Hemagglutination-Inhibition Method and Immunofluorescence Staining with Venezuelan Equine Encephalomyelitis Virus

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ABSTRACT

SHEPEL, MICHAEL (Fort Detrick, Frederick, Md.). Hemagglutination-inhibition method and immunofluorescence staining with Venezuelan equine encephalomyelitis virus. Appl. Microbiol. 14:346-352. 1966.—Hemagglutination and fluorescent antibody (FA) are compared for the direct detection of virus devoid of host cells. A determination was made of the minimal number of tissue plaque-forming units of Venezuelan equine encephalomyelitis virus that could be detected by the hemagglutination technique. Similar concentrations of the virus in bovine albumin borate saline, Brain Heart Infusion broth (Difco), and demineralized water were tested by the FA technique. Somewhat higher concentrations of the virus in bovine albumin borate saline were used in the hemagglutination-inhibition test. The quantitative hemagglutination procedure employed for these studies was carried out at 37 C for 75 min with variations in concentration of goat red cells. As a result of lowering the red cell concentration, smaller concentrations of virus were detected. The direct FA staining procedure applied to slide preparations containing known numbers of tissue culture plaque-forming units of virus was negative. Adsorbed viral antigen on agglutinated goat erythrocytes was visualized by direct and indirect FA techniques.

The purpose of this investigation was to compare the hemagglutination-inhibition (HAI) test with fluorescent antibody (FA) in the detection of Venezuelan equine encephalomyelitis (VEE) virus relatively free from host cells. Data relating to FA staining of VEE virus adsorbed on goat erythrocytes are also presented.

The existence of hemagglutinins in a number of arthropod-borne viruses (arboviruses) was initially shown by Sabin and his associates (6, 7, 8, 29, 30, 36). Since Porterfield (26) first demonstrated that goose erythrocytes could be agglutinated by arboviruses, goose cells were thereafter routinely used by investigators (4, 11) in hemagglutination (HA) and HAI tests. The use of the HAI reaction in classification of arboviruses has been reported by Casals and Brown (5) and Casals (4). The use of immunological characteristics for classification of arboviruses has also been reported by Porterfield (26, 27), Clarke and Casals (10), and Clarke and Theiler (12).

The present study, directed toward utilization of HA technique for arboviruses, was influenced by the fact that the HA test is simple and rapid, and it is best performed in the absence of host cells. The FA procedure up to the present time has been applied successfully only to infective tissue with viruses (3, 19, 21). There is a lack of data in the literature concerning the use of FA techniques in the detection of arboviruses free from host cells. In this study, the sensitivity of the HA reaction is shown to be related to the quantity of red cells employed.

MATERIALS AND METHODS

Virus. The origin of the Trinidad strain of VEE virus employed in this study was previously described by Hardy (17). The plaque technique on chick embryo monolayers described by Dulbecco (14) with Western equine encephalomyelitis virus was used for quantitative assay of plaque-forming units (PFU) in the stock virus. Pooled infectious culture fluids were centrifuged at 1,300 × g for 30 min, and the supernatant fluid was then filtered through a membrane.
filter (HA) to insure the removal of all particulate matter. This material served as a source of virus for this investigation.

Chemical reagents and erythrocytes employed for HA and HAI test. A full description of the source and preparation of goose erythrocytes and preparation of all reagents has been given by Clarke and Casals (11).

Direct counting of erythrocytes. The total number of cells used for conducting HA and HAI tests was counted directly with the Levy-Hauser, double-ruled Neubauer blood-cell counting chamber.

Immune sera for the HAI test. Specific rabbit VEE antiserum was prepared according to the method of Shepel and Klugerman (33). The serum gave a HAI titer of 1:10,240. Before the serum was used, it was treated with kaolin and adsorbed with erythrocytes to remove nonspecific inhibitors and agglutinins by the method of Clarke and Casals (11).

Detection tests. To afford a basis for reasonable comparable quantitative results, the same stock VEE virus preparation was used in the HA test first and then in the preparation of smears for FA staining with labeled VEE antibody globulin. The effect of red blood cell concentration on the number of virus PFU that could be detected in the HA test was done simultaneously.

The HA test with goose red cell suspension, which varied in concentration from 7,000,000 to 1,500,000 cells per milliliter, was a modification of that described by Clarke and Casals (11). Twofold dilutions of tissue culture fluid containing VEE virus were prepared in 0.2% bovine albumin borate saline (BABS, pH 9.0). A total of four dilutions were utilized (0.5 ml per dilution). Each dilution of virus was combined with different concentrations of goose red cells (0.5-ml volume). The resulting mixtures (pH 6.4) were shaken, and the reaction was allowed to proceed at 37°C for approximately 75 min. The final tube: showing complete agglutination, manifest by a uniform, thin, translucent shield of cells covering the lower surface of the tube, was taken as the end point. An incomplete shield combined with a dark ring of cells indicated partial HA. Ordinarily, the transition from complete agglutination to the absence of agglutination was rather abrupt, with one tube showing partial agglutination between these extremes (Fig. 1, 2). The test was not considered valid unless the erythrocyte cell controls were completely negative.

The HAI test was conducted according to the method of Clarke and Casals (11). The homologous immune rabbit serum was used in a dilution of 1:64 in 0.2% BABS (pH 9.0). An appropriate volume (0.5 ml) of goose red cell suspension was added to each serum-virus mixture. A serum control was prepared by combining equal volumes of serum and BABS. The serum control assured that nonspecific agglutinins were absent. Specific identification of virus was indicated by a complete HAI reaction, with red cells accumulating as sharply demarcated discs. In the virus control, the cells agglutinated and settled as a thin, uniform translucent shield covering the entire bottom of the tube.

The direct FA technique was essentially the same as that used by most workers in the field.

Chemical reagents. Fluorescein isothiocyanate (FITC, BBL) was used to label globulins in the direct method, and lissamine rhodamine RB #200 (BBL) was used for counterstaining.

FITC-labeled antibody. Rabbit antiserum used was the same as that mentioned previously in the HA test. The immune globulin was precipitated from whole serum by the methanol method of Dubert et al. (13), and was reconstituted to five-eighths of the original volume. This globulin solution yielded an HAI titer of 1:5,120. Total protein was determined by the micro-Kjeldahl procedure.

The conjugated globulin fraction was prepared according to a modification (suggested by M. R. Klugerman) of the method of Marshall, Eveland, and Smith (20). The powdered FITC was dissolved in a mixture of unbuffered saline and 0.5 M carbonate-bicarbonate buffer (pH 9.0) prior to adding globulin solution (preadjusted to pH 9.0 with 0.1 M NaOH). The serum-dye mixture contained 10% buffer and 0.04 mg of FITC per mg of protein. The final pH of the mixture was 9.0. The mixture was allowed to react to 4°C for 18 hr with gentle agitation. Untagged dye was removed by passage through a Sephadex column (G-25, medium grade) and eluted with physiological saline. The antiserum used was 1:1,280, as determined by HAI test. The concentration of bound FITC was 28.2 μg/mg of globulin. This was determined from standard calibration curves relating FITC concentrations in 0.1 N NaOH to absorbance at 490 μm, and protein content (biuret reaction) to absorbance to 560 μm.

Preparation, fixation, staining, and examination of smears. A quantity of virus PFU in 0.05 ml was deposited within a 1-cm square on a glass slide (3 by 1 inch). The slides were dried and kept in the refrigerator at 4°C until processed. Smears were fixed in 10% formalin (21, 35) for 10 min at room temperature (25°C), and were washed twice in buffered saline (pH 7.2). Preparations were dried at 37°C for 20 min. Four slide preparations were used with each concentration of virus tested. Two of the preparations were counterstained with lissamine rhodamine RB #200. Smears were stained directly with a drop of VEE fluorescein-labeled antibody solution for 30 min in a moist chamber at room temperature. After this, the slides were rinsed and washed for 10 min in carbonate-bicarbonate buffer (0.5 M, pH 9.0) and mounted in glycerol adjusted to pH 9.0 with carbonate-bicarbonate buffer (25). Labeled globulin was used undiluted and diluted 1:2 with neutral buffered saline. Specifications for the fluorescence lamp and microscope used have been described previously (25). Slides were examined for specific staining of viral particles, and fluorescent reactions were graded on the basis of lowest to highest estimated intensity (1+ to 4+).

Immunofluorescence techniques in staining of VEE virus adsorbed on goose erythrocytes. The agglutination of erythrocytes by VEE virus suggested exploration of the possibility of using FA to visualize virus particles attached to the surface of goose erythrocytes and to compare direct and indirect FA staining methods.
Prior to use of FA staining, the appearance of erythrocytes agglutinated by the viral antigen was observed. The HA test was conducted as follows. A quantity of $6 \times 10^6$ goose erythrocytes was added to $35 \times 10^7$ tissue culture plaque-forming units (TCPFU) of virus in BABS. Virus-erythrocyte suspensions were shaken continuously in a flat-bottomed plastic petri dish (50 mm) for 10 min. The effect of virus adsorption on goose erythrocytes was examined both macroscopically and microscopically (Fig. 3). Photomicrographs were taken on panatomic X film at an exposure of $1/25$ sec through a Zeiss microscope equipped with a 12× ocular and 10× objective of NA 0.25.

Smears were prepared from the agglutinated erythrocyte-virus mixture, allowed to air-dry, and then fixed in formalin. Specific whole rabbit anti-VEE serum was used for direct and indirect staining of the erythrocyte-virus complex. A portion of the unlabeled globulin fraction was also used. A fluorescein-conjugated goat anti-rabbit serum used for indirect staining was obtained commercially (BBL). The direct-staining technique used in this study was that described previously for smears made from infectious fluids. The indirect technique consisted of covering the smears with one or two drops of appropriate dilutions of either immune whole serum or globulin. After incubation for 30 min at room temperature, the smears were washed by immersion in carbonate-bicarbonate buffer (pH 9.0) for 10 min. Preparations were then stained with FITC-labeled goat anti-rabbit globulin for 1 hr at room temperature. After staining, slides were again washed for 10 min, and smears were mounted in buffered glycerol. Reactions were viewed by fluorescence microscopy. Photographs were taken on 35-mm Tri-X film with a Leitz fluorescent microscope equipped with OSRAM HBO-200-W fluorescent lamp and 20× photo ocular and 40× objective. Filters used were Schott BG-12 exciter filter (3 mm) in combination with barrier filter OG-1 (2 mm). Controls consisted of examining (i) unstained smears of erythrocytes treated with VEE virus; (ii) erythrocytes without virus treated with normal rabbit globulin and stained with fluorescent-labeled, goat anti-rabbit γ globulin; and (iii) infected erythrocytes used for inhibition of fluorescence in the one-step blocking test (16). In this test, smears were stained in the usual manner with mixtures of equal parts of labeled antibody and unlabeled antiserum. Proper dilutions of each reagent were used.

**Results**

It can be seen from Fig. 1 that, as the concentration of red cells decreases, the HA test becomes more sensitive, and fewer infective particles are detected. The highest level of sensitivity (least number of viral particles detected) was obtained with approximately 1,500,000 red blood cells (RBC; Table 1). Small concentrations of virus could be detected with an equally small concentration of RBC. Larger numbers of RBC tended to mask the presence of virus. A mixture containing $6 \times 10^6$ RBC and $3.5 \times 10^3$ TCPFU did not produce hemagglutination (row 2), but a RBC suspension of $1.5 \times 10^6$ resulted in complete agglutination (row 4). Experimental results tend to indicate a direct relationship between the lowest virus concentration that can be detected and the number of indicator cells used in the test. When the RBC concentration was increased from $6 \times 10^6$ to $7.5 \times 10^6$, the lowest detectable concentration of virus was 14,250 TCPFU (row 1). The results showed that low concentrations of virus can be detected with small numbers of RBC. However, the use of very low concentrations of RBC was precluded by limits of visibility. When RBC were

![Table 1. Sensitivity of the hemagglutination test with various concentrations of red blood cells](https://aem.asm.org/)

<table>
<thead>
<tr>
<th>Row no.</th>
<th>Total RBC concn</th>
<th>Tissue culture PFU in tube no. †</th>
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<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>7,500,000</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>6,000,000</td>
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<tr>
<td>3</td>
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<td>+</td>
</tr>
<tr>
<td>4</td>
<td>1,500,000</td>
<td>+</td>
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</table>

* Row numbers correspond with those in Fig. 1. † Tube numbers correspond with those in Fig. 1; + = complete agglutination; ± = partial agglutination; 0 = no agglutination.
utilized in concentrations below $1.5 \times 10^6$, reactions could not be accurately assessed because button formation was not easily observed. In consequence of these findings, red cell concentrations of $1.5 \times 10^4$ to $3 \times 10^4$ were selected for routine dilution tests.

As seen in Fig. 2, the quantities of the test virus used were completely inhibited, as indicated by the cells' settling as sharply demarcated red discs. Agglutination was observed in tubes without serum. The third tube in the virus-cell control showed an incomplete shield combined with a dark ring of cells, indicating partial HA (Table 2).

In comparative assays of FA and HA procedures, the direct FA method failed to show any evidence of specific fluorescence with virus deposited on slides. On the other hand, virus concentrations of 28,500, 14,200, 7,100, and 3,500 were detected with the HA test.

Figure 3 shows goose erythrocytes with and without adsorbed VEER virus. Treated cells in photograph B characteristically display aggregates of red cells forming large dark masses of agglomerated cells, whereas untreated cells in A show absence of aggregates. Photomicrographs A1 and B1 demonstrate the agglutination effect more clearly.

A comparison of the direct and indirect FA staining method for visualization of VEER virus adsorbed on goose erythrocytes can be seen in Fig. 4. Photograph A shows positive fluorescence at the periphery of red cells. Brilliant staining of viral aggregates was obtained by indirect staining (photographs B and C). The fluorescent reagent, applied after covering the smears with whole serum and serum fraction, stained virus-agglutinated cells with equal intensity. Areas of autofluorescence are seen in photograph D, but the specific reactions appeared brighter, with color definitely yellow-green and more distinct.

**Discussion**

The results presented in Fig. 1 and 2 show that lower concentrations of VEER virus can be detected by reducing the number of goose erythrocytes. The HA test can detect the presence of VEER virus in 75 min. The settling patterns of cells served as the index of detection. Positive detection was evident by a shield of cells covering the entire lower surface of the tube. In no detection, the cells settled as dark, red central buttons.

An attempt to demonstrate the presence of naked virus in smear preparations by direct immunofluorescence failed with all virus concentrations tested (28,500 to 3,562 TCPFU). This result could be expected, since the virus is not sufficiently large for visualization under the ordinary light microscope. The average particle size of arbovirus in group A is approximately 50 nm (9, 22, 23, 24, 28, 31, 32, 37). To visualize particles of this size, it is necessary to use the electron microscope.

The amount of FA reacting with an individual virus particle is undoubtedly too low to visualize in the fluorescent microscope. In experiments with radiiodinated sera, Boursnell, Coombs, and Rizk (1) showed that measurement of antibody was difficult when the amount of antibody com-

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**Figure 2. Top view of tubes shows patterns of sedimented cells.** Virus without the specific antisera is in the top row and the specific antisera plus virus in the bottom row. The tube in the upper row at the extreme right is the 0.2% bovine albumin borate saline cell control. The two tubes in the right side of the lower row represent the saline cell control and serum cell control, respectively. Rows are numbered from top to bottom and correspond with Table 2.

**Table 2. Hemagglutination-inhibition test**

<table>
<thead>
<tr>
<th>Tube contents</th>
<th>Tissue culture PFU in tube no.</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Virus-cell (row 1) ‡</td>
<td>14,250</td>
<td>+</td>
</tr>
<tr>
<td>Virus-cell-specific anti-</td>
<td>1,825,000</td>
<td>912,500</td>
</tr>
<tr>
<td>serum (row 2)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Total RBC concentration was 3,000,000.
† Tube numbers correspond with those in Fig. 2.
‡ Row numbers correspond with those in Fig. 2.
bined with antigen was small. In addition, viral particles in smear preparations were scattered and not confined to a localized area. In contrast, infected tissue cells contain large amounts of antigen in discrete foci as a result of virus multiplication (18). Therefore, these foci can be readily observed when stained with specific fluorescein-labeled antibody.

In theory, FA seems to be a sensitive technique for detecting virus. However, some investigators have reported a lack of correlation between FA reactions in tissue culture and other standard tests. It is known from the work of Hatch, Kaltter, and Ajello (18) that a positive neutralization test was not always correlated with a positive FA reaction. They compared neutralization and FA reactions for identification of poliovirus in tissue cells. Unsuccessful attempts to detect rubella virus in monkey kidney cells with direct (2) and indirect (34) staining have been reported. These failures to demonstrate virus may have resulted from a low rate of virus multiplication.

The employment of goose erythrocytes provides a convenient method for concentrating and visualizing VEE virus aggregates by fluorescent microscopy. A similar procedure has been used by Ewy and Lui (15) and involved fluorescent staining of influenza virus adsorbed on chick red blood cells. In the current study, approximately $35 \times 10^5$ TCPFU of VEE virus and $6 \times 10^6$ goose red blood cells were employed. This was the limit of virus quantity examined. The indirect FA method appeared to produce better visualization of adsorbed virus. Reactions were less discernible with the direct technique.

Data presented in this report show the possibility of detecting certain arboviruses belonging to group A, with antisera containing specific antibodies. In addition, the results tend to indicate that the HA test is more sensitive than FA in the detection of VEE virus. This may possibly be explained on the basis that small scattered virus particles (50 mμ or less) free of tissue cells are beyond visualization in the fluorescent micro-

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**FIG. 3.** Photograph of goose erythrocytes treated and untreated with VEE virus. (A) Untreated red cells observed macroscopically, demonstrating absence of cell aggregates (X 1.7). (B) Treated red cells examined macroscopically, showing the presence of aggregates of cells (X 1.7). (A, and B1) Higher magnification of the agglutination effect (X 110).
Fig. 4. Photographs of goose erythrocytes (6 × 10⁶)-VEE virus (35 × 10⁶ TCPFU) complexes stained by the direct and indirect methods of immunofluorescence. (A) Direct staining with fluorescein-tagged whole anti-VEE rabbit serum. (B) Virus agglutinated cells treated with whole anti-VEE rabbit serum and counterstained with fluorescein-labeled goat anti-rabbit globulin (indirect method). (C) Virus agglutinated cells treated with rabbit anti-VEE globulin fraction and counterstained with fluorescein-labeled goat anti-rabbit globulin (indirect method). (D) Erythrocytes (without virus) treated with normal rabbit globulin and counterstained with fluorescein-labeled goat anti-rabbit globulin.

scope. The use of goose erythrocytes for concentrating virus may be a valuable adjunct to FA staining procedures.

Acknowledgments

I thank Maxwell R. Kluger for determining fluorescein-to-protein ratios of tagged conjugates. Appreciation is also extended to Frank Wagner and Donald Keller for photographing various preparations.

Literature Cited


4. Casals, J. 1957. The arthropod-borne group of