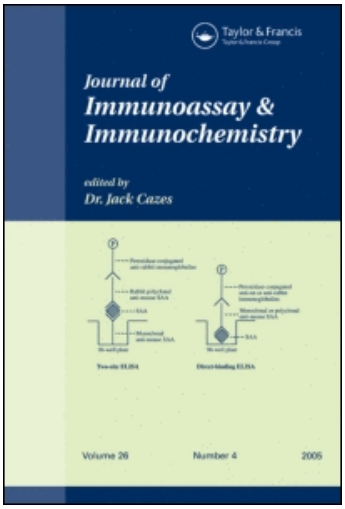

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PEROXIDASE-LABELLED MONOCLONAL ANTIBODIES FOR USE IN ENZYME IMMUNOASSAY

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ABSTRACT

Desirable characteristics of enzyme-antibody conjugates for use in enzyme immunoassay are labelling uniformity, permanent availability and stability. The use of monoclonal antibodies (McABS) for preparation of enzyme conjugates, in place of polyclonal antibodies, ensures labelling uniformity and permanent availability. The problem of stability still exists. Monoclonal antibody-horseradish peroxidase (McAB-HRPO) conjugates produced in our laboratory showed variable stability. After extensive testing of McAB-HRPO conjugates it became obvious that sodium borohydride, used as a reducing agent, did not result in the production of stable conjugates without enzyme pretreatment with fluorodinitrobenzene (FDNB). Ascorbic acid or ethanolamine used as the reducing agent, resulted in McAB-HRPO conjugates which were stable for periods of ten months or more when stored filter sterilized at 4 °C.

INTRODUCTION

Mouse monoclonal antibodies from ascites fluid are frequently conjugated with horseradish peroxidase (HRPO) for use in enzyme immunoassay. The periodate-oxidation method of conjugation developed by Nakane and Kawaoi is often used (1). These authors reported that under optimal conditions, 68% of the enzyme activity and 99% of the immunoglobulin were incorporated into the

conjugate. Efficiency of conjugation reported by other investigators using this method is considerably lower (2,3,4). This may be partly due to the sodium borohydride reduction step used to stabilize the cross-linkage between the enzyme and the immunoglobulin. Antibody activity of HRPO-labelled McABs produced by this method was reduced by 60% two weeks post conjugation. Different reducing agents were therefore investigated, including sodium borohydride (1), ascorbic acid (5) and ethanolamine (6). Fluorodinitrobenzene treatment of peroxidase prior to the conjugation procedure was performed, to prevent self-coupling of the enzyme was performed (1).

Some commercial antibody-HRPO conjugates along with some prepared in our laboratory lost all antibody activity within six months, when stored at -20°C. Others both polyclonal and monoclonal antibody-HRPO conjugates lost between 10-80% activity. Therefore the effects of storage at 4°C and -20°C with numerous freeze-thaw cycles was also investigated.

The above parameters are of considerable consequence when reagents are utilized in mass serological diagnostic procedures in which reproducibility and variation both are important for assay accuracy.

MATERIALS AND METHODS

Monoclonal antibody production

Monoclonal antibodies to the light chain of bovine immunoglobulin were produced following a modification of the method of

Kennett (7). The monoclonal antibody selected for this study was IgG_1 isotype.

Purification of monoclonal antibody from ascites fluid

Ascites fluid was clarified at 52,000 x g/10 minutes to remove lipids and fibrin clots. Antibodies were then precipitated from the clarified supernatant fluid by the addition of an equal volume of saturated ammonium sulphate $[(NH_4)_2\ SO_4]$. The precipitate was dissolved in 0.15 M NaCl and dialysed against 0.15 M NaCl for 12-16 hours at 4°C. Protein concentration was estimated on the dialysate by ultraviolet spectrophotometry at 280 nm using $E^{1\%}_{1\ cm} = 13.7$.

Fluorodinitrobenzene treatment of peroxidase

The blocking of HRPO with FDNB prior to oxidation with sodium meta-periodate was done by the method of Nakane and Kawaoi (1) using 1% 1-fluoro-2,4-dintrobenzene (Sigma Chemical Co., St. Louis, MO).

Peroxidase conjugation

Conjugation of the McAB to HRPO was carried out by a modification of the method of Nakane and Kawaoi (1). Ten milligrams of peroxidase (type VI-Sigma Chemical Co., St. Louis, MO) was dissolved in 2.5 ml of distilled water and mixed with 0.5 ml of 0.1 M sodium meta-periodate for 20 minutes at room temperature. The aldehyde was dialysed against 0.001 M sodium acetate buffer, pH 4.4. at 4°C for 18 hours. Fifty microliters of 0.2 M sodium carbonate buffer pH 9.5 was added to 10 mg HRPO followed

immediately by the addition of 2 milligrams of purified monoclonal antibody. The mixture was stirred gently at room temperature for 2 hours and the reaction was stopped by the addition of one of the following reducing agents: 0.25 ml ascorbic acid (4 mg/ml), 0.25 ml sodium borohydride (4 mg/ml) or 10% v/v 2 M ethanolamine pH 9.5 (final concentration of 0.2 M).

Conjugates of 2.0 mg of protein were left standing for 24-48 hours at 4°C to stabilize and were then filter sterilized (0.22 mμ) and stored at 4°C or at -20°C until used. Larger batches were found to take 5-10 days to stabilize. Different amounts of peroxidase (1,2,5,10,12,15 or 20 mg) were used in the conjugation procedure. From the data obtained, 10 mg peroxidase conjugated with 2 mg of protein by the above technique yielded the optimal coupling efficiency. This ratio was used for conjugation of other monoclonal antibodies of IgG_1 isotype with similar results.

Enzyme immunoassay

Immunologic and enzymatic activity of HRPO-labelled McABs were demonstrated in an indirect enzyme immunoassay for bovine serum antibody to Brucella abortus (8). In brief, 96 well polystyrene plates (Linbro, McLean, VA) were coated with 200 μl per well of 0.06 M carbonate buffer, pH 9.6, containing 200 ng of B. abortus smooth lipopolysaccharide (9). After 18 hours of incubation at RT, plates were washed four times in 0.01 M phosphate buffer pH 7.2 containing 0.15 M NaCl and 0.05% Tween 20

(PBS-T). A serum obtained from a cow (shown to be infected with B. abortus by culture of the organism from milk) was diluted to contain sufficient antibody in 200 µl to saturate the antigenic determinants passively adsorped to the polystyrene and added to each well for one hour at RT. After four washes in PBS-T 200 µl of doubling dilutions of McAB-HRPO containing 500 to 15.6 ng/well of antibody were added for one hour at RT. After an additional four washes in PBS-T, 200 µl of substrate/chromogen (4.0 mM hydrogen peroxide and 1.0 mM 2,2'-azinobis (3-ethylbenzthiazoline sulfonic acid) diamonium salt in 0.05 M citrate buffer, pH 5.0) was added. The plate was shaken on an orbital shaker and the optical density was measured at 414 nm in a spectrophotometer after 10 minutes. Antibody-enzyme conjugates were tested at regular intervals for 10 months.

A capture assay, used for the estimation of the bound enzyme present in the conjugates, was performed using a polyclonal, sheep a-mouse antibody to trap dilutions of the mouse immunoglobulin of the McAB-HRPO conjugate. An assay was performed to estimate total enzyme concentration present in the McAB-HRPO conjugates. Both tests were developed with the substrate/chromogen described above.

RESULTS AND DISCUSSION

Actual optical density measurements of the enzyme-antibody conjugate reduced with ascorbic acid and tested monthly over a

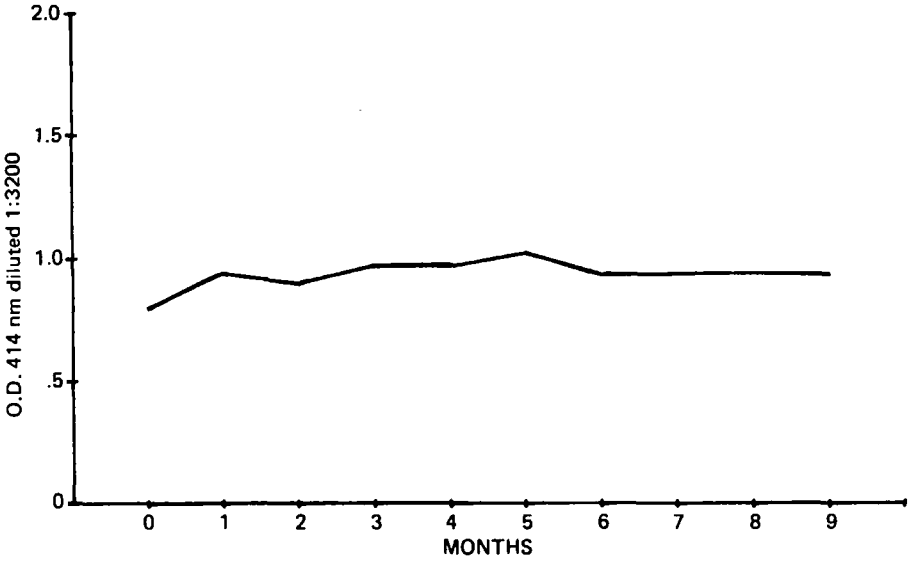

FIGURE 1

Monthly spectrophotometric reading of McAB-HRPO conjugate using ascorbic acid as the reducing agent without prior treatment of the enzyme with FDNB. The readings were taken at a 1:3200 dilution of the reagent at 10 min of development.

period of ten months are presented in Figure 1. It is obvious that the reagent is highly stable over the test period, with variation in all cases less than 10%. To minimize test to test variation, this enzyme-antibody conjugate was chosen as the standard and minor variations in optical density measurements were mathematically corrected to correlate optical density values of other reagents to this standard. Thus the standard enzyme-antibody conjugate is represented by the horizontal line (1) in

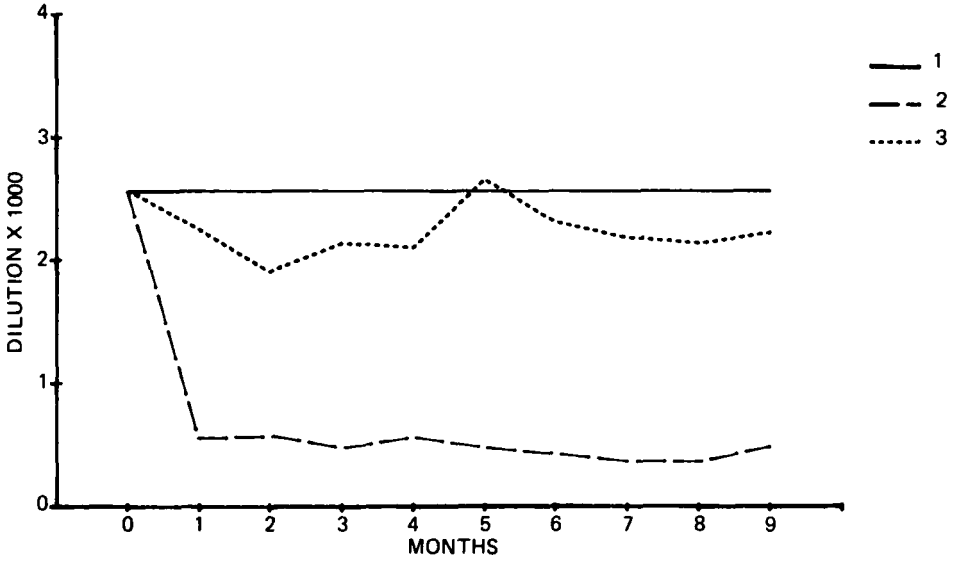

FIGURE 2

Stability of McAB-HRPO conjugates over a ten month period using three different reducing agents (1) ascorbic acid, (2) sodium borohydride, (3) ethanolamine. Conjugate binding is expressed as that dilution which gives an optical density reading of 1.0 spectrophotometrically at 414 nm, after a ten minute incubation period with substrate/chromogen.

Figures 2,3 and 4 with the other curves corrected by the same values.

The results show that when sodium borohydride was replaced by ascorbic acid or ethanolamine in the final step of the periodate method of conjugating McABs with HRPO, the antibody-enzyme conjugates maintained a high level of stable antibody activity for at least ten months. Sodium borohydride on the other hand caused an 80% decrease in activity over the initial two months (Figs. 1,2).

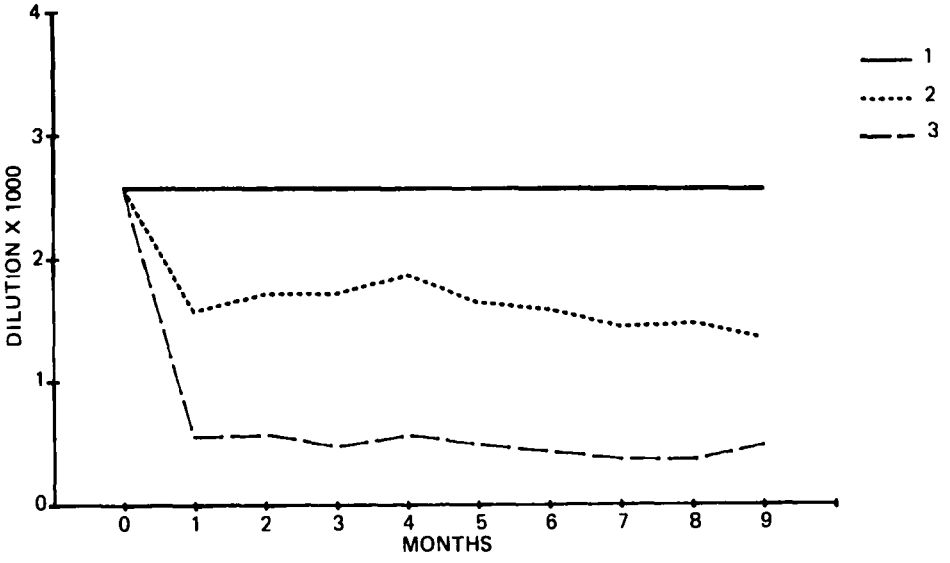

FIGURE 3

Comparison of stability of conjugate using HRPO with or without FDNB treatment (1) ascorbic acid as a reducing agent with no FDNB treatment of HRPO, (2) sodium borohydride used as a reducing agent with FDNB treated HRPO and (3) sodium borohydride as a reducing agent with no FDNB treatment of HRPO. The conjugate binding is as described in FIGURE 2.

When ethanolamine was used in the procedure, however, higher background absorbance was demonstrated in the buffer controls of the enzyme immunoassay indicating non-specific interaction (data not shown).

When HRPO was treated with FDNB to block α and ϵ amino groups and hydroxy groups of HRPO, conjugation with mouse McABS using sodium borohydride resulted in a reagent that was stable for ten months (Fig. 3). However, when ascorbic acid was used as

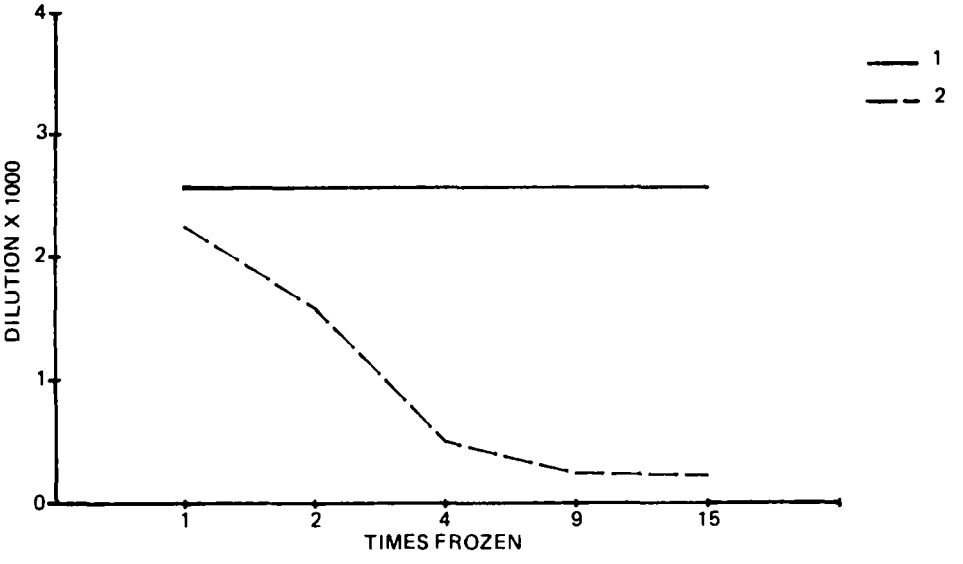

FIGURE 4
The effect of freezing and thawing on stability of McAB-HRPO conjugates prepared with ascorbic acid (1) refrigerated conjugate, (2) conjugate that has been repeatedly frozen and thawed.

the reducing agent in the presence of FDNB, the antibody activity of the conjugate was unpredictable.

Estimation of concentration of bound enzyme on conjugates prepared showed that a ten-fold reduction in captured antibody activity had occurred when sodium borohydride had been used as the reducing agent compared to conjugates using ascorbic acid and ethanolamine. Similarly the conjugate made using sodium borohydride as the reducing agent and FDNB treated HRPO showed a two-fold reduction in the captured antibody activity. Total enzyme

estimations on all conjugates were very similar in concentration. It therefore appears that there is a breakdown of the antibody-enzyme complex in the conjugates treated with sodium borohydride and that this is a direct result of the antibody being reduced by the sodium borohydride.

Freezing and thawing of the McAB-HRPO conjugate also proved to affect stability (Fig. 4). After multiple freezing and thawing cycles no antibody activity could be detected in numerous conjugates produced in the laboratory.

Bovine albumin is quite often added to commercial conjugates as a stabilizer. However this was not desirable as the conjugates produced were used in testing bovine serum samples.

In diagnostic serology, antibody-enzyme conjugate stability is important to minimize day to day test variation and to maintain cost-effectiveness. We suggest that the conjugation procedure replacing sodium borohydride with ascorbic acid as the reducing agent and the storage of conjugate at 4°C may therefore provide a useful means for enzyme-antibody stabilization.

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