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Evaluation of dithiothreitol (DTT) for inactivation of IgM antibodies[1]

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SUMMARY A newly introduced sulphydryl compound, dithiothreitol (DTT), is evaluated for its optimal conditions of inactivation of IgM antibodies. The maximal effects of DTT reagent are observed when its final concentrations are between 0·0025 M and 0·005 M, pH between 7·0 and 8·0, and incubation at 37°C. Concentrations over 0·01 M, pH values greater than 8·0, and an incubation temperature over 40°C resulted in a gel formation of the specimen. Examination of both 'cold' and 'warm' type antibodies demonstrated that the results obtained by the DTT reagent are in close agreement with those obtained by 2-mercaptoethanol reagents and DEAE Sephadex treatment. Since the procedure is simple and rapid and lacks offensive odour, DTT is recommended for routine use in blood banking for the inactivation of IgM antibodies.

The identification of red cell antibodies as IgM or IgG immunoglobulins often provides useful information in blood banking (Hasekura, 1974; Moor and Steane, 1976). IgM immunoglobulin may be inactivated by treatment with sulphydryl compounds such as 2-mercaptoethanol (2-ME). The drawbacks are its obnoxious odour and the need for dialysis of the specimen in some cases (Pirofsky and Rosner, 1974; Moor and Steane, 1976). Dithiothreitol (DTT) treatment does not have these objectionable characteristics and is a simple, rapid procedure. DTT was evaluated for its clinical usefulness and its optimal conditions of inactivation of IgM antibodies.

Material and methods

Specimens of red cell antibodies were obtained from the North Suburban Blood Center and the Lutheran General Hospital Blood Center. Rh immunoglobulin (RhoGam) and blood grouping reagents were provided by Ortho Diagnostics, Raritan, New Jersey. 2-ME was obtained from Sigma (St. Louis, Missouri). The conventional procedure for cleavage of immunoglobulins by 2-ME was used (American Association of Blood Banks, 1974). Isolation of IgG from other immunoglobulin classes was obtained by using DEAE Sephadex ASO (Sigma, St. Louis, Missouri) (Webb, 1972).

DTT was obtained from Sigma (St. Louis, Missouri). The procedure used was essentially the same as that described by Pirofsky and Rosner. Briefly, 0·01 M DTT is prepared in pH 7·4 phosphate buffered saline (PBS). After addition of 0·25 ml DTT in PBS to 0·25 ml of test serum, resulting in a final concentration of 0·005 M DTT, the mixture is incubated at 37°C for two hours. For evaluation of the optimal conditions of DTT inactivation of IgM antibodies, changes of molarity of DTT were made by appropriately diluting the stock solution in phosphate buffered saline. The pH of the solution was altered by changing appropriately the ratio of Na_2HPO and KH_2PO_4.

The titration of antibodies was scored according to the procedure set out by the American Association of Blood Banks 1974.

Results

The effects of incubation time, incubation temperature, and concentrations and pH of DTT were evaluated by using high-titre group O sera in the saline phase. The pretreatment titration of the sera ranged from 32 to 128. The effects of incubation temperature and incubation time on the reduction of titration scores by DTT are shown in Figure 1. A marked reduction was seen after a 15-minute

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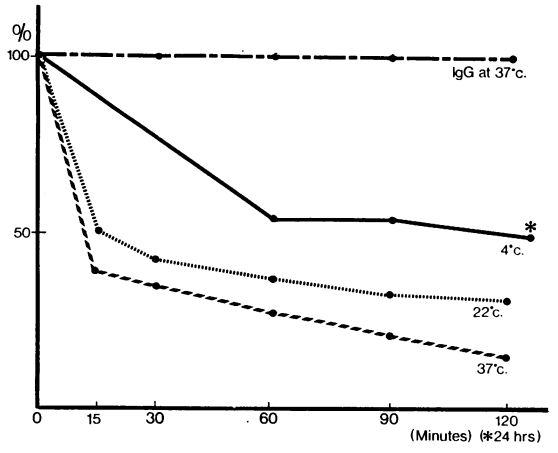

Fig. 1 *Effects of incubation temperature and incubation time on reduction of titration scores (expressed in percentage in ordinate) by DTT using group O sera in saline agglutination, except for IgG at 37°C where antiglobulin test is used.*

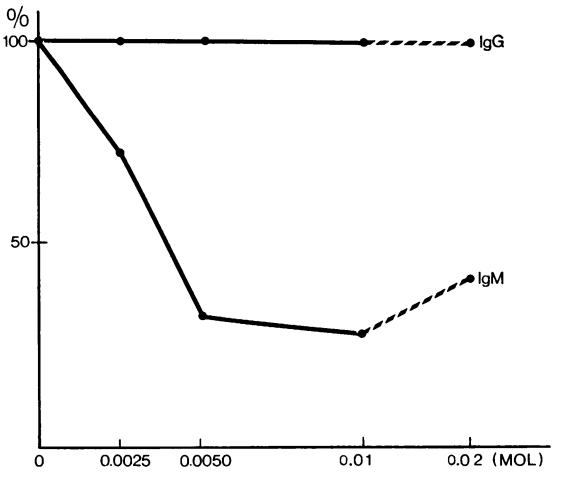

Fig. 2 *Effects of concentrations of DTT (expressed in molarity in abscissa) on reduction of titration scores (expressed in percentage in ordinate). Antiglobulin test was used for IgG antibodies using Rh immunoglobulin, while the saline agglutination test at 22°C was used for IgM antibodies using group O sera. Broken lines show gel formation.*

incubation at both 22°C and 37°C in the saline phase of agglutination. A specimen incubated at 4°C showed far fewer effects by DTT at 15 minutes. At this temperature reduction was completed within 60 minutes of incubation. No additional change was observed at the end of a 24-hour incubation period. IgG antibody in antiglobulin phase using Rh immunoglobulin showed no reduction in titration scores.

Variations in the concentration of DTT showed significant effects on the reduction of the titration scores of IgM antibodies (Fig. 2). While IgG anti-Rh antibodies in the antiglobulin test showed no changes in the titration scores, high titred group O sera in saline agglutination at 22°C showed a marked reduction in titre when treated with 0·0025 to 0·005 M DTT in the final concentrations. When the DTT concentration exceeded 0·005 M the solution became a gel, and an accurate observation could not be made.

The effects of pH are shown in Figure 3. For this study, group O sera with titres ranging from 64 to 128 were used for saline agglutination at 22°C. Since the titration scores of the control specimen varied at different pHs, the reduction of the titration scores is also expressed as the reduction rate (%). DTT significantly reduced the titres over a wide pH range. The solution above pH 8·0 showed gel formation by DTT.

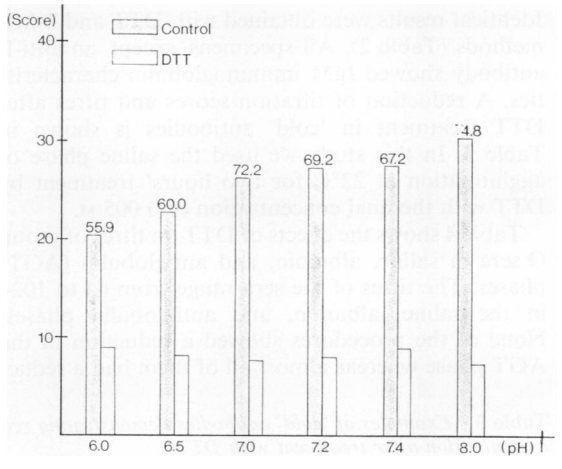

Fig. 3 *Effects of pH of DTT on reduction of titration scores. Saline agglutination tests at 22°C using group O sera with titres ranging from 64 to 128 were used. The figures above the columns indicate percentage of reduction.*

A total of 23 irregular 'warm' red cell antibodies were studied using DTT, 2-ME, and Sephadex (Table 1). All of the specimens in this category were tested by the antiglobulin technique. In this table the range of titres before the treatment of DTT was also shown. None of the three IgG Rho (D) antibodies showed reduction, nor did the three anti A_1 antibodies found in A_2B blood donors.

A total of 40 specimens of 'cold' type red cell antibodies were evaluated by saline titration at 22°C.

Table 1 *Effects of DTT, 2-ME, and Sephadex on 'warm' antibodies (using antiglobulin test)*

Antibody	*Number tested*	*Pre-treatment titres*	*Reduction of titration scores*		
			DTT	*2-ME*	*Sephadex*
Rh_o (D)	7	8-64	0	0	1
Rh_o (D)*	3	32	0	0	0
rh_o (C)	4	8-16	2	0	2
rh_s (E)	1	8	0	0	0
K	3	8-16	0	0	0
Fy^a	1	8	1	1	1
M	1	8	1	1	1
A_1	3	8-128	0	0	0
Total	23		4	2	4

*Rh immune globulin (Rhogam)

Table 2 *Effects of DTT, 2-ME, and Sephadex on 'cold' antibodies (saline agglutination test)*

Antibody	*Number tested*	*Pre-treatment titres*	*Reduction of titre by*		
			DTT	*2-ME*	*Sephadex*
Le^a	3	8-16	3	3	0
Le^b	4	4-8	4	4	3
P	3	4-16	2	2	2
A	15	32-128	15	15	15
B	15	16-64	15	15	15
Total	40		39	39	35

Identical results were obtained with DTT and 2-ME methods (Table 2). All specimens except an anti-P antibody showed IgM immunoglobulin characteristics. A reduction of titration scores and titres after DTT treatment in 'cold' antibodies is shown in Table 3. In this study we used the saline phase of agglutination at 22°C for two hours' treatment by DTT with the final concentration of 0·005 M.

Table 4 shows the effects of DTT on titres of group O sera in saline, albumin, and antiglobulin (AGT) phases. The titres of the sera range from 64 to 1024 in the saline, albumin, and antiglobulin phases. None of the procedures showed a reduction in the AGT phase whereas almost all of them had a reduction in the saline phase. These results were observed in both anti-A and anti-B antibodies; Table 5 shows an example of such results. While a marked reduction of titres by DTT and Sephadex was observed in the saline phase agglutination, no substantial decrease in titres was seen after treatment by these reagents.

Discussion

There appears to be disagreement regarding the optimal conditions, such as incubation period, incubation temperature, or pH, for the 2-mercaptoethanol method of inactivating IgM antibodies (Freedman *et al.*, 1976), and only a few studies on dithiothreitol reagent are available in the literature (Pirofsky and Rosner, 1974; Knight, 1978). The procedure for DTT is generally the one described by Pirofsky and Rosner. Our studies demonstrate that the original procedure can be accepted reliably for clinical use although several modifications and cautions can be cited. Our studies show, for example, that the incubation time can be extended to over 60 minutes while the original procedure calls for a 15-minute incubation. Over 60% reduction of IgM antibodies' activities is observed within 15 minutes of incubation, and the maximum reduction of the activities is seen at the end of two hours. Two-hour incubation, however, did not deteriorate the activities of the IgG antibodies. The optimal temperature was found to be 37°C. This is in agreement with that already reported (Freedman, 1976). It is interesting to note, however, that at 4°C the effects of DTT were not significantly changed even after a 24-hour incubation. The concentration of DTT showed a marked effect on the reduction of IgM antibody activities. The final concentration between 0·0025 M and 0·005 M showed maximal effects, while a concentration exceeding 0·005 M resulted in a gel formation of the specimen. No significant changes were noted on IgG antibodies at these concentra-

Table 3 *Examples of 'cold' antibodies demonstrating reduction of titration scores and titres in saline agglutination after treatment with DTT*

Antibody	*Specimen No.*		*Titre*						*Score*
			1	2	4	8	16	32	
anti-Le^a	5	Before DTT	2+	1+	+W	0	0	0	9
		After DTT	0	0	0	0	0	0	0
	17	Before DTT	3+	2+	1	+W	0	0	17
		After DTT	1+	W	0	0	0	0	4
anti-Le^b	4	Before DTT	2+	2+	1+	+W	0	0	14
		After DTT	1+	+W	0	0	0	0	4
anti-p	12	Before DTT	3+	2+	2+	2+	+W	0	24
		After DTT	1+	+W	0	0	0	0	4
	32	Before DTT	2+	1+	+W	+W	0	0	10
		After DTT	1+	+W	0	0	0	0	4

Table 4 *Effects of DTT on group ABO antibodies* in different reaction media*

	Number tested	Reduction of titre in phases of		
		Saline	Albumin	AGT
DTT	20	19	14	0
2-ME	20	18	14	0
Sephadex	20	19	14	0

*Pre-treatment titres of specimen ranging from 64 to 1024
AGT = antiglobulin test

Table 5 *Reduction of anti-A titres by DTT, 2-ME, and Sephadex in group 0 serum*

	Pre-treatment	Post-treatment by		
		DTT	2-ME	Sephadex
Saline	256	64*	128	64*
AGT	1024	1024	1024	512

*More than twofold reduction

tions. The study on the effects of pH showed maximal reductions of the titration at between 7·0 and 8·0. The pH exceeding 8·0 again resulted in a gel formation of the specimen. Since a substantial reduction of IgM titres is observed at a wide range of pH (as low as pH 6·0) and the pH of the specimen does not vary greatly, it appears that the pH is not critical in this procedure.

The mechanism of inactivation of IgM antibodies by sulphydryl compounds is generally thought to be by cleavage of disulphide bonds, especially those of an IgM molecule. The degree of cleavage varies, depending on the types of the disulphide bonds. It is noted, for example, that intersubunit disulphide bonds are more labile than interchain disulphide bonds (Moore and Steane, 1976). It is not clear whether an IgM globulin subunit after the sulphydryl treatment can still retain the antigen binding activity. It has been shown that anti-A agglutinins treated with sulphydryl compounds would inhibit agglutination although the treated agglutinin no longer agglutinates the cells. Similarly, they are shown to give a positive antiglobulin test with appropriate cells (Moore and Steane, 1976; Knight, 1978). It is interesting to note in our studies (Table 4) that no changes of scores (neither increase nor decrease) are observed in the antiglobulin phase of reaction after DTT treatment. Further studies on the antigenicity of the cleavaged IgM antibodies are needed.

In a total of 63 'warm' and 'cold' type antibodies, it was demonstrated that the results of DTT treatment were in agreement with those obtained by 2-ME and Sephadex. In cold type agglutinins (IgM), the results are in virtually complete agreement (Table 3). Our studies have shown that no effects on IgG antibodies were demonstrated by DTT treatment. It is interesting that all three anti-A_1 obtained in A_2B blood showed no reduction by sulphydryl reagents, indicating IgG characteristics.

It can be concluded from our studies that the DTT reagent is reliably used for inactivation of IgM antibodies and has little or no effect on IgG antibodies, and the results are in close agreement with those for 2-ME and Sephadex. Several precautions are needed with these reagents. High concentrations of DTT over 0·01 M or a high pH over 8·0 resulted in a gel formation. The optimal incubation period may be between 60 and 120 minutes although most of the reduction was observed within 15 minutes.

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