

Neutralizing Antibody Response of Rabbits and Goats to Caprine Arthritis-Encephalitis Virus

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Rabbits were immunized with purified caprine arthritis-encephalitis virus and examined for neutralizing activity. Analysis of virus-antiserum interaction at 37°C demonstrated little loss of viral infectivity after incubation with heat-inactivated rabbit antiserum for 60 min. However, sensitization of virus (as assessed by the addition of complement) occurred almost immediately and was 95% complete after 10 min. The complement-dependent neutralizing activity was associated with the immunoglobulin G fraction of rabbit antiserum. Addition of goat anti-rabbit immunoglobulin G to the immune rabbit serum-caprine arthritis-encephalitis virus mixture also resulted in neutralization of infectivity when unbound antibody was removed before addition of the anti-immunoglobulin. Serum from most caprine arthritis-encephalitis virus-infected goats contains antibody activity to the core protein p28, as demonstrated by immunodiffusion and enzyme-linked immunosorbent assay. However, attempts to demonstrate neutralizing activity in the serum of goats up to 1.5 years post-inoculation or in serum of hyperimmunized goats were unsuccessful when the sera were examined alone or in combination with complement or rabbit anti-goat immunoglobulin or both.

A retrovirus, caprine arthritis-encephalitis virus (CAEV), causes a persistent disease of domestic goats most often manifested as two syndromes (6, 7): chronic proliferative synovitis and periartthritis in adult goats and leukoencephalomyelitis in young goats. CAEV can be isolated from multiple tissues of infected goats, although there seems to be a predilection for the tissues of the synovium and central nervous system (7, 13). Virus has been isolated from experimentally infected goats for up to 4 years post-inoculation (D. S. Adams, unpublished data) and from goats naturally infected for many years (8). After initial exposure to the virus, goats mount a vigorous humoral antibody response as well as a cell-mediated immune response to the major viral core protein, p28 (1, 25).

The progressive inflammatory arthritis caused by CAEV resembles rheumatoid arthritis (2, 7). Although conclusive evidence for an etiological relationship is lacking, viral infection is currently considered to be a likely initiator of the inflammatory and immunological processes involved in rheumatoid arthritis (17). Thus, the caprine arthritis-encephalitis syndrome affords an opportunity to examine a virus-host relationship which results in chronic connective tissue disease.

One important mechanism of host defense against virus infection is the ability of the im-

mune response to mediate virus neutralization. Therefore, in the present work, we examined the neutralizing response of experimental (rabbit) and natural (goat) hosts to CAEV.

MATERIALS AND METHODS

Cell cultures. Cell strains of synovial membrane explants from Caesarian-derived, specific pathogen-free goat kids (2) were grown in Dulbecco modified Eagle medium (DMEM) with 20 mM bicarbonate-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid) buffer supplemented with 2 mM glutamine, 100 µg of streptomycin per ml, 100 U of penicillin per ml and 5% heat-inactivated fetal bovine serum.

Virus cultivation. The prototype strain of CAEV, 75-G63, isolated from a goat with spontaneous arthritis (7), or virus cloned from this stock by three terminal dilutions in synovial cell cultures was used for immunization and in the neutralization assays. Infectious virus was harvested from synovial cell culture medium, clarified (600 × *g*, 30 min), and stored at -70°C. The infectivity titer of the parent virus stock was 10^{6.5} 50% tissue culture infectious doses (TCID₅₀) per ml, and the titer of the cloned virus stock was 10^{6.7} TCID₅₀ per ml. Cloned virus for goat and rabbit hyperimmunization was concentrated approximately 500 times by ultracentrifugation and sucrose gradient purification as previously described (4).

Infectious virus titration. CAEV infectivity was quantitated by terminal twofold dilutions in synovial cell cultures grown in 96-well microtiter plates (0.1 ml per well; Linbro, Van Nuys, Calif.), using cytopathic

effect (CPE) to detect virus infectivity (13). Fifty percent endpoints were determined 14 days postinfection (p.i.) by the method of Reed and Muench (24).

Antisera. Serum was collected from specific pathogen-free goat kids experimentally inoculated with the parent stock of CAEV. Six kids were injected intravenously with 1 ml of virus, and 0.5 ml of the same inoculum was injected into the right tibiotarsal and the left radiocarpal joints. Four additional goats were hyperimmunized by repeated inoculations of cloned CAEV concentrated by differential centrifugation. This protocol involved one intramuscular inoculation of CAEV suspended in complete Freund adjuvant, followed by five intraperitoneal inoculations without adjuvant.

Two groups of rabbits were also immunized with cloned CAEV as follows. Rabbits 1, 2, and 5 through 10 were inoculated in the footpads with gradient-purified CAEV (4) suspended in complete Freund adjuvant, followed by four intraperitoneal inoculations of virus suspended in physiological buffered saline. Rabbits 3 and 4 were inoculated intravenously with gradient-purified virus suspended in physiological buffered saline, followed by four intraperitoneal inoculations. All antisera were collected 7 to 10 days after the last challenge, stored at -70°C , heat inactivated at 56°C for 30 min, and adsorbed with uninfected goat synovial cells before use. Sera were checked for infectious CAEV, and all were negative.

Complement. Serum from specific pathogen-free goats was collected within 3 h of bleeding, stored at -70°C , and used as a source of goat complement. Guinea pig complement was obtained commercially from Flow Laboratories, Inc., Rockville, Md. The hemolytic activity of the goat complement was ascertained with sensitized rabbit erythrocytes before use in the neutralization tests.

Anti-immunoglobulin serum. Rabbit anti-goat immunoglobulin was prepared by injection of a mixture of goat immunoglobulin G, subclass 1 (IgG1), IgG2, and IgM purified as described for bovine immunoglobulins (19). Goat anti-rabbit IgG was prepared by injecting rabbit IgG separated from serum by dialysis and column chromatography on DEAE-cellulose in 0.01 M phosphate (pH 8.0). The purity of the IgG was verified by immunoelectrophoresis with goat anti-rabbit immunoglobulin serum. Reactivity of the anti-goat immunoglobulin and anti-rabbit immunoglobulin sera was verified by immunodiffusion and immunoelectrophoresis. Sera were heat inactivated at 56°C for 30 min and filter sterilized before use.

Neutralization assays. Twofold dilutions of antiserum and the appropriate normal serum controls were made in prewarmed DMEM to which was added an equal volume of a constant virus dilution (200 TCID₅₀ per 0.1 ml). This mixture was incubated at 37°C for 30 min and assayed for viral infectivity. When appropriate, complement or anti-immunoglobulin was added, and incubation was continued for an additional 30 min before the infectivity assay. The neutralization titer was the highest dilution of serum which completely inhibited virus infectivity in 50% of the inoculated wells.

A neutralization kinetics assay was performed by mixing equal volumes of antiserum or normal serum with 10^5 TCID₅₀ of cloned CAEV. At preselected intervals, 0.1-ml samples were transferred to a dilution tube containing a 0.9-ml volume of DMEM with or

TABLE 1. Neutralizing activity in sera of rabbits immunized with CAEV in the presence and absence of complement

Rabbit no.	Neutralization titer ^a	
	Without complement	With complement
1	0	0
2	4	120
3	0	0
4	0	0
5	0	20
6	2	60
7	0	20
8	0	80
9	4	100
10	0	40

^a Reciprocal of geometric mean antibody titer causing a 50% reduction in the number of wells with CPE in the presence and absence of guinea pig complement. Titers were similar in sera tested at both 8 and 14 weeks after the first inoculation.

without complement or anti-immunoglobulin. The tubes were held at 37°C for an additional 15 min, rapidly cooled in ice, diluted, and plated to determine the amount of surviving infectious virus.

RESULTS

Complement requirement for CAEV neutralization by rabbit antisera. The kinetics of CAEV neutralization by antiserum raised in rabbits was examined, as initial attempts to detect neutralizing activity in serum from CAEV-inoculated goats were unsuccessful. Of 10 rabbits immunized, 7 had sera that were able to neutralize CAEV in the presence of guinea pig complement (Table 1). Two of the three rabbits which did not respond also did not receive complete Freund adjuvant. The neutralization titer of immune rabbit serum (IRS) was dependent upon increasing concentrations of complement up to 8% complement (final concentration; data not shown). The complement-dependent neutralization titer could be further increased two- to threefold by incubation of the antiserum-virus suspension overnight at 4°C before the addition of complement, but this did not result in increased titers in the absence of complement (data not shown).

In lieu of complement, the ability of goat anti-rabbit IgG to complete the neutralization of IRS-sensitized virus was examined. Goat anti-rabbit IgG, added to the IRS-CAEV mixture after unbound immunoglobulin was removed via differential centrifugation, resulted in a 20-fold decrease in CAEV titer as compared with normal rabbit serum (NRS) controls (data not shown).

Kinetics of CAEV sensitization by rabbit antiserum. The kinetics of IRS sensitization of virus

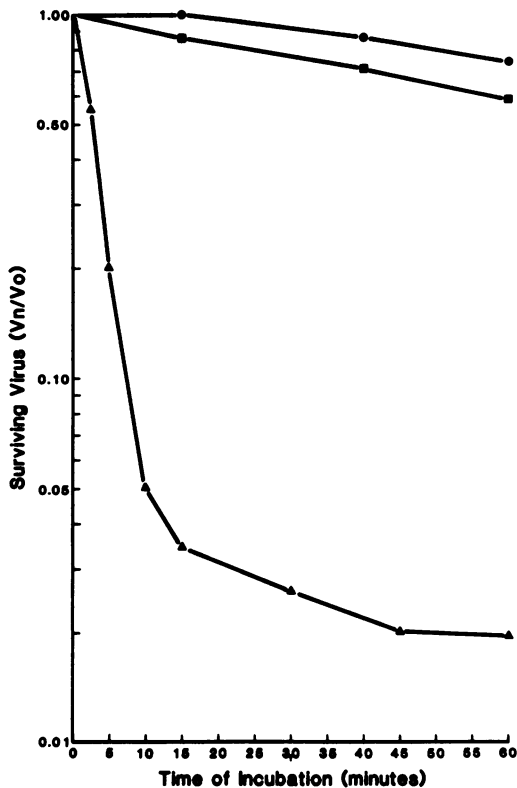


FIG. 1. Kinetics of CAEV sensitization by IRS as detected by virus inactivation with the addition of complement. At intervals, samples of the virus-serum mixtures were transferred to tubes containing DMEM with or without guinea pig complement and then diluted and assayed for infectious virus. ●, NRS (1:15) plus complement; ■, IRS (1:15) plus diluent; ▲, IRS (1:15) plus complement; Vn/Vo, virus surviving fraction at various times (Vn) relative to input virus (Vo).

was examined by measuring virus infectivity after the addition of guinea pig complement (Fig. 1). The decrease in CAEV infectivity in the presence of IRS alone did not differ significantly from that seen in the presence of NRS. However, IRS did sensitize the virus to subsequent neutralization after the addition of complement. Neutralization of CAEV in the presence of complement began immediately after exposure to the rabbit antiserum and was 95% complete after 10 min. When lower antibody concentrations were employed, the complement-dependent neutralizing reaction proceeded at a proportionately slower rate (data not shown).

Neutralizing activity associated with the IgG fraction of rabbit antiserum. The IgG fractions were separated from immune and normal rabbit serum, examined for neutralizing activity, adjusted to 0.8 mg/ml of protein, serially diluted, and mixed with CAEV in the presence and absence of guinea pig complement. The neutral-

izing activity associated with immune IgG was complement dependent, with a titer of 1:100 when assayed at 37°C and a titer of 1:200 after incubation overnight at 4°C (data not shown). Normal IgG had no inhibitory effect on CAEV infectivity. These results indicate that the complement-dependent neutralizing activity of the IRS was mediated by serum IgG and not non-immunoglobulin serum components (11).

Examination of serum from experimentally infected goats for CAEV neutralizing activity. Serum withdrawn at periodic intervals from goats experimentally inoculated with the parent stock of CAEV was examined for neutralizing activity. Assays involving a constant amount of virus mixed with various dilutions of antiserum, as well as constant antibody (1:4) with various dilutions of virus, were performed. Inhibition of CAEV-specific CPE was monitored in the presence and absence of homologous goat complement (Table 2) and guinea pig complement (data not shown). Neutralization titers of greater than 1:4 were not observed in serum from infected goats through 39 weeks p.i. (Table 2). However, only titers greater than 1:4 could be considered significant, as titers ranging from 0 to 1:4 were obtained for sera from five specific pathogen-free goats. All of the inoculated goats demonstrated CAEV-induced arthritis, and the ability or inability to reisolate CAEV from synovial fluid samples had no discernible effect on the neutralizing capabilities of the host serum (Table 2). In these same CAEV-infected animals, antibody measured by enzyme-linked immunosorbent assay and directed principally to the viral core protein, p28, appeared by 3 weeks and was maximal at 8 to 12 weeks p.i. (1, 25).

Examination of serum from hyperimmunized goats for CAEV neutralizing activity. As serum from goats given a single injection of CAEV did not contain neutralizing activity, four goats were hyperimmunized by multiple inoculation of concentrated, cloned CAEV in an attempt to stimulate neutralizing antibody. All of the goats developed antibody to p28, as detected by immunodiffusion. However, no neutralizing activity was detected in any of the four hyperimmunized goat sera (HGSs) assayed with or without complement or anti-immunoglobulin enhancement or both in neutralization kinetic assays.

Results obtained with acid elution techniques to reactivate complexed antibody suggested that neutralizing antibody is not bound in goat serum as antigen-antibody complexes. Sera were acid eluted by mixing undiluted serum with an equal volume of Hanks balanced salt solution (pH 1.0), resulting in a final pH of 2.4 to 2.6, and held at room temperature for 15 min (16). Reactivation of neutralizing antibody from known virus-antibody complexes (IRS plus CAEV) was

TABLE 2. Examination of CAEV-infected goats for neutralizing activity^a

Goat no.	Neutralization titer ^b at following time (weeks) p.i.:					Reisolation of virus ^c
	2	7	19	24	39	
78G56	0	0	0	0	0	+
75G59	2	2	2	0	4	-
78G61	ND ^d	0	2	0	0	-
78G65	2	0	0	0	2	+
78G77	4	2	4	0	0	+
78G88	0	0	0	0	0	+

^a Neutralization assay with the parental CAEV stock at 37°C in the presence of 8% homologous goat complement (similar titers were obtained without complement).

^b Reciprocal of geometric mean antibody titer causing a 50% reduction in number of wells with CPE. Five goats used as controls had neutralization titers ranging from 0 to 4; there was no reisolation of virus from these animals.

^c Synovial fluid samples taken 1 year p.i. and cocultivated with synovial cells in vitro.

^d ND, Not done.

shown to occur with this procedure (Table 3). Acid elution of serum from each of the hyperimmunized goats before the addition of CAEV to the reaction mixture did not result in detectable neutralizing activity (Table 3).

The lack of detectable CAEV neutralization by goat serum may represent an absence of antibody directed towards virus surface antigens. Therefore, to determine whether HGS contained antibody able to bind to the virus surface, HGS or normal goat serum adjusted to similar concentrations of IgG1 and IgG2, as determined by radial immunodiffusion, was mixed with 10^{5.3} TCID₅₀ of cloned CAEV and incubated at 37°C for 30 min. Rabbit anti-goat immunoglobulin at a concentration that would precipitate 100% of the immunoglobulin present was added to the reaction mixtures and incubated overnight at 4°C. The precipitated immunoglobulin was removed from the reaction mixture by centrifugation, and the supernatants were examined for the amount of remaining infectious virus. The data shown in Table 4 indicate that HGS contains antibodies which bind to the surface of the CAEV particle, as immunological specific precipitation of immunoglobulin caused a 3- to 50-fold decrease in CAEV infectivity. A decrease in infectivity of 7-fold (0.8 log₁₀) is significant at the 95% confidence level, as assessed by the Student *t* test. A loss of infectivity was not seen when normal goat serum was added in place of HGS or NRS in place of rabbit anti-goat immunoglobulin (Table 4). These results indicated that three of four HGSs contain antibody which binds to the virus surface; therefore, anti-goat immunoglobulin was added to the HGS-sensitized virus to determine its effectiveness in mediating neutralization. HGS or normal goat serum and virus were incubated at 37°C, and antibody-virus complexes were removed from unbound antibody via differential centrifugation. Rabbit anti-goat immunoglobulin or NRS

was added to the complexes, and remaining CAEV infectivity was determined. HGS did not contain antibodies capable of neutralizing virus infectivity after the addition of rabbit anti-goat immunoglobulin (Table 4).

DISCUSSION

The present study demonstrates that rabbits produce neutralizing antibodies against CAEV, the retrovirus responsible for caprine arthritis-encephalitis syndrome. Complete neutralization of CAEV infectivity by all rabbit antisera tested was, however, complement dependent. Although kinetic assays demonstrated rapid

TABLE 3. Effect of acid elution of immune sera on CAEV neutralization

Sample	Neutralization titer ^a
NRS + CAEV + acid	—
NRS + CAEV + acid + CAEV + C ^b	0
IRS + CAEV + acid + CAEV + C	120
IRS + CAEV + diluent + CAEV + C ^c	4
NGS ^d + acid + CAEV + C	0
4 HGS + acid + CAEV + C ^e	0-4

^a Reciprocal of geometric mean antibody titer causing 50% reduction in number of wells with CPE. —, No infectious virus remaining (acid sensitive).

^b Undiluted NRS or IRS was saturated with virus (10^{4.5} TCID₅₀ of CAEV per ml) for 30 min at 37°C, acid eluted, and then either assayed for viral infectivity or adjusted for pH and serially diluted. An equal volume of 200 TCID₅₀ per 0.1 ml of CAEV was then added for neutralization in the presence of guinea pig complement (C).

^c Neutral Hanks balanced salt solution was added in place of acid Hanks balanced salt solution.

^d NGS, Normal goat serum.

^e HGSs from four animals in separate tests were used, each was acid eluted, and then an equal volume of 200 TCID₅₀ per 0.1 ml of CAEV was added for neutralization in the presence of goat complement.

TABLE 4. Examination of HGS for antibody to virus surface antigens, using rabbit anti-goat immunoglobulin

Reaction mixture	Precipitation with anti-immunoglobulin (TCID ₅₀ /ml) ^a	Neutralization with anti-immunoglobulin (TCID ₅₀ /ml) ^b
Control sera		
NGS ^c + CAEV + anti-immunoglobulin	10 ^{5.10}	10 ^{4.94}
1-4. HGS + CAEV + NRS	10 ^{5.04d}	10 ^{4.73d}
Immune sera		
1. HGS + CAEV + anti-immunoglobulin	10 ^{3.80}	10 ^{4.66}
2. HGS + CAEV + anti-immunoglobulin	10 ^{4.06}	ND ^e
3. HGS + CAEV + anti-immunoglobulin	10 ^{4.48}	ND
4. HGS + CAEV + anti-immunoglobulin	10 ^{3.30}	10 ^{4.80}

^a Amount of infectious virus remaining in the supernatant after precipitation with rabbit anti-goat immunoglobulin at a concentration precipitating 100% of the immunoglobulin present as described in the text.

^b HGS or normal goat serum plus CAEV was reacted for 30 min at 37°C, then virus-antibody complexes were pelleted by differential centrifugation in a Beckman SW50.1 rotor (35,000 rpm, 60 min). The supernatant containing unbound antibody was discarded, and the virus pellet was resuspended in DMEM. An equal volume of undiluted rabbit anti-goat immunoglobulin or NRS was added for 30 min at 37°C, and then the reaction mixture was assayed for infectious virus.

^c NGS, Normal goat serum.

^d Average values. The range for precipitation controls was 10^{4.91} to 10^{5.15}; the range for neutralization controls was 10^{4.65} to 10^{4.80}.

^e ND, Not done.

CAEV sensitization to complement neutralization, a residual fraction of about 2% remained infectious. This has been noted in other virus systems, and it has been suggested that this fraction represents (i) reversible complexes, (ii) viral aggregates which cannot be completely neutralized, (iii) complexing with blocking, non-neutralizing antibody, or (iv) a genetically resistant subpopulation (9, 18). Complement-dependent neutralization has been demonstrated for a number of viruses, especially enveloped viruses (27, 28). Whether neutralization of sensitized CAEV after the addition of complement involved "virolysis" (22) or other mechanisms, including aggregation or steric hindrance of virus adsorption caused by the binding of complement components, was not determined. IRS was also able to sensitize CAEV to neutralization by goat anti-rabbit IgG, but only if unbound immunoglobulin was removed via centrifugation before the addition of anti-IgG. Neutralization in the presence of anti-IgG most likely involves steric hindrance or stabilization of the antibody-virus complexes (15).

It has been reported that oncornavirus p15E can activate serum complement, resulting in antibody-independent, complement-mediated virolysis (3). Furthermore, infectivity of mouse xenotrophic viruses can be neutralized by circulating serum lipoproteins independent of antibody or complement (11). However, the neutralizing activity described here was associated with purified IgG from IRS and not mediated by non-immunoglobulin serum components.

Our attempts to demonstrate neutralizing activity in serum from natural hosts (goats) have

been unsuccessful. No neutralizing activity was found in serum from animals inoculated with a single dose of CAEV or in hyperimmunized animals. However, all animals eventually developed antibodies directed against the major viral core protein, p28. All animals developed arthritis, and virus could be reisolated from the synovial cavity of the majority of these animals. As infection by CAEV results in the establishment of persistent disease, the possibility that neutralizing antibody could be present in the serum but bound as virus-antibody or envelope glycoprotein-antibody complexes must be considered. However, preliminary observations with acid elution to reactivate antibody from complexes did not support this possibility.

Sera from three of four hyperimmunized goats did contain antibodies which presumably bound antigens exposed on the surface of the virion, since it was shown that rabbit anti-goat immunoglobulin precipitation of HGS-virus mixtures resulted in a 10- to 50-fold decrease in the amount of infectious virus. The fact that the antibody directed against these surface antigens did not result in neutralization even after the addition of anti-goat immunoglobulin may be due to infrequent spacing of the antigenic determinants involved in infectivity or antibody directed against irrelevant antigen(s) or both.

Other explanations may account for the lack of detectable neutralizing antibody in CAEV-infected goats. First, the lack of detectable neutralizing activity may reflect a lack of immunogenicity of the relevant virus envelope antigen(s) or insufficient antigen stimulus or both. Second, virus infection of the immune reactive cells at a

critical induction period may influence the production of neutralizing antibody (20). In this regard, CAEV readily infects cultured peripheral blood monocytes (12), and virus can be recovered from mononuclear cells in the synovial fluid and peripheral blood of infected animals (Klevjer-Anderson et al., manuscript in preparation). Third, all available neutralizing antibody may be bound to virus antigens expressed on cell surfaces. Although CAEV can be recovered from numerous tissues in goats, it is unknown whether expression of virus antigens on cell surfaces (with or without a productive virus infection) occurs to the extent necessary to bind available antibody as has been reported for mouse mammary tumor virus in certain strains of mice (26). Fourth, the caprine species may intrinsically produce little or low-avidity (or both) neutralizing antibody to the particular antigen(s) involved in neutralization of CAEV infectivity. In conjunction with this, blocking antibodies of high avidity may be generated which compete with the binding of neutralizing antibody but which would themselves not neutralize virus infectivity (18).

The lack of neutralizing activity against viruses causing persistent disease occurs in other systems and may have important pathogenetic implications. Failure to elicit neutralizing antibodies during persistent infection by Aleutian disease virus and African swine fever virus may involve the ability of these viruses to infect immune reactive cells (5, 23). Very low or no neutralizing activity has also been associated with several murine retroviral infections (10).

In any case, the lack of neutralizing activity can only enhance the ability of CAEV to establish a persistent infection. Other factors which may be important in the establishment of persistent disease include (i) the ability of this virus to integrate its genomic information into host cell DNA; (ii) the capacity to remain infectious in macrophages after phagocytosis; and (iii) the potential for antigenic variation, as is the case for other lentiviruses, i.e., visna virus and equine infectious anemia virus (14, 21).

Continued virological and immunological studies are necessary before the pathogenetic mechanisms responsible for this chronic connective tissue disease are completely understood. In regard to CAEV neutralization, a more fundamental understanding of the antigenic determinants responsible for virus infectivity will be obtained when the effects of monoclonal antibody to individual viral glycoproteins are examined.

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