Archives of Virology 58, 35—43 (1978)

Archives of Virology

Differentiation Between Specific and Nonspecific Reactions of Bovine Sera and Foot and Mouth Disease Virus (FMDV) in Immunodiffusion Tests

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With 3 Figures

Accepted March 13, 1978

Summary

The precipitating and neutralizing activities of normal bovine sera with FMDV were studied and compared.

Twenty-two out of 79 normal bovine sera gave a positive reaction in micro neutralization tests with FMDV type O, while six did so with type A. In RID tests 32 sera were positive with type O and 28 with type A virus. Almost all of the 79 sera gave a positive reaction in the RID with trypsin treated virus of both types. After three to four fold concentration most sera also gave visible reactions in ID tests when tested against complete virus.

When O virus was used the ID patterns produced by most normal sera clearly differed from those obtained with early and late convalescent sera from FMDV infected steers. When type A materials were employed this was also the case but to a lesser extent. The patterns obtained with concentrated normal sera showed, in general a strong line with trypsin treated virus and no line or a weaker one with complete virus. The substances in normal bovine sera precipitating trypsin treated O virus were different from those reacting with trypsinized virus of type A.

Introduction

Normal sera of a number of species are known to contain substances reacting with a variety of viruses (13).

Frequently these activities, a.o. those directed against FMDV, were found associated with the 19S macroglobulin fraction (1, 3, 8, 9). For poliovirus antiviral activities were also found to be associated with IgG and IgA (T) globulins (15).

0304-8608/78/0058/0035/$ 01.80

Differentiation of the nonspecific reactions caused by these substances from specific ones induced by contact with FMDV antigens was only achieved until now by either setting a level below which all reactions were considered nonspecific or by heating the serum.

In this report qualitative differences between nonspecific and specific reactions observed in immuno diffusion (ID) tests are described. The method used presents a more rational approach to the differentiation of nonspecific and specific reactions with FMDV.

Materials and Methods

Virus and Trypsin Treated Virus

Virus preparations of type A subtype 10, strain Holland and of O subtype 1, strain BFS 68 were prepared by the Frenkel culture method, purified, concentrated and trypsin treated as described (11) with the exception that instead of sucrose gradients, linear CsCl gradients (1.30—1.50) were used. All virus preparations were applied in a concentration of 300 μg/ml.

Sera

Sera of 79 eighteen months to two years old steers of Irish origin, that had never been in contact with FMD, were obtained one or two days after arrival of the animals at the laboratory. The steers were kept in quarantine and three weeks later sera were again collected at random from 38 of these 79 steers. Homologous early (7 d. p.i.) and late (28 d. p.i.) convalescent steer sera against A en O virus were used in the ID test to obtain specific patterns. Homologous hyperimmune steer sera served as reference for the radial immuno diffusion (RID) test. Sera were stored at —20° C.

For use in the ID test sera were concentrated by a modification of the method described by WARRINGTON (14). One ml of serum was mixed with 0.16 ml of a 50 per cent PEG-6M (Hoechst) solution and left overnight at 4° C.

The precipitate which had a volume between 0.05 and 0.1 ml, was collected by centrifugation for 5 minutes at 3000 rpm and resuspended in 0.2 ml PBS.

Radial Immuno Diffusion (RID)

The RID was carried out essentially as described by COWAN (4) with the minor modification that serum-agarose gels were made in cups with a diameter of 20 mm and a depth of 2 mm, the central well had a diameter of 3 mm (12). The sera were tested in a final serum dilution of ½.

Immuno Diffusion Test

The ID test described by Ouchterlony was used with some minor modifications as reported (11). All normal sera were tested after three to fourfold concentration. The early convalescent sera were tested in a ½ and the late convalescent sera in a ¼ dilution. The unstained patterns were photographed. The effect of different NaCl concentrations on the ID reaction was investigated by adding NaCl to the ID buffer system up to final concentrations of 0.05 and 0.25 M of NaCl.

Micro Neutralization Test (MNT)

Neutralizing activity was determined by the constant virus-varying serum dilution method, using microtiter equipment and secondary pig kidney cells (10). About 100 ID_{50} of virus was used in every well.

Endpoints were determined according to Reed and Muench. Titers are expressed as the negative logarithm of the serum dilution endpoint.

Sera are considered positive if their titers are 0.45 or more.

Results

As shown in Table 1 more sera produced a positive reaction in the RID test than in the MNT. Almost all reacted with trypsin treated virus. Not necessarily the same sera gave a positive reaction with both virus types or reacted positive in both the RID and MNT. The precipitation lines obtained in the ID tests with concentrated normal sera tented to disappear with increasing NaCl concentration, while the specific precipitation lines improved slightly. NaCl was therefore not used in the gels.

Table 1. *Number of positive reactions of 79 bovine sera in the micro neutralization test (MNT) and the radial immunodiffusion test with complete virus (RID) and trypsin treated virus (RID-tryp)*

	virus type O		virus type A
MNT	22	(5)	6
	(11)[a]		(2)
RID	32	(14)	28
RID-tryp	76		78

[a] Between brackets the number of sera are shown that gave either positive reactions in the same test with both types of virus (FMDV type A and type O) or positive reactions in both tests with the same virus type

The different patterns observed in the ID tests are shown in Figure 1. With type A virus a whole spectrum of patterns was obtained. Fourteen of the 79 sera reacted almost exclusively with trypsin treated virus (group I), 29 showed a slight reaction with complete virus, with the trypsin treated virus spurring over the complete virus (group II). Crossing lines were found in six cases (group III) while complete virus was spurring in four cases over trypsin treated virus (group IV). No crossing or spurring was seen with 7 sera (group V). A vague precipitation was observed with 17 sera (group VI), while the remaining 2 sera produced no precipitation at all. With type O virus patterns of group I, II and III were observed with respectively 35, 40 and 3 sera. One serum produced no reaction.

The patterns I and II of normal sera differ clearly from the specific ones shown in Figure 2.

Strong precipitation lines are produced with trypsin treated virus, whereas only minor lines or no lines at all are formed with complete virus. It is not clear whether these minor lines in group II cross or are partially identical with the main ones. The specific patterns obtained using early sera usually show the complete virus spurring over the trypsin treated virus. Representative samples are shown in Figure 2. These patterns were also observed with convalescent sera raised in other species. Occasionally however patterns with crossing lines were observed (not shown). The patterns obtained with late sera produced lines of identity of which representative samples are shown in Figure 2.

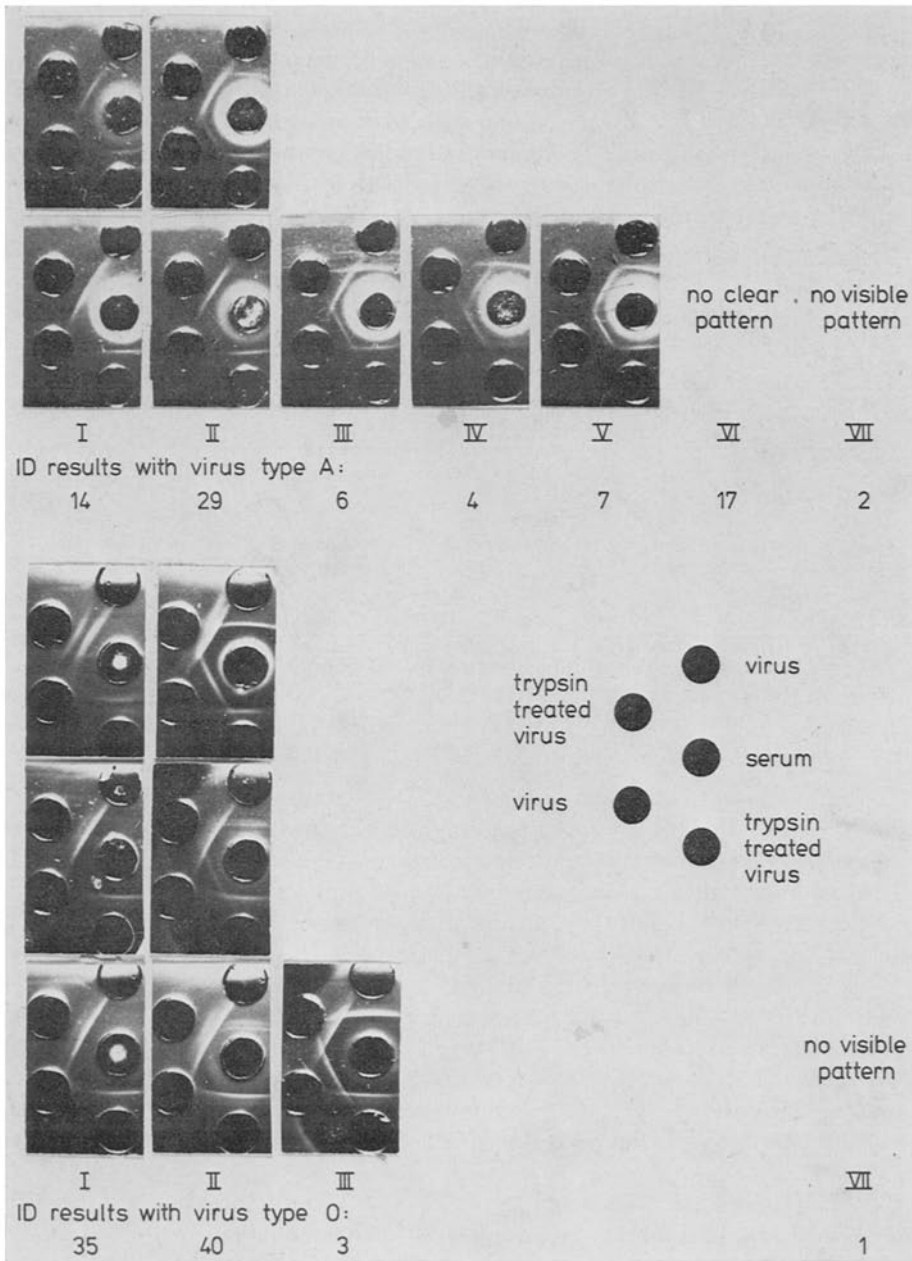

Fig. 1. ID results of 79 concentrated sera tested against complete and trypsin treated FMDV type O and A. The results are grouped according to the patterns obtained of which representative samples are shown

In order to learn if the main ID reactions, with trypsin treated type A and O virus were caused by the same serum component, all sera were retested in one test against trypsin treated preparations of A and O. The resulting patterns show strong lines with O and weaker ones with A virus (Fig. 3). Twenty-eight sera gave crossing lines, while in case of 51 sera the line with trypsin treated virus type A was too weak to tell whether it crossed or showed partial identity with the O virus line (Fig. 3). A number of normal sera were tested side by side with early and late convalescent sera against complete and trypsin treated virus type O. The precipitation lines between complete virus and normal sera were rather weak if present at all.

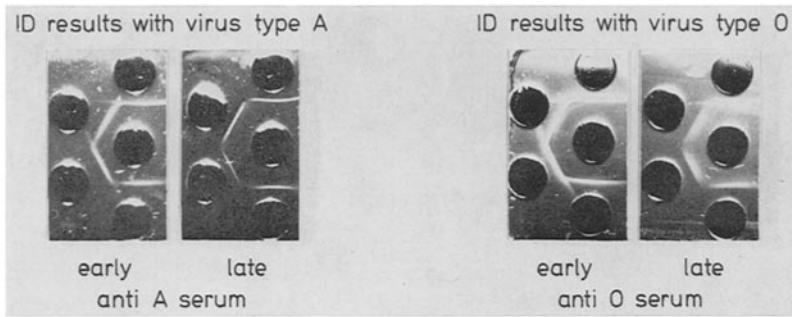

Fig. 2. ID results of early and late convalescent sera anti A and anti O tested against complete and trypsin treated FMDV of both types. The wells were filled with antigens and sera in the same way as in Figure 1

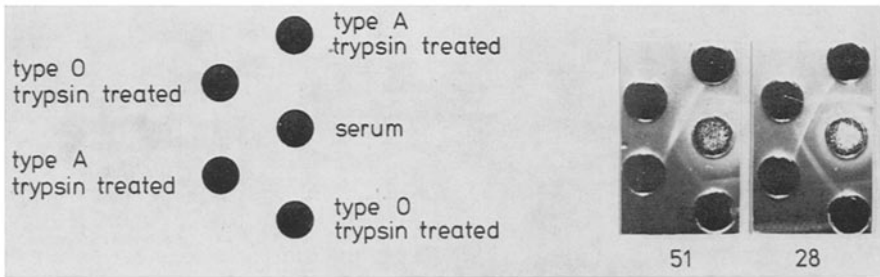

Fig. 3. ID results using concentrated sera tested against trypsin treated type A and O FMDV. Two kinds of patterns were obtained of which representative samples are shown. The number of sera producing either one pattern are presented

With trypsin treated virus normal sera produced precipitation lines that spurred on the lines formed by late sera and were identical with those formed by early sera.

Three weeks after arrival of the steers 34 of them were bled again and the sera obtained were concentrated and tested by ID against complete O and trypsin treated O virus. The resulting patterns were compared with the ones produced with sera collected directly after arrival. It was found that a quarter of the sera having group I patterns changed during the three weeks to sera exhibiting group II

patterns and vice versa, all the patterns were however still distinct from the specific ones. The over all distribution over the three groups after three weeks was more or less the same as that shown in Figure 1, obtained directly after arrival of the steers. The same observation was done when all the sera were retested.

A number of control experiments were done. Instead of concentrated sera, increasing concentrations of PEG (1 per cent, 2 per cent and 4 per cent) were put in the centre well. No precipitation lines developed.

As shown before (2) the purification procedure produces virtually pure virus. Because bovine tonque epithelium not infected with virus did not react, when put through the purification procedures with normal bovine sera, the precipitation lines obtained between normal bovine sera and virus must be virus specific. Further the early and late convalescent sera were concentrated with PEG as described and tested by ID both before and after dilution to the appropriate concentration. If not too much distorted by the unbalance of the system, the patterns of the concentrated sera were the same as those of the diluted sera and of the untreated sera.

Discussion

In our laboratory sera from the FMD susceptible Irish cattle used for FMD-work have in the past years routinely been tested in the RID test immediately after arrival of the animals in Holland. With undiluted sera positive reactions were observed with complete and trypsin treated FMDV, more or less as shown in Table 1. The number of sera reacting with trypsin treated FMDV exceeded that of the ones reacting with complete virus and the reactions were more intensive. This prompted us to investigate this phenomenon, especially since it seemed to offer a possibility to differentiate qualitatively between specific and nonspecific reactions.

Nonspecific activity has mainly been found in the 19S serum fraction (3, 8, 9). This is in agreement with unpublished results obtained in our laboratory with a number of normal sera fractioned on Sephadex G-200.

The present finding (Table 1) that not necessarily the same sera give positive reactions either in the RID and MNT or with both virus types is in agreement with the results of ANDERSON (1). The high number of positive reactions found in both tests are probably due to the higher serum concentrations used by us as compared with those employed by ANDERSON (1).

Since only a few sera showed visible ID patterns with complete and trypsin treated virus, all sera were concentrated by PEG precipitation. The method applied concentrates both specific 19S and 7S antibodies, as shown by WARRINGTON (14). If one only wants to study the substances present in the 19S serum fraction 4 per cent PEG is sufficient. Normal sera concentrated with 4 per cent PEG gave the same ID patterns as sera concentrated with 7 per cent PEG, in accordance with the observation that the nonspecific reactions are found in the 19S serum fraction (3, 8, 9). A 4 per cent PEG concentration has the additional advantage of precipitating less protein, which makes the use of a higher concentration-factor possible.

However if one wants to differentiate between specific and nonspecific reactions it is undesirable to remove part of the 7S antibodies.

As reported before (1) the RID cannot differentiate between a specific and nonspecific precipitation reaction. ANDERSON (1) proposed therefore to make this differentiation by establishing the antibody class in which the precipitating activity occurs. If after four or five weeks the activity were still found in the 19S serum fraction, this would indicate a nonspecific reaction. Serum fractionation is however too laborious to apply in serologic surveys. The only suitable method left for differentiating between 19S and 7S globulins is the reduction of 19S macroglobulins by β-mercapto ethanol. In our hands this method destroyed some of the 7S antibody activity in bovine sera, as reported earlier by COWAN (5).

Our method of defining the nature of the precipitating agents by ID test with the aid of complete and trypsin treated virus and concentrated sera seems therefore at least in case of type O, much more suitable for large scale application, since it can easily be applied to a large number of sera.

The observation that the sera almost always reacted in the RID test with trypsin treated virus but only occasionally with complete virus was confirmed in ID tests with the concentrated sera. It suggests that trypsin treated virus exhibits reactive groups not present on the complete virion which are recognized by normal sera. This is in agreement with the ID results obtained when normal sera and late convalescent sera were tested side by side against trypsin treated virus: precipitation lines of normal sera spurred over those of late sera. Why under these conditions early sera give lines of identity remains to be explained.

The partial inhibition of the formation of nonspecific patterns by NaCl probably reflects the less stable binding between the virus and the precipitating substances as compared to the binding between virus and specific 19S antibodies.

The high "specificity" of the precipitating substances is evident from their ability to react, sometimes even exclusively, with trypsin treated virus. This high "specificity" could further be reflected by the multiple lines sometimes formed in ID patterns (Figs. 1 and 3). These lines could be due to breakdown products of complete or trypsin treated virus, as shown, to some extent, in the specific ID patterns (Fig. 2). However, since the intensities and number of the lines in the nonspecific reactions clearly differ from the ones in the specific reactions, one might speculate that the precipitating substances recognize slightly different virus particles present in this virus preparation, that are not observed with specific antibodies. The high specificity of some of these substances is also shown by the crossing lines obtained with 28 sera when tested against trypsin type A and O (Fig. 3).

In their apparent nonspecificity, combined with a high specificity for certain chemical structures on the virion these components of normal sera resemble specific 19S antibodies. According to COWAN (5) 19S antibodies show extensive cross reaction but are also very specific for certain viral antigens. This might be due to the presence of several highly specific antigenic determinants on the virion that are either not recognized by or are not able to react with 7S antibodies, perhaps as a consequence of steric or conformational factors.

The low binding strength between the precipitating substances in normal sera and the virus and the observation that the presence of these substances is not limited to a short period support the hypothesis (9, 16) that the substances are the result of an unrecognized infection of the animals with some unrelated agent. IgM

possessing similar properties was produced by stimulations of mice with a bacterial endotoxin. It reacted with bovine gamma globulin, had a low avidity for this antigen and remained present during a long period (6). However the other possibility, that these substances are part of some kind of inherited surveillance mechanism (7), cannot be excluded. The observed "specific" nature of the precipitating agents in normal bovine sera does not seem to be restricted to bovine sera and FMDV, but might reflect a more general phenomenon since it has been shown that normal equine sera react in a similar "specific" manner with strains of polio virus (16, 17).

This study substantiates the occurrence of specific reacting serum components, which may be applied for immunological analyses, for instance ID results reported here suggests that the trypsin treated virion exhibits reactive groups not present on the complete virion. This has been shown before but in a more tedious way (2).

Acknowledgement

I thank Dr. J. G. van Bekkum and Dr. S. J. Barteling for critically reading the manuscript, Mr. Jan Briaire for his excellent technical assistance and Mr. Wim Tiessink for doing the micro neutralization test.

References

1. ANDERSON, A. A.: Cross-reactions of normal bovine serums to FMDV in plaque-reduction neutralization and radial immunodiffusion. Amer. J. vet. Res. **36**, 979—983 (1975).
2. BACHRACH, H. L., MOORE, D. M., MCKERCHER, P. D., POLATNICK, J.: Immune and antibody response to isolated capsid protein of FMDV. J. Immunol. **115**, 1636—1641 (1975).
3. BÖGEL, K., MATTHAEUS, W., STROHMAIER, K.: Thermostabiler Inhibitor im Schweine-Serum gegenüber dem MKS-Virus III. Electrophoretische und chromatographische Untersuchungen. Z. Bl. vet. Med., Series B **14**, 221—223 (1967).
4. COWAN, K. M., WAGNER, G. G.: Immunochemical studies of FMD virus VII. Detection and quantitation of antibody by radial immunodiffusion. J. Immunol. **105**, 557—566 (1970).
5. COWAN, K. M.: Antibody response to viral antigens. Adv. Immunol. **17**, 195—247 (1973).
6. DRESSER, D. W., POPHAM, A. M.: Induction of an IgM anti-(bovine-)IgG in mice by bacterial lipopolysaccharide. Nature **264**, 552—554 (1976).
7. IHLE, J. N., DOMOTOR, J. J., BENGALI, K. M.: Characterization of the type and group specificities of the immune response in mice to murine leukemia viruses. J. Virol. **18**, 124—131 (1976).
8. KALMAR, E., PELEG, B. A., MODAN, M.: Non-specific neutralising activity of bovine sera against various types of FMD virus. II genetic control of non-specific neutralising activity in calf sera. Refuah. Vet. **29**, 91—102 (1972).
9. LONGWORTH, J. F., ROBERTSON, J. S., TINSLEY, T. W., ROWLANDS, D. J., BROWN, F.: Reaction between an insect picornavirus in naturally occurring IgM antibodies in several mammalian species. Nature **242**, 314—316 (1973).
10. LEEUW, P. W. DE: In preparation.
11. MELOEN, R. H.: Localisation on Foot and Mouth Disease Virus (FMDV) of an antigenic deficiency induced by passage in BHK cells. Arch. Virol. **51**, 299—306 (1976).
12. MELOEN, R. H.: In preparation.

13. PATTY, R. E.: Inhibition of FMDV by normal bovine serum. Amer. J. vet. Res. **31**, 165—171 (1970).
14. WARRINGTON, R. E., MORGAN, O. O.: FMD virus: Purification of 19S gamma globulin. Arch. ges. Virusforsch. **33**, 134—144 (1971).
15. URASAWA, S., URASAWA, T., KANAMITSU, M.: Radioimmuno electrophoretic identification of poliovirus inhibitors and their characteristic mode of action. Arch. ges. Virusforsch. **33**, 113—125 (1971).
16. URASAWA, S., URASAWA, T., KANAMITSU, M.: Analysis of specificity of antibodies contained in antiserum against poliovirus with mutants resistant to inhibitors in equine serum. J. Immunol. **113**, 537—542 (1974).
17. URASAWA, S., URASAWA, T., KANAMITSU, M.: Further studies of specificity of antibodies contained in antiserum against poliovirus. Japan. J. Microbiol. **20**, 11—16 (1976).

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Received November 1, 1977