Passive Hemagglutination-Inhibition Test for Typing Foot-and-Mouth Disease Virus

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In addition to currently used serological tests for the occurrence of foot-and-mouth disease virus (FMDV), a specific "passive" hemagglutination-inhibition (HAI) test has been developed as a supplement. Serial twofold dilutions of antiserum (0.05 ml) were mixed with 0.05 ml of a constant concentration of FMDV. After incubating for 30 min at 37°C, agglutinating antibodies were determined by adding 0.1 ml of 2.5% virus-sensitized erythrocytes. The minimum concentration of antiserum required to agglutinate the erythrocytes defined the inhibition in the HAI test. Similar tests using different concentrations of virus to inhibit antibodies were carried out in parallel fashion. The relationship between the logarithm of the HAI titer and the concentration of inhibiting virus was nearly first order (P > 0.25). The slope was used as a measure of the relative specificities of the antigen-antibody interaction and was independent of concentration. The HAI test was type-, subtype-, strain-, and variant-specific with the viral antigens used. In particular, typing was performed directly on bovine antisera.

Previous reports (13, 15) have described the development and uses of a passive hemagglutination (HA) test to detect antibodies to foot-and-mouth disease virus (FMDV) in guinea pig and bovine sera. Type-specific reactions were obtained with guinea pig antibodies to FMDV, types A, O, and C, whereas good type specificity was obtained only with antibody of the 19S immunoglobulin M (lgM) class from immunized steers.

Recently, a number of reports have been published on the subject of typing by a passive hemagglutination-inhibition (HAI) test (2, 3, 8, 12). These workers stressed the advantages to be gained from a HAI test for typing β-lipoprotein, leukemia, herpesvirus, and rhi-

and establish the applicability of HAI testing for the typing and subtyping of FMD viruses.

**MATERIALS AND METHODS**

*Diluents.* The procedure for sensitization of erythrocytes has been described (13). In summary, the sensitization was performed in the presence of phosphate buffer (0.15 m Na₂HPO₄·7H₂O, 0.15 m KH₂PO₄ at pH 7.2). All other operations were carried out in a buffer containing 0.05% (w/v) gelatin, 0.5% (w/v) dextrose, 0.03% (w/v) bovine serum albumin dissolved in 0.075 m phosphate buffer containing 0.001 m MgCl₂·6H₂O, 0.001 m CaCl₂, and 0.15 m NaCl (ADGP).

*Antigens.* Preliminary experiments with the HAI test were carried out with FMDV, type A, subtype 12, strain 119, variant "b" (A₁₂; more recent designation by the FMDV World Reference Laboratory, Pirbright, England, for type A-119) derived from infective tissue culture fluid and purified virus from baby hamster kidney (BHK) cells.

The activities of types A₁₂, O, subtype 1, strain Caseros (O₁-Cas), and C, subtype 3, strain Resende (C₃) from BHK cells were compared.

For comparing subtype activities, the following viruses were selected: type A₁₂, type A, subtype 24, strain Cruzeiro (A₂₄), and type A, subtype 25, strain Argentina 1959 (A₂₅).

Strains O₁-Cas and O, Brugge were derived from Brazil and Belgium, respectively.

Comparative studies of variants were made with type A₁₂, large-plaque (ab), small-plaque (b), and "b" variants from BHK cells. Large and small
plaque variants were obtained by plaque cloning (J. S. Martinsen, personal communication), and their antigenic designations followed Cowan's nomenclature (5). The "b" variant was obtained by absorbing out the large-plaque variant with guinea pig 19S IgM antibodies (5) from a mixture of these in high-passage virus (150 passages in calf kidney cultures). The variants were related antigenically and were assumed to share common antigenic determinants, but the "b" variant was antigenically more pure.

Virus was purified by the procedures of Polatnick and Bachrach (11) and Bachrach et al. (1). Some virus preparations were purified by precipitation with 6% (w/v) polyethylene glycol instead of alcohol (14). Concentrations of virus were determined by spectrophotometry assuming an extinction coefficient of $E_{260nm}$ = 76. Purified virus was dialyzed against, stored in, and subsequently diluted with 0.2 M NaCl containing 0.05 M sodium phosphate, pH 7.5.

Bovine sera. Bovine sera were obtained as follows. (i) Normal serum was obtained from uninfected steers. (ii) Antisera were obtained by infecting the tongues of three steers with FMDV, type $A_{11}$ (wild type), type $O_{1}$-Cas, type $C_{2}$, or type $A_{24}$.

Guinea pig sera. Guinea pig sera were obtained as follows. (i) Normal serum was obtained from uninfected guinea pigs. (ii) Antisera were produced by plantar pad inoculation of the same viruses. Blood samples were collected at periodic intervals. (iii) Hyperimmune sera were obtained by infecting the pads as in (ii). In addition, antisera were prepared against types $A_{11}$, and $A_{24}$. One to three months later, they were inoculated intramuscularly with 1.0 ml of 10% FMDV-infected vesicular fluid from guinea pigs, and blood samples were collected 10 days later (6). To inactivate the complement, all sera were heated at 56 C for 30 min before use and then were absorbed with sheep erythrocytes to eliminate normal agglutinins (13).

Sensitization of erythrocytes. Sheep erythrocytes from a single donor were sensitized with the different viruses in the presence of 0.25% glutaraldehyde [a stock solution of 25% (v/v) aqueous glutaraldehyde was used (Fisher Scientific Co., New York, N.Y.) by the procedure previously described (13, 15)]. The sensitized erythrocytes were then washed twice with ADGP (pH 7.2) and pelleted by centrifugation at 510 × g for 10 min.

Standard HAI test. Two dimensional or block assays were performed by combining 0.05 ml of serial twofold dilutions of antigen with 0.05 ml of serial twofold dilutions of the sera in ADGP buffer in glass tubes (13 by 100 mm). Mixtures were incubated for 30 min at 37 C. Tests were also performed at 25 C (5 hr) and 4 C (18 hr), but inhibition was of a lower order. Excess hemagglutinating antibodies were detected by adding 0.1 ml of 2.5% virus-sensitized erythrocytes to each tube. Mixtures were shaken once and then incubated for 18 hr at 4 C. The titer of the serum was defined as the reciprocal of the highest dilution giving a definite agglutination pattern. This titer was also used as a measure of inhibition. In practice, the logarithm of the titer was plotted graphically against the concentration of the antigen used to inhibit the antiserum (Fig. 1). Regression lines were computed for each set of data. Any deviation from first-order relationship was small ($P > 0.25$). The slopes of these lines were a measure of the inhibition activity (HAI) expressed as the change in log hemagglutination titer per microgram of virus added ($\Delta$HA/HA concentration).

Density gradient purification of 19S IgM and 7S immunoglobulin G (IgG) antibodies from bovine antisera. The procedure has been described (15) using KBr-NaNO$_3$ density gradient centrifugation. Antiserum was centrifuged for 18 hr at 101,000 × g at 4 C in a 40.2 rotor in a model L ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.). One-milliliter fractions were collected dropwise from the bottom of the tube.

**FIG. 1.** Relationship between hemagglutination-inhibition activity expressed as log hemagglutination titer and the weight in micrograms of FMDV, types $O_{1}$, $A_{11}$, and $C_{2}$ (concentration) used to inhibit bovine antisera (11 DPI) produced from each of these types. (A) $C_{2}$ antisera; (B) $O_{1}$-Cas antisera; (C) $A_{11}$ antisera. Regression lines were drawn between the points. Symbols: (C) FMDV, type $A_{11}$; (+) FMDV, type $O_{1}$-Cas; (•) FMDV, type $C_{2}$.

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RESULTS

Development of the HAI test. Foot-and-mouth disease virus was grown in three different host cells and tested for its ability to inhibit antisera. Infective guinea pig vesicular fluid, infective tissue culture fluid, and purified virus inhibited antisera, but only the latter two viruses inhibited specifically. Although infective guinea pig vesicular fluid demonstrated the highest inhibitory activity, 5% of this activity was due to nonspecific effects. Therefore, to preclude nonspecific effects, the present work was carried out exclusively with infective tissue culture fluids and purified viruses.

To optimize the test procedure, it was necessary to determine whether inhibition was influenced significantly by the concentration of virus used to sensitize the erythrocytes. Table 1 compares the slopes of the inhibition regression lines for the different concentrations of sensitizing virus. The determined slopes were independent of the virus concentration within the limits of error of the experiment. For sensitization of erythrocytes, virus concentrations giving high HA titers, but free from spontaneous or nonspecific agglutination, were selected.

Type specificity. The HAI activities of FMDV types A, O, and C, with homologous and heterologous bovine antisera were examined (Fig. 1, Table 2). Inhibition of HA was type-specific and little cross-inhibition was apparent.

Subtype specificity. The HAI activities of three serological subtypes of FMDV, type A, were compared with homologous and heterologous guinea pig hyperimmune sera (Table 3). Two of these subtypes (A12 and A14) were also tested with "early" [14 days postinoculation (DPI)] bovine antisera (Table 4). All reactions were subtype-specific.

Variant specificity. The HAI activities of FMDV, O1-Cas, and O2 Brugge were compared with guinea pig, bovine, or swine antisera (Table 5). The activities were strain-specific. In Table 6, inhibitions of fractionated "early" (11 DPI) bovine antisera to FMDV, type A12, by three variants of type A12-119 virus are compared. Antibodies had been prepared by infecting a steer with uncloned FMDV, type A12-119, so that no specificity could be assigned to 19S IgM or 7S IgG. The 19S IgM antibodies are more discriminating than 7S IgG antibodies (no distinction was made between 7S IgG, and 7S IgG4 immunoglobulins). This discrimination is consistent with the relatively high cross-reactivity reported for 7S IgG bovine antibodies (15) by the direct HA test.

Similar tests were carried out with "early" (7 DPI) guinea pig antiserum and hyperimmune guinea pig serum. Since these antisera have been shown to contain virtually pure specific 19S IgM and 7S IgG antibodies, respectively (R. E. Warrington and Y. Kawakami, unpublished data), no further fractionation was considered necessary in tests for differentiating between variants. Antibody losses had also been previously experienced during fractionation (R. E. Warrington and Y. Kawakami, Bacteriol. Proc., p. 184, 1971). The HAI activities of these antisera were compared with FMDV A12-119 variants (Table 7). Only the inhibition of 19S IgM antibodies by large-plaque variant was high compared to other activities with this serum, whereas inhibitions of 7S IgG antibodies by both "b" and large-plaque variants were high.

DISCUSSION

The present data show that the HAI titer of immune bovine sera is essentially independent of the concentration of purified virus used to sensitize erythrocytes. Thus, any error in the sensitization of erythrocytes due to variations of virus concentration is unlikely to be reflected as an error in the degree of inhibition.

The data also show that FMDV types, subtypes, and variants may be differentiated by the inhibition test. Types and subtypes could be distinguished by "early" bovine antiserum and guinea pig hyperimmune sera. FMDV A12-119 variants could be distinguished with 19S IgM bovine antibodies, but certain anomalies occurred with guinea pig antiserum (Table 7) which may possibly be explained by the work of Cowan (5) from immunodiffusion studies with FMDV, type A12-119 variants. According to his postulate, 19S IgM antibodies, which predominate in "early" guinea pig antiserum, have specificities directed to distinct a- and b-subdeterminant groupings, whereas 7S IgG antibodies in hyperimmune sera have specificities to the ab-determinant. Consequently, the relatively high inhibitory activity of "early" serum by large-plaque (ab) variant (postulated to contain more a- than b-determinants) could be explained by assuming that all a-specific antibodies were absorbed, thus leaving some b-specific antibodies to react poorly with predominantly a-specific, large-plaque (ab) variant-sensitized erythrocytes. The relatively low inhibitory activity in other reactions with such serum could be explained in two ways. First, the "b" variant will only inhibit b-specific antibodies, leaving a-specific antibodies free
Table 1. Passive hemagglutination-inhibition reactions between FMDV types A₁₂ and O₁-Cas and their bovine antisera 11 days postinoculation

| Erythrocytes sensitized with FMDV | Bovine antisera (11 DPI) | Inhibiting virus | Erythrocytes sensitized with FMDV, types A₁₂ and O₁-Cas* |
|----------------------------------|-------------------------|------------------|---------------------------------------------------------|
|                                  |                         |                  | 10 μg/ml | 20 μg/ml | 30 μg/ml | 40 μg/ml |
| A₁₂                             | anti-A₁₂               | A₁₂              | 31.9 ± 4.8 | 25.5 ± 12.0 | 25.5 ± 12.0 | 18.7 ± 9.0 |
| O₁-Cas                          | anti-O₁-Cas            | O₁-Cas           | 24.7 ± 7.6 | 21.8 ± 5.4 | 30.2 ± 7.2 | 30.2 ± 7.2 |

* Different concentrations of purified foot-and-mouth disease virus (FMDV) were used to sensitize the erythrocytes.

The hemagglutination-inhibition is defined as the change of log hemagglutination titer per microgram of virus added to inhibit antibodies x 100.

Table 2. Passive hemagglutination-inhibition reactions between FMDV type A₁₂, O₁-Cas, or C₃ and their bovine antisera

| Bovine antisera (11 DPI) | Inhibiting virus | Sensitized erythrocytes |
|--------------------------|------------------|-------------------------|
|                          |                  | A₁₂⁺ (20 μg/ml) | O₁-Cas (30 μg/ml) | C₃ (60 μg/ml) |
| anti-A₁₂                 | A₁₂              | 18.1 ± 1.3         | 3.7 ± 1.0         | 3.3 ± 1.0 |
| O₁-Cas                   |                  | 2.8 ± 1.4          | 19.6 ± 5.3        | 2.8 ± 1.4 |
| C₃                       |                  | 4.4 ± 1.2          | 2.8 ± 1.4         | 20.3 ± 2.9 |

* Foot-and-mouth disease virus (FMDV), type A, subtype 12, strain 119, variant "b" was used.

The hemagglutination-inhibition is defined as the change of log hemagglutination titer per microgram of virus added to inhibit antibodies x 100.

Table 3. Passive hemagglutination-inhibition reactions between FMDV type A₁₂, A₁₄, or A₁₅, and their guinea pig antisera

| Guinea pig hyperimmune serum | Inhibiting virus | Sensitized erythrocytes |
|------------------------------|------------------|-------------------------|
|                              |                  | A₁₂⁺ (40 μg/ml) | A₁₄ (50 μg/ml) | A₁₅ (30 μg/ml) |
| anti-A₁₂                    | A₁₂              | 17.5 ± 3.7      | 0.0 ± 0.0      | 2.8 ± 1.4      |
| A₁₄                         |                  | 4.4 ± 1.2       | 20.3 ± 2.9     | 4.4 ± 1.2      |
| A₁₅                         |                  | 2.8 ± 1.4       | 0.0 ± 0.0      | 13.7 ± 2.3     |

* Foot-and-mouth disease virus (FMDV), type A, subtype 12, strain 119, variant "b" was used.

The hemagglutination-inhibition is defined as the change of log hemagglutination titer per microgram of virus added to inhibit antibodies x 100.

Table 4. Passive hemagglutination-inhibition reactions between FMDV type A₁₂ or A₁₄ and their bovine antisera 14 days postinoculation

| Bovine antisera (14 DPI) | Inhibiting virus | Sensitized erythrocytes |
|--------------------------|------------------|-------------------------|
|                          |                  | A₁₂⁺ (30 μg/ml) | A₁₄ (40 μg/ml) |
| anti-A₁₂                 | A₁₂              | 14.3 ± 1.8        | 0.0 ± 0.0      |
| A₁₄                      |                  | 4.4 ± 1.2         | 18.1 ± 5.2     |

* Foot-and-mouth disease virus (FMDV), type A, subtype 12, strain 119, variant "b" was used.

The hemagglutination-inhibition is defined as the change of log hemagglutination titer per microgram of virus added to inhibit antibodies x 100.

to react with large-plaque (ab) virus sensitized erythrocytes. Second, the large plaque (ab) variant will again absorb all a-specific antibodies leaving a few b-specific antibodies free to react with "b" variant-sensitized erythrocytes.

In the case of hyperimmune serum, however, 7S IgG antibodies were ab-specific. Both large-plaque (ab) and "b" variants could inhibit the antibodies to a similar extent. The small variations between these figures (Table 7) could be due to steric factors occurring between antibody molecules and their attachment to b- and ab-combining sites.

The fact that 19S IgM bovine antibodies are especially adapted for measuring antigenic differences is consistent with our previous work (15). Unfortunately, the specificities of the antibodies from this steer were not known since they had been induced by uncharacterized A₁₂-119 virus from tongue epithelium and may have contained a mixed population of variants. According to the discussion by Davie (7), subtype differentiation of FMDV by complement fixation requires antisera against two viruses that are being compared. This test was re-
TABLE 5. Passive hemagglutination-inhibition reactions between FMDV type O1-Cas or O1, Brugge and their guinea pig, bovine, and swine antisera

| Antiserum | Inhibiting virus | Sensitized erythrocytes |
|-----------|------------------|-------------------------|
|           |                  | O1-Cas | O1, Brugge |
|           |                  | 30 µg/ml | 40 µg/ml | 50 µg/ml |
| O1-Cas    | O1-Cas           | 18.1 ± 5.3 |   |   |
| HIS* (GP) | O1, Brugge       | 6.0 ± 2.7 |   |   |
| O1, Brugge| O1-Cas           | 6.0 ± 2.7 |   |   |
| HIS (GP)  | O1, Brugge       | 20.4 ± 2.9 |   |   |
| O1-Cas    | O1-Cas           | 15.9 ± 3.7 |   |   |
| 11 DPI (bovine) | O1-Cas |   |   |
| O1, Brugge | O1, Brugge     | 6.0 ± 2.7 |   |   |
| O1, Brugge| O1-Cas           | 3.8 ± 1.6 |   |   |
| 89 DPV* (swine) | O1, Brugge |   |   |

* The hemagglutination-inhibition is defined as the change of log hemagglutination titer per microgram of virus added to inhibit antibodies ×100.
* HIS, hyperimmune serum; GP, guinea pig.
* DPV, days postvaccination.

TABLE 6. Passive hemagglutination-inhibition reactions between FMDV type A12-119 variants “b,” small-plaque or large-plaque, and 19S IgM and 7S IgG bovine antibodies

| Bovine 11 DPI antiserum antibody class | Inhibiting virus variant type A12-119 | Sensitized erythrocytes (30 µg/ml) |
|--------------------------------------|--------------------------------------|----------------------------------|
|                                      | “b” Variant                          | Small-plaque variant (b')        |
|                                      | Small plaque (b')                    | 13.4 ± 1.9                       |
|                                      | Large plaque (ab)                    | 7.1 ± 0.9                        |
|                                      |                                      | 6.8 ± 1.3                        |
| 19S                                  |                                      | 7.0 ± 2.3                        |
|                                      |                                      | 13.6 ± 2.3                       |
|                                      |                                      |                                  |
|                                      | “b” Variant                          | Large-plaque variant (ab)        |
|                                      | Small plaque (b')                    | 13.4 ± 1.9                       |
|                                      | Large plaque (ab)                    | 9.0 ± 2.1                        |
|                                      |                                      | 8.6 ± 1.5                        |
| 7S                                   |                                      |                                  |
|                                      |                                      | 10.5 ± 2.3                       |
|                                      |                                      | 8.3 ± 1.9                        |
|                                      |                                      | 10.5 ± 2.3                       |

* The hemagglutination-inhibition is defined as the change of log hemagglutination titer per microgram of foot-and-mouth disease virus (FMDV) added to inhibit antibodies ×100.

TABLE 7. Passive hemagglutination-inhibition reactions between FMDV type A12-119 variants “b,” small-plaque or large-plaque, and guinea pig antisera

| A12-119 guinea pig antiserum | Inhibiting virus variant type A12-119 | Sensitized erythrocytes |
|------------------------------|--------------------------------------|-------------------------|
|                              | “b” Variant                          | Large-plaque variant(ab) |
|                              | (40 µg/ml)                           | (ab) variant(60 µg/ml) |
| 7 DPI                        | “b” Variant                          | 9.8 ± 1.4               |
|                              | Large plaque(ab)                     | 6.9 ± 1.9               |
|                              |                                      | 15.5 ± 3.3              |
| Hyperimmune                  | “b” Variant                          | 16.6 ± 2.3              |
|                              | Large plaque(ab)                     | 13.7 ± 2.9              |
|                              |                                      | 15.9 ± 3.7              |

* The hemagglutination-inhibition is defined as the change of log hemagglutination titer per micrograms of foot-and-mouth disease virus (FMDV) added to inhibit antibodies ×100.

ferred to as a “two-way test.” Thus, in the HAI test for variants, a two-way test would require specific antisera against small-plaque, large-plaque, and “b” variants. Close examination of the data obtained with variants suggests a need for a two-way test before full characterization of antigens and antibodies can be made. However, a limited one-way test in which the two viruses are compared with a common type-specific antiserum should be reliable enough for differentiating between subtypes when time is the limiting factor.

The nature of the antigens involved in the HAI reaction has not been studied extensively (10). For certain animal and plant viruses, intact virus apparently is responsible for coating tanned erythrocytes. For others (e.g., adenoviruses and myxoviruses), the antigens are separable from the infective particle (9). In prelim-
inary work with purified 12S subunits from FMDV type A, it has been found that erythrocytes sensitized with up to 120 μg of this antigen per ml failed to detect significant levels of antibodies in bovine antisera. The subunits could, however, inhibit specific antibodies in these antisera in the HAI test. Thus, under the conditions used here, only the 140S FMDV particles attached to erythrocytes can detect antibodies.

It should be emphasized that HA patterns and end points were less distinct when erythrocytes had been sensitized with virus precipitated by 6% (w/v) polyethylene glycol or 50% (w/v) ammonium sulfate due to interference or adsorption of these compounds onto erythrocytes. Therefore, until means have been found to eliminate these factors from the test, the use of concentrated, purified virus will be continued. This is being investigated.

In a previous report (15), the problem of cross-reaction between FMDV immune bovine sera was alleviated by separation of the less specific 7S IgG from the highly specific 19S IgM antibodies. The HAI test demonstrates the feasibility of increasing the specificity of antisera by inhibiting or absorbing out heterologous and cross-reacting antibodies from it. The exact mechanism of this absorption is not known, but a preferential elimination of poorly specific 7S IgG, leaving only 19S IgM antibodies would explain the highly specific character of the HAI test. However, we could assume the model of Bradish et al. (4), in which antigen-antibody complexes possess amphoteric properties, being both infective and capable of combining with additional antibodies. These highly specific complexed antibodies could be potent agglutinators of sensitized erythrocytes. Each of these explanations is relevant at this stage.

In previous studies on the differentiation of subtypes of FMDV (7), use was made of the complement-fixation test. It was found to be more precise than the cross-neutralization or cross-immunity tests. However, considerable cross-reactions occurred between guinea pig antisera to different subtypes. It was suggested that, although this cross-reaction was an advantage for the diagnosis of FMD, specific immune serum against each unknown subtype from the field was essential for complete differentiation. This procedure is time-consuming, especially in the event of a field outbreak of the disease. The present results suggest the use of a one-sided HAI test in which only the unknown virus from the field would be used to inhibit various well-characterized antisera. It should be possible to specify the subtype of this virus without further antisera preparation and within a short period of time.

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