Passive Hemagglutination Enhancement by Periodate, Low pH, and Freeze–Thaw Treatment of Aldehyde Processed Cells (33992)

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Previously we published on a passive hemagglutination procedure which is highly sensitive and reproducible. Erythrocytes were stabilized by treatment with pyruvic aldehyde and formaldehyde. A variety of protein and polysaccharide antigens adsorbed firmly on the stabilized cells without recourse to coupling agents (1). Subsequently we reported that the sensitivity of the hemagglutination (HA) reaction could be substantially increased by freezing and thawing of antigen-coated erythrocytes. The reproducibility was ensured by storing antigen-coated cells in liquid nitrogen (2). Extending the study, we found that the HA reaction could be enhanced substantially by various means which alter the characteristics of the erythrocyte surface.

Preparation of stabilized rabbit erythrocytes (FPRE). Rabbit erythrocytes (RE) were stabilized by sequential treatment with pyruvic aldehyde and formaldehyde, then stored in liquid nitrogen (2).

Antigen-coated FPRE. Frozen FPRE were thawed and coated with the antigen, bovine serum albumin (BSA) as described previously (1, 2). Briefly, FPRE were stirred for 2 hr in 0.1 *M* acetate buffer, pH 4, containing 0.1 mg of BSA/ml. The antigen-coated cells (BSA-FPRE) were washed, resuspended as 10% in 0.1 *M* phosphate buffer, pH 7.2, and stored in liquid nitrogen.

HA titration procedure. Rabbit anti-BSA serum was diluted in serial twofold dilutions by pipettes in test tubes. The diluent comprised 0.1 *M* phosphate buffer, pH 7.2, containing 1 mg of gelatin/ml. Twenty-five μl of diluted samples were transferred to titration trays. Disposable polystyrene trays with 96 "V" shaped wells, 0.3-ml capacity, were used (catalog no. IS-MVC-96, Linbro Chemical Co., New Haven, Connecticut). Twenty-five μl of 0.25% BSA-FPRE were added to 25 μl of antiserum dilutions, the trays were covered and shaken once for 20 sec. The cells were allowed to settle for 18 hr at room temperature (24°) after which the HA titers were recorded.

Periodate oxidation of BSA-FPRE. Periodate was dissolved in distilled water, then diluted in half by 0.1 *M* phosphate buffer, pH 7.2. Five ml of 0.7 m*M* sodium periodate in 0.05 *M* phosphate buffer, were added to 0.02 ml of packed BSA-FPRE and stirred with a magnetic mixer for 15 min. The cells were washed 5 times and resuspended as 0.25% in 0.1 *M* phosphate buffer.

The HA titer was increased from 160,000 without the treatment to 2,400,000 after the periodate oxidation. The HA titers using 4 separate cell preparations matched within one well. Titers in normal sera were less than 10. Similar results were obtained with 0.05 to 2 m*M* periodate treatment. When the periodate concentration was increased to 16 m*M*, the cells turned to greenish-brown from the original brown color and the control buttons were poorly defined.

Similar enhancement in HA titer was obtained when FPRE were oxidized by periodate and then coated with BSA. The reproducibility, however, was poor (4 well differences in titers among 4 cell preparations) as compared to the oxidation of BSA coated FPRE.

Freezing and thawing of FPRE. The FPRE were allowed to thaw at room temperature without shaking, resuspended by shaking, and quick frozen in liquid nitrogen. The process was repeated once per week. At intervals, cells were coated with BSA at pH 4 and titered with a standard antiserum. With repeated freezing–thawing of FPRE, the passive HA titers increased as much as 30-fold (Table I). Storage of FPRE at —20° also

TABLE I. Enhancement of Passive HA Titers by Freezing and Thawing FPRE.

Conditions of FPRE storage	HA titer (10^3 units) / cells coated at pH 4.0 for 2 hr; duration of storage (weeks)				
	0	1	4	8	35
—20° with freeze–thaw[a]	40[b]	80	320	1280	ND
without freeze–thaw	40	ND[c]	ND	320	640
—196° without freeze–thaw	40	ND	ND	80	160

[a] Freezing and thawing once per week.
[b] HA titer of 40 in 10^3 units = 40,000.
[c] Not done.

increased the passive HA titer but the rate of enhancement was not as rapid. Of 5 FPRE prepared from different rabbits, 4 responded to freeze–thaw treatment as shown in Table I; with one preparation, the duration required for the titer rise was three times the other four. The HA titers with normal sera were less than 10.

Exposure of FPRE to acid prior to coating with antigen. Two ml of 0.1 *M* acetate buffer, pH 3.6 or 4, were added to 0.02 ml of packed FPRE and stirred for varying lengths of time. The cells were centrifuged and coated with BSA at various pH. Acetate buffer, 0.1 *M*, was used for pH 3.6, 4, and 5; 0.11 *M* phosphate buffer was used for pH 6 and 7.2.

Treatment of cells at low pH prior to coating enhanced the HA titer (Table II). Even with this acid treatment, however, low titers were obtained when the coating pH was high. The enhancement was slightly greater at pH 3.6 than at 4.0. The effects of freeze–thaw and low pH treatments were additive.

Discussion. Previously we reported that the sensitivity of the HA reaction can be enhanced by freezing and thawing of antigen-coated FPRE. Since the effect was reproduced in the present study by freezing and thawing FPRE prior to coating, it appears that the alteration of the cell surface, and not the antigen, was responsible for the enhancement. It is likely that the enhancement by periodate oxidation and low pH also resulted from alteration of the cell surface.

The optimal cell preparation is that which gives a maximum HA titer with an arbitrarily selected antiserum (1,280,000 for this serum) without appreciable nonspecific agglutination titer in normal serum (4–8). When the treatment of FPRE with freeze–thaw, periodate oxidation, or low pH was prolonged beyond the optimal condition, the HA titers continued to increase but so did those in normal sera.

TABLE II. Enhancement of HA Reaction by Exposing FPRE to Low pH Prior to Coating.

Treatment prior to coating		Coating		
pH	Duration (hr)	pH	Duration (hr)	HA titer (10^3 units)
—	—	4.0	1	40
—	—	4.0	2	60
4.0	1	4.0	1	160
4.0	5	4.0	1	320
4.0	16	4.0	1	160
—	—	3.6	1	80
—	—	3.6	2	96
3.6	1	3.6	1	320
3.6	1	4.0	1	160
3.6	1	5.0	1	40
3.6	1	6.0	1	0.25
3.6	1	7.2	1	<0.02

In the FPRE system, 4 procedures enhanced the passive HA reaction: repeated freezing and thawing of antigen-coated FPRE (2), low pH treatment of FPRE prior to coating, periodate oxidation of antigen-coated FPRE, and repeated freezing and thawing of FPRE prior to coating. The last procedure is probably the method of choice since the antigen is not subjected to possible deleterious treatments after coating. Low pH treatment is also performed before coating but the degree of enhancement was not as high as

that attained by the freeze–thaw procedure.

Summary. The sensitivity of the passive hemagglutination reaction was increased considerably by subjecting stabilized rabbit erythrocytes to periodate oxidation, repeated freezing and thawing, or to low pH prior to coating with the antigen BSA. Cells were stabilized by pyruvic aldehyde followed by formaldehyde and then coated with antigen at low pH.

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Received Feb. 26, 1969. P.S.E.B.M., 1969, Vol. 131.

Demonstration of Blood Group Substance A Bound to *Pasteurella pestis* (33993)

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Recently it was reported that high titer anti-A agglutinins and hemolysins occurred in human blood Group O donors immunized against plague with *Pasteurella pestis* vaccine. It was suggested that this result was due to the presence of blood Group A antigen in the vaccine injected (1). In view of the significance of this observation relative to obvious potential problems inherent in a relationship between *P. pestis* vaccine and blood group antigens, it is important to determine the source and extent of this antigenic relationship.

In the present study, sera from rabbits that were given primary immunizing doses of human Group A red cell stromata were quantitatively assayed for hemolysins against human A, B, and O red cells at periodic intervals following injection. The animals were then placed into four subgroups and reinjected with: human type A red cell stromata, intact commercial *P. pestis* vaccine containing trace amounts of beef heart extracts and porcine peptones and peptides (vaccine I), washed *P. pestis* bacilli from vaccine I, and vaccine I cell free suspending medium. At periodic intervals following injection, the serum from each rabbit was assayed to determine the secondary immune response for hemolysins against human A, B, and O red cells elicited by each preparation.

In a second experiment one group of rabbits was initially injected with human type A red cell stromata, while a second group was initially injected with washed *P. pestis* bacilli harvested from a commercial vaccine containing trace amounts of beef heart extract and soya peptones and peptides (vaccine II). Each of these two groups was subsequently divided into two subgroups, and each of the subgroups was then reinjected with human A red cell stromata, or washed *P. pestis* bacilli (vaccine II), respectively.

The data from these experiments showed that hemolysins against all human red cell types are elicited in a typical primary and secondary immune response when rabbits are immunized with human A red cell stromata. Washed *P. pestis* bacilli stimulated anti-A hemolysins only when they were prepared from a vaccine containing porcine peptones and peptides in addition to beef heart extract (vaccine I). It is concluded that *P. pestis,* cultured or maintained in an environment containing meat extracts or meat peptones and peptides, binds blood group A specific substance, from these sources, on its cell surface or incorporates it as an integral part of the cell.

Materials and Methods. Preparation of antigens, P. pestis *vaccine. P. pestis* vaccine I was a commercial preparation (Cutter Laboratories) containing 2×10^9 formalin killed *P pestis* bacilli/ml of suspending medium which