity of myeloma protein bound to the Sepharose was estimated by determining the amount of protein consumed.

### Alloantisera

Anti allotype antisera were prepared as previously described ( 2 ).

### Anti-DNP Sera

C3H.SW (Ig<sup>a</sup>) and CWB (Ig<sup>b</sup>) mice were primed with an intraperitoneal injection of 100 µg of dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH) on alum with  $2 \times 10^9$  B. pertussis organisms. Six weeks later, they were boosted intravenously with 10 µg of aqueous DNP-KLH and were bled after five days.

### Affinity Chromatography Radioiodination

The column used to hold the immunoabsorbant consisted of a Beckman microfuge tube (400 µl capacity) with its bottom tip cut off and plugged with glass wool. Sephadex G-25 was packed over the glass wool to a bed volume of 100 µl. Sepharose-myeloma protein containing about

50  $\mu$ g of protein (10  $\mu$ l bed volume) was layered onto the Sephadex. For maximal specificity of labeled reagents, the volume of Sepharose-myeloma protein should be limiting with respect to the amount of antibody used. The Sephadex G-25 was used to create a manageable bed volume and to achieve a faster separation of eluted protein from the acidic solution used for elution.

Immediately before use, the column was washed with 1-2 ml of 0.2 M glycine-HCl, pH 2.3, and neutralized with phosphate buffered saline (PBS), pH 7.5. Alloantiserum (500  $\mu$ l) was passed through the column (4 drops/min) to saturate all binding sites on the immunoabsorbant. The column was then thoroughly washed with 3-5 ml of PBS (14 drops/min). Gentle positive pressure was applied when needed to achieve the drop rate.

The chloramine-T method ( 3 ) was used to radioiodinate the bound antibody. Chloramine-T (20  $\mu$ g) with 1 mCi of  $^{125}$ I (IMS30, Amersham/Searle Corp., Arlington Hts., IL) in 35  $\mu$ l of 0.05M phosphate buffer, pH 7.5, was layered onto the column. The Sepharose and the top of the Sephadex bed were mixed in this "cocktail" for 15-30 sec, then sodium metabisulfite (100  $\mu$ g in 25  $\mu$ l of 0.05 M phosphate buffer, pH 7.5) was added to the slurry and the column washed immediately with 3-4 ml of PBS.

The labeled antibody was eluted with 0.2 M glycine-HCl containing 1% bovine serum albumin (BSA), pH 2.3 (14 drops/min). Two-drop fractions (approximately 60  $\mu$ l/drop) were collected into 120  $\mu$ l of 0.3 M borate buffer, containing 1% BSA and 0.2% sodium azide, pH 8.6. The percentage of protein-bound  $^{125}$ I in each fraction was determined by precipitation with trichloroacetic acid in the presence of 1% carrier BSA. In some instances the eluted radio-

iodinated antibody was further purified by binding and elution from a second immunoabsorbant column.

#### Solid Phase Radiometric Assay for Allotype-Marked Anti DNP Antibodies (4)

A solution of 1% BSA, 0.005 M EDTA, 0.1%  $\text{NaN}_3$ , PBS (pH 7.6) was used for washing and dilution. All steps were performed at room temperature. Flexible polystyrene, U-bottom microtitre plates (Cooke Engineering, Alexandria, VA) were coated with DNP by incubating 50  $\mu\text{l}$  of DNP-BSA (2 mg/ml) per well for an hour. Test sera or standard anti-DNP sera at various dilutions were added to the coated wells (20  $\mu\text{l}$ /well) and incubated for 3 hours. The plate was then washed twice and radioiodinated anti-allotype antibody (20  $\mu\text{l}$ /well, approximately  $3 \times 10^4$  cpm) was added and incubated for another 3 hours. Finally, the plates were washed twice and the wells separated and counted in a well-type gamma counter.

To conveniently separate the wells, the adhesive plastic sheet usually used to seal microtitre plates was applied to the bottom of the plate. The top of the plate was then sliced away with a hot wire (mounted horizontally, 10 mm high, on a lucite table) leaving the liberated wells adhering to the bottom sheet in their original position and ready for easy transfer to tubes for counting.

### Quantitative Determination of Allotype Levels

Immunoglobulin allotype levels were determined by an antibody consumption type assay. Fifteen  $\mu\text{l}$  of radioiodinated anti-allotype antibody (approximately  $2.5 \times 10^4$  cpm) were mixed with 15  $\mu\text{l}$  of a known myeloma protein (standard) or a test serum and incubated for 4 hours at room temperature. Aliquots of 20  $\mu\text{l}$  were then transferred into microtitre wells previously coated with a myeloma protein of the same allotype for 90 min at room temperature (0.1 to 1 mg/ml). The plates were washed twice with diluent and the microtitre wells cut as above and counted.

For maximal specificity in this assay, the amount of myeloma protein coated on the wells should be adjusted so as to be just sufficient to obtain maximal binding of the amount of antibody used in the assay.

## RESULTS

### Radiolabeling of Anti Allotype Antibody

We report the results of the radioiodination and use of one alloantiserum, anti-Ig-1a, in this paper. This serum reacts with IgG<sub>2a</sub> immunoglobulins in Ig<sup>a</sup> allotype mice. Similar results were obtained with the other antisera, e.g., anti-Ig-1b, anti-Ig-4a, rabbit anti-mouse IgG.

A Sepharose Ig-1a myeloma protein (GPC-8) column was saturated with 500  $\mu$ l of anti-Ig-1a alloantiserum filtered before use through a Millipore filter (0.45  $\mu$ ). Radioiodination was carried out as described. The column was then washed with PBS. Free <sup>125</sup>I (non-TCA precipitable) passed rapidly through the column appearing mainly in the first 2 wash fractions (approximately 250  $\mu$ l). After 2 ml of PBS had passed through the column, the PBS was replaced with glycine-HCl buffer (pH 2.3) to elute the bound antibody. The first three fractions of acid-eluted material contained more than 75% of eluted counts. More than 95% of these eluted counts were protein bound, i.e., TCA precipitable.

To determine the percentage of active <sup>125</sup>I-labeled anti-Ig-1a antibody obtained, and to further purify the labeled antibody, the acid-eluted material was pooled and applied to a second Sepharose-GPC-8 column. Forty percent of the counts were bound and subsequently recovered by elution. The recovered material bound completely to GPC-8 myeloma protein (Ig-1a), and not at all to C.BPC101 (Ig-1b) myeloma protein. This preparation contained 2-3% of the <sup>125</sup>I originally used for labeling.

Data from labelings of a number of alloantisera using this procedure show that 40-70% of the acid-eluted, TCA-precipitable

material recovered from the first column is active, labeled anti-allotype antibody. Although, as shown above, the labeled antibody may be further purified by binding and eluting from a second immunoadsorbant column, this additional purification has proved unnecessary when the labeled reagents are used in the binding assays described below.

#### Quantitation of Allotype-Marked Anti-DNP Antibodies

Data in Fig. 1 show the specificity, sensitivity and working range of a binding assay using the  $^{125}\text{I}$ -labeled reagent described above to detect Ig-1a anti-DNP antibodies. Serial dilutions of CSW(Ig<sup>a</sup>) anti-DNP, CSW non-immune and CWB(Ig<sup>b</sup>) anti-DNP sera were tested (see Methods). As the data show, the assay with these reagents is specific for Ig<sup>a</sup> anti-DNP antibody since significant amounts of radioactivity were bound only to the wells incubated with CSW anti-DNP. The anti-DNP antibody in as little as 3  $\mu\text{l}$  ( $3 \times 10^{-9}$  ml) of antiserum was detectable. Below 100  $\mu\text{l}$  of antiserum the amount of radioactivity bound was proportional to the amount of anti-DNP antiserum added making the assay quite sensitive in this range. No significant binding was observed with CSW non-immune serum nor with CWB(Ig<sup>b</sup>) anti-DNP. Similar specificity and sensitivity were found in assays using other  $^{125}\text{I}$ -labeled anti-allotype preparations



Quantitation of Allotype-Marked Immunoglobulins

Data in Fig. 2 show that as little as 1 ng of Ig-1a immunoglobulin (myeloma protein GPC-8) is specifically detectable in an antibody consumption test using  $^{125}\text{I}$ -labeled anti-Ig-1a antibodies. Residual antibody was measured by binding to wells coated with Ig-1a myeloma protein (see Methods section). As the figure shows, below 34 ng the amount of  $^{125}\text{I}$  anti-Ig-1a consumed is essentially proportional to the log of the amount of Ig-1a myeloma protein added. Minimal labeled antibody was absorbed by an Ig-1b myeloma protein (C.PBC101).

## DISCUSSION

The radioiodination method presented here allows the efficient purification and radio-labeling of small amounts of specific antibody, yielding preparations which are minimally contaminated with inactive labeled proteins. We have demonstrated the utility of such labeled anti-allotype antibody in a solid phase radioimmune assay and an antibody consumption assay, showing that such labeled antibody preparations allow a high degree of specificity and sensitivity. These results suggest that this radioiodination method could be useful in any assay where specifically purified radioiodinated antibody is required, as long as the target antigen can be coupled to Sepharose, e.g., in the autoradiographic detection of cell surface immunoglobulins.

Preliminary results in our laboratory indicate that the principle of labeling described here for  $^{125}\text{I}$  can be extended to coupling other types of labels to antibodies, e.g., fluorescein or rhodamine. Thus this method may provide a useful short-cut for preparing tagged antibody reagents for use in a variety of assay systems.

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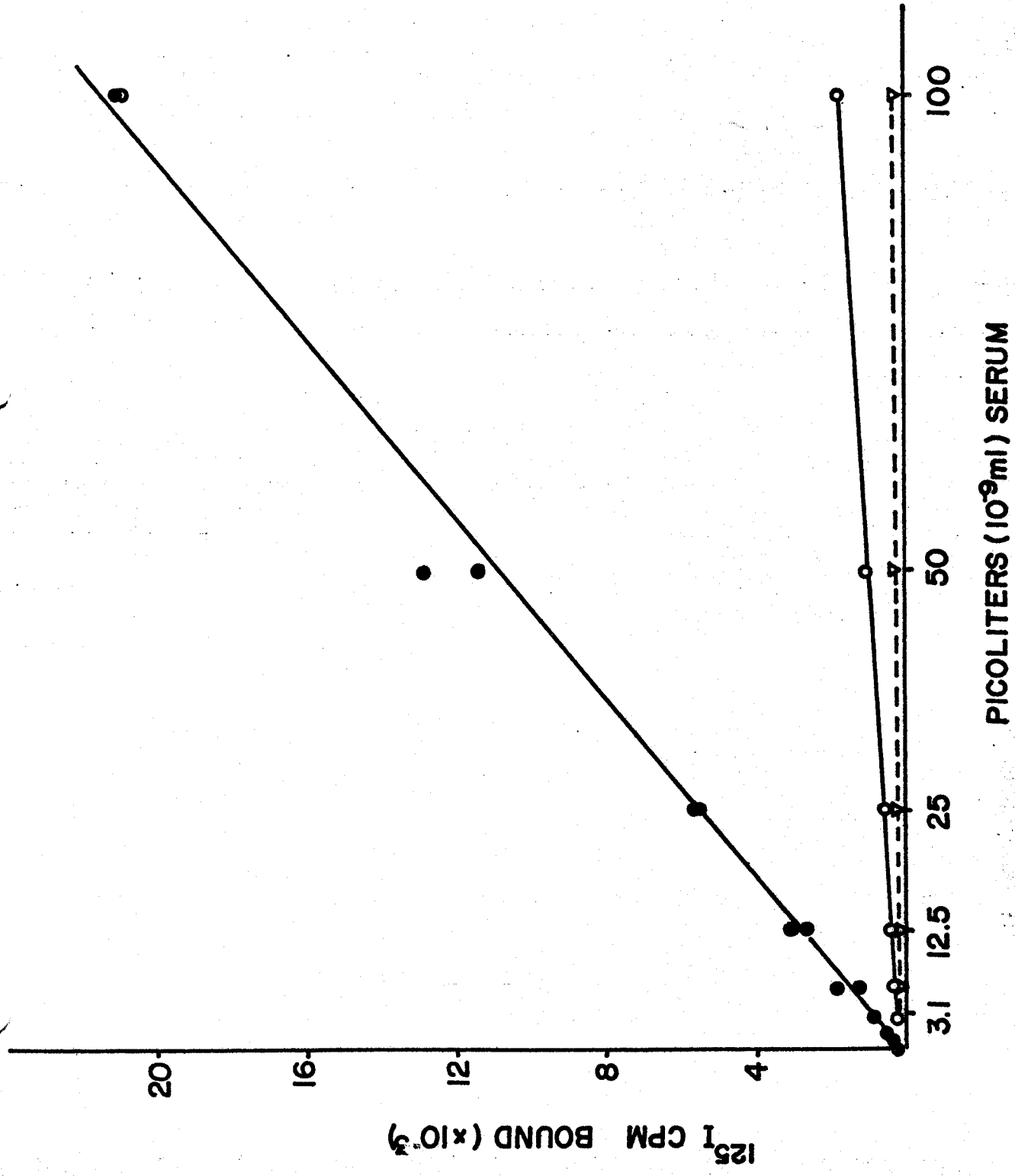
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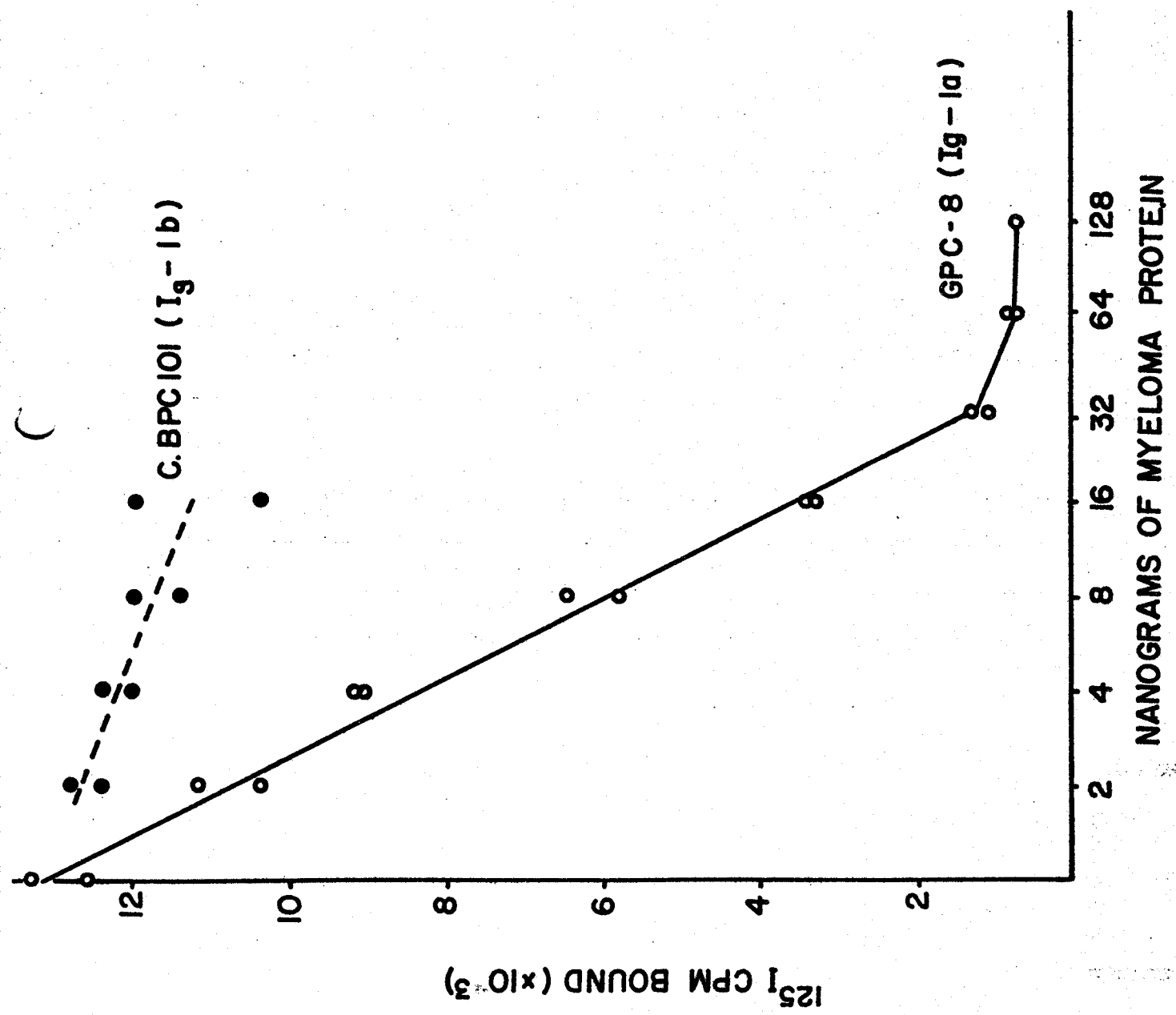
LEGENDS FOR FIGURES

Figure 1. Solid-phase immunoradiometric assay for allotype marked anti-DNP antibody.

- CSW (Ig<sup>a</sup>) anti-DNP serum
- CSW (Ig<sup>a</sup>) non-immune serum
- ▼ CWB (Ig<sup>b</sup>) anti-DNP serum

Figure 2. Antibody consumption assay for allotype levels.





C. BPC 101 (Ig-1b)

GPC-8 (Ig-1a)

<sup>125</sup>I CPM BOUND (x10<sup>3</sup>)

NANOGRAMS OF MYELOMA PROTEIN