Complement-Fixing And Neutralizing Antibody Response To Bovine Viral Diarrhea And Hog Cholera Antigens

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SUMMARY

In calves inoculated with bovine viral diarrhea (BVD) viruses and soluble antigen, the complement-fixing (CF) antibodies appeared before serum-neutralizing (SN) antibodies and remained at high levels throughout the test period. A rapid rise in SN antibodies occurred after challenge with homologous virus with no apparent effect on CF antibody levels.

The CF antibody responses in calves infected with cytopathogenic NADL-MD and noncytopathogenic CG-1220 viruses were similar whereas SN antibody responses indicated strain specificity by reciprocal cross-neutralization tests.

The CF antibody levels in 5 hog cholera (HC) antisera were assayed using the soluble antigen of NADL-MD BVD virus. No demonstrable SN antibodies were present in four HC antisera tested against NADL-MD virus, but a significant titer was present in the commercially prepared antiserum.

Virus was reisolated from animals infected with BVD viruses by buffy coat culture technique during 3 weeks postinoculation, even when significant levels of CF and SN antibodies were present.

Bovine viral diarrhea (BVD) and hog cholera (HC) viruses are responsible for two different diseases in cattle and swine respectively. These two viruses share a common soluble antigen, however, as demonstrated by agar double diffusion studies (1). For this reason it was logical to apply the complement fixation test in an attempt to quantitatively assay antibody response in animals exposed to these viruses. Such a test also could be useful to further characterize the relationship between the two viral agents.

This is a report on the application of the complement fixation (CF) and serum neutralization (SN) tests utilizing soluble antigen and infective BVD viruses; to compare antibody responses in calves inoculated with BVD viruses or soluble antigen; to relate cytopathogenic and noncytopathogenic BVD viruses; and to assay antibody levels in sera from swine hyperimmunized against HC viruses. In addition, buffy coat culture technique was used to correlate virus reisolations with the appearance of antibodies after exposure to BVD viruses.

Materials and Methods

PREPARATION OF SOLUBLE ANTIGEN

The soluble antigen of BVD virus, NA DL-MD, was used in the CF test. Preparation of the soluble antigen was as described (1) with the following modifications: concentration of ultracentrifuged supernatant tissue culture fluid was accomplished by pressure dialysis at 4°C without the use of 20M Carbowax, a nonspecific sensitizing agent for sheep erythrocytes (2); and chloroform was added to the concentrated soluble antigen to a final concentration of 5%. This emulsion was gently mixed for 30 minutes at room temperature to inactivate any remaining infective virus particles. Excess chloroform was removed by centrifugation at 2,000 G for 20 minutes in a refrigerated centrifuge. The supernatant fluid was dialyzed against frequent changes of veronal-buffered saline (3) at 4° C for 4 to 5 days. Small amounts of precipitate were removed by centrifugation and the supernatant fluid was stored at -40° C.

COMPLEMENT FIXATION TECHNIQUE

Fresh guinea pig complement, hemolysin and sheep erythrocytes were prepared and standardized according to the methods of Schmidt and Lennette (4). The CF technique was essentially the same as described by these authors with the following modifications: all dilutions of reagents and sera were made in veronal-buffered saline

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solution containing Ca and Mg ions (3); procomplementary activity of swine test sera was removed by formalin as described by Cowan (5); and unheated specific pathogen-free (SPF) bovine serum was added to optimally diluted complement to enhance fixation of complement to the antigen-antibody system (6). The normal unheated bovine serum, at a concentration of 5%, had neither anticomplementary nor nonspecific activity with the test antigen.

Bovine test sera used in this study had negligible anticomplementary activity. Serum titer was expressed as the reciprocal of the initial dilution giving 50% lysis.

Neutralization Technique

The SN test was conducted using a technique described for cytopathogenic strains of virus (1). Modifications of this technique were necessary for SN tests with noncytopathogenic strains of virus. The principle of interference, described by Gillespie *et al.* (7), was used for titration and neutralization of noncytopathogenic virus in tissue culture systems. For titrations, cultures containing dilutions of noncytopathogenic virus were incubated 72 hours and then to each was added approximately 10^3 $TCID_{50}$ of cytopathogenic NADL-MD virus. If a noncytopathogenic virus had propagated in the culture there was almost 100% interference with the cytopathogenic virus. For SN tests, it was necessary to aspirate the maintenance fluid from each culture after 96 hours' incubation and to replace it with maintenance medium containing approximately 10^3 $TCID_{50}$ NADL-MD virus. The presence of unneutralized noncytopathogenic virus could then be detected by its interference with NADL-MD virus. Fifty percent neutralization endpoints were calculated by the method of Reed and Muench (8).

Preparation of Test Sera

Sera were collected prior to inoculation and at weekly intervals from four SPF calves that had been given the following treatment:

Calf number 1 was given two intravenous injections of 5 ml (10^6 $TCID_{50}$) NADL-MD virus 12 weeks apart.

Calf number 2 was given daily intravenous injections for 5 days of 5 ml concentrated (7.5 x 10^7 $TCID_{50}$) NADL-MD virus.

Calf number 3 was given two intradermal injections of 2½ ml concentrated (100 fold) soluble antigen of NADL-MD virus with equal parts Freund's type adjuvant a week apart; ten ml concentrated soluble antigen and adjuvant intramuscularly on the third week; and ten ml (2 x 10^6 $TCID_{50}$) virulent NADL-MD virus intravenously on the 6th and 8th week.

Calf number 4 was given two intravenous injections of 5 ml (10^6 $TCID_{50}$) noncytopathogenic CG-1220 virus (isolated by the authors) 7 weeks apart.

Five hog cholera antisera used in the agar double diffusion studies (1) were also used in this study; one prepared against standard hog cholera virus in a SPF pig; three antisera prepared against variant, lapinized and standard HC virus respectively; and one commercially prepared antiserum. Untreated SPF swine and bovine sera served as controls. All test sera were heat inactivated at 56° C for 30 minutes prior to their addition to the tests.

Virus Isolation Technique

Buffy coat cultures were prepared from infected calves by a procedure similar to that used for propagating swine leukocytes (9). Approximately 25 ml of blood was collected aseptically into 5 ml of a 10% sodium citrate solution. After centrifugation at 2,000 G for 30 minutes, the plasma was carefully aspirated and the buffy coat transferred to another centrifuge tube containing 5 ml phosphate-buffered saline (PBS) devoid of Ca and Mg ions. After resuspending the leukocytes, an additional 25 ml of PBS was added and the cell suspension centrifuged at 2,000 G for 30 minutes. The washing procedure was repeated two to three times, care being taken to exclude as many erythrocytes as possible from each recovery. After the last centrifugation, buffy coat cells were resuspended into two types of medium; (1) 20% fetal bovine serum and 80% Eagle's basal medium (EBM) and (2) 20% simultaneously collected serum from infected animals and 80% EBM. The cell suspension was planted in tissue culture tubes or on occasion directly into primary bovine embryonic kidney (BEK) cell cultures. Cultures were incubated 5 to 7 days and then reinoculated into new BEK cultures. If noncytopathogenic strains of virus were involved, the cultures were reinoculated

Table I — Reciprocal Cross-Neutralization Tests of Cytopathogenic NADL-MD and Noncytopathogenic CG-1220 Viruses

Antisera	Neutralization Titers	
	NADL-MD virus	CG-1220 virus*
NADL-MD calf 1		
4 week serum.......	1150**	256**
6 week serum.......	5400	1530
CG-1220 calf 4		
4 week serum.......	320	1140
6 week serum.......	512	1320

*Interference test to detect CG-1220 virus utilizing NADL-MD virus.
**Titers expressed as reciprocal of final serum dilution.

Table II — Complement Fixation and Neutralization Tests of Hog Cholera Antisera Utilizing NADL-MD Bovine Viral Diarrhea Virus Antigens

Antisera	CF Titers		SN Titers
	without bovine serum	with bovine serum	
Standard HC (SPF).....	40*	640*	< 4*
Standard HC..........	20	320	< 4
Variant HC............	20	320	< 4
Lapinized HC..........	<10	80	< 4
Commercial HC........	10	160	512
Untreated control (SPF)	<10	< 10	< 4
Untreated control (SPF)	<10	< 10	< 4

*Titers expressed as reciprocal of serum dilution.

with cytopathogenic NADL-MD virus after 72 hours' incubation as previously outlined.

Results

Antibody Responses in Calves

The CF and SN antibody responses in calves inoculated with BVD viruses and soluble antigen are presented graphically in figures 1, 2, 3, and 4. The CF antibodies appeared before the SN antibodies and remained at high levels throughout the test period. Both CF and SN antibody levels reached a peak approximately 6 weeks after inoculation (figures 1, 2 and 4). A high level of CF antibodies with a relatively low level of SN antibodies was produced in calf 3, given a series of injections of soluble antigen and adjuvant (figure 3). A rapid rise in SN antibodies occurred after challenge inoculation with virulent homologous virus with no apparent effect on CF antibody levels (figure 1, 3 and 4).

Relationship of Cytopathogenic NADL-MD and Noncytopathogenic CG-1220 Viruses

The CF antibody responses were similar

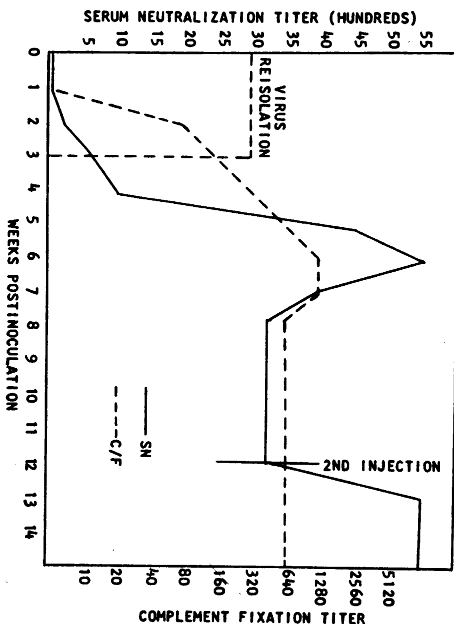

FIGURE 1. Antibody response in calf 1 after two intravenous injections of cytopathogenic NADL-MD virus 12 weeks apart.

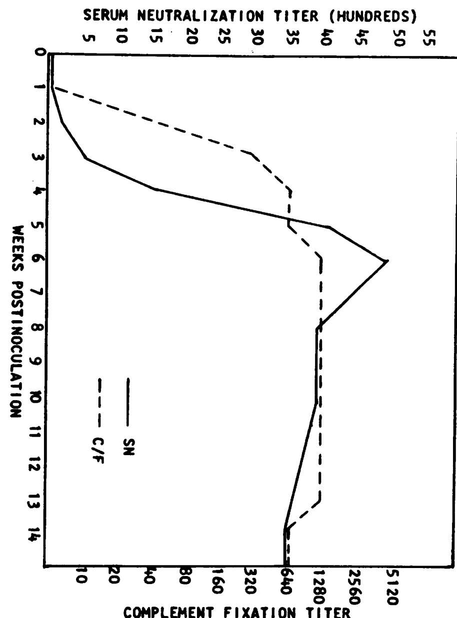

FIGURE 2. Antibody response in calf 2 after 5 daily intravenous injections of concentrated NADL-MD virus.

in calves 1 and 4 inoculated with cytopathogenic NADL-MD and noncytopathogenic CG-1220 virus respectively. The SN antibody response was distinctly higher in calf 1 than calf 4 when tested against NADL-MD virus (figures 1 and 4).

Results of reciprocal cross-neutralization tests between the two viruses, utilizing 4 and 6 week postinoculation sera from calves 1 and 4, are presented in table 1.

Complement Fixation and Neutralization Tests of Hog Cholera Antisera

The CF tests of HC antisera, utilizing soluble antigen of NADL-MD BVD virus, resulted in fixation of complement to the antigen-antibody system. Addition of normal unheated bovine serum to the test markedly enhanced fixation of complement resulting in significantly higher titers (table II).

With the exception of the commercially prepared HC antiserum, no demonstrable SN antibodies were present in the hog cholera or control swine sera when tested against NADL-MD BVD virus (table II).

Virus Isolation

Virus was reisolated during 3 weeks postinoculation from calf 1 when bovine fetal serum was incorporated in the medium for propagating buffy coat cells (figure 1). Noncytopathogenic CG-1220 virus, as determined by interference tests, was reisolated during 2 weeks postinoculation of calf 4 (figure 4). Virus was reisolated 1 week postinoculation from calves 1 and 4 when simultaneously collected sera from the infected animals were incorporated into the medium for propogating buffy coat cells. No virus was reisolated from calf 3 after injections of soluble antigen and challenge with virulent virus.

Discussion

It was demonstrated that CF antibodies appeared before SN antibodies in calves given BVD viruses; a sequence that also occurs in myxovirus (10) or arbovirus (11) infections. This could be interpreted as a basis for assuming that different types of antibodies are produced at different stages of infection or that different antigens are concerned in the tests. The latter interpretation seems more plausible because the inoculation of comparatively crude soluble antigen preparations resulted primarily in a CF antibody response (figure 3). Soluble antigen may well represent a precursor or by-product of viral synthesis.

Reciprocal cross-neutralization tests per-

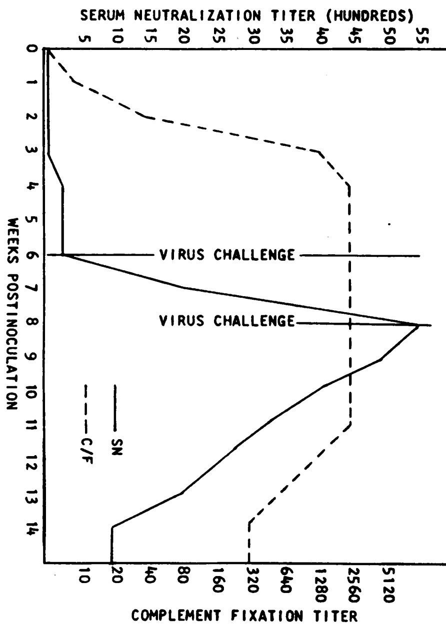

FIGURE 3. Antibody response in calf 3 after a series of injections, 2 intradermally and 1 intramuscularly, of soluble NADL-MD antigen with adjuvant. The calf was given challenge inoculations 6 and 8 weeks later of virulent NADL-MD virus.

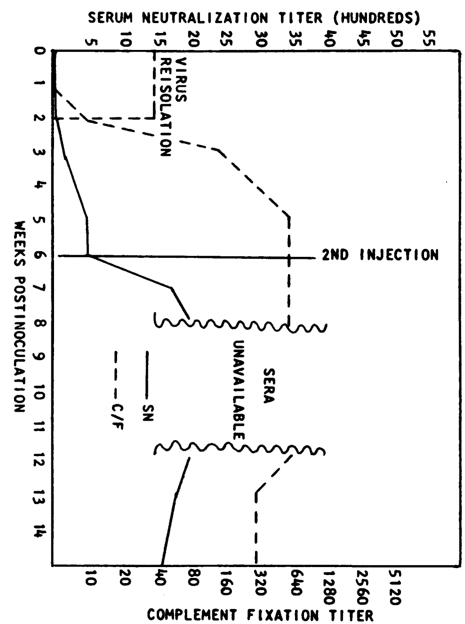

FIGURE 4. Antibody response in calf 4 after 2 intravenous injections of noncytopathogenic CG-1220 virus 6 weeks apart. Complement-fixation and serum neutralization tests were conducted with NADL-MD virus antigens.

formed with NADL-MD and CG-1220 antisera indicated some degree of strain specificity although the CF titers were identical. Neutralizing antibodies may therefore be directed against more specific viral antigen(s). Additional BVD isolates could evidence group relationships based on the complement fixation test and strain specificity on reciprocal cross-neutralization test. This would correlate with other observed differences such as cytopathogenic and noncytopathogenic properties. Hog cholera virus which shares a common soluble antigen with BVD viruses (1) and stimulates the production of CF (table II) but not reciprocal neutralizing antibodies (12) may well represent the extreme divergence.

The BVD virus probably existed intracellularly for 2 to 3 weeks postinoculation, because it was possible to make recoveries of virus from buffy coat cells even though significant CF and SN serum titers were present (figures 1 and 4). Removal of SN antibodies from the cells by repeated washings and implanting them in a medium containing fetal bovine serum enhanced the probability of successful virus reisolation. Using serum from the infected animal in the culture medium is therefore inadvisable since it may neutralize virus released from the cells.

Application of techniques using unheated bovine serum in the antigen-antibody systems to enhance fixation of complement and the treatment of swine serum with formalin to remove the procomplementary activity has made the CF test a useful aid in studying the relationship between BVD and HC viruses. It is deficient as a single test for HC because swine infected with BVD viruses could develop CF antibodies without being exposed to HC viruses. Yet, if swine sera contained CF antibody against BVD soluble antigen in the absence of an SN titer it would be presumptive evidence of earlier exposure to HC virus.

Soluble group-specific antigen common to known types of virus have been useful in classifying new isolates. The inclusion of new isolates into the adenovirus group has in part been based upon their production of a soluble CF antigen (13, 14). Members of the myxovirus group also are known to produce a "soluble" CF antigen common to all strains of a given type in addition to strain-specific antigens (10, 15). Since isolates of BVD and HC viruses have similar physical and chemical properties (16) and cross react in immunofluorescence (17), they may be classified as belonging to the same group according to a common soluble antigen (1) and into strains by neutralization test or as yet unrecognized antigens.

REFERENCES

1. GUTEKUNST, D. E., MALMQUIST, W. A., Can. J. Comp. Med. and Vet. Sci, 1963; 27, 121.
2. COWAN, K. M., Thesis, 1954, The Johns Hopkins University.
3. KABAT, E. A., MAYER, M. M., Experimental Imnunochemistry, 2nd Ed., 1961, 149.
4. SCHMIDT, N. J., LENNETTE, E. H., J. Exp. Med., 1955, 102, 133.
5. COWAN, K. M., J. Immunology, 1961, 86, 467.
6. BOULANGER, P., BANNISTER, G. L., J. Immunology, 1960, 85, 368.
7. GILLESPIE, J. H., MADIN, S. H., DARBY, N. B., Proc. Soc. Exp. Biol. and Med., 1962, 110, 248.
8. REED, L. J., MUENCH, H., Am. J. Hyg., 1938, 27, 493.
9. MALMQUIST, W. A., MAY, D., Am. J. Vet. Res., 1960, 21, 104.
10. BURNET, F. M., Principles of Animal Virology, 2nd Ed., 1960, 283.
11. PORTERFIELD, J. S. Advances in Virus Research, 1962, 9, 130.
12. SHEFFY, B. E., COGGINS, L., BAKER, J. A., Proc. Soc. Exp. Biol. and Med., 1962, 109, 349.
13. ROWE, W. P., HARTLEY, J. W., HUEBNER, R. J., Proc. Soc. Exp. Biol. and Med., 1956, 91, 260.
14. GINSBERG, H. S., Virology, 1962, 18, 312.
15. LENNETTE, E. H., HORSFALL, F. L., Jr., J. Exp. Med., 1941, 73, 581.
16. DINTER, Z., Zbl. f. Bakt. I. Orig., 1963, 188, 475.
17. MENGELING, W. L., GUTEKUNST, D. E., FERNELIUS, A. L., PIRTLE, E. C., Can. J. Comp. Med. and Vet. Sci., 1963, 27, 162.

ERRATUM

The following caption should have accompanied Fig. 1 on Page 270, November 1963 issue, as part of the article "Metabolism and Distribution of ^{131} I-labelled Albumin in the Pig", by John Dich and Knud Nielsen:

"Fig. 1. Experimental data from albumin turnover studies with pig No. 7. Q_{Ret}: Fraction of the dose retained in the body. Q_{Ev}: Fraction of the dose in the extravascular compartment. Q_P: Fraction of the dose in the intravascular compartment. Dotted line indicates equilibration time. Columns indicate daily urinary and fecal excretion (per cent of injected dose).