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Chemical Linkage of Erythrocytes and Viral Antigen in the Hemolysis-in-Gel (HIG) Test for Viral Antibodies

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The sensitivity of the hemolysis-in-gel (HIG) test with rubella antigen is not improved by chemical linkage of the virus to the erythrocytes, and after such modification, IgM specific antibodies are not detectable. In the influenza HIG test with tetraazotized o-dianisidine (TOD), chromic chloride and potassium periodate as coupling reagents, increased sensitivity was observed with allantoic fluid of infected eggs as antigen. If Tween-ether treated hemagglutinin is used in the HIG test, zones of hemolysis are detectable only after treatment of the erythrocytes with TOD, chromic chloride and potassium periodate.

Key words: hemolysis-in-gel test — IgM antibodies — linkage of viral antigen

Introduction

The hemolysis-in-gel (HIG) test is widely used for mass screening for antibodies to certain viral antigens, mainly rubella and influenza (Oxford et al., 1979; Skaug et al., 1981). In these 2 HIG tests the effect of chemical linkage between erythrocytes and viral antigen, which has been used for passive hemagglutination tests and has been adapted by some workers (Väänänen and Vaheri, 1979, Champsaur et al., 1980) merits study in order to increase sensitivity. Since the main limitation of the rubella HIG test seems to be its inability to detect rubella-specific immunoglobulin M antibodies, and seeing that after chemical linkage stereometric changes may occur in the erythrocyte membrane, we have attempted to detect rubella IgM antibodies in the HIG test after treatment of the erythrocytes with different coupling reagents.

Material and Methods

Erythrocytes

Sheep erythrocytes were washed 3 times with veronal dextrose gelatine (DGV) buffer and stored at 4°C.
**Sera**

A number of sera with different titers against rubella and influenza virus were selected from the routine diagnostic service. Sera were heated at 56°C for 30 min before use in the HIG test.

**Serum fractionation**

To investigate antibody activity in different Ig classes, serum was layered on 4.5 ml gradients of 12.5–37% w/v sucrose in DGV buffer and centrifuged for 16 h at 36,000 rpm in a Spinco SW 50.1 rotor at 4°C. Fractions were collected dropwise from the bottom and tested by both rubella hemagglutination inhibition (HI) and the HIG test. The purity of fractions was tested by single radial immunodiffusion.

**Antigen in the HIG test**

Commercial lyophilized rubella HA antigen was obtained from Behringwerke (Marburg) and used in the HIG test. Influenza virus A/Port Chalmers/73 (H3N2) was propagated by allantoic inoculation in embryonated hen's eggs by standard procedures. A part of the allantoic fluid was treated with 0.01% Tween 80 and ether at 4°C for 18 h and the water phase collected.

**HIG test**

The HIG plates were made essentially according to the method of Grillner and Strannegard (1976). Briefly, 0.5 ml of 10% washed sheep erythrocytes in DGV buffer were mixed with 0.5 ml antigen corresponding to 5260 HA units hemagglutinin, and incubated for 30 min at 4°C. After centrifugation the erythrocytes were resuspended in 7.0 ml DGV buffer and mixed with 6.0 ml 1.0% agarose in DGV buffer previously cooled to about 50°C. The prepared plates could be stored at 4°C for 1–2 weeks before use. Wells were punched in the gel, and control plates without antigen were prepared in the same way. Each well was filled with 5 µl serum and the plates were incubated at 4°C for 24 h. The gels were then flooded with a 1:5 dilution of guinea-pig complement and incubated in a humidified atmosphere at 37°C for 3 h. Zone diameters were measured to the nearest 0.1 mm with a magnifying calibrated zone reader.

**Coupling procedure**

Four different coupling procedures were used for chemical linkage of erythrocytes and viral antigen. In one experiment the concentrations of the coupling chemicals were varied in order to obtain optimal attachment.

(a) **Chromic chloride method** (Faulk et al., 1971). An aliquot of 0.5 ml of 10% erythrocytes were mixed with 0.5 ml antigen and 0.05 ml 0.1% chromic chloride in 0.15 M NaCl and incubated for 20 min at 20°C with gentle mixing. After 3 washes, the erythrocytes were suspended in 1.0 ml DGV buffer.

(b) **Cyanuric chloride method** (Avrameas et al., 1969). An aliquot of 0.5 ml of 10% erythrocytes were mixed with 0.5 ml antigen and 0.03 ml of 0.3% cyanuric chloride in acetone for 30 min at room temperature, and washed 3 times.

(c) **Tetraazotized o-dianisidine (TOD) method** (Avrameas et al., 1969). An aliquot
of 0.5 ml of 10% erythrocytes were mixed with 0.5 ml antigen and 0.05 ml of 0.5% tetraazotized o-dianisidine in 0.15 M NaCl at 20°C for 20 min and washed 3 times.

(d) Glutaraldehyde method (Avrameas et al., 1969). An aliquot of 2.5 ml of 2% erythrocytes were mixed with 0.075 ml of 0.25% glutaraldehyde at 20°C for 15 min. After washing 3 times, the erythrocytes were resuspended in 1 ml DGV buffer, and 0.5 ml antigen was added. The mixture was incubated at 4°C for 30 min, and after centrifugation the erythrocytes were resuspended in 1.0 ml DGV buffer.

Potassium periodate method

For the influenza HIG test the method used was similar to that of Russell et al. (1975). An aliquot of 0.5 ml of 10% sheep erythrocytes were mixed with an equal volume of $5 \times 10^{-4}$ M potassium periodate in phosphate buffered saline and kept at room temperature for 10 min with gentle mixing. Then 0.5 ml virus, in the form of allantoic fluid or Tween–ether treated hemagglutinin, was added and the mixture kept at 4°C for 10 min. The sensitized erythrocytes were washed 3 times and made up to a 5% suspension in DGV buffer.

Hemagglutination inhibition test

The hemagglutination inhibition test (HI test) for determining rubella antibodies was carried out according to Stewart et al. (1967) and the HI test for influenza antibodies performed as recommended by WHO (Palmer et al., 1975).

Results

For the determination of rubella antibodies, dilutions of a positive serum with a titer of 1:128 in the HI test were tested in both the HI and the HIG test. The HIG test was performed using 2 different techniques of coupling the virus to the erythrocytes, by hemagglutination and by chemical linkage with reagents in different concentrations. Table I shows that the coupling techniques used produced no improvement in sensitivity. Both with and without chemical linkage, the edge of the hemolysis ring was sharp, allowing exact measurement of diameters. With high concentrations of TOD, chromic chloride and cyanuric chloride, spontaneous hemolysis occurred, whereas with glutaraldehyde in high concentration, no zones of hemolysis were detectable.

In different sucrose gradient fractions of a serum positive for rubella-specific IgM, no antibodies of IgM class could be detected in the HIG test by either method of attachment (Fig. 1).

For determination of influenza antibodies the HIG test was carried out with 2 different kinds of antigen, allantoic fluid and Tween–ether hemagglutinin. In addition, the erythrocytes were treated with potassium periodate.

The results with allantoic fluid as antigen are shown in Table II. With TOD, chromic chloride and periodate the sensitivity is increased and HI titers of 1:8 were detectable by the HIG test. With cyanuric chloride and glutaraldehyde as coupling reagents no improvement was seen.
| HI titer | HIG test (area of hemolysis, mm) |
|---------|---------------------------------|
|         | Without coupling reagent | TOD | Cyanuric chloride | Chromic chloride | Glutaraldehyde |
|         | 0.2% | 0.5% | 1.5% | 0.05% | 0.3% | 0.7% | 0.1% | 0.4% | 1.2% | 0.025% | 0.5% | 1.5% | 10% |
| 1: 4    | 0   | 0    | x   | 0   | 0    | x   | 0   | 0   | x   | 0   | 0    | 0   | 0   | 0   |
| 1: 8    | 7.1 | 0    | x   | 0   | 0    | x   | 0   | 0   | x   | 0   | 0    | 0   | 0   | 0   |
| 1: 16   | 9.5 | 9.4  | 9.2 | x   | 9.3  | 9.4 | x   | 9.3 | 9.6 | x   | 9.3  | 9.3 | 9.5 | 0   |
| 1: 32   | 11.0| 10.9 | 10.7|x   | 11.1 | 10.8|x   | 11.2| 11.1|x   | 10.8 | 11.3| 11.0| 0   |
| 1: 64   | 12.3| 12.4 | 12.3|x   | 12.1 | 12.2|x   | 12.4| 12.5|x   | 12.4 | 12.3| 12.1| 0   |
| 1: 128  | 13.5| 13.5 | 13.4|x   | 13.2 | 13.5|x   | 13.6| 13.5|x   | 13.6 | 13.7| 13.3| 0   |

* 0 = no hemolysis.
* x = complete hemolysis.
Fig. 1. Results with 7 sucrose gradient fractions of a rubella IgM-positive antiserum in the HI (×) and the HIG test with and without chemical linkage of the antigen. 1, without coupling reagents; 2, glutaraldehyde; 3, cyanuric chloride; 4, chromic chloride; 5, tetraazotized o-dianisidine.

With Tween-ether treated hemagglutinin as viral antigen, without chemical linkage no zones of hemolysis were seen in the plates. After addition of TOD, chromic chloride and potassium periodate sharp zones occurred which had the same diameters as the zones of hemolysis obtained with allantoic fluid as antigen (Table III).

### TABLE II

| HI titer | HIG test (area of hemolysis, mm) |
|----------|---------------------------------|
|          | Without coupling reagent | TOD | Cyanuric chloride | Chromic chloride | Glutaraldehyde | Periodate |
| 1: 4     | 0 a                          | 0   | 0                 | 0                 | 0               | 0        |
| 1: 8     | 0                            | 7.0 | 0                 | 6.0               | 0               | 7.2      |
| 1: 16    | 6.0                          | 10.0| 5.2               | 7.6               | 6.2             | 9.6      |
| 1: 32    | 7.5                          | 11.5| 7.7               | 10.8              | 7.5             | 11.0     |
| 1: 64    | 11.1                         | 14.0| 9.9               | 13.4              | 10.4            | 12.9     |

a 0 = no hemolysis.
TABLE III
HI AND HIG TESTS FOR INFLUENZA ANTIBODIES WITH TWEEN–ETHER TREATED HEMAGGLUTININ

| HI titer | HIG test (area of hemolysis, mm) |
|----------|---------------------------------|
|          | Without coupling reagent | TOD cyanuric chloride | Chromic chloride | Glutaraldehyde | Periodate |
| 1: 4     | 0\(^a\) 0 0 0 0 0 0 |
| 1: 8     | 0 5.4 0 5.6 0 6.1 |
| 1:16     | 0 8.1 0 8.0 0 8.5 |
| 1:32     | 0 11.4 0 11.5 0 11.8 |
| 1:64     | 0 14.0 0 13.8 0 13.9 |

\(^a\) 0 = no hemolysis.

Discussion

The HIG test is simple to work with and is considered well suited to routine laboratory diagnosis. It has been used with many antigens and allows large scale testing of immunity to many viruses. With chemical coupling of the viral antigen to erythrocytes, the HIG test may be performed, at least in principle, with any virus group. Many coupling reactions have been used in the passive hemagglutination test, but in the latter high concentrations of coupling reagents can be used because the erythrocytes have been previously stabilized with glutaraldehyde. In the HIG test, determination of antibodies against a nonhemagglutination corona virus was made using chromic chloride (Hierholzer and Tannöck, 1977), which is known to bind non-specifically immunoglobulins to erythrocytes (Gold and Fudenberg, 1967).

With hemagglutinating viruses such as rubella and influenza, there is no evidence as to the effect of coupling reagents in the HIG test. Usually the rubella HIG test is performed without attachment of the antigen to the erythrocytes by chemical reagents, although Champsaure et al. (1980) used chromic chloride. Our results show that introducing different coupling techniques used in passive hemagglutination confers no advantage in the rubella HIG test. This is in agreement with Klingeborn and Dinter (1978) who attached the antigen by chromic cation for the HIG test with the equid herpes virus 1 (EHV 1) and found no difference. Rubella IgM antibodies are not active in the HIG test with chemical linkage. The fact that IgM antibodies to viral antigens do not usually fix complement (Schneweis et al., 1974) may explain this.

Chemical linkage and treatment of the erythrocytes with potassium periodate, so that after adsorption no spontaneous elution of the virus occurs (Fazekas the St. Groth, 1949), increases the sensitivity of the influenza HIG test. In the past, both chromic chloride (Väänänen et al., 1976) and potassium periodate (Russell et al., 1975) have been used.
Our results show that TOD can be used with the same effect as chromic chloride and potassium periodate for sensitization of the erythrocytes. Cyanuric chloride and glutaraldehyde as coupling reagents with Tween–ether treated hemagglutinin gave no detectable zones of hemolysis, for reasons remaining unclear. There was no difference between allantoic fluid and Tween–ether treated hemagglutinin as antigens in the influenza HIG test, so both may be used in this test.

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