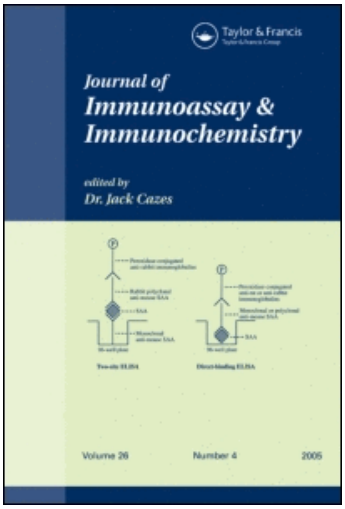

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Treatment of Antibodies to Reduce NON-Specific Binding in Immunoassays Using the Avidin-Biotin Complex

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TREATMENT OF ANTIBODIES TO REDUCE NON-SPECIFIC BINDING IN IMMUNOASSAYS USING THE AVIDIN-BIOTIN COMPLEX

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ABSTRACT

Three different methods to reduce non-specific binding of antibodies to avidin coated plates in an immunoassay are described and compared: The use of a blocking agent, antiserum adsorption on avidin-agarose and antiserum adsorption to avidin by a so called pre-incubation method. The pre-incubation method proved to be highly efficient, fast and simple. Further applications of this method to different immunoassay systems are proposed in the discussion section.

KEY WORDS: Antibody, avidin, biotin, enzyme immunoassay, non-specific binding.

INTRODUCTION

The tenacious interaction between the vitamin biotin and the glycoprotein avidin has been applied in many immunological systems for the isolation, localization and visualization of various antigens. Additionally, it has been used in drug delivery, lymphocyte stimulation and immunoassays (1). In immunoassays the avidin-biotin system has been introduced to amplify the sensitivity (2,3). We have developed a competitive antigen-immobilized enzyme linked immunosorbent assay (ELISA) system where immuno plates are coated first with avidin and subsequently with biotinylated estradiol. In this system, however, the performance of the assay is limited by non-specific binding (NSB) of the antibody to avidin. In the present work we describe different methods to reduce this NSB and compare their performance.

MATERIALS AND METHODS

Supplies

Avidin, peroxidase conjugated goat anti-rabbit immunoglobulin G fragment (PO-F(ab')$_2$) and avidin-agarose were purchased from Sigma, St. Louis, MO, USA. Bovine non-fat dry milk (Rapilait) was from a local store (Migros, Switzerland). Anti-estradiol serum was collected from a rabbit immunized with bovine serum albumin-6-carboxymethyloxime-estradiol (Steraloids, Wilton, N.H., USA). Microtiter plates (Immuno Plates I, 96 flat-bottom wells) were from Nunc, Roskilde, Denmark.

Microtiter plate coating

Microtiter plates were treated with 5 µg/ml avidin dissolved in borate coating buffer (10 mM, pH 9.6) containing 10 mM sodium chloride and 0.01% thimerosal (200 µl/well). After an overnight incubation at room temperature, the coating solution was discarded and the plates were washed three times with blocking buffer (Tris-HCl, 10 mM, pH 5.5, containing 0.1% bovine non-fat dry milk, 10 mM ethylenediaminetetra-acetate and 140 mM sodium chloride). Antigen was bound to the avidin coated plates by incubation of 150 µl biotinylated estradiol (15 ng/ml) in assay buffer (phosphate-buffered saline (PBS), containing 0.05% bovine non-fat dry milk and 0.01% thimerosal) at room temperature for 1 h. The antigen solution was discarded, the plates were washed five times, and used directly or stored at 4 °C.

Antibody treatment

Anti-estradiol serum was treated in three different ways:

1. Milk blocking buffer (PBS/"milk"): Antiserum was diluted suitably in PBS containing 0.05% non-fat dry milk.
2. Adsorption on avidin-agarose: Aliquots of antiserum (1:10) in PBS/"milk" were incubated with different amounts of avidin-agarose (0.001 - 10%, w/v, calculated for the avidin moiety) for 1 h at room temperature with agitation. The suspension was centrifuged for 10 minutes at 3000 x g and the supernatant was further diluted in PBS/"milk" to the titer required for the assay.
3. Pre-incubation with avidin: Aliquots of antiserum (1:10) in PBS/"milk" were

incubated with avidin (0.001 - 10%, w/v) for 1 h at room temperature. The mixture was suitably diluted and used in the assay without any additional separation step.

Controls were prepared by diluting antiserum in PBS.

Immunoassay

The protocol of the assay is described in detail in (4). In brief, 200 µl of the antibody preparations were incubated in the wells of the microtiter plates coated with avidin and biotinylated estradiol for 1 h at room temperature. The plates were washed five times (Tris-HCl 50 mM, pH 7.8 containing 0.1% (v/v) Tween 20), incubated with 200 µl of PO-F(ab')$_2$ (dilution 1:10,000) for 1 h at room temperature and washed again. The peroxidase activity was monitored with tetramethylbenzidine/H_2O_2. NSB was measured in the presence of an excess (10 µg/ml) of estradiol (calculated as % of the total binding).

RESULTS AND DISCUSSION

The non-specific binding of antibodies is a serious problem common to all immunoassays. This NSB can be minimized by saturating adsorptive surfaces with "blocking" proteins (5), a collective term for various protein additives that play no active role in the immunochemical reaction. A second well known and commercially used method to decrease NSB is the treatment of the antiserum with an adsorptive component present in the immunogen (e.g. human serum albumin) followed by harvesting the free antibody fraction. We applied these two methods to avidin to reduce NSB of the antiserum and compared these results with those obtained by the above described pre-incubation method. In this third method the untreated antiserum was pre-incubated with avidin for adsorption of components causing the NSB. The separation of these avidin adsorbed components was integrated in the wash step of the ELISA protocol. The presence of additional avidin in the antiserum solution did not interfere with the immobilized biotinylated estradiol.

TABLE I

Avidin Concentrations for Antiserum Treatment to Reduce Non-Specific Binding

Avidin concentration (% w/v)		Non-specific binding (%)	
in stock solution	in application solution	antiserum pre-incubated with avidin	antiserum adsorbed on avidin-agarose
10	0.2	11 ± 1	17 ± 5
1	0.02	8 ± 2	16 ± 3
0.1	2×10^{-3}	10 ± 1	25 ± 5
0.01	2×10^{-4}	14 ± 2	50 ± 2
0.001	2×10^{-5}	31 ± 2	54 ± 3

Table I: Stock solutions of antiserum (dilution 1:100) were treated with different concentrations of avidin, as indicated in column 1. The corresponding concentrations in the final antibody solution (dilution 1:5,000) are given in column 2. The resulting non-specific binding was measured in the presence of free estradiol in excess and calculated as % of total activity. Means and standard deviations are calculated from three independent determinations with six replicates.

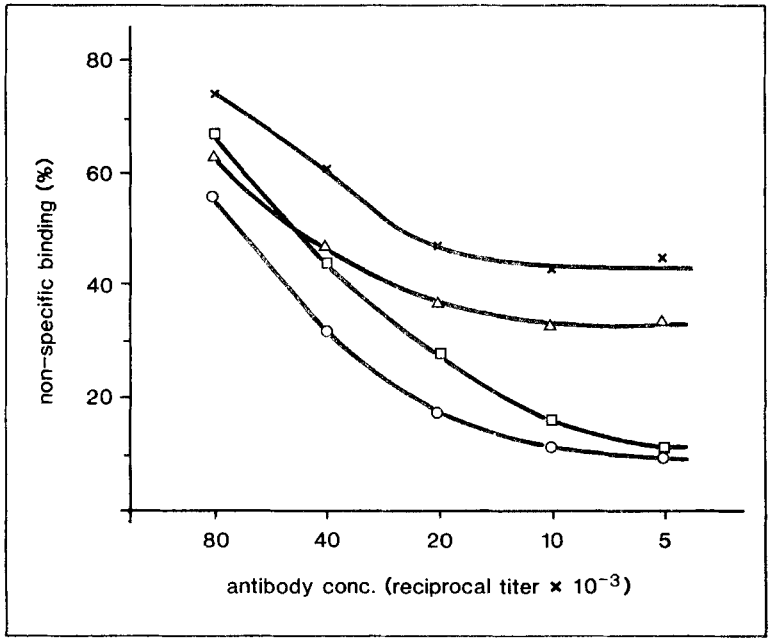

FIGURE 1

Reduction of the non-specific binding achieved by three different methods

Non-specific binding is calculated as % of the maximum signal and plotted versus the antiserum concentrations (inversely proportional to titer). Antiserum was diluted in PBS containing 0.05 % "milk" (△) or in PBS/"milk" including 0.002 % avidin (○). Adsorption to avidin-agarose (□) was performed in the same PBS/"milk" buffer. Controls (×) were diluted in PBS.

The avidin concentrations tested for the antiserum treatment are indicated in Table 1, both for the pre-incubation and the avidin-agarose adsorption method. Optimal results were achieved with 0.002% or 0.02% of avidin in the final antibody solution, respectively. Maximum adsorption was attained after only 20 minutes.

The influence of avidin treatment on the NSB and the concomitant loss of antibody are shown in Figure 1. Compared to the controls, the optimal titer of anti-estradiol shifted after both avidin treatment procedures from 1:10,000 to 1:5,000, i.e. a double concentration of antiserum was required.

A reduction of the NSB depended very much on the procedure applied. With an antibody titer of 1:5,000, the NSB was 45 $\pm$ 6% for the controls, 34 $\pm$ 7% after dilution of the antiserum with PBS/"milk", 11 $\pm$ 3% after antiserum treatment with avidin-agarose and 10 $\pm$ 4% after pre-incubation of the antiserum with avidin (mean $\pm$ SD, from 3 independent determinations with 6 replicates). Considering the high efficiency and the simplicity, the pre-incubation method is best suited to reduce the NSB. An analogous treatment might also be applied to immunoassay systems based on antigens attached to plastic surface using carrier molecules such as bovine serum albumin, lectins or thyroglobulin instead of avidin.

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