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Antibody Class and Complement Requirement of Neutralizing Antibodies in the Primary and Secondary Antibody Response of Cattle to Infectious Bovine Rhinotracheitis Virus Vaccine

By

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Summary

Calves responded to a single intramuscular injection of an attenuated strain of infectious bovine rhinotracheitis virus by producing IgM followed by IgG antibody. Both IgM and IgG antibody produced during the first month were primarily complement-requiring neutralizing antibody (CRNAb), especially IgM antibody. After a month, IgG had replaced IgM as the predominant immunoglobulin, and titers with and without complement (C') decreased in both IgG and IgM fractions. The largest decrease was in the IgM CRNAb fraction.

Seven days after a second injection given on day 196, calves responded with an anamnestic IgG response in which CRNAb titers were 1 or 2 two-fold dilutions higher than non-CRNAb titers. One calf developed an IgM response similar to its primary response, whereas inhibition of the IgM response occurred in the other 3 calves which had much lower IgM antibody titers than those attained in the primary response. Twenty-eight days after the second injection the titers of IgG were the same or only a 2-fold dilution less than their 7-day secondary titers, whereas IgM titers generally decreased considerably more than this. Guinea pig and rabbit sera were equally effective as C' sources in potentiating CRNAb, whereas bovine serum was a poor C' source.

Introduction

Many viruses elicit production of complement-requiring neutralizing antibodies (CRNAb) which appear at different phases of the immune response. The virus, host, and possibly method of immunization are variables that influence the production of CRNAb. For example, herpes simplex virus elicited CRNAb in early

rather than late serum, irrespective of the host in which the antisera was produced (19, 20), whereas equine arteritis virus elicited CRNAb in the horse and several laboratory animals in the late phase of immunization (12). Although murine cytomegaloviruses elicited CRNAb in rabbits during the early, but not late, phase of immunization (9), the behavior of cytomegaloviruses in primates was found to be different. Two strains of monkey cytomegaloviruses elicited only non-CRNAb (NCRNAb), whereas 2 human strains elicited CRNAb during early and late phases of immunization (7). Variable responses have occurred with different strains of human cytomegaloviruses; in rabbits CRNAb was primarily associated with early antisera, whereas 2 other strains elicited CRNAb which was essentially limited to late antisera. Humans infected with cytomegaloviruses have not exhibited enhanced titers with complement (C') (1). Much of the difference between C' dependence and independence in studies with cytomegaloviruses has been related to the immunoglobulin class of antibody which was produced, immunoglobulin M (IgM) being much more C' dependent than immunoglobulin G (IgG) (1).

We previously reported that cattle immunized with a vaccine strain of infectious bovine rhinotracheitis (IBR) virus produced an early primary response consisting predominantly of CRNAb. Complement dependence diminished much sooner than the antibody titer during the first month. In response to a second injection C' dependence was much less prominent so that C' dependence could be used to distinguish a primary from a secondary response (16). This investigation is concerned with the relationship between antibody class, CRNAb and their appearance in the immune response, and some of the factors necessary for demonstrating CRNAb.

Materials and Methods

Test Materials

A vaccine strain of IBR virus was used for immunization of cattle, whereas the virulent Cooper strain of IBR virus was used in neutralization tests.

Sera used in this study for determination of immunoglobulin class of antibodies and CRNAb were from 6-month-old calves given $10^{6.6}$ mean tissue culture infective doses of IBR virus intramuscularly on days 0 and 196 as previously described (16), and from 2 yearling heifers (no. 104 and 106) 6 days after a single intramuscular injection of $10^{8.2}$ mean tissue culture infective doses of IBR virus. These calves had no detectable antibody on day 0. Sera from healthy cattle were obtained and screened for antibody to IBR virus as previously described (16).

Sources of C' were rabbit and guinea pig sera (GIBCO, Grand Island, NY), and bovine serum obtained from 2 yearling calves so as to preserve their C' activity. Complement was stored at —80° C. All sera were tested for the presence of antibody to IBR virus with rabbit C'. Several guinea pig sera were found to contain CRNAb, but not NCRNAb, to IBR virus. Complement was usually used at a 1:3 dilution. Heat inactivation of sera was at 56° C for a half hour.

Separation of IgM from IgG was done on Sephadex G-200 Superfine. The efficiency of separation was determined on column eluates by radial immunodiffusion with antisera specific for IgG and IgM. Samples were concentrated to their original volume by dialysis against polyethylene glycol, molecular weight 20,000, in Hanks' balanced salt solution.

Neutralization Tests

Unless otherwise indicated, neutralization tests were conducted as follows: 0.2 ml of serial 2-fold dilutions of test sera was mixed with 0.2 ml of a constant amount of virus and incubated for an hour at 37° C; 0.1 ml C' or heat-inactivated serum was

added and the mixtures incubated for another hour at 37° C, diluted 1000-fold, the residual virus assayed on bovine embryonic lung monolayers and titers determined as previously described (16).

In determining the effect of 2 sera as sources of complement on the CRNAb titer, incubation of the first serum was included in the serum-virus mixture. An hour later the second serum was added and the mixture was incubated another hour at 37° C.

In studies on the effect of temperature on detection of CRNAb, serum-virus mixtures were incubated at 37° C for an hour. The mixtures were either placed in an ice water bath and allowed to cool, C' added and the mixture incubated for another hour at 5° C, or C' added and the mixture incubated for an hour at 37° C. Both groups were then immediately diluted in cold Hanks' balanced salt solution and assayed for virus.

Results

Comparison of Incubation Procedures

A comparison was made between incubating serum, virus and C' together at 37° C for an hour with incubating serum and virus together at 37° C for an hour, then adding C' and incubating at 37° C for another hour. Higher titers with and without C' were obtained with the 2 hour incubation procedure than when all 3 components were incubated at the same time. Furthermore, the separate incubation periods seemed to be more effective in demonstrating CRNAb than the simultaneous incubation of the 3 components (Table 1). Further investigation of the 2 hour incubation using all permutations of fresh and heat-inactivated guinea pig serum, bovine serum or diluent were conducted on a single serum in duplicate experiments. The greatest neutralization was obtained when diluent was used with antiserum and virus for the first incubation and fresh guinea pig serum used in the second incubation. All other permutations of these reagents resulted in at least one 2-fold reduction in neutralization, and in the case of using heated and fresh bovine serum along with the serum-virus mixture, the reduction was two and three 2-fold dilutions, respectively.

Table 1. *Comparison between 2 incubating procedures on serum neutralizing titers determined with and without C'*

	Serum, virus, C' together[a]		Serum and virus incubated together, then C' added and mixture incubated[b]	
Serum	Heat inactivated rabbit serum	Fresh rabbit serum	Heat inactivated rabbit serum	Fresh rabbit serum
A	16	512	16	1024
B	4	64	8	512
C	2	64	4	256

[a] Serum, virus and C' were incubated at 37° C for an hour and assayed for virus

[b] Serum and virus were incubated at 37° C for an hour, then C' added and the mixture incubated for another hour before assaying for virus

Comparison of Different Sera as Sources of Complement

Different lots of guinea pig, rabbit, and bovine sera used as sources of C' were tested for their effectiveness in demonstrating CRNAb. Representative results, with a single antiserum, show that rabbit and guinea pig sera were equally effective, whereas bovine serum was a poor enhancer of CRNAb (Table 2).

Table 2. *Comparison of sera of several species as sources of C' in enhancing CRNAb*

Source of C'	Heat inactivated serum	Fresh serum
Guinea pig	4[a]	256
Rabbit	4	256
Bovine	4	16

[a] Serum neutralizing titer

Effect of Incubation Temperature on Demonstrating CRNAb

The temperature dependence of C' enhancement was studied using 9 and 23 day sera from immunized calves and sera from healthy cattle previously found to have antibody to IBR virus. Of 10 samples tested, no sample exhibited CRNAb at 5° C, whereas all samples did at 37° C. Titers at 5° C with C' were the same as titers without C' at 37° C.

Primary Response

Six-day samples from heifers 104 and 106 had antibody only in the IgM fraction and this was predominantly CRNAb. Non-CRNAb and CRNAb titers for heifer 104 were 1 and 256, respectively, and less than 4 and 64, respectively, for heifer 106. Results from sera of 6-month-old calves showed that immunoglobulin early in the primary response was CRNAb in the IgM fraction. This rapidly declined along with IgM NCRNAb. Thirty-two days after inoculation most antibody was in the IgG fraction, and most of this was CRNAb (Table 3).

Table 3. *Neutralizing antibody titers of calves determined with and without C'*

Calf	Test serum	Days postinoculation													
		Primary response[a]								Secondary response[b]					
		0		4		9		32		0		7		28	
		HC'[c]	C'	HC'	C'	HC'	C'	HC'	C'	HC'	C'	HC'	C'	HC'	C'
	whole	NT[d]	<1	NT	<1	1	64	NT	NT	2	4	32	128	32	64
14	IgM	NT	NT	NT	NT	1	32	<1	2	<1	<1	8	16	1	2
	IgG	NT	NT	NT	NT	1	4	4	8	2	4	32	64	32	64
	whole	NT	<1	<1	<1	8	256	4	32	2	4	256	2048	128	512
23	IgM	NT	NT	NT	NT	4	256	1	8	<1	1	16	256	2	8
	IgG	NT	NT	NT	NT	2	16	4	16	2	4	128	1024	64	256
	whole	NT	<1	<1	<1	32	256	4	32	4	8	64	128	64	128
41	IgM	NT	NT	NT	NT	16	512	1	8	<1	<1	2	4	2	4
	IgG	NT	NT	NT	NT	4	16	8	32	8	16	64	128	32	128
	whole	NT	<1	NT	<1	8	512	16	64	8	8	256	512	128	256
48	IgM	NT	NT	NT	NT	8	256	<4	8	<1	<1	8	32	4	8
	IgG	NT	NT	NT	NT	2	8	16	64	4	4	128	256	128	256

[a] Single injection given on day 0
[b] Second injection given 196 days after first
[c] Heated complement
[d] Not tested

Secondary Response

An anamnestic IgG response occurred following the second injection in each calf. Complement enhanced titers one or two 2-fold dilutions. Calf 23 had an IgM response similar to that produced after primary inoculation. The 3 other calves had IgM responses which were much lower than after the first inoculation. Complement enhanced titers one or two 2-fold dilutions. By 28 days after the second injection, most antibody was found in the IgG fraction, a very small amount was found in the IgM fraction, and C' still enhanced the titers of both the IgG and IgM antibodies by one or two 2-fold dilutions (Table 3).

Discussion

The kinetic response of cattle to antigens with respect to antibody class has not been studied very extensively. Quantitation of immunoglobulins has shown that calves vaccinated with *Brucella abortus* strain 19 developed IgM antibodies which rose sharply after vaccination and thereafter declined, whereas IgG levels peaked later and persisted longer. In adult cattle, the IgM and IgG responses of vaccinated cattle were found to be similar, both reaching high levels and persisting at high levels until declining around the 64th day. Cattle naturally infected had most of their antibody in the IgG class (3). The response of cattle to *Anaplasma marginale*, an organism known to persist in the host for a considerable period after the acute infection, was found to consist of production of IgM quickly followed by IgG, with IgM persisting along with IgG (10).

There are only a few reports on the class of antibody produced in cattle in response to viruses. Inoculation of cattle with Murray Valley encephalitis virus caused an IgM response which was quickly followed and replaced by IgG. The secondary response was almost entirely IgG (17). In very young calves, Murray Valley encephalitis, reovirus 1, Getah and bovine parainfluenza 3 viruses elicited almost no IgM in the secondary response, and almost no IgM in the primary response to Murray Valley encephalitis and Getah viruses while producing substantial IgG antibody to all these viruses in the primary and secondary responses (18).

We found the response of calves to IBR virus followed the IgM-IgG sequence in which IgM was detected before IgG and the latter persisted longer than the former. A typical anamnestic IgG response was elicited to a second injection given 196 days after the first in all 4 calves. However, the IgM response was similar to the primary response in one calf, whereas the other 3 calves had minimal IgM responses. This is similar to results of SANDERSON, who found calves inoculated with several viruses produced minimal IgM responses to a second injection (17, 18). Although IgM memory is usually considered to be short lived, HAMMER *et al.* (8), using a protein antigen, reported that although no anamnestic IgM response occurred in cattle in the early stages of immunization, a second injection given 7 months after the first elicited a pronounced anamnestic type IgM response. It is, therefore, possible that injection and examination of the calves after a longer interval would have produced a different type secondary response.

Under condition of continual antigenic stimulation, as occurs in *Brucella* and *Anaplasma* infections, the bovine continues to produce both IgM and IgG. Possibly, if sufficient IBR viral antigen were continually present, IgM would be

continually synthesized. However, in the latent type of infection which often accompanies herpesvirus infections, there is apparently insufficient antigen to maintain IgM synthesis.

The use of C' was especially useful in detecting the early IgM response to IBR virus, although CRNAb was present in both IgM and IgG fractions. Others have found that the faster bovine gamma globulin, IgG1 fixes C' whereas the slower gamma globulin, IgG2, probably lacks this ability (5). Potgieter, using neutralization kinetics, was unable to demonstrate CRNAb in the IgG fraction (11). Although we obtained only a one or two 2-fold difference between IgG titers with and without C', in no instance was the titer without C' greater than that with C' as could be expected if the variation were due technical factors. Furthermore, as shown in Table 1, CRNAb is probably best demonstrated when serum and virus are allowed to incubate before addition of C'. Since neutralization kinetics are performed with all 3 reagents mixed simultaneously, this may explain the difference between our results and those of Potgieter (11), and suggests the greater sensitivity of the end point neutralization as compared to the neutralization kinetic test in demonstrating CRNAb.

Antibodies to viral antigens in bovine serum have been reported to react poorly in standard C' fixation tests in which guinea pig serum is used as the source of C', and it has often been necessary to add unheated normal bovine serum in addition to guinea pig serum to conduct C' fixation tests (4). Although we found rabbit and guinea pig sera, as opposed to bovine serum, equally effective for C' enhanced neutralization, guinea pig serum often contained low titers of CRNAb, but not NCRNAb, making certain lots unsuitable. In contrast to C' fixation tests in which addition of bovine serum enhances detection of antibody, we found that bovine serum added before guinea pig serum resulted in significantly less antibody detected than when it was omitted, i.e., CRNAb titers were not maximum. One explanation of the inhibition of the 2 sera may be that C' components from different sources are not completely interchangeable (14) and interference may occur.

Another explanation for the poor enhanced neutralization produced by bovine serum may be related to the mechanisms of enhancement by C' of viruses sensitized by antibody. Studies with both herpes simplex and equine arteritis viruses have shown that when optimal concentrations of the early C' components are used, the enhancement which occurs is probably due to piling up of C' components on the surface of the virion so as to prevent an early function of the infectious process (6, 13). Addition of late C' components causes virolysis with release of nucleic acid (13). An insufficient amount of certain C' components in bovine serum could result in poor enhancement.

Although bovine C' is weakly hemolytic in standard C' fixation tests with sheep erythrocytes sensitized with rabbit antiserum, it has been demonstrated that under the proper conditions of ionic strength, Ca^{++} and Mg^{++} concentrations and with guinea pig erythrocytes that high hemolytic titers can be obtained (2). Barta and Barta (2) have suggested that since conglutination occurs when sensitized sheep, but not guinea pig, erythrocytes are treated with bovine serum, that different intermediate complexes are formed so that the former reacts with conglutination whereas the latter activates the lytic pathway without conglutination. Although the effect of conglutinin on C' enhanced neutralization has not been studied, a case

could be made both for or against its enhancement of neutralization after reaction of sensitized virus with early C' components.

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