Effect of dithiotheritol dtt concentration

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Immunoglobulin M is the first antibody produced in an immune response and is a pentameric structure held together by sulphide bonds in the J-chain. Dithiothreitol (DTT) cleaves inter-chain and intra-chain sulphide bonds at different concentrations abolishing the haemagglutination property of IgM. Varying concentrations of DTT were examined and it was concluded that concentrations of DTT higher than 0. 006 mol/L, completely denatured the structure of IgM leading to loss of agglutination activity.

INTRODUCTION

In collaboration with factors of the innate immunity, natural Immunoglobulin M (IgM) provides a first line of defense against invading antigens. IgM is pentameric and is found in serum. In addition to its natural presence, IgM could potentially bind to 10 antigenic determinants per molecule which also enables it to react with a broad spectrum of antigens simultaneously (Boes et al., 1998). The five identical monomers of IgM are made up of two heavy and two light chains that are held together by inter-chain sulphide bonds. The inter-subunit J-chain sulphide bonds hold the four-chain units together forming the larger IgM pentamer (Delves et al., 2011). J-chain is a 15-kDa

glycoprotein that is covalently associated by disulfide bonds with IgM (Koshland, 1985).

The reactions of antibody with a multivalent antigen results in the cross linking of the various antigen particles by the antibodies. This eventually results in the clumping of antigen particles by antibodies and is known as agglutination (Coico and Sunshine, 2009). The voluminous IgM antibodies (molecular weight of 970 kDa) have large Fab areas that are far enough apart and thereby facilitate the bridging of red blood cells separated by the zeta potential. This property, and the pentavalence of IgM antibodies is the major cause for the effectiveness of IgM antibodies in interacting with the blood type antigens (RhD) on the surface of erythrocytes resulting in agglutination (Coico and Sunshine, 2009).

Reducing agents such as Dithiothreitol (DTT) inactivates IgM antibody and abolishes agglutination activity (Okuno and Kondelis, 1978). This study was aimed at evaluating and understanding the concentration dependent activity of DTT in the inactivation of IgM.

MATERIALS AND METHODS

Sample Preparation: A stock solution of 0. 01 mol/L dithiothreitol (DTT) was appropriately diluted with saline (0. 9%), in ten fresh sterile tubes, resulting in dilutions ranging from 0. 01mol/L to 0. 001mol/L. One drop of each of the aforementioned dilution and one drop of diluted anti-D IgM were mixed in further ten tubes. Saline was used as control.

The reaction tubes were incubated in a 37°C water bath for 20 minutes.

Two drops of RhD positive Red Blood Cells (RBCs) were added to each tube, gently mixed and incubated at 20°C (room temperature) for 20 minutes.

- Observation of agglutination: The reaction tubes were centrifuged at 800g for one minute and were gently shook over a white background and examined for agglutination.
- Addition of second antibody: Two drops of anti-IgM antibody were added to tubes that contained non-agglutinated samples, and were incubated in a water bath at 37°C for 20 minutes.

The samples were then centrifuged at 800g for one minute and were examined for agglutination against a white background.

RESULTS

Variations in the concentrations of DTT showed varied levels of agglutination (Table 1). Tubes bearing DTT of concentrations between 0. 001 mol/L and 0. 006 mol/L and the control tube, showed agglutination. Still, the degree of agglutination observed was much lesser in higher concentrations (0. 006 mol/L) compared to lower concentrations (0. 001 mol/L). No agglutination was observed at DTT concentrations higher than 0. 006 mol/L. No additional change was observed despite addition of anti-IgM antibody to tubes with DTT at concentrations ranging from 0. 007 mol/L to 0. 01 mol/L, as shown in Table 2.

DISCUSSION

Inactivation or fragmentation of IgM can be achieved by treatment with reducing agents such as, 2-mercaptoethanol (2-ME) (Pirofsky and Rosner, 1974), reductant Tris(2-carboxyethyl) phosphine (TCEP) (Getz et al, 1999), or

even enzymes such as pepsin (Kishimoto et al., 1968). Obnoxious odour, requirement for dialysis, cost and time factors are certain drawbacks associated with such methods (Getz et al, 1999) Thus treatment with DTT (Okuno & Kondelis, 1978) is a simple, rapid and hence the widely preferred method as it lacks undesirable qualities.

DTT is a potent reducing agent that cleaves the inter-subunit di-sulphide bonds of IgM molecules, thereby abolishing agglutination property (Rudmann, 2005). Intra-subunit bonds are less sensitive to reduction than inter-subunit bonds and therefore mild reduction of the IgM pentamer leads to the release of the J chain producing IgM subunits (Tomasi, 1973), and this will result in loss of agglutinating activity. Though these subunits will retain antigen binding property, the affinity may be very low. This rationale explains the low degree of agglutination in tubes (5 & 6) with increasing concentration of DTT (Table 1).

A second antibody with affinity for the IgM subunits bound to antigen can lead to agglutination. More rigorous reduction will result in the fracture of the intra-subunit bonds and lack of antigen binding property. Accordingly, no agglutination was observed in tubes 7-10 (Table 2).

This study exploited the ability of an IgM anti-D (Rh) antibody to agglutinate RhD positive RBCs. Treatment of the anti-D antibody with DTT lead to the loss of agglutinating activity. Addition of an anti-IgM antibody which could have agglutinated these cells, if the IgM subunits had still retained antigen binding activity, showed no marked change indicating complete reduction of IgM involving cleavage of the intra-subunit sulphide bridges. Thus, it is

understood that the second antibody will just bind to free heavy chains in solution and no agglutination will occur. This phenomenon was clearly depicted in the observations in Table 2. It is evident from this experiment, that a DTT concentration above 0. 006 mol/L is lethal to the core structure of the IgM antibody.

Remington et al., (1968) demonstrated the use of IgM antibodies in the diagnosis of acute congenital toxoplasmosis. Studies have been conducted on the use of IgM antibody responses in the diagnosis of primary infections to measles, rubella, mumps, and M. Parainfluenzae viruses (Bringuier et al., 1978). The technique used in this study is of major importance in transfusion medicine. DTT can be used to remove IgM coated on RBCs which may lead to spontaneous agglutination (Hillyer et al., 2009). This method of inactivating IgM antibodies is of value in investigating cases of hemolytic disease of the newborn (Olson et al., 1970). and in studying sera containing mixtures of warm and cold red cell antibodies (Olson et al., 1970; Ashford et al ., 1985; Knight, 1978). Khodadai et al., (2006) have used this method to inactivate IgM but not IgG, in the crossmatch assay to help sensitized patients have the chance for successful transplantation. Studies show that pre-treatment of patient sera with DTT enhances IgG HLA antibody identification testing by single antigen beads (SAB) (Villatoro et al., 2010).