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From the Department of Microbiology, Dental Faculty, University of Oslo, Norway, and the Department of Physiology, Veterinary College of Norway.

CRYOPRESERVATION OF SHEEP RED BLOOD CELLS

4. TITRATIONS OF THE FIRST COMPLEMENT COMPONENT WITH FROZEN EAC4 CELLS

By

Veslemøy Myhrvold

MYHRVOLD, VESLEMØY: *Cryopreservation of sheep red blood cells. 4. Titrations of the first complement component with frozen EAC4 cells.* Acta vet. scand. 1980, *21*, 291—298. — The cryoprotection of the sheep erythrocyte intermediate EAC4 cells, used as reagent in titration of the first complement component, C1, was investigated. The cryoprotective agents tested were untreated polyvinylpyrrolidone (PVP), purified PVP, neutralized PVP and a hydroxyethylated potato starch of high viscosity, Avelex 1030, hydrolyzed for 40 min. Recovery of EAC4 cells after thawing was 80—90 %, with best results using untreated, purified or neutralized PVP. The EAC4 cells frozen in the presence of untreated PVP showed, however, increased susceptibility to the hemolytic action of C1, whereas cells frozen with purified or neutralized PVP or with Avelex 1030 gave titers similar to that obtained with fresh cells. C1 titrations with frozen and thawed EAC4 cells gave more reproducible results than those obtained when titrations were performed with fresh separately prepared cells.

sheep erythrocyte intermediate; EAC4 cells; freezing at —196°C; storing at —90°C; titrations of the first complement component, C1.

The term complement (C) refers to a group of nine serum factors (C1-C9), constituting a cytotoxic reaction system, which plays a role in various immunological reactions. Titration of the individual complement components is based on the ability of a given component to convert the appropriate intermediate product of immune hemolysis to the next intermediate product in the reaction sequence. One of the intermediate products, EAC4, is

composed of sheep erythrocytes (E), hemolytic antibody (A) and the fourth component of complement (C4). This complex is stable, and has kept the ability to C1 fixation and is therefore used in C1 titrations, provided C1 is the limiting factor. For C1 titrations the titration mixture is added excess of other components necessary for the hemolytic action of complement.

Spurlock et al. (1974) have shown that EAC4 cells could be kept frozen at —80°C for at least 45 days with hydroxyethyl starch as the cryoprotective agent. Cell recoveries varied from 70 to 95 %, and the activity of the thawed cells in the C1 assay was unchanged. Previous experiments (*Myhrvold* 1979 c) have shown that neutralized polyvinylpyrrolidone (PVP), purified PVP and a hydroxyethylated potato starch of high viscosity, Avelex 1030, are satisfactory cryoprotective agents for sensitized sheep erythrocytes (EA). In the present paper EAC4 cells recovered after freezing at —196°C and storage at —90°C were used for C1 assays and compared with results obtained using fresh EAC4 cells. The effect of using various cryoprotectants was investigated.

MATERIALS AND METHODS

Reagents

Preparation of partly purified guinea pig serum C1. For purification of guinea pig serum C1, necessary for the preparation of EAC4 cells, the procedures of *Linscott* (1968) and *Tamura & Nelson* (1968) were followed. Guinea pig serum (25 ml) was adjusted to pH 7.5 by addition of 0.15 M-HCl (about 0.35 ml), the ionic strength was reduced to 0.04 by addition of 2.75 volumes cold distilled water, and the mixture was stirred for 30 min in the cold. The euglobulin fraction was collected by centrifugation for 45 min at 14000 × g. The precipitate was suspended in 15 ml gelatin-veronal-buffered saline containing 0.15 mM-Ca and 1 mM-Mg ($GVBSM^{++}$) (*Myhrvold* 1979 c) diluted to an ionic strength of 0.04 and centrifuged as before. The final precipitate was dissolved in 25 ml of cold $GVBSM^{++}$, and this partially purified material was dialyzed against 2 l of 0.005 M sodium phosphate buffer pH 7.5. The buffer was changed once. After dialysis the material was centrifuged at 34800 × g for 30 min, the precipitate washed twice with 0.005 M sodium phosphate buffer pH 7.5, resuspended in 25 ml $GVBSM^{++}$ and titrated to calculate the C1 activity.

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

C'EDTA for EAC4 cells. Guinea pig serum (5 ml) was mixed with 45 ml of GVBS-EDTA buffer. The mixture was heated for 15 min at 37°C and cooled to 0°C.

Preparation of EAC4 cells. Twenty ml of a preparation of sensitized sheep erythrocytes (EA) containing 5×10^8 cells/ml was cooled to 0°C. To convert the cells to EAC1, 2 ml of partially purified C1 supplying about 400 molecules of C1/cell was added (*Rapp & Borsos* 1970). The suspension was well stirred and held at 0°C for 10 min. Fifty ml cooled C'EDTA (see above) was rapidly added to the ice-cold sensitized red cells. The mixture was held at 0°C for 15 min and was stirred occasionally to keep the cells in suspension. By this procedure C4 binds to EAC1, yielding the intermediate product EAC14. C1 dissociates from EAC14 and is destroyed by the EDTA in the buffer. Therefore and because of the lack of active Mg^{++} C2 will not be fixed and subsequently the other components (C3-C9) will also not be fixed. The red cells were then collected by centrifugation, washed first with GVBS-EDTA buffer and then three times with $GVBSM^{++}$.

Freezing procedure

EAC4 cells were centrifuged and resuspended in gelatin-veronal-buffered saline-sucrose containing 0.15 mM-Ca and 1 mM-Mg ($GVBSM^{++}$-sucrose) (*Myhrvold* 1979 c) to a concentration of 4×10^9 cells/ml. One volume of the cryoprotective agent was then added quickly to one volume of the cells. Preparations of the cryoprotectants were made as previously described (*Myhrvold* 1979 b). 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. The cells were washed with $GVBSM^{++}$-sucrose only (one of the buffers used for C1 assays). Loss of cells was calculated from degree of hemolysis (*Myhrvold* 1979 a). The cells were suspended to a concentration of 1.5×10^8 cells/ml in $GVBSM^{++}$-sucrose and used for C1 titrations.

C1 titration

GVBS-sucrose-EDTA buffer for C1 titration. Nine parts of gelatine-veronal-buffered saline-sucrose without Ca and Mg were mixed with one part of 0.1 M trisodium ethylendiaminetetraacetate (EDTA) (*Myhrvold* 1979 c).

C'EDTA for C1 titration. This reagent supplies the test system with the complement components C3-C9 and a small but not sufficient amount of C2. One ml of guinea pig serum and 36.5 ml of GVBS-sucrose-EDTA were mixed and heated for 15 min at 37°C immediately before use.

C2 for C1 titration. Ten ml of guinea pig serum was dialyzed against 4 l of 0.008 M EDTA at 4°C for 20 h. pH was adjusted to 5.4 with 5 M-NaOH (about 0.5 ml). The dialyzed material was centrifuged for 10 min at 12000 × g at 4°C. The supernatant was brought to 30 ml with distilled water, pH adjusted to 6.0 with 0.5 M-NH_4OH, and the volume brought to 35 ml with distilled water.

45 ml 4 M-$(NH_4)_2SO_4$ containing 2 mM EDTA and with pH adjusted to 6.0 was added. Final concentration of $(NH_4)_2SO_4$ was 2.25 M. After stirring at 4°C for 30 min the solution was centrifuged for 15 min at 16300 × g at 4°C.

51 ml 4 M-$(NH_4)_2SO_4$ containing 2 mM EDTA and with pH adjusted to 6.0 was added to the supernatant (68 ml), making the final concentration of $(NH_4)_2SO_4$ 3.0 M. The suspension was stirred at 4°C for 30 min and centrifuged as before.

The precipitate was suspended in 10 ml of 0.02 M acetate buffer containing 2 mM EDTA and with pH adjusted to 6.0 and dialyzed at 4°C for 17 h against 4 l of this same buffer to remove $(NH_4)_2SO_4$. The solution (11 ml) was adjusted to ionic strength 0.15 with 10 % NaCl and pH was adjusted to 7.4 with 1 M-NH_4OH.

The C2 mixture was titrated and the effective number of C2 molecules per ml was calculated (*Borsos et al.* 1961). According to *Rapp & Borsos* (1970) a minimum of 50 effective molecules of C2 per cell should be used in the C1 titration.

Titration. Fresh and frozen EAC4 cells were used for C1 titrations as shown below. All dilutions were made in GVBSM^{++}-sucrose.

Tube no.	1	2	3	4	5	6	7	8	9
EAC4 (1.5×10^8/ml), ml	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
C1 (conc.$\times10^{-3}$), 0.5 ml	1/20	1/40	1/80	1/160	1/320	—	—	—	—
GVBSM^{++}-sucrose, ml	—	—	—	—	—	0.5	1.0	0.5	—
	Incubated for 10 min at 30°								
C2 (1:25), ml	0.5	0.5	0.5	0.5	0.5	0.5	—	0.5	—
	Incubated for 10 min at 30°								
C'EDTA (1:37.5), ml	1.5	1.5	1.5	1.5	1.5	1.5	1.5	—	—
GVBS-sucrose-EDTA, ml	—	—	—	—	—	—	—	1.5	—
Water, ml	—	—	—	—	—	—	—	—	2.5
	Incubated for 90 min at 37° with occasional agitation								
0.15 M-NaCl, ml	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0

The tubes were then centrifuged at 800 × g for 10 min and the degree of lysis in each tube determined by OD readings at wave length 412 nm in Beckman DU spectrophotometer.

RESULTS AND DISCUSSION

Earlier experiments (*Myhrvold* 1979 a) showed that preparations of PVP K 15, with an average molecular weight of 10000, were better suited as cryoprotectant of sheep red cells than PVP preparations of higher molecular weights.

The present experiments showed (Table 1) that recovery of EAC4 cells was the same whether untreated PVP, purified PVP or neutralized PVP was used as cryoprotectant, and that the different PVP preparations tested were all more effective than Avelex 1030 (about 10 %).

The activity of the thawed cells in C1 assay was unchanged when cells frozen with purified or neutralized PVP or with Avelex 1030 were used for C1 titrations, and both recovery and activity were unchanged after storage for at least 37 days. Cells frozen with untreated PVP showed, however, increased hemolytic action of C1 (about 60 %).

Preparing EAC4 cells in large amounts and freezing in small portions eliminate the batch-to-batch variability seen when titrations were performed with fresh separately prepared EAC4 cells (see controls in Table 1).

Recovery of EAC4 cells after freezing was about 5 % better than recovery of EA cells as shown earlier (*Myhrvold* 1979 c). In those experiments best recovery of EA cells was seen when the suspending buffer and the first washing buffer were

Table 1. Cryoprotection of EAC4 cells. C1 titrations with frozen and fresh (control) cells.

Type of cryoprotectant[1]	Storage days	Recovery %	CH50 titer	CH50 titer/ control titer
PVP untreated	20	90.1	80×10^3	1.66
	28	88.7	76×10^3	1.58
Control			48×10^3	1.00
PVP purified	1	89.9	77×10^3	1.10
	15	90.7	78×10^3	1.11
	30	89.4	74×10^3	1.06
Control			70×10^3	1.00
PVP neutralized	1	92.0	103×10^3	1.14
	7	90.7	96×10^3	1.07
	20	89.7	101×10^3	1.12
	37	89.8	99×10^3	1.10
Control			90×10^3	1.00
Avelex 1030[2]	8	78.0	145×10^3	1.00
	20	79.8	160×10^3	1.10
Control			145×10^3	1.00

[1] Final concentrations of the cryoprotectants 10 %.
[2] A hydroxyethylated potato starch, hydrolyzed for 40 min.

GVBSM^{++}-sucrose. Because complement titrations take place with reagents suspended in GVBSM^{++} buffer with no sucrose added, it was necessary that the EA cells were washed with this buffer before use. The loss by this step in the cell preparation was about 5 %. C1 titrations, however, take place with the EAC4 cells diluted in GVBSM^{++}-sucrose. Accordingly washing with GVBSM^{++} was omitted in these experiments, and about 5 % better recovery was obtained.

Spurlock et al. (1974) obtained best results (recovery 73—95 %) when 14 % hydroxyethyl starch was used with a final cell concentration of 1.5×10^8 cells/ml. Raising the cell concentration to 5×10^8 and 1×10^9 cells/ml lowered the cell recovery to 64 and 47 %, respectively. In our experiments the cells were frozen in a final concentration of 2×10^9 cells/ ml. Recovery with the different PVP preparations was about 90 %, whereas recovery with the hydroxyethylated starch product Avelex 1030, the starch found best in earlier experiments (*Myhrvold* 1979 b), was about 80 %.

The data indicate that PVP is a better cryoprotectant than the various starch products, including the hydroxyethyl starch used by *Spurlock et al.*, if higher cell concentrations than 1.5×10^8 cells/ml are desirable. Another advantage concerning neutralized PVP compared with purified PVP and the starch products is its much easier preparation.

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SAMMENDRAG

Beskyttelse av røde sauеblodlegemer ved frysing. 4. Titrering av første komplementkomponent med frosne EAC4 celler.

Bevaring af EAC4 celler som reagens for titrering av første komplementkomponent, C1, ble undersøkt etter nedfrysing til —196°C. Som kryoprotektive midler ble brukt ubehandlet polyvinylpyrrolidone (PVP), renset PVP, nøytralisert PVP og en hydroksyetylert stivelse (Avelex 1030) hydrolysert i 40 min. Cellegjenvinningen var uavhengig av hvilken PVP som ble brukt, mens Avelex 1030 ga litt dårligere resultater enn PVP. Den hemolytiske virkning af C1 økte ved bruk av EAC4 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 tilnærmet samme titre i C1 titreringer som de ufrosne cellene. C1 titreringer med frosne EAC4 celler ga mer konstante resultater enn når forskjellige prepareringer av ufrosne celler ble brukt som reagens.

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Reprints may be requested from: Veslemøy Myhrvold, the Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo 3, Norway.