Application of Freeze-Dried, One-Day-Old Chick Erythrocytes to Viral Hemagglutination and Hemagglutination-Inhibition Tests

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Erythrocytes collected from 1-day-old chicks were stabilized by fixation with formaldehyde and by freeze-drying after treatment with carbon monoxide. Suspensions of freeze-dried erythrocytes in distilled water or physiological saline had a homogeneous bright reddish-purple color. Freeze-dried erythrocytes were compared with fresh erythrocytes for hemagglutination and hemagglutination-inhibition tests for various viruses including rubella, Japanese encephalitis, influenza, mumps, Newcastle disease, and Sendai viruses. After storage at 4°C for 1 year or more, freeze-dried erythrocytes maintained their original appearance and sensitivity to hemagglutination antigens.

The hemagglutination-inhibition (HI) test has been widely used for the estimation of titers of specific antibody in the sera of individuals infected with certain viruses. Erythrocytes collected from chicks, geese, and turkeys have been routinely used. However, it is difficult to preserve these erythrocytes for long periods so that they are available in an emergency or for incidental use. We attempted to stabilize erythrocytes of 1-day-old chicks so that they could be stored for long periods and used in routine assays (3, 5, 6, 9, 10).

Blood collected from unfed chicks by heart puncture within 24 h after hatching was pooled in acid-citrate-dextrose solution. The pooled erythrocytes were washed three times with phosphate-buffered saline (PBS), pH 7.2 (7). Then erythrocyte hemoglobin was converted into carboxyhemoglobin, and the reddish color was intensified by exposure of the blood to carbon monoxide by the method of Fauconnier (8). The erythrocytes were suspended to a concentration of 10% in PBS with 10% formalin and allowed to stand at 4°C for 7 days for fixation. They were then washed six times with 20 volumes of distilled water to remove unabsorbed formalin and suspended in distilled water to a concentration of 10%. This suspension was rapidly frozen in a mixture of solid carbon dioxide

The togavirus hemagglutination (HA) antigens were prepared by procedures cited previously (4, 12). Myxo- and paramyxoviral HA antigens were prepared by cultivating the Tokyo 16/73 strain of influenza A virus, the Enders strain of mumps virus, the MN strain of Sendai virus, and the Tokyo strain of New-

castle disease virus in the allantoic cavity of hen's eggs (11).

Sera from mice, rats, or rabbits immunized with the respective inactivated viral antigens were used as antibody.

HA and HI tests were carried out in microplates as described Sever (16). The following diluents were used. (i) For rubella virus, HEPES-saline-albumin-gelatin, pH 6.2 (0.025 M HEPES [*N*-2-hydroxyethyl piperazine-*N'*-2'-ethanesulfonic acid], 0.14 M NaCl, 10⁻³ M CaCl₂, 0.2% bovine serum albumin, and 0.001% gelatin [14]); (ii) for Japanese encephalitis virus, 0.1 M PBS at pH 6.2 as a diluent for the erythrocyte suspension, and borate-buffered saline at pH 9.0 with 0.2% bovine serum albumin as a diluent for HA antigen (4); (iii) for both myxo- and paramyxoviruses, PBS with bovine serum albumin at a concentration of 0.2%. Non-specific inhibitors were removed from the serum by treatment with heparin and MnCl₂ for rubella viral HI tests (13), by acetone extraction for Japanese encephalitis viral HI tests, and by digestion with receptor-destroying enzyme (Takeda Chemical Industries, Ltd., Osaka, Japan) for myxo- or paramyxoviral HI tests (1, 2).

The freeze-dried erythrocytes were found to retain as vivid a red color as the fresh erythrocytes and showed no clumping. Table 1 shows the HA titers of rubella, Japanese encephalitis, influenza, mumps, Sendai, and Newcastle disease viruses measured comparatively by use of fresh and freeze-dried chick erythrocytes. There was no significant difference between HA titers obtained with fresh and freeze-dried

TABLE 1. *Stability of freeze-dried erythrocytes collected from 1-day-old chicks (CRBC): compared with fresh CRBC in viral HA tests*

Virus	HA titer ^a		
	Fresh CRBC	Freeze-dried CRBC	
		Stored for:	
		6 months	2 years
Rubella	32	32	32
JE ^b	128	128	128
Influenza	160	320	160
Mumps	64	64	64
Sendai	160	160	320
ND ^c	320	640	640

^a Reciprocal of antigen titer.

^b Japanese encephalitis.

^c Newcastle disease.

erythrocytes, and when stored at 4°C for 2 years, freeze-dried erythrocytes maintained their original sensitivity to various viral HA antigens. The HI titers of mouse, rat, and rabbit sera containing specific viral antibody were compared using fresh and freeze-dried chick erythrocytes, and there was no difference in the HI titer of antiserum with the two types of erythrocytes.

In contrast to fresh erythrocytes, freeze-dried erythrocytes retained their red color, intensified by conversion of hemoglobin into carboxy-hemoglobin in response to exposure to carbon monoxide and also their sensitivity to viral hemagglutinins for 1 year or more. For the routine fixation of erythrocytes, treatment with a high concentration of formalin followed by vigorous stirring at 37°C was used to complete fixation rapidly within several hours. In the present experiments, erythrocytes were suspended in 10% formalin and allowed to stand at 4°C for 7 days. This sequence probably resulted in less damage of the specific membrane receptors of erythrocytes to which viral antigens attach. This was suggested by the increased resistance of freeze-dried erythrocytes to hypo- and hypertonic solutions.

Successful stabilization of chick erythrocytes without loss of sensitivity in viral HA reactions made erythrocytes more readily available for routine viral HA and HI tests. In addition, the stabilized chick erythrocytes are expected to be useful for the standardization of HI tests for the detection of specific antibody in sera. Further-

more, freeze-dried chick erythrocytes should be useful for the biological purification by viruses by alternate absorption and elution and for passive hemagglutination reactions when coated with various antigens and antibodies.

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