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CRYOPRESERVATION OF SHEEP RED BLOOD CELLS

3. COMPLEMENT TITRATIONS WITH FROZEN SENSITIZED CELLS

By

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MYHRVOLD, VESLEMØY: *Cryopreservation of sheep red blood cells. 3. Complement titrations with frozen sensitized cells.* Acta vet. scand. 1979, *20*, 537—545. — The effect of different suspending and washing procedures for recovery of sensitized sheep erythrocytes (EA) after freezing at —196°C was investigated. Best results were obtained using gelatin-veronal-buffered saline-sucrose containing 0.15 mM-Ca and 1 mM-Mg (GVBSM^{++}-sucrose) as the suspending and first washing buffer. The cryoprotective agents tested were polyvinylpyrrolidone (PVP), neutralized PVP, purified PVP and a gum product, Avelex 1030. All PVP preparations tested gave good results as cryoprotectants in terms of cell recovery after thawing whereas Avelex 1030 was less satisfactory. The EA cells frozen in the presence of untreated PVP showed, however, increased susceptibility to the hemolytic action of complement, whereas cells frozen with purified or neutralized PVP gave titers similar to that obtained with fresh cells. Good results were also obtained with Avelex 1030. Complement titrations with frozen EA cells were more reproducible than titrations with fresh cells.

sensitized sheep erythrocytes (EA); freezing at —196°C; storing at —90°C; complement titrations.

Sensitized sheep erythrocytes (EA) are used for complement titrations. *Spurlock et al.* (1974) have shown that such cells could be kept frozen at —80°C for at least 45 days with hydroxyethyl starch (HES) as the cryoprotective agent. Cell recoveries varied from 70 to 95 %, and the activity of the thawed cells in the complement assay was unchanged.

Earlier experiments (*Myhrvold* 1979b) showed that polyvinylpyrrolidone (PVP) and several gum products were satis-

factory cryoprotective agents for sheep erythrocytes (E) kept at —196°C. This paper describes similar experiments with EA cells. The effect of storing at —90°C was also investigated and complement titrations using fresh cells were compared with cells which had been frozen at —196°C and stored at —90°C.

MATERIALS AND METHODS

Reagents

Acid-citrate-dextrose buffer (ACD). 14.7 g glucose, 13.2 g trisodium citrate (dihydrate) and 4.8 g citric acid were dissolved in 1000 ml distilled water.

Gelatin-veronal-buffered saline-sucrose containing 0.15 mM-Ca and 1 mM-Mg ($GVBSM^{++}$-sucrose). 486.1 g sucrose, 5.095 g Na-5,5 diethyl barbiturate and 18 g NaCl were dissolved in about 1.5 l of distilled water. pH was adjusted to 7.35 ± 0.05 with 1 M-HCl, the acid being added slowly with vigorous mixing of the solution. Five g gelatin dissolved in distilled water and 2.5 ml of a solution containing 1.0 M-$MgCl_2$ and 0.15 M-$CaCl_2$ were added. The volume was brought to 2 l with distilled water. This stock solution was kept at —20°C and diluted with 2½ volumes of distilled water before use.

Gelatin-veronal-buffered saline containing 0.15 mM-Ca and 1 mM-Mg ($GVBSM^{++}$). 83.0 g NaCl and 10.19 g Na-5,5 diethyl barbiturate were dissolved in about 1.5 l of distilled water. pH was adjusted to 7.35 ± 0.05 with 1 M-HCl, the acid being added slowly with vigorous mixing. Ten g gelatin dissolved in distilled water, 5 ml of a solution containing 1.0 M-$MgCl_2$ and 0.15 M-$CaCl_2$ and enough distilled water to make 2 l were added. This stock solution was kept at —20°C and diluted with five volumes of distilled water before use.

0.1 M trisodium ethylendiaminetetraacetate (0.1 M EDTA). 37.2 g of disodium ethylenediaminetetraacetate (Na_2H_2 EDTA) was dissolved in about 800 ml of distilled water. pH was adjusted to 7.65 ± 0.05 with freshly prepared 2 N-NaOH, and the volume was brought to 1 l with distilled water. The solution was stored at about 4°C for maximum three weeks.

GVBS-EDTA buffer. Nine parts of gelatin-veronal-buffered saline without Ca and Mg was mixed with one part of 0.1 M EDTA.

Preparation of EA cells. Sheep blood (100 ml) was drawn aseptically into 400 ml acid-citrate-dextrose buffer (ACD) (*Myhrvold* 1979a) with constant mixing. After storage at 4°C for at least one week, an appropriate volume of blood was removed and transferred to centrifuge tubes. The red cells were packed by centrifugation for 10 min at about 700 × g, the supernatant fluid and the buffy coat removed by aspiration, the cells resuspended in GVBS-EDTA buffer, and the mixture incubated at 37°C for 10 min. The cells were then washed once with ten volumes GVBS-EDTA buffer and three times with $GVBSM^{++}$. The sedimented washed cells were suspended in approx. 18 volumes $GVBSM^{++}$ to make an approx. 5 % suspension. One ml of the suspension was hemolysed with 14 ml of water and the optical density of the lysate measured at a wave length of 541 nm in a 1 cm light path using a Beckman DU spectrophotometer. An optical density (OD) of 0.680 corresponds, in terms of the hemoglobin concentration, to 1×10^9 erythrocytes per ml of cell supsension, provided the sheep blood is derived from animals in healthy condition (*Mayer et al.* 1948). A suspension containing 10^9 cells per ml were sensitized with an equal volume of hemolysin made by inoculation of rabbits with boiled sheep erythrocyte stromata (*Mayer* 1967). The hemolysin was used in a concentration twice the optimal level of hemolysin used for titration of whole complement.

Complement

Guinea pig serum (GPS) as complement source was obtained from blood drawn by heart puncture, and the serum was distributed in tubes and stored at —90°C.

Freezing procedure

EA cells were centrifuged and suspended in a concentration of 4×10^9 cells/ml in one of the suspending solutions. One volume of the cryoprotective agent was added to one volume of the suspended cells. Purification of PVP, neutralization of PVP and preparation of hydrolyzed Avelex 1030 were made as described (*Myhrvold* 1979b). The tubes were stoppered and plunged into liquid nitrogen (—196°C) and stored at —90°C. Thawing was done in a 43°C water bath the tubes being shaken by hand. For investigation of different suspending solutions and washing procedures for recovery (Table 1) the thawed cells were

washed either with the suspending solution or with GVBSM^{++}-sucrose until the supernatants were colorless. Because the diluent used in the complement titration was GVBSM^{++}, the cells were resuspended and washed with this buffer. With GVBSM^{++}-sucrose as suspending buffer the thawed cells were washed with GVBSM^{++}-sucrose and with GVBSM^{++}. With GVBSM^{++} as suspending buffer the thawed cells were washed with this buffer only. Loss of cells was calculated from degree of hemolysis (*Myhrvold* 1979a). All washings were performed until the supernatants were colorless. Then the cells were suspended in GVBSM^{++} to a concentration of 5×10^8 cells/ml and used for complement titrations.

Complement titration

Fresh and frozen EA cells were used for complement titrations as shown below.

Tube no.	1	2	3	4	5	6	7	8	9	10
EA (5×10^8/ml), ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0
GVBSM^{++}, ml	3.0	2.75	2.5	2.25	2.0	1.75	1.25	0	3.25	1.75
GPS (1:200), ml	0.25	0.5	0.75	1.0	1.25	1.5	2.0	0	0	2.0
Water, ml	0	0	0	0	0	0	0	3.25	0	0

The tubes were then incubated at 37 °C for 60 min with occasional agitation, centrifuged at $800 \times g$ for 10 min and the degree of lysis in each tube determined by OD readings at wave length 541 nm in Beckman DU spectrophotometer. The OD reading of the supernatant from the tube with complete hemolysis (tube 8) was corrected by subtraction of the OD value of the cell blank (tube 9), and the OD readings of the supernatant fluids from all the tubes were corrected by subtraction of the OD values of the blanks (tubes 9 and 10). The fraction of cells lysed in each tube was calculated by dividing its corrected OD value by the corrected OD of tube 8. The reciprocal of the dilution of serum yielding 50 % hemolysis of the erythrocytes was defined as the complement titer for that serum (CH50-complement hemolysis 50 %).

RESULTS

It was shown (Table 1) that the buffer used for suspending the EA cells before freezing as well as the washing procedure

Table 1. Cryoprotection of sensitized sheep erythrocytes at —196°. Importance of suspending solution and washing procedure. Cryoprotective agent: 10 % untreated polyvinylpyrrolidone (K 15, average molecular weight 10 000).[1]

Suspending solution before freezing	Loss by thawing %	Loss by washing in susp. sol. %	Loss by washing in GVBSM^{++}–sucrose %	Loss by washing in GVBSM^{++} %	Recovery %
GVBSM^{++}[2]	10.1	31.8			58.1
	10.1		8.3	4.4	77.2
0.15 M-NaCl	9.5	35.5		0.6	54.4
	9.0		10.2	7.1	73.7
ACD	9.7	30.8		2.6	56.9
	9.4		7.6	2.5	75.5
GVBSM^{++}–[3] sucrose	6.2	4.2		4.1	85.5

[1] For freezing, each tube contained 0.75 ml EA cells in suspending buffer and 0.75 ml 20 % PVP in 0.15 M-NaCl.

[2] Gelatin-veronal-buffered saline containing 0.15 mM-Ca and 1 mM-Mg.

[3] Gelatin-veronal-buffered saline sucrose containing 0.15 mM-Ca and 1 mM-Mg.

for the thawed cells, were of importance for the cell recovery. Best results were seen when the EA cells were suspended in GVBSM^{++}-sucrose before freezing. In these experiments PVP had been dissolved in 0.15 M-NaCl before addition to the suspended cells. When PVP was dissolved in GVBSM^{++}-sucrose, the loss by thawing increased to 19.3 %. With GVBSM^{++}-sucrose and no cryoprotectant the loss by thawing was 30.9 %. When the suspending solution before freezing was GVBSM^{++}, 0.15 M-NaCl or ACD, good results were seen with GVBSM^{++}-sucrose as washing buffer. The complement titrations take place with reagents dissolved in GVBSM^{++} buffer with no sucrose added. It is therefore necessary that the EA cells are washed with this buffer before use. It is seen (Table 1) that the loss by this step in the cell preparation was rather small. The experiments also showed (Tables 2 and 3) that recovery of EA cells was the same whether untreated, purified or neutralized PVP was used as the cryoprotective agent, and that the different PVP preparations tested were all more effective than Avelex 1030. Avelex 1030 was unfit

Table 2. Cryoprotection of sensitized sheep erythrocytes (EA). Complement titration with frozen and fresh (control) cells. Cryoprotective agents: Untreated, purified and neutralized polyvinylpyrrolidone and hydrolyzed Avelex 1030. Suspending buffer before freezing and buffer used for washing: GVBMS^{++}.

Type of cryoprotectant[1]	Storage days	Recovery %	CH50 titer	CH50 titer/control titer
	19	51.7	250	1.30
PVP untreated	26	47.9	247	1.28
	56	44.1	250	1.30
Control			192	1.00
PVP purified	1	49.5	217	1.01
	20	42.5	218	1.01
Control			215	1.00
	12	48.9	200	1.03
PVP neutralized	26	46.4	198	1.02
	36	40.0	204	1.05
Control			194	1.00
Avelex 1030	11	21.4		
hydrolyzed 40 min	26	24.8		

[1] Final concentrations of the cryoprotectants 10 %.

for use when the suspending buffer and the washing buffer was GVBSM^{++} (recovery 20—25 %, Table 2).

The activity of the thawed cells in the complement assay was unchanged when cells frozen with purified PVP, neutralized PVP or Avelex 1030 were used for complement titrations (Tables 2 and 3). When EA cells frozen with untreated PVP were used for complement titrations, the hemolytic action of complement increased (about 30 %). The deleterious effect of storage of frozen EA cells at —90°C for intervals up to 36 days was small both in terms of recovery of intact cells and in terms of the usefulness of the cells in complement titrations (Tables 2 and 3). Further experiments indicate that the storage time can be prolonged.

DISCUSSION

Sensitized sheep erythrocytes (EA cells) are used routinely in many laboratories for the assay of complement. Normal function of these cells can be expected for only about a week when

Table 3. Cryoprotection of sensitized sheep erythrocytes (EA). Complement titration with frozen and fresh (control) cells. Cryoprotective agents: Untreated, purified and neutralized polyvinylpyrrolidone and hydrolyzed Avelex 1030. Suspending buffer before freezing: GVBSM^{++}-sucrose. Buffer used for washing: GVBSM^{++}-sucrose and GVBSM^{++}.

Type of cryoprotectant[1]	Storage days	Recovery %	CH50 titer	CH50 titer/ control titer
	13	84.6	250	1.32
PVP untreated	27	82.2	247	1.31
	57	79.6	253	1.34
Control			188	1.00
PVP purified	1	80.2	220	1.01
	20	79.9	218	1.00
Control			217	1.00
	1	83.7	204	1.04
PVP neutralized	12	82.0	198	1.01
	26	81.9	206	1.05
	36	81.1	200	1.02
Control			196	1.00
Avelex 1030	11	63.5	226	0.98
hydrolyzed 40 min	26	65.1	234	1.01
Control			230	1.00

[1] Final concentrations of the cryoprotectants 10 %.

stored at 40°C, requiring weekly preparations. More important, however, has been the great variations in the CH50 titers when the titrations were performed with different EA cell preparations. These variations were probably due to the EA cells, which represented the only individually prepared reagent. The other reagents were kept in small portions at —90°C and used immediately when thawed.

Earlier experiments (*Myhrvold* 1979a, b) showed that the molecular weight and concentration of PVP were of importance for the cryoprotective effect on unsensitized sheep erythrocytes with best result using PVP K 15 (average molecular weight 10 000) in a final concentration of 10 %. Accordingly this was the type and concentration of the PVP used in these experiments. Cell recovery was also dependent on type, concentration and time for hydrolysis of different starches (*Myhrvold* 1979b), and Ave-

lex 1030 hydrolyzed for 40 min and in a final concentration of 10 % was preferred for these experiments.

The experiments showed that preservation of EA cells by freezing with PVP and Avelex 1030 was simple and reliable. The preparation of neutralized PVP was more simple than preparing purified PVP and hydrolyzed Avelex 1030 and should therefore be recommended. Indefinite storage of fully functional cells is probably possible. The problem in utilizing low temperatures for preservation of living cells is likely to be found in determination of the cooling and thawing procedure more than in the low temperature storage as such (*Mazur* 1976).

Preparations of EA cells in large amounts and freezing in small portions are time-saving and eliminate batch to batch variability due to the short life span of EA cells stored at 4°C. The above experiments show that complement titration with EA cells kept frozen at —90°C give more reproducible results than those obtained with freshly prepared EA cells.

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SAMMENDRAG

Beskyttelse av røde sauеblodlegemer ved frysing.
3. Komplementtitreringer med frosne sensibiliserte celler.

Betydningen av suspensjonsmiddel og vaskeprosedyre for gjenvinning av sensibiliserte røde sauеblodlegemer (EA) etter nedfrysing til —196°C ble undersøkt. Best resultat ble oppnådd med gelatin-

veronalbuffer-sukrose med 0.15 mM Ca og 1 mM Mg (GVBSM^{++}-sukrose) som suspensjonsmiddel for cellene før frysing og som vaskebuffer etter tining. Som kryoprotektive midler ble brukt polyvinylpyrrolidone (PVP), renset PVP, nøytralisert PVP og et gummiprodukt, Avelex 1030. Cellegjenvinningen var uavhengig av hvilken PVP som ble brukt, mens Avelex 1030 ga dårligere resultat enn PVP. Den hemolytiske virkning av komplement økte ved bruk av EA celler som hadde vært frosset i nærvær av ubehandlet PVP, mens celler som hadde vært frosset med renset eller nøytralisert PVP eller med Avelex 1030 ga samme titre i komplementtitreringer som ufrosne celler. Komplementtitreringer med frosne EA celler ga mer konstante resultater enn når ufrosne celler preparert separat ble brukt som reagens.

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