Archives of Virology 51, 299—306 (1976)

Localisation on Foot-and-Mouth Disease Virus (FMDV) of an Antigenic Deficiency Induced by Passage in BHK Cells

By

R. H. Meloen

Central Veterinary Institute, Virology Department, Lelystad, The Netherlands

With 14 Figures

Accepted March 30, 1976

Summary

Passage of FMDV in BHK suspended cells was confirmed to induce an antigenic deficiency on the virion. By immunodiffusion experiments with complete virus, with trypsin-treated virus and with 12S virus fraction it was shown that the induced antigenic deficiency is located on the trypsin-removable part of the virion. These results were confirmed by absorption experiments.

Introduction

Cowan *et al.* (11) showed that passage of FMDV (type Asia 1) in BHK cells can change the antigenic properties of the virus, depending upon the line of BHK cells, the culture method applied, and the passage history of the virus. Upon passage, the morphology of the plaques changed and the virus became antigenically deficient compared with the initial strain, as was demonstrated in the immunodiffusion (I.D.) test. When the original virus was tested with a convalescent serum prepared against the original virus, it exhibited a spur in comparison with the BHK passaged virus. A vaccine prepared with the BHK adapted virus proved less effective in protecting cattle against challenge by the original virus than a vaccine prepared with a virus strain which was more closely related to the original strain. In our institute (more similar or less the same) observations were made when FMD type O_1 virus, obtained from bovine tongue epithelium (Frenkel) culture, was compared with the same strain after adaptation to BHK-suspension cultures.

The experiments of Wild *et al.* (15) have demonstrated, that the antigen mainly responsible for inducing immunity to challenge is removed by trypsin treatment.

It therefore seemed of interest to learn whether the antigenic changes taking place upon passaging FMDV in BHK suspension cultures were associated with the trypsin sensitive part of the virion. Since Brown and Smale (8) showed that IgM of guinea pigs is mainly directed against this trypsin-sensitive part, selected early convalescent serum raised in guinea pigs was used in this study. In addition, sera raised in steers and pigs were also tested.

Materials and Methods

Viruses

The FMD-virus, used in I.D. and serum absorption experiments was type 0, subtype 1, strain Brugge. The virus had been maintained by intra-dermolingual passages in steers. This strain was passaged 2 to 10 times in bovine tongue epithelium (Frenkel) culture and was designated O_{Fr}. The same strain was also passaged once in BHK monolayers and five times in BHK suspended cells before finally being cultured in BHK suspended cells as described elsewhere (6), and was designated O_{BHK}. All animals were infected with FMDV type O_1, strain Weerseloo, a field strain isolated from a pig during the Dutch 1966 epizoötic. This strain was passaged a few times in cattle before being used as the laboratory challenge strain. In our laboratory this strain proved to be practically identical to O_1-Brugge, which as a vaccine produces good protection against challenge with O_1-Weerseloo.

Virus Concentration and Purification

Virus was concentrated and purified as described previously (5) but with some modifications. Virus was concentrated 200 fold by two consecutive PEG precipitations and purified on a 10 to 25 per cent sucrose gradient. After centrifugation in the SW 25.1 rotor (Beckman Instruments) for 2.5 hours at 25,000 rpm, the virus fractions were detected spectrophotometrically at 254 nm, pooled and concentrated by ultra-filtration (Amicon Corporation, filter XM 300). Virus concentration and relative purity were determined spectrophotometrically using $E^{1\%}_{260} = 76$ and $\frac{E260}{E240} = 1.42$ respectively (1). Usually virus preparations had an $\frac{E260}{E240}$ value of approximately 1.30. No correction was made for the $E^{1\%}_{260}$.

For I.D. tests the final concentration of the virus was adjusted to 350 μg/ml. To all virus, trypsin treated virus and 12S virus fraction preparations, sodium azide was added to a concentration of 0.1 per cent. The preparations were stored at 4° C.

Trypsin Treatment of the Virus

The method was essentially the one described by WILD and BROWN (15). To stop trypsinization of the purified virus soybean inhibitor (Sigma) was added to a concentration of 1.5 mg/ml. Trypsin treated virus was again purified as described for the complete virus. Trypsin treatment generally reduced infectivity, as measured in the agar-cell suspension plaque assay (4), by 3 log. O_{BHK} and O_{Fr} trypsin treated-virus preparations are designated O^{tryp}_{BHK} and O^{tryp}_{Fr} respectively.

Preparation of the 12S Protein Subunit

To purified and concentrated virus approximately 1 mg/ml RNase (grade IIIa, Sigma) was added to a concentration of 60 μg/ml. The suspension was then dialysed for 1 hour at 37° C and for 16 hours at 4° C against 0.05 M phosphate buffer containing 0.2 M NaCl. The pH was adjusted to 5.3 by addition of concentrated acetic acid. The preparation was clarified by centrifugation for 10' at 10,000 rpm at 4° C. The quantity of 12S was determined spectrophotometrically by using $E^{1\%}_{280} = 11.1$ (2). The concentrated 12S material was adjusted to 500 μg/ml for I.D.

Antiserum Preparations

Seven days post infection serum was obtained from FMD susceptible pigs, cattle and guinea pigs infected with O_1-Weerselo, by footpad, intradermolingual and hindpad inoculation respectively. From each group of at least three animals one antiserum giving a clear I.D. reaction was selected. Hyperimmune serum was prepared in guinea pigs infected with O_1-Brugge, by footpad, subcutaneous and intravenous injection successively during a 3 month period. All antiserum preparations and antiserum fractions were stored at —20° C. The I.D. results shown were obtained with the undilut-

ed steer serum and 1:2 diluted pig serum and guinea pig serum. The hyperimmune guinea pig serum was used in a dilution of 1:4.

Fractionation of Antisera

The antisera were fractionated on Sephadex G-200 columns. Five ml of each serum was loaded onto a 100×3 cm column and eluted with a 0.1 M Tris-HCL buffer, pH 7.4 containing 1 M NaCl using a flow rate of 20 ml/hour. The fractions within the first and second U.V.-absorbing peaks were collected and concentrated by ultra-filtration. The fractions of the 19 and 7S peaks were collected and the final volume adjusted to 5 ml with phosphate buffered saline.

Absorption of the Antiserum Fraction

To remove preciptable antibodies, antiserum fractions were absorbed onto an excess of virus or trypsin-treated virus for three days at 4° C. The amount of antigen required was ascertained in the following way: small aliquots of antiserum were incubated with increasing amounts of antigen for three days at 4° C, and then tested in I.D. against both serum and antigen. The precipitation pattern showed whether equivalent amounts or excess antigen had been added to the antiserum (10).

Unreacted antigen and immune precipitates were removed after incubation, by centrifugation of the mixture on a 10 per cent sucrose cushion in the SW 50.1 rotor (Beckman Instruments) for 1 hour at 45,000 rpm. At the end of the run the antiserum was removed from the interface above the sucrose cushion.

Immunodiffusion Test

The immunodiffusion method described by OUCHTERLONY (14) was used. Agarose (3) gels (1 per cent) were prepared in 0.5 M glycine-HCL, pH 7.8, containing 0.025 M sodium barbital and 0.1 per cent sodium azide. The wells had a diameter of 3 mm and the distance between the wells was also 3 mm. Each well was filled with approximately 15 µl serum or antigen preparation. The precipitation reactions were developed at 4° C for 7 days. The gels were then washed, dried, stained with amido-black and photographed.

Assay of Neutralizing Antibodies

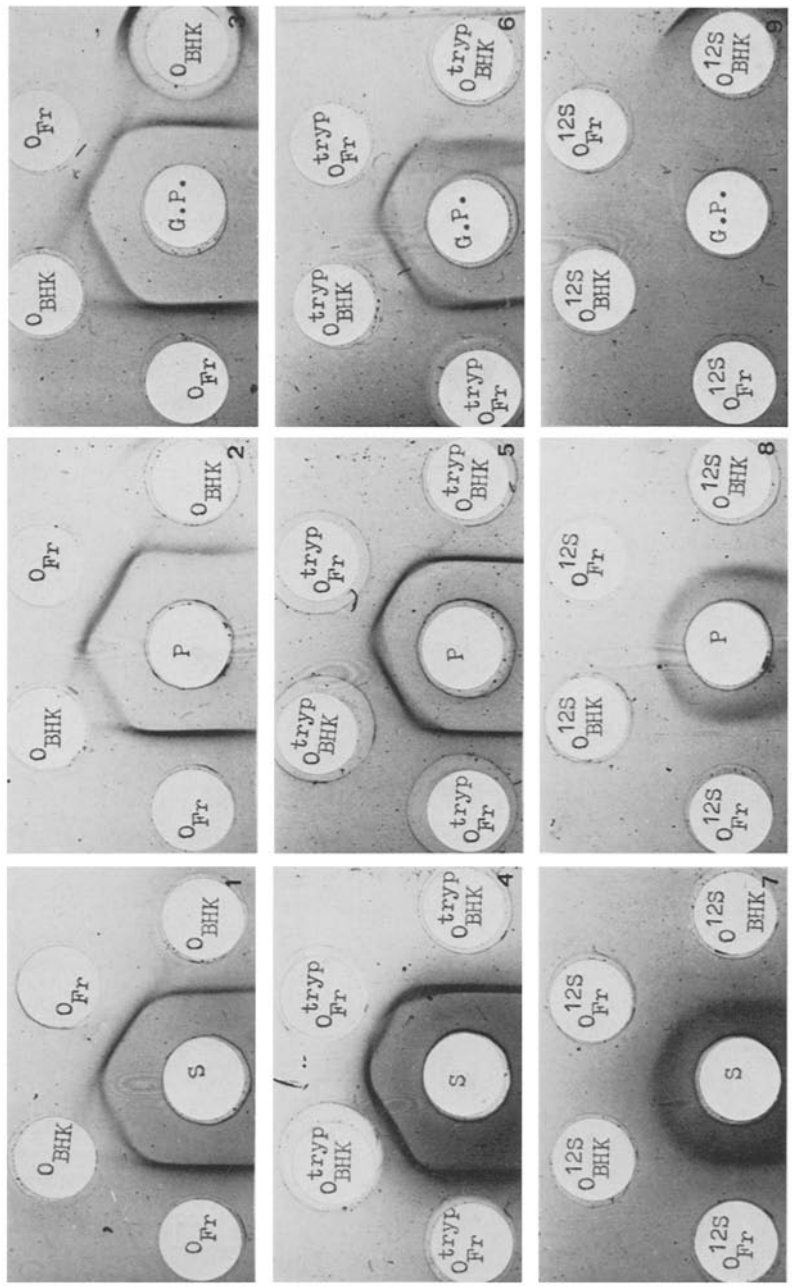
A modification (12) of the metabolic inhibition test, as described by MARTIN and CHAPMAN (13) was used.

Results

Early sera of cattle, pigs and guinea pigs were collected and screened for precipitating activity in I.D. tests. Those sera giving the clearest I.D. pattern were screened on the presence of precipitable 7S antibodies by Sephadex G-200 gel filtration. The first, second and third eluted peaks were collected, concentrated to the original volume by ultra-filtration and screened for precipitable antibodies. Precipitation was only obtained with the first (19S) peak and not with the second and third peak, thus all the precipitating activity of antibodies used in this study is of the "19S" class.

The selected early convalescent sera of all three species showed spurring of the O_{Fr} and the O_{BHK} in the I.D. test (Figs. 1, 2 and 3), indicating that O_{BHK} is antigenically deficient compared with O_{Fr}. In order to locate this antigenic deficiency on the virion, all possible combinations of antigens O_{Fr}, O_{BHK}, O_{Fr}^{tryp} and O_{BHK}^{tryp} were tested against the three antisera.

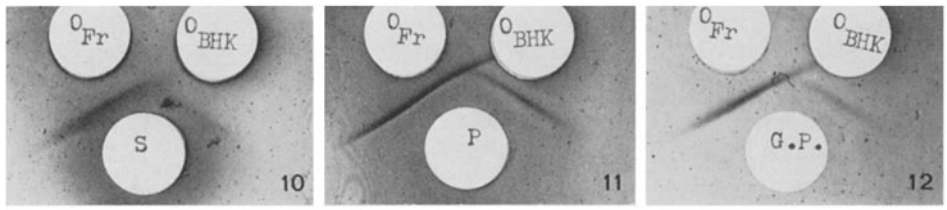
The relevant results are shown in Figures 1 to 9. No spur was evident in the combination O_{Fr}^{tryp} and O_{BHK}^{tryp} using the steer serum (Fig. 4) while a faint spur was obtained using the pig or the guinea pig serum (Figs. 5 and 6). This spur was absent when the 12S subunits were tested. This suggested that the trypsin treat-



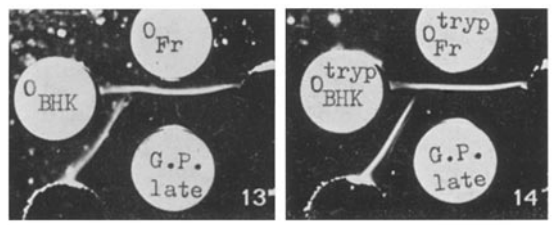
Figs. 1—9. I.D. results of early convalescent antisera raised against FMDV type O_1 in a steer (S), a pig (P) and a guinea pig (G.P.), and tested against O_{BHK}, O_{Fr}, O^{tryp}_{BHK}, O^{tryp}_{Fr}, O^{12S}_{BHK} and O^{12S}_{Fr}

ment removed the site on the virion where the antigenic change was located. Absorption experiments were performed to check this suggestion. The I.D. results show (Figs. 10, 11, and 12) that the sera absorbed onto O_{Fr}^{tryp} and O_{BHK}^{tryp} still produced spurs, while they also contained a considerable amount of their initial neutralising activity (Table 1).

In contrast to the early sera, the guinea pig hyperimmune serum gave large spurs in both combinations of O_{Fr}/O_{BHK} and $O_{Fr}^{tryp}/O_{BHK}^{tryp}$ (Figs. 13 and 14). Using late convalescent sera raised in different species, we obtained results similar to those found with guinea pig hyperimmune serum.



Figs. 10—12. I.D. results of early convalescent antisera raised against FMDV type O_1 in a steer (S), a pig (P) and a guinea pig (G.P. and absorbed onto O_{Fr}^{tryp}. These absorbed sera were tested against the combination O_{Fr} and O_{BHK}



Figs. 13, 14. I.D. results of late hyperimmune guinea pig antiserum tested against O_{Fr}, O_{BHK}, O_{Fr}^{tryp} and O_{BHK}^{tryp}

Table 1. *Neutralizing activity and I.D. pattern of early antisera absorbed with* O_{Fr}, O_{Fr}^{tryp}, O_{BHK} *and* O_{BHK}^{tryp}

Absorbed with	Antisera: Steer M.I.T.[b]	Steer I.D.[c]	Pig M.I.T.	Pig I.D.	Guinea pig M.I.T.	Guinea pig I.D.
——[a]	3.90	$O_{Fr} > O_{BHK}$	3.45	$O_{Fr} > O_{BHK}$	n.d.[e]	$O_{Fr} > O_{BHK}$
O_{Fr}	<0.90	—[d]	1.50	—	n.d.	—
O_{Fr}^{tryp}	2.10	$O_{Fr} > O_{BHK}$	2.85	$O_{Fr} > O_{BHK}$	n.d.	$O_{Fr} > O_{BHK}$
O_{BHK}	0.90	—	0.90	—	n.d.	—
O_{BHK}^{tryp}	3.45	$O_{Fr} > O_{BHK}$	3.45	$O_{Fr} > O_{BHK}$	n.d.	$O_{Fr} > O_{BHK}$

[a] —— unabsorbed sera
[b] M.I.T. metabolic inhibition test, antibody titers expressed as negative logaritm of the serum dilutions
[c] I.D. immuno diffusions experiments, $O_{Fr} > O_{BHK}$ means that O_{Fr} spurs on O_{BHK}
[d] — no precipitation detected
[e] n.d. not done

Discussion

By culturing FMDV in BHK suspended cells the virus may lose an antigenic component as shown by COWAN *et al.* (11). His vaccination experiments suggest a relationship between this loss and the loss of immunogenicity of the virus when incorporated into a vaccine. BROWN and SMALE (8) demonstrated that FMDV has three antigenic sites and that the site removed by trypsin seemed to be the one that is mainly responsible for the immunogenicity. This antigenic loss first reported by Cowan might therefore be associated with the trypsin sensitive part of the virion. This possibility was investigated by using 19S antibody. BROWN and SMALE (8) showed that guinea pig anti FMDV 19S antibody is mainly directed against the trypsin-sensitive part, while COWAN (9) observed in I.D. tests that minor strain differences could be detected with 19S antibodies on the complete virion, but not on the trypsin-treated virion. With the aid of 19S antibodies from three different species we have demonstrated that the antigenic deficiency, due to passaging in BHK, is located on the trypsin-sensitive part of the virion, since the spur shown in I.D. by the combination O_{Fr}/O_{BHK} practically disappeared in the combination $O_{Fr}^{tryp}/O_{BHK}^{tryp}$. The absorption experiments confirmed these I.D. results and showed in addition that a considerable amount of the neutralizing 19S antibodies was directed against this trypsin sensitive part. The I.D. tests of the absorbed sera also indicate that the antibodies directed against the trypsin sensitive part of the virion, detect two antigens on the O_{Fr} and one on the O_{BHK}. It remains possible that the antigen which is missing on O_{BHK}, has been changed to one that is not detected in the present system.

Using late antiserum in I.D. tests it was impossible to determine whether the antigenic change was located on the complete or incomplete virion, for in contrast to early antiserum, late antiserum detects an antigenic deficiency on both the complete and trypsin treated particle. This is consistent with the results of WILD *et al.* (16) who demonstrated that a very small amount of late antibody is exclusively directed against the trypsin sensitive site. Thus early antiserum seems the most effective for detecting antigenic differences on the trypsin-sensitive part of the virion, as was also shown by COWAN (9).

In accordance with the observations of COWAN (9) we did find precipitation with O^{tryp} and early antisera. As pointed out in the results section it seems quite improbable that our early sera contained specific 7S antibodies, since not even faint precipitation was observed with the second (7S) peak obtained by G200 gel filtration of the sera. Furthermore, if the O^{tryp} precipitation line obtained with early guinea pig sera had been due to 7S contamination, we should also have found precipitation with the 12S virus protein subunit.

However, according to BROWN and SMALE (8) the precipitation line between O^{tryp} and early antisera is caused by 7S antibodies present in their early sera, because sucrose gardient fractionation of these sera eliminated this line from the 19S fraction. The difference between their observations and ours might be that they lost their 19S anti O^{tryp} antibodies due to their fractionation procedures since the anti O^{tryp} antibodies were a minor constituent in their early sera.

The faint spur visible in Figures 5 and 6 must also be caused by 19S antibodies. These antibodies sometimes detect an antigenic deficiency on the trypsin-treated

virion though to a lesser extent than on the complete virion. This property is also evident from the results of the absorption experiments (Table 1): O_{Fr}^{tryp} absorbs some neutralizing activity of the early sera in contrast to O_{BHK}^{tryp}. This is shown more clearly by the guinea pig hyperimmune serum: O_{Fr}^{tryp} spurs on O_{BHK}^{tryp} (Figs. 13 and 14).

BROWN (7) could not show precipitation between either early guinea pig serum or early cattle serum with 12S virus protein sub-unit. Our results differ from his with respect to early cattle serum. We are unable to offer an explanation for this discrepancy.

Nonetheless, the antigenic loss "seen" on the complete virus but not on the trypsin treated virus nor on the 12S virus protein subunit is in complete accordance with the FMDV model proposed by BROWN and SMALE (8).

The results presented in this paper offer an explanation for the fact, that loss of an antigen due to passaging of FMDV in BHK cells may result in a reduced immunogenicity of a vaccine prepared with the virus. It is shown to be due to an altered trypsin sensitive site on the virion, the site that is considered to be mainly responsible for immunogenicity of inactivated FMDV (15).

Acknowledgments

I wish to thank Mr. Jan Briaire for excellent technical assistance and Miss Ella Fanoy for carrying out the metabolic inhibition tests.

References

1. BACHRACH, H. L., TRAUTMAN, R., BREESE, S. S.: Chemical and physical properties of virtually pure foot and mouth disease. Amer. vet. Res. **25**, 333—342 (1964).
2. BACHRACH, H. L., VAN DE WOUDE, G. F.: Amino acid composition and C-terminal sequence of foot and mouth disease virus protein. Virology **34**, 282—289 (1968).
3. BARTELING, S. J.: A simple method for preparation of agarose. Clin. Chem. **15**, 1002 (1969).
4. BARTELING, S. J.: The use of frozen cells in the agar-cell suspension titration technique. Arch. ges. Virusforsch. **38**, 271—273 (1972).
5. BARTELING, S. J., MELOEN, R. H.: A simple method for the quantification of 140S particles of foot and mouth disease virus. Arch. ges. Virusforsch. **45**, 362—364 (1974).
6. BARTELING, S. J.: Use of polyethylene glycol-treated serum for production of foot and mouth disease (FMDV) in growing BHK-suspended cell cultures. Bull. Off. int. Epiz. **81**, 1243—1254 (1974).
7. BROWN, F.: A β-globulin antibody in the sera of guinea pigs and cattle infected with foot and mouth disease. J. Immunol. **85**, 298—303 (1960).
8. BROWN, F., SMALE, C. J.: Demonstration of three specific sites on the surface of foot and mouth disease virus by antibody complexing. J. gen. Virol. **7**, 115—127 (1970).
9. COWAN, K. M.: Immunochemical studies of foot and mouth disease. V. Antigenic variants of virus demonstrated by immune diffusion analyses with 19S but not 7S antibodies. J. exp. Med. **129**, 333—350 (1969).
10. COWAN, K. M.: Immunochemical studies of foot and mouth disease. VI. Differences in antigenic determinant site recognition by guinea pig 19S and 7S antibodies. J. Immunol. **104**, 423—431 (1970).
11. COWAN, K. M., EROL, N., WHITELAND, A. P.: Heterogenity of type Asia-1 foot and mouth disease virus and BHK-21 cells and the relationship to vaccine preparation. Bull. Off. int. Epiz. **81**, 1271—1298 (1974).

12. FISH, R. C., VAN BEKKUM, J. G., LEHMANN, R. P., RICHARDSON, G. V.: Immunologic response in Dutch cattle vaccinated with FMDV vaccins under field conditions. Neutralising antibody responses to O, A and C types. Amer. J. vet. Res. **30**, 2115—2123 (1969).
13. MARTIN, W. B., CHAPMAN, W. G.: Tissue culture colour test for assaying the virus and neutralising antibody of foot and mouth disease and its applications to the measurement of immunity in cattle. Res. vet. Sci. **2**, 53—61 (1961).
14. OUCHTERLONY, O.: Antigen—Antibody reactions in gels. Arkiv. för Kemi, Mineralogi och Geologi **26 B**, 1 (1949).
15. WILD, T. F., BROWN, F.: Nature of inactivating action of trypsin on foot and mouth disease virus. J. gen. Virol. **1**, 247—250 (1967).
16. WILD, T. F., BURROUGHS, J. N., BROWN, F.: Surface structure of Foot-and-Mouth Disease virus. J. gen. Virol. **4**, 313—320 (1969).

Author's address: Dr. R. H. MELOEN, Central Veterinary Institute, Virology Department, 39 Houtribweg, Lelystad, The Netherlands.

Received January 15, 1976