PRESERVATION OF ANTIGEN-COATED SHEEP ERYTHROCYTES BY FREEZING FOR USE IN INDIRECT HEMAGGLUTINATION PROCEDURE

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ABSTRACT

Hubert, Earl G. (Veterans Administration Center, Los Angeles, Calif.), George M. Kalmanson, and Lucien B. Guze. Preservation of antigen-coated sheep erythrocytes by freezing for use in indirect hemagglutination procedure. J. Bacteriol. 86:569-572. 1963.—Preservation of sheep erythrocytes, coated with various protein and lipopolysaccharide antigens, was accomplished by rapid freezing and storage at −70 C in a dextrose-lactose solution. The stored cells were tested at intervals in an indirect hemagglutination procedure. Sensitivity of these cells was maintained, and antibody titers were consistently reproducible throughout the period of testing. These observations suggest that sheep erythrocytes may be coated with a variety of antigens and may be employed satisfactorily in an indirect hemagglutination test, even after storage at −70 C for a period up to 6 months.

The ability of erythrocytes to adsorb polysaccharide antigens and of tanned erythrocytes to adsorb protein antigens and become specifically agglutinatable by homologous antibody has led to use of the indirect hemagglutination test in a wide variety of research and clinical procedures (Neter et al., 1956a; Stavitsky and Arquilla, 1958; Boyden, 1960).

Certain drawbacks to the technique are apparent. The antigen coating of the erythrocytes must be performed daily by rather laborious procedures, which involve repeated washing of the red cells. In addition, comparisons of results of antibody titrations, performed at different times, may be difficult due to occasional variation in different lots of erythrocytes. It would therefore be desirable to have available a readily prepared, standardized and stable preparation of antigen-coated erythrocytes. McKenna (1957) and Csizmas (1960) reported that formalin-treated red blood cells would adsorb antigens and remain stable for prolonged periods of time; however, in our laboratory, the interpretation of tests employing formalized cells has not been consistently successful because of the tendency of these cells to agglutinate spontaneously.

Since Strumia, Colwell, and Strumia (1960a, b) demonstrated that blood for transfusion purposes may be frozen in dextrose and lactose, and preserved at temperatures between −68 and −78 C, it was thought that antigen-coated erythrocytes might be similarly preserved for use in the indirect hemagglutination test.

MATERIALS AND METHODS

Antigens. Two different bacterial protein antigens, two bacterial lipopolysaccharide antigens, and two human tissue antigens were obtained as follows.

(i) Diphtheria toxoid, lot 42929-225 (obtained from Arthur W. Tallman, Lederle Laboratories, Pearl River, N.Y.), was used as an example of a bacterial exotoxin. It contained approximately 820 Lf/ml of toxoid and its purity was approximately 1790 Lf/mg of protein nitrogen.

(ii) The second bacterial protein antigen was prepared from a culture of Streptococcus faecalis by the acid extraction procedure which Lancefield and Perlman (1952) used to prepare M protein from group A streptococci. This was used as an example of an intracellular bacterial antigen. This preparation contained 2.07 mg of protein/ml and was stored at −20 C until used.

(iii) Bacterial antigens were prepared from two different cultures of Escherichia coli by the heat extraction method of Neter et al. (1956a). The cultures employed for antigen preparation were isolated from the urine of patients with pyelonephritis. These crude antigens were probably composed of lipopolysaccharides (Neter et al., 1956b).
(iv) Saline extracts of human kidney cortex and liver were employed as representative tissue protein antigens. Thin slices of kidney cortex and liver were washed repeatedly with cold distilled water to remove as much blood as possible. The tissues were then homogenized in sterile 0.85% saline in a ratio of 2 parts saline to 1 part tissue, and the mixtures were allowed to stand overnight at 4 C. The supernatants were separated by centrifugation and stored at −20 C. The kidney cortex antigen preparation contained 7.69 mg of protein/ml, and the liver antigen preparation contained 12.69 mg of protein/ml.

Antisera. Antisera against the bacterial protein antigens and against the human tissue antigens were prepared by immunizing groups of rabbits. Equal volumes of undiluted antigen and Freund’s complete adjuvant were emulsified and 0.5 ml was injected intramuscularly into each of two sites; the injections were repeated 1 week later. Blood samples were taken 14 to 21 days after immunization.

Homologous human sera were employed in titrations with the E. coli antigens. These sera were obtained from the patient at the time isolation of the organism was made from the urine specimen. All sera were stored at −20 C.

Immunological methods. Sheep erythrocytes collected in an equal volume of Alsever’s solution and stored at 4 C for 48 hr were used in these experiments.

The method of Stavitsky and Arquilla (1958) was used in performing the indirect hemagglutination test. In brief, for sensitization with protein antigens, thrice-washed erythrocytes in 2.5% suspension in buffered 0.85% saline (pH 7.2) were tanned by mixing with an equal volume of tannic acid diluted 1:5000 in 0.85% saline and incubated for 15 min at 37 C. The tanned erythrocytes were coated with protein antigen in buffered 0.85% saline (pH 6.4) for 30 min at 37 C, washed once in 1:100 normal rabbit serum in 0.85% saline, and resuspended to a 2.5% suspension in 1:100 normal rabbit serum in 0.85% saline. Cells to be sensitized with the crude E. coli antigens were not tanned, but were washed three times with 0.85% saline and suspended to a 2.5% suspension in the same solution. The erythrocytes were then coated with crude antigen in 0.85% saline by incubation for 30 min at 37 C, washed once in 1:100 normal rabbit serum in 0.85% saline, and resuspended to a 2.5% suspension in the same solution. To carry out the antibody titration, the antisera were serially diluted in twofold dilutions in 0.5-ml amounts of 1:100 normal rabbit serum in 0.85% saline, and then 0.05 ml of antigen-coated erythrocytes was added to each serum dilution. Controls consisted of coated cells plus 1:100 normal rabbit serum in 0.85% saline, uncoated cells plus serial dilutions of antiserum, and uncoated cells plus 1:100 normal rabbit serum in 0.85% saline. The mixtures were allowed to remain undisturbed overnight at room temperature and were read by pattern of sedimentation the next morning. None of the sera agglutinated uncoated cells, and the normal rabbit serum control did not agglutinate either coated or uncoated sheep erythrocytes.

Preservation was performed by dispensing 2.5% suspensions of antigen-coated cells and uncoated control cells in 0.75-ml samples in 5-ml Kimble Neutrugas Ampuls. This amount was sufficient to test a single 12-tube row of serially diluted antiserum. To the cells was added an equal volume of a mixture of 10% dextrose and 15% lactose freshly prepared in distilled water. This mixture was then shell frozen rapidly with constant rotation in an ethanol-CO₂ bath at −70 C and immediately placed in a Dry Ice (−70 C) chest for storage.

To reconstitute the frozen cells, they were rapidly thawed by agitating in a water bath (37 C) for several seconds and transferred to test tubes (12 by 75 mm). The cells were then mixed with an equal volume of 1:100 normal rabbit serum in 0.85% saline, and the supernatant was separated by centrifugation for 1 min in a Clay-Adams serofuge. Hemolysis was measured at 576 mλ in a Beckman spectrophotometer, and cell loss was computed from a standard curve prepared at 576 mλ with known concentrations of sheep red blood cell hemoglobin. If a significant loss of cells due to lysis was observed, two or more vials of reconstituted cells were pooled to give 0.75 ml of a 2.5% suspension of cells, sufficient for each 12-tube row of serum dilutions tested. The cells were washed two more times in 1:100 normal rabbit serum in 0.85% saline and finally diluted in the same solution to a 2.5% suspension of cells ready to be used.

Antibody titrations were performed prior to freezing of the cells to determine the baseline antibody titer in each case. The frozen cells were then retested at 1 week and at monthly intervals thereafter for 6 months.
TABLE 1. Serum hemagglutination titers, using different protein and lipopolysaccharide antigens, at varying intervals after frozen storage of coated erythrocytes

<table>
<thead>
<tr>
<th>Coating antigen</th>
<th>Titer after storage of cells for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 weeks*</td>
</tr>
<tr>
<td>Diphtheria toxoid</td>
<td>256,000†</td>
</tr>
<tr>
<td>Streptococcus protein</td>
<td>640</td>
</tr>
<tr>
<td>Escherichia coli 143</td>
<td>320</td>
</tr>
<tr>
<td>E. coli 337</td>
<td>20,480</td>
</tr>
<tr>
<td>Human kidney cortex</td>
<td>8000</td>
</tr>
<tr>
<td>Human liver</td>
<td>2560</td>
</tr>
</tbody>
</table>

* Zero time represents the hemagglutination titer obtained before cells were frozen.
† Titers are expressed as the reciprocal of the dilution.

RESULTS AND DISCUSSION

The coated cells maintained their sensitivity throughout the 24-week period of testing, and antibody titers were clearly reproducible within one serial dilution step (Table 1).

Upon reconstitution, a 15% loss of frozen cells due to lysis was found after 1 week of storage. This cell loss remained constant (between 15 to 19%) throughout the first 12 weeks of storage. At 24 weeks of storage, the loss of stored cells increased to 23%. Adjustment for this loss has already been described. This increased fragility of cells did not appear to affect the quality of the patterns, and the definition of end points throughout the entire period of testing was comparable with those obtained with freshly prepared cells.

These data suggest that sheep erythrocytes may be coated with a variety of antigens and employed satisfactorily in the indirect hemagglutination test, with consistently reproducible results, even after storage at −70° C for a period up to 24 weeks.

A limitation to the use of this procedure is the occurrence of increasing susceptibility to lysis with prolonged storage. Although the exact cause of this increased fragility is not known, certain factors have been reported which may contribute to this deterioration. Lovelock (1953) showed that excessive exposure to the critical temperature range −3 to −40° C during freezing and thawing may result in increased susceptibility of the cells to damage during storage and at the time of reconstitution. Damage to the cells may also be provoked by the growth of ice crystals in the frozen cells during storage. Such crystal growth occurs at temperatures near the melting point and continues at temperatures below that employed in this study (Burton and Oliver, 1935; Pryde and Jones, 1952).

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LITERATURE CITED


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