Detection of rubella haemagglutination-inhibition (HAI) and virus-specific IgM antibody using trypsin-treated human group O erythrocytes in the HAI test

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SUMMARY The modification of the standard rubella haemagglutination-inhibition (HAI) test using trypsin-treated human group O erythrocytes instead of chick erythrocytes was evaluated. In a comparative study we found that, of 816 samples tested by both methods, the titres of 807 (98.9%) sera were in close agreement within an acceptable twofold difference. Trypsin-treated human group O erythrocytes usually provided titres that were twofold higher than those obtained with chick erythrocytes. In general, a very good correlation between the two methods was obtained. Data are presented that emphasise the importance of trypsin treatment of human group O erythrocytes before use in the HAI method. Furthermore, we found that trypsin-treated human group O erythrocytes can be stored for periods of up to 30 days and used in the HAI test without any appreciable loss of sensitivity or specificity. Moreover, we replaced chick erythrocytes with trypsin-treated human group O erythrocytes in the sucrose density gradient/HAI method used for the detection of rubella virus-specific IgM and found it to be a very satisfactory method. In view of these findings we recommend that trypsin-treated human group O erythrocytes should replace chick erythrocytes in the standard rubella HAI test since the former provided not only a more sensitive, more economic, and less time-consuming method but also a technique which is as specific as that using chick erythrocytes.

The rubella haemagglutination-inhibition (HAI) test using day-old chick erythrocytes is the most widely used technique for both diagnostic and epidemiological studies on rubella. However, in most developing countries, such as Kuwait, a continuous supply of day-old chick cells from a commercial source is not usually available, and the use of this technique for such purposes is therefore limited.

Quirin *et al.* (1972) and Iwakata *et al.* (1974) provided preliminary data, which suggested that day-old chick erythrocytes could effectively be replaced by trypsin-treated human group O erythrocytes in the HAI method without affecting the sensitivity or specificity of the test. This encouraged us to investigate this possibility further, and in this paper we report our results. Furthermore, we have extended our investigation to show that trypsin-treated human group O erythrocytes may be used

instead of day-old chick cells to detect rubella virus specific IgM by HAI after serum fractionation on sucrose density gradients (Vesikari and Vaheri, 1968; Best *et al.*, 1969), since such an application may provide an alternative method when chick erythrocytes are not available.

Material and methods

PREPARATION OF TRYPSIN-TREATED HUMAN GROUP O ERYTHROCYTES

A slight modification of the method described by Iwakata *et al.* (1974) was followed. Fresh or recently collected human group O positive blood was obtained from volunteers or blood donors. Erythrocytes were washed three times in phosphate buffered saline (PBS) before a 10% suspension was prepared from the packed cell volume in veronal buffered saline.

A 5% trypsin (Wellcome Reagents, Ltd) prepara-

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tion was added to the 10% erythrocyte suspension in a ratio of 0.05 ml trypsin per ml of cell suspension, the mixture being shaken and incubated at room temperature for one hour. Erythrocytes were then washed three times in PBS and stored in Alsever's solution at 4°C as a 10% suspension until required. Before use, erythrocytes were washed three times in PBS and resuspended in HAI buffer (96 ml of veronal buffered saline, 1 ml of 10% solution of calcium chloride, 1 ml of an 8% magnesium sulphate solution, and 2 ml of a 10% solution of bovine plasma albumin, pH adjusted to 6.5-7.0). A final 0.25% suspension was used in the test proper.

SERA

Sera were obtained from women attending antenatal clinics in Kuwait and from female nurses and university students. Sera tested for rubella virus-specific IgM were collected from patients who had experienced naturally acquired infection as well as from susceptible nurses and female medical students at St. Thomas' Hospital, London, who were given RA27/3 or Cendehill vaccine. Samples were normally stored at -20°C for periods of two to three months before being tested.

To remove non-specific serum inhibitors, a 0.2-ml serum sample was diluted with 0.6 ml of HAI diluent and mixed with 0.1 ml of heparin (5000 units/ml) and 0.1 ml of MnCl₂ (1M). The mixture was incubated at 4°C for 20 minutes before precipitate was separated by centrifugation.

Samples to be tested by the HAI method using day-old chick erythrocytes were absorbed overnight at 4°C with 0.1 ml of a 50% suspension of chick erythrocytes to remove serum agglutinins. However, this step was found to be unnecessary if human group O erythrocytes were used.

ANTIGEN

A commercially obtained lyophilised preparation of haemagglutinin (Wellcome Reagents, Ltd) was used in the test. Incubation temperatures during antigen titrations were 4°C for one hour and 37°C for one hour when chick and human erythrocytes were used, respectively.

Antigen was used at 4-8 haemagglutinating units (HAU) in the test when either type of erythrocyte was employed. A back titration of antigen was included in each test.

HAI METHOD

The method of Cooper *et al.* (1969) was followed using microtitre equipment throughout. However, although the incubation temperature of antigen and serum dilutions when either chick or human erythrocytes were used was the same (room temperature for

one hour), the incubation temperatures after addition of erythrocytes varied according to the type of erythrocyte used, being 4°C for one hour for chick erythrocytes and 37°C for one hour for trypsin-treated human group O erythrocytes. These conditions were found to provide the best results for the test.

Each test was controlled by including a standard high (1:128) and low (1:8) rubella HAI antibody positive and negative (< 1:4) control serum in each plate which was subjected to the same treatment as those of the test sample. All the specimens were coded, this being broken at the end of the study.

DETECTION OF RUBELLA VIRUS-SPECIFIC IgM

The long-incubation procedure in which serum fractions and antigen were incubated overnight at 4°C before addition of red cells, as described by Al-Nakib *et al.* (1974), was followed except that trypsin-treated human group O erythrocytes were substituted for chick erythrocytes. However, since human erythrocytes were used it was unnecessary to remove non-specific serum agglutinins, thereby eliminating the stage of absorption of sera with erythrocytes.

Results

DETECTION OF RUBELLA HAI ANTIBODY

The sensitivity and specificity of the HAI method, employing trypsin-treated human group O erythrocytes and day-old chick erythrocytes, were compared using the same units of antigen. Table 1 shows that

Table 1 Comparison of sensitivity of trypsin-treated human group O erythrocytes with that of day-old chick erythrocytes in the rubella HAI test

Samples	No of samples showing close agreement* with chick erythrocytes/total no. tested	Percentage
Showing close agreement*	807/816	98.9
Showing a four-fold higher titre with trypsin-treated human group O erythrocytes	9/816	1.2

*Within a two-fold difference.

of 816 serum samples tested during various occasions, the HAI titres of 807 (98.9%) samples were in close agreement (ie, within a twofold difference) when either type of erythrocyte was employed. The results in Table 2a illustrate that both trypsin-treated group O rhesus-positive and group O rhesus-negative erythrocytes can be used in the HAI test satisfactorily, although group O rhesus-negative erythrocytes appear to provide slightly higher titres

Table 2a *Detection of rubella HAI antibody when trypsin-treated human group O rhesus positive and group O rhesus negative erythrocytes were used and sensitivity compared with that of day-old chick erythrocytes*

Type of human group O erythrocytes	No. of samples showing close agreement* with chick erythrocytes/total no. of samples tested	Percentage
Trypsin-treated		
group O rhesus-positive	77/80	96.3
group O rhesus-negative	56/80†	70

*Within a twofold difference.

†24/80 (30%) samples showed a fourfold higher titre than that obtained with chick erythrocytes.

than group O rhesus-positive erythrocytes when both were compared with chick erythrocytes.

However, when trypsin-untreated human erythrocytes were used instead of trypsin-treated erythrocytes, only 15.7% of the samples tested provided titres equivalent to those obtained with chick erythrocytes, the majority having titres of $\geq 1:256$ (Table 2b). This particular finding clearly emphasised the importance of trypsin-treatment of human erythrocytes before use in the HAI test.

Table 2b *Detection of rubella HAI antibody when trypsin-treated and untreated human group O erythrocytes were used and the sensitivity compared with that of day-old chick erythrocytes*

Human group O erythrocytes	No. of samples showing close agreement* with chick erythrocytes/total no. of samples tested	Percentage
Trypsin-treated	115/115	100
Trypsin-untreated	18/115†	15.7

*Within a twofold difference

†Majority of samples showing titres $\geq 1:256$.

The Figure clearly shows the good correlation obtained between HAI titres of the various serum samples tested in our series when either day-old chick or trypsin-treated human group O erythrocytes were used. However, although both showed that the majority of samples had titres of 1:32, the method in which trypsin-treated human group O erythrocytes were used detected titres of $\geq 1:64$ in a higher proportion of sera. Furthermore, it detected low levels (1:4-8) of rubella HAI antibody in 4/31 (12.9%) samples found to be devoid of any detectable rubella HAI activity when chick erythrocytes were used. This particular result was found to be reproducible on many occasions. In general, titres obtained with trypsin-treated human group O erythrocytes were twofold higher.

Figure *Proportion of sera according to rubella HAI titre when either type of erythrocyte was used in the HAI test.*

In order to avoid the necessity of having to prepare a fresh batch of trypsinised human group O erythrocytes every time a test is to be conducted we investigated the effect of storage on trypsinised erythrocytes for periods of up to 30 days and compared their activity with that of freshly trypsinised human group O erythrocytes and day-old chick erythrocytes. The data presented in Table 3 show

Table 3 *Effect of storage on sensitivity of trypsin-treated human group O erythrocytes when compared with that of freshly trypsinised human erythrocytes and day-old chick erythrocytes in the rubella HAI method*

Period of storage between trypsinisation and testing in the HAI method	No. of samples showing close agreement with freshly trypsinised human erythrocytes*/total no. of samples tested	No. of samples showing close agreement with day-old chick erythrocytes*/total No. of samples tested
5 days	27/27 (100%)	54/63† (85.8%)
10 days	25/27 (92.6%)	60/63 (95.3%)
15 days	27/27 (100%)	58/63 (92.1%)
30 days	—	33/40‡ (82.5%)

*Agreement within a twofold difference.

†9/63 (14.3%) samples showed a fourfold higher titre with human erythrocytes.

‡7/40 (17.5%) samples showed a fourfold higher titre with human erythrocytes.

that trypsinised human group O erythrocytes can be stored for periods of up to 30 days and used in the HAI test without an appreciable loss of activity. Thus, when compared with freshly trypsinised human erythrocytes, an agreement within a twofold difference was obtained with 92.6% to 100% of samples after storage of trypsinised human erythrocytes for periods of five to 15 days. Furthermore, compared with day-old chick erythrocytes, agreement within a twofold difference was also obtained with 82.5% to 95.3% of samples after storage for periods of up to 30 days. The other samples showed a fourfold higher titre. However, it was noticed that a twofold higher antigen concentration was required in the HAI test when trypsinised human group O erythrocytes were stored for 30 days than after shorter periods of storage or when freshly prepared erythrocytes were used.

DETECTION OF RUBELLA VIRUS-SPECIFIC IgM USING TRYPSIN-TREATED HUMAN O ERYTHROCYTES INSTEAD OF CHICK ERYTHROCYTES

Our preliminary data showed that human erythrocytes could satisfactorily replace day-old chick erythrocytes in the HAI method used in the detection of virus-specific IgM after sera fractionation on sucrose density gradient. Thus, virus-specific IgM was detected in 52 of 56 (92.9%) samples obtained from patients with naturally acquired rubella and from vaccinees who received either RA27/3 or Cendehill vaccine, for periods of up to eight weeks after infection. However, details of these samples and virus-specific IgM immune responses have already been reported (Banatvala *et al.*, 1977).

Discussion

The data presented in this paper show that trypsin-treated human group O erythrocytes can effectively replace chick erythrocytes in the standard rubella HAI test without any loss of sensitivity or specificity. In addition, the use of human erythrocytes presents a number of advantages over chick erythrocytes. Firstly, they are more readily available, being easily obtained from blood banks as expired blood or fresh from volunteers. This, we feel, is of particular importance in developing countries where a commercial source of day-old chick erythrocytes is not always available. Secondly, the use of human group O erythrocytes avoids the necessity of having to absorb sera with chick erythrocytes to remove non-specific serum agglutinins. As a result, the technique becomes not only more economic since a 50% suspension of chick erythrocytes is usually required to achieve satisfactory removal of serum agglutinins, but also less time-consuming since absorption periods ranging

between two and 18 hours at 4°C are usually necessary to remove most serum agglutinins. Finally, we have shown that erythrocytes can be trypsinised, stored for periods of up to 30 days in Alsever's solution, and used in the HAI test without any significant loss in sensitivity or specificity. Iwakata *et al.* (1974) also found this for periods of up to 15 days, although they did not study the effect of storage for more prolonged periods. Prolonged storage makes the technique extremely practicable and avoids the need for erythrocytes to be freshly trypsinised every time a test is conducted.

Although our results are in close agreement with those obtained by Iwakata and co-workers and confirm the feasibility of using trypsin-treated human group O erythrocytes instead of chick erythrocytes in the HAI test, we have used a slightly different HAI method, and one which we think is less time-consuming. Thus, we used a shorter incubation period of erythrocytes with antigen/antibody reactants, being one hour at 37°C instead of overnight at room temperature. Nevertheless we were able to show that agreement in titre of sera tested by either method approaches 98%, that the use of trypsin-untreated human group O erythrocytes results in non-specific reactions, and that trypsin-treated erythrocytes, when employed, generally result in a two-fold increase in sensitivity of the HAI technique over that using chick erythrocytes.

Our preliminary data on the detection of virus-specific IgM also suggest that trypsin-treated human group O erythrocytes could replace chick erythrocytes in the long-incubation method of sucrose density gradient/HAI technique (Al-Nakib *et al.*, 1974) used in the detection of rubella virus-specific IgM. Results obtained on some 56 samples from cases of naturally acquired rubella and of infection induced by RA27/3 and Cendehill vaccines indicate that virus-specific IgM could be detected in 52 (92.9%) sera for periods of up to eight weeks after primary infection (Banatvala *et al.*, 1977). This further emphasises the practicability of using such erythrocytes in the HAI test and extends its use to rubella virus-specific IgM detection.

In view of these findings and those of other investigators (Quirin *et al.*, 1972; Iwakata *et al.*, 1974), we feel that trypsin-treated human group O erythrocytes should replace chick erythrocytes in the standard rubella HAI test, and that these results should encourage more laboratories, especially in developing countries where a continuous supply of chick erythrocytes may not always be available, to employ this technique. It may be of interest to note that, recently, the Center for Disease Control at Atlanta, Georgia, has recommended the use of trypsin-treated human group O erythrocytes in

preference to chick erythrocytes in the standard rubella HAI test (Center for Disease Control, 1977).

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