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Stability of monoclonal IgM antibodies freeze-dried in the presence of trehalose

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Abstract

We describe the use of the disaccharide trehalose for stabilization of mouse monoclonal IgM antibodies during freeze-drying and prolonged storage at elevated temperatures. Spent culture media, ascitic fluids and isolated immunoglobulins were freeze-dried in the presence of trehalose, stored at different temperatures, and tested after rehydration for their binding to their corresponding antigens. Antibodies, directed against various types of antigens, effectively recovered their binding efficiency as tested in enzyme-linked immunoassays, flow cytometry and immunofluorescence. Application of trehalose for freeze-drying of labile monoclonal IgM antibodies permits convenient long-term storage of large quantities of antibodies, facilitates their transport at ambient temperature and simplifies the construction of pre-aliquoted kits based on such antibodies.

Keywords: IgM; Monoclonal antibody; Trehalose; Freeze-drying

1. Introduction

One of the essential steps in hybridoma technology is adequate storage of monoclonal antibodies (mAbs). Mouse mAbs, in the form of spent tissue culture supernatants, ascitic fluids or purified antibodies, are usually stored as small aliquots at −20°C or −70°C. Alternatively, they are stored at 2–4°C with added preservatives to retard microbial growth, or they are freeze-dried. Particular mAbs, however, have individual characteristics and they may therefore differ greatly in their susceptibility to damage by environmental factors. Freeze-thaw cycles are potentially damaging particularly to mAbs of the IgM class. IgM mAbs are also prone to aggregation after prolonged storage at 4°C. Moreover, when ascitic fluids are not delipidated, insoluble lipoprotein precipitates are formed in samples stored for a long period at 4°C. As IgM is less robust than IgG, many IgM mAbs are irreversibly denatured by freeze-drying (Goding, 1986). In our experience the binding activities of some IgM mAbs were substantially lower after storage for a few weeks at 4°C in the presence of preservatives. Several studies have shown that sugars with

Abbreviations: ELISA, enzyme-linked immunosorbent assay; mAbs, monoclonal antibodies; PBS, phosphate-buffered saline pH 7.4; PEG, polyethylene glycol; SDS, sodium dodecyl sulphate; SwAM, swine anti-mouse immunoglobulin antibody; SwAM-FITC, fluorescein isothiocyanate-conjugated SwAM.

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known cryopreservative properties have the capability to protect proteins under dehydration-induced stress (Crowe et al., 1987; Crowe et al., 1990; Carpenter and Crowe, 1988). In particular the disaccharide trehalose (α-D-glucopyranosyl-α-D-glucopyranoside) has a remarkable ability to preserve labile proteins during dessication (Carpenter and Crowe, 1988; Rudolph and Cliff, 1990; Israeli et al., 1993) and subsequent storage at higher temperatures (Roser, 1991). Trehalose is highly soluble, non-reducing, non-hygroscopic and belongs to the most chemically unreactive sugars. To overcome problems with long-term storage of large amounts of labile mAbs and costly transportation of such mAbs in insulated boxes, we tested the stabilizing effect of trehalose on the binding activities of IgM mAbs.

2. Materials and methods

2.1. Antibodies

The following mouse IgM class mAbs were used in this study: TU-14 against tubulin (Dráber et al., 1989), VI-01 against vimentin (Dráberová et al., 1986), and TEC-01 against the carbohydrate epitope of teratocarcinoma stem cells (Dráber and Pokorná, 1984). Spent culture supernatants contained 10% foetal calf serum. The mAbs were precipitated from ascitic fluids by polyethylene glycol (PEG) and purified by hydroxyapatite chromatography (Stanker et al., 1985). The antibody purity attained was higher than 80% as assessed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). The mAbs in the form of supernatants, ascitic fluids or purified immunoglobulins were stored at 4°C in the presence of 0.05% sodium azide or in small aliquots without any additive at −70°C. Fluorescein isothiocyanate-conjugated swine anti-mouse immunoglobulin antibody (SwAM-FITC) and horseradish peroxidase-conjugated SwAM were obtained from Sevac (Prague, Czech Republic).

2.2. Freeze-drying

Supernatants, ascitic fluids or purified mAbs were freeze-dried (LYOVAC GT3, Leybold-Heraeus, Germany) in the presence of trehalose (Sigma Chemical Co., St. Louis, MO) at final concentrations of 0.05, 0.10, 0.25, 0.30 or 0.40 M. For the convenient handling of small amounts of freeze-dried mAbs, the samples containing trehalose were frozen as 50 μl aliquots in liquid nitrogen. Alternatively, ascitic fluids were freeze-dried in the presence of 0.05 M trehalose and 1% PEG 8000 (Sigma). Ascitic fluids were also freeze-dried in the presence of the disaccharides sucrose, maltose, lactose and the monosaccharides glucose and galactose (all from Sigma) at final concentrations of 0.25 and 0.4 M. Before freeze-drying the purified mAbs were dialysed against 5 mM sodium phosphate buffer pH 7.5 and diluted to concentrations of 1, 0.1 or 0.01 mg/ml. Freeze-dried preparations were stored at 4°C and at ambient temperature for various time periods or at 50°C for 14 days. Freeze-dried samples were reconstituted in distilled water to the original antibody volume, centrifuged at 12000 × *g* for 5 min and used for the binding assays.

2.3. ELISA

The reactivity of TU-14 preparations was assessed by a quantitative enzyme-linked immunosorbent assay (ELISA) essentially as previously described (Landsdorp et al., 1980) with 50 μg/ml of phosphocellulose purified porcine brain tubulin (Weingarten et al., 1975) adsorbed on 96-well plastic plates (NUNC, Roskilde, Denmark). Bound mAbs were detected with SwAM antibody conjugated with horseradish peroxidase and *o*-phenylene-diamine as chromogen. Optical density at 490 nm was measured with a Microelisa Mini Reader (Dynatech Laboratories, Alexandria, VI). Three measurements were used for each test point. Standard errors of the means of triplicate absorbance measurements were less than 3%.

2.4. Indirect immunofluorescence and flow cytometry

Immunofluorescence staining of intermediate filaments on 3T3 cells, fixed for 10 min in methanol followed by 6 min in acetone at −20°C, was performed according to Dráber et al. (1989)

with undiluted VI-01 supernatants and secondary SwAM-FITC antibody diluted 1/30. P19X1 embryonic carcinoma cells, characterized by large differences in the densities of TEC-01 epitopes on individual cells, were cultured as described (Dráber and Malý, 1987). Cells were harvested with 0.02% ethylenediaminetetraacetic acid in phosphate-buffered saline pH 7.4 (PBS), washed twice and incubated for 40 min with TEC-01 antibody diluted 1/500. After three 5 min washes, the cells were incubated for 40 min with SwAM-FITC diluted 1/20, washed again and examined by flow cytometry using FACS 440 (Becton Dickinson, Mountain View, CA). Normal mouse serum diluted 1/500 was used as a negative control. PBS was used for all dilutions and washings.

3. Results

The inclusion of 0.25 M trehalose facilitated the freeze-drying of large amounts of labile mouse IgM mAbs that would otherwise have quickly lost binding activity on 4°C storage or become partially denatured during freezing and thawing. Recovery of the binding efficiency after rehydration

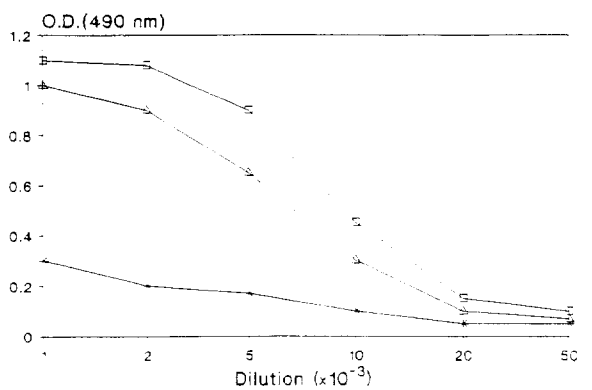

Fig. 1. Effect of trehalose on recovery of the binding activity of IgM monoclonal antibody freeze-dried as ascitic fluid. Binding of anti-tubulin TU-14 antibody was detected by ELISA with immobilized tubulin. □, control, freshly collected ascitic fluid; △, ascitic fluid freeze-dried in the presence of 0.25 M trehalose; ⋆, ascitic fluid freeze-dried without trehalose. Freeze-dried samples were reconstituted to the original volume.

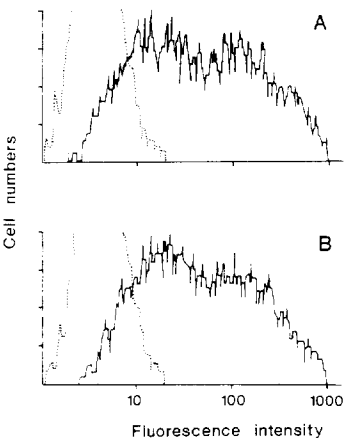

Fig. 2. Effect of trehalose on recovery of the binding activity of freeze-dried IgM monoclonal antibody as detected by flow cytometric analysis. Cytometric analysis of TEC-01 antigen expression on the cell surface with control, freshly collected ascitic fluid containing TEC-01 antibody (*A*), and with ascitic fluid freeze-dried in the presence of 0.25 M trehalose and reconstituted to the original volume (*B*). Continuous line: binding of TEC-01 antibody; stippled line: binding of normal mouse serum.

was observed in ascitic fluids and spent culture supernatants as well as in purified antibodies freeze-dried in the presence of trehalose. A comparison of the binding activities of reconstituted ascitic fluids with anti-tubulin activity freeze-dried in the presence or absence of trehalose is shown in Fig. 1. The binding activity recovered was similar at trehalose concentrations of 0.25, 0.30 or 0.40 M, but was lower at 0.05 and 0.10 M. Trehalose at 0.25 M was therefore routinely used for freeze-drying IgM mAbs. More than 30 ascitic fluids containing different IgM mAb, directed against various antigenic determinants were freeze-dried, and in all cases trehalose protected the binding activities of the corresponding antibodies. For example, flow cytometric analysis showed a similar binding activity of freshly prepared or reconstituted freeze-dried TEC-01 antibody over a large range of densities of cell-surface TEC-01 antigen in embryonic carcinoma cells (Fig. 2). The binding activities of freeze-dried IgM mAbs containing trehalose were retained, without any observable decrease, following two years of storage at 4°C or at ambient tempera-

ture. The stabilizing effect of trehalose was observed even when the freeze-dried samples were stored for 14 days at 50°C. In contrast, the nonreducing disaccharide sucrose provided substantially lower protection at this temperature. The reducing disaccharides maltose and lactose as well as the reducing monosaccharides glucose and galactose did not protect the binding activities of freeze-dried antibodies at high temperatures. The same results were obtained with samples freeze-dried in the presence of sugars used at 0.25 or 0.4 M. The thermostabilization properties of some sugars are shown in Fig. 3.

The stabilizing effect of trehalose was demonstrable not only in ascitic fluid where the typical antibody level is 1–15 mg/ml, but also in unconcentrated culture supernatants where the concentration of antibodies is in the range of 5–50 μg/ml, depending on the individual clone and cell density (Goding, 1986). As an example Fig. 4 shows immunofluorescence staining of intermediate filaments of the vimentin type in fixed 3T3 cells with fresh supernatant containing VI-01 antibody and with rehydrated supernatants freeze-dried in the presence or absence of trehalose. A typical distribution of vimentin filaments was observed both with the fresh supernatant and the

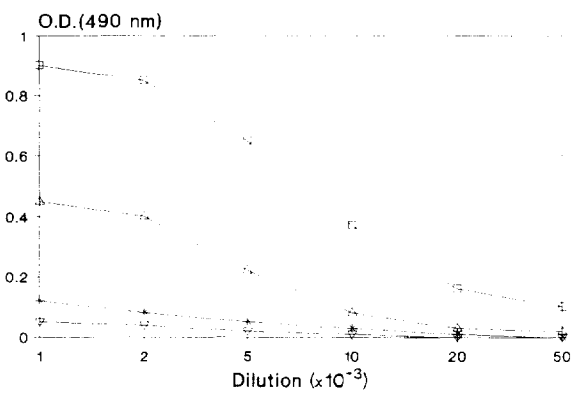

Fig. 3. Recovery of binding activity of IgM monoclonal antibody freeze-dried as ascitic fluid in the presence of various sugars and subsequently stored for 14 days at 50°C. Binding of anti-tubulin TU-14 antibody was detected by ELISA with immobilized tubulin. □, trehalose; △, sucrose; ★, lactose; ▽, glucose. Ascitic fluids were freeze-dried in the presence of 0.25 M sugars and reconstituted to the original volume.

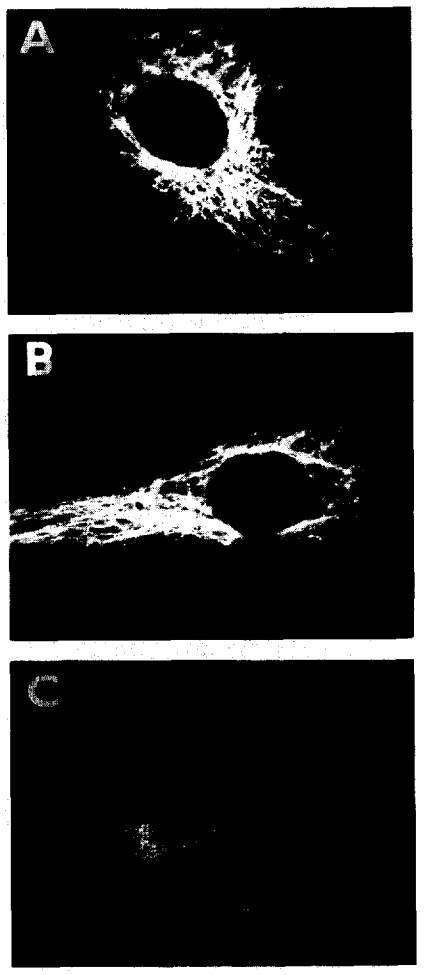

Fig. 4. Effect of trehalose on recovery of the binding activity of IgM monoclonal antibody freeze-dried as supernatant. Binding of anti-vimentin antibody VI-01 to intermediate filaments was detected by indirect immunofluorescence staining of fixed 3T3 cells. *A*: control, freshly collected supernatant, ×600; *B*: supernatant freeze-dried in the presence of 0.25 M trehalose, ×600; *C*: supernatant freeze-dried without trehalose, ×400. Freeze-dried samples were reconstituted to the original volume.

supernatant freeze-dried in the presence of trehalose (Figs. 4A and 4B), whereas supernatants freeze-dried in the absence of trehalose gave only nonspecific staining (Fig. 4C). The stabilizing effect of trehalose was also observed with purified antibodies that had been freeze-dried at various protein concentrations. Binding activity was recovered even with mAbs freeze-dried at a con-

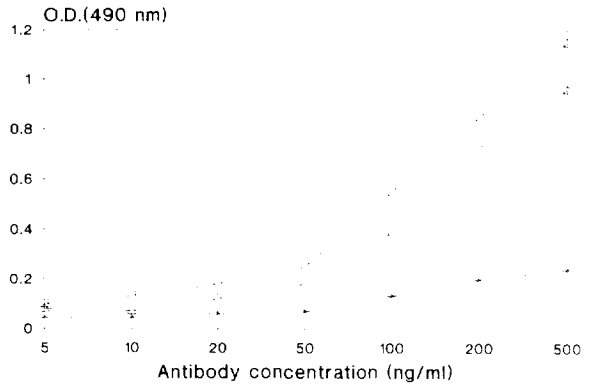

Fig. 5. Effect of trehalose on recovery of the binding activity of freeze-dried purified IgM monoclonal antibody. Binding of anti-tubulin TU-14 antibody was detected by ELISA with immobilized tubulin. □, control, freshly purified antibody; △, antibody freeze-dried in the presence of 0.25 M trehalose; ⋆, antibody freeze-dried without trehalose. Antibodies were freeze-dried at a concentration of 10 μg/ml. Freeze-dried samples were reconstituted to the original volume.

centration of 10 μg/ml. Binding of purified TU-14 antibody, freeze-dried in the presence of 0.25 M trehalose, to immobilized tubulin is shown in Fig. 5. Trehalose can, therefore, substitute for the carrier proteins that are usually added when the concentration of purified antibodies is lower than 0.1 mg/ml. We also noted that the stability of rehydrated samples containing trehalose was higher than that of the original antibodies or rehydrated antibodies containing the other sugars, after 1 month of storage at 4°C. When ascitic fluids containing 0.25 M trehalose were not freeze-dried, but stored at −20°C and repeatedly freeze/thawed, the binding activities of the corresponding labile IgM mAbs were also partially preserved. The effect of trehalose was, however, comparable with the other cryoprotective sugars (not shown).

4. Discussion

One significant issue that must be addressed in the use of labile IgM mAbs is the long-term stability of these reagents either in the form of unpurified ascitic fluids and supernatants or as isolated mAbs. To deal with this problem we have investigated the possibility of stabilizing such IgM mAbs in the freeze-dried state. Many proteins are conformationally unstable when subjected to freezing and subsequent dehydration, and must, therefore, be stabilized. The requirements for protection against dehydration are usually more specific and many effective cryoprotectants have no stabilizing effect during dehydration which takes place in two phases. Primary drying removes frozen water through sublimation and secondary drying removes non-frozen 'bound' water (Arakawa et al., 1993). In this report we show that trehalose provides effective stabilization during freeze-drying of IgM mAb and such preparations can be stored at elevated temperatures.

Unique thermostability conferred to biomolecules by trehalose has been demonstrated for liposome-encapsulated haemoglobin (Rudolph and Cliff, 1990), restriction enzymes (Roser, 1991) and air-dried antibodies (Blakeley et al., 1990). In this case small volumes of titrated antibodies against human blood groups were desiccated at 37°C in 4-well immunoplates. However, for large-scale production and storage of mAb, freeze-drying is often the method of choice. Although we did not observe any changes in the binding activities of more than 30 different trehalose-protected IgM mAbs, it would be advisable to check the recovery of binding activity of a particular antibody in a test sample before processing large amounts. As expected, the stabilizing effect of trehalose was also demonstrable in freeze-dried preparations of more stable IgG antibodies (not shown). From the practical point of view, the freezing of antibodies as small 50 μl spheres, facilitated convenient handling of the final freeze-dried preparations.

Of the other sugars tested, only the non-reducing disaccharide sucrose (β-D-fructofuranosyl-α-D-glucopyranoside) that is often used as a cheap protectant during the freeze-drying of proteins provided some protection for freeze-dried IgM mAb stored at high temperatures. However, its protective efficiency did not reach that of trehalose. Sucrose is stable as a pure substance but in the presence of chemically reactive amino groups of proteins it splits into the reducing

monosaccharides glucose and fructose. The prolonged storage of susceptible dried proteins in reactive sugars can thus lead to chemical damage in the proteins (Roser, 1991). The relative chemical stability and non-reducing nature of trehalose may thus be the most significant feature in the stability of freeze-dried IgM mAbs at high temperatures. It has been found that trehalose serves as a 'water substitute' when the hydration shell of the protein is removed and hydrogen bonding occurs between the dried proteins and stabilizing sugar (Carpenter and Crowe, 1989).

Although we added trehalose to a final concentration of 0.25 M, even lower concentrations may possibly be used in combination with other protein-stabilizing compounds. Recently Carpenter et al. (1993) have reported the recovery of phosphofructokinase activity after rehydration of samples freeze-dried in the presence of 50 mM trehalose and 1% PEG 8000. The role of polymer is to stabilize protein during the freezing event, while the sugar is required to provide hydrogen bonding to polar residues in the protein in the dry state. Our data with a limited number of purified IgM mAbs indicate that 50 mM trehalose in combination with 1% PEG of average molecular weight 8000 gives a slightly lower protective effect than 0.25 M trehalose alone (not shown). One can, therefore, consider whether it is better, for a particular purpose, to choose the more expensive but well defined trehalose or use a cheaper combination of a lower concentration of trehalose with less well defined PEG.

In conclusion, our results clearly indicate that trehalose is a suitable additive for the preservation of various forms of labile IgM mAbs during freeze-drying and their long-term storage. The thermostability of such preparations facilitates their storage at ambient temperature, and the need for equipment to maintain low temperatures is thus eliminated. Trehalose-protected antibodies could simplify the transport of bioreagents and the standardization of immunoassays.

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