Detection of Foot-and-Mouth Disease Virus Antibodies

II. Use of Fractionated Bovine Antisera for Improving the Specificity of a “Passive” Hemagglutination Test

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Because 7S immunoglobulin (Ig) G antibodies of low type specificity were present in mixtures with highly specific 19S IgM antibodies, many bovine antisera to foot-and-mouth disease virus (FMDV) type A, strain 119 cross-reacted with type O of FMDV and to some degree with type C in the passive hemagglutination (HA) test. After 19S IgM antibodies were separated by density gradient centrifugation or precipitated with 4% (w/v) polyethylene glycol, the antigen could be determined with "block" HA tests. Such tests used several antigen concentrations in the titration of each antiserum. Adding 4% (w/v) polyethylene glycol to the serum was especially convenient for rapid precipitation of 19S IgM antibodies for the test. Similar results were obtained with bovine 19S IgM antibodies to FMDV type O, subtype 1, strain Caseros and type C strain Rezende.

A method of coupling foot-and-mouth disease virus (FMDV) to sheep erythrocytes with glutaraldehyde was described in a previous report (10). Glutaraldehyde both stabilizes erythrocytes and couples the virus. In a passive hemagglutination (HA) test, sensitized cells were used to detect antibodies in bovine, swine, and guinea pig antisera. However, guinea pig hyperimmune sera containing only 7S immunoglobulin (Ig) G antibodies and guinea pig antisera [7 days post-infection (DPI)] containing only 19S IgM antibodies (reference 10; Warrington, unpublished observations) were more specific to FMDV types A, O, and C than bovine and swine antisera containing varying proportions of 19S IgM and 7S IgG antibodies.

By using FMDV type A, subtype 12, strain 119 (A-119) large plaque variant, Cowan (3) has also demonstrated by immunodiffusion that 19S IgM antibodies induced in guinea pigs by this strain can distinguish between three variants, whereas the 7S IgG antibodies from hyperimmunized guinea pigs could not distinguish between them and were considered less specific.

The experiments to be described in this report are an attempt to resolve the cross-reaction which occurs among bovine antisera by fractionating the 19S IgM antibodies and measuring the specificity against three different antigenic types. In this way it may be possible to use the HA test to assist in the rapid serological typing of FMDV antibodies in bovine antisera.

MATERIALS AND METHODS

Glutaraldehyde. Experiments were carried out with a stock solution of 25% aqueous glutaraldehyde (Fisher Scientific Co., New York, N.Y.).

Diluents. The sensitization of erythrocytes with antigen was performed in the presence of phosphate buffer (PB; 0.15 M NaHPO4, 7H2O, 0.15 M KH2PO4 at pH 7.2). All other operations were carried out in 0.15 M PB (pH 7.2) mixed with an equal quantity of 0.3 M sodium chloride containing 0.06% (w/v) gelatin, 1.0% (w/v) dextrose, 0.06% (w/v) bovine serum albumin (ADGP; 0.002 M MgCl2, 6H2O, 0.002 M CaCl2). This buffer was similar to that used by Dold and Northrop (5). A general enhancement of agglutinating activity in its presence was found in this laboratory compared to the use of the phosphate-buffered saline containing 0.1% (w/v) gelatin (PBSG; 0.15 M NaHPO4·7H2O, 0.15 M KH2PO4, 0.15 M NaCl at pH 7.2) by using the same antisera as was previously reported (reference 10 and Fig. 1).

Antigens. All HA tests were carried out with FMDV type A, subtype 12, strain 119 (A-119); type O, subtype 1, strain Caseros (O, Cas); type C strain Rezende (C-Rez) which were produced and purified.

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by the procedures of Polatnick and Bachrach (8) and Bachrach et al. (1). Two variants of type A-119 were employed. The large plaque variant was closely related or identical to the wild-type virus and was used only to induce antibodies in a steer. The "b" variant was obtained by adsorbing out the large plaque variant with guinea pig 19S IgM antibodies (3) from a mixture of these in high-passaged virus (150 passages in calf kidney cultures). The variants were related immunologically and were assumed to share common antigenic determinants (3), but the "b" variant was more antigenically pure. Their concentrations were measured by spectrophotometry assuming an extinction coefficient of $E_{254}^{\text{nm}} = 76$. The purified virus was dialyzed against, stored in, and subsequently diluted with 0.2 M NaCl containing 0.05 M sodium phosphate, $pH$ 7.5 (1).

**Antisera.** Antisera were obtained by infecting a steer with FMDV type A-119 large plaque variant on the tongue and bleeding at intervals thereafter. Antisera were obtained from guinea pigs after infecting them with FMDV in the footpads. Later (1 to 3 months) they were inoculated intramuscularly with 1.0 ml of 10%, FMDV-infective vesicular fluid from guinea pigs, and blood samples were obtained 10 days later (4). All sera were heated at 56 °C for 30 min before use to inactivate the complement.

**Preparation of materials for HA test.** Sheep erythrocytes from a single donor were collected and sensitized and the antisera were preabsorbed by the procedures previously described (10) by using ADGP buffer as a diluent.

**Standard test.** The HA test was run essentially as before (10), except that ADGP buffer replaced PBSG and 0.1 ml of 2.5% virus-sensitized erythrocytes was mixed with 0.1 ml of antiserum dilution and incubated as before for 18 hr at 4 °C. Routine tests were carried out in test tubes (13 by 100 mm). Sheep erythrocytes were sensitized with FMDV types A, O, and C in the presence of 0.25% glutaraldehyde at concentrations determined by preliminary titration of antigens with antisera. Only those antigen concentrations were chosen which demonstrated acceptably low, nonspecific reaction with normal serum, a negligible agglutination in the absence of serum and maximal titers. The titer was defined as the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern.

**Density gradient purification of 19S IgM and 7S IgG antibodies.** A method by using KBr-NaNO$_3$ density gradients was essentially the same as that described by Cowan and Trautman (4). A volume of 1.5 ml of 65% saturated NaNO$_3$ (v/v) was layered onto 2.5 ml of 75% saturated KBr (v/v) and stirred gently with a saw-toothed wire. Serum (1 ml) was overlaid and then 1.2 ml of light oil was added. The tubes were centrifuged for 18 hr at 10,000 × g at 4 °C by using a 40.2 rotor in the model L ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.). The bottom of the tube was punctured and 1-ml fractions were collected dropwise. Fractions were dialyzed against phosphate-buffered physiological saline (PBS) adjusted to $pH$ 7.6. Previous experience has shown that fraction 1 contains predominantly 19S IgM antibodies, whereas fraction 3 contains mostly those of the 7S IgG class.

**Precipitation of antibodies from antisera with polyethylene glycol (PEG).** Three solutions of PEG (molecular weight, 20,000) were made up in PBS with concentrations such that when 3 ml was added to 2 ml of antisera, the final concentrations were 4, 5, or 6% (w/v). The solutions contained in lusteroid tubes were held at room temperature for 30 min before centrifuging at 12,100 × g for 30 min in the SS-34 rotor of the Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). After decanting the supernatant fluid, the precipitate was resuspended in 2 ml of PBS. The precipitates contained different amounts of 19S IgM antibodies (Warrington and Morgan, Arch. Gesamte Virusforsch., in press). The supernatant fluids were not tested for antibody because the presence of high levels of PEG interfered with the HA test.

**RESULTS**

Use of ADGP buffer during the agglutination reaction. In Table 1 are compared the HA titers of antisera from guinea pigs hyperimmunized with FMDV types A, O, and C by using ADGP buffer during agglutination. Much lower HA titers were obtained with the same antisera in

| GP antisera        | FMDV type | A        | O        | C        | No virus |
|--------------------|-----------|----------|----------|----------|----------|
| Anti-A-119         | 10,240    | (45.0)$^b$ | <20 (<0.1) | <20 (<0.1) | <20 |
| Anti-O, Caseros    | 40 (0.1)  | 2,560 (11.2) | 20 (<0.1)  | <20 (1.2)  | <20 |
| Anti-C-Rezende     | <40 (<0.1)| 1,280 (4.3) | 1,280 (4.3) | <20 (4.3) | <20 |

$^a$ Expressed as the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern minus the titer obtained with normal GP serum. Amounts used were: 30 μg/ml, type A (A-119, variant b); and 70 μg/ml, type C. These were the maximum permissible concentrations above which nonspecific agglutination occurred.

$^b$ Increase in hemagglutination titer caused by an increase of 1 μg of antigen concentration per ml assuming a linear relationship (see Fig. 1).
preliminary work (10) when PBSG buffer for agglutination and larger amounts of reactants were employed. The use of only 0.1 ml of reactant and improved buffer have increased the sensitivity of the test considerably. All subsequent tests were therefore carried out under the new conditions.

Factors contributing to nonspecific interactions between bovine antisera and FMDV types A, O, and C. In Table 2 are compared the HA activities of untreated and PEG-precipitated bovine antisera to FMDV A-119, variant b, at 9 and 24 DPI by using sheep erythrocytes sensitized with FMDV types A, O, and C. The selected bleedings were representative of that period of time after infection when nonspecific reactions are most

**Table 2. Determination of the specificity by hemagglutination of foot-and-mouth disease virus antibodies differentially precipitated with polyethylene glycol (PEG)**

| Bovine anti-A 119 | FMDV type | A | O | C | No virus |
|------------------|-----------|---|---|---|---------|
| **Antiserum (9 DPI)** |           |   |   |   |         |
| Untreated        |           | 20,480 (80) | 10,240 (80) | 10,240 (40) | 10 (<10) |
| 6% PEG-treated   |           | 2,560 (<10) | 1,280 (<10) | 2,560 (<10) | 10 (<10) |
| 5% PEG-treated   |           | 1,280 (<10) | 640 (<10)   | 160 (<10)   | 10 (<10) |
| 4% PEG-treated   |           | 2,560 (<10) | 160 (<10)   | 160 (<10)   | 10 (<10) |
| **Antiserum (24 DPI)** |           |   |   |   |         |
| Untreated        |           | 2,560 (<10) | 2,560 (<10) | 1,280 (<10) | 10 (<10) |
| 6% PEG-treated   |           | 1,280 (<10) | 640 (<10)   | 320 (<10)   | 10 (<10) |
| 5% PEG-treated   |           | 1,280 (<10) | 320 (<10)   | 320 (<10)   | 10 (<10) |
| 4% PEG-treated   |           | 320 (<10)   | 80 (<10)    | 80 (<10)    | 10 (<10) |

* Days postinfection.

b Expressed as the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern. Types A119 strain 119 (variant b) O and C were used at 40 μg/ml. These were not the maximal permissible concentrations for each virus but were used for convenience in this comparative test.

c Normal bovine serum was treated in parallel with antiserum. The hemagglutination titers of the treated normal bovine sera are quoted in parentheses.

**Table 3. Determination of the specificity by hemagglutination of foot-and-mouth disease virus (FMDV) antibodies differentially precipitated with 4% polyethylene glycol (PEG)**

| Bovine antiserum | Days postinfection | 4% PEG-treated or untreated antiserum | FMDV type | A | O | C |
|------------------|-------------------|--------------------------------------|------------|---|---|---|
| Anti-A 119       | 9                 | UT*                                  | 5,100      | 5,100 | 5,100 |
|                  | 9                 | 4% PEG                               | 1,280      | 160  | 160 |
|                  | 24                | UT                                   | 2,540      | 2,540 | 1,260 |
|                  | 24                | 4% PEG                               | 320        | 80   | 80 |
| Anti-C-Rezende   | 14                | UT                                   | 300        | 620  | 1,260 |
|                  | 14                | 4% PEG                               | 20         | 10   | 40 |
|                  | 28                | UT                                   | 620        | 300  | 140 |
|                  | 28                | 4% PEG                               | 20         | <10  | 40 |
| Anti-O1-Caseros  | 7                 | UT                                   | 300        | 1,260 | 620 |
|                  | 7                 | 4% PEG                               | <10        | 320  | 10 |
|                  | 21                | UT                                   | 2,540      | 1,260 | 2,540 |
|                  | 21                | 4% PEG                               | <10        | 160  | 40 |

* Untreated.

b Expressed as the reciprocal of the highest antiserum dilution still giving a definite agglutination pattern minus the titer obtained with normal serum. Types A119 strain 119 (variant b) O at 10 μg/ml and type C at 40 μg/ml were used. These were the maximal permissible concentrations above which non-specific agglutination occurred.
frequent with the HA test (Warrington and Kawakami, unpublished data). Also, for diagnostic purposes, early antisera would be of major interest. It is evident that antibody precipitated with 4% (w/v) PEG was more type specific than untreated antiserum or precipitate obtained with higher concentrations of polymer. Indeed, this concentration was shown to be optimal for separating 19S IgM and 7S IgG antibody classes to FMDV type A-119 large plaque (Warrington and Morgan, Arch. Gesamte Virusforsch., in press).

In Table 3 are compared HA titers of bovine antisera to FMDV types A, O, and C before and after treatment with 4% (w/v) PEG only. The specificity of antibodies to FMDV types A, O, and C was improved by precipitation with 4% (w/v) PEG. The observed increase in specificity could have been due to the removal, by PEG treatment, of a nonspecific factor from the antibody suspension. Normal bovine serum was tested for the presence of a possible factor by mixing a sample with PEG-precipitated 19S IgM antibodies to A-119 virus and titrating with erythrocytes sensitized with FMDV types A, O, or C. The normal serum had a negligible effect on both titer and type specificity of PEG-precipitated antibodies. Nevertheless, this does not eliminate the possibility that some factor could have been induced during the infection or immunization process.

The minimum concentration of antigen required to sensitize and agglutinate erythrocytes in the absence of antiserum is variable, depending upon factors such as cell batch and virus used. It was therefore considered necessary to select several antigen concentrations in the range 10 to 60 μg/ml to define the titer more precisely and reproducibly. In general, the HA titer (reciprocal units) is proportional to the antigen concentration (Fig. 1). The least squares best fit lines were drawn between points. The slope of both graphs had standard deviations of about 10% and calculated Student’s t values confirmed that the relationships did not differ significantly from linearity (P > 0.25). The slope represents the increase in titer per unit increase in antigenic mass. This measurement, being independent of the absolute concentration of antigen, should reflect the specificity or avidity of antibody. All antisera may be titrated by such block tests and compared with suspensions of 19S IgM and 7S IgG antibodies used as standards for all tests.

The existence of possible differences of specificity between 19S IgM and 7S IgG antibodies was therefore investigated as follows. Block titrations (Fig. 2) were carried out with bovine antisera fractionated by density gradient centrifuga-

![Fig. 1. Relationships between HA titers (reciprocal of dilution) of a preparation of 7S IgG antibodies to FMDV type A-119, variant b, and the amount of antigen required to sensitize sheep erythrocytes (μg/ml). The best-fit lines from linear least-squares analysis were drawn. Their slopes had standard deviations of ± 10%. The Student's t values confirmed that the relationships did not differ significantly from linearity (P > 0.25). Best-fit line "a" (○) from "early" bovine bleeding. Best-fit line "b" (●) from unfractionated hyperimmune guinea pig serum.](image-url)
of unity would have indicated equality of reactivity of anti-A-119 antibodies with both antigens. Figure 3B shows that when FMDV (C-Res) was compared with FMDV A-119, variant b, in their abilities to detect anti-A-119 antibodies, little distinction could be made between 7S IgG and 19S IgM antibody classes (anti-A-119). The reactivity of FMDV (C-Res) was low in all cases.

DISCUSSION

Previous work (10) has shown that the presence of 0.9% (w/v) NaCl in PB stabilized the HA reaction. A similar buffer was used by Boyden (2) and Stavitsky (9) in their first HA procedures. The inclusion of Mg\(^{2+}\) and Ca\(^{2+}\) ions, dextrose, bovine serum albumin, and gelatin in the medium suggested by Dold and Northrop (5), who concluded that a mixture of Ca\(^{2+}\) and Mg\(^{2+}\) ions was essential for developing good HA patterns. However, concentrations were not critical.

A more distinct HA pattern and enhancement of titer were observed by us with ADGP buffer. Both the 19S IgM and 7S IgG antibodies from guinea pigs infected with FMDV types A, O, and C were shown to be highly type specific (reference 10; Warrington, unpublished data). The observed cross-reactivities among most bovine antisera were caused by the presence of 7S IgG antibodies with limited type specificities mixed with highly type specific 19S IgM antibodies. In the review by Pike (7), evidence was presented that 19S IgM antibodies had a higher capacity to agglutinate sensitized erythrocytes than did 7S IgG antibodies, due to differences of valency. This might suggest an explanation for the observed specificity differences. However, on closer examination of the data illustrated in Fig. 2, this could not explain the higher reactivities of FMDV types O and C with 7S IgG antitype A-119 antibodies compared to those observed with 19S IgM antibodies. This explanation was consequently eliminated. It must be emphasized that no attempt has been made at this stage to identify poor specificity with any particular immunoglobulin, e.g., 7S IgG\(_1\) or 7S IgG\(_2\), since this would require a more detailed immunochemical investigation which is beyond the scope of the practical aspects of the present study.

The isolation of 19S IgM antibodies from sera by precipitating with 4% (w/v) PEG would facilitate typing procedures during diagnostic work. It must be pointed out, however, that there was little difficulty in deciding upon the antigenic type (in the case of FMDV type A-119, variant b), as long as block tests were performed on each antiserum (Fig. 2). To generalize the rule, it will be necessary to perform further block tests with other FMDV types and antisera from other cattle. There was some evidence for the improvement of type specificity of 7S IgG antibodies with time.

![Graph](image-url)
Fig. 3 (A). Each point of this plot has an ordinate representing the ratio of the computed slopes (ΔHA) of the HA titer-virus concentration kinetics, examples of which are illustrated in Fig. 2 for each virus used. In the present figure, different concentrations of FMDV antigenic types O1 Caseros (ΔHA0) and A-119, variant b (ΔHAΔ), are compared as to their abilities to detect purified anti-A-119, 19S IgM, 7S IgG antibodies, and antibodies in unfractionated bovine antisera. Statistical analysis has demonstrated that the level of significance of this difference was good (P > 0.5) for all bleedings. (B) Similar to A except that FMDV type C-Rezende (ΔHAΔ) was compared to type A-119 (ΔHAΔ). There was little significant difference between the type specificities of 19S IgM and 7S IgG antibody classes for the antigen after infection. These conclusions are consistent with those reported by Hampar et al. (6) by using herpesvirus system, except that we demonstrated no significant improvement of type specificity of 19S IgM antibodies with time after infection. In fact, there was a tendency for the specificity to diminish.

Recent experience has shown that to sustain a high level of sensitivity and reproducibility for the test, purified virus stocks must be frequently titrated for their optimal sensitizing concentration with bovine, guinea pig, and swine antisera, especially after renewal of sheep erythrocyte pools. Also, it is essential to prepare fresh ADGP buffer at intervals of a week to reduce the possibility of spontaneous precipitation of buffer and its resultant effects on the test.

So far, attempts to produce a stabilized pool of sensitized erythrocytes have failed due to the occurrence of spontaneous agglutination after a few days of storage at −60 C. Success in this area could improve standardization of the test and facilitate faster diagnostic procedures.

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