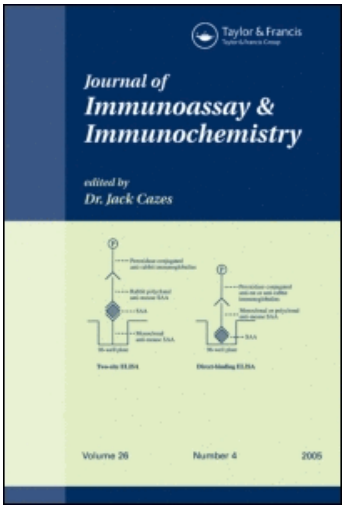

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STABILITY OF FREEZE DRIED HORSERADISH PEROXIDASE CONJUGATED MONOCLONAL ANTIBODIES USED IN DIAGNOSTIC SEROLOGY.

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ABSTRACT

Two murine monoclonal antibodies, an IgG_1 isotype specific for the heavy chain of bovine IgG_1, the second an IgG_3 isotype and specific for an epitope of the O-polysaccharide of **Brucella abortus** were conjugated with horseradish peroxidase. The conjugated antibodies were freeze dried in the presence of a number of additives to preserve activity and tested for stability over an 18 month period. Addition of 0.3M trehalose or 0.8% lactalbumin and 3.2% sucrose resulted in the lowest loss of activity if the conjugated antibodies were freeze dried in glass vials. Freeze drying in polypropylene vials resulted in a more rapid rate of deterioration with some additives or an accelerated rate of decline after an initial plateau of lesser loss of activity. The use of polypropylene vials and lactalbumin and sucrose were included in this study because of their low cost compared to glass vials and trehalose. While each antibody behaved differently, addition of trehalose or lactalbumin and sucrose to the conjugated antibodies aided in the preservation of enzyme activity. Both additives provide a suitable method for long term storage of freeze dried or freeze dried and reconstituted monoclonal antibody-enzyme conjugates. (KEY WORDS: Monoclonal antibodies, enzyme conjugation, stability, serology).

INTRODUCTION

As the number of enzyme immunoassays (ELISA) used in diagnostic serology increases so does the demand for universally available stable enzyme conjugated reagents. In order to standardize diagnostic tests, more and more assays use monoclonal antibodies because of their uniformity of specificity and the continuous supply of identical reagents. Once developed, the monoclonal antibodies may be purified and conjugated by a variety of methods to a variety of enzymes. One of the more commonly used enzymes is horseradish peroxidase and

the periodate procedure (1,2) has been widely used for conjugation of this enzyme to antibodies. Enzyme conjugated monoclonal antibodies can be stored for extended periods at 4°C if filter sterilized with minimal loss of activity. Freezing of some conjugated monoclonal antibodies has been shown to cause loss of activity (2). This loss of activity may be overcome by addition of 50% (final volume) of glycerol to prevent crystal formation, however, distribution of materials maintained at -20°C or below is not as convenient as freeze dried materials in promoting universal usage of reagents. Freeze drying in some instances causes a considerable loss in activity and the method used for reconstitution may also be of importance for long term storage.

In this communication, a variety of chemicals were tested for their ability to preserve activity of freeze dried enzyme conjugated monoclonal antibodies. The stability of these conjugated reagents was tested over a period of about 18 months.

MATERIALS AND METHODS

Monoclonal Antibodies: Two murine monoclonal antibodies, both used routinely in diagnostic procedures, were selected. One antibody, M23, was specific for a heavy chain epitope of the bovine IgG_1 molecule and is used in several indirect ELISA procedures for bovine antibody to infectious agents. This antibody was a murine isotype IgG_1 with a kappa light chain. The second monoclonal antibody was a specific for an epitope present on the O-polysaccharide of lipopolysaccharide of **Brucella abortus**. This antibody was a murine isotype IgG_3 with a kapppa light chain. Both antibodies were purified from ascites fluid by anion exchange high pressure liquid chromatography using a Bio Gel TSK DEAE-5-PW column (3) and conjugated with horseradish peroxidase, type VI (4) the method of described in (2). Several batches of each monoclonal antibody were prepared, purified and conjugated, however, data from only one batch will be reported.

Treatment of Enzyme Conjugated Monoclonal Antibodies: Prior to freeze drying, the conjugated monoclonal antibodies were dialized against 0.05M phosphate buffer, pH 7.0 and containing 0.15M NaCl. At least 20 vial of monoclonal antibody-enzyme conjugate were prepared by each procedure and then freeze dried:

1. Untreated.
2. Dialized against distilled water.
3. Addition of 0.3M trehalose (5).
4. Addition of 0.8% lactalbumin and 3.2% sucrose.
5. Addition of 0.5% lactalbumin and 2% sucrose
6. Addition of 0.05M tris-HCl, pH 8.0 and 0.5% bovine serum albumin.
7. Addition of 0.05M tris-HCl, pH 8.0 and 2% bovine serum albumin.
8. Addition of 1% bovine serum albumin.
9. Addition of 3% bovine serum albumin.
10. Addition of 3% polyethylene glycol MW 6,000.

All concentrations are final and contained in 1.0 ml of conjugated material in polypropylene and amber glass vials, both screw cap with a 2.0 ml capacity. Dilution factors were considered when testing the conjugated antibody.

After freeze drying, polypropylene or glass vials of each treatment were reconstituted to 1.0 ml with distilled water or distilled water and glycerol 1:1.

For control purposes, glass and polypropylene vials containing conjugated antibody treated by each method were filter sterilized and maintained at 4°C.

Testing of Enzyme Conjugated Monoclonal Antibodies: All assays were performed every 30 +/- 3 days. A glass and a polypropylene vial containing conjugated antibody of each treatment was reconstituted with 1.0 ml of distilled water or distilled water and glycerol 1:1. These vials were maintained at 4°C throughout the study. From time to time, when the activity of a treatment decreased to less than 25% of its original activity, a new vial of conjugated antibody treated the same way was reconstituted and tested. Vials (glass and polypropylene) containing conjugated antibody treated with 0.8% lactalbumin and 3.2% sucrose or 0.3M trehalose were reconstituted every 60 days and tested in the regular schedule.

The M23 conjugated monoclonal antibody was tested by two different methods. Firstly, a direct binding assay using bovine IgG_1 passively immobilized to polystyrene 96 well plates (6), each well coated with 100 ul containing 100 ng of bovine IgG_1 in 0.06M carbonate buffer, pH 9.6 overnight at 4°C and then frozen at -20°C. Plates were thawed at 37°C for 30 minutes before washing four time with 0.1M tris buffer, pH 8.0, containing 0.15M NaCl and 0.05% tween 20 (tris/tween). After washing, 100 ul of conjugated monoclonal antibody, diluted 1:1,000; 1:10,000; 1:100,000 and 1:1,000,000 in tris/tween were added in duplicate and incubated for 1 hour at 20°C. Following an additional four wash cycles, 100 ul of substrate and chromogen (1mM hydrogen peroxide and 4mM ABTS in 0.05M citrate buffer, pH 4.5) to each well. The plate was shaken continuously for 10 minutes and optical density readings were obtained at 414 nm using a spectrophotometer (7).

Secondly, an indirect ELISA for antibody to **Brucella abortus** was used to test the M23 antibody-enzyme conjugate. This assay has been described in detail elsewhere (8). The only modification was that in the place of serum, an excess of bovine IgG_1 antibody to Brucella abortus was added. Optical density readings were obtained after continuously shaking the substrate/chromogen buffer for 10 minutes as above.

The enzyme conjugated monoclonal antibody to **Brucella abortus** O-polysaccharide was tested by a direct assay as the M23 enzyme conjugate above except 100 ng per well of **Brucella abortus** lipopolysaccharide was immobilized on polystyrene plates (9) and used as antigen.

From the optical density readings obtained with the various treatments, the dilution calculated to give an optical density of 1.0 was determined. The calculated dilutions were used for comparing the various treatments by dividing the value obtained with the equivalent value obtained with the control (non-freeze dried, sterilized) preparation. Thus if the dilutions were similar, a value of 1

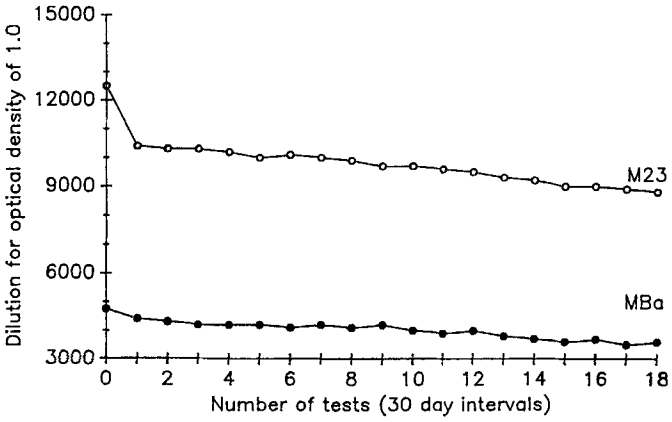


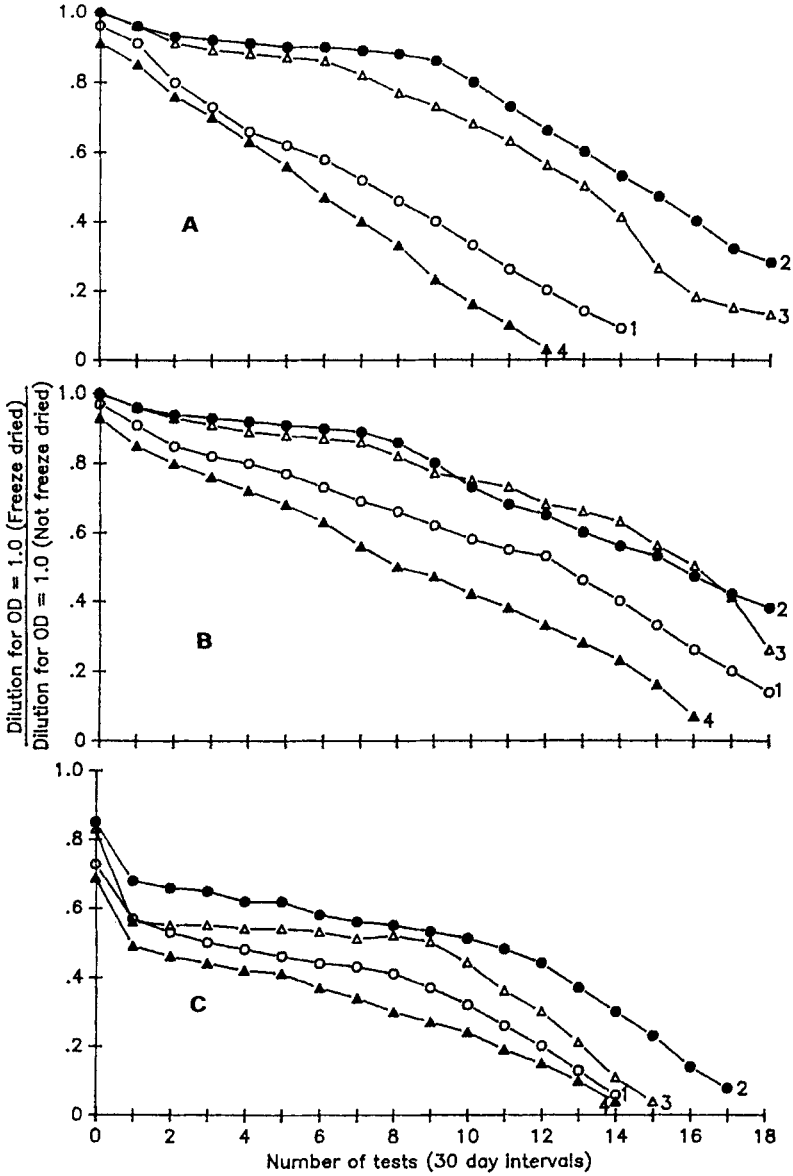
FIGURE 1: Optical density at 414 nm of horseradish peroxidase conjugated monoclonal antibody specific for bovine IgG_1 heavy chain (M23) and a **Brucella abortus** O-polysaccharide epitope in direct ELISAs over time.

would be obtained, however, if the freeze dried material lost activity, the value would decrease from 1.

RESULTS

Loss of 99% activity in the initial 30 day period was apparent in the conjugated materials freeze dried in distilled water or stored in either distilled water or distilled water and glycerol. Similarly, treatment with bovine serum albumin; treatment with polyethylene glycol; treatment with 0.05M tris and 0.5% bovine serum albumin and 0.5% lactalbumin and 2% sucrose resulted in high loss of activity of the freeze dried material. Testing of these conjugated antibodies was therefore discontinued.

Figure 1 represents the activity of the untreated, non-freeze dried, filter sterilized conjugated monoclonal antibodies, stored in amber glass vials, over the 18 months test period. Both conjugated antibodies were tested in the direct ELISAs only. These graphs show a gradual loss in activity of both enzyme-antibody conjugates. The anti-bovine IgG_1 antibody conjugate lost 16.8% activity in the



initial 30 days. Subsequently, over the next 17 tests there was a 12.8% loss in activity for a total loss of 29.6% of activity. The anti-**Bruclla abortus** antibody conjugate lost an initial 7.4% of its activity followed by a gradual loss of 16.9% over the next 17 tests for a total loss in activity of 24.3%. These graphs represent the minimal loss of activity to which other treatments were compared.

Testing of the freeze dried conjugated antibodies untreated, treated with 0.3M trehalose, with 0.8% lactalbumin and 3.2% sucrose or with 0.05M tris, pH 8 with 2% bovine serum albumin was continued and the results from 18 tests (approximately 18 months) are presented in Figure 2, A, B and C for the M23 antibody, direct and indirect ELISAs and the **Brucella abortus** direct ELISA, respectively, when stored in polypropylene vials and in Figure 3, A, B and C when stored in amber glass vials. Reconstitution with distilled water and glycerol (1:1) increased the viscosity of the conjugated antibody which already contained trehalose, sucrose and lactalbumin and bovine serum albumin making it difficult to pipette accurately. Therefore, freeze dried conjugated antibody preparations were reconstituted in distilled water only. Table 1 shows the percentage loss after reconstitution and the second test and between the second test and the 18^{th} test. Freeze drying did not cause over 10% loss of activity of the M23 enzyme conjugate initially whether stored in

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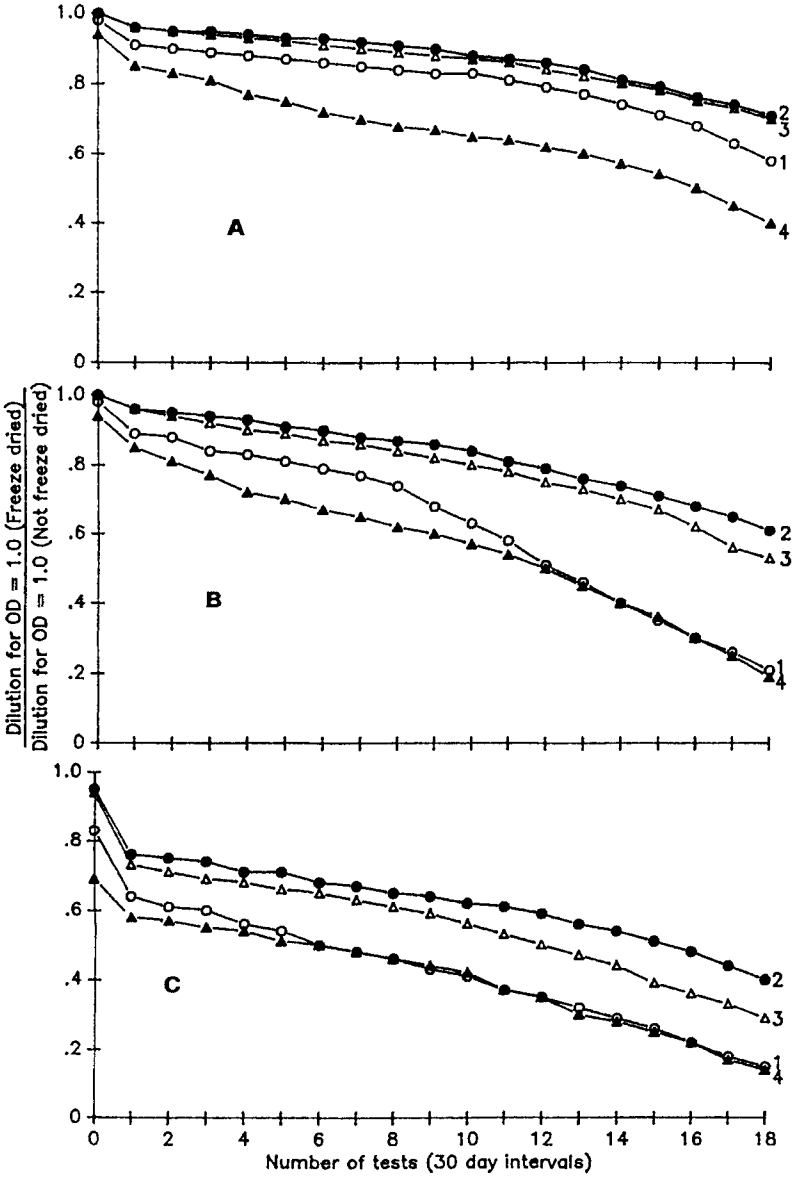
FIGURE 2: Ratio of the optical density at 414 nm that gave an optical density of 1.0 of freeze dried conjugated antibody and the optical density required to give an optical density of 1.0 of the non-freeze dried antibody conjugate control plotted against time (number of tests).

Various treatments were used: 1: no additives; 2: 0.3M trehalose; 3: 0.8% lactalbumin and 3.2% sucrose; 4: 0.05M tris, pH 8.0 with 2% bovine serum albumin. All concentrations are final.

A. Conjugated M23 monoclonal antibodies were stored in polypropylene vials and activity was measured by a direct ELISA.

B: Conjugated M23 monoclonal antibodies were stored in polypropylene vials and activity was measured by an indirect ELISA.

C: Conjugated MBa monoclonal antibodies were stored in polypropylene vials and activity was measured by a direct ELISA.



polypropylene or glass and independent of the preservatives used. Long term storage of the M23 monoclonal antibody-enzyme conjugate in polypropylene vials reduced the activity from 62 to 100%. It is apparent that 0.3M trehalose and 0.8% lactalbumin with 3.2% sucrose protect the reagent best and according to Figures 2A and B, there is a definite plateau lasting for 6 to 8 months during which little activity is lost while the untreated and the conjugated antibody to which tris and bovine serum albumin were added lost activity in a nearly linear fashion. Storage in amber glass vials seemed to protect the conjugated M23 antibody and after the initial loss of activity of less than 10%, there was only a 30-60% loss in activity over the long term when activity was assessed in the direct ELISA. It is of interest to note that the loss of activity measured by the indirect ELISA was considerably greater with all the various treatments (Table 1). The monoclonal antibody specific for **Brucella abortus** conjugated with horseradish peroxidase lost 20 to 32.5% activity between reconstitution and the test at 30 days and if this reagent was stored in polypropylene, all activity was lost by the end of the test period. If stored in amber glass vials, there was a total loss of about 60% activity when trehalose was added with the other treatment resulting in greater losses (Table 1). It is of interest to note that the MBa conjugate when stored in polypropylene and pre-

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FIGURE 3: Ratio of the optical density at 414 nm that gave an optical density of 1.0 of freeze dried conjugated antibodyand the optical density required to give and optical density of 1.0 of the non-freeze dried antibody conjugate control plotted against time (number of tests).

Various treatments were used: 1: no additives; 2: 0.3M trehalose; 3: 0.8% lactalbumin and 3.2% sucrose; 4: 0.05M tris, pH 8.0 with 2% bovine serum albumin. All concentrations are final.

A: Conjugated M23 monoclonal antibodies were stored in amber glass vials and activity was measured by a direct ELISA.

B: Conjugated M23 monoclonal antibodies were stored in amber glass vials and activity was measured by an indirect ELISA.

C: Conjugated MBa monoclonal antibodies were stored in amber glass vials and activity was measured by a direct ELISA.

TABLE 1: Percent loss of activity of two horseradish peroxidase conjugated monoclonal antibodies over time with or without preserving additives. The conjugated reagents were stored in either polypropylene or amber glass vials. The M23 antibody conjugate was tested by a direct and an indirect ELISA while the MBa antibody conjugate was tested by a direct ELISA.

Antibody/ Assay[1]	Additive[2]	Container[3]	% loss of activity[4] Initial	Long term	Total	Det.[5]
M23/ Direct	None	Polypro.	5.2	94.8	100	L
	Trehalose		4.0	68.0	72.0	P
	Lac.+ sucr.		4.0	83.0	87.0	P
	Tris + BSA		6.6	93.4	100	L
	None	Glass	7.1	33.7	40.8	L
	Trehalose		4.0	25.0	29.0	L
	Lac.+ sucr.		4.0	26.0	30.0	L
	Tris + BSA		9.6	47.9	57.5	L
M23/ Indirect	None	Polypro.	6.2	80.2	86.4	L
	Trehalose		4.0	58.0	62.0	P
	Lac.+ sucr.		4.0	70.0	74.0	P
	Tris + BSA		8.6	91.4	100	L
	None	Glass	9.2	69.4	78.6	P
	Trehalose		4.0	35.0	39.0	L
	Lac.+ sucr.		4.0	43.0	47.0	L
	Tris + BSA		9.6	70.2	79.8	P
MBa/ Direct	None	Polypro.	21.9	78.1	100	P
	Trehalose		20.0	80.0	100	P
	Lac.+ sucr.		32.5	67.5	100	P
	Tris + BSA		29.0	71.0	100	L
	None	Glass	22.9	59.0	81.9	L
	Trehalose		20.0	37.9	57.9	L
	Lac.+ sucr.		22.3	46.8	69.1	L
	Tris + BSA		15.9	63.8	79.7	L

[1] Monoclonal antibody cojugated with horseradish peroxidase, tested by the direct or indirect ELISA procedure.

[2] Additive refers to the preserving agent added prior to freeze drying the conjugated antibody; no additives; 0.3M trehalose; 0.8% lactalbumin and 3.2% sucrose; 0.05M tris, pH 8.0 with 2% bovine serum albumin. All concentrations are final.

[3] Containers for lyophilizing were screw cap 2 ml capacity polypropylene or amber glass vials.

[4] Percent loss of activity represents the loss on reconstitution of the freeze dried conjugated antibody. The initial loss was between the time of reconstitution (time 0) and the first test (day 30). Long term loss is the loss after the 30 day test and up to the end of the test period (test 18). Total loss is the sum of the initial and long term losses.

[5] Det. refers to the rate of deterioration of the reagents. Under some conditions, loss of activity was near linear over the test period (L) while in other cases, a plateau with a lower rate of deterioration was in evidence during the first half of the study, followed by a more rapid rate of decline in activity (P).

served with trehalose or lactalbumin and sucrose and to a lesser extent unpreserved antibody conjugate gave a plateau effect lasting up to about 10 months during which activity declined less rapidly. This was followed by a more rapid decline (Figure 2C). The reason for the plateau or the onset of accelerated deterioration is not understood, but it may relate to loss of enzyme activity and loss of immunological reactivity of the antibody which contribute approximately equally to the total decrease in activity. Antibody conjugate treated with tris and bovine serum albumin deteriorated in a near linear fashion when stored in polypropylene vials (Figure 2C). If stored in amber glass vials, the MBa-enzyme conjugate decreased in activity in a near linear fashion.

In Figure 4A and B, glass vials containing monoclonal antibody-enzyme conjugate M23 and anti-Brucella abortus, respectively, treated with 0.3M trehalose were reconstituted every 60 days for 12 months and their activities are compared by the direct ELISAs. There was little or no apparent loss of activity after storing the conjugated M23 antibodies in a freeze dried form and the kinetics if the decay after reconstitution did not change. A small amount of deterioration of the anti-**Brucella abortus** antibody was apparent in the initial stage after reconstitution with time but the kinetics of the activity decrease did not change over the test period. Nearly identical data was obtained when 0.8% lactalbumin and 3.2% sucrose was added to the conjugated monoclonal antibodies (data not shown).

DISCUSSION

Preservation and storage on monoclonal antibodies conjugated with enzymes for use in diagnostic operations is a very important aspect of enzyme immunoassay standardization. Horseradish peroxidase conjugated monoclonal antibodies are generally quite stable if filter sterilized, stored at 4°C and handled aseptically (Figure 1). There was a 25 to 30% total loss of activity over the 18 months test period. Based on experience, this is the minimum amount of loss normally associated with storage of monoclonal antibody conjugated with

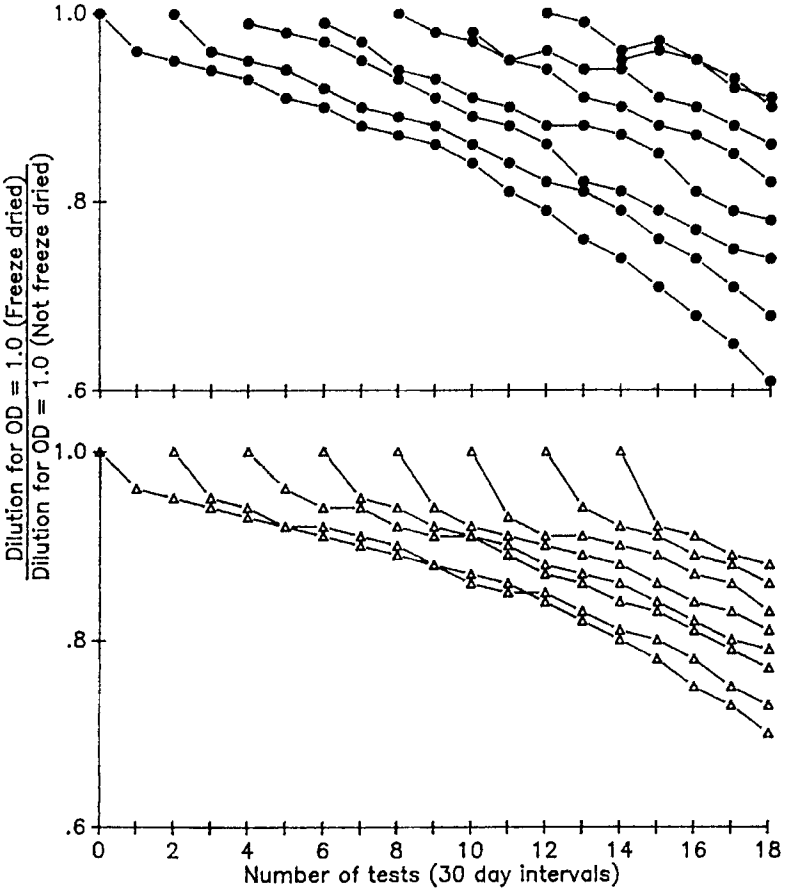

FIGURE 4: Ratio of freeze dried monoclonal antibody conjugate dilution required to give an optical density of 1.0 and the dilution of non-freeze dried monoclonal antibody required to give an optical density of 1.0 plotted against time.

A: Glass vial of M23 monoclonal antibody conjugate with 0.3M trehalose (final concentration) added was reconstituted every 60 days and tested until the end of the test period.

B: A similar preparation of monoclonal antibody MBa conjugate was tested.

enzyme. However, in the past, it proved difficult to ship conjugated reagents in a liquid form and freezing and thawing destroys activity very quickly (2). Therefore, lyophilization would be a more suitable method of storage and of considerable advantage for shipping, especially over long distances.

The data presented suggests that each monoclonal antibody behaves differently and should therefore be treated as individual cases. In this study, one antibody conjugate, a mouse IgG_3 isotype specific for an epitope of the O-polysaccharide of **Brucella abortus**, showed considerable loss in the initial stages after reconstitution but then stabilized (Table 1). Loss of activity was decreased by two factors. Firstly, storage in amber glass vials decreased long term loss of activity. From Figure 2C and 3C, it may be observed that the rate of decrease of activity is approximately the same whether the antibody conjugate is stored in glass or in polypropylene vials for the initial 6 to 8 months, then subsequently, the reagent stored in polypropylene vials deteriorated at an accelerated rate. This is particularly noticeable with antibody conjugate treated with 0.3M trehalose (Graph 2, Figure 2C and 3C). The second factor was addition of a preserving agent. A number of different methods of perservation were attempted, some resulting in total loss of activity while others aided in preserving activity. The most useful additives used in this study were 0.3M trehalose and a combination of 0.8% lactalbuminand 3.2% sucrose (Graphs 2 and 3, Figure 2C and 3C).

In contrast, the second monoclonal antibody, a mouse isotype IgG_1 with specificity for the heavy chain of bovine IgG_1, lost minimal activity after reconstitution, less than 10%. Addition of trehalose or lactalbumin and sucrose slower deterioration over the next 6 to 8 months (Graph 2 and 3, Figure 2A). This was followed by a more rapid decline in activity, paralleling that observed if no preservative or tris and bovine serum albumin were added (Graph 1 and 4, Figure 2A). Alternately, if the reagent was stored in amber

glass vials, deterioration of activity was slow, linear and similar except where tris and bovine serum albumin was added as a preservative (Figure 3A). It is should be noted that two different methods of measuring the activity of this conjugated monoclonal antibody gave different stability data. Thus when activity was measured in a direct ELISA procedure (Figure 2A and 2B), results were generally higher than when activity was measured by an indirect ELISA (Figure 3A and 3B), especially when trehalose or lactalbumin and sucrose were used as preservatives (Graph 12 and 3, Figure 2A, 2B, 3A and 3B).

Of the preservatives used in this study, 0.3M trehalose provided the greatest stability of the freeze dried conjugated antibodies, followed closely by a combination of 0.8% lactalbumin and 3.2% sucrose. The trehalose reagent is preferable because of its solubility, however, it is somewhat more expensive. Further trial with this method of preserving enzyme conjugated monoclonal antibodies will be carried out but for the time being, a suitable method of lyophilizing two monoclonal antibody-enzyme conjugates with only a small loss in activity over an 18 months test period has been described.

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