Trypsinized Human Group O Erythrocytes as an Alternative Hemagglutinating Agent for Japanese Encephalitis Virus

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Trypsinized human group O erythrocytes were found to be a suitable alternative to gander cells in hemagglutination (HA) and hemagglutination inhibition (HAI) tests for Japanese encephalitis (JE) virus. In the HAI test, no cross-reactions against JE virus were observed with immune sera containing antibody to taxonomically related or unrelated viruses, with mouse brain antigen, or with nonantibody serum inhibitors; specific antibody rise could be detected in an immunized rabbit. Gander and trypsinized human group O cells gave comparable titers in the HAI test, but the latter were preferable since (i) they required less challenging HA antigen, being more sensitive to agglutination by JE virus, and (ii) all human and some animal sera investigated were devoid of natural agglutinins for these cells, thereby eliminating or reducing the need for prior adsorption with packed cells.

The hemagglutination inhibition (HAI) test has found wide application because of its simplicity in the detection and quantitation of antibody produced against hemagglutinating viruses. Certain viruses agglutinate only particular species of erythrocytes, and in consequence most diagnostic virus laboratories often have to carry a wide range of cell species. Recently, however, Biddle (1) has shown that human erythrocytes that are not normally agglutinated by rubella virus are rendered agglutinable by trypsinization. Some preliminary evidence was obtained that morphologically related arboviruses might behave in like manner. This communication reports the agglutination of the trypsinized human erythrocytes by Japanese encephalitis (JE) virus, a group B arbovirus, and shows that these cells may be used as an alternative to gander and 1-day-old chicken cells.

MATERIALS AND METHODS

Virus antigens. The Nakayama strain of JE virus hemagglutinin in the form of a lyophilized acetone-ether extract (Takeda Chemical Industries, Osaka, Japan) was reconstituted with distilled water and diluted an additional ten times with borate saline (pH 9.0) containing 0.4% bovine serum albumin (BSBA; Dade Reagents, Inc., Miami). The extract was kindly provided by Akira Oya, National Institutes of Health, Japan. Rubella virus antigen (Baylor strain, Grand Island Biological Co., Oakland, Calif.) was reconstituted with distilled water and diluted as required in HSAG buffer, pH 6.2 (0.6% HEPES, 0.8% NaCl, 0.01% CaCl₂·2H₂O, 1% bovine albumin Fraction V, 0.003% gelatin) as described by Leibhaber (5).

Red cells. Gander, fowl, 1-day-old chicken, and human group O or A red cells were stored in Alsever solution and prepared for use as 0.5% (vol/vol) suspensions in 0.15 M NaCl in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, (Tris-saline) or 0.2 M phosphate buffer, pH 6.4, as required.

Trypsinization. Red blood cells suspended in Tris-saline were treated with 1:250 trypsin (Difco) at a concentration of 1 mg/ml for 1 h at 37°C. These conditions were found to be optimal, although the time could be extended to 75 min; thereafter the trypsinized cells were susceptible to autoagglutination. The cells were washed three times in Tris-saline and then resuspended in 0.2 M phosphate buffer, pH 6.4, as were the untreated cells, for hemagglutination (HA) and HA inhibition (HAI) titrations using JE virus. The trypsinized cells were stable for 1 to 3 days, after which they tended to autoagglutinate. Red cells for rubella virus titrations were similarly processed and resuspended at a 0.5% (vol/vol) concentration but in HSAG buffer.

HA and HAI titrations. These were performed by the usual procedures in "V" microplates (Cooke Engineering Co., Alexandria, Va.) with final volumes reduced to 100 µl. In the HA test, virus was diluted in 50-µliter volumes, whereas in the HAI test, sera or serum inhibitor fractions were diluted in 25-µlitter volumes and then challenged with 4 agglutinating doses of HA antigen in 25 µl. In each case 50 µlitters of red cell suspension was added, and 50% end points were read. All titrations were performed in duplicate. For titrations involving JE virus, all dilutions were carried out in BSBA; after adding red cells the plates were held at room temperature for 1 h. In the case of rubella virus titrations, the dilutions were carried out in HSAG buffer, and the plates were
incubated at 4 C for 2 h after adding the red cells. In the HAI titrations, virus-antibody or inhibitor mixtures were held at room temperature for 15 min before adding red cell suspensions. The HAI test for influenza virus was performed as previously described (2) with volumes proportionately reduced for microtiter plates.

Antisera. Single samples of immune human sera from clinically and serologically confirmed cases of JE or rubella were kindly provided by W. K. Chang. Antisera against JE virus hemagglutinin were prepared in rabbits by inoculating 1 ml of hemagglutinin, freshly reconstituted as described above, into the marginal ear vein. Antisera were similarly prepared in rabbits against A/Asia/57 (H2N2) influenza virus and crude mouse brain antigen. This latter antigen was prepared from three adult mouse brains that were suspended in 5 volumes of borate saline, pH 9.0, and homogenized in a Dounce homogenizer followed by three successive cycles of freezing (−70 C) and thawing.

Serum fractions. Partially purified lipoprotein nonspecific inhibitors (NSI) of JE virus hemagglutination in human (Shortridge and Ho, J. Gen. Virol., in press) and cobra (Naja naja) (Yip and Shortridge, unpublished data) sera were prepared by high-speed sequential flotation centrifugation at specific density intervals (4).

Animal sera. These were titrated for their natural agglutinin content by the micro-method and included porcine, toad, rhesus, dogfish, gibbon, horse, camel, patas and spider monkey, hedgehog, rat, sheep, chimpanzee, dog, wild boar, and calf sera. These samples were obtained through the generosity of D. E. Bidwell of the Nuffield Institute of Comparative Medicine, Zoological Society of London, and constituted portions of sera taken for routine diagnostic purposes. Salmon serum was obtained from the Ministry of Agriculture, Fisheries and Food, Weymouth, England, and human, duck, and cobra sera were obtained locally.

Serum treatment. Nonspecific inhibitors of JE or rubella virus hemagglutination were removed from sera or partially purified NSI preparations by kaolin treatment (3). Influenza virus NSI was destroyed by diluting the serum 1:5 with Vibrio cholerae neuraminidase (Burroughs Wellcome, Beckenham, England) and holding at 37 C overnight. Enzymatic activity was ceased by heating at 56 C for 30 min. When required, agglutinins were removed by adding packed cells at 4 C for 1 h and the cells were removed by low-speed centrifugation.

RESULTS AND DISCUSSION

Effect of trypsin on red cells. Trypsinization of human group O erythrocytes clearly increased the sensitivity of these cells to JE virus HA activity (Table 1). Furthermore, there was a 10- to 16-fold increase in sensitivity over 1-day-old chicken cells and a 2- to 4-fold increase over gander cells, the cell species commonly used for JE virus HA. Human group A erythrocytes did not appear to be as sensitive after trypsinization as group O cells.

Susceptibility of trypsinized red cells to natural agglutinins. One of the drawbacks of using gander erythrocytes for detecting JE virus antibody in human sera by the HAI test is the need to carry out prior adsorption of the sera to remove natural agglutinins to this species of erythrocytes, as their presence may mask specific antibody. As a possible alternative hemagglutinating agent, trypsinized human cells were investigated for their susceptibility to natural agglutinins in human and nonhuman vertebrate sera. A spectrum of these latter sera was included, as this laboratory is currently engaged in investigating a number of animals as possible reservoirs of JE virus. All human sera contained agglutinins for gander erythrocytes, whereas 35 (64%) had them for untreated or trypsinized human group A cells to high titer (Table 2). No agglutinins were detected in human sera with untreated or trypsinized human group O cells. Almost all animal sera had agglutinins for gander erythrocytes; this activity was not as pronounced with human cells, as 10 and 4 of the sera were agglutinin positive for group A and group O cells, respectively. Trypsinization of human cells apparently resulted in the exposure of more receptors, but there were still fewer sera manifesting agglutinin reactivity towards the group O cells. The average agglutinin titer of positive sera for the trypsinized group O cells

| Table 1. Effect of trypsin on the hemagglutination of various erythrocyte species by JE virus |
|---------------------------------|-----------------|-----------------|-----------------|---------|
| Erythrocyte species             | Erythrocyte condition | JE virus HA titer |     |
|                                |                  | Expt 1           | Expt 2          | Expt 3    | Expt 4  |
| Gander                          | Untreated        | 256              | 1,280           | 256      | 384     |
| 1-day-old chicken               | Untreated        | 64               | 320             | 48       | NTa     |
| Human group A                   | Untreated        | 48               | 20              | 4        | 16      |
| Human group A                   | Trypsinized      | 384              | 1,280           | 256      | 192     |
| Human group O                   | Untreated        | 48               | 40              | 8        | 32      |
| Human group O                   | Trypsinized      | 1,024            | 3,840           | 512      | 768     |

*NT, Not tested.
was, in addition, lower than those recorded for all other cells.

From this point, all further studies were made on trypsinized human group O cells and their performance as a reliable hemagglutinating indicator for JE virus compared with gander cells.

**Behavior of trypsinized human group O red cells in the HAI test.** Preliminary observations showed that trypsinized human group O cells were comparable to gander cells for monitoring the presence or absence (after kaolin treatment) of nonantibody NSI activity in primate (human) or reptile (cobra) serum lipoprotein fraction concentrates (Table 3). It would seem unlikely that trypsinized human cells were behaving anomalously in the HAI test towards NSI. These observations were extended to include the cells’ behavior in the HAI test for the detection of specific JE virus antibody. Rubella virus antigen was included as a control to evaluate the specificity of the virus antigen-antibody reaction, since rubella virus that is grouped with JE virus as a togavirus is also sensitive to agglutination by trypsinized human group O red cells (1). Results are shown in Table 4.

No cross-reaction between any host component that may be present in the JE virus antigen extract and an antiserum prepared against crude mouse brain antigen was observed with the trypsinized human group O cells. An antiserum prepared against JE virus HA antigen showed specific antibody reactivity towards JE virus and manifested a greater than fourfold rise in specific antibody level over the preimmunization serum. On the other hand, an antiserum prepared against a taxonomically unrelated influenza virus was devoid of specific antibody reactivity for JE virus (and rubella virus) after kaolin treatment. When trypsinized human group O cells were used for the detection of specific JE virus antibody in human immune sera (patients nos. 173, 336, and 1311), they were as sensitive as gander cells, and there was no cross-reactivity with rubella virus antigen. Conversely, similar considerations apply to the detection of rubella virus antibody where the

### Table 2. Distribution and content of erythrocyte agglutinins in vertebrate sera

| Serum group tested | Erythrocyte species | Erythrocyte condition | Samples tested | Serum agglutinin examination |
|--------------------|---------------------|-----------------------|---------------|-----------------------------|
|                    |                     |                       |               | Samples positive | Titer range | Average titer for positive sera |
| Human              | Gander              | Untreated             | 55            | 55              | 20-1,280 | 60 |
|                    | Human group A       | Untreated             | 55            | 35              | 10-1,280 | 355 |
|                    | Human group O       | Trypsinized           | 55            | 35              | 10-1,920 | 603 |
|                    |                     | Untreated             | 55            | 0               | 0        | 0 |
|                    |                     | Trypsinized           | 55            | 0               | 0        | 0 |
| Non-human vertebrate | Gander              | Untreated             | 19            | 18              | 20-640  | 60 |
|                    | Human group A       | Untreated             | 19            | 10              | 10-320  | 58  |
|                    | Human group O       | Trypsinized           | 19            | 16              | 10-480  | 69  |
|                    |                     | Untreated             | 19            | 4               | 10-80   | 29  |
|                    |                     | Trypsinized           | 19            | 12              | 10-80   | 25  |

* A positive sample has an agglutinin titer of 10 or greater.
* Duck serum was only negative. Full list of sera examined given in c or d.
* Sera agglutinin positive: porcupine, wild boar, calf, and sheep. Sera agglutinin negative: salmon, dogfish, cobra, toad, duck, hedgehog, spider monkey, rhesus monkey, patas monkey, chimpanzee, rabbit, rat, dog, horse, and camel.
* Sera agglutinin positive: toad, spider monkey, patas monkey, rhesus monkey, chimpanzee, rabbit, porcupine, dog, horse, wild boar, calf, and sheep. Sera agglutinin negative: salmon, dogfish, cobra, duck, hedgehog, rat, and camel.

### Table 3. Use of trypsinized cells for detecting the NSI activity of lipoprotein concentrates against JE virus in the HAI test

| Erythrocytes | HAI titer against JE virus |
|--------------|---------------------------|
|              | Cobra lipoprotein mixture | Human lipoprotein mixture |
|              | Untreated | Kaolin treated | Untreated | Kaolin treated |
| Gander       | 3,840     | < 10          | 640       | < 10          |
| Trypsinized human group O | 3,840 | < 10          | 640       | < 10          |
TABLE 4. Use of trypsinized cells for detecting JE virus or rubella virus antibody in immune sera or specific antiserum

| Serum                                | Erythrocytes | HAI titer with JE virus or rubella virus antigens |
|--------------------------------------|--------------|--------------------------------------------------|
|                                      |              | JE virus                                      | Rubella virus |
|                                      |              | Untreated serum | Kaolin treated serum | Untreated serum | Kaolin treated serum |
| Rabbit antiserum against mouse brain antigen, bleed B<sup>a</sup> | Gander      | 10             | <10  | 10             | <10  |
| Rabbit antiserum against JE virus HA antigen, bleed A<sup>b</sup> | Trypsinized human group O | 10             | <10  | 10             | <10  |
| Rabbit antiserum against JE virus HA antigen, bleed B<sup>c</sup> | Gander      | 20             | <10  | 10             | <10  |
| Rabbit antiserum against A/Asia/57 influenza virus<sup>d</sup> | Trypsinized human group O | 80             | 60   | 10             | <10  |
| Patient no. 173, JE virus-immune serum | Gander      | 20             | <10  | 10             | <10  |
| Patient no. 336, JE virus-immune serum | Trypsinized human group O | 20             | <10  | 15             | <10  |
| Patient no. 1311, JE virus-immune serum | Gander      | 60             | 30   | 20             | <10  |
| Patient no. 1318, rubella virus-immune serum | Trypsinized human group O | 40             | 20   | 20             | <10  |
|                                      | Gander      | 120            | 80   | 20             | <10  |
|                                      | Trypsinized human group O | 160            | 80   | 20             | <10  |
|                                      | Gander      | 160            | 160  | 80             | <10  |
|                                      | Trypsinized human group O | 160            | 120  | 60             | <10  |
| Patient no. 1318, rubella virus-immune serum | Gander      | 20             | <10  | 320            | 160  |
|                                      | Trypsinized human group O | 20             | <10  | 320            | 160  |

<sup>a</sup> Bleed B, 20 days post-immunization bleed.
<sup>b</sup> Bleed A, pre-immunization bleed.
<sup>c</sup> Bleed B, 20 days post-immunization bleed.
<sup>d</sup> Homologous HAI titer after NSI inactivation by bacterial neuraminidase was 240 by using fowl or gander erythrocytes.

reaction appeared specific for that virus only (patient no. 1318).

All in all, the work reported here clearly shows that trypsinized human group O erythrocytes may be used as an alternative cell species to gander (and 1-day-old chicken) cells for the quantitation of JE virus antigen or its antibody. Apart from its intrinsic interest in the study of virus-cell interaction, this observation may find application in laboratories where there are space and budget limitations for the maintenance of a variety of animals that are used solely for the purpose of providing cells for the titration of hemagglutinating viruses. The two- to fourfold increase in sensitivity of trypsinized human group O erythrocytes over gander erythrocytes to agglutination by JE virus means that less of the specially prepared HA antigen is required for JE virus antibody titrations. Additionally, human serum samples for JE virus antibody titration in the HAI test do not require prior adsorption with packed cells for removal of natural agglutinins. Some animal sera may require adsorption.

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